Abstract

Detection and characterization of HIV-1 specific T cell responses amongst exposed and unexposed HIV-1 seronegative individuals

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Thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy

The primary aim of this thesis was to examine whether HIV-1 specific T cell responses in HIV-1 exposed seronegative (HESN) subjects could be firstly detected and if so, conferred protection against productive infection from HIV-1.

Cultured IFN-γ ELISpot found 38.7-60% of HESN subjects had detectable HIV-1 specific T cell responses. HIV-1 specific T cell responses could be titrated, were typically mediated by $CD4^+$ T cells and tended to map to previously defined, promiscuous epitopes. In a statistically powered, retrospective study, no evidence was found to support a role for pre-existing HIV-1 specific T cell responses in either protection against or risk of HIV-1 infection.

Exposure to HIV-1 impacted upon detection of pre-existing HIV-1 specific T cell responses, in terms of frequency ($p=0.01$), magnitude ($p=0.02$) and maintenance of response. This suggests, that exposure to HIV-1 was truly priming HIV-1 specific T cell responses. However, exposure to HIV-1 was not a prerequisite and HIV-1 specific T cell responses were detectable amongst HIV-1 unexposed seronegative (HUSN) donors. Similar to HESN, HIV-1 specific T cell responses detected amongst HUSN, could be mapped to the peptide level, titrated and were predominately mediated by $CD4^+$ T cells. These T cells were shown to be detectable amongst memory $CD4^+$ T cell subsets, suggesting they were not the result of *in vitro* priming. Whilst sample size was low, HIV-1 specific T cell responses, detected amongst HUSN donors were shown to be oligoclonal in TCRVβ usage.

The detection of memory $CD4^+$ HIV-1 specific T cell responses amongst HUSN opens a broader debate about the ontogeny of HIV-1 specific T cell responses, the inherent properties of the adaptive immune system and its preponderance toward degeneracy and cross reactivity. Greater understanding of these fundamental immunology questions should, improve our understanding of T cell ontogeny and hopefully prove beneficial in the design of a novel therapeutic vaccine for HIV-1.

Acknowledgements

I feel I have been incredibly privileged not only to have studied for my DPhil at Oxford University, but to have had the opportunity to work with some truly fantastic people. First and foremost I wish thank my supervisors Professor Sir Andrew McMichael and Dr Nilu Goonetilleke for their on-going support and encouragement. I feel honoured to be able to say I was Andrew's student, he is an inspiring mentor!

None of the work in this thesis would have been possible without access to a number of clinical cohorts. I wish to thank Professor Shannon Galvin for giving me access to her Malawian retrospective cohort. Peter Gilbert, for granting access to the VAX004 trial samples, and for performing the calculations required to power the study. Nilu Goonetilleke, Kuba Kopycinski and Adam Ritchie for access to the CHAVI002 cohort and additional low risk control samples from the IAVI016 cohort. Thanks are also given to the many clinical staff and study participants including the many lab members I bled during this thesis for assay optimization and control data!

Science is truly about collaboration, and it is with great thanks to Professor Sir Andrew McMichael that I have been fortunate to have had the opportunity to collaborate with a number of groups. I wish to thank Professor Federica Sallusto, Tess Brodie and Astrea Rossetti for their help and advice with the peptide library technique – In 5 weeks I learnt more than I could have imagined -ringrazia milione! Professor David Price, James McLaren and Kristin Ladell for persevering whilst we optimized a way to sequence TCR from short term cell lines and for helping me navigate my way through the TCR multiplex technique! Professor Clive Gray, Catherine Riou and Mandla Mlotshwa for the opportunity to run multiparameter flow panels on acute HIV-1 patients and the experience of working in South Africa! As is often the way with science, a flippant conversation over lunch one sunny day in Jo'burg led me to discover the TCRVβ antibody kit that would prove so useful in later studies! Kuba Kopycinski who remains my flow guru! Thank you for ALL your help, from teaching me the basics on the CyANs to letting me spend some time on the IAVI machines when the Oxford LSR2 was playing up! Mike Liu for the many Atlas and general lab questions you solved. Victoria Whale for technical assistance and more importantly for celebratory or commiseratory glasses of wine when experiments worked / failed! Dr Jo Roberts for letting me spend SO much money and ensuring that there was always cake!! Kati DiGleria and Zhanru Yu for help and advice with mass spectrometry, peptide synthesis and HPLC purification. Simon Brackenridge and Tim Rostrum for help with DNA extractions and the CCR5Δ32 PCR. TC my fellow student and chief phlebotomist- 4 years went too fast…Lucy Dorrell for many inspiring conversations and always good advice! Brasenose College for fun nights out, amazing dinners, interesting lectures and importantly college port! And finally all members of the CHAVI and Dorrell groups not already mentioned, "Clutters",

Gem, HB and Steve thank you for making my time in the lab so fun and memorable!

Just 48 hours before submitting this thesis my computer decided it had had enough- leaving me with a blue screen of doom and a nasty crunching noise as my hard drive irrecoverably crashed. I wish to say a very big thank you to Matt Brechin, the IT star who managed to salvage my endnote file when I feared all was lost and who loaned me a computer for those final 48hours!

On a more personal note I wish to thank my boyfriend John-Paul Jukes. At every high and every low you've been there for me- your support, love and understanding mean more than words can say and I would not have made it through without you!! THANK YOU. Finally, I wish to thank my family. In particular my mum who is always there for me when I needed a chat, a girly shopping spree or simply a hug! And who even proof-read this thesis! My Dad for supplying the vast amounts of fudge that helped make "the write up" a little easier and more importantly for instilling a paranoid "must back it up" attitude into me- never has your advice been so appreciated!! THANK YOU. My Grandparents, who were incredibly special people and a true inspiration, I wish more than anything you were here to share this moment. There aren't really the words to thank a family who mean so much. Thank you for believing in me, and supporting me in all that I have done. Whatever I achieve in life I owe to your love and support. Somehow "Thank You" doesn't seem enough. I can only hope I make you proud.

Thank You!

Abbreviations

aa	Amino acid
Ab	Antibody
ABC	Avidin biotin complex
AEC	3-Amino-9-ethylcarbazole
AIDS	Acquired autoimmune deficiency syndrome
ANOVA	Analysis of variance
APC	Antigen presentation cells
APOBEC	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like
ART	Antiretroviral therapy
BCG	Bacillus Calmette-Guerin
Bkgd	Background
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CCR5	Cysteine-cysteine linked chemokine receptor 5
CDR	Complementarity-determining regions
CFP-10	Culture filtrate protein 10
CFSE	Carboxyfluorescein succinimidyl ester
CLIP	Class II associated invariant-chain peptide
CHAVI	Centre for HIV-1 and Aids Vaccine Immunology
CLP	Common lymphoid progeniotors
CM	Central memory
CMV	Cytomegalovirus
CPM	Counts per minute
CPS	Cryopreservation solution
CRF	Circulating recombinant form
D	Diversity chain
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DTNPs	deoxynucleotide triphosphates
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EAE	Experimental autoimmune encephalitis
EBV	Epstein-Barr virus
ELISA	enzyme linked immunooassy
ELISpot	Enzyme linked immunospot assay
ELP	Early lymphoid progenitor cell
EM	Effector memory
EMRA	Effector memory like T cells (CCR7−CD45RO−CD45RA+)
ER	Endoplasmic reticulum
ESAT-6	Early secreted antigenic target -6
FACS	Fluorescence-activated cell sorting

FDA	Food and Drug Administration
FEC	Influenza virus, Epstein Bar Virus, Cytomegalovirus optimal epitopes
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate, green
GALT	Gut associated lymphoid tissue
GEE	Generalized estimating equations
GUD	Genital ulcer disease
HA	Hyaluronan
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCL	Hydrochloride
HCV	Hepatitis C Virus
HD	Helper deficient
HESN	HIV-1 exposed seronegative
HIV	Human immunodeficiency syndrome
HIV-1	Human immunodeficiency syndrome type 1
HIV-2	Human immunodeficiency syndrome type 2
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HUSN	HIV-1 unexposed seronegative
IAVI	International Aids Vaccine Initiative
ICS	Intracellular cytokine staining
IDU	Intravenous drug user
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL	Interleukin
IS	Immunological synapse
J	Joining chain
KIR	Killer cell immunoglobulin like receptor
KLH	Keyhole limpet hemocyanin
LANL	Los Alamos National Laboratory
LCMV	Lymphocytic Choriomeningitis Virus
LMMP	Lymphoid primed multipotent myeloid precursor cells
LTNP	Long term non progressor
LTBI	Latent tuberculosis infection
LYVE-1	lymphatic vessel endothelial hyaluronan receptor
mAb	Monoclonal antibody
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-β	Macrophage inflammatory protein–beta
µl	Microlitre
ml	Millilitre
Mrna	Messenger ribonucleic acid
MS	Multiple sclerosis
MSM	Men who have sex with men

Mtb	Mycobacterium tuberculosis
MVA	modified vaccinia virus Ankara
NAT	Nucleic acid test
Nef/Acc	Nef and accessory proteins (Vpr, vpu, vif, tat, rev)
ND	Not done
NK	Natural killer
NKT	Natural killer T cell
OXTREC	The Oxford Tropical Research Ethics Committee
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phaseolus vulgaris (red kidney bean) Phytohemagglutinin
PPD	Purified protein derivative
PTID	Patient identification number
QC	Quality control
QFT-IT	QuantiFERON-TB Gold In-Tube
R10	RPMI media with 10% heat inactivated bovine serum albumin
R-AB-10	RPMI media with 10% heat inactivated human AB serum
RANTES	Regulated upon activation of normal T cells, expressed and secreted
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SCHARP	Statistical centre for HIV/AIDS research and prevention
SFU	Spot forming units
SFU/10^6	Spot forming units per million cells
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
STD	Sexually transmitted disease
STCL	Short term cell line
STI	Sexually transmitted infection
TAP	Transporter associated with antigen processing
TB	Tuberculosis
TCM	Central memory T cell
TCR	T cell receptor
TEM	Effector memory T cell
TFH	T follicular helper cell
ThPOK	T-helper inducing POZ-Kruppel factor
TLR	Toll like receptore
TMA	Transcription mediated amplification
TNFα	Tumor necrosis factor alpha
TReg	Regulatory T cell
TRIM5α	Tripartite motif-containing protein 5α
TST	Tuberculin skin test
TT	Tetanus toxin
USA	United States Of America
UK	United Kingdom
V	Variable chain
VV	Vaccinia virus

VZV	Varicella zosta virus
WHO	World health organization
ZAP-70	Zeta chain-associated protein kinase of 70kD

Contents Page

ABSTRACT ..... **I**

ACKNOWLEDGEMENTS ..... **II**

ABBREVIATIONS ..... **IV**

CONTENTS PAGE **1**

CHAPTER 1: INTRODUCTION **6**

1.1 BRIEF HISTORY OF HIV-1 6
1.2 VIRAL HETEROGENEITY 6
1.3 HIV-1 TRANSMISSION 9
1.4 CLINICAL STAGES OF HIV-1 INFECTION 10
1.4.1 Acute HIV-1 10
1.4.2 Chronic infection and progression to AIDS 12
1.4.2.1 HIV-1/TB co-infection 13
1.4.2.2 Impact of HIV-1 upon TB 13
1.4.2.2.1 Impact of TB upon HIV-1 infection 14
1.5 CLINICAL DIAGNOSIS OF HIV-1 INFECTION 15
1.6 HETEROGENEITY IN HIV-1 INFECTION 15
1.7 IMMUNE RESPONSES AS CORRELATES OF PROTECTION 16
1.7.1 Innate immunity 16
1.7.1.1 Soluble factors 16
1.7.1.2 Intrinsic cellular factors 17
1.7.1.3 Cellular immune response 18
1.7.1.3.1 NK cells 18
1.7.1.3.2 Dendritic cells 19
1.7.2 Adaptive immunity 20
1.7.2.1 Humoral response 20
1.7.2.2 T cell response 21
1.7.2.2.1 Antigen processing and presentation 21
1.7.2.2.1.1 HLA molecules 21
1.7.2.2.1.2 HLA class I processing and presentation 22
1.7.2.2.2 HLA class II processing and presentation 23
1.7.2.2.3 HLA associations with HIV-1 disease progression 24
1.7.2.3 The TCR 25
1.7.2.4 Thymic selection and development of T cells 26
1.7.2.4.1 $CD4^+/CD8^+$ lineage commitment 28
1.7.2.5 Homeostasis of naïve T cells 28
1.7.2.6 T cell activation 29
1.7.2.6.1 Requirement for co-receptors 29
1.7.2.6.2 Peptide-HLA-TCR interaction 30
1.7.2.6.3 Successful T cell activation and subsequent signalling cascade 31
1.7.2.6.4 Immune activation and HIV-1 31
1.7.2.7 Differentiation into memory T cells 32
1.7.2.7.1 Impact of HIV-1 upon immunologic memory 34
1.7.2.8 Effector function 35
1.7.2.8.1 $CD4^+$ T cells 35
1.7.2.8.2 $CD8^+$ T cells 37
1.7.2.9 T cell effector function and HIV-1 38
1.7.2.9.1 $CD8^+$ T cells 38
1.7.2.9.2 $CD4^+$ T cells 38
1.7.2.9.3 T cells and acquisition of HIV-1 39
1.8 THESIS AIMS 40

CHAPTER 2: MATERIALS AND METHODS **41**

2.1 STUDY SUBJECTS 41
2.1.1 HIV-1 exposed seronegative cohorts 41
2.1.1.1 GALVIN cohort 41
2.1.1.1.1 Ethical considerations and sample access 41
2.1.1.1.2 Enrolment criteria 41
2.1.1.1.3 HIV-1 status and risk of HIV-1 infection 45
2.1.1.2 IAVI 016 45
2.1.1.3 Ethical considerations and sample access 46
2.1.1.4 CHAVI002 cohort 46
2.1.1.4.1 Inclusion criteria 46
2.1.1.4.1.1 Ethical considerations, sample access and work contributions 47
2.1.1.5 VAX004 cohort 47
2.1.1.5.1 Ethics considerations and sample access. 47
2.1.1.5.2 Enrolment criteria 48
2.1.1.5.3 Assessment of HIV-1 status and risk of infection 48
2.1.1.1 Study powering 49
2.1.2 Acute HIV-1 infection samples 49
2.1.2.1 Definition of acute HIV-1 infection 50
2.2 MEDIA AND WASH BUFFERS 52
2.2.1.1 Cell culture media 52
2.2.1.2 Complete media + /- IL-2 for T cell library technique 52
2.2.1.3 Wash buffer 52
2.2.1.4 MACS buffer 52
2.2.1.5 Cryopreservation solution 52
2.2.2 Peptide synthesis 52
2.2.2.1 Creation of master and working stocks 53
2.2.2.2 Preparation of peptide pools and antigen “screening” plates 53
2.2.2.3 Preparation of peptide mapping plates 54
2.2.2.4 Quality control 54
2.3 CELL CULTURE TECHNIQUES 55
2.3.1 Isolation of peripheral blood mononuclear cells (PBMC) from whole blood 55
2.3.1.1 MACS micro bead isolation of $CD14^+$ and $CD4^+$ T cells 56
2.3.2 Thawing PBMC 57
2.3.3 Cell counting 57
2.3.4 Irradiation of allogeneic feeder cells 58
2.3.5 Cryopreservation 58
2.3.5.1 Isolated PBMC and short-term cell lines 58
2.3.5.2 Cryopreservation of $CD14^+$ cells 58
2.3.6 Cell culture 58
2.3.6.1 Short-term cell culture 58
2.3.6.2 T cell library technique 59
2.4 CELL SORTING 59
2.4.1 Isolation of $CD4^+$ Naïve, central and effector memory T cells 59
2.4.2 Antigen specific T cells 60
2.5 FUNCTIONAL AND PHENOTYPIC ANALYSIS 61
2.5.1.1 Flow cytometry 61
2.5.1.2 Staining protocol 66
2.5.1.3 Compensation Controls 67
2.5.1.4 Flow Analysis 67
2.5.2 Tritiated thymidine incorporation 74
2.5.2.1 Criteria for positive response by [^{3}H] Thymidine assay 74
2.5.3 ELISpot assays 74
2.5.3.1 Visualization ELISpot assays 75
2.5.3.2 Criteria for positive/negative response 75
2.6 MOLECULAR BIOLOGY TECHNIQUES 76
2.6.1 TCR Vβ clonotyping 76
2.6.1.1 Proteinase K digest 76
2.6.1.2 Heminested touch down polymerase chain reaction (PCR) 76

2.6.1.3 Ligation 79
2.6.1.3.1 Bacterial transformation 79
2.6.1.3.1.1 Preparation of LB-AIX plates 79
2.6.1.3.2 Plating bacteria 79
2.6.1.3.3 Colony picking 79
2.6.1.3.4 Colony PCR 80
2.6.1.3.5 Amplification check 80
2.6.1.3.6 Sequencing 80
2.6.2 Examination of CCR5Δ32 82
2.6.2.1 DNA Extraction 82
2.6.2.2 CCR5 Δ32 PCR 82
2.6.3 Transcription mediated amplification assay (TMA) 83
2.7 STATISTICAL ANALYSIS 83
2.8 ONLINE DATABASE AND SEARCH TOOLS 84
2.8.1 Basic Local Alignment Search Tool (BLAST) 84
2.8.2 Los Alamos National Laboratory (LANL) HIV-1 database 84

CHAPTER 3: CD4$^+$ T CELLS IN ACUTE HIV-1 INFECTION 85

3.1 INTRODUCTION 85
3.2 CHAPTER AIMS 89
3.3 RESULTS 90
3.3.1 Cohort Characteristics 90
3.3.3 Memory T cell subsets 91
3.3.4 Kinetics of HIV-1 specific memory CD4$^+$ and CD8$^+$ T cell responses 91
3.3.5 Magnitude and maintenance of CD4$^+$/CD8$^+$ T cell responses 91
3.3.6 Functionality of CD4$^+$ and CD8$^+$ T cell responses 92
3.3.7 Activation status of gag specific IFN-γ$^+$ memory CD4$^+$ and CD8$^+$ T cells 92
3.3.8 Memory phenotype of gag specific IFN-γ$^+$ T cells 93
3.4 DISCUSSION 94

CHAPTER 4: DETECTION OF LOW FREQUENCY AND MAGNITUDE T CELL RESPONSES 114

4.1 INTRODUCTION 114
4.2 CHAPTER AIMS: 117
4.3 RESULTS 118
4.3.1 *Ex vivo* detection of latent Mycobacterium tuberculosis infection 118
4.3.2 Improved sensitivity afforded by cultured IFN-γ ELISpot 118
4.3.3 Cultured IFN-γ ELISpot assay specificity 119
4.3.4 T cell response titration and reproducibility 119
4.3.5 Epitope mapping and CD4$^+$/CD8$^+$ T cell specificity 119
4.3.6 Cryopreservation does not significantly decrease sensitivity 120
4.4 DISCUSSION 121

CHAPTER 5: DETECTION OF HIV-1 SPECIFIC T CELL RESPONSES IN SERONEGATIVE INDIVIDUALS. 132

5.1 INTRODUCTION: 132
5.2 CHAPTER AIMS 135
5.3 COHORTS STUDIED 135
5.4 RESULTS: 137
5.4.1 Detection of HIV-1 specific T cell responses in seronegative donors by cultured IFN-γ ELISpot 137
5.4.2 Magnitude of HIV-1 specific T cell responses in HESN and HUSN 138
5.4.3 No overall proliferative defect 138
5.4.4 Maintenance of HIV-1 specific T cell responses 139
5.4.5 Absence of low titre virus 139
5.4.6 CCR5Δ32 mutation 140
5.4.7 HIV-1 specific T cell responses are typically mediated by CD4$^+$ T cells 140

5.4.8 HIV-1 specific T cell responses typically map to previously defined, promiscuous HLA binding epitopes 140
5.4.9 Exclusion of peptide impurities and titration of mapped responses 141
5.4.10 Design and validation of a novel "promiscuous peptide" pool 141
5.4.11 Detection of HIV-1 specific T cell responses amongst HESN and HUSN donors was not improved by inclusion of promiscuous epitope pool..... 142
5.5 DISCUSSION 143

CHAPTER 6: HIV-1 SPECIFIC T CELL RESPONSES IN SERONEGATIVE DONORS, THE RESULT OF CROSS REACTIVITY? 169

6.1 INTRODUCTION 169
6.2 CHAPTER AIMS: 174
6.3 RESULTS: 175
6.3.1 Attempts to directly assess T cell cross reactivity: 175
6.3.1.1 Sequence homology to other pathogens 175
6.3.2 Indirect methods to look for T cell cross reactivity: 175
6.3.2.1 Examination of TCR clonality: 175
6.3.2.1.1 Flow cytometric analysis 175
6.3.2.1.1.1 Defining polyclonal and oligoclonal TCR responses 175
6.3.2.1.1.2 Polyclonal TCR response to *Mycobacterium tuberculosis* (Mtb) peptides 176
6.3.2.1.1.3 HESN and HUSN predominately oligoclonal 176
6.3.2.1.2 TCR sequencing..... 177
6.3.2.1.2.1 Isolation of antigen specific T cells for TCR sequencing..... 177
6.3.2.1.2.1.1 IFN-γ capture assays lack specificity 177
6.3.2.1.2.1.2 Background TNF-α production high..... 177
6.3.2.1.2.2 Multiplex analysis of TCR clonality using fixed cells..... 178
6.3.2.2 Ontogeny of pre-existing HIV-1 specific T cell responses..... 178
6.3.2.2.1 Establishment of T cell libraries 179
6.3.2.2.2 T cell library screening 179
6.3.2.2.3 What are the precursor frequencies of HIV-1 specific T cell responses in memory and naïve subsets? 180
6.3.2.2.4 Can responses detected by cultured IFN-γ ELISpot be correlated with T cell library data? 180
6.4 DISCUSSION 181

CHAPTER 7: DO PRE-EXISTING HIV-1 SPECIFIC T CELLS PROTECT? 208

7.1 INTRODUCTION 208
7.2 CHAPTER AIMS 211
7.3 RESULTS 212
7.3.1 Study power..... 212
7.3.2 Detection Of HIV-1 Specific T Cell Responses 212
7.3.3 Magnitude of HIV-1 specific T cell responses 213
7.3.4 Maintenance of responses 213
7.3.5 Epitope mapping and $CD4^+$/$CD8^+$ specificity 213
7.3.6 Impact of HIV-1 infection 214
7.4 DISCUSSION 215

CHAPTER 8: DISCUSSION 228

8.1 ADDRESSING THE THREE MAIN LIMITATIONS OF PREVIOUS HESN STUDIES 229
8.1.1 Defining a HESN 229
8.1.2 A robust, reproducible and sensitive detection method..... 232
8.1.3 Controlled studies..... 236
8.2 DETECTION OF HIV-1 SPECIFIC T CELL RESPONSES AMONGST HESN 236
8.3 WHY WERE PRE-EXISTING HIV-1 SPECIFIC T CELL RESPONSES PREDOMINATELY MEDIATED BY $CD4^+$ T CELLS?..... 238
8.4 WHAT ROLE, IF ANY, COULD PRE-EXISTING $CD4^+$ HIV-1 SPECIFIC T CELL RESPONSES PLAY IN THE ACQUISITION OF HIV-1 INFECTION? 239

8.4.1 Direct role in protection? 240
8.4.2 Indirect role in protection? 241
8.4.3 Detrimental effect upon HIV-1 acquisition? 243
8.4.4 Merely markers of exposure? 243
8.4.5 Direct assessment of pre-existing HIV-1 specific T cells and acquisition of HIV-1 244
8.5 CORRELATES OF PROTECTION: IF NOT T CELLS THEN WHAT? 245
8.6 DETECTION OF HIV-1 SPECIFIC T CELL RESPONSES AMONGST HUSN DONORS 248
8.7 ARE ALL PRE-EXISTING HIV-1 SPECIFIC T CELL RESPONSES THE RESULT OF CROSS REACTIVITY? 251
8.8 DESIGNING THE NEXT HIV-1 VACCINE 252
8.9 CONCLUSION 253

CHAPTER 9: REFERENCES 255

APPENDIX I: TMA DATA I

APPENDIX II: CHAVI002 MAPPING DATA IIIV

APPENDIX III: DESIGN OF A NOVEL PROMISCUOUS EPITOPE POOL IVII

CHAPTER 1:

INTRODUCTION

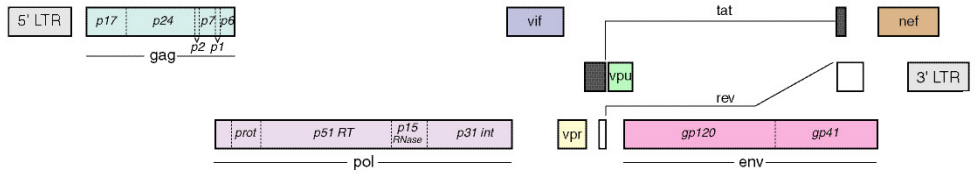
Chapter 1: Introduction

1.1 Brief History of HIV-1

In 1981 a cluster of young, previously healthy, homosexual males presented to the clinic with unusual infections and cancers such as Pneumocystis jiroveci (carinii) pneumonia and Kaposi's sarcoma[1,2]. These patients were found to have a profound loss of $CD4^+$ T cells and were rapidly described as having acquired immunodeficiency syndrome (AIDS). Subsequent documentation of cases of AIDS amongst individuals with haemophilia, blood transfusion recipients and heterosexual intravenous drug abusers and their sex partners suggested that a transmissible agent was the primary cause of the immunologic defects associated with AIDS. Just two years later in 1983, a retrovirus from the lentivirus family, human immunodeficiency virus type 1 (HIV-1), was identified as the etiologic agent of AIDS[3,4]. Nearly thirty years on HIV-1 is a global pandemic, with an estimated 33.2 million people living with HIV-1 infection and more than 8 million AIDS related deaths per year[5].

1.2 Viral heterogeneity

HIV has a complex viral genome consisting of 9 structural *(Gag Pol and Env),* regulatory (*Tat* and *Rev*) and accessory genes (*Vpu, Vpr, Vif* and *Nef*) [6-8](Figure 1.1). HIV displays considerable genetic variation, owing to the combined effects of its rapid replication rate, the error prone nature of reverse transcriptase (0.2–2 mutations per genome per cycle)[9] and high rates of recombination and mutation (Reviewed in[10-12]). On the basis of phylogenetic analysis HIV has been classified into two types referred to as HIV-1 and HIV-2 which differ by approximately 40% (reviewed in[10,11]). HIV-2 is the least pathogenic, and is believed to have been transmitted to humans around 1932 from a related simian immunodeficiency virus (SIV) in sooty mangabeys (SIVsm)[13-17]. Primarily found in West African nations (reviewed in[14,18]), the instances of HIV-2 appear to be on the decline, falling from 8.9% in 1987 to less than 4.4% in 2006[15]. By contrast, HIV-1 is the main circulating strain and is responsible for the global HIV pandemic. HIV-1 is believed to have been transmitted to humans on at least 3 separate occasions



Gag	Matrix (p17)	Important for viral assemble and targets gag to plasma membrane
	Core/capsid (p24)	Divided into two functional domains, the N-terminal domain contributes to core formation and C terminal domain is critical for capsid dimerization and virion assembly.
	Nucleocapsid (p7 and p6)	P7 is responsible for encapsulation of 2 copies of viral RNA in assembling virion. P6 binds to vpr and incorporates vpr into virion assembly
Pol (Polymerase)	Protease	Critical role in maturation of virus by cleaving the gag-pol polyprotein prior to viral assembly
	Reverse transcriptase	Catalyzes reverse transcription of viral RNA genome into double stranded DNA. Many antiretroviral drugs target this enzyme
	Integrase	Catalyzes insertion of proviral DNA into host genome by removal of 3' nucleotides from each strand of viral DNA and then by linkage to 5' end.
Env *(Envelope)*	Gp160	Gp160 is rapidly cleaved by cellular proteases to form gp120 (surface) and gp41 (transmebrane) glycoproteins. Gp120 and gp41 form highly glycosylated trimeric complexes upon surface of HIV-1 virion and mediate attachment and fusion to host cell via interactions with co-receptors CCR5/CXCR4 and CD4.
Tat *(Transcription trans-activator)*		Enhances levels of proviral RNA transcription
Rev *(regulator of expression of virion proteins)*		Binds to un-spliced viral transcripts generated in nucleas and transports them to cytoplasm where the full complement of viral proteins are translated prior to viral assembly.
Nef *(Negative factor)*		Localizes to cell membrane, down regulates CD4 and HLA class I molecules and up-regulates cellular expression of Fas ligand. Nef has also been shown to activate cellular protein kinases and thereby interfering with cell signalling.
Vpr *(Viral protein R)*		Mediates the transportation of viral nucleoprotein to host cell nucleus after viral entry and un-coating in cytoplasm. Vpr can also induce G2 cell cycle arrest and sustained expression causes T cell apoptosis. Also suggested to reactivate latent HIV-1 in cell lines
Vpu *(Viral protein U)*		Viral membrane protein that binds CD4 in the ER and targets it for proteolysis, permitting gp120 egress from ER.
Vif *(Viral infectivity factor)*		Important for production of highly infectious virions. Vif stabilizes viral nucleoprotein via interation with 5' region of genomic RNA, and can modulate reverse transcriptase through its C terminal

Figure 1.1 Schematic representation of the HIV-1 proteome (A) and accompanying table giving role of each gene (B).

around 1908 from an SIV found in chimpanzees (SIVcpz)[17,19]. Based upon high levels of genetic heterogeneity, predominately within the envelope, HIV-1 can be divided into three groups; M (main), N (non-M, non-O) and O (outlier) that can be as much as 40% divergent[10,17]. Group M accounts for the majority of HIV-1 cases worldwide and can be further categorized into nine subtypes or clades referred to as A-D, F-H, J and K. Genetic variation within a subtype can be of the order of 15-20%, whereas variation between subtypes is approximately 25-35%, depending on the subtypes and genome regions examined[10]. Despite considerable inter clade variation (up to 10%) some sequences are conserved across all clades and consensus sequences for each clade have been defined[11,20,21]. In addition to the main clades there are also 48 circulating recombinant forms (CRF), denoted by numbers (ascending in order of discovery) followed by letters of the parental subtypes[11,22]. CRFs appear to be playing an increasing role in the HIV-1 pandemic, accounting for 16% of all new HIV-1 infections between 2004-2007[11]. Each HIV-1 clade has a distinct geographical localization (Figure 1.2)[22] which appears to be relatively stable. Clade C is known to dominate in sub-Saharan Africa and accounts for nearly half (48%) of all global infections[11].

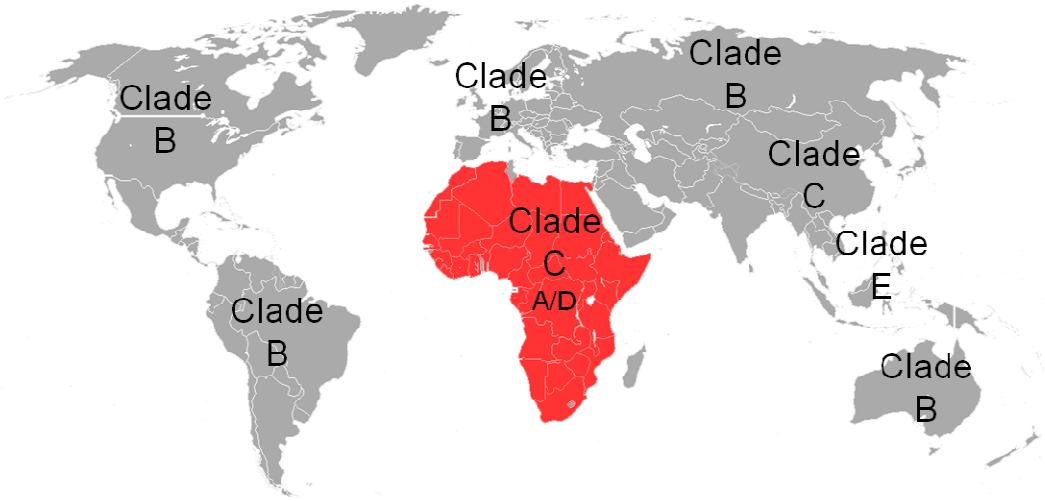


Figure 1:2

Map of the world showing the geographical distribution of the main circulating clades of HIV-1 virus. Africa harbours the highest incidence of HIV-1 and shows the greatest diversity in circulating strains. Whilst clade B, is the most common subtype in the Americas and Western Europe, clade C dominates in Africa.

1.3 HIV-1 transmission

HIV-1 infection is transmitted through contact with infected bodily fluids, most commonly through sexual intercourse. It remains unclear whether HIV-1 is transmitted as a free or cell-associated virus, evidence from *in vivo* macaque studies[12] and *in vitro* experiments using human cervical explants[23], suggest SIV/HIV-1 can be transmitted in either form. The mechanism by which HIV-1 crosses the genital mucosal epithelium is also unclear, hindered by inconsistent findings largely derived from *in vitro* culture experiments and the use of cell lines. HIV-1 virions that are initially free, or those that are released from infected donor seminal cells, interact with epithelial cells and traverse the mucosal epithelium through several pathways, including transcytosis[24], endocytosis[25] and subsequent exocytosis, or merely by penetrating through gaps in the epithelium, to make initial cell contact with underlying mucosal Langerhans cells (Reviewed in[25-28]).

The risk of infection following a single exposure to HIV-1 is estimated to be low, at around 1 infection per 1000 contacts for heterosexual transmission[29-32]. The crossing of the mucosal epithelia by HIV-1 is therefore, likely to represent a rare event. Although, infectiousness and susceptibility to HIV-1 has been shown to be highly influenced by a number of co-factors, not least, the presence of sexually transmitted infections (STIs) which disrupt the mucosal surfaces[30-34]. In addition the transmission route also affects infectivity, with penile-anal having a higher infectivity compared to penile-vaginal exposure, presumably owing to the relative fragility of the anal mucosa compared to the genital tract[34-36]. The type of sexual act and viral load of the HIV-1 infected partner have also been shown to affect infectivity[29,30,36,37]. In instances where one or more co-factors are present, infectivity can exceed 1 infection per 3 contacts for penile-anal exposure or 1 infection per 10 contacts for penile-vaginal exposure[31].

Genetic studies using single genome amplification of HIV-1 RNA in plasma or HIV-1 DNA in blood lymphocytes, suggests a single virion, referred to as the "founder virus", is responsible for around 80% of HIV-1 infections[26,38-43]. However, in the absence of a mucosal barrier to transmission, such as in the case of

injection drug users, as many as 16 transmitted virions have been found to be responsible for productive infection[44]. Infectious molecular clones derived from primary founder viruses, are almost exclusively CD4 and CCR5 T cell tropic variants[39,43]. This differs from the virus quasispecies that arise later in HIV-1 infection which have been shown to infect monocytes, macrophages and $CD4^+$ T cells with equal efficiency. The small size of the infected founder population does not imply that the transmitted virus itself is inherently easier to contain. Indeed there is evidence to suggest that founder virus is particularly resistant to fusion inhibitors, (e.g. T1249) and broadly neutralizing antibodies compared to virus isolated from chronically infected individuals[39,42,43]. The transmission of a limited number of founder viruses has been referred to as a genetic "bottle-neck"[26,39,41], providing a finite window of potential vulnerability, that could be exploited by vaccination strategies to prevent viral dissemination.

1.4 Clinical stages of HIV-1 infection

Following successful penetration of the mucosal barrier, HIV-1 enters host cells by the binding of the viral envelope glycoprotein (env) to the CD4 molecule on target cells, along with a chemokine co-receptor (either CCR5 or CXCR4)[45,46]. Almost complete resistance to HIV-1 infection can be conferred by a homozygous 32base pair deletion in CCR5 which abrogates surface expression[46-48]. The clinical course of HIV-1 is then established, and despite considerable heterogeneity in clinical prognosis, HIV-1 infection can be classified into three clinical phases (Figure 1.3), known as acute/primary, chronic and the final stage of immune collapse.

1.4.1 Acute HIV-1

Prior to the detection of viral RNA in plasma, there is a period of around 10 days, typically referred to as the eclipse phase (reviewed in[49,50]), which suggests a requirement for local, mucosal viral amplification prior to viral dissemination and lymphatic spread[26,50]. During the eclipse phase low levels of viral replication occur, primarily in "resting memory" $CD4^+$ T cells. Resting memory cells are metabolically active, express low level CCR5 but do not express classical activation markers. Once viral replication reaches a "threshold" level HIV-1 is rapidly transported into draining lymphoid tissue and ultimately gut-associated

lymphoid tissue (GALT), where a plentiful supply of activated $CD4^+$ T cells promotes near exponential viral replication and dissemination throughout the body[40,51-55]. At this time, viral load is seen to peak in the plasma at around 10^7-10^8 copies per ml[49,50,56], and the majority (50-70%) of subjects experience clinical symptoms, often described as "flu like" (reviewed in[57]).

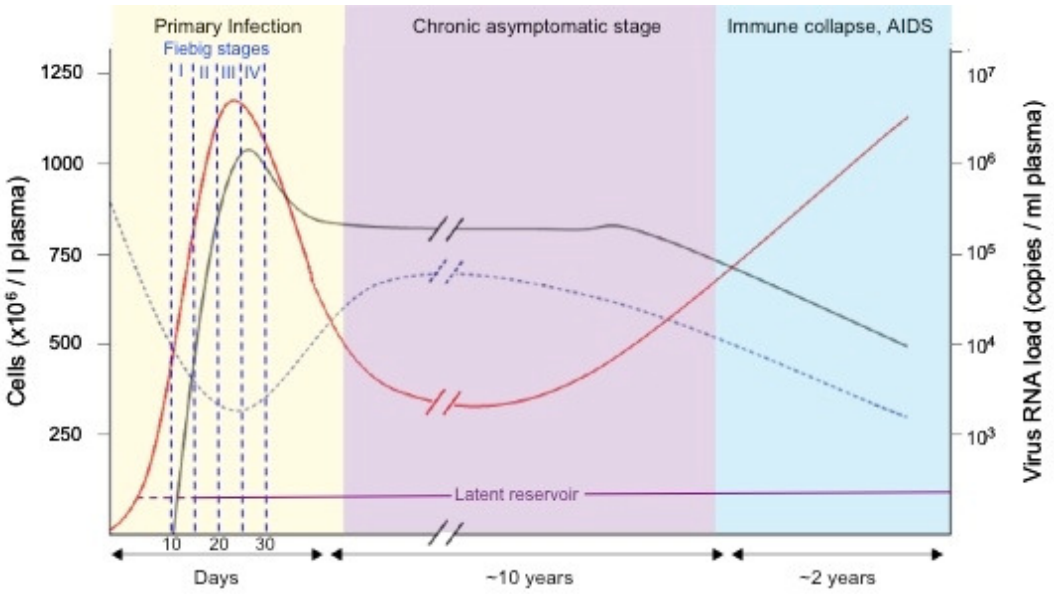


Figure 1:3

Schematic representation of the typical course of HIV-1 infection, acute/primary, chronic asymptomaptic and eventual immune collapse and progression to AIDS defining illness. Viral load is shown in red, CD8+ T cell responses in black and Absolute CD4+ T cell count in blue. The establishment of a latent viral reservoir is represented by a purple line. The earliest stages of infection are further divided into Fiebig stages I-V, depicted by vertical blue lines.

The precise mechanism by which HIV-1 is transported to the GALT remains unclear. It is most likely to be augmented by dendritic cells (DC), which "capture" and internalize HIV-1 through the interaction of DC-specific C-type lectin, DC-SIGN (CD209) and HIV-1 envelope glycoprotein gp120[58-60]. DC can then transmit HIV-1 to $CD4^+$ T cells, thus directly facilitating the spread of HIV-1[60]. Recent studies have also shown that HIV-1 envelope can interact with the gut homing receptor integrin $\alpha4\beta7$ on $CD4^+$ T cells[41,55]. Unlike CCR5 and CD4, $\alpha4\beta7$ is not required for viral entry or replication *in vitro*[55]. However, the locality of $\alpha4\beta7$

$CD4^+$ T cells at the genital mucosa does provide HIV-1 with a means by which to gain entry to the GALT, facilitating viral cell to cell transmission[54,55]. During peak HIV-1 viremia there is a profound loss of $CD4^+$ T cells within the GALT which is mirrored in the periphery (Figure 1.3). Detailed histological, and flow cytometric analysis of tissue samples obtained from the acute stages of SIV infection suggest that 24-48% of all circulating and lymph node associated, memory $CD4^+$ T cells are eliminated between days 10-14 of infection[53].

The profound depletion of $CD4^+$ T cells is mediated by both direct cytopathic effects of HIV-1 infection and extensive bystander killing and apoptosis, which result in around 80% of $CD4^+$ T cells in the GALT being depleted in the first 3 weeks of HIV-1 infection[61-64]. Detection of HIV-1 in either tissue sections or by flow cytometric analysis suggests that GALT remains a primary reservoir for HIV-1 throughout the course of HIV-1 infection, with pools of latently infected cells maintaining low level viral replication in spite of antiretroviral therapy (ART)[57,64,65].

1.4.2 Chronic infection and progression to AIDS

Following the peak in plasma viremia, viral load typically declines 10-200 fold achieving a steady state, referred to as viral set point[49,56,57,66,67]. The decline in viremia occurs in the absence of therapeutic intervention and is concomitant with the emergence of HIV-1 specific $CD8^+$ T cell responses, and partial recovery of $CD4^+$ T cell counts. Subjects generally remain within this chronic asymptomatic stage (Figure 1.3) for around 10 years, during which time viral load is kept in check by a dynamic equilibrium of virus clearance, virus production and host immune responses[38,63,68-82]. In the absence of ART sustained hyper-activation and progressive immune dysfunction in chronic infection contributes to progressive decline in $CD4^+$ T cell count and in most patients results in immune collapse and progression to AIDS[3,47,83,84]. This third phase is associated with a rise in plasma viremia and decline of $CD4^+$ lymphocyte numbers to below 200/µl blood. The combined effects of profound $CD4^+$ T cell loss and immune dysregulation leaves HIV-1 seropositive individuals susceptible to multiple co-infections such as,

pneumonia, hepatitis C virus (HCV) and tuberculosis (TB)[85-96]. It is estimated that ~25% of HIV-1 infected individuals are co-infected with HCV. HIV-1 exacerbates HCV, with progression to fibrosis occurring twice as fast in HIV-1/HCV co-infected individuals[97]. However, the leading cause of morbidity and mortality amongst HIV-1 infected individuals is TB[98-102].

1.4.2.1 HIV-1/TB co-infection

Since its discovery in 1882 by Koch, TB has continued to pose a global public health concern[103]. Current estimates suggest that one third of the world's population is infected with TB and that infection rates are continuing to rise[5]. HIV-1 is the strongest known risk factor for TB, with sub-Saharan Africa hit hardest. Caused by the respiratory pathogen *Mycobacterium tuberculosis* (Mtb), TB is transmitted through the inhalation of aerosolized droplets containing Mtb (Reviewed in[87]). For the majority of healthy, immunocompetent individuals, Mtb infection is contained, and progression to active disease averted by a highly orchestrated $CD4^+$ T cell specific and T helper-1 (Th1) dominant immune response[87]. These individuals are said to be latently infected with Mtb (LTBI) and have a 10% lifetime risk of developing active TB disease[87,104-106]. In the presence of HIV-1 co-infection, progression to active disease is over 20 times more likely (10% annual risk compared to 10% lifetime risk)[102,107,108]. Not only are HIV-1 seropositive individuals at greater risk of developing Mtb, they have higher mortality rates from active TB than HIV-1 seronegative individuals[83,87,99,107-109].

1.4.2.2 Impact of HIV-1 upon TB

Mtb is an intracellular pathogen that resides within membrane bound phagosomes in macrophages for the majority of its life cycle (Reviewed in[87]). Inaccessible to antibodies, Mtb specific T cell responses have been implicated as primary correlates of immune control[98]. The critical role of $CD4^+$ T cell mediated immune control of Mtb has been highlighted in depletion studies performed in murine and macaque models, which show an absence of $CD4^+$ T cells leads to poor control of Mtb growth in the lungs and accelerated death[110]. Further evidence to support a

role of $CD4^+$ T cells in control of TB can be gleamed from the inverse correlation between $CD4^+$ T cell counts amongst HIV-1 infected individuals and the incidence of disseminated or extra pulmonary TB[111,112].

Control of Mtb appears to be dependent upon both $CD4^+$ T cells and a number of cytokines in particular interferon gamma (IFN-γ)[109,111,113-115]. Individuals with mutations in the gene encoding IFN-γ receptor, have severe and/or recurrent TB disease[116,117]. HIV-1 seropositive individuals also display a diminished ability to secrete IFN-γ upon stimulation with Mtb antigens in *ex vivo* enzyme linked immunospot (ELISpot) assays[114,118-120]. Indeed, unlike most opportunistic infections, which do not arise until profound depletion of $CD4^+$ T cells has occurred, TB can present at any stage of HIV-1 infection (reviewed in[107]). Tentative evidence to explain why HIV-1 infected individuals are so susceptible to active TB has come from a limited number of studies that suggest HIV-1 preferentially infects Mtb specific T cells[121,122]. It is likely the combined effects of a loss of $CD4^+$ T cells and dysregulation of the remaining $CD4^+$ T cells accounts for the increased susceptibility to active TB. To date it remains unclear whether the increased risk of active TB in HIV-1 infected individuals is due to reactivation of LTBI or *de novo* infection. Detailed genetic studies comparing the genomic characteristics of latent Mtb with bacteria detected during active disease would be required to fully address this.

1.4.2.2.1 Impact of TB upon HIV-1 infection

Not only does HIV-1 infection increase the likelihood of developing active TB infection, but TB infection accelerates the course HIV-1 infection[92,102,107,123-126]. The development of TB is associated with increased HIV-1 replication, which is detectable not only in plasma viral load but also at the site of TB infection (the lungs)[87,92,102,124,126]. It remains unclear whether the enhanced viral replication associated with TB co-infection is due to a specific effect of TB upon HIV-1 or whether it is a result of the pro-inflammatory response that accompanies active TB. *In vitro* stimulation with Mtb antigens dramatically increases viral production by $CD4^+$ T cells from HIV-1 infected individuals, an increase that is dependent on the

prior sensitization of the donor to Mtb[102]. Furthermore there is *in vitro* evidence to show Mtb activates HIV-1 transcription and enhances viral entry by up-regulating the HIV-1 co-receptor CXCR4[124].

1.5 Clinical diagnosis of HIV-1 infection

Conventional screening for HIV-1 infection relies upon the detection of IgG antibodies within the plasma, typically by enzyme linked immunosorbant assay and confirmed using Western Blot. Patients do not typically return a positive Western Blot result until 3-4 weeks post HIV-1 transmission by which time viral load is generally on the decline. In the absence of prior knowledge of an individual's risk of exposure to HIV-1 the symptoms associated with acute HIV-1 infection are often too vague or non-specific to lead to diagnosis. Risk of HIV-1 transmission has been shown to be greatest during acute HIV-1, presumably owing to the extremely high viral loads and relative homogeneity of HIV-1. Evidence from Macaque studies has shown plasma from acutely infected animals is 750 times as infectious, on a per virion basis, as plasma from animals with chronic infection[127]. Detection of acute HIV-1 infection can be achieved using assays for HIV-1 RNA, p24 antigen, and third generation enzyme immunoassay antibody tests capable of detecting IgM antibodies. However, owing to a lack of clearly discernable symptoms it is often difficult to capture subjects prior to seroconversion. Given the increased transmission risk associated with acute HIV-1 infection, the ultimate goal of diagnostic strategies is therefore to identify HIV-1 at the earliest possible time point.

1.6 Heterogeneity in HIV-1 infection

Whilst the course of HIV-1 infection can be classified into three clinical phases (Figure 1.3) marked heterogeneity exists in an individual's clinical prognosis following HIV-1 exposure (reviewed[49,56]). Peak viral load and depletion of $CD4^+$ T cells can vary by 1000 fold between subjects. Moreover, whilst some individuals rapidly progress to AIDS defining illness around 2-5% of all HIV-1 infected individuals maintain near undetectable viral loads and normal $CD4^+$ T cell counts in the absence of ART for >10years and are referred to as long term non-progressors (LTNP)[76,78,79,128-133]. There is also heterogeneity in individual's risk of

acquiring HIV-1[47,134-141]. Whilst, the risk of HIV-1 acquisition varies according the route of transmission and presence of co-factors (Section 1.3), there are rare individuals known to have been highly exposed to HIV-1 who remain concordantly seronegative[47,134-136,139,142-147]. Referred to as HIV-1 exposed seronegative (HESN), these "high risk" individuals can be found in a number of cohorts, including; sero-discordant couples[136,147,148], commercial sex workers from areas with high prevalence of HIV-1[135,143,149-153], men who have sex with men (MSM)[136,137,154-157], infants born to HIV-1 infected mothers[158-162] and health care workers exposed to contaminated blood products[138,163,164]. Arguably the best evidence to support the existence of HESN comes from the study of haemophiliacs who received HIV-1 infected blood clotting factor concentrates prior to wide scale HIV-1 screening[165-167]. These individuals were subjected to high dose, intravenous infusion of HIV-1 contaminated blood products, whilst the majority seroconverted, a minority remained seronegative. It is hoped that the study of these rare individuals will provide insight into the immune correlates of protection and ultimately aid in the design of a novel therapeutic vaccine.

1.7 Immune responses as correlates of protection

1.7.1 Innate immunity

1.7.1.1 Soluble factors

Proteomics based approaches have shown the first signs of HIV-1 infection to be the detection of acute phase proteins such as serum amyloid A and alpha1-antitrypsin[168]. Detectable, in the plasma just 3-5days post transmission, acute phase proteins precede systemic detection of viremia and detectable increases in plasma cytokines[102,168-173]. As viral load peaks there is a sequential increase in systemic levels of multiple cytokines and chemokines which is often referred to as a "cytokine storm"[172,174]. The study of acute plasma donor samples obtained at pre- and post seroconversion time points has shown the earliest systemic cytokine elevations include, rapid and transient increases in interferon alpha (IFNα) and interleukin (IL)-15, a rapid but more sustained increase in TNFα, and a slightly slower but also more sustained increase in IL-18, accompanied by elevations in multiple other pro-inflammatory cytokines/chemokines, and a late-peaking increase

in IL-10[172]. The precise cellular sources of acute-phase cytokine production and the role of DC in initiating the acute-phase cytokine storm have not been fully determined. *In vitro* studies have shown IFNα to interfere with viral growth, acting early, possibly during the initiation or the nucleic acid intermediates of reverse transcription[175]. In addition, IFNα potentiates the development and maintenance of T cell responses by activating STAT 4 transcription factor and preventing activated T cells from undergoing apoptosis[176-180]. Although positive effects of cytokines detected during the acute cytokine storm have been described it is likely that the cytokine storm also contributes to harmful immune activation and loss of $CD4^+$ T cells.

1.7.1.2 Intrinsic cellular factors

In order to complete their lifecycle and generate virus progeny, retroviruses such as HIV-1 have to hijack the host cells machinery. Under the selective pressure of numerous pathogenic infections, a variety of restriction factors have evolved to block retroviral replication[181-187]. Three of these factors have been associated with control of HIV-1 infection, including APOBEC (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like), tripartite motif-containing protein 5α (TRIM5α), and tetherin (also known as BST-2 or CD317) [181-187]. Each factor acts at a different stage in HIV-1 infection, with TRIM5α and APOBEC negatively affecting viral replication[181-186] and tetherin impairing release of mature HIV-1 particles from infected cells, and preventing cell to cell transmission of HIV-1[187]. APOBEC3G is one of the best studied intrinsic factors. It is packaged within viral particles which, upon entry to a new cell, induce excessive D to A hyper mutations resulting in instability of the nascent viral transcripts or lethal mutation[181-184]. A recent *in vitro* study showed that the pool of defective viruses generated by APOBEC3G represents a source of viral antigens that can induce potent $CD8^+$ T cell responses, showing the innate and adaptive immune systems working together to clear the virus[184].

Expression of APOBEC3G has also been linked to HIV-1 exposure, with APOBEC3G mRNA shown to be at higher levels in 27 HESN recruited from a

serodiscordent couples cohort, compared to 26 age matched control individuals[181,183]. *In vitro* experiments have shown PBMC susceptibility to infection with the HIV-1(Ba-L) R5 strain is reduced by high mRNA[181] expression of APOBEC3G. However, the role of APOBEC3G in conferring protection against establishment of HIV-1 is questionable, since viral infectivity factor (Vif) protein is known to trigger degradation of APOBEC3G and counteracts its antiviral effect[182].

1.7.1.3 Cellular immune response

1.7.1.3.1 NK cells

NK cells were originally characterized by their ability to spontaneously kill tumour and virus-infected cells. They usually become activated in the acute stages of viral infection and assist in T cell polarization and DC maturation through the secretion of immunomodulatory and antiviral cytokines[54,142,188-196]. The functional activities of NK cells are regulated by a fine balance between the engagement of inhibitory and activating receptors such as NK-cell immunoglobulin like (KIR) receptors[39,142,188,191-193,196].

NK cells are activated in acute HIV-1 infection and, *in vitro* have been shown to lyse HIV-1 infected target cells[195]. However, the precise mechanisms behind this activation remain unknown. The role of NK cells in infection appears to be a delicate balancing act between ligand interactions with inhibitory and activating KIR receptors[142,188,191-193,197]. KIR receptors are known to interact with HLA alleles and enhanced NK cell activity has been associated with protective HLA alleles[142,188,191-193,196,197]. The potential role of NK cells in control of HIV-1 was recently highlighted in a genome wide association study which identified a 35kb single nucleotide polymorphism upstream of HLAC as being a major determinant in viral load set point[198]. Notably, HLA-C allotypes dominate over HLA-A and HLA-B as a source of ligands for KIR, and preferentially interact with inhibitory KIR receptors[196,199].

NK cells have also been implicated to play a role in protection against the establishment of HIV-1, with a number of HESN cohorts showing increased NK

activity, both in terms of cell lysis and cytokine production[142,189-191,200]. Furthermore, HESN cohorts appear to be enriched for individuals homozygous for the activating NK receptor KIR3DS1[142].

1.7.1.3.2 Dendritic cells

Resident DCs are often the first cell type encountered by HIV-1 and are responsible for the secretion of many of the early cytokines[201]. DCs constitute a heterogeneous population of cells, present in minute numbers, in various tissues, just beneath the dermis or mucosal layer. They form a first-line defence against viruses and other pathogens and are often considered "sentinels" of the immune system (Reviewed in[27]). HIV-1 is "captured" by the DC-specific C-type lectin, DC-SIGN, which binds to the HIV-1 envelope glycoprotein gp120[58,60]. *In vitro* experiments demonstrate that "captured" HIV-1 particles can be efficiently processed and presented upon either HLA class II or class I molecules[58-60,202]. In addition to processing and presenting HIV-1 antigens to T lymphocytes, DCs have been shown to be involved in the transmission of HIV-1 and *in vitro* experiments have shown blocking DC SIGN reduces viral replication[60,203]. It is hypothesized that DCs facilitate viral dissemination by transporting captured HIV-1 virions to secondary lymphoid organs, where there are a high number of target cells[58,60].

DCs are rapidly depleted during HIV-1 infection and have been shown to be dysfunctional during chronic HIV-1 infection, exhibiting impaired antigen presentation and cytokine responses[204,205]. However, DC number can be restored by ART where they serve as $CD4^+$ T cell count and viral load independent indicators of disease progression[205]. Indeed, macaque data suggests a loss of blood myeloid DCs at virus set-point predicts disease progression, whereas an increase predicts long term absence of disease, independent of viral load or $CD4^+$ T cell counts[204].

Despite a robust innate immune response HIV-1 does not undergo sequence mutation during the earliest stages of HIV-1 infection. This suggests any effects innate immune responses have are insufficient to dive viral escape mutation.

1.7.2 Adaptive immunity

1.7.2.1 Humoral response

Humoral responses encompass a family of B cell secreted immunoglobulins (Ig) antibodies that show differential localization and function. Neutralizing antibody (nAb) responses after both natural infection and vaccination are an important component of protection from many viral infections. The initial antibody response to HIV-1 is primarily directed at non-neutralizing epitopes on the envelope glycoprotein and is detectable 6-12 weeks post infection. Whereas neutralizing antibody responses are typically delayed occurring several months following the transmission event[206] (reviewed in[207-209]). This antibody response has been described as ineffective, because autologous nAbs often lag behind genetic changes in the virus, rarely target the contemporaneous virus and are generally not associated with control of viremia[206,207,210-215]. However, there is evidence of Ab driven viral escape mutation and an inverse correlation between detection of nAbs and viral load exists[207-209,213,216]. nAbs fall into 2 categories: antibodies that bind to variable regions of HIV-1 and that neutralize a relatively narrow group of isolates and those that bind to conserved regions of the virus and that can neutralize a wider range of isolates, including those in different subtypes or from diverse geographical regions. The majority of nAb responses fall into the first category, with just 20% of patients developing antibodies with some degree of neutralization to heterologous virus strains, years after infection (reviewed in[49,209]). Part of the reason why nAb are ineffective is a result of the intrinsic properties of HIV-1 env which not only displays extraordinary genetic diversity, but also obscures the conserved regions such as the CCR5/CD4 binding site through heavy glycosylation and the interactions of externally orientated variable loops (V1, V2, V3 and V4)[39,207,212] .

In addition to the complications involved in nAb targeting of HIV-1 env, B cells rapidly decline in number during acute HIV-1 infection and their activity has been shown to be dysregulated[63,217-219]. In addition to impaired proliferative capacity (evidenced by Ki67 staining), and enhanced activation status ($CD86^+$, $CD38^+$), B cells show a loss of CD21 expression, which is believed to be indicative of exhaustion[217,219].

A number of vaccine strategies have attempted to illicit antibody responses with the hope that these may confer protection against the establishment of HIV-1[208,220-223]. However the majority have failed to confer protection[211,220-222]. The recent Rv144 vaccine trial, used a recombinant canary pox priming immunization followed by an envelope protein boosting immunization and was shown to generate modest (31% vaccine efficacy) protection against the acquisition of HIV-1 infection[209,224]. Since this vaccine regimen elicited non-neutralizing antibody and $CD4^+$ T cell responses, attention is turning to the possibility that non-neutralizing antibodies may have mediated the protection[208,209]. Attempts to recapitulate these results in primate studies have generated mixed reports. A plasmid DNA prime/recombinant adenovirus serotype 5 boost vaccine regime was shown to be capable of conferring protection, in the form of a 1 log reduction in viral load, against subsequent SIVsmE660 challenge[186]. This protection was associated with low levels of neutralizing antibodies and envelope-specific $CD4^+$ T cell responses[186]. However, a study by Burton *et al*, showed that passive transfer or vaginal application of non-neutralizing monoclonal antibodies directed against the CD4 binding site were unable to confer protection against the establishment of (SHIV)-162P4 to macaques, with some evidence to suggest they may actually enhance infection[215]. In contrast application of strongly nAb b12 vaginally provided sterilizing immunity in seven of seven animals[215]. Further research is required to fully understand the correlates of immune protection achieved by the Rv144 trial.

1.7.2.2 T cell response

T cells can be broadly classed into two subsets, those expressing the CD4 co-receptor and those expressing CD8. Both classes of T cell are believed to play critical roles in the host immune response to infection. Initiation of a T cell response begins with the recognition of processed and presented antigen by human leukocyte antigen molecules (HLA) to the T cell receptor (TCR).

1.7.2.2.1 Antigen processing and presentation

1.7.2.2.1.1 HLA molecules

The seminal work of Zinkernegel and Doherty in 1974 identified HLA as a key factor in restricting antigen specific T cell responses[225,226]. In a series of elegant experiments $CD8^+$ T cells were shown to only recognize and lyse chromium labelled, Lymphocytic Choriomeningitis Virus (LCMV) infected, target cells (fibroblasts/macrophages) if they shared one or more HLA class I alleles[225,226].

Encoded on the short arm of chromosome 6, the HLA locus is highly polymorphic, with over 5300 allelic variants mapped[227]. The result of this extensive variability is that a vast array of peptides can be accommodated and presented to the immune system. HLA can be broadly classified into two groups denoted as HLA Class I and HLA Class II. HLA Class I molecules are present on the surface of all nucleated cells, can act as ligands to KIR receptors and typically present antigenic peptides processed by the endogenous pathway, to $CD8^+$ T cells[199,228-233]. By contrast HLA Class II molecules are expressed by specialised antigen presenting cells (APC), such as DC/macrophages and present exogenous antigen to $CD4^+$ T cells[234-239]. Both classes of HLA are heterodimeric, membrane bound glycoproteins that bind peptide in a small pocket referred to as the binding groove. The structural properties of the binding groove denote peptide specificity and can be used to distinguish between HLA class I and class II molecules. Peptide binding to the HLA occurs within 6 specialized binding pockets denoted as A-F. The binding pockets of B and F, which accommodate side chains from amino acids in position 2 and 9 are far less variable and are typically referred to as dominant, anchor residues[240,241]. Amino acids in these positions are critical for HLA-peptide binding.

1.7.2.2.1.2 HLA class I processing and presentation

HLA class I molecules can be further differentiated into three classical class I molecules (HLA-A, -B and –C) and three conserved, non-classical molecules (HLA-E, -F and –G). All of these glycoproteins share a common invariant β_2 microglobulin chain and a highly polymorphic heavy chain. The binding groove is comprised of a "floor" of eight antiparallel beta sheets and a wall of two alpha helices (α1 and α2). A third alpha chain (α3) interacts with the T cell co-receptor CD8 and is far more conserved than α1 and α2 (reviewed in[242,243]). Importantly the binding groove is closed at each end, and therefore is restricted to the binding of

short peptide fragments, typically between 8-11 amino acids long. Most peptides that bind HLA class I molecules are derived from the endogenous presentation pathway. This pathway begins with proteolytic degradation of intracellular proteins within the proteosome, prior to transportation to the endoplasmic reticulum (ER) by transporter associated with antigen-processing (TAP) proteins. Peptides are then further "trimmed" to between 8-10 amino acids in length by an ER amino peptidase. In order for successful peptide loading, partially folded class I molecules are transported to the lumen of the ER where they associate with the chaperone protein calnexin. The beta2-microglobulin associates with the HLA class I molecule displacing calnexin and replacing it with a complex of chaperone proteins, calreticulin and Erp57. The unloaded HLA class I molecule then associates with TAP proteins, overseen by the adaptor protein tapesin, completes the folding process, and peptide is loaded into the binding groove. The stabilized peptide-HLA (pHLA) complex is then transported to the cell surface via the Golgi apparatus (reviewed in[242]) were it awaits recognition by TCR of $CD8^+$ T cells.

In addition to presentation of peptides derived from endogenous presentation, HLA class I molecules can also present peptides derived from exogenous processing (See Section 1.7.3.2.3). The precise mechanisms behind cross-presentation are not fully understood, although there is evidence for and against a cytosolic step (reviewed in[202]).

The half-life of pHLA complexes upon the surface of DC has been estimated to be short (~10hours). Therefore in order to achieve successful presentation there must be sustained and continuous synthesis of HLA class I molecules and loading of antigenic peptide (Reviewed in[244]).

1.7.2.2.2 HLA class II processing and presentation

HLA class II molecules can be differentiated into HLADR, -DP and –DQ. Unlike HLA class I molecules, the peptide binding groove is open at both ends allowing longer (15-20 amino acid) peptides to bind in multiple registers[238,245,246]. The lack of rigid allele-specific binding motifs permits broader binding specificity and has

been used to explain the higher frequency with which promiscuous and cross reactive $CD4^+$ T cell responses are detected compared to $CD8^+$ T cell responses[247,248]. HLA class II molecules present peptides derived from exogenous antigens.

HLA class II α and β chains assemble with a third non-HLA encoded glycoprotein chain, the invariant chain, early during biosynthesis in the ER. The invariant chain enhances localization of class II molecules in endosomes/lysosomal compartments, the site of peptide acquisition, where it is eventually degraded by endosomal proteases, leaving a small remnant, termed CLIP, within the antigen-binding groove of class II. Also in endosomal compartments, peptide fragments of either foreign or self-antigens are generated from HLA proteolysis and become available for HLA class II binding. In endosomes, CLIP is replaced by antigenic peptides, a reaction that is catalysed by an HLA-encoded protein "DM". DM, is a class II-like heterodimeric molecule that binds to class II molecules in the low pH of the endosomes and promotes both peptide release and peptide binding. In acidic endosomes, DM and class II interactions are initiated, leading to catalytic release of CLIP from class II molecules and subsequent loading of antigenic peptide. Current models for DM suggest that binding to class II molecules stabilizes an "open" conformation of the peptide-binding pocket of class II that allows the exchange reaction to occur rapidly. Antigen presented upon HLA class II molecules has an extremely long half-life, estimated to be around 100hours. This mechanism allows DCs to retain pHLA complexes for several days post encounter with antigen, ensuring maximal time for presentation to cognate $CD4^+$ T cells (Reviewed in[249]).

1.7.2.2.3 HLA associations with HIV-1 disease progression

A number of studies have shown that specific HLA class I alleles are associated with the rate of HIV-1 disease progression. In particular HLA alleles HLA-B*5701, HLA-B*5703, HLA-B*5801, HLA-B27 and HLA-B51 have all been associated with good control of the virus and a slower progression to AIDS[188,236,250-252]. The reason for this apparent role in control is believed to be partly due to the epitopes presented by these alleles which are typically focused

toward conserved regions of the viral gag protein[70,74,77,250]. In a recent genome-wide association study, the strong protective influence of a single nucleotide polymorphism (SNP) located 35kilobases upstream of the HLA-C locus confirmed the association of HLA-B57 with a low viral set point[198]. The role of HLA-C in conferring protection is not well understood, however, a number of groups have demonstrated that surface expression levels of HLA-C are a key component in the control of HIV-1, with higher surface expression associating with slower disease progression (reviewed in[199]). As well as a role in control of viremia, expression of particular HLA class I alleles, such as HLA-B35, have been associated with rapid disease progression[252]. The mechanism behind this negative association remains unknown.

There is limited evidence to suggest a role in control for HLA class II alleles in control of HIV-1 viremia[129,133,236,252]. Recent studies have suggested *DRB1*13* alleles correlate with reduced viral load, non-progressive HIV-1 infection, and potent, polyfunctional $CD4^+$ T cell responses at the rectal mucosa[133,236]. Interestingly, DRB1*13 has also been associated with the viral clearance and protection from vertical transmission of Hepatitis C and B[253].

1.7.2.3 The TCR

The recognition of processed and presented antigenic peptides by T cells is mediated by the T cell receptor (TCR). The TCR is a heterodimeric, membrane integral protein, consisting of 2 polypeptide chains (typically α and β although around 10-15% are γδ)[227,254,255]. For the α and γ locus, random splicing and recombination occurs between variable (V) and joining (J) segments. For the δ locus and the β locus, there is recombination between V and J segments, but also the inclusion of one of two short diversity (D) segments. Antigen specificity is conferred by the three hyper variable complementarity-determining regions (CDRs) encoded within the V gene segments of both α and β chains (*TRAV* and *TRBV* respectively). Whilst CDR1 and 2 regions are germ line-encoded, the CDR3 region is only generated after V(D)J recombination, and therefore contains substantial non-germ line variability (Reviewed in[227]). Further complexity is

generated through the deletion of germ line-encoded bases and the addition of random non-templated bases. The resulting hypervariable sequences of the CDR3 enables recognition of a diverse array of peptide-HLA (pHLA) complexes. It should be noted that V(D)J recombination is not entirely random, and the prevalence of specific gene segments and combinations of gene segments shows marked variation in the repertoire[227,255]. Contributions to this bias are introduced even before thymic selection, through variation in the efficiency of recombination of different gene segments. The random biases introduced during V(D)J recombination may in part explain the phenomena of public TCR clonotypes, that is distinct TCR clonotypes that are shared amongst multiple individuals[249,255-260].

The available pool, for the human αβ TCR consists of 42 V and 61 J segments in the α locus and 37V, 2 D and 13 J segments in the β locus (Reviewed in[233]). The V(D)J recombination system combined with the random addition of non-template nucleotides by terminal deoxynucleotidyl transferase and random α/β chain pairing, enables a theoretical 10^{15}-10^{20} unique TCR to be generated[233,245,261-263]. However, due to physical constraints of the human lymphatic system the full recombination potential of the TCR repertoire cannot be realised[262]. Thymic selection (Section 1.7.2.4), is believed to reduce the peripheral TCR repertoire to around $2.5x10^7$ unique TCR through the elimination of autoreactive T cells[262-264]. Total TCR diversity has been estimated using gene amplification and sequencing of the TCR-CDR3 regions, which show circulating naïve T cells contain more than 10^6 different β chains, each pairing with at least 25 different α chains[265].

1.7.2.4 Thymic selection and development of T cells

Very little is known about the earliest progenitor cells of T lymphocytes. It is thought that very few cells from the bone marrow derived progenitor cells go on to populate the thymus and therefore experiments aimed at providing detailed classification of these cells are technically very difficult and the majority of research in this field has been conducted in murine models[266,267]. Upon arrival in the thymus cells undergo an ordered sequence of lineage exclusions mediated by

notch signalling before becoming fully committed to the T cell lymphocyte lineage by a stage termed double negative 3 (DN3). The DN3 stage is marked by VDJ recombination within the β chain, which associates with a surrogate α chain and is expressed as a "pre-TCR" on the cell surface[261]. This stage represents the initial TCR checkpoint, with cells that successfully complete β-chain rearrangement surviving and migrating to the subcapsular zone where they undergo rapid proliferation prior to returning to the cortex and differentiating into double positive (DP) cells, whilst those that fail die. DP cells then undergo sequential rearrangement of the V and J regions alpha chain, until a TCR α chain is formed that can associate with the ready-formed β chain to complete TCR development. At this stage DP cells undergo the second TCR checkpoint, known as positive selection. Of the DP cells that produce a TCR, only those with TCR capable of low affinity binding to a self/pHLA on thymic epithelial cells survive positive selection[267-269]. Any cell that does not express low affinity self/pHLA TCR dies through neglect. Negative selection, and subsequent activation induced apoptosis occurs should the DP cells have a high-affinity interaction with self/pHLA[267-269]. If cells possessing high affinity self/pHLA TCR were not eliminated, they could differentiate into autoreactive lymphocytes, leading to the development of peripheral autoimmune disease[268,269]. All positively selected cells transition to become single positive $CD4^+$ or $CD8^+$ T cells and migrate within the medulla where they are further screened for high-affinity self/pHLA interactions. During this time tissue specific self-antigens are expressed by thymic epithelial cells under the regulation of AIRE transcription, and cross presented by medullary DC[269]. During the final stages of T cell development thymocytes migrate frequently and rapidly to make contact with DC, they are particularly susceptible to pro-apoptotic stimuli and may be subjected to negative selection following TCR encounter with high affinity self-pHLA[267]. Should the thymocyte survive this final screening, they undergo terminal differentiation to become functionally competent and relatively refractory to apoptosis[267]. The final stage in development is exit the thymus, to populate peripheral lymphoid organs.

1.7.2.4.1 $CD4^+/CD8^+$ lineage commitment

Lineage commitment to single $CD4^+$ or $CD8^+$ T cells is mediated by the ability of the developing thymocytes TCR to recognize antigen in the context of HLA Class II or class I molecules respectively. The precise mechanisms behind what determines a cell's fate as $CD4^+$ or $CD8^+$ remains incompletely understood. A number of hypotheses have been generated, predominately based on genetic manipulation in mouse models with a number of signalling molecules implicated[267,270,271]. For $CD8^+$ T cell differentiation a short TCR interaction and abundance of the tyrosine kinase zeta chain-associated protein kinase of 70 kD (Zap70) was recently suggested to be required[272]. For $CD4^+$ T cell differentiation, ThPOK (T-helper inducing POZ-Kruppel factor; also known as Zbtb7b and cKrox) was identified as a master regulator following identification of a mutation in ThPOK as the causative factor in the helper deficient (HD) mouse model[273]. Downstream GATA3 has also been implicated and knock in GATA-3-GFP mouse models show transcription levels to be elevated during $CD4^+$ T cell development, forming a positive feedback loop that ensures commitment to the $CD4^+$ lineage during differentiation[274]. In addition to transcription factors the lck transduction molecule has also been implicated[270]. Transgenic mouse studies have shown that thymocytes carrying a class II-restricted TCR develop into functional CD8 T cells when Lck activity is reduced.

1.7.2.5 Homeostasis of naïve T cells

BrdU labelling studies in mice have shown that release of newly formed naive T cells from the un-manipulated thymus is very low and that naïve T cells can persist for prolonged periods in thymectomized mice[275]. It is considered that the requirement of positively selected T cells to have weak TCR interaction with self-peptide is critical to survival of naïve T cells. Indeed ablation of TCR or removal of major histocompatibility complex (MHC- the murine equivalent of HLA) class I causes $CD8^+$ T cells rapidly die[276]. This is less clear cut for $CD4^+$ T cells with contradictory studies showing $CD4^+$ T cells rapidly die in MHC class II deficient hosts, whilst others suggest $CD4^+$ T cells can persist for several months[249,277]. So called "tonic TCR signalling" alone is insufficient to maintain T cells and there is a

requirement of interaction with common γ chain cytokines, in particular IL-7[278,279]. IL-7 knock out studies performed in mice have shown that naïve $CD4^+$/$CD8^+$ die within 1-2 weeks[278]. A recent study using adoptive transfer of naïve $CD8^+$ T cells into lymphopenic mice and exogenous addition of IL-7, suggests that IL-7 not only maintains naïve T cell homeostasis but that it is also impacts upon the diversity of naïve T cells through differential expression of the IL-7 receptor upon naïve T cells[279].

1.7.2.6 T cell activation

1.7.2.6.1 Requirement for co-receptors

Interaction between pHLA and TCR is insufficient to fully activate T cells and there is a requirement for a second signal in the form of co-stimulatory molecules (Figure 1.4)[280-283]. Co-stimulatory signals exert their function by augmenting production of IL-2, promoting cell cycle progression, inducing effector cytokine production, suppressing cell death and enhancing memory T cell development (Reviewed in[282,284]). In the absence of co-stimulatory molecules T cells encountering antigen either fail to respond and die by apoptosis or become anergic[281]. The best characterized co-stimulatory molecule is CD28 which is responsible for sustained T cell activation. CD28 is constitutively expressed upon T cells[285] and interacts with its ligand either B7-1 (CD80) or B7-2 (CD86) which are up-regulated upon APC during activation[280]. The importance of CD28 mediated signals was first emphasized by the immunodeficiency observed in CD28-deficient mice[286]. These mice are compromised in an array of immune functions, including deficient germinal centre formation and immunoglobulin isotype class switching, reduced delayed hypersensitivity responses, and reduced helper cell differentiation. $CD8^+$ T cell responses are also impaired, although not completely abrogated[286]. In addition to providing stimulatory signals, co-stimulatory molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4 or CD152), have been shown to play an inhibitory role, and are important in the regulation of T cell tolerance and autoimmunity (reviewed in[281]).

1.7.2.6.2 Peptide-HLA-TCR interaction

The interaction of peptide loaded HLA (pHLA) and TCR occurs at a site known as the immunological synapse (IS; reviewed in[287]). Through the localization of TCR to lipid raft structures, the IS permits TCR to come into close contact with pHLA and engage in serial signalling, thereby potentiating signal transduction[288]. However, it remains controversial as to whether IS development is cause or consequence of TCR signalling, with some experimental evidence to suggest TCR signalling occurs outside the central "cluster" of TCR and signalling molecules created by the IS [287].

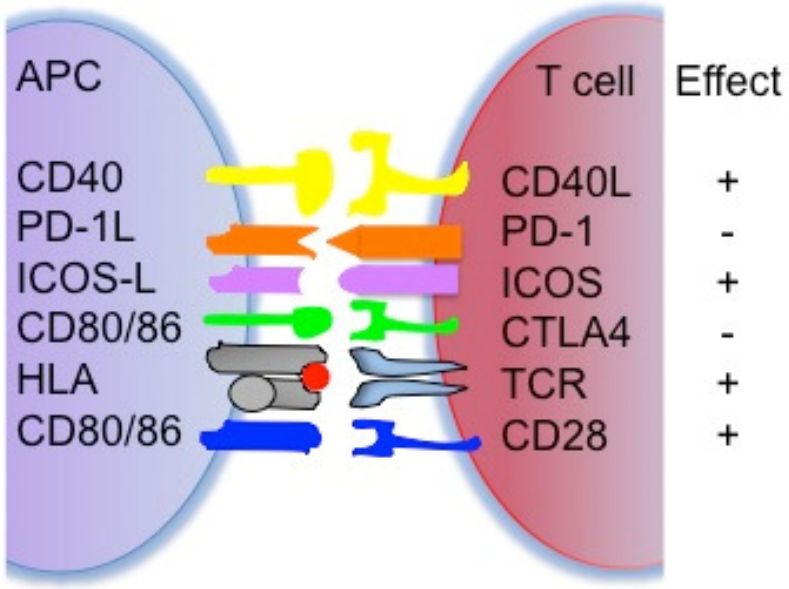


Figure 1.4

Schematic representation depicting the co-stimulatory receptors involved in T cell activation and their ligands. Notably, co-stimulatory molecules may serve to either potentiate or diminish peptideHLA-TCR signalling and the effect of each receptor ligand interaction is shown on the right hand side of this diagram.

Quantified using TCR down regulation following *in vitro* stimulation of T cell clones with bacterial superantigen, HLA and anti-CD3 antibodies, $CD4^+$ T cells have been shown to require the engagement of ~8000 T cell receptor (TCR) prior to activation. Although this number can be reduced to ~1500 in the presence of co-stimulatory molecules[282]. In contrast to $CD8^+$ T cells, $CD4^+$ T cells have a

requirement for prolonged TCR contact with the peptide-HLA complex[289]. During the sustained interaction between peptide-HLA and TCR serial signalling can occur, whereby a single peptide-HLA complex can trigger ~200TCR[290]. Using quantitative immunoprecipitation of radiolabelled APC with immunogenic hen egg-white lysozyme protein, the number of peptide-HLA complexes capable of stimulating T cell hybridomas was quantified[291]. Together with the use of anti-CD3 antibodies bound to latex beads, Harding *et al* demonstrated how serial triggering enables the sensitive detection of low numbers (200-300) of peptide-HLA complexes on the cell surface[291,292]. $CD8^+$ T cells require only brief TCR interaction, and have been shown to degranulate at extremely low antigen concentrations (10^{-12}-10^{-15}M) with seemingly incomplete activation, whilst induction of Ca^{2+} flux and cytokine secretion requires much higher concentrations of antigen (10^{-9}M)[292].

1.7.2.6.3 Successful T cell activation and subsequent signalling cascade

Following successful engagement of TCR with cognate antigen and in the presence of co-stimulatory molecules T cells become activated. Signalling is sustained for the duration of TCR contact with pHLA and ceases immediately following withdrawal of antigen[293]. The TCR does not have intrinsic enzymatic activity however, the CD3 complex (γ,δ and ε chain) and TCRζ chain dimers contain cytosolic components with a unique motif, known as immunoreceptor tyrosine based activation motifs (ITAM) that becomes phosphorylated during T cell activation by protein tyrosine kinase, Lck and initiates a cascade of signalling molecules to result in T cell activation (reviewed in[270,294]). Both the quality and duration of TCR interaction has been shown to affect the subsequent immune response[244,282,289].

1.7.2.6.4 Immune activation and HIV-1

Chronic cellular immune activation is increasingly being recognized as a critical factor in an individual's susceptibility to HIV-1 infection[144,200,295,296]. During productive HIV-1 infection the measurements of viral load alone does not

accurately predict disease progression. Analysis of immune activation status has proved to be a more reliable means by which to predict disease prognosis independent of $CD4^+$ T cell count and viral load[297-299]. Two activation markers in particular, HLADR and CD38, have been extensively studied. HLADR is constitutively expressed upon antigen presenting cells. Although not typically expressed on T cells, a proportion of activated T cells become $HLADR^+$ during an immune response[200,295,300,301]. The reason or consequence of HLADR expression upon T cells remains unknown. By comparison CD38 is constitutively expressed by naïve T cells and is rapidly up regulated on activated T cells[200,301-306]. Physiologically, CD38 expression and its ligation have been associated with increased levels of cytokine production and $CD4^+$ T cell proliferation[304]. In addition to the well characterized correlation of CD38/HALDR expression with disease prognosis[297,298], a recent study of 466 subjects all within the first 225 days post seroconversion, suggested the expression of Ki67, a surrogate marker for proliferation, correlated with disease progression, independent of $CD4^+$ count or viral load[299]. It is hypothesized that activated T cells may contribute to a poor prognosis by supporting higher levels of viral replication and/or by causing inflammation-associated-damage to the immune system and other organ systems.

Immune activation appears particularly relevant during the acute stages of HIV-1 infection, where immune activation at the mucosal surface, as a result of sexually transmitted infection (STI)[33] or reaction to microbicide[141], has been shown to enhance HIV-1 transmission. Within HESN cohorts there have been mixed reports as to whether generalized immune activation[191] or a diminished activation[144] profile affords protection against the establishment of HIV-1.

1.7.2.7 Differentiation into memory T cells

Immunological memory is a key component of the adaptive immune response. It is defined by the ability of antigen specific T cells to persist following exposure to antigen and rapidly respond to secondary challenge. Memory T cells achieve rapid response to antigen on account of two factors; increased precursor frequency and a lower activation threshold[307-310]. Naïve T cells, require antigen stimulation for around 20hours in order to become committed to proliferate[311]. However, memory

T cells have a lower activation threshold, because their signalling machinery is fully coupled, and a short TCR stimulation of around 30 minutes in the absence of CD28 engagement is sufficient to trigger proliferation and cytokine response in $CD4^+$ T cells[311,312].

The generation of memory T cell responses can be broadly classified into three stages (reviewed in[311]). The first is the expansion phase where naïve T cells undergo successive rounds of replication. Through the combined effector functions of $CD4^+$ and $CD8^+$ T cells the acute pathogen is rapidly cleared. Memory T cells then enter the second phase known as "contraction phase" where the majority (>90%) of effector T cells die[311,313]. The surviving T cells enter the third stage- the memory phase in which they persist for several months or even years[275,309,314,315].

In 1999 Sallusto *et al,* identified two main subsets within the memory compartment of $CD4^+$ and $CD8^+$ T cells based on two distinct criteria: the absence or presence of immediate effector function and the expression of homing receptors, which allow for migration to secondary lymphoid tissues[316]. Known as central memory T cells (TCM) and identifiable by surface expression of $CCR7^+$, $CD45RA^-$.[316] TCM produce mainly IL-2 but after proliferation they efficiently differentiate into effector cells and produce large amounts of IFN-γ or IL-4[317-319]. TCM retain of CCR7 expression enabling them to migrate to secondary lymphoid organs. The second memory subset is known as effector memory T cells (TEM) identifiable by a lack of $CCR7^-CD45RA^-$[316]. TEM are typically referred to as terminally differentiated, they are capable of rapid cytokine production following antigenic stimulation, but have poor proliferative capacity and an increased propensity to undergo apoptosis[308,309].

The pathways leading to generation of TCM and TEM remain controversial[311,320]. The most widely accepted model is the linear differentiation pathway[311,321,322]. This model suggests that following encounter with antigen T cells differentiate into TCM prior to terminal differentiation into TEM[311]. This model suggests that T cells differentiate along a one way linear pathway with TCM acting as differentiation

intermediates, capable of retaining proliferative capacity and the ability to differentiate[311,316]. Progression through this pathway is determined by the cumulative strength of stimulation received by T cells, the stochastic interaction with APC and the different concentrations of various cytokines to which the cell is exposed to[307,321,323,324]. There is considerable support for this model within the literature, with perhaps the best evidence coming from adoptive transfer experiments in mice which show TEM to have limited reconstitution capacity compared to TCM[275,309]. Moreover, there have been several *in vitro* experiments to show that TCM possess the ability to rapidly proliferate and spontaneously differentiate into TEM[275,314]. In contrast, TEM have been found to show limited proliferative capacity in the presence of IL-7 and IL-15, but fail to fully expand due to a high degree of spontaneous apoptosis[314].

1.7.2.7.1 Impact of HIV-1 upon immunologic memory

CD4+ TCM are particularly affected during HIV-1 infection and have been shown to be rapidly depleted during the acute stages of HIV-1, and all but absent during chronic infection[23,66,317,325-328]. Notably preservation of the CD4+ TCM subset has been correlated with protection in non-human primates, and long-term non-progression in humans[318,329].

Within CD8+ T cell subset, there is evidence that immune response is driven towards a short lived effector phenotype, with limited generation of TCM or highly differentiated phenotypes[66,229,325,330]. Indeed CD8+ TCM that do persist into chronic HIV-1 infection have been shown to have a reduced lifespan[330]. The mechanisms behind this skewed memory phenotype are incompletely understood and likely to be multifaceted including, rapid consumption of terminally differentiated CD8+ T cells as a result of increased turnover[325,331], a lack of cognate CD4+ T cell help[332,333] or impaired homeostasis due to reduced IL-7R (CD127) surface expression[278,334].

1.7.2.8 Effector function

1.7.2.8.1 $CD4^+$ T cells

$CD4^+$ T cell activation leads to differentiation into distinct subsets known as Th1, Th2, Th17, Th9, TFH (T follicular helper cells) and Treg (regulatory T cells) cells which are discernable by their ability to secrete a range of different cytokines and differentiated by unique transcription factors (Figure 1.5)[335]. Th1/2 subsets were first to be described and are characterized by the secretion of IFN-γ, IL-2, TNFα and TNFβ for Th1 and IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 for Th2. Th1 responses are primarily thought to be involved in immune response to bacteria/viral infection resulting in activation and enhanced microbial killing of macrophages, proliferation and differentiation of $CD8^+$ T cells[335]. In contrast, Th2 cells promote B cells to produce IgG and IgE antibodies are typically associated with parastic, helminth infection or allergic diseases such as asthma[313].

Commitment to a Th lineage does not appear to be definitive/absolute and there appears to be a degree of flexibility in $CD4^+$ T cell differentiation[335]. This is best exemplified by *in vitro* experiments that have shown T cells committed to a particular lineage, e.g. Th1, when cultured under polarizing conditions for their opposite phenotype (e.g. Th2 driver IL-4) "switch" to a Th2 phenotype secreting IL-4. These experiments suggest T cells keep their "options open" prior to becoming fully committed to a particular lineage.

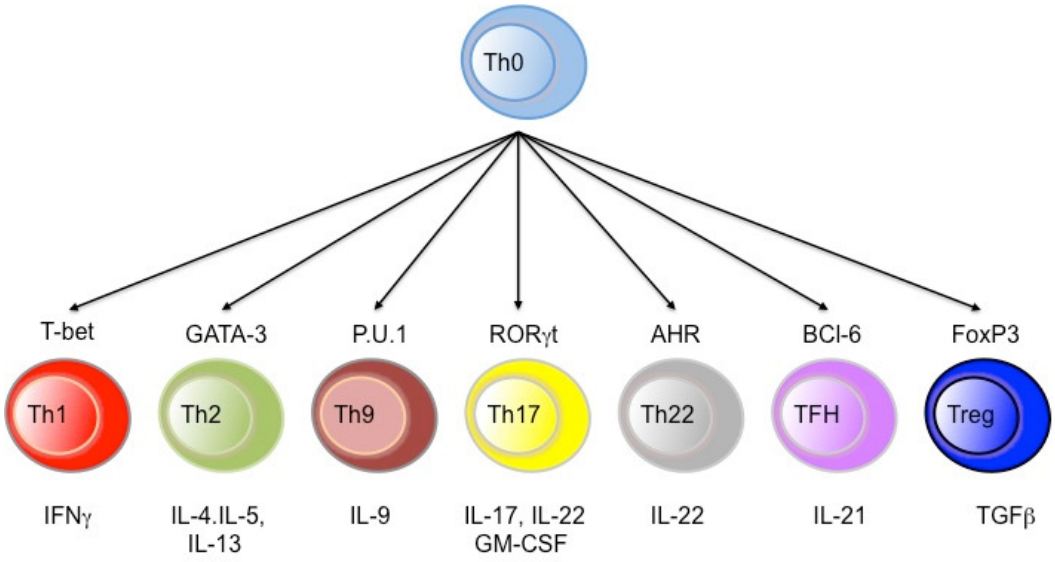


Figure 1.5

Following successful activation undifferentiated (Th0) $CD4^+$ T cells can differentiate into a number of possible effector phenotypes. Discernable by the range of cytokines produced, seven $CD4^+$ effector subsets have been described; Th1, Th2, Th9, Th17, Th22, T follicular helper (TFH) and regulatory T cells (Treg). Represented above each $CD4^+$ subset is the master transcription regulator involved in subset differentiation, and below the principal cytokines produced by each subset.

$CD4^+$ T cells are believed to play both a direct and indirect role in protective immune responses to pathogen. A direct protective effect is mediated through the secretion of pro-inflammatory cytokines such as IFN-γ, or even via perforin mediated killing of target cells, which can be detected during sustained chronic infection such as HIV, hepatitis or cytomegalovirus infection[173,336,337]. The primary role of $CD4^+$ T cells is to provide help to both the adaptive and innate immune response, and indeed $CD4^+$ T cells are commonly referred to as T helper cells[338,339]. Murine studies have shown $CD4^+$ T cells are required for the successful generation and reactivation of memory $CD8^+$ T cells during viral infection[333,340-343]. Seminal work by Matloubian *et al*, showed using a mouse model of LCMV infection, that $CD4^+$ T cells play a critical role in sustaining $CD8^+$ T cell responses[340]. Shedlock *et al* confirmed this observation, showing that depletion of $CD4^+$ T cells during the primary phase of infection leads to impaired $CD8^+$ T cell memory response upon secondary challenge, a deficiency that could be restored by the transfer of memory $CD8^+$ T cells generated from $CD4^+$ competent mice[341]. In addition to providing "help" to $CD8^+$ T cells, specialised subsets of $CD4^+$ T cells referred to as T

follicular helper cells (TFH), have been shown to provide vital "help" to antibody producing B cells[344,345]. Identifiable by the cell surface phenotype, $CD4^+CD57^+CXCR5^+$ (circulating phenotype $CD4^+ICOS^{high}PD1^{high}$) and the cytokine profile IL-21, IL-6 and IL-27 (Reviewed in[344]), TFH cells are believed to act as "tolerance keepers" in germinal centres. Their primary effector functions include the induction of germinal centre (GC) formation, B cell differentiation, somatic hypermutation and immunoglobulin class-switch recombination[346,347] (Reviewed in[344]). A key player mediating these effector functions is CD40L (CD154), which is expressed at high levels of the surface of TFH[346]. Murine studies have shown that an absence of CD154 signalling typically results in abortive B cell responses and a failure to generate germinal centres and long term $CD8^+$ T cell memory[283,348].

1.7.2.8.2 $CD8^+$ T cells

Successful $CD8^+$ T cell activation requires a third signal, which can be derived from IL-12 or from inflammatory signals supplied by TLR ligands and IFNs[349](reviewed in[350]). IL-12 enhances cytotoxicity and promotes production of IFN-γ[349]. Type 1 IFNs promote survival by directly acting on the developing $CD8^+$ T cells[350]. Cytotoxic T cells are predominately required for eliminating target cells infected by viruses or intracellular bacteria, by programming them to undergo apoptosis. The principal cytokine secreted by $CD8^+$ T cells is IFN-γ, although they can also produce substantial amounts of IL-2 and TNFα which enhances their proliferation or cytotoxic effect respectively[75,351-353]. Polychromatic flow cytometry studies suggest that production of multiple cytokines is preferential for successful disease outcome and protection[352,354,355]. The basic principle of action is the calcium-dependent release of lytic granules upon target cells via TCR and LFA1/ICAM-1 interactions, or the engagement of Fas by Fas ligand (reviewed in[301,356]). Exocytosis of perforin leads to the formation of pores in the target cell membrane and serine proteases called granzymes translocate to the cytosol of the infected target cells and induce apoptosis[357]. Granzyme B is required for the induction of target cell death through the activation of apoptotic pathways and induction of nuclear DNA fragmentation[357].

1.7.2.9 T cell effector function and HIV-1

1.7.2.9.1 $CD8^+$ T cells

$CD8^+$ T cell responses are thought to be the major contributors to the control of HIV-1 replication[38,63,206,252,358,359]. The temporal emergence of $CD8^+$ T cell responses and concomitant decline in HIV-1 viremia provided the first evidence for the critical role of $CD8^+$ T cells in HIV-1 infection[206,359]. Later macaque studies showed that depletion of $CD8^+$ T cells has a dramatic effect upon the pathogenesis of SIV causing failure to control peak viremia during primary infection and a rapid increase in viral load during chronic asymptomatic stage[358,360]. Despite impaired $CD4^+$ T cell help the $CD8^+$ T cell response to HIV-1 is vigorous and potent, resembling $CD8^+$ T cell responses in other persistent viruses such as Epstein Barr virus and CMV[325,361]. $CD8^+$ T cells have been shown to kill HIV-1 infected cells both directly and indirectly, through the secretion of a variety of effector molecules including perforin, MIP1β, IFN-γ, TNFα and IL-2[63,66,75,80,82,352,362,363]. Whilst it remains unclear which of these effector functions play the pivotal role in controlling HIV-1 infection, multiparametic flow cytometry analysis suggests that polyfunctional T cell responses (single cells capable of simultaneous secretion of multiple effector molecules) are superior to monofunctional responses in the control of HIV-1 viremia[75,133,352,354,364,365].

The importance of cytolytic and cytokine mediated actions of $CD8^+$ T cells in control of HIV-1 is best exemplified by the presence of "viral escape" mutants which occur within weeks of acute HIV-1 infection and often associate with a fitness cost to the virus[38,68-73,366,367]. Coupled with HLA class I associations with disease progression (Section 1.7.2.2.4) there is strong evidence to suggest $CD8^+$ T cells are critical for immune control of HIV-1, yet despite potent effector functions HIV-1 manages to overcome the immune pressure exerted by HIV-1 viral control is lost and the host succumbs to immune collapse/AIDS defining illness.

1.7.2.9.2 $CD4^+$ T cells

A hallmark characteristic of HIV-1 infection is the profound depletion of $CD4^+$ T cells mediated by direct $CD8^+$ mediated cytotoxic killing, cytopathic effects of

HIV-1 infection and bystander apoptosis[53,368-372]. Despite profound depletion, $CD4^+$ T cell responses are detectable during chronic HIV-1 infection[327,373,374] These residual $CD4^+$ T cells appear to be deregulated, showing reduced proliferative capacity as evidenced by Ki67 staining and BrdU incorporation, impaired IFN-γ secretion, reduced IL-7R (CD127) expression and increased expression of markers such as PD1 and CD57 which are associated with exhaustion and senescence[16,114,118,300,302,303,317,375-378]. Accumulating evidence suggests that preservation of functional HIV-1 specific $CD4^+$ T cell responses may be critical for maintenance of effective $CD8^+$ T cell responses and subsequent control of viral replication[16,130,131,318,329,342,379].

Similar to $CD8^+$ HIV-1 specific T cell responses, the functionality of $CD4^+$ T cell responses (particularly their ability to secrete both IL-2 and IFN-γ) has been correlated with delayed progression of HIV-1 infection and improved prognosis following antiretroviral therapy[79,133,380,381]. Whether maintenance of $CD4^+$ T cell responses is cause or effect of reduced viral loads and improved prognosis is not understood.

1.7.2.9.3 T cells and acquisition of HIV-1

In addition to providing immune control during HIV-1 infection, both $CD4^+$[137,138,145,148,150,295,296,382] and $CD8^+$[135,147,151,156-158,163,203,296,383,384] HIV-1 specific T cell responses have been detected amongst HESN individuals from a variety of high risk cohorts. The detection of HIV-1 specific T cell responses amongst HESN has led to speculation that they may confer a degree of protection against the establishment of HIV-1[385-387]. However, in contrast to protective, vaccine induced, T cell responses in primate models[186,388-390], HIV-1 specific T cell responses in HESN tend to be weak, often transient and typically restricted to single epitopes[135,155,156]. Furthermore not all studies have been able to detect T cell responses amongst HESN individuals[134,155], adding further controversy as to their role as correlates of protection.

1.8 Thesis aims

The primary aim of this thesis is to examine some of the areas of controversy surrounding detection of HIV-1 specific T cell responses amongst HESN individuals. Specifically we aim to assess whether pre-existing HIV-1 specific T cell responses can be reproducibly detected amongst HESN subjects.

To achieve this goal this thesis aims to:

- Standardize highly sensitive detection methods by which to accurately detect and characterize low magnitude and frequency T cell responses. We hypothesize that the failure of some studies to detect pre-existing HIV-1 specific T cell responses may be a result of pre-existing T cell responses falling below the limits of detection by standard *ex vivo* assays.

- Conduct a large and detailed study of well-defined HESN individuals, appropriately controlled by the inclusion of low risk, HIV-1 unexposed seronegative individuals. We hypothesize, that much of the discrepancy in the study of HESN cohorts can be accounted for by the small sample size and recruitment of ill-defined HESN subjects.

Finally, should HIV-1 specific T cell responses be detectable amongst HESN subjects a statistically powered, retrospective study will be employed to directly address the role, if any, pre-existing HIV-1 specific T cell responses have upon the acquisition of HIV-1.

CHAPTER 2:

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Study subjects

2.1.1 HIV-1 exposed seronegative cohorts

Peripheral blood mononuclear cell (PBMC) samples were obtained from four retrospective cohorts referred to herein as the GALVIN, IAVI016, CHAVI002 and VAX004 cohorts. In addition laboratory members and Buffy Coat samples were used for assay optimization and development. Informed consent was obtained from all laboratory volunteers and all studies were approved by local ethics committees. Clinical criteria used to group participants into three groups; HIV-1 infected, HIV-1 exposed, seronegative (HESN) and HIV-1 unexposed, seronegative (HUSN) individuals is summarized in table 2.1.

2.1.1.1 GALVIN cohort

A longitudinal cohort based in Malawi, established to determine the prevalence and durability of HIV-1 specific cellular responses amongst both HIV-1 positive individuals and HIV-1 seronegative individuals. Co-ordinated by Professor S. Galvin (University of North Carolina, USA), the cohort consisted of sexually transmitted infection (STI) clinic attendees in central Lilongwe. A total of 196 subjects were recruited into the study, 60 HIV-1 positive individuals, 126 HIV-1 seronegative individuals defined as being at “high risk” from sexually transmitted HIV-1 infection, and 10 HIV-1 negative “low risk” individuals.

2.1.1.1.1 Ethical considerations and sample access

The study was approved by review boards of the University of North Carolina Medical School and Malawian National Health Sciences Research Committee. Retrospective study of 32 HIV-1 positive, 50 HESN and 10 HUSN participants was granted by The Oxford Tropical Research Ethics Committee (OXTREC).

2.1.1.1.2 Enrolment criteria

Study inclusion criteria included age greater than 18years and haemoglobin greater than or equal to 7gm/dL. There was no exclusion for ethnicity. Volunteers were excluded from the study if they were unable/un-willing to give consent or if their

Cohort Name	Country of origin	Original Study design	Total no. subjects enrolled	No. subjects available for study	Specific Inclusion Criteria	Studies performed using this cohort
GALVIN	**Malawi** Central Lilongwe	Longitudinal study to determine prevalence and durability of HIV-1 specific T cell responses amongst HIV-1 seropositive and seronegative individuals.	**196** 60 HIV-1^+ 126 HESN 10 HUSN	**92** 32 HIV-1^+ 50 HESN 10 HUSN	**Criteria for HIV-1^+:** Chronically infected HIV-1^+ Nucleic acid test (NAT) and serology positive **Criteria for HESN:** NAT negative >1STI in a clinical setting where HIV-1 has a 47% prevalence. **Criteria for HUSN:** NAT Negative In a monogamous relationship with HIV-1 seronegative partner Self-reports low risk behaviour.	• Detection latent Mtb infection + TCRVβ analysis of Mtb specific T cell responses (Chapter 4+6). • Detection and characterization of HIV-1 specific T cell responses amongst HIV-1 exposed and unexposed individuals (Chapter 5)
IAVI016	**UK** Oxfordshire	Randomized, double blinded, placebo controlled, phase one trial to characterize HIV-1 specific T cell responses induced following heterologous prime boost vaccination.	**24** 16 Vaccine 8 Placebo	**14** 8 Placebo 6 Pre-vaccination	Pre-vaccination and placebo recipients only studied. **Criteria for HUSN:** Concordantly seronegative Self-report a monogamous relationship with known HIV-1 seronegative partner.	• Controls for use of cultured ELISpot assay in the detection of latent Mtb infection (Chapter 4). • Detection and characterization of HIV-1 specific T cell responses amongst HIV-1 unexposed individuals (Chapter 5).

Continued

Cohort Name	Country of origin	Original Study design	Total no. subjects enrolled	No. subjects available for study	Specific Inclusion Criteria	Studies performed using this cohort
CHAVI002	**UK** St Mary's Clinic, London	Statistically powered study for the detection and characterization of HIV-1 specific T cell responses amongst HIV-1 seronegative subjects.	**76** 24 HIV-1^+ 24 HESN 28 HUSN	**76** 24 HIV-1^+ 24 HESN 28 HUSN	**Criteria for HIV-1^+:** >12 months documented HIV-1 sero-status at enrolment. Western blot/ELISA positive **Criteria for HESN:** Documented (Western Blot or ELISA positive) HIV-1 seropositive sexual partner. NAT/p24 test negative >25 unprotected sexual acts in preceding 12months with first act occurring at least 10months prior to screening. **Criteria for HUSN:** HIV-1 antibody and NAT/p24 test negative. Monogamous relationship with documented HIV-1 seronegative partner for at least 12 months prior to study enrollment.	• Detection and characterization of HIV-1 specific T cell responses amongst HIV-1 unexposed individuals (Chapter 5). • Analysis of TCRVβ usage in HIV-1 specific T cell responses detected amongst seronegative individuals.

Continued:

Cohort Name	Country of origin	Original Study design	Total no. subjects enrolled	No. subjects available for study	Specific Inclusion Criteria	Studies performed using this cohort
VAX004	**North America**	Randomized double blinded, placebo controlled vaccine trial to test the efficacy of a bivalent vaccine containing recombinant gp120 antigens.	**5403** 1801 Placebo 3602 Vaccine	**87 Placebo** 49 remained HIV-1 seronegative. 38 subsequently seroconverted to become HIV-1^+	**Criteria for HESN:** Male only subjects recruited from placebo arm of the trial. Self report having had anal sex during preceding 12months. Excluded if in a continuously monogamous sexual relationship with HIV-1 uninfected partner for greater than or equal to 12months. Or if they had injected drugs in previous 3 years. **Criteria for HIV-1^+:** HIV-1 antibody test positive.	• Powered study to assess whether pre-existing HIV-1 specific T cell responses confer a 3-4 fold increased risk of HIV-1 infection amongst HESN individuals (Chapter 7). • Immunogencity test of novel pool of promiscuous epitopes for improved sensitivity in detection of HIV-1 specific T cell responses amongst HIV-1 seronegative individuals (Chapter 5).

Table 2.1:

Summary of cohorts studied within the context of this thesis and the criteria used to group study participants as HIV-1 positive, HIV-1 exposed seronegative(HESN) and HIV-1 unexposed seronegative (HUSN).

haemoglobin count was below 7gm/dL. Volunteers were required to attend a study visit every 3 months for 1 year and received payment of 300kwacha per visit.

2.1.1.1.3 HIV-1 status and risk of HIV-1 infection

HIV-1 status was assessed at each study visit using two parallel rapid tests; Determine (Abbott Laboratories, USA) and Unigold (Trinity Biotech, Ireland). Plasma samples from subjects with discordant or duel negative tests were tested for HIV-RNA (Roche HIV-1 Amplicor Monitor, version 1.5; USA) and in accordance with World Health Organization Criteria (WHO) subjects with discordant rapid tests with detectable RNA were tested by standardized Western Blot (Bio-Rad Laboratories, USA).

Risk of HIV-1 infection was assessed by an interview based behavioural questionnaire at each study visit. Subjects were assigned a risk score according to their responses to questions regarding their sexual behaviours over the past two months and the presence of sexually transmitted infections (STIs)/genital ulcer disease (GUD).

2.1.1.2 IAVI 016

Sponsored by the International Aids Vaccine Initiative (IAVI) 016 was a relatively small scale, randomized, double blinded, placebo controlled, phase one study. It was primarily aimed at characterizing the T cell responses elicited following heterologous prime boost regime with a recombinant DNA and modified vaccinia virus Ankara (MVA) vaccine expressing a consensus clade A gag protein together with a string of common $CD8^+$ T cell epitopes[391].

A total of 24 participants were enrolled in the study and randomly distributed to receive vaccine or placebo at a 2:1 ratio. Study participants were healthy, HIV-1 negative, aged between 18 and 60 years. All study participants were referred to as "low risk" for HIV-1 infection, conforming to a monogamous relationship with a HIV-1 uninfected partner, and concordantly testing HIV-1 seronegative for the duration of the study. Exclusion criteria included pregnancy, lactation, active syphilis, infection with hepatitis B or C, as well as history of immunosuppression or severe reaction to any vaccine.

2.1.1.3 Ethical considerations and sample access

Cryopreserved PBMC samples were available at pre-vaccination time points for 14 HUSN. Ethical approval for the study of pre-existing HIV-1 specific T cell responses amongst this population was granted by OXTREC.

2.1.1.4 CHAVI002 cohort

CHAVI002 was a specifically designed, statistically powered study for the detection and characterization of HIV-1 specific T cell responses amongst HESN subjects. A total of twenty-four serodiscordant and 14 HUSN were enrolled at St. Mary's Hospital, London, United Kingdom. They ranged in age from 19-75 years (median 33years) and were predominately white males. Subjects were followed longitudinally across 4 study visits at 3 month intervals. Some participants were lost to follow-up; 12/14 HUSN and 17/24 serodiscordant couples completed all 4 visits.

2.1.1.4.1 Inclusion criteria

Serodiscordant couples were eligible for enrolment if they reported at least 25 unprotected anal, vaginal, and/or oral sex acts in the preceding 12 months, with the first sex act occurring at least 10 months prior to the screening. HESN donors must test HIV-1 antibody and nucleic test (NAT)/p24 negative at each study visit. HIV-1 infected partner must be western blot or HIV-1 enzyme linked immunosorbant assay (ELISA) positive at enrolment with documented HIV-1 sero-status for >12months prior to study enrolment. The use of antiretroviral therapy (ART) was not an exclusion criteria and 17/24 HIV-1 infected participants enrolled were on ART at the time of study enrolment. Of the 7/24 HIV-1 seropositive donors not on ART plasma viral loads ranged from 1,656–34,0581 copies/ml. Plasma viral loads for 15/17 HIV-1 infected subjects on ART were below the limits of detection (<50copies/ml) 2/17 had plasma viral loads of 56 and 170 copies/ml.

Inclusion criteria for HUSN couples were HIV-1 antibody and NAT/p24 negative. In addition both partners must be negative for Neisseria gonorrhoeae, syphilis, and Chlamydia trachomatis, and self-report that they have been in a monogamous relationship for at least 12 months prior to study enrolment.

2.1.1.4.1.1 Ethical considerations, sample access and work contributions

The CHAVI002 study was approved by local and NIH AIDS ethics committees and overseen by Dr N Goonetilleke and two post docs within the laboratory (Dr A Ritchie and Dr K Kopycinski). I was involved in assay development (specifically, intracellular cytokine staining of short-term cell lines and epitope mapping) and T cell assays performed at early study visits. Independent statistical analysis was performed on data generated from the CHAVI002 cohort, by Dr Z Moodie at the statistical centre for HIV-1 and AIDS research and prevention (SCHARP). In addition genetic analysis of CCR5Δ32 genotype for CHAVI002 subjects was performed by Dr S Brackenridge.

2.1.1.5 VAX004 cohort

Initiated in 1998 by VaxGen Inc., VAX004 was a randomized, double blinded, placebo-controlled trial. Conducted at multiple sites in North America and the Netherlands it was originally designed to address the efficacy of bivalent vaccines containing recombinant HIV-1 envelope glycoprotein 120 (rgp120) antigens. A total of 5403 individuals were recruited into the study, all of whom were referred to as "high risk" for HIV-1 infection through sexual activity. During the course of the study 368 (6.8%) of subjects became infected with HIV-1, giving an annual incidence rate of 2.6% (2.7% in men and 0.8% in women) [392].

In addition to evaluating vaccine efficacy, VAX004 also aimed to examine immune correlates of protection. As such a retrospective research proposal was submitted to conduct a statistically powered study, to address whether pre-existing HIV-1 specific T cell responses, detectable in a proportion of HIV-1 uninfected individuals, conferred a 3-4 fold increased risk of HIV-1 infection (Power calculations kindly performed by Dr P Gilbert (The Statistical Centre for HIV/AIDS Research & Prevention; SCHARP)).

2.1.1.5.1 Ethics considerations and sample access.

VAX004 trial was conducted in accordance with the Declaration of Helsinki and local institutional review board[211,220,392]. Retrospective samples were obtained following approval by OXTREC. A total of 87 subjects from the placebo arm of

VAX004 were available for study, 49 of whom remained HIV-1 seronegative for the duration of study (3 years) and 38 who subsequently seroconverted.

2.1.1.5.2 Enrolment criteria

Volunteers were healthy, 18-62 years old, HIV-1 seronegative, non-injection drug users, the majority of whom were men who have sex with men (MSM). All subjects studied within the context of this thesis were male. Inclusion criteria for male subjects were having had anal sex during the preceding 12 months. Subjects were excluded from the study if they had been in a continuously monogamous sexual relationship with a HIV-1 uninfected partner for greater than or equal to 12 months, or had injected drugs during the previous 3 years[220].

Additional inclusion criteria were stipulated for the 87 subjects studied in this thesis. For the 49 subjects who remained HIV-1 uninfected they had to have been followed for the full 3 year follow up period, and have attended study visits at months 6, 12, 18 and 24. The 38 subjects who became HIV-1 positive during the course of the study must have attended study visits at months 1 and 6; been diagnosed with HIV-1 infection after the month 6 study visit; not started antiretroviral therapy within 6 months of infection diagnosis and have attended at least 2 visits in the first 6 months post infection diagnosis.

2.1.1.5.3 Assessment of HIV-1 status and risk of infection

Subjects were randomly distributed to receive either vaccine or placebo (alum) at a 2:1 ratio. Intramuscular injection of either vaccine or placebo (600 occurred at months 0, 1, 6, 12, 18, 24 and 30 [220,392]. HIV-1 status was assessed at each study visit and at a follow up visit at 36 months, by standard ELISA (Abbott HIV-1 EIA; Abbott Laboratories, USA) and confirmatory immunoblot (Novapath HIV-1 Immunoblot; Bio-Rad, Hercules, CA). Self-reported risk behaviours such as drug use, occurrence of STDs and sexual activity were assessed by standardized, interview based questionnaires which enabled a risk score to be assigned (Table 2.2). Risk score was defined as the total number of risk factors reported, and categorized into one of three groups ; Low Risk (Risk score 0), Medium Risk

(Risk score 1-3) or High Risk (Risk score 4-7) [220,392]. All samples studied within this thesis fell within the medium–high risk groups.

2.1.1.1 Study powering

The study had 80% power to detect a 3-4 fold increase in risk of HIV-1 infection amongst subjects with pre-existing HIV-1 specific T cell responses.

	Self-Reported Risk Behaviours
1.	Unprotected receptive anal sex with a HIV-1 positive, male partner
2.	Unprotected insertive anal sex with a HIV-1 positive male partner
3.	Unprotected receptive anal sex with a HIV-1 negative male partner
4.	≥ 5 acts of unprotected receptive anal sex with a male partner of unknown HIV-1 status
5.	Greater than 10 sex partners
6.	Anal herpes
7.	Hepatitis A
8.	Use of alkyl nitrates (“poppers”)
9.	Use of amphetamines

Table 2.2

Self-reported risk behaviors in the VAX004 cohort.

2.1.2 Acute HIV-1 infection samples

Subjects in the acute stages of HIV-1 infection were obtained from a multi-centre, prospective, observational, cohort study referred to as CHAVI 001. The aim of the study was to characterize the HIV-1 virus, the host response and genetic factors that determine HIV-1 transmission and viral set point. Inclusion criteria included >18years old, haemoglobin >10.0g/dL and a willingness to receive HIV-1 test results. Exclusion criteria were prior use of antiretroviral therapy (ART), previous or current participation in HIV-1 vaccine trial or any pre-existing condition that in the mind of the investigator would make participation in the study unsafe or otherwise interfere with the outcome of the study. The study was approved by the Prevention Sciences Review Committee Division of Acquired Immunodeficiency Syndrome.

2.1.2.1 Definition of acute HIV-1 infection

Acute HIV-1 infection was defined as having negative/discordant HIV-1 antibody tests (simultaneous testing with at least two different rapid tests) but positive plasma p24 antigen or nucleic acid test (NAT) at screening visit (Fiebig stage I/II)[393]. Subjects were bled at multiple time points and followed over a two year period. Table 2.3 provides a summary of the clinical and demographic information for all 6 subjects studied.

Patient Identification (PTID)	Gender	Age at screening	Ethnicity	Fiebig Stage at screening	Viral load at screening (copies/ml)	Absolute $CD4^+$ Count at screening	Median CD4+ count	Viral Load set point (copies/ml)
CHAVI067	Female	32	Black	I/II	639000	1580	1557	40
CHAVI162	Male	42	Black	I/II	>10000000	928	384	114039
CHAVI164	Male	27	Black	I/II	23600	700	454	571762
CHAVI185	Female	20	Black	I/II	20449	242	307	40795
CHAVI198	Male	33	Black	I/II	>10000000	599	673	1177
CHAVI470	Male	17	White	I/II	840933	324	538	23710

Table 2.3

Clinical and Demographic data for CHAVI 001 Acute HIV-1 infection subjects

2.2 Media and wash buffers

2.2.1.1 Cell culture media

10% heat inactivated, human AB serum (Sigma, UK) (R-AB-10) or heat inactivated, sterile filtered, fetal calf serum (FCS) (Gemcell^{TM}, Gemini Bio Products) (R-10) was added to RPMI-1640 media (Sigma, UK) containing 1% sodium pyruvate (Sigma, UK), penicillin streptomycin (Sigma, UK), HEPES buffer (Sigma, UK) and L-glutamine (Sigma, UK). Media was stored at 4°C and R-AB-10 was sterile filtered using a 0.22μM filter (Millipore, USA) prior to each use.

2.2.1.2 Complete media + /- IL-2 for T cell library technique

RPMI 1640 (Gibco, Invitrogen, UK) supplemented with 1% glutamine (Gibco, Invitrogen, UK), 1% pyruvate (Gibco, Invitrogen, UK), 1% non-essential amino acids (Gibco, Invitrogen, UK), 1% penicillin streptomycin (5U/ml) (Gibco, Invitrogen, UK), 0.0005% MB-mercaptoethanol (Gibco, Invitrogen, UK) and 5% human serum (Swiss Red Cross) with or without 500IU/ml IL-2 (San Giovanni Hospital (Bellinzona, Switzerland).

2.2.1.3 Wash buffer

RPMI 1640 (Gibco Invitrogen, UK) supplemented with 10% complete media (as previously described 2.2.1.2) without IL-2.

2.2.1.4 MACS buffer

2mM EDTA (Invitrogen) and 3% sterile filtered, fetal calf serum (FCS; Sigma, UK) were added to PBS (Gibco, Invitrogen, UK). Media was filtered through a vacuum driven 0.45μm filter (Stericup, Millipore, UK) prior to use.

2.2.1.5 Cryopreservation solution

90% foetal calf serum (sterile filtered, Gemcell) and 10% Dimethyl sulfoxide (DMSO; Sigma, UK)

2.2.2 Peptide synthesis

Predominately commercially synthesised peptides, obtained from Sigma genosis were used throughout this thesis. However, additional stocks were occasionally synthesized “in house”. Quality control (QC) checks ensured peptide of interest was one of the 3 major peaks by mass spectrometry. With the exception of

CHAVI002 samples, all peptides used were 2004 clade C consensus (CHAVI002 used clade B consensus peptides)[20,21]. Consensus peptides spanned the entire HIV-1 proteome and were synthesized as 18mers overlapping by 10 amino acids. In addition a complete set of previously defined, HIV-1 specific optimal cytotoxic T lymphocyte (CTL) epitopes (2005, LANL) were also synthesized[20,21]. Additional peptide stocks were created for the study of *Mycobacterium tuberculosis* (Mtb) specific responses. Peptides spanning early secreted antigenic target-6 (ESAT-6), culture filtrate protein–10 (CFP-10)[104] along with a pool of peptides common to many Mycobacterium (85A) [394,395] were synthesized as 18mers (overlapping by 10 amino acids). In addition a novel "promiscuous peptide pool" was designed within the context of this thesis (Section 5.4.10). Novel consensus promiscuous peptides were synthesized as 18mers together with previously published peptides ranging in length from 8-24 amino acids (Table 5.4).

2.2.2.1 Creation of master and working stocks

Lyophilized peptide stocks were reconstituted in 100% DMSO (Sigma, UK) to a final concentration of 40mg/ml. Working stocks were prepared using sterile dH_20 to dilute the peptides to 4mg/ml. For all cellular assays peptides were used at a final concentration of 2µg/ml and re-suspended in R-AB-10 media (2.2.1.1). DMSO concentration in the final assay was never greater than 0.05%.

2.2.2.2 Preparation of peptide pools and antigen "screening" plates

For screening purposes peptides were grouped into "pools" (Table 2.3). Peptides were typically used at a final concentration of 2µg/ml (env peptides used at a final concentration of 1.5µg/ml) and the DMSO concentration of each pool did not exceed 0.5%. 10x stocks of each pool were prepared (peptide concentration 20µg/ml) for use in ICS assays or culture set up.

In order to screen for HIV-1 specific/Mtb specific T cell responses antigen plates were prepared at 2x final concentration (peptide concentration 4µg/ml) and aliquoted into round bottom 96 well microtitre plates (Nunc, Denmark). To

minimize non-specific/background IFN-γ production R-AB-10 media was used in the preparation of all antigen plates.

2.2.2.3 Preparation of peptide mapping plates

Optimal peptide matrices were designed using "Deconvolute This!"[396]. This programme tests all possible peptide configurations against a varying number of potential positive responses. From these simulations an optimal configuration with low number of potential re-tests (ambiguity ≤1) was selected and peptide pools prepared accordingly. The configurations chosen had "coverage" of 3, meaning that each peptide occurred on the plate 3 times. Following first round mapping responses were deconvoluted and individual peptides "confirmed" using customized ELISpot plates and ICS.

2.2.2.4 Quality control

In house QC was performed for all peptide pools and antigen plates. Peptide stocks and antigen plates were prepared using sterile, filter tips. Sterility was assessed by diluting 1µl of peptide/peptide pool into 100µl R-AB-10 media and culturing at 37°C/5%CO_2 for 24–72 hours. *Ex vivo* PBMCs from either a healthy volunteer or buffy coat were used to test the plates to ensure that pools of interest had not become contaminated with positive control antigens. All plates and pools were stored at -80°C prior to use and freeze thaw cycles of peptide pools minimized.

Pool Name	Description	Number of peptides in pool	Final peptide concentration
Gag	Consensus clade C peptides spanning the Gag region of HIV-1	61	2µg/ml
Env	Consensus clade C peptides spanning the Env region of HIV-1	104	2µg/ml
Nef/Acc	Consensus clade C peptides spanning Nef and accessory (Vif, Tat, Rev, Vpu and Vpr) region of HIV-1	97	2µg/ml
Pol	Consensus clade C peptides spanning the Pol region of HIV-1	126	2µg/ml
CTLA CTLB	Pools of previously defined HIV-1 specific optimal CTL epitopes.	CTLA 109 CTLB 97	2µg/ml
ESAT-6	Peptides spanning a secreted antigen that is highly specific to Mtb and absent from all strains of BCG.	11	2µg/ml
CFP-10	Peptides spanning culture filtrate protein-10, a secreted antigen specific to Mtb and absent from all strains of BCG.	12	2µg/ml
85A	Peptides spanning antigen 85A, a highly conserved antigen present in all mycobacterial species and strains of the BCG vaccine	41	2µg/ml
Prom	Novel set of peptides previously defined as "promiscuous" based on their ability to be recognized in the context of multiple HLA.	101	2µg/ml
Mock	Negative control containing media and 0.05% DMSO	0	N/A
FEC	Peptide control. A pool of commonly recognized epitopes from flu, Epstein Barr and cytomegalovirus	32	1.5µg/ml
PHA	Positive control Phytohemagglutinin		10µg/ml
PPD	Purified protein derivative (PPD) a poorly defined mycobacterial antigen not specific to Mtb and present in BCG as well as environmental mycobacteria.		10µg/ml

Table 2.3

Peptide pools and control antigens used within this thesis.

2.3 Cell culture techniques

2.3.1 Isolation of peripheral blood mononuclear cells (PBMC) from whole blood

Fresh whole blood was either taken from laboratory volunteers or Buffy coat samples obtained from the National Blood Service (UK). Blood was carefully overlaid onto leucospe^{TM} tubes (Greiner®, UK) pre-loaded with 15ml histopaque-

1077 (Sigma-Aldrich, UK) and centrifuged at 800g for 30 minutes without braking. The tubes were removed from the centrifuge and the PBMC layer carefully removed (Figure 2.1) before transferal to a new 50ml falcon tube (BD Biosciences, UK). The volume was made up to 45ml using HBSS (Sigma, UK) prior to being

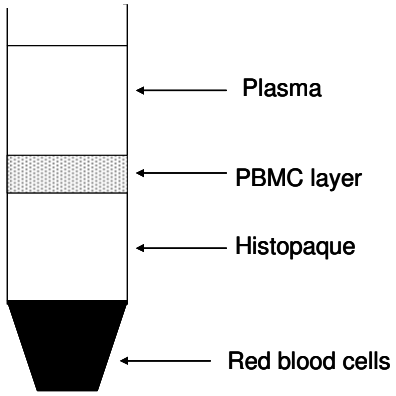


Figure 2.1
Peripheral blood mononuclear cell (PBMC) isolation from whole blood.

centrifuged at 350g for 10 minutes (fast acceleration and brake). The supernatant was discarded and pellet re-suspended in 45ml HBSS (Sigma, UK) and washed twice more by centrifugation at 350g 10minutes. After the final wash the pellet was re-suspended in 5-10ml R-AB-10 and rested 37°C /5% Co_2 for a minimum of 30minutes prior to cell counting.

2.3.1.1 MACS micro bead isolation of $CD14^+$ and $CD4^+$ T cells

3 MACS micro columns per subject were pre-wet with 4ml MACS buffer (2.2.1.4), and flow through discarded. Isolated PBMC were washed once in 10ml MACS buffer and centrifuged at 1500rpm for 5 minutes. Supernatant was decanted and the pellet re-suspended in 2ml MACS buffer. 240µl of $CD14^+$ MACS beads were added (Miltenyi Biotec, UK) and incubated on ice for 20 minutes. Cells were washed once with MACS buffer, centrifuged at 1500 rpm for 5 minutes. Supernatant was decanted, pellet re-suspended in 3ml MACS buffer and filtered prior to addition to MACS column (Miletnyi Biotec, UK). Columns were washed with 9ml MACS buffer and flow through collected in a 15ml falcon labelled $CD14^-$. The negative fraction was then passed through a second column to ensure complete isolation of all $CD14^+$ monocytes. $CD14^+$ cells were eluted by removal of

the column from the magnet and flushing with 9ml MACS buffer. Positive and negative fractions were centrifuged 1500rpm for 5 minutes. The positive fraction was cryopreserved for use as allogeneic antigen presenters. The negative fraction was re-suspended in 2ml MACS buffer with 240ul $CD4^+$ micro beads. The cells were incubated, on ice, for 20 minutes prior to washing with 10ml MACS buffer. After centrifugation at 1500rpm for 5 minutes the supernatant was decanted and the pellet re-suspended in 3ml MACS buffer. Cell suspension was passed through micro column and flow through collected in a tube labelled $CD4^-$. Following 3x 3ml washes of the column the positive fraction was eluted by removal of the column from the magnet and flushing through 9ml MACS buffer, collecting flow through in a 15ml falcon tube labelled $CD4^+$. Positive and negative fractions were centrifuged 5 minutes at 1500rpm. $CD4^-$ negative fraction discarded and $CD4^+$ fraction supernatant decanted and pellet re-suspended in 5ml complete media.

2.3.2 Thawing PBMC

Cryopreserved PBMC / STCL were transferred from the liquid nitrogen store on dry ice and immediately placed in a 37°C water bath. Samples were allowed to thaw until just a small ice crystal remained, prior to transfer to a 15ml falcon (Fisher Scientific, UK). Drop wise R-10 (2.2.1.1) supplemented with 50units/ml benzonase (Novagen, UK) was added and samples centrifuged at 350g for 10 minutes. Supernatant was decanted and the pellet re-suspended in 15ml R-10 (2.2.1.1) for two further washes, with each centrifugation at 350g 10 minutes. Following the final wash, supernatant was decanted and the pellet re-suspended in 5ml R-AB-10 (2.2.1.1) and left to rest at 37°C/5%$C0_2$ for 2-4 hours.

2.3.3 Cell counting

Total cell counts were made using an automated Z1 particle counter (Beckman Coulter). Briefly, 20ul cell suspension was added to 10ml isoton solution (Beckman coulter). Prior to use the Z1 particle counter was primed using Coulter Isoton II diluent (Beckman coulter, UK) and a background count made using 10ml Coulter Isoton II diluent (Beckman Coulter, UK). To pass internal quality control checks the background count should be <50 and duplicate counts within 10% of each other. Viability was assessed using a 1:1 dilution of cells:trypan blue (SIGMA, UK)

2.3.4 Irradiation of allogeneic feeder cells

Isolated PBMC (typically ~100x10^6) were re-suspended in 5ml complete media in a 15ml Falcon tube (BD Biosciences, UK). Cells were irradiated at 45Gy (Biobeam 8000) for 14 minutes 21 seconds. Following irradiation cells were washed twice in 10ml MACS buffer (2.2.1.4), centrifugation at 1500rpm 5 minutes.

2.3.5 Cryopreservation

2.3.5.1 Isolated PBMC and short-term cell lines

PBMC were centrifuged at 350g for 10 minutes, supernatant decanted and the pellet re-suspended. Using an ice cold stratacooler (Fisher scientific, UK) the cells were re-suspended at an optimal concentration of 1x10^7 PBMC/ml and transferred in 1ml aliquots to internally threaded cryovials (Thistle scientific, UK). The stratacooler (Fisher scientific, UK) was then transferred to a -80°C freezer, which allowed cells to cool at a rate of 1°C/minute, thereby preserving cell viability. After 24-36 hours the cells were transferred to a vapour phase liquid nitrogen store (VWR International, UK) for long term storage prior to use.

2.3.5.2 Cryopreservation of $CD14^+$ cells

Freezing media consisting of 40% human sera (Sigma, UK), 50% RPMI (Gibco, Invitrogen, UK) and 10% DMSO (Sigma, UK) prepared and chilled to 4°C. Cells washed in 10ml MACS (2.2.4,1) buffer and centrifuged at 1500rpm for 5 minutes. Supernatant decanted and pellet re-suspended in 1ml freezing media. Cell suspensions were transferred to pre-labelled cryovials 1ml per subject.

2.3.6 Cell culture

2.3.6.1 Short-term cell culture

PBMCs were cultured at an optimal seeding density of 1.5x10^6/ml in 1ml R-AB-10 (2.2.1.1) supplemented with 25ng/ml IL-7 (R&D systems, UK). Lines were stimulated with the appropriate peptide pool (final concentration 2μg/ml) in a 12 well culture dish (Corning Inc. USA) at 37°C/5% CO_2. For cultures set up at <1.5x10^6cells/ml a 24 well plate (Corning Inc. USA) was used. Exogenous IL-2 (Proleukin, John Radcliffe Hospital Pharmacy, UK) was added to a final concentration of 1.8x10^3 units/ml on days 3 and 7 of culture. In addition, 1ml of R-

AB-10 media (2.2.1.1) was added at day 7 and, if required, day 8 or 9. On day 10 cultures were transferred to a 15ml falcon (Fisher Scientific,UK) using Pasteur pipette (Fisher Scientific, UK) and washed 4 times with 15ml sterile phosphate buffered saline (PBS) (Sigma, UK). STCL were rested for 30-34 hours in 1ml R-AB-10 media (2.2.1.1.) at 37°C/5% CO_2.

2.3.6.2 T cell library technique

T cell libraries were set up with sorted, naïve, effector memory and central memory populations in 384 well plate format, in the presence of $2.5x10^4$ irradiated (45Gy) allogeneic feeder cells and IL-2 (500IU/ml). Naïve populations were seeded at 1000 cells/well whilst memory populations were seeded at 500 cells/well. All cultures were polyclonally stimulated with 1μg/ml PHA (Remel, Thermo Fisher, Switzerland). After 5 days cells were transferred to 96well U bottom plates (Nunc, Denmark) by pooling two wells of the initial plate. Three days later cells were split into 48well format, expanded and after 3 days transferred to 24 well plates. Throughout the culture period cells were supplemented with exogenous complete media containing IL-2 (500IU/ml).

2.4 Cell sorting

2.4.1 Isolation of $CD4^+$ Naïve, central and effector memory T cells

Naïve ($CD4^+$ $CD45RA^+CCR7^+CD25^-$), Central memory ($CD4^+CD45RA^-CCR7^+$ $CD25^-$) and effector memory ($CD4^+CD45RA^-CCR7^-CD25^-$) populations were sorted using BD FACS Aria (Beckman Dickenson, UK). All stages of cell staining were performed using MACS buffer (2.2.4.1). Briefly, PBMC were washed 1x centrifuged 1500rpm 5 minutes, Supernatant decanted and pellet re-suspended in 300μl. Cells were incubated with 1.5μl of purified, un-conjugated CCR7 antibody for 20 minutes on ice, then washed once in final volume of 10ml. Supernatant was decanted and the pellet re-suspended at 300μl. 82.5μl surface marker mastermix (Table 2.4) was added per subject and incubated 20minutes on ice. Cells were then washed once in 10ml, re-suspended in 500μl MACS buffer (2.2.4.1) and filtered prior to acquisition upon BD FACS Aria (cell sorting performed by Dr D. Jarrossy, IRB, Switzerland).

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CCR7	150503	R&D systems	Un-conjugated	1.5ml
IgG2a		Southern Biotec	PE	1.5ml
CD45RA	ALB11	Beckman Coulter	FITC	30ml
CD4	SK3	BD Pharmingen	PECy7	15ml
CD8	B9.11	Beckman Coulter	PECy5	6ml
CD56	N901 (NHK-1)	Beckman Coulter	PECy5	15ml
CD25	B1.49.9	Beckman Coulter	PECy5	15ml

Table 2.4

Antibody clones and dilutions used for Cell Sort Naive/Memory $CD4^+$ T cells for T cell library technique.

2.4.2 Antigen specific T cells

HIV-1 specific T cells were cell sorted using BD FACS Aria (Beckman Dickenson, UK) based upon ICS with IFN-γ (Table 2.5). Cells were sorted to 96% purity, run at 50% maximal flow rate (2000 events/second). Cells were sorted into a dry collection tube, spun at 16000g for 1minute prior to elution of supernatant. Dry cell pellet was stored at -80°C until use (Cell sorting performed by Dr K. Ladell, Cardiff University, UK).

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CD3	SK7	BD Biosciences	APCCy7	5µl
CD4	S3.5	Invitrogen	PECy 5.5	0.2µl
CD8	FR261 HH	Invitrogen	QD705	10µl
CD14	TüK4	Invitrogen	Pacific Blue	4 µl
CD19	SJ25-C1	Invitrogen	Pacific Blue	2µl
CD16	3G8	Biolegend	Pacific Blue	2µl
CD56	MEM188	Biolegend	Pacific Blue	4µl
IFN-γ	B27	BD Biosciences	FITC	1µl
Live Dead	N/A	Invitrogen	APC	1µ/ml

Table 2.5

Antibodies used for isolation of antigen specific T cells for TCR clonotyping experiments.

2.5 Functional and phenotypic analysis

2.5.1.1 Flow cytometry

Five flow cytometry panels were designed for use either with a CyAn flow cytometer (Dako Inc, UK) (Panel 1 only), or LSRII (Beckton Dickenson, UK) (Panels 2-5). The LSRII cytometer configuration is shown in Figure 2.2. Panel One enabled $CD4^+/CD8^+$ T cell specificity to be examined, it was used with samples obtained from CHAVI002, IAVI 016, GALVIN and VAX004 cohorts. Panel Two was designed for assessment of T cell receptor (TCR) diversity and was used with subjects from GALVIN and CHAVI002 cohorts. Panel 3 was used to screen whether IFN-γ^+HIV-1 specific T cell responses co-produced TNFα and was used exclusively with samples from the CHAVI002 cohort. Panel 4 was designed to examine whether HIV-1 specific T cell responses detected at pre-infection time points were maintained post seroconversion and used exclusively with samples from VAX004. Panel 5 was designed and validated by Dr C Riou (National Institute of Communicable Disease, South Africa) and used to perform detailed functional and phenotypic analysis of gag specific T cell responses detected during acute HIV-1 infection, it was used exclusively with sample from CHAVI001. All staining was performed in a final volume of 100µl. Antibodies were titrated in accordance with recommendations made by Lamoreaux *et al* . Tables 2.6–2.10 list the antibody clone and volume used for each of the five panels.

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CD3	UCHT1	BD Biosciences	Pacific Blue	2.5µl
CD4	RPA-T4	BD Biosciences	PE	10µl
CD8	Sk1	BD Biosciences	PerCP	5µl
CD56	B159	BD Biosciences	PE-CY7	1.5µl
CD14	M5E2	BD Biosciences	PE-CY7	4µl
CD19	SJ25C1	BD Biosciences	PE-CY7	4µl
IFN-γ	B27	BD Biosciences	FITC	1µl
Live Dead	N/A	Invitrogen	APC	1µl/ml

Table 2.6

Antibody clones and dilutions used for panel one: Intracellular cytokine staining for determination of $CD4^+/CD8^+$ Specificity.

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CD3	S4.1	Invitrogen	QD 655	1µl
CD4	RPA-T4	BD Biosciences	APC	5µl
CD8	FR261 HH	Invitrogen	QD705	1µl
CD14	TüK4	Invitrogen	Pacific Blue	4µl
CD19	SJ25-C1	Invitrogen	Pacific Blue	2µl
CD16	3G8	Biolegend	Pacific Blue	2µl
CD56	MEM188	Biolegend	Pacific Blue	4µl
IFN-γ	B27	BD Biosciences	AF700	0.5µl
TCRVβ	N/A	Beckman Coulter	PE + FITC	20µl
Live Dead	N/A	Invitrogen	Pacific Blue	1/1000

Table 2.7

Antibody clones and dilutions used for Panel two: TCR diversity study used with samples from the CHAVI002 and GALVIN cohorts.

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CD3	S4.1	Invitrogen	QD 655	1µl
CD4	RPA-T4	BD Biosciences	APC	5µl
CD8	FR261 HH	Invitrogen	QD705	1µl
CD14	TüK4	Invitrogen	Pacific Blue	4µl
CD19	SJ25-C1	Invitrogen	Pacific Blue	2µl
CD16	3G8	Biolegend	Pacific Blue	2µl
CD56	MEM188	Biolegend	Pacific Blue	4µl
IFN-γ	B27	BD Biosciences	AF700	0.5µl
TNF-α	MAb11	BD Biosciences	PECY7	
Live Dead	N/A	Invitrogen	Pacific Blue	1/1000

Table 2.8

Antibody clones and dilutions used for panel three: TNFα functional study – used with CHAVI002 subjects.

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CD3	S4.1	Invitrogen	QD 655	1µl
CD4	S3.5	Invitrogen	PECy 5.5	0.5µl
CD8	FR261 HH	Invitrogen	QD705	1µl
CD14	TüK4	Invitrogen	Pacific Blue	4µl
CD19	SJ25-C1	Invitrogen	Pacific Blue	2µl
CD16	3G8	Biolegend	Pacific Blue	2µl
CD56	MEM188	Biolegend	Pacific Blue	4µl
IFN-γ	B27	BD Biosciences	AF700	0.5µl
IL-2	MQ1-17H12	BD Biosciences	PE	5µl
CD45RA	MEM56	Invitrogen	QD605	2µl
CCR7	3D12	BD Biosciences	PECY7	0.5µl
CD27	1A4CD27	Beckman Coulter	PECy5	10µl
Live Dead	N/A	Invitrogen	Pacific Blue	1/1000

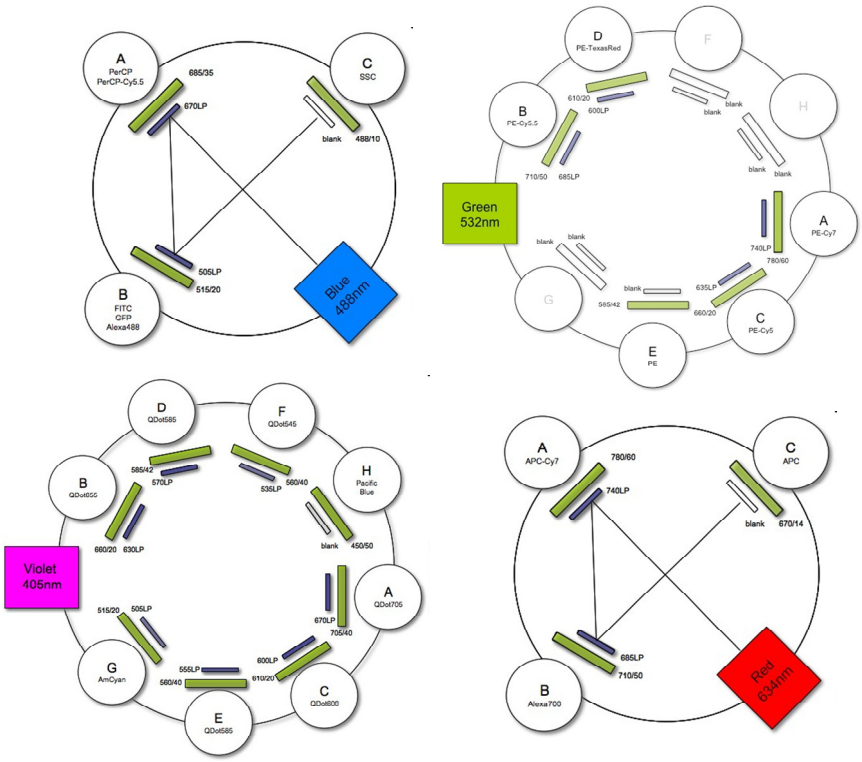
Table 2.9

Antibody clones and dilutions used for panel four: examination of maintenance of pre-existing HIV-1 specific T cell responses post HIV-1 seroconversion- used with VAX004 samples.

Antibody	Clone	Supplier	Fluorochrome	Volume Used
CD3	S4.1	Invitrogen	QD655	1µl
CD4	S3.5	Invitrogen	PECy 5.5	0.2µl
CD8	FR261 HH	Invitrogen	QD705	10µl
CD14	PNIM2707U	Beckman Coulter	ECD	3µl
CD19	PN1M2708U	Beckman Coulter	ECD	3µl
V-Amine	N/A	Invitrogen	Red Amine	1/200
CD45RA	MEM56	Invitrogen	QD605	10µl
CCR7	3D12	BD Biosciences	PECY7	4µl
CD57	Custom conjugate*	University of Pennsylvania	QD565	10µl
CD27	1A4CD27	Beckman Coulter	PECy7	4µl
HLADR	L243	Biolegend	APCCy7	5µl
CD38	HB7	BD Bioscience	APC	7µl
IL-2	MQ1-17H12	BD Bioscience	PE	5µl
IFN-γ	B27	BD Biosciences	AF700	0.2µl
Ki67	B56	BD Bioscience	FITC	20µl
TNFα	MAB11	eBioscience	Pacific Blue	2.5µl

Table 2.10

Antibody clones and dilutions used for panel five: Functional and phenotypic analysis of $CD4^+$ gag specific T cell responses in acute HIV-1 infection- Used with CHAVI 001 subjects.* CD57 custom conjugate antibody was a kind gift from Dr M Betts, University Of Pensylvania.



Laser	PMT	Fluorochrome	Dichroic mirror	Bandpass filter	Range, nm
Violet	A	QD705	670LP	705/40	685-725
	B	QD655	630LP	660/20	650-670
	C	QD605	600LP	610/20	600-620
	D	QD585	570LP	585/42	570-600
	E	QD565	557LP	560/40	557-580
	F	QD545	535LP	560/40	540-557
	G	AmCyan	505LP	515/20	505-525
	H	Pacific Blue	-	450/50	425-475
Blue	A	PerCP, PerCP-Cy5.5	670LP	685/35	670-703
	B	FITC, AF488	505LP	515/25	505-528
	SSC		-	488/10	483-493
Green	A	PE-Cy7	740LP	780/60	750-810
	B	PE-Cy5.5	685LP	710/50	685-735
	C	PE-Cy5	635LP	660/20	650-670
	D	PE-TexRed	600LP	610/20	600-620
	E	PE	-	585/42	564-606
Red	A	APC-Cy7, APC-AF750, APC-H7	740LP	780/60	750-810
	B	AF700	685LP	710/50	685-735
	C	APC	-	670/14	663-677

Figure 2.2

LSRII flow cytometer technical specification.

Staining was performed on 1-1.5 million PBMC, with a minimum of 100,000 events acquired. Stimulation for ICS assays took place in 1ml R-AB-10 media (2.2.1.1) in polystyrene FACS tubes (Becton, Dickinson, UK). Cells (except those used with panel 1) were stimulated in the presence of co-stimulatory molecules CD49d and CD28 (BD Bioscience, UK) with either a pool of peptides or individual peptide at a final concentration of 2µg/ml. For all experiments an unstimulated control tube for each participant was also included, this tube received co-stimulatory molecules and a DMSO control (0.05% DMSO in R-AB-10 media; 2.2.1.1). Staining was performed either in a V bottom, 96 well plate (Corning Inc, USA), or polystyrene FACS tube (Becton Dickinson, UK).

2.5.1.2 Staining protocol

1µl of co-stimulatory molecules CD28 and CD49d (BD Biosciences, UK) and 1ml Golgi stop (BD Biosciences, UK) were added simultaneously with peptide/DMSO control. Following 6 hour incubation at 37°C/5% CO_2 samples were washed with 4ml PBS (Sigma, UK), centrifuged at 350g for 10 minutes, supernatant decanted and pellet re-suspended. For panels 2-3 1ml PBS 0.5%BSA and 1µl Live dead stain (Invitrogen, UK) was added and left to incubate for 30 minutes at 4°C prior to two washes with 4ml PBS 0.5% BSA. For panel 5 (Table 2.10) live/dead cells were stained using 50µl of 1/200 dilution of red amine incubated at room temperature 10 minutes prior to washing with PBS.

A "mastermix" of appropriate surface markers (see tables 2-6 to 2-10) was added to each well and left to incubate at room temperature, protected from light, for 30 minutes. Samples were washed in 150µl (V bottom plate) or 3ml PBS (4ml polystyrene FACS tube) and centrifuged at 400g for 5 minutes. The pellet was re-suspended and cells incubated for 20 minutes at room temperature with 100µl fixation buffer (Caltag reagent A, Caltag Laboratories, USA or BD Cytofix, BD Biosciences, USA). Samples were washed in 150µl/3ml PBS and centrifuged 400g for 10 minutes. Supernatant was discarded, cell pellet re-suspended in 100µl permeabilization buffer (Caltag reagent B, Caltag Laboratories, USA or Cytofix-cytoperm BD Biosciences, USA) and master mix of intracellular antibodies added.

Antibodies were left to incubate at 4°C for 1 hour prior to two washes with PBS and re-suspension in 100µl fixation buffer (BD Cytofix, BD Bioscience, USA). All samples were stored on ice prior to acquisition, for a maximum of 24 hours.

2.5.1.3 Compensation Controls

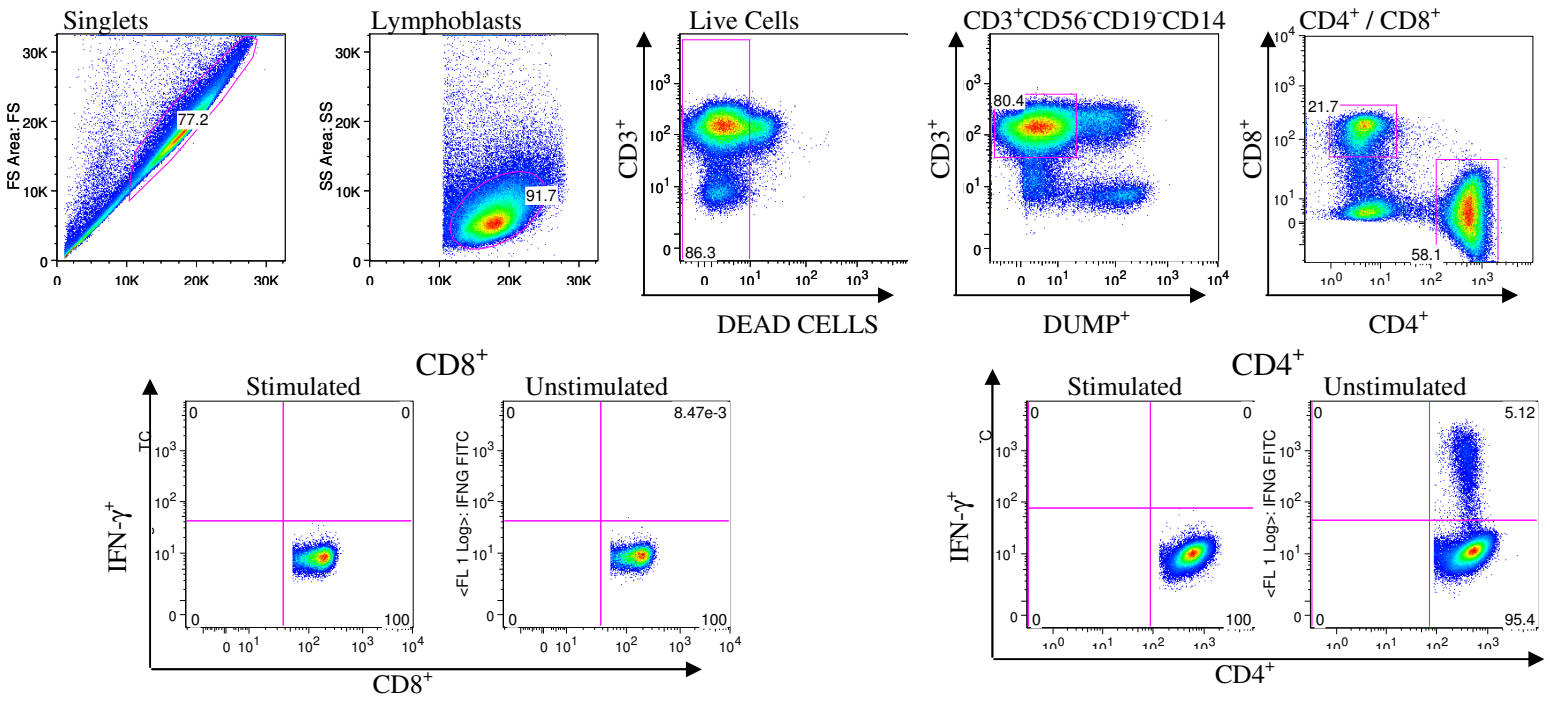
To correct for spectral overlap between the fluorochromes, compensation controls were set up for each experiment. Anti-rat/Mouse Igκ BD™ CompBeads Compensation Particles (BD Biosciences, UK), which bind to any κ light chain bearing immunoglobulin, and negative Control (FBS) particles that have no binding capacity were vortexed for 20 seconds and 1 drop of each added to polystyrene FACS tubes (BD Bioscience, USA). The appropriate volume of each antibody used in the panel was added to the beads and left to incubate at room temperature for 15 minutes. The beads were washed in 4ml PBS, centrifuged at 350g for 10 minutes and re-suspended in 100µl fixation buffer (BD Cytofix, BD Biosciences, UK). Beads were stained at the same time as cell samples and stored at 4°C prior to acquisition.

2.5.1.4 Flow Analysis

Flow cytometry data was analysed using FlowJo Version 8.7.3 (Treestar Inc. USA) the gating strategy used for each panel is shown in Figures 2.3-2.7. A positive response to antigen was defined as >0.05% $CD4^+/CD8^+$ T cells producing IFN-γ, and 2x background for panels 1-3. For panel 5 positive gag specific T cell responses were defined as >0.02% $CD4^+/CD8^+$ T cells producing IFN-γ (criteria based upon negative control data generated using HIV-1 seronegative donors stimulated with irrelevant peptide, by Dr C Riou, South Africa). Accurate gating strategy was achieved using fluorescence minus one (FMO) controls and back-gate analysis[398].

To minimize artificial bias introduced in assessment of TCRVβ dominance, standardised gating was employed to ensure an equal numbers of $CD4^+IFN\text{-}\gamma^+$ cells were analysed across all 8 tubes. The additional gating strategy is illustrated in Figure 2.4. A minimum of 200 IFN-γ^+ T cells were analysed and >20 IFN-γ^+ T cells had to express the TCRVβ for it to be considered positive. This artificial cut off criteria was based upon observations made in control, unstimulated samples.

A

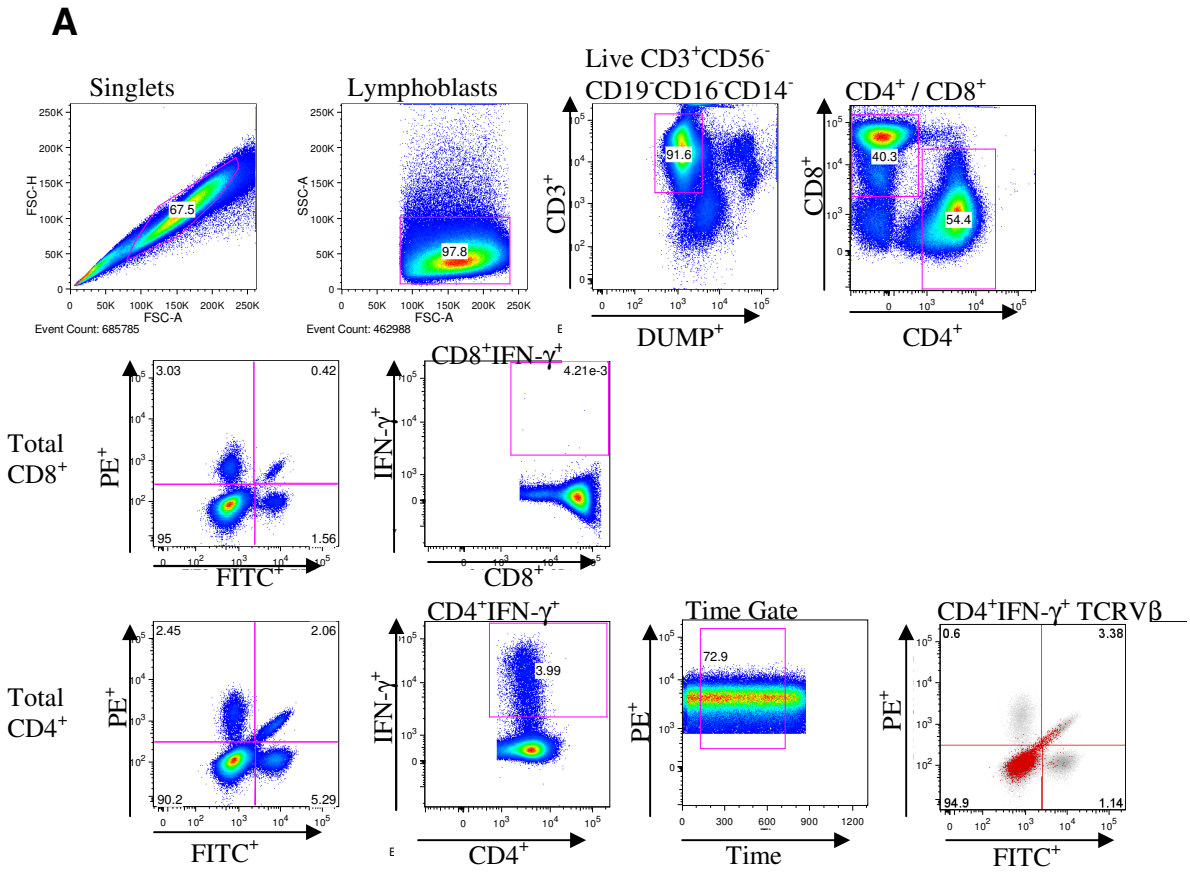


B

	FL 1 Log	FL 2 Log	FL 4 Log	FL 5 Log	FL 6 Log	FL 8 Log
FL 1 Log		26.53	2.556	0	0	0
FL 2 Log	0.754		12.7	1.533	0	0
FL 4 Log	0	0		9.711	0	19.96
FL 5 Log	0.114	3.892	0.933		0	0.269
FL 6 Log	0	0	0	0		0
FL 8 Log	0	0	0.481	0	0.0862	

Figure 2.3

Gating strategy used with flow panel one, to discern whether antigen specific T cell responses were mediated by $CD4^+$ or $CD8^+$ T cells. Stained samples were acquired using a CyAn cytometer (Dako instruments) at a flow rate of <900events per second. A minimum of 100,000 per sample were acquired and compensation performed using compensation beads. The compensation matrix applied to analysis is shown in B).

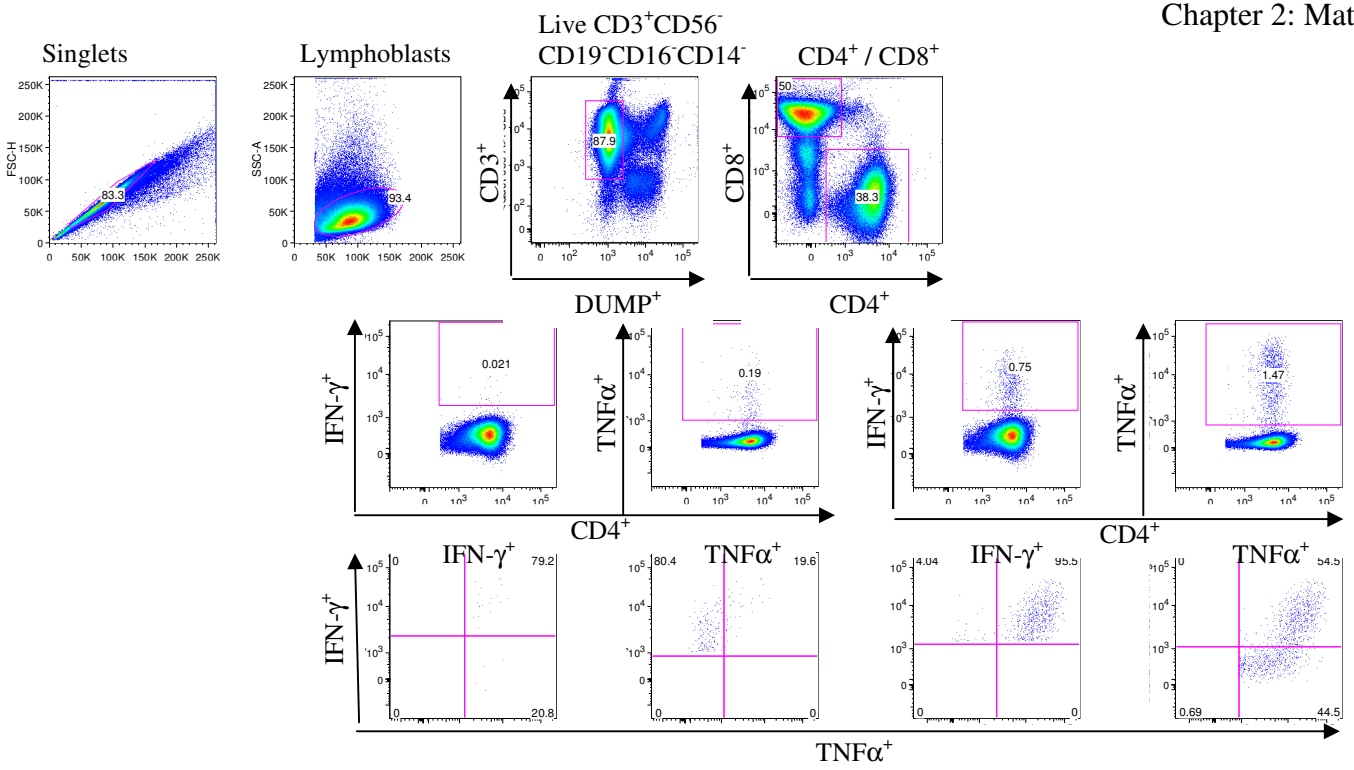


	Alexa Fluor 700-A	APC-A	FITC-A	Pacific Blue-A	PE-A	Qdot 655-A	Qdot 705-A
Alexa Fluor 700-A		2.51	0.117	0	0	0.122	13.4
APC-A	31		0	0	0	9.37	3.86
FITC-A	0	0		0	0.0641	0	0
Pacific Blue-A	0	0	0.127		0	0.132	0.0884
PE-A	0	0	0.588	0		3.36	1.93
Qdot 655-A	0.563	8.26	0	0	0		6
Qdot 705-A	11	4	0	0	0	7.44	

Figure 2.4

The gating strategy used with flow panel two, designed to examine TCRVβ usage in antigen specific T cell responses (A). Stained samples were acquired using an LSRII cytometer (BD Biosciences) at a flow rate of 1000events per second. A minimum of 100,000 per sample were acquired and compensation performed using compensation beads. To ensure equal numbers of IFN-γ^+ T cells were analysed across each of the eight tubes a time gate was applied prior to analysis of TCRVβ staining pattern. The compensation matrix applied to analysis is shown in B).

A



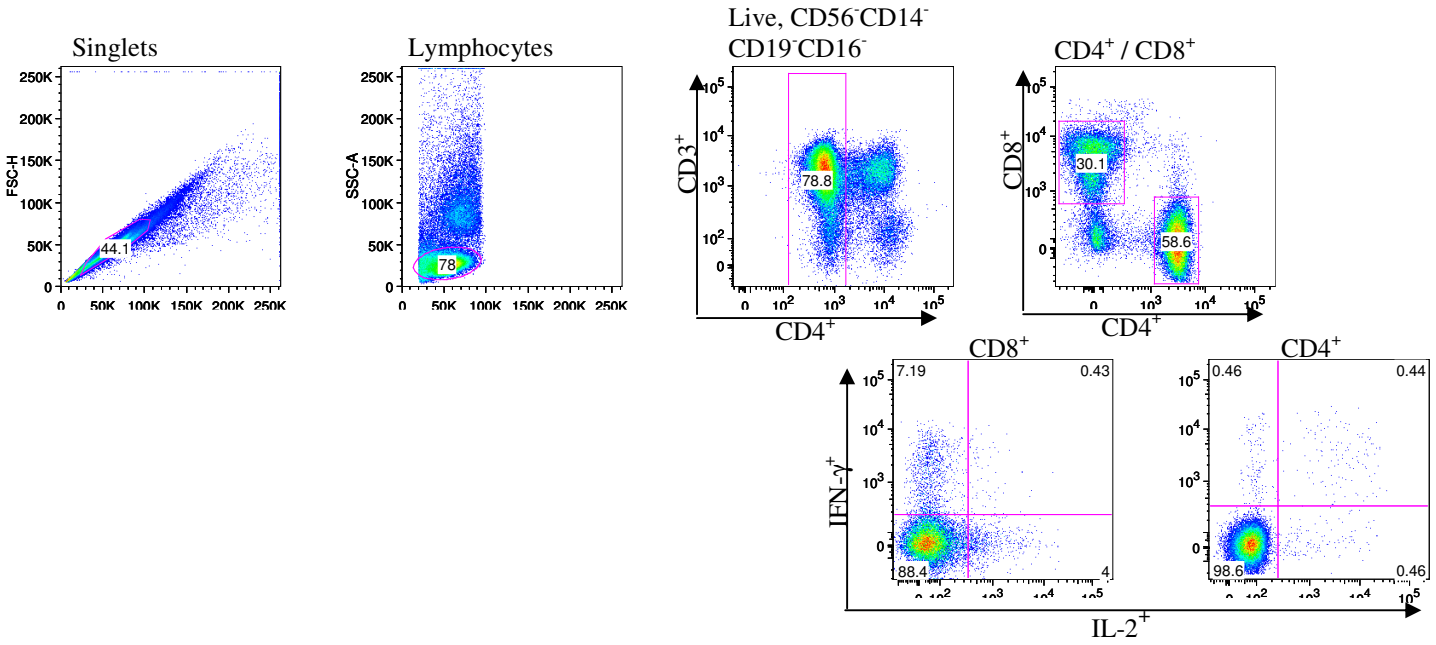
B

	Alexa Fluor 700-A	APC-A	FITC-A	Pacific Blue-A	PE-A	Qdot 655-A	Qdot 705-A
Alexa Fluor 700-A		4.49	0.776	0	0	0.181	12
APC-A	24		0	0	0	7.1	2.93
FITC-A	0	0		0	0.0585	0	0
Pacific Blue-A	0	0	0.0978		0	0.149	0.0813
PE-A	0	0	0.7	0		2.58	1.45
Qdot 655-A	0.595	11.2	0	0	0		7.39
Qdot 705-A	13	7.59	0	0	0	12.3	

Figure 2.5

Gating strategy used with flow panel three, designed to assess whether IFN-γ+ HIV-1 specific T cell responses co-produced TNFα (A). Stained samples were acquired using an LSRII cytometer (BD Biosciences) at a flow rate of 1000events per second. A minimum of 100,000 per sample were acquired and compensation performed using compensation beads. To ensure equal numbers of IFN-γ+ T cells were analysed across each of the eight tubes a time gate was applied prior to analysis of TCRVβ staining pattern. The compensation matrix applied to analysis is shown in B).

A

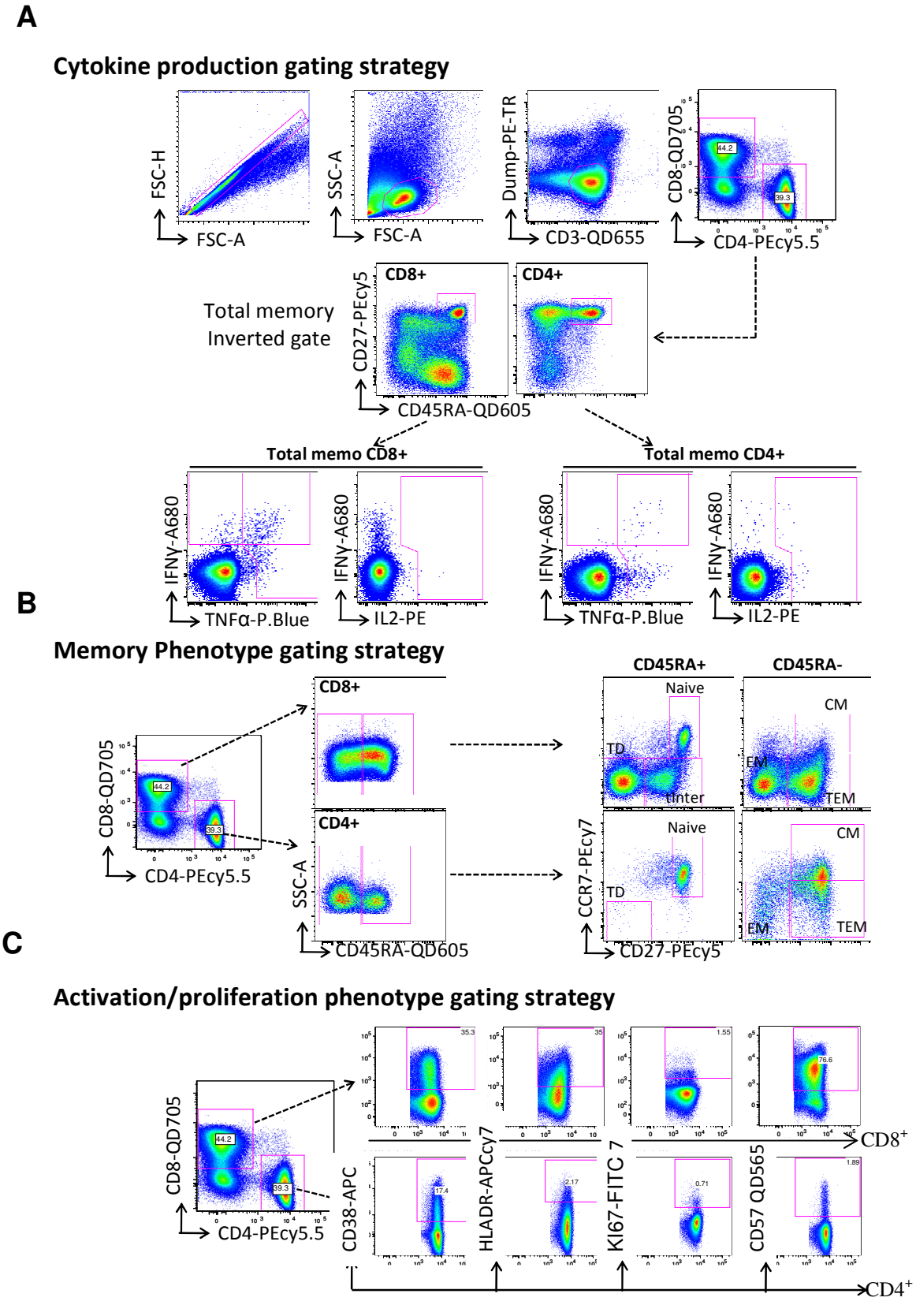


B

	Alexa Fluor 700-A	Pacific Blue-A	PE-A	PE-Cy5-5-A	PE-Cy5-A	PE-Cy7-A	Qdot 605-A	Qdot 655-A	Qdot 705-A
Alexa Fluor 700-A		0	0	13.66	0	10.33	0	0	12.64
Pacific Blue-A	0		0	0	0	0	0.227	0	0
PE-A	0	0		10.91	18.21	3.764	70.79	2.945	1.758
PE-Cy5-5-A	11.03	0	7.066		6.762	58.27	1.247	0.482	12.96
PE-Cy5-A	29.98	0	1.499	90.46		45.26	0	9.279	9.645
PE-Cy7-A	0	0	0.787	0.69	0.211		0	0	0
Qdot 605-A	0	0	0.288	0	0	0		0.17	0
Qdot 655-A	0.429	0	0	1.346	21.79	0	19.49		7.309
Qdot 705-A	11.94	0	0	16.39	2.493	9.385	0	11.54	

Figure 2.6

Gating strategy used with flow panel four, designed to screen HIV-1 seropositive subjects from the VAX004 trial for maintenance of pre-existing HIV-1 specific T cell responses (A). Stained samples were acquired using an LSRII cytometer (BD Biosciences) at a flow rate of 1000events per second. A minimum of 100,000 per sample were acquired and compensation performed using compensation beads. The compensation matrix applied to analysis is shown in B).


A
Cytokine production gating strategy
FSC-H
FSC-A
SSC-A
FSC-A
Dump-PE-TR
CD3-QD655
CD8-QD705
CD4-PEcy5.5
44.2
39.3
Total memory
Inverted gate
CD8+
CD4+
CD27-PEcy5
CD45RA-QD605
Total memo CD8+
Total memo CD4+
IFNγ-A680
TNFα-P.Blue
IL2-PE
B
Memory Phenotype gating strategy
CD8+
CD4+
CD8-QD705
CD4-PEcy5.5
SSC-A
CD45RA-QD605
CD45RA+
CD45RA-
Naive
CM
TD
EM
tInter
TEM
CCR7-PEcy7
CD27-PEcy5
C
Activation/proliferation phenotype gating strategy
CD38-APC
HLADR-APCcy7
KI67-FITC 7
CD57 QD565
CD8+
CD4+

D

	Alexa 680-A	APC-A	APC-Cy7-A	FITC-A	Pacific Blue-A	PE-Cy5-5-A	PE-Cy5-A	PE-Cy7-A	PE-Green-A	QDot 565-A	QDot 605-A	QDot 655-A	QDot 705-A	TR-PE-A
Alexa 680-A		0.308	49.71	0.114	0.09	2.306	0.0644	1.38	0	0	0	0	6.328	0.0652
APC-A	68.63		25.1	0	0	2.265	9.674	0.792	0	0	0	4.771	6.247	0
APC-Cy7-A	2.77	2.934		0	0	0.0894	0.327	4.196	0	0	0	0.136	0.268	0
FITC-A	0	0	0		0	0	0	0	0.21	1.634	0.478	0.0618	0.151	0.114
Pacific Blue-A	0.132	0	0.0651	0		0	0	0	0	5.328	1.415	0.216	0.354	0
PE-Cy5-5-A	52.18	1.29	18.03	0.567	0		8.452	35.33	10.01	1.122	0.771	0.552	22.29	6.27
PE-Cy5-A	69.38	49.79	20.72	0.243	0.0582	52.06		14.8	1.566	0.156	0.105	6.453	17.78	1.208
PE-Cy7-A	0.191	0.0512	11.31	0.708	0	1.03	2.469		11	1.378	1.002	0.173	0.502	6.993
PE-Green-A	0.0602	0	0	5.457	0	6.616	19.66	1.319		14.95	11.46	1.88	2.697	62.64
QDot 565-A	0	0	0	9.401	0	0	0	0	4.708		3.825	0	0.284	0.198
QDot 605-A	0	0	0	0	0	0	0.202	0	0.467	0.687		0.701	0.222	18.95
QDot 655-A	1.869	20.11	0.0834	0	0	1.588	43.29	0.0592	0	0	9.285		39.88	1.785
QDot 705-A	35.1	1.868	16.8	0	0.213	10.99	2.605	4.475	0	0	0.153	5.986		0
TR-PE-A	0.628	0.439	0.145	0.613	0	19.15	44.5	4.757	8.521	0.716	15.16	3.893	7.453	

Figure 2.7

A-C shows the gating strategy used with flow panel five, designed to examine the function and phenotyped of gag specific T cell responses amongst subjects acutely infected with HIV-1. Stained samples were acquired using an LSRII cytometer (BD Biosciences) at a flow rate of ~1000events per second. A minimum of 700,000 events per sample were acquired and compensation performed using compensation beads. The compensation matrix is shown in D.

2.5.2 Tritiated thymidine incorporation

$2.5x10^5$ T cells were cultured in the presence of irradiated autologous monocytes ($2.5x10^4$) which were either pulsed of un-pulsed for 3 hours with peptide pools spanning HIV-1 proteome (clade C consensus). Positive control stimulation was set up in parallel with tetanus toxin (provided by G Galli Novartis Vaccines, Siena, Italy). Proliferation was measured on day 4 after 16 hours incubation with 1μCi/ml [^{3}H] Thymidine (GE Healthcare). Briefly cells were harvested using Micro96 harvester (Bucher Biotec, Switzerland), dried at 56 degrees for a minimum of 1hour, placed in a microbeta sample bag (Perkin Elmer) with 1450 microbeta scintillation fluid and sealed using Wallac heat sealer (Wallac, USA). Proliferation was measured using 1450 Microbeta liquid scintillation and luminescence counter (Wallac Trilux, USA).

2.5.2.1 Criteria for positive response by [^{3}H] Thymidine assay

Criteria for a positive response was defined as a stimulation index of >5 and a delta value (cpm in response to antigen-pulsed monocytes-cpm in response to unpulsed monocytes) of greater than $3x10^3$cpm. These criteria were based upon observations made across multiple negative and positive samples assessed by T cell library technique and represented the 99th percentile of delta cpm obtained from unstimulated samples studied within Professor F Sallusto's laboratory[308].

2.5.3 ELISpot assays

96 well Multiscreen HPTS plates (Millipore, USA), were pre-wet with 70% ethanol, 50μl per well, for 1-3 minutes prior to washing with 180μl/well sterile PBS (Sigma, UK) four times. Interferon gamma (IFN-γ monoclonal antibody (mAb), 1DIK (Mabtech, Sweden) was prepared at 0.5mg and added 100μl per well. Plates were wrapped in Saran Wrap (Dow Chemical Company, UK) and stored at 4°C prior, for a maximum of 1 week prior to use. Prior to addition of cells, pre-coated plates were washed once with sterile PBS (180μl per well) and blocked for 2 hours with 180μl per well R-10 media (2.2.1.1) at 37°C 5% CO_2. PBMC were re-suspended at $4x10^6$ cells/ml (*ex vivo* assays) or $0.8x10^6$ cells/ml (cultured assays) using R-AB-10 media (2.2.1.2) and added 50μl/well to plate. Cells were rested 30-60 minutes at 37°C/5% CO_2 prior to addition of antigen. Following 18-20 hour

incubation at 37°C/5% CO_2, cells were discarded and plates washed 8 times with PBS 0.05% tween 20 (Sigma, UK) (referred to herein as PBS Tween). 100µl of sterile filtered, biotinylated, anti IFN-γ mAb (7-B6-1 biotin, Mabtech, Sweden) (diluted to 0.5µg/ml using PBS 0.1% bovine serum albumin (BSA) Sigma, UK) was added to each well and incubated overnight at 4°C.

2.5.3.1 Visualization ELISpot assays

Vectorstain Elite avidin biotin complex (ABC) (Vector laboratories Inc, UK) was prepared at 1 drop avidin, 1 drop biotin per 5ml PBS Tween and left to conjugate at room temperature 2 hours. Plates were washed 8x with PBS Tween prior to addition of pre-conjugated ABC (100µl/well). Following 1 hour incubation at room temperature, plates were washed 8x with PBS tween (Sigma, UK), followed by 4 PBS (Sigma, UK) washes. Acetate solution consisting of 43.7ml water (Sigma, UK), 3.5ml 0.1M Acetic Acid (Sigma, UK), 280µl sodium acetate buffer (Sigma, UK), was mixed with 3-Amino-9-ethylcarbazole (AEC) (Sigma, UK) solution (1 AEC tablet dissolved in 2.5ml Dimethylformamide (DMF)). Filtered using 0.22µm filter (Millipore, USA), 25µl hydrogen peroxide solution (Sigma, UK) was added and the solution immediately added to plates at 100µl per well. The reaction was halted after 4 minutes by immersion in water, and plates left to air dry. The number of spot forming units (SFU) was counted using an automated ELISpot plate reader (AID Systems, Germany) and expressed as SFU/million PBMC.

2.5.3.2 Criteria for positive/negative response

A positive ELISpot response was defined as 4x mock and/or greater than 300 SFU/million (cultured assays) / 50 SFU/million (*ex vivo* assays). As an additional control the response had to be observed in both the "All" pool and one or more of the sub pools (sub pool rule). The assay was classed as "failed" and data excluded from analysis should the mock wells have greater than 250 SFU/million. These stringent criteria were based upon observations made in healthy volunteers enrolled in an IAVI sponsored vaccine trial[391] and represented >99th percentile, of background subtracted T cell responses in pre-vaccination samples[391].

2.6 Molecular biology techniques

2.6.1 TCR Vβ clonotyping

2.6.1.1 Proteinase K digest

Endogenous RNases and DNases were inactivated and DNA isolated using proteinase K. Proteinase K (Roche,UK) was diluted to 100µg/ml using 10mM Tris-hydrochloride (HCL) pH 8 diluted in Tris water (Sigma, UK). 10µl of 100µg/ml Proteinase K (Roche, UK) was layered onto 100,000 cells and vortexed prior to incubation at 56°C for 1 hour. Following centrifugation at 16000g for 1 minute the proteinase K was inactivated by incubating at 95°C for 10 minutes.

2.6.1.2 Heminested touch down polymerase chain reaction (PCR)

TCRBV and TCRBJ primers, spanning all 23 TCRBV and 13TCRBJ primers were divided into 3 tubes (see Table 2.11) to reduce primer dimerization, cross priming and non-specific amplification. As previously published[399] PCR cycling conditions were as follows: 1x HiFi buffer, 3mM $MgSO_4$, 200µM dNTPs, 0.056U platinum Taq Hi-Fi DNA polymerase (Invitrogen, USA) and 10pmol of each primer and 7µl cell lysate in a final volume of 50µl. The first PCR reaction used external TCRBJ and BV primers (Table 2.11). Following activation at 95°C for 5 minutes cycling conditions were as follows:

{95^{o}C / 30seconds} + {68^{o}C / 60seconds} x2
{95^{o}C / 30seconds} + {65^{o}C / 30seconds} + {68^{o}c / 30seconds} x3
{95^{o}C / 30seconds} + {60^{o}C / 30seconds} + {68^{o}c / 30seconds} x30

The PCR product was purified using nucleospin Extract II (Fisher, UK) kit as per the manufacturer's instructions, and used as a template for the second PCR reaction. The second PCR reaction used internal primers (Table 2.11), under the same conditions as the first. PCR product was visualized upon a 2% agarose gel, using cyber gold (Invitrogen, UK), as a 250-300bp band (Figure 2.8A). The band was cut from the gel and DNA extracted using NucleoSpin Extract II (Fisher, UK) as per manufacturers' instructions. Briefly, gel was dissolved at 56°C for 10 minutes in 2 volumes NT buffer to 1 volume gel. 600ml NT buffer was added to

binding column and spun 11000g for 1minute. Flow through was discarded and 600µl NT3 buffer added before being centrifuged at 11,000g for 1 minute. The flow through was discarded and the tube spun dry at 11000g for 1 minute. The DNA was then eluted by adding 30µl of NE buffer and centrifuging at 30µl/ml. DNA was stored 4°C until use.

Primer Name	Sequence	Primer Name	Internal TCRBJ primers	External TCRBJ primers
BV2	ATACTTCTATTGGTACAGACAAATCT	BJ1.1	CTTSCCTACAACTGTGAGTCTGGTG	AAAAATGTCTTACCTACCTACAACTG
BV3	CGCTATGTATTGGTATAAACAG	BJ1.2	CTTACCTACAACGGTTAACCTGGTC	CCCAGCCTTACCTACAAC
BV4	CGCTATGTATTGGTACAAGCA	BJ1.3	CTCACCTACAACAGTGAGCCAACTT	TGACTTACTCACCTACAAC
BV5	CAGTGTGTCCTGGTACCAACAG	BJ1.4	CATACCCAAGACAGAGAGCTGGGTTC	TCTTTTACATACCCAAGAC
BV6a	ATACATGTACTGGTATCGACCAACAG	BJ1.5	CTTACCTAGGATGGAGAGTCGAGTC	TTCTGCAACTTACCTAGGAT
BV6b	GGCCATGTACTGGTATCGACAAG	BJ1.6	CATACCTGTCACAGTGAGCCTG	GAGCCCCCATACCTGTCAC
BV6c	GTATATGTCCTGGTATCGACAAGA	BJ2.1	CCTTCTTACCTAGCACGGTGA	CCTGGAGCCCCCTTCTTAC
BV7a	AACCCTTTATTGGTACCGACA	BJ2.2	CTTACCCAGTACGGTCAGCCT	CCGCCTCCTTACCCAGTAC
BV7b	AACCCTTTATTGGTATCAACAG	BJ2.3	CCCGCTTACCGAGCACTGTCA	AGCCCCCGCTTACCGAGCAC
BV7c	ATCCCTTTTTTGGTACCAACAG	BJ2.4	CCAGCTTACCCAGCACTGAGA	GCCCCAGCTTACCCAGCAC
BV11	TACCCTTTACTGGTACCGGCAG	BJ2.5	CGCGCTCACCGAGCAC	AGCCCGCGCTCACCGAGCAC
BV12a	CTCCCGTTTTCTGGTACAGACAGAC	BJ2.6	CTCACCCAGCACGGTCAGCCT	GCGAAAACTCACCCAGCAC
BV12b	CACGGTCTACTGGTACCAGCA	BJ2.7	CTCACCTGTGACCGTGAGCCTG	GCCCGAATCTCACCTGTGAC
BV14	TAACCTTTATTGGTATCGACGTGT			
BV15	CGTCATGTACTGGTACCAGCA			
BV18	TCATGTTTACTGGTATCGGCAG			
BV19	GGCCATGTACTGGTACCGACA			
BV20	AACTATGTTTTGGTATCGTCA			
BV21	TTATGTTTACTGGTATCGTAAGAAGC			
BV23	TTATGTTTATTGGTATCAACAGAATCA			
BV25	CAAAATGTACTGGTATCAACAA			
BV29	CACGATGTTCTGGTACCGTCAGCA			
BV30	CAACCTATACTGGTACCGACA			

Table 2.11
TCRBV and TCBJ primers used for multiplex PCR. The primers were split into three tubes, each tube contained all 23 TCRBV, TCRBJ primers shown in red were added to tube A, primers shown in blue were in tube B and primers shown in black were pooled into tube C. All primers were used at a final concentration of 10pmol.

2.6.1.3 Ligation

PCR4. TOPO (Invitrogen) used as per manufacturer's instructions. Briefly, 4µl DNA/water was added to 1µl salt solution (NaCl+MgCl) and 1ml Topo vector. The tube was gently flicked to mix and left 30 minutes at room temperature before being stored on ice prior to bacterial transformation.

2.6.1.3.1 Bacterial transformation

50µl DH5α competent cells (Invitrogen, UK) was added to each ligation, and heat shocked at 42°C for 30seconds prior to return to ice. Using sterile technique 950µl of SOC media (Invitrogen, UK) was added to each transformation tube and placed in a 37°C thermomixer 750rpm for 1.5 hours.

2.6.1.3.1.1 Preparation of LB-AIX plates

200mg IPTG (Invitrogen, UK) were dissolved in 1ml deionised water. 50mg X-GAL (Invitrogen, UK) was dissolved in 2.5ml dimethyl formamide (DMF). Dissolve amplicillin to 100mg/ml (Sigma, UK). 32 capsules of LB Agar Medium (Qbiogene, UK) were added to 800ml water and sterilized at 121°C. After cooling to ~60°C, 800µl ampicillin, 2ml XGAL, and 200ml IPTG was added. Plates were poured and stored 4°C until use.

2.6.1.3.2 Plating bacteria

200µl transformed cells was added to dry AIX plate and evenly spread with a glass rod. Once all plates were spread they were stored upside-down at 37°C for 16-18 hours. Plates were stored at 4°C until colony picking.

2.6.1.3.3 Colony picking

Colonies that have successfully incorporated the CD3 amplicon are white, blue colonies are negative. 95 white colonies were picked using a sterile toothpick. Briefly the toothpick was used to stab the centre of the colony and to transfer to PCR well containing PCR mastermix (Table 2.12). The last well of the PCR plate acted as a negative control, and therefore had a blue colony picked.

Reagent	Supplier	Volume to add per well
10x HiFi Buffer	Invitrogen	2.5µl
MgSO4	Invitrogen	1µl
DTNP	Invitrogen	0.5µl
M13 forward primer (5µM)	Invitrogen	1µl
M13 reverse primer (5µM)	Invitrogen	1µl
Platinum Taq	Invitrogen	0.14µl
Water	Sigma	18.86µl

Table 2.12

Colony PCR mastermix

2.6.1.3.4 Colony PCR

Cycling conditions for the PCR reaction were as follows:

{95°C / 30seconds} + {68°C / 60seconds} x2

{95°C / 30seconds} + {65°C / 30seconds} + {68°c / 30seconds} x3

{95°C / 30seconds} + {60°C / 30seconds} + {68°c / 30seconds} x30

2.6.1.3.5 Amplification check

Colony PCR product was visualized on 1% agarose gel as a 750bp band. Briefly 5µl colony PCR product was mixed with 1µl Track it Cyan/Orange buffer (Invitrogen) loaded into gel and run 200mA for 40 minutes. The gel was stained with SYBR Gold (Invitrogen, UK) for 25 minutes and visualized under UV light (Figure 2.8B).

2.6.1.3.6 Sequencing

TCR sequencing was performed by Beckman Coulter Genomics (USA). For clonotype classification the International Immunogenetics Information System (IMGT)[400] nomenclature was used.

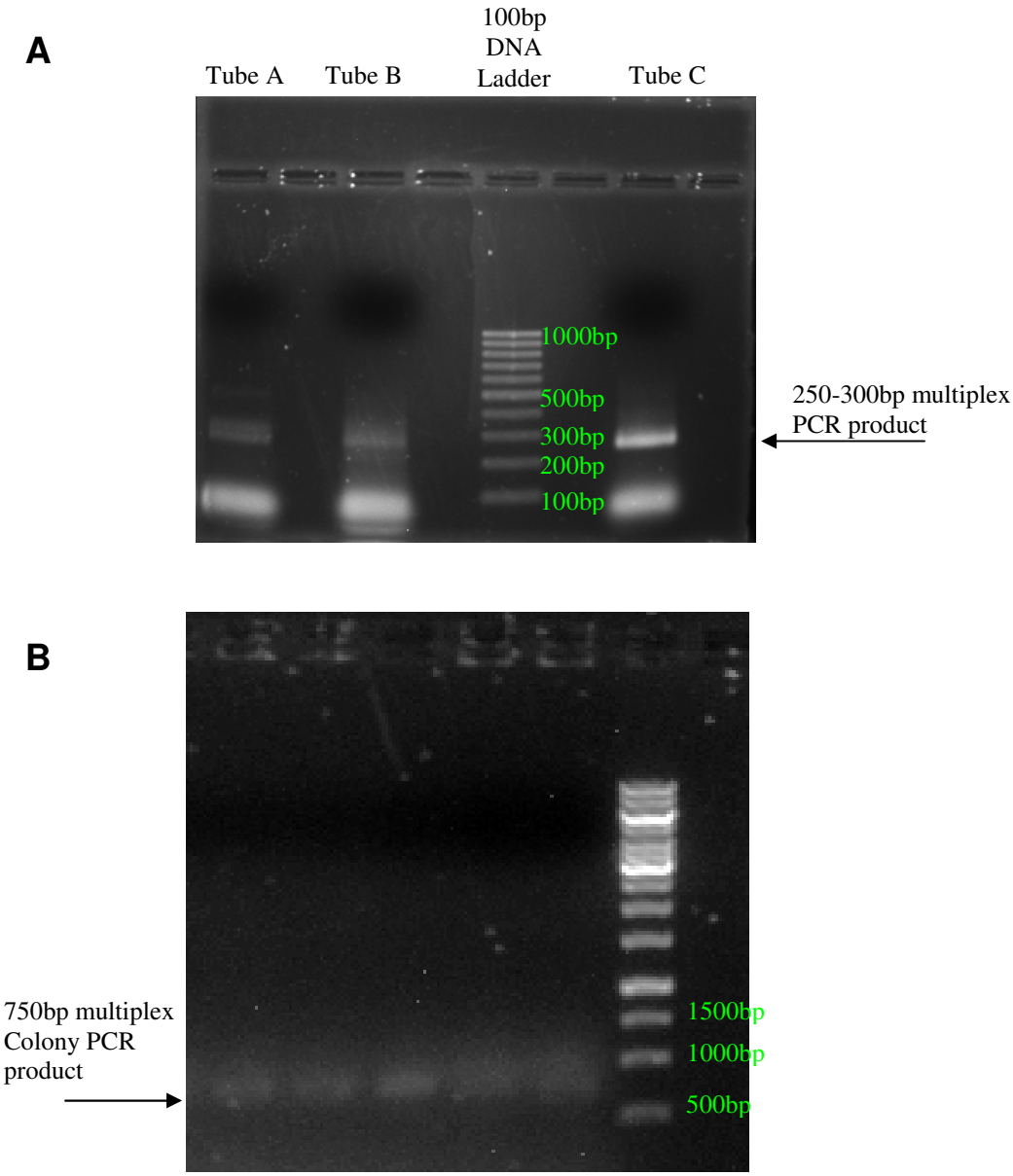


Figure 2.8

TCR multiplex PCR products were visualized on a 2% agarose gel using cyber gold, as a 250-300bp band (A). The band was cut from the gel and DNA extracted using NucleoSpin Extract II bands and ligated into DH5α competent cells prior to colony PCR. To confirm amplification, colony PCR product was visualized on 1% agarose gel, using cyber gold, as a 750bp band (B).

2.6.2 Examination of CCR5Δ32

2.6.2.1 DNA Extraction

DNA was extracted from participant samples using 5 PRIME Achieve Pure DNA kit (Prima scientific Co, Ltd, Thailand) as per manufacturer's recommendations. The purity and quantity of DNA extracted was assessed using a Nanodrop (ND-1000, Thermo scientific Inc, USA) and only DNA with a 260/280 ratio of 1.8 -2.0 was used.

2.6.2.2 CCR5 Δ32 PCR

A stock mix of primers, deoxynucleotide triphosphates (DTNPs), magnesium chloride ($MgCl_2$) and PCR buffer was prepared (Figure 2.9A) and stored at -20°C in 1ml aliquots. Prior to use the required volume of stock mix was defrosted and 5µl/ml BioTAQ (BioTAQ Inc, USA) added to create a working stock. 2µl of genomic DNA from each sample was added to a PCR plate followed by 23µl of the working stock. Each well was then layered with 10µl of mineral oil, sealed and briefly centrifuged prior to cycling on a PCR machine (Figure 2.9C)

A)

3µl 10x PCR buffer
240µl 100mM dNTPs
1.2µl 50mM $MgCl_2$
300µl of 4mM stocks of CCR5 primers

B)

CCR5 Primer 1	GAA GTT CCT CAT TAC ACC TGC AGC TCT C
CCR5 Primer 2	CCT CTT CTC ATT TCG ACA CCG AAG CAG AG

C) {96^{o}C / 50seconds} + {70^{o}C / 50seconds} + {72^{o}C / 50seconds} x5
{96^{o}C / 50seconds} + {61^{o}C / 50seconds} + {72^{o}C / 50seconds} x15
{96^{o}C / 50seconds} + {55^{o}C / 50seconds} + {72^{o}C / 50seconds} x10

Figure 2.9

(A) PCR stock mix recipe (B) CCR5 Primer Sequences and (c) cycling conditions used in CCR5 Δ32 PCR.

PCR product was resolved on a 2% agarose gel and imaged using UVP Biospectrum® AC imaging system (Ultra-Violet Products Ltd, UK). The presence of a band at 174bp indicated that the individual is "wildtype" and does not possess the CCR5Δ32 mutation. A single band at 142bp indicated the individual is homozygous for the

CCR5Δ32 mutation, whilst two bands at 174bp and 142bp respectively indicated heterozygous genotype.

2.6.3 Transcription mediated amplification assay (TMA)

Blinded, low titre virus detection was performed by Professor E. Delwart's laboratory (University of California) using isothermal transcription mediated amplification assay (TMA). TMA (Aptima; Gen-Probe, San Diego, CA) has a 50% detection limit of 3.6 to 14 copies of RNA/ml when performed in singlicate[401,402]. Plasma volumes of 0.5-1ml were tested a medium of 6 times, across multiple study visits. The output is a signal-to-cut off (S/Co) ratio (range, 0 to 30), with S/Co ratios of <1.0 considered HIV RNA "negative" and S/Co ratios of ≥1.0 considered HIV RNA "positive".

2.7 Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software (GraphPad Software). P values of <0.05 were considered significant. Data examined using D'Agostino and Pearson omnibus normality test to determine whether it followed a normal/non parametric distribution and statistical test chosen accordingly (Table 2.13).

Power calculations performed for the VAX004 cohort were kindly performed by Dr P Gilbert at The Statistical Centre for HIV/AIDS Research & Prevention (SCHARP).

The precursor frequency of antigen specific $CD4^+$ T cells was calculated based on the number of negative wells from T cell library assays using Poisson distribution[308,403]. Briefly the calculation was as follows:

% Cloning efficiency calculated as (LN(number of negative wells/total number of wells tested)) / total number of cells seeded per wellx100. *Where LN = natural logarithm.* Next the % cloning efficiency was translated to the number of cells that would need to be screened in order to detect 1 antigen specific cell (1 cell per) using the equation (1 / % cloning efficiency) x100. The number of antigen specific cells per $1x10^6$ cells was then extrapolated using the equation ($1x10^6$ / 1 cell per)x100.

For example:

Within the naïve $CD4^+$ T cell subset of Leuk 3, 19 cell lines out of 192 showed a positive proliferative response to TT. Cells were seeded at 2000 cells/well

% Cloning efficiency = ((LN(173/192))/192 x100) 0.005210189

1 cell per = ((1/0.005210189)x100) 19193.16216

Precursor frequency = 52.1 Naïve $CD4^+$ TT specific cells per $1x10^6$ naïve $CD4^+$ T cells

Data from the CHAVI002 cohort was independently analysed by Dr Z Moodie (SCHARP). To account for the correlation in the repeated measurements over time, generalized, estimating equations with Wald analysis were applied to HESN/HUSN data to look for significance.

Analysis to be performed	Gaussian Distribution	Non Gaussian Distribution
Comparison 2 unpaired groups	Unpaired T test	Mann Whitney U test
Comparison 2 paired observations	Paired T test	Wilcoxon test
Comparison 3 or more un paired observations	One way ANOVA	Kruskal-Wallis test
Comparison 3 or more paired observations	Repeated-measures ANOVA	Friedman test

Table 2.13

Rationale behind the application of statistical tests to data generated within this thesis.

2.8 Online database and search tools

2.8.1 Basic Local Alignment Search Tool (BLAST)

Mapped HIV-1 specific T cell responses were screened using the NCBI BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). 5000 non-redundant protein sequences were searched using an expected threshold of 10. No compositional adjustments were made.

2.8.2 Los Alamos National Laboratory (LANL) HIV-1 database

The HIV-1 Molecular Immunology database (http://www.hiv.lanl.gov/content/immunology)[404] was used to screen mapped HIV-1 peptides for putative CD4/CD8 T cell epitopes.

The HIV-1 sequence database (www.hiv.lanl.gov.)[21] was used to perform calculations of Shannon entropy and to align promiscuous epitope sequences to consensus/ancestral sequences.

CHAPTER 3:

$CD4^+$ T CELLS IN ACUTE

HIV-1 INFECTION

Chapter 3: CD4+ T cells in acute HIV-1 infection

3.1 Introduction

To gain insight into the types of acute human immunodeficiency virus (HIV-1) specific T cell responses that may be detected amongst HIV-1 exposed seronegative (HESN) individuals it would seem important to understand the cellular immune response that occurs during acute HIV-1. HIV-1 infection is characterized by a profound loss of $CD4^+$ T cells and high viral loads, typically peaking at around 10^7-10^8 copies per ml[53,405]. Generally, acute viremia falls 10-200 fold within 12-20 weeks, achieving a steady state, referred to as viral set point[49,169,170,405]. Early observations showed a temporal association between the emergence of $CD8^+$ T cell responses and decline in peak HIV-1 viremia leading to the presumption that $CD8^+$ T cell responses play a critical role in exerting immune pressure upon HIV-1[206]. In a seminal paper by Schmitz *et al*, the artificial depletion of $CD8^+$ T cells in simian immunodeficiency virus (SIV) was shown to result in a dramatic rise in viremia, a failure to achieve viral set point and accelerated clinical disease progression[358]. More recently the rapid evolution of viral mutations in $CD8^+$ T cell targeted regions[38,71,73,146,406-408] and correlations between particular human leukocyte antigen (HLA) class 1 alleles and non-progressive HIV-1 infection[74,251,252,409] have been made. Although circumstantial, these observations underscore the hypothesis that $CD8^+$ T cells play a critical role in control of HIV-1 viremia.

By contrast, the role of $CD4^+$ T cell responses in HIV-1 infection remains poorly understood, and relatively understudied. Known for their "helper" qualities, the synergistic interaction between the different $CD4^+$ T cell subsets, B cells and cytotoxic $CD8^+$ T cells have been shown to play a critical role in the clearance of a number of viral infections[338,340,367,410-412]. Moreover, murine studies have shown $CD4^+$ T cells are required for the successful generation and reactivation of memory $CD8^+$ T cells during viral infection[332,340,341,343,367,411,413]. It is possible therefore, that prior to their profound depletion, $CD4^+$ T cells may play an important role in the control of HIV-1 viremia by providing cognate help to $CD8^+$ T cells and helping to orchestrate the humoral response to HIV-1. Indeed, potent, maintained HIV-1 specific, $CD4^+$ T cell responses have been associated with improved control of viral replication and

delayed progression to acquired immunodeficiency syndrome (AIDS) defining illness[16,76,414,415], although it remains unknown whether the detection of maintained $CD4^+$ T cell responses is cause or effect of improved viral control.

The study of $CD4^+$ T cells during acute HIV-1 infection is inherently complicated. Not least due to the fact that around 24-48% of all circulating and lymph associated memory $CD4^+$ T cells are eliminated between days 10-14 of infection[53]. Furthermore obtaining blood samples from subjects in the acute stages of HIV-1 infection is notoriously difficult, since acute HIV-1 infection is often asymptomatic and the majority of patients do not come to medical attention until weeks/months post infection. In a retrospective study performed using longitudinal plasma samples obtained from 51 subjects Fiebig *et al*, defined the time points of appearance of HIV-1 markers and viral load concentrations during primary HIV infection[393], into what is now commonly referred to as Fiebig staging. In summary, Fiebig stage 0 is the eclipse phase which is characterized by undetectable viral markers in blood samples and is reported to last on average up to 11 days from viral exposure. At stage I HIV-1 is present in blood samples but only RNA assays are positive. At stage II RNA and HIV-1 p24 antigen tests are positive but antibody enzyme linked immunoassay (EIA) is non-reactive. By stage III RNA, HIV-1 antigen and HIV Immunoglobulin (Ig) M-sensitive EIA are reactive, but Western blot is negative. By stage IV an indeterminate Western blot result is obtained, that is to say there are HIV-1 specific Western blot bands present to either p24, gp41, gp120/160, however, they fail to meet the criteria for a positive classification as defined by the USA Food and Drug Administration. Western blot reactivity to two of the 3 HIV-1 proteins is detected at stage V. At stage VI there is Western blot reactivity to p24, gp41, gp120/160 and p31[393].

Of the few studies made during primary HIV-1 infection (defined as 22 days post onset symptoms-first 12months of infection), $CD4^+$ T cells have been shown to be highly activated ($CD38^{+++}HLADR^+$) and proliferating ($Ki67^+$)[303,328,416]. There is growing evidence to suggest that immune activation plays a critical role during HIV-1 infection. Indeed recent studies have shown that expression of activation markers such as HLADR and CD38 provide a more reliable means by which to predict disease progression independent of $CD4^+$ T cell count and viral load[298-300,304-306,417-419]. It is hypothesized that activated T cells may contribute to a poor prognosis by supporting

higher levels of viral replication and/or by causing inflammation-associated-damage to the immune and other organ systems. The role of $CD4^+$ T cell activation is clearly a double edged sword, in order for an effective immune response, HIV-1 specific T cells must be activated, yet activation itself leads to increased viral replication and correlates with a worsening clinical outcome[299,304,417,420]. This suggests a delicate balancing act must be achieved to limit excessive immune activation yet maintain HIV-1 specific T cell responses.

Additional studies of primary HIV-1 infection have shown $CD4^+$ T cells to secrete a range of cytokines, including IFN-γ and IL-2 as well as cytotoxic molecules such as perforin and granzyme B[303,363]. Viral escape from $CD4^+$ T cell targeted regions is rare, suggesting $CD4^+$ T cells are unlikely to be major contributors to the control of viremia[421,422]. Despite an initial burst of $CD4^+$ HIV-1 specific T cell immunity, $CD4^+$ T cell responses rapidly decline as infection progresses[303,373]. This contraction of the $CD4^+$ T cell response pool is most likely to be the result of preferential infection by HIV-1[368] and direct/bystander killing by $CD8^+$ T cells. However, contraction of the $CD4^+$ T cell response pool has been detected in other chronic viral infections such as Sendai virus and cytomegalovirus (CMV) suggesting $CD4^+$ T cell responses often naturally contract following the initial burst of viremia[423-425]. Coupled with the on-going $CD4^+$ T cell depletion associated with HIV-1 infection, this contraction phase, compounds the need to obtain acute HIV-1 samples if the role of $CD4^+$ T cell responses are to be fully understood.

The decline in $CD4^+$ T cell responses and proliferative capacity during chronic HIV-1 infection is accompanied by an increased expression of exhaustion or senescence markers, and has been linked to a loss of control of HIV-1 viremia and T cell dysfunction[426,427]. Indeed markers such as programmed death-1 (PD1) and CD57 have been associated with reduced proliferative capacity[428,429], and increased susceptibility to apoptosis[426,429,430]. In non-human primate models the blockade of PD-1 has been shown to significantly reduce viral load, increase SIV specific T cell responses and correlates with improved survival times[431].

Where detected in chronic HIV-1 infection, CD4+ T cell responses are of extremely low magnitude and often narrowly focused[373]. The narrow targeting of CD4+ T cell responses is not unique to HIV-1 and a marked hierarchy of immunodominant CD4+ T cell responses has been seen during Epstein Barr Virus (EBV) infection[432]. Whilst during acute HIV-1 infection relatively broad and potent CD4+ T cell responses can be detected[433], a number of studies have identified Gag as the primary target of CD4+ T cell responses, particularly during chronic stages of infection[373,374]. Not only is gag proposed to have the highest epitope density compared to other HIV-1 proteins[373,374] but CD4+ T cell responses to gag have been correlated with viral control[76,374,414,434,435].

HIV-1 infection is also known to cause perturbations in the memory compartment. Central memory CD4+ T cells (TCM; CCR7+, CD45RA-)[316] are particularly affected during HIV-1 infection and have been shown to be rapidly depleted during the acute stages of HIV-1[317,420], and all but absent during chronic infection[317,436]. To date no association between memory CD4+ T cell subsets and viral load has been detected[299,328]. Although, preservation of the CD4+ TCM subset, has been correlated with protection in non-human primates, and long-term non-progression in humans[318,329,415,437,438].

In summary, the cellular immune events that occur during acute HIV-1 play an important role in determining the subsequent course of HIV-1 infection. Due to the difficulties associated with obtaining blood samples from subjects in the acute stages of HIV-1 infection the functional and phenotypic profile of the earliest HIV-1 specific T cell responses, particularly those mediated by CD4+ T cells, are yet to be fully characterized. A greater understanding of CD4+ T cell events during acute HIV-1 should prove beneficial not only in the design of a novel therapeutic vaccine, but may also aid in the development of improved diagnostics or simply improve the timing of initiation of current ART.

3.2 Chapter Aims

HIV-1 T cell research has focussed on CD8$^+$ T cells, which are widely believed to play a critical role in HIV-1 infection. However, there appears to be a discrepancy between the quality of HIV-1 specific CD8$^+$ T cell responses during acute HIV-1 infection and those that manifest later in the disease. Whilst the emergence of HIV-1 specific CD8$^+$ T cell responses correlates with initial fall in peak viremia, subsequent expansion of CD8$^+$ T cell responses fails to confer further protection. Given CD4$^+$ T cells are known to provide "help" for both the initiation and reactivation of memory CD8$^+$ T cells, it is possible that impairment and loss of the CD4$^+$ T cell subset is associated with the failure to control HIV-1 viremia.

Relatively little is known about the kinetics or functional profile of HIV-1 specific CD4$^+$ T cell responses that occur during acute HIV-1 infection. With access to acutely infected, clade C subjects and in a study performed in collaboration with Professor C Gray (National Institute of Communicable Diseases (NICD), Johannesburg, South Africa), this chapter aims to examine both the kinetics and functionality of HIV-1 specific CD4$^+$ T cell responses during acute HIV-1 infection. Multiparameter flow panels designed and optimized by Dr C Riou (NICD, Johannesburg, South Africa) will be employed to enable changes in memory CD4$^+$ T cell subsets, activation status and poly-functionality to be longitudinally assessed across 5 study visits. Based on a limited number of samples (n=6) this work will primarily serve as a hypothesis generating study. A greater understanding of the HIV-1 specific T cell responses detectable during acute HIV-1 infection may provide indication of protective immune responses and could provide insight into the types of HIV-1 specific T cell responses that may be detected amongst HESN individuals.

I performed all staining, acquisition and analysis of samples presented herein with supervision from Prof C Gray and Dr C Riou.

3.3 Results

3.3.1 Cohort Characteristics

Six subjects acutely infected with HIV-1 clade C were recruited within CHAVI 001 cohort. All subjects were within Fiebig stage I/II at screening, that is to say they had detectable HIV-1 RNA in plasma and were p24 antigen test positive[393]. For the purposes of data analysis, time post infection was adjusted to days post Fiebig stage I/II[393]. On average the first available time point for study was 35 days post Fiebig I/II (range 19-68 days) and subjects were followed longitudinally for an average of 548 days post Fiebig I/II (range 447-628 days, Figure 3.1). Greater than 700,000 events were acquired per subject and functional and phenotypic analysis of gag specific IFN-γ^+ T cells was performed on no less than 30 events.

All subjects were treatment naïve for the duration of study, and all were male aged from 17-42 years (Full cohort details available Chapter 2 Section 2.1.2). For the majority of subjects, viral load was on the decline at the first time point analysed and profound loss of CD4$^+$ T cells had already occurred, with subjects on average having an absolute CD4$^+$ T cell count of just 665cells/μl (Figure 3.1).

3.3.2 Generalized immune activation

In the first analysis generalized immune activation status was assessed by surface expression of CD38 and HLADR upon total CD4$^+$/CD8$^+$ T cells (Figure 3.2). The overall immune activation status of the CD4$^+$ T cell compartment (Figure 3.3) was lower than that of CD8$^+$ T cells (Figure 3.4), with on average 24% of CD4$^+$ T cells expressing CD38 compared to 39% of CD8$^+$ T cells. For both CD4$^+$ and CD8$^+$ T cell subsets the proportion of activated T cells appeared to decline with time (Figure 3.3-3.4). The proportion of proliferating CD4$^+$/CD8$^+$ T cells was assessed using the surrogate marker of proliferation, Ki67. On average 6.5% of CD4$^+$ T cells expressed Ki67 (Figure 3.3) compared to 34.7% CD8$^+$ T cells (Figure 3.4). As for CD38 and HLADR expression, both CD4$^+$Ki67$^+$ (Figure 3.3) and CD8$^+$Ki67$^+$ (Figure 3.4) T cells appeared to wane with time.

3.3.3 Memory T cell subsets

$CD4^+$ and $CD8^+$ T cells were phenotyped according to memory subset based upon surface expression of CD45RA, CCR7 and CD27 (Figure 3.5). Central memory T cells (TCM), transitional effector memory (TTEM) cells and effector memory (TEM) T cells of both $CD4^+$ (Figure 3.6) and $CD8^+$ (Figure 3.7) memory subsets remained constant over time. For $CD4^+$ T cells the main circulating memory subset is typically TCM[316], yet for 3/6 subjects the TEM and TTEM populations were the main circulating memory subsets (Figure 3.6). Pre-infection timepoints would be required to ascertain whether this is indicative of an early loss of the TCM subset.

3.3.4 Kinetics of HIV-1 specific memory $CD4^+$ and $CD8^+$ T cell responses

The first analysis relied upon detection of gag specific T cell responses, based solely upon their ability to produce IFN-γ upon stimulation with a pool of autologous peptides spanning gag (Figure 3.8). Although there was a wide variation in the magnitude of gag specific IFN-γ^+ CD4 T cell responses, for 4/6 donors $CD4^+$ T cell responses were found to precede $CD8^+$ T cell responses (Figure 3.8B-C, E-F). For the remaining 2/6 subjects, $CD4^+$ and $CD8^+$ IFN-γ^+ gag specific T cell responses were detected simultaneously (Figure 3.8A, D). The median time post Fiebig stage I/II at which gag specific, IFN-γ^+ T cell responses were detected was 51.5 days for $CD8^+$ T cell responses and 31 days for $CD4^+$ T cells (Figure 3.8).

3.3.5 Magnitude and maintenance of $CD4^+$/$CD8^+$ T cell responses

The magnitude of gag specific IFN-γ^+ T cell response made by $CD8^+$ T cells ranged from 0.024-0.91% (Average 0.27% $CD8^+$ T cells) (Figure 3.8) and was significantly higher than that of $CD4^+$ T cells (paired T test p=0.042; Figure 3.8). The magnitude of $CD4^+$IFN-γ^+ gag specific T cell response ranged from 0.021-0.135% (average 0.056% $CD4^+$IFN-γ^+ Figure 3.8). The true maintenance of $CD4^+$/$CD8^+$ gag specific T cell responses could not be fully assessed owing to the use of pools of overlapping peptides rather than known mapped epitopes. However, for 2/6 subjects $CD4^+$IFN-γ^+ gag specific T cell responses became undetectable <200 days post Fiebig I/II (Figure 3.8). By contrast $CD8^+$ T cell responses persisted for the duration of study (>450days post Fiebig I/II), in all 6 donors (Figure 3.8).

3.3.6 Functionality of CD4$^+$ and CD8$^+$ T cell responses

To avoid complications associated with the high background levels of IL-2 and TNFα observed in African cohorts[439,440], Boolean gating was not employed, and assessments of cytokine production were represented as proportional expression of TNFα/IL-2 within IFNγ$^+$ T cells. Gag specific IFN-γ$^+$CD4$^+$ T cell responses were more polyfunctional (Figure 3.9) than CD8$^+$ T cell responses during acute HIV-1 (Figure 3.10). This discrepancy was consistent over time with on average 76.9% of CD4$^+$IFN-γ$^+$ T cells secreting 2 or more cytokines (Figure 3.9), whilst just 23.9% of CD8$^+$IFN-γ$^+$ T cells produced more than 1 cytokine (Figure 3.10)

The proportion of total memory CD4$^+$/CD8$^+$ T cells that solely produced IFN-γ remained constant over time (Figure 3.9-3.10). For CD8$^+$IFN-γ$^+$ total memory T cells IL-2/TNFα co-production remained fairly constant over time (Figure 3.10). Whilst the proportion of CD4$^+$IFN-γ$^+$IL-2$^+$ T cells remained constant over time (2.33% CD4$^+$IFN-γ$^+$ >100 days post Fiebig I/II) (Figure 3.9), the proportion of CD4$^+$IFN-γ$^+$TNFα$^+$ cells was shown to increase over time (Figure 3.9), with a median of 41.5% CD4$^+$IFN-γ$^+$ T cells within the first 100 days post Fiebig I/II, and 61% CD4$^+$IFN-γ$^+$ at <100 days post Fiebig I/II (Figure 3.9).

3.3.7 Activation status of gag specific IFN-γ$^+$ memory CD4$^+$ and CD8$^+$ T cells

For the next analyses total memory gag-specific CD4$^+$/CD8$^+$ IFN-γ$^+$ T cells were assessed for activation status (Figure 3.11-3.12). On average 39.3% CD4$^+$IFN-γ$^+$ T cells were HLADR$^+$CD38$^+$ (Figure 3.11 red line), with 61.7% CD4$^+$IFN-γ$^+$ T cells expressing CD38 (Figure 3.11 mauve line) and 20% expressing HLADR (Figure 3.11 orange line). On average 24% CD4$^+$IFN-γ$^+$ T cells were replicating, as evidenced by Ki67 staining (Figure 3.11 blue line). Very few CD4$^+$IFN-γ$^+$ gag specific T cells expressed the senescence marker CD57 (range 0-14.6% Figure 3.11 black line).

The patterns of immune activation were similar for CD8$^+$IFN-γ$^+$ gag specific T cells. On average 63.5% of CD8$^+$IFN-γ$^+$ T cells were HLADR$^+$CD38$^+$ (Figure 3.12 red line), 41.4% expressed CD38$^+$ (Figure 3.12 mauve line) and 43.3% expressed

HLADR (Figure 3.12 orange line). The proportion of proliferating (Figure 3.12 blue line) gag specific CD8$^+$IFN-γ^+ T cells ranged from 0-71.6% (average 16% CD8$^+$IFN-γ^+ T cells). As for CD4$^+$IFN-γ^+gag specific T cells, very few CD8$^+$IFN-γ^+gag specific T cells expressed CD57 (Range 0-34%, average 8.9% CD8$^+$IFN-γ^+ T cells; Figure 3.12 black line).

3.3.8 Memory phenotype of gag specific IFN-γ^+ T cells

Gag specific CD4$^+$IFN-γ^+ T cells were predominately from the central and transitional memory subsets with on average just 17% (range 8-42%) pertaining from terminally differentiated effector memory cells (Figure 3.13). By contrast gag specific CD8$^+$IFN-γ^+ T cells were almost exclusively from the terminally differentiated effector memory and transitional memory subsets with just 8% of gag specific CD8$^+$IFN-γ^+ T cells being from the central memory subset (range 0-34.4%; Figure 3.14). The memory subsets remained stable over time (Figure 3.13-3.14).

3.4 Discussion

With the use of overlapping pools of peptides spanning autologous gag sequences $CD4^+$ T cell responses were assessed for 6 subjects during acute HIV-1 infection. Owing to the limited number of subjects studied this work was primarily hypothesis generating, and has subsequently been expanded with the analysis of additional subjects within the acute stage of HIV-1 infection together with the inclusion of HIV-1 seronegative, control, subjects (Dr C Riou and Professor C Gray personal communication). All 6 subjects studied within this chapter were at Fiebig stage I/II at enrolment with the first available time point for study a median of 35 days post Fiebig I/II (range 19-68 days). Subjects were followed across 4-5 study visits all of which occurred within the first 630 days (Range 447-628 days) post Fiebig I/II (Figure 3.1). The availability of samples within such early time points post HIV-1 infection sets this study apart from previous literature reports, which have often loosely defined acute HIV-1 infection as being within the first year of seroconversion, or following the onset of symptoms (Fiebig stage IV-V[393])[303,328,416].

In accordance with previous studies[303,328,416], $CD4^+$ T cell responses were readily detected amongst all 6 donors in the acute stages of HIV-1 infection (Figure 3.8) but quickly waned, becoming undetectable in <200 days post Fiebig I/II for 2/6 subjects (Figure 3.8). Rapid decline of $CD4^+$ T cell responses has been noted previously and a natural contraction of $CD4^+$ T cell responses detected in other viral infections. There are several mechanisms that may account for the decline/loss of $CD4^+$ T cells in the acute stages of HIV-1. Douek *et al* showed that HIV-1 specific $CD4^+$ T cell responses are preferentially infected by HIV-1 and have a reduced lifespan[368,441]. We noted that the proliferative capacity of both gag specific IFN-γ^+ $CD4^+$ T cells (Figure 3.9) and total memory $CD4^+$ T cells declined with time (Figure 3.3). To address whether the decline of $Ki67^+CD4^+$ T cells was associated with an overall increase in senescence, CD57 expression was assessed (Figure 3.3). Whilst the precise role of CD57 is unknown, it is linked to dysfunction of $CD4^+$ T cell responses, and increased susceptibility to apoptosis[428,429]. The expression of CD57 upon total memory $CD4^+$ T cells was shown to be low (average 3%) and did not vary with time (Figure 3.3). Furthermore, there was no evidence to suggest an enrichment of $CD57^+$ cells within

gag specific CD4+IFN-γ+ T cells (Figure 3.10). Whilst the samples obtained for analysis in this study were within the first 35 days post Fiebig stage I/II the viral ramp up was missed and further study is required to address whether CD57+ expression was up regulated at earlier time points.

In accordance with previous studies, gag specific CD4+IFN-γ+ T cell responses were significantly lower in magnitude than CD8+IFN-γ+ gag specific T cell responses[373] (Figure 3.8). However, gag specific CD4+ T cell responses were shown to precede gag specific CD8+ T cell responses in 4/6 subjects (Figure 3.8). Asynchronous kinetics of CD4+ T cells have also been noted in CMV infection where the delayed occurrence of CD4+ T cell responses has been correlated with symptomatic infection[442]. It is interesting to speculate that the asynchronous emergence of CD4+/CD8+ T cell responses is indicative of cognate CD4 "help" to CD8 T cells. However, this interpretation must be treated with caution. Not only is the sample size small but this study is limited to gag specific T cell responses. Whilst gag has been shown to be an immunodominant target for CD4+ T cells[373,374], the earliest CD8+ T cell responses have typically been shown to target other HIV-1 proteins, in particular Nef[443-445]. It is most likely that the asynchronous kinetics observed within this study are purely reflective of the delayed emergence of gag specific CD8+ T cell responses in comparison to other HIV-1 proteins, and further study is now required to see whether divergent kinetics of CD4+/CD8+ T cell responses can be detected for other proteins.

Whilst most likely to reflect the delayed emergence of CD8+ T cell responses to gag protein, a further explanation for the failure to detect early gag specific CD8+ T cell responses lies in this studies reliance upon IFN-γ+ T cells to detect antigen specific T cell responses. Whilst for all subjects viral load was on the decline at the earliest study visit, the viral load was still high at a mean of 3.67×10^6 copies/ml (Figure 3.1). Within Hepatitis C virus (HCV) infection and Lymphocyte Choriomeningitis Virus (LCMV) infections a dysfunctional or "stunned" phenotype has been described to occur, where antigen specific T cells momentarily fail to secrete IFN-γ in the presence of excessive antigen[446-449]. Human leukocyte class 1 or II tetramers would be required to address this hypothesis further.

In agreement with previous studies[303,305], gag specific CD4+IFN-γ+ T cells detected in acute infection were highly activated (CDD38+HLADR+) and proliferating (Ki67+) (Figure 3.9). The detection of activated/proliferating CD4+ IFN-γ+ gag specific T cell responses rapidly waned (Figure 3.9). The expression of CD38 and Ki67 has been shown to represent a CD4+ count/viral load independent means by which to predict disease progression[298-300,304-306,417-419]. However, the subjects within this study were only followed for the first 2 years of HIV-1 infection and so correlations between the expression levels of activation markers and disease progression could not be made.

To examine whether activation status caused an imbalance in CD4+ T cell memory lineage naïve, central memory, effector memory and transitional memory subsets were examined. Prior studies have shown that memory CD4+ T cell subsets[53,326] are preferentially infected by HIV-1 and it was predicted that CD4+ memory subsets would rapidly decline in acute HIV-1 infection. TCM is normally the dominant circulating memory subset amongst CD4+ T cells, and is particularly affected during HIV-1 infection[316,420]. Indeed for the 6 subjects studied the proportion of CD4+ TCM cells was typically low (Range 1.3-60.2%) and did not vary with time/viral load (Figure 3.6). The most likely explanation for the low frequency of TCM is preferential killing by HIV-1, since TCM retains surface expression of the HIV-1 co-receptor CCR7[316]. However, in order to accurately draw this conclusion analysis of pre-infection samples would be required to ascertain baseline levels of each memory subset. The analysis of samples from HIV-1 seronegative individuals is also required to demonstrate the natural fluctuations in each subset with time. Previous flow cytometric studies of memory CD4+ T cell subsets have suggested that memory differentiation and activation profiles within the CD4+ compartment are established early after HIV infection attaining "set-point" by 3 months post infection[328]. It is possible that the early changes in CD4+ memory subsets had already occurred prior to the first sampling time point available, indeed for all subjects peak viral load was on the decline toward set point at the first time point available for study (Figure 3.1). These observations highlight the requirement to study CD4+ T cells at the earliest possible time point, and suggest that the majority of changes to memory subsets occur during viral ramp up.

The functional response of gag specific $CD4^+$ T cells shifted from primarily IFN-$\gamma^+TNF\alpha^-$ towards IFN-$\gamma^+TNF\alpha^+$ with time (Figure 3.9). TNF-α production is known to be involved in the pathogenesis of HIV-1 infection, correlating with worsening clinical outcome[450]. However, the exact role in HIV-1 infection remains incompletely understood. TNFα production has been correlated with an increased risk of apoptosis[176] and *in vitro* studies with cell lines have shown that TNFα production can actively enhance HIV-1 replication[451,452]. It is possible therefore that the increasing proportion of $CD4^+$IFN-$\gamma^+TNF\alpha^+$ T cells represents progressive dysregulation of the $CD4^+$ T cell subset as disease progresses. In future experiments we will investigate whether the increase in $CD4^+$IFN-$\gamma^+TNF\alpha^+$ T cells correlates with an increase in apoptosis markers such as PD1.

Very few $CD4^+$ T cells were capable of co-producing IL-2 (Figure 3.9). IL-2 is known to be required for the on-going proliferation of memory $CD4^+$ T cells and the detection and maintenance of IL-2 producing HIV-1 specific T cells correlated with improved immune control of HIV-1 viremia[317,318,329,437]. Should IL-2^+CD4^+ T cell responses play a role in control of viremia during acute HIV-1 infection, it is likely that the peak $CD4^+$IL-2^+ T cell response occurs during viral ramp up and may have been missed or on the decline at the time points studied.

In summary this chapter provides a detailed phenotypic analysis of gag-specific T cell responses performed over time from 6 untreated, subtype C HIV-1 infected individuals. It is unique from previous studies of HIV-1 infection owing to the early time points available to study, typically just an average of 35 days post Fiebig I/II. Gag specific $CD4^+$ and $CD8^+$ T cell responses appeared to have asynchronous kinetics, divergent functionality and a loss of proliferative potential. The rapid appearance of gag specific $CD4^+$ T cells during acute infection was relatively short-lived and of low magnitude compared to $CD8^+$ T cells. Both the $CD4^+$ and $CD8^+$ T cell memory subsets appeared relatively stable over time. Together these data suggest that whilst the samples obtained for this study were within the acute phase of HIV-1 infection, typically just as peak viral load was on the decline, it is likely that the peak $CD4^+$ T cell response and memory subset alterations occur much earlier in infection, most likely during viral ramp up. These observations suggest that there is a short,

finite window of time when CD4$^+$ T cells are potentially functional. Future studies are now required to see whether these observations are supported for all HIV-1 proteins, and across multiple individuals. Additional experiments are also required to study the normal range of fluctuation in activational status and memory subsets that occur over time in HIV-1 seronegative individuals. Whilst the number of subjects studied herein is limited, the observations made in this chapter have been supported by follow up studies in an additional 6 acute HIV-1 infected subjects (Dr C Riou and Professor C Gray, personal communication).

[I] Data generated in this chapter, together with additional studies made in a further 6 acute HIV-1 infected subjects and seronegative subjects was accepted for publication in the Journal of Immunology.

Riou, C., Ganusov,V.V., Campion, S., Mlotshwa, M., Liu, M.K., Whale, V., Goonetilleke, N., Borrow, P., Ferrari, G., Betts, M. R., Haynes, B.F., McMichael, A.J. & Gray, C.M. Distinct kinetics of Gag-specific CD4$^+$ and CD8$^+$ T cell responses during acute HIV-1. Journal of Immunology

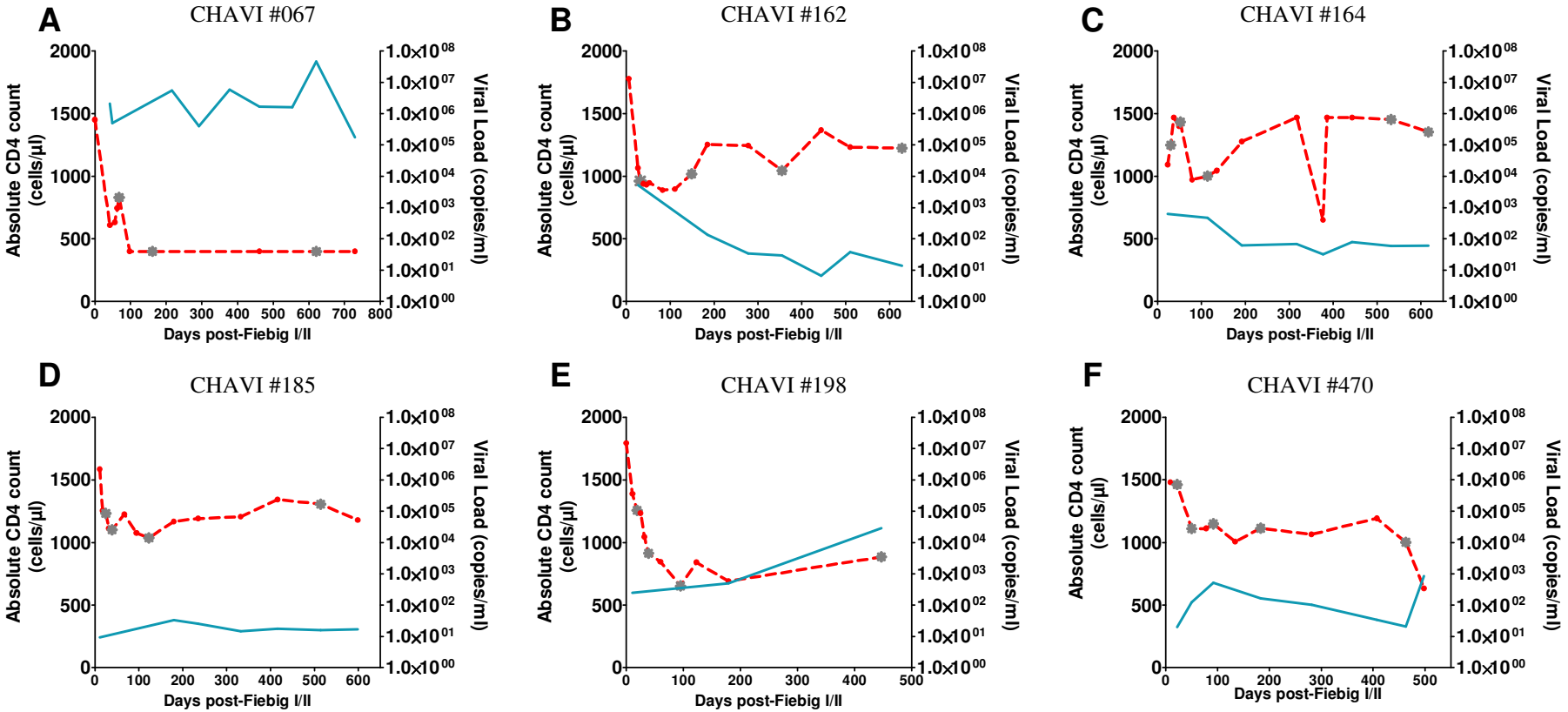


Figure 3.1
Six acute HIV-1 clade C infected subjects recruited within CHAVI 001 cohort were available for study. A-F presents the viral load (red) and absolute $CD4^+$ T cell counts (blue) for each subject. The time points available for study by flow cytometry are marked with an ✱.

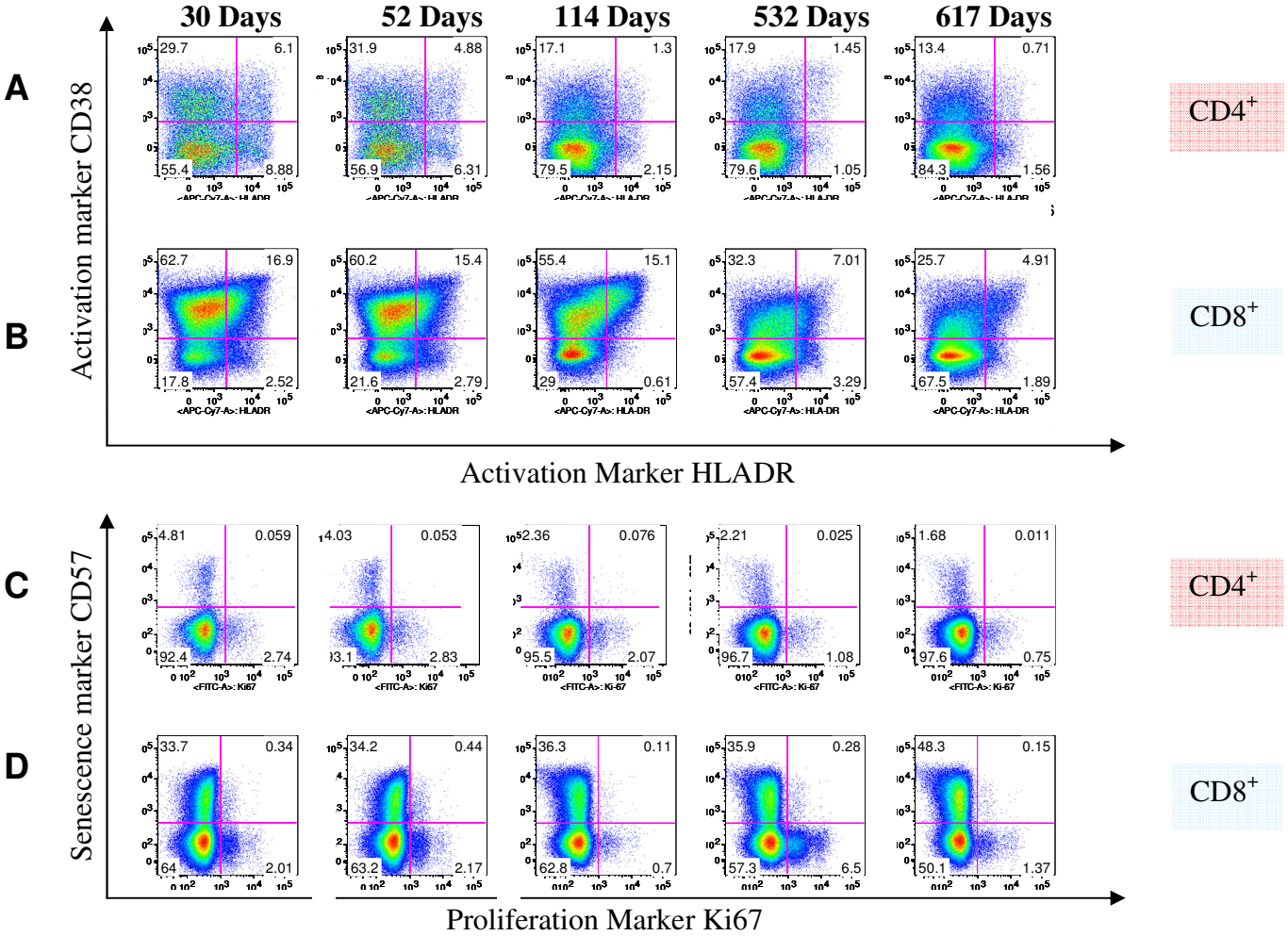


Figure 3.2

Representative FACS plots from the $CD4^+$ and $CD8^+$ T cell compartments of 1/6 HIV-1 infected donors showing changes in the surface expression of activation markers CD38 and HLADR with time (A-B). T cell proliferation and senescence was also examined using the markers Ki67 and CD57 respectively (C-D). The numbers shown in each quadrant corner represent the $\%CD4^+/CD8^+$ T cells expressing marker. The days post Fiebig I/II are shown above the FACS plots.

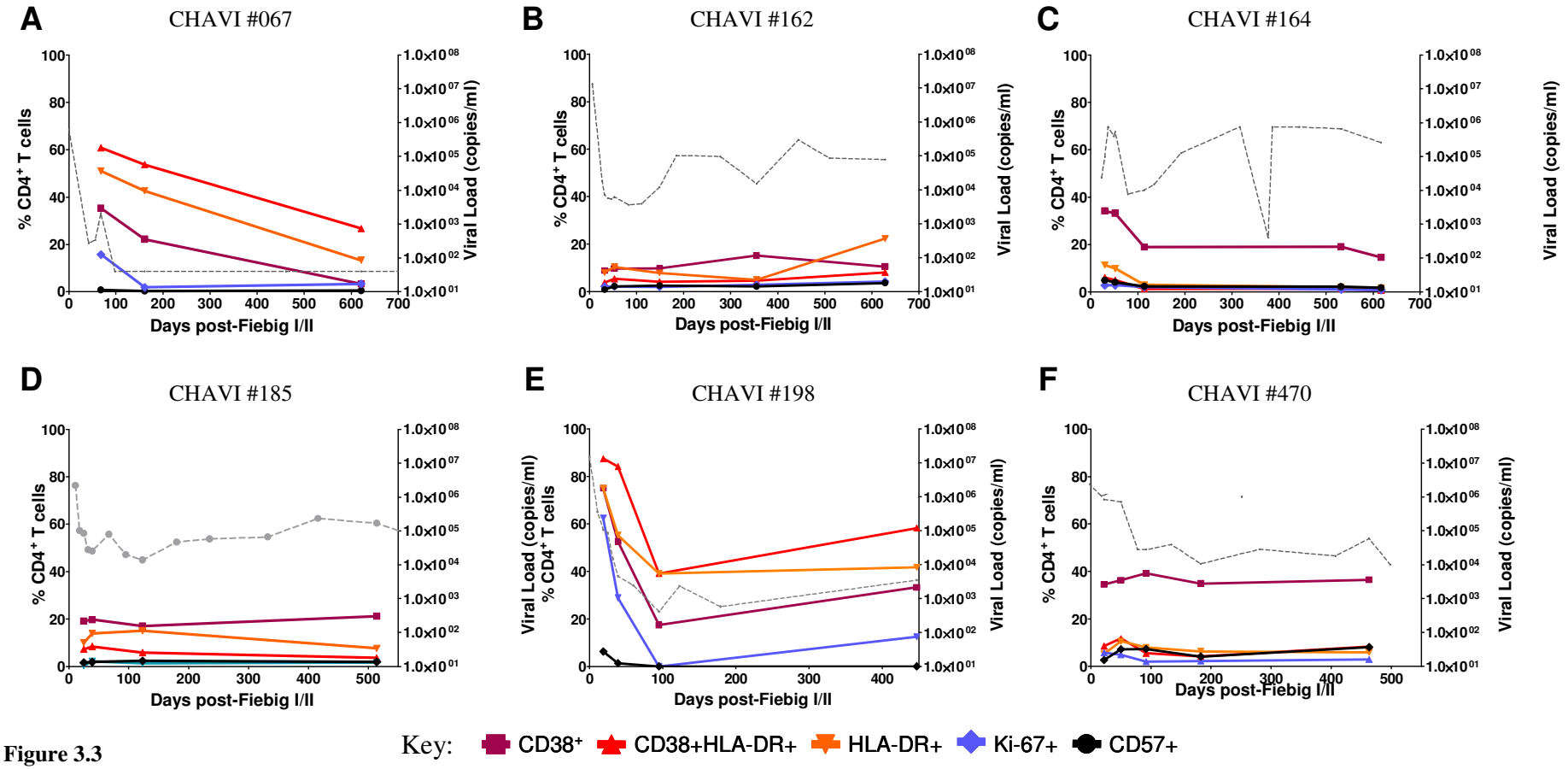


Figure 3.3

The generalized activation status of CD4+ T cells was assessed by surface expression of CD38+HLADR (red line), CD38 (mauve line), HLADR (orange line). In addition the proliferative response was examined using Ki67 staining (blue line) and the levels of senescence by CD57 staining (black line). All live, $CD56^{-}CD14^{-}CD19^{-}CD16^{-}CD3^{+}CD4^{+}$ T cells included in analysis. For reference each subject's viral load is plotted on right hand axis (grey dotted line).

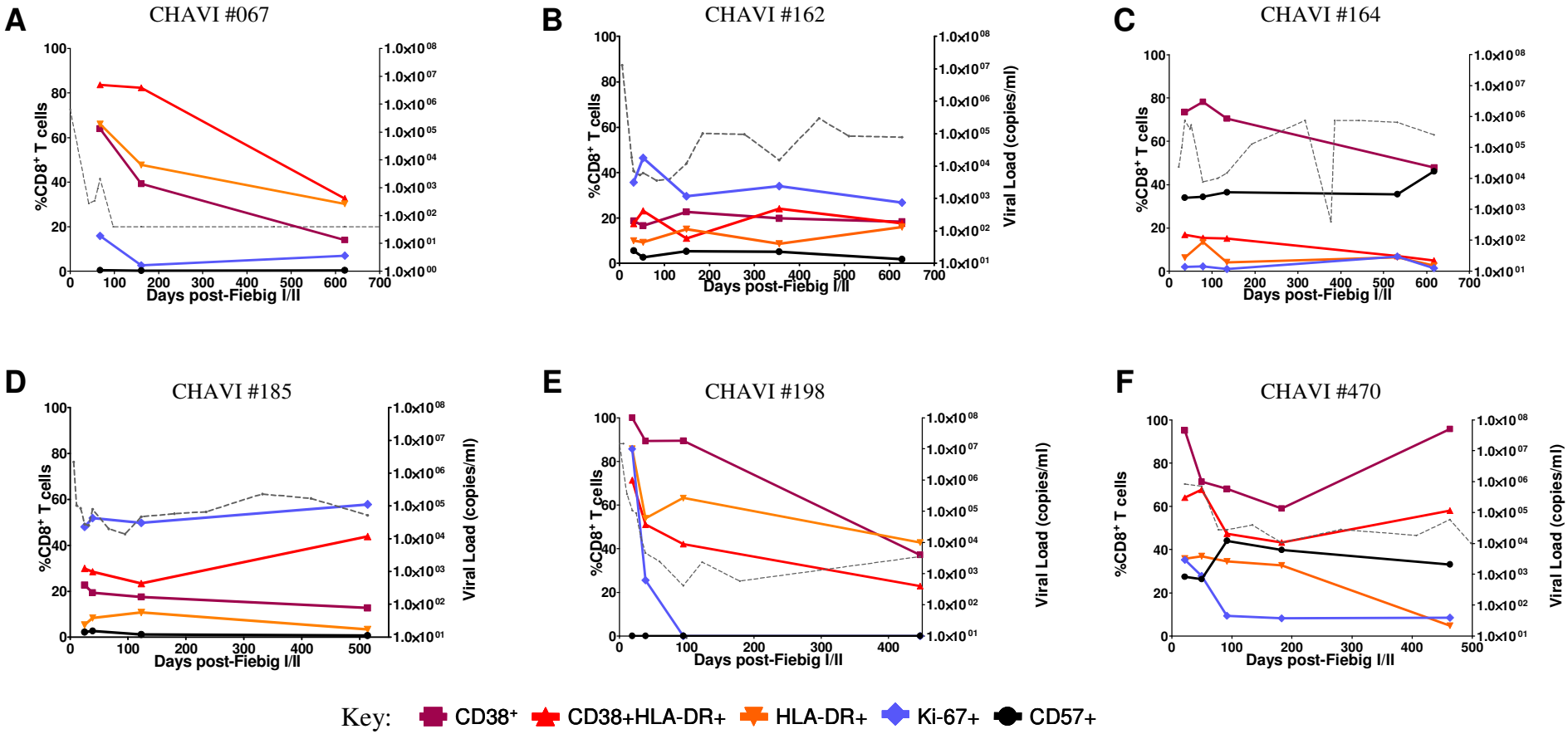


Figure 3.4

The generalized activation status of $CD8^+$ T cells was assessed by surface expression of CD38+HLADR (red line), CD38 (mauve line), HLADR (orange line). In addition the proliferative response was examined using Ki67 staining (blue line) and the levels of senescence by CD57 staining (black line). All live, $CD56^-CD14^-CD19^-CD16^-CD3^+CD8^+$ T cells included in analysis. For reference each subject's viral load is plotted on right hand axis (grey dotted line).

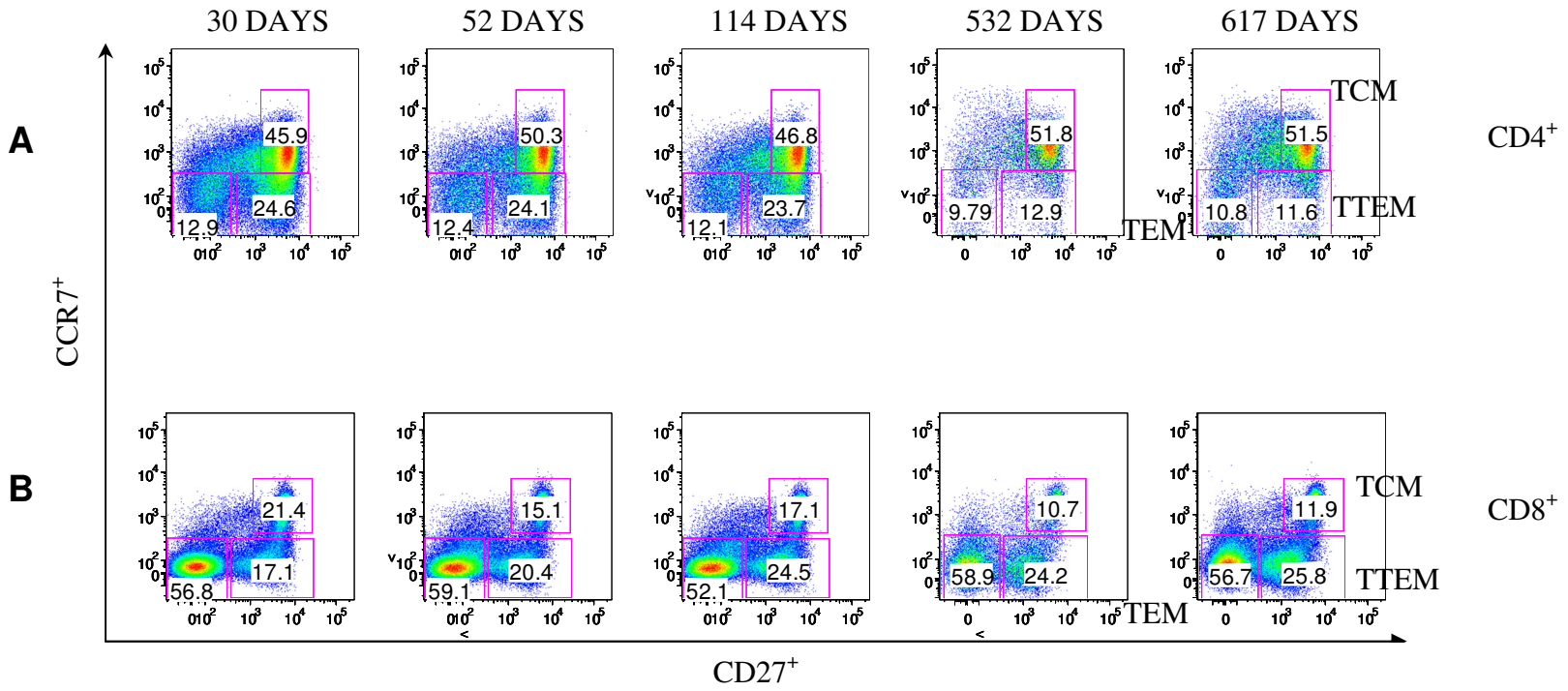


Figure 3.5

Representative FACS plots showing the changes in the composition of $CD4^+$ (A) and $CD8^+$ (B) memory T cell subsets over time. The days post Fiebig stage I/II are shown above each plot and the gate names given on the far right plot. Central memory (TCM), transitional effector memory (TTEM) and effector memory (TEM) subsets were identified based upon surface expression of CD27, CCR7 and an absence of CD45RA. For the purposes of these analysis all live, $CD56^-CD14^-CD19^-CD16^-CD3^+CD4/8^+CD45RA^-$T cells were included.

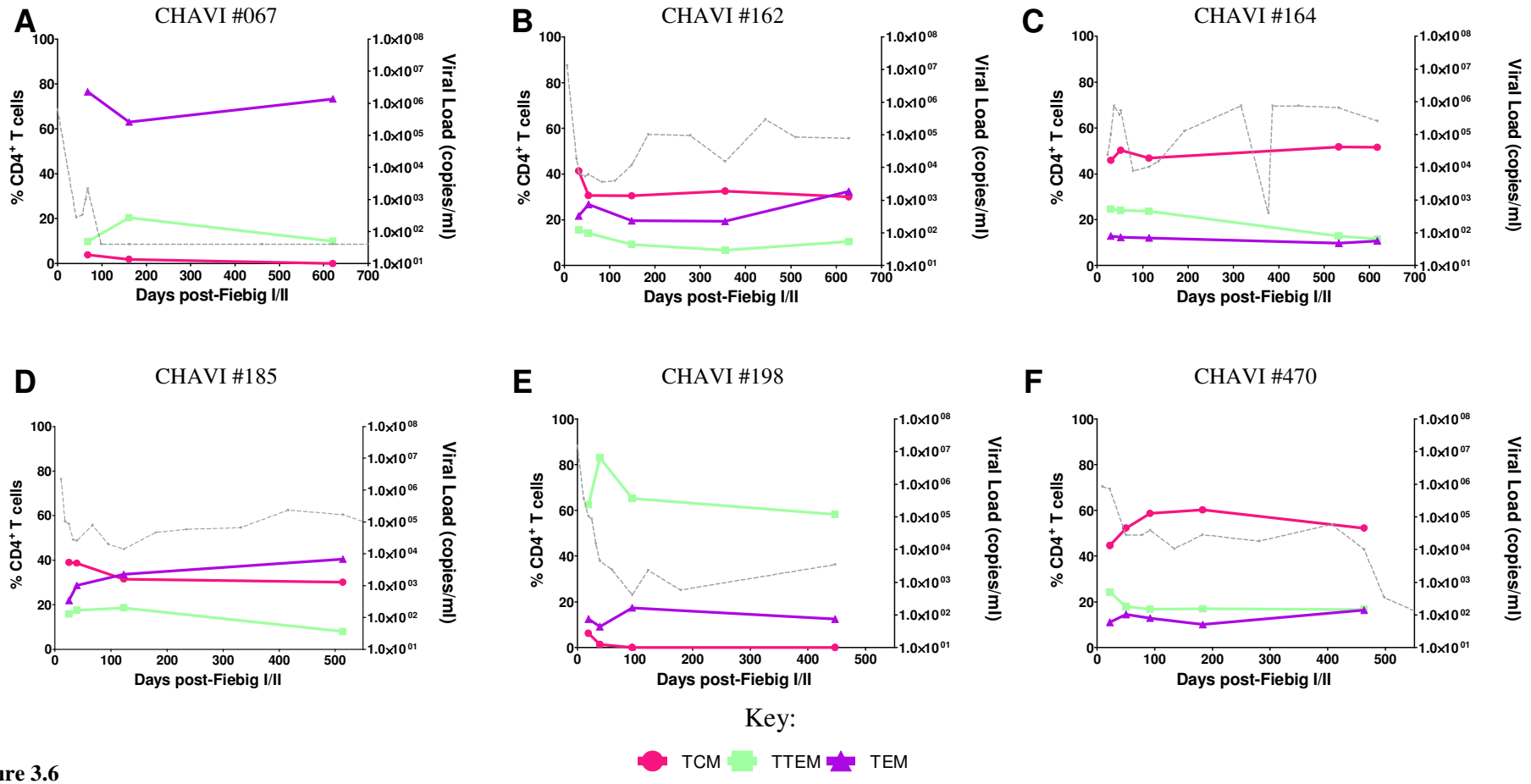


Figure 3.6

The changes in the relative frequencies of central memory (pink line), transitional memory (green line) and effector memory (purple line) phenotype of $CD4^+$ T cells was assessed over time post Fiebig stage I/II. All live, $CD56^-CD14^-CD19^-CD16^-CD3^+CD4^+CD45RA^-$T cells were included in analysis. For reference each subject's viral load is plotted on right hand axis (grey dotted line).

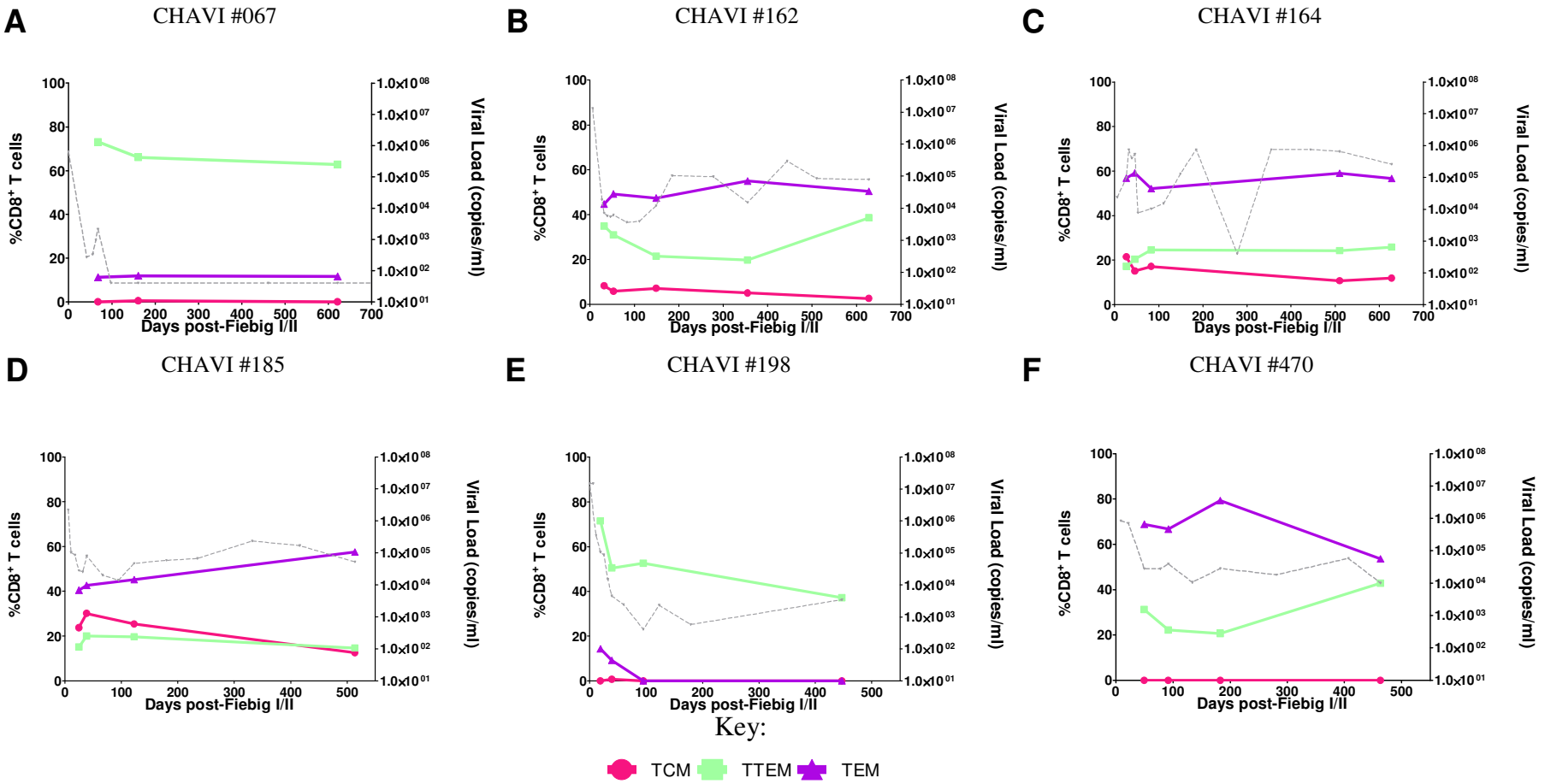


Figure 3.7

The changes in the relative frequencies of central memory (pink line), transitional memory (green line) and effector memory (purple line) phenotype of $CD8^+$ T cells was assessed over time post Fiebig stage I/II. All live, $CD56^-CD14^-CD19^-CD16^-CD3^+CD8^+CD45RA^-$T cells were included in analysis. For reference each subject's viral load is plotted on right hand axis (grey dotted line).

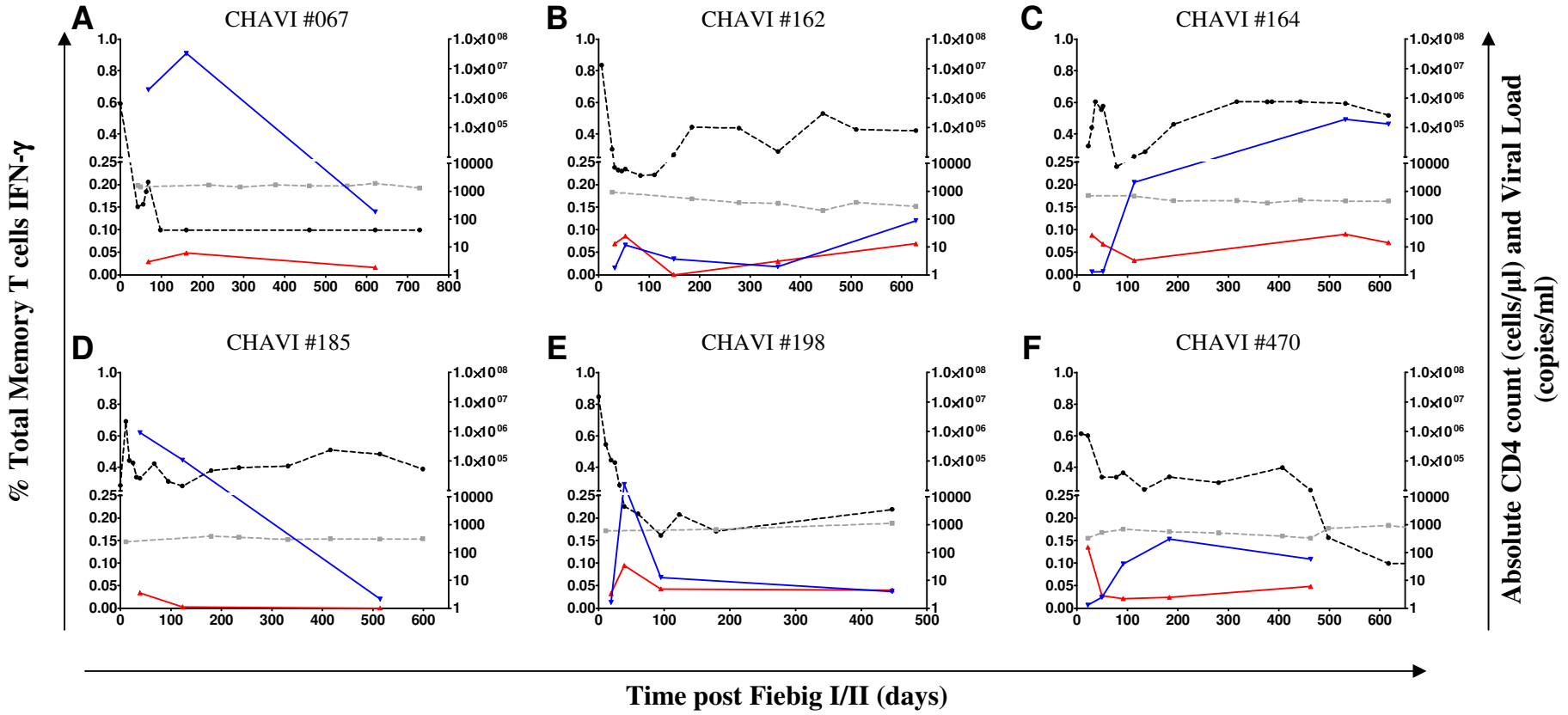


Figure 3.8

The kinetics of gag-specific IFN-γ^+ $CD4^+$ (red line) and $CD8^+$ (blue line) memory T cell responses were assessed for six acutely infected HIV-1 positive subjects (A-F). IFN-γ responses (left Y-axis) were expressed as % of total memory $CD4^+$ or $CD8^+$ T cells following background subtraction. Criteria for a positive IFN-γ^+ T cell response was defined as >0.02% following background subtraction. Memory T cells were identified by the exclusion of $CD45RA^+CD27^+$ cells. Viral load is represented by a black dotted line and absolute CD4 counts as grey dashed line (both plotted on right Y-axis). Subjects were studied across a minimum of 4 study visits, dated according to time post Fiebig stage I/II[393].

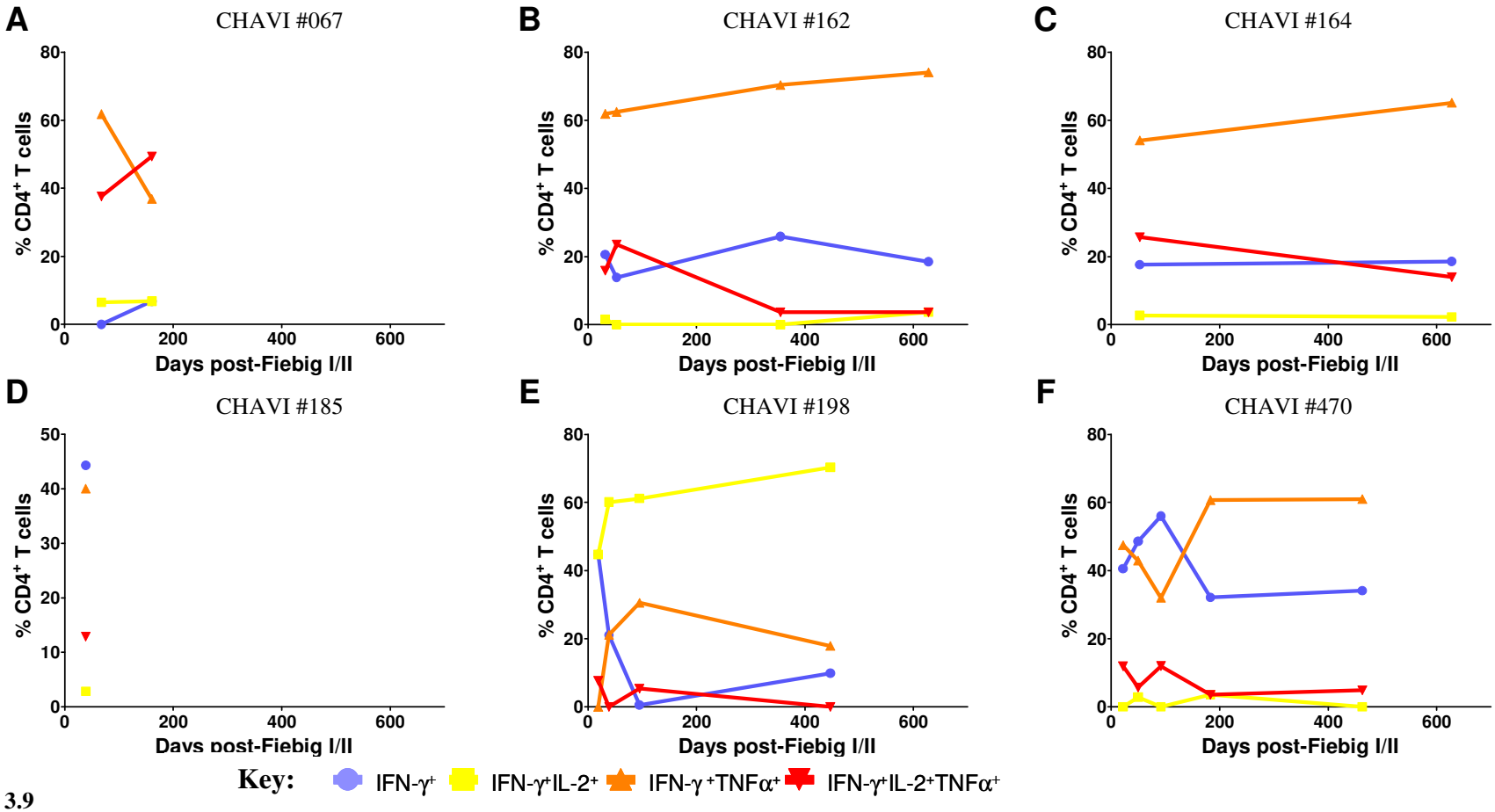


Figure 3.9

The functionality of total memory CD4$^+$ IFN-γ$^+$ T cells was compared for all six subjects over time. Notably for 2/6 of the subjects gag specific CD4$^+$IFN-γ$^+$ T cell responses were rapidly lost (A,D). The percentage of total memory IFN-γ$^+$ cells that did not co-produce IL-2 or TNFα appeared to remain stable over time (blue line). Similarly the proportion of gag specific CD4$^+$IFN-γ$^+$IL-2$^+$ (yellow line) did not appear to vary with time. However the proportion of CD4$^+$IFN-γ$^+$TNF-α$^+$ appeared to increase with time (orange line) whist the proportion of gag specific CD4$^+$ T cells capable of producing all three cytokines (red line) appeared to decrease with time.

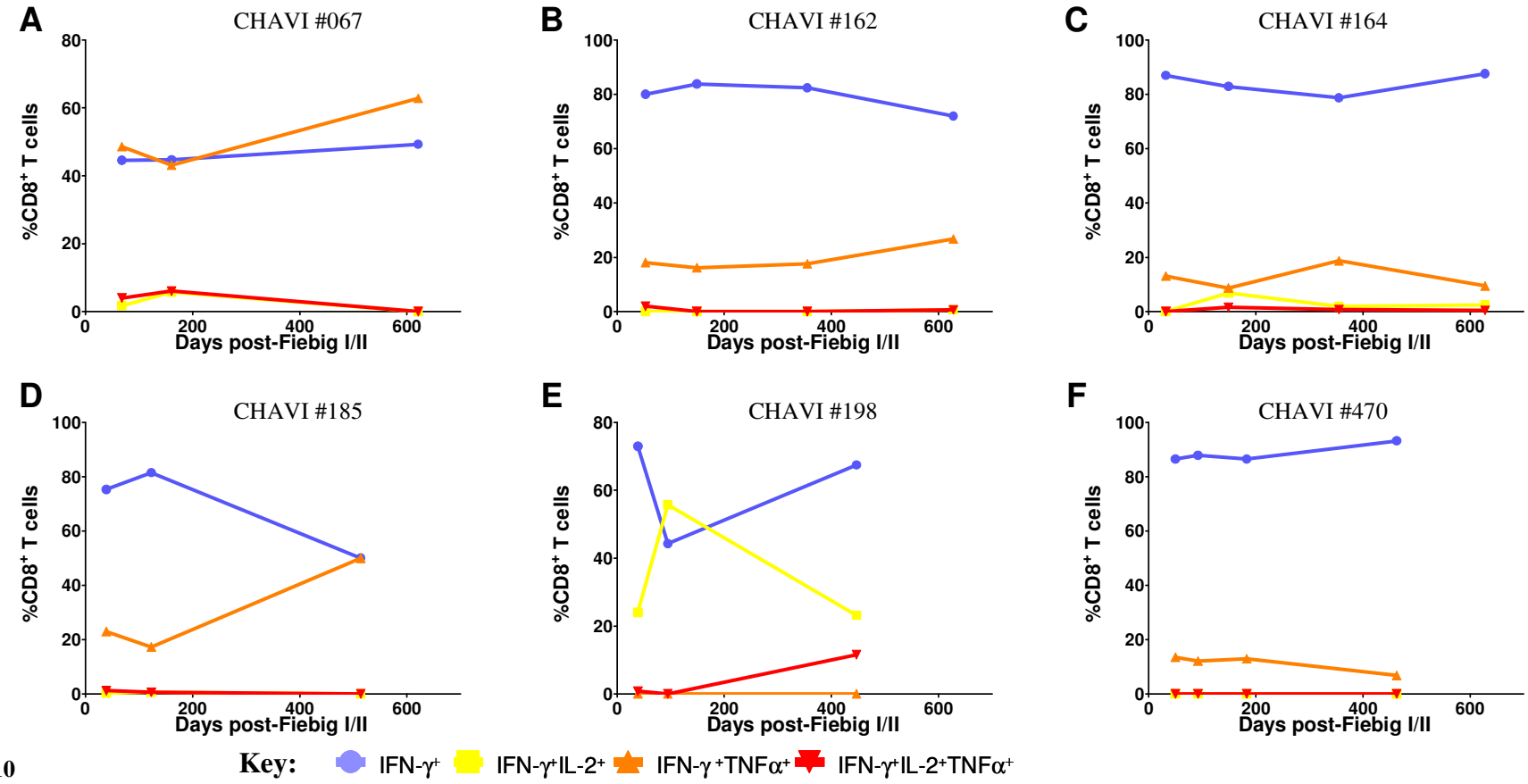


Figure 3.10

The functionality of total memory CD8+ IFN-γ+ T cells was compared for all six subjects over time. For all subjects gag specific CD8+ IFN-γ+ T cells persisted throughout the duration of study. The percentage of total memory IFN-γ+ cells that did not co-produce IL-2 or TNFα appeared to remain stable over time (blue line). Similarly the proportion of gag specific CD8+ IFN-γ+ IL-2+ (yellow line) and CD8+IFN-γ+TNF-α+ appeared to increase with time (orange line) whist the proportion of gag specific CD4+ T cells capable of producing all three cytokines (red line) appeared to decrease with time.

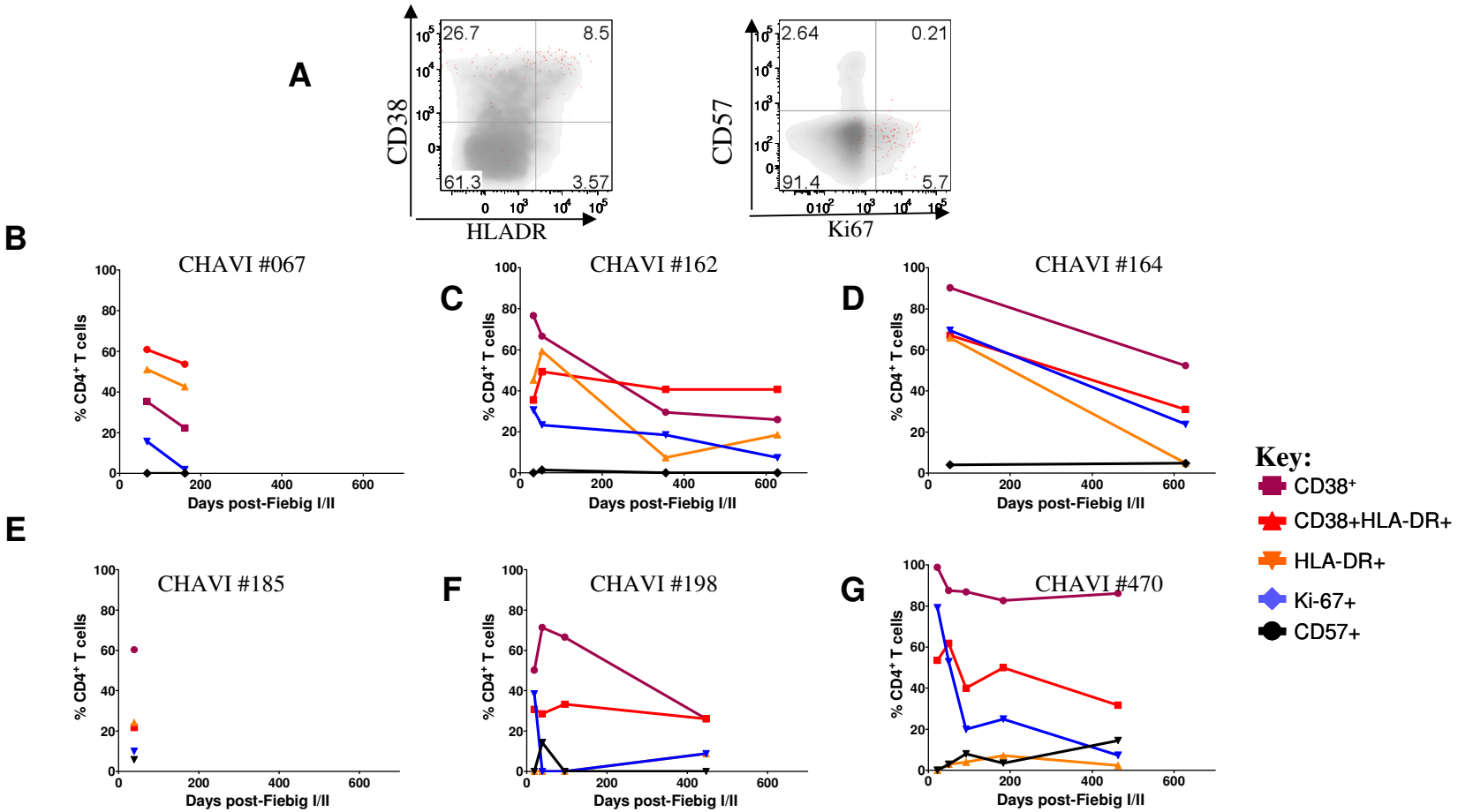


Figure 3.11

The activation status of CD4+IFN-γ+ T cells was assessed for all 6 donors by examination of surface expression of HLADR (orange line), CD38 (red line) individually as well as the % IFN-g+ cells expressing both HLADR+CD38 (dark purple line). In addition proliferative capacity was assessed by Ki67 staining (blue line) and senescence by CD57 (black line). A) Shows representative FACS plots of CD4+IFN-γ+ T cells (shown in red) overlaid on total CD4+ activation profiles (grey density plot). B-G show the % CD4+IFN-γ+ T cells expressing marker(s) for each subject over time (days post Fiebig) I/II. Only gag specific IFN-γ+ T cells were included in these analyses. Criteria for a positive IFN-γ+ T cell response was >0.02% CD4+ T cells following background subtraction.

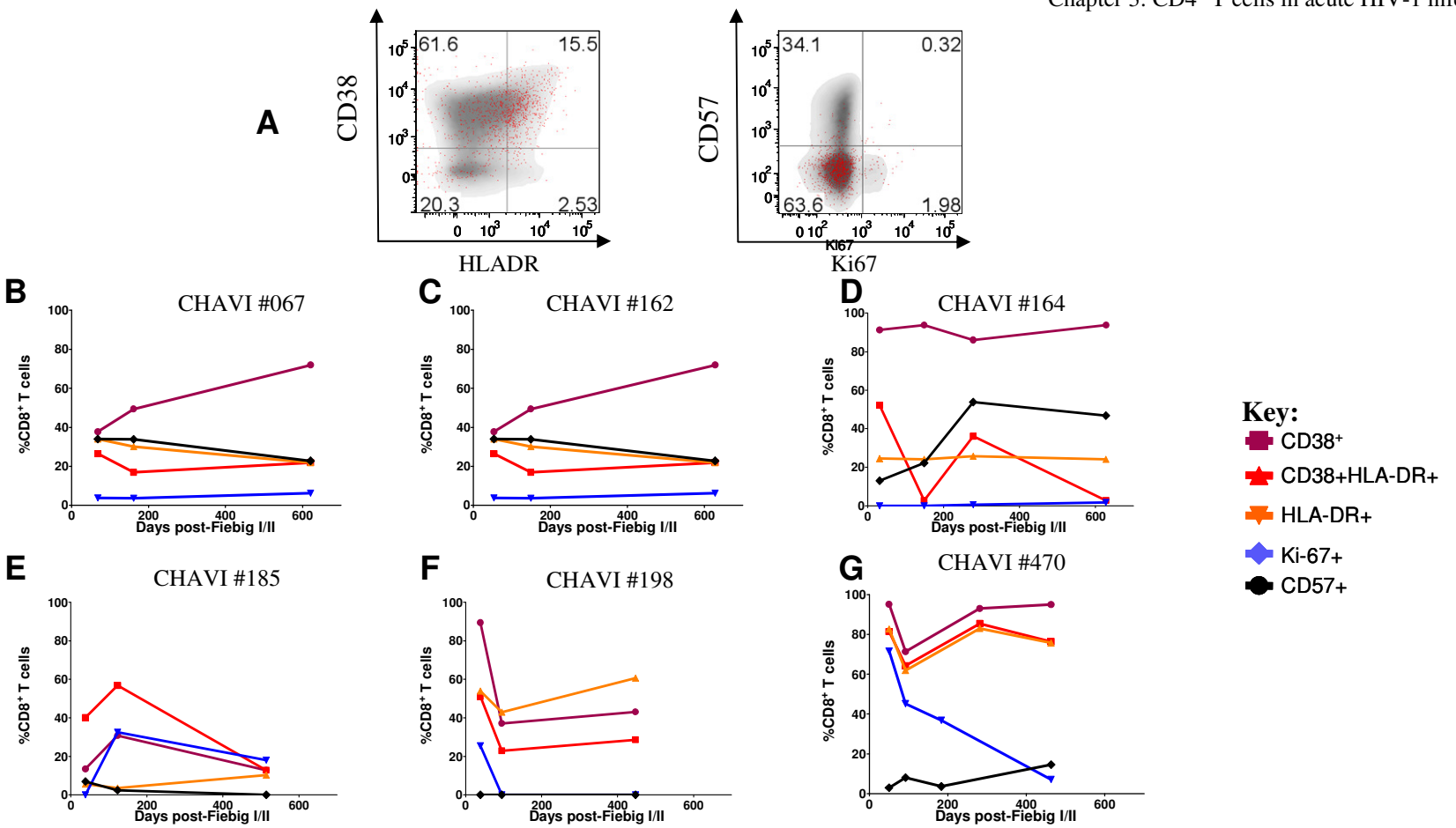


Figure 3.12

The activation status of CD8 IFN-γ+ T cells was assessed for all 6 donors by examination of surface expression of HLADR (orange line), CD38 (red line) individually as well as the % IFN-γ+ cells expressing both HLADR+CD38 (dark purple line). In addition proliferative capacity was assessed by Ki67 staining (blue line) and senescence by CD57 (black line). A) Shows representative FACS plots of CD8+IFN-γ+ T cells (shown in red) overlaid on total CD4+ activation profiles (grey density plot). B ZA0058 -G show the % CD4+IFN-γ+ T cells expressing marker(s) for each subject over time (days post Fiebig) I/II. Only gag specific CD8+IFN-γ+ T cells were included in these analyses. Criteria for a positive IFN-γ+ T cell response was >0.02% CD8+ T cells following background subtraction.

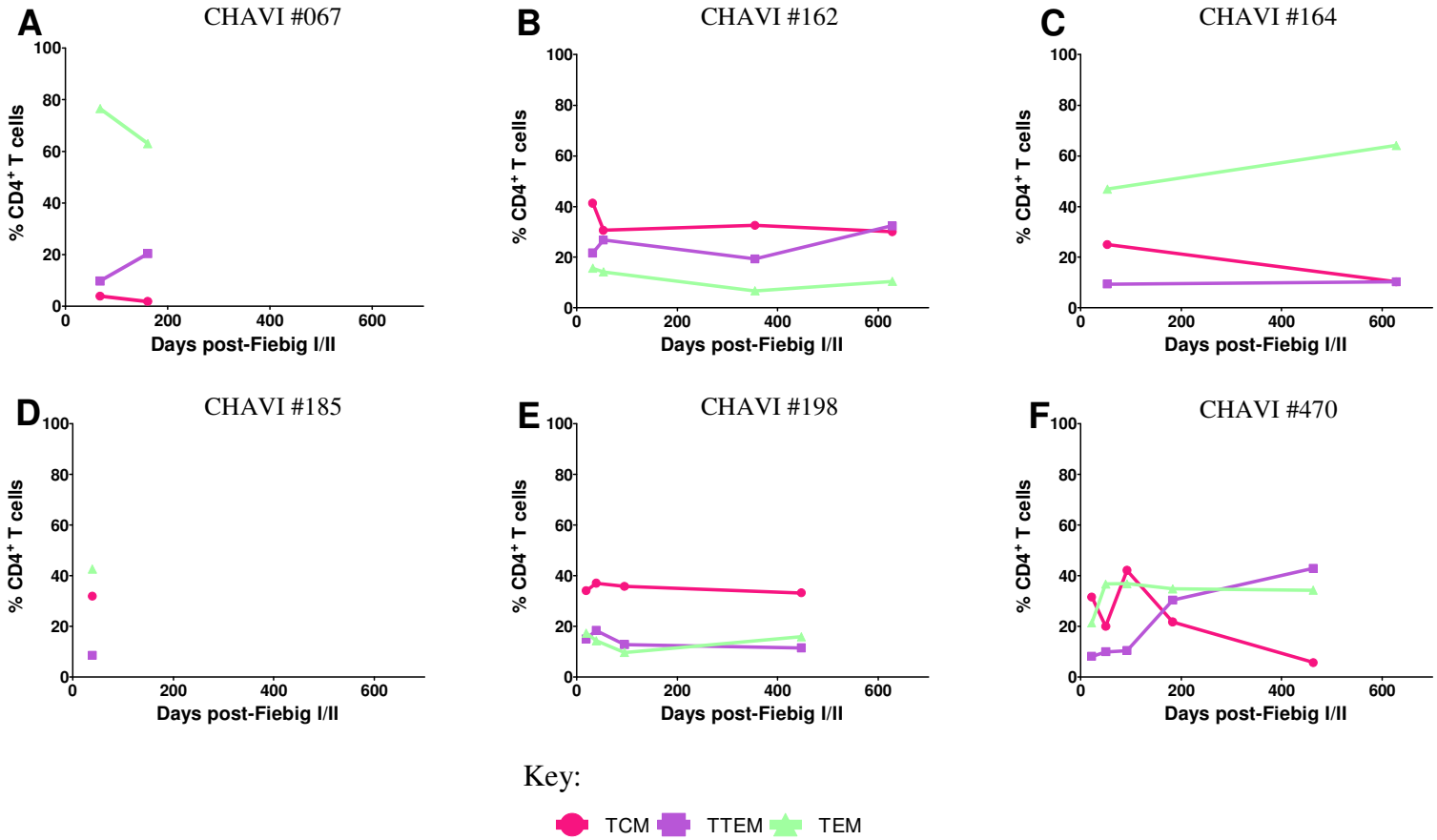


Figure 3.13

The ontogeny of gag specific CD4$^+$IFN-γ^+ memory T cell responses was assessed for all 6 donors by examination of surface expression of CCR7 and CD27, and an absence of CD45RA. Gag specific CD4$^+$IFN-γ^+ memory T cell responses were a mix of central memory (TCM pink line) and transitional effector memory (TTEM green line) subsets. Few gag specific CD4$^+$IFN-γ^+ originated from the effector memory subset (TEM purple) Graphs A-F show the % CD4$^+$IFN-γ^+ T cells from each memory subset over time (days post Fiebig) I/II. Only gag specific CD4$^+$IFN-γ^+ T cells were included in these analyses. Criteria for a positive IFN-γ^+ T cell response was >0.02% CD4$^+$ T cells following background subtraction.

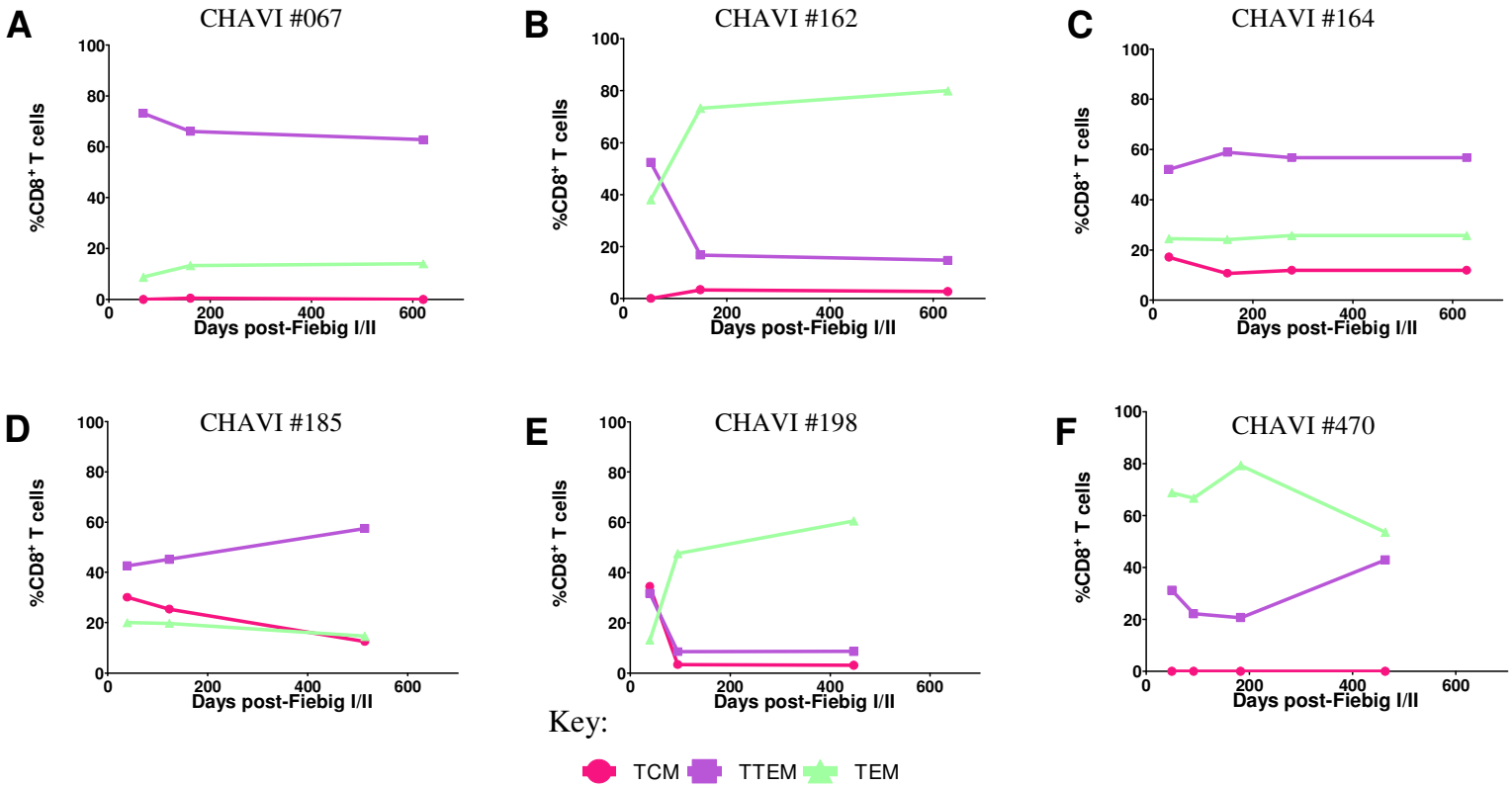


Figure 3.14

The ontogeny of gag specific CD8$^+$IFN-γ$^+$ memory T cell responses was assessed for all 6 donors by examination of surface expression of CCR7 and CD27, and an absence of CD45RA. Gag specific CD8$^+$IFN-γ$^+$ memory T cell responses were predominately from the transitional memory (TTEM green line) and effector memory (EM purple line) subsets. Very few central memory (TCM pink line) gag specific CD8$^+$IFN-γ$^+$ T cell responses were detected. Graphs A-F show the % CD4$^+$IFN-γ$^+$ T cells from each memory subset over time (days post Fiebig) I/II. Only gag specific CD8$^+$IFN-γ$^+$ T cells were included in these analyses. Criteria for a positive IFN-γ$^+$ T cell response was >0.02% CD8$^+$ T cells following background subtraction.

CHAPTER 4:

DETECTION OF LOW FREQUENCY AND MAGNITUDE T CELL RESPONSES

Chapter 4: Detection of low frequency and magnitude T cell responses

4.1 Introduction

Originally described more than 20 years ago[453] the enzyme linked immunospot (ELISpot) assay has emerged as a robust, reliable and reproducible method by which to conduct large throughput studies of HIV-1 vaccine candidates[454,455]. With a detection limit estimated to be around 1 in 10,000-20,000 cells[456], ELISpot is one of the most frequently applied methodologies to the investigation of T cell responses, including the detection of HIV-1 specific T cells in HIV-1 exposed seronegative (HESN) individuals[135,145,147,382,383]. However, similar to HIV-1 specific T cell responess detected during acute HIV-1 infection (Chapter 3), pre-exisiting HIV-1 specific T cell responses are typically narrowly focused and of low frequency and magnitude[135,152,155]. We hypothesize that, antigen specific T cell responses may exist below the limits of detection by conventional ELISpot analysis and that it would be advantageous to have a more sensitive means by which to screen for pre-exisiting HIV-1 specific T cell responses.

Another setting where improved sensitivity would advantageous is in the detection of latent *Mycobacterium tuberculosis* infection (LTBI)[114,457,458]. *Mycobacterium tuberculosis* (Mtb) is a respiratory pathogen and the etiologic agent of tuberculosis (TB). In conjunction with the HIV-1 pandemic[17,459], TB remains a global health concern and a leading cause of morbidity and mortality amongst HIV-1 positive individuals[5,99,108]. For the majority of immunocompetent individuals, Mtb infection is contained, and progression to active disease averted by a highly orchestrated $CD4^+$ T cell specific and T helper-1 (Th1) dominant immune response[98]. These individuals are said to be latently infected with Mtb (LTBI) and have a 10% lifetime risk of developing active TB disease[460]. In the presence of HIV-1 co-infection, progression to active disease is over 20 times more likely (10% annual risk)[460]. Not only are HIV-1 seropositive individuals at greater risk of developing Mtb, they have higher mortality rates following active TB than HIV-1 seronegative individuals[100,101,108]. The interaction of Mtb and HIV-1 has been shown to be bi-directional and synergistic, not

only does HIV-1 infection increase the likelihood of developing active TB infection, but TB infection accelerates the course HIV-1 infection[99] by actively inducing viral replication[92,99,102] .

Given current estimations that one third of the world's population is infected with Mtb and that annual HIV-1 infection rates are increasing[5] it is imperative that we are able to accurately diagnose both active and LTBI in HIV-1 positive individuals. Yet, to date there is no gold standard for the diagnosis of LTBI. Tuberculin skin test (TST) has traditionally been used to measure delayed hypersensitivity reaction following intra-dermal injection of purified protein derivative (PPD)[461]. Obtained from the crude precipitate of Mtb culture supernatant, PPD contains more than 200 proteins widely shared amongst mycobacteria, including M.bovis, Bacilli Calmette-Guérin (BCG) and common environmental mycobacteria[462]. Consequently its use in the diagnosis of LTBI lacks specificity and sensitivity[461]. More recently, recognition of the critical role played by interferon gamma (IFN-γ) in regulating cell mediated responses to Mtb, led to the development of IFN-γ release assays (IGRA)[463,464]. Current FDA approved IGRA, QuantiFERON-TB Gold In-Tube (QFT-IT) and T-SPOT.TB assay[465] are based upon the enzyme-linked immunosorbent assay (ELISA) or ELISPOT techniques respectively[464].

IGRAs have been shown to greatly improve the specificity of LTBI diagnosis by measuring cytokine production to Mtb-specific proteins, that are absent from BCG and most other environmental mycobacteria[464]. Early secreted antigenic target-6 (ESAT-6) and culture filtrate-10 (CFP-10) are the two best studied and can improve sensitivity of LTBI diagnosis to 96% compared to 69% sensitivity achieved by TST [464]. In addition to T cell activation, ESAT-6 and CFP-10 have been shown to play a major role in Mtb virulence[466-468]. Restoration of CFP-10 and ESAT-6 expression in BCG, increases the virulence and immunogenicity of the bacterium[469,470], as evidenced by a change in colony morphology and growth rate in the lungs and spleen of SCID mice, and also by an increased degree of splenomegaly[469,470]. Moreover, deletion of the genes encoding CFP-10 and ESAT-6 has been shown to substantially attenuate virulence, enabling live vaccine to be administered with little to no pathological evidence of disease[471].

Despite the advantages of using IGRA over TST questions still remain as to the suitability of their use in HIV-1 positive individuals[457,458]. Poor concordance between the different IGRA[114], an overall reduction in proliferative response and IFN-γ secreting capacity of Mtb-specific CD4 T cells in HIV infection[472] has prompted the Centre for Communicable Diseases (USA) to recommend caution in their use in HIV-infected persons[473,474].

To address sensitivity issues arising from the application of the standard *ex vivo* ELISpot to the detection of low frequency and magnitude T cell responses, a number of modifications have been described[155,156,475-478]. One such modification involves a brief *in vitro*, peptide stimulated culture period to preferentially expand antigen specific cells. In addition to peptide, PBMC are also supplemented with anti-apoptotic and proliferative cytokines (Interleukin-7 and interleukin-2 respectively)[391]. Referred to as the cultured ELISpot, this modified version of the standard ELISpot assay, has been shown to greatly improve sensitivity in a number of disease settings[81,391,479-481], but has yet to be applied to detection of LTBI.

4.2 Chapter Aims:

The focus of this chapter will be to assess whether the cultured IFN-γ ELISpot technique can be applied to enhance the sensitivity of detection of LTBI in both HIV-1 infected and uninfected donors. Moreover it aims to assess whether cells generated from the brief *in vitro* culture period can be used for more detailed functional and phenotypic studies.

In light of previous observations suggesting that where detectable, HIV-1 specific T cell responses in HESN individuals tend to be weak and often transient[135,151,155]. Standardization of the cultured IFN-γ ELISpot as a sensitive, robust and reproducible method will provide not only a clinically relevant observation as to whether current diagnostic tests are underestimating the true prevalence of LTBI. But moreover, it will provide justification for the use of cultured IFN-γ ELISpot, in detection and characterization of HIV-1 specific T cell responses in HESN individuals, the primary focus of this thesis.

4.3 Results

The criteria used to define a positive T cell response, by either cultured or *ex vivo* ELISpot, was based upon observations made in healthy, HIV-1 seronegative, individuals enrolled in the IAVI 016 vaccine trial[391]. Briefly, a positive response was defined as greater than 4x mock and/or greater than 300 spot forming units/million cells (SFU/10^6) in cultured assays or >50 SFU/10^6 in *ex vivo* assays. This threshold represented >99th percentile, of background subtracted T cell responses in pre-vaccination samples[391]. As an additional control, each peptide was tested twice, once in an "All" pool of peptides and again in 1 of 3 sub pools. To be defined as positive, T cell responses had to be detected in both the "All" pool and one or more of the sub pools. The assay was classed as "failed" and data excluded from analysis should the mock wells have greater than 250 SFU/10^6.

4.3.1 *Ex vivo* detection of latent Mycobacterium tuberculosis infection

Using a laboratory-modified version of the commercial T-Spot.TB assay, cryopreserved peripheral blood mononuclear cells (PBMC) isolated from 27 HIV-1 seronegative and 27 HIV-1 seropositive, Malawian individuals were assessed for the *ex vivo* detection of latent Mtb infection. Detection of positive T cell responses to Mtb specific antigens, ESAT-6 and/or CFP10, suggests 13/27 (48.1%) HIV-1 seronegative donors were latently infected with Mtb (Figure 4.1A). In contrast only 3/27 (11.1%) HIV-1 seropositive individuals had detectable Mtb specific T cell responses (Figure 4.1B). The HIV-1 positive individuals were still capable of responding to commonly presented, $CD8^+$ epitopes from flu, Epstein Barr Virus (EBV) and Cytomegalovirus (CMV) (peptide pool referred to as FEC) and the mitogenic control phytohaemagglutinin (PHA) (Figure 4.1B).

4.3.2 Improved sensitivity afforded by cultured IFN-γ ELISpot

Ex vivo responses detected in a large subset of these participants (26 HIV-1 negative and 22 HIV-1 positive) were compared with those observed following the cultured IFN-γ ELISpot technique (Figure 4.2). The cultured ELISpot assay was shown to increase the magnitude of Mtb specific responses >20 fold in both HIV-1 negative

and positive donors (Figure 4.2A,B). The frequency at which Mtb specific T cell responses were detected amongst HIV-1 seronegative individuals increased to 65% (Figure 4.2A). More strikingly, a greater than 6 fold increase in the detection of Mtb specific T cell responses was observed in HIV-1 seropositive individuals (Figure 4.2B). Of the 22 HIV-1 positive individuals tested, 7/22 had detectable Mtb specific T cell responses following cultured IFN-γ ELISpot compared to just 1/22 using standard *ex vivo* ELISpot analysis (Figure 4.2B).

4.3.3 Cultured IFN-γ ELISpot assay specificity

Cultured Mtb-specific T cell responses were compared between a population in which Mtb infection is rare (UK – incidence 3%) and a population in which Mtb is endemic (Malawi – 68%)[5,83]. Following cultured ELISpot 26/41 (63%) HIV-1 seronegative, Malawian participants produced Mtb-specific T cell responses (Figure 4.3A) whilst only 1/14 (7%) UK participants tested positive (Figure 4.3B). However, T cell responses to 85A and FEC peptides were comparable between each group. Notably the background IFN-γ production for these assays was low (data not shown).

4.3.4 T cell response titration and reproducibility

Intra assay variability was assessed in four subjects. For each donor multiple (n=3-6) short-term cell lines were generated using stimulation with tuberculosis peptides. Each short-term cell line was independently screened using cultured IFN-γ ELISpot for detection of tuberculosis specific T cell responses. The IFN-γ response to tuberculosis peptides did not significantly vary between cultured lines generated from the same donor (p=>0.01 for each donor tested) (Figure 4.4 A). In addition, antigen specific T cell responses detected by cultured ELISpot could be titrated both at the start (Figure 4.4 B) and end (Figure 4.4C) of the culture period, suggesting responses were not the result of *in vitro* priming, or due to saturating peptide concentrations.

4.3.5 Epitope mapping and $CD4^+/CD8^+$ T cell specificity

A three-step approach was used to map Mtb specific T cell responses to the peptide level in 10 HIV-1 seronegative subjects. Initial screening by cultured IFN-γ ELISpot assay identified the protein to which responses were targeted (Figure 4.5A). Next a three dimensional matrix enabled likely peptide candidates to be identified (Figure

4.5B). Peptide responses were confirmed and characterized as being mediated by either $CD4^+$ or $CD8^+$ T cells using a combination of ELISpot (data not shown) and intracellular cytokine staining (ICS) methods (Figure 4.5C).

4.3.6 Cryopreservation does not significantly decrease sensitivity

Sixteen short-term cell lines generated from different donors were compared for total IFN-γ production against a pool of Mtb peptides pre- and post-cryopreservation (Figure 4.6). Recovery of viable T cells ranged from 45-98%. Paired t-test showed no significant decrease in sensitivity of detection of Mtb specific T cell responses following revival of cryopreserved short-term cell lines, as compared to data generated from lines analysed directly after culture ($p=0.554$).

4.4 Discussion

One of the aims of this thesis is to examine the detection of HIV-1 specific T cell responses in HIV-1 exposed, seronegative (HESN) individuals. Previous literature reports have suggested that where detectable, HIV-1 specific T cell responses in HIV-1 seronegative donors are of low magnitude and frequency[135,151,155]. Therefore, in order to accurately study these T cell responses we felt it essential to employ a highly sensitive detection method.

The ELISpot assay has a long history as a robust and reproducible means by which to identify antigen specific T cell responses[135,145,147,382,383,453]. However, its recent application to the detection of latent Mtb infection amongst HIV-1 infected subjects has raised questions regarding its sensitivity[473,474]. Within this chapter a modified version of the conventional *ex vivo* ELISpot assay, referred to as the cultured ELISpot, was assessed for its ability to improve the sensitivity of detection of tuberculosis specific T cell responses, without compromising specificity.

A laboratory modified version of the T.Spot TB assay[464] was used to screen HIV-1 seropositive and seronegative Malawian individuals for latent Mtb infection. Similar to the commercially available T.Spot TB assay the laboratory modified version contained pools of peptides spanning Mtb specific genes deleted from BCG and most environmental mycobacteria, ESAT-6 and CFP-10[482,483]. In addition, a pool of peptides spanning the 85A complex was also included. 85A is the major protein secreted in mycobacterium culture filtrate and is common to several mycobacteria[395], it therefore acted as a $CD4^+$ peptide control. In addition to the standard mitogenic, positive control (PHA), a $CD8^+$ peptide control of commonly presented flu, EBV and CMV epitopes (FEC)[484] was also included.

Initially 27 HIV-1 seronegative and 27 seropositive Malawian individuals were screened for *ex vivo* Mtb specific T cell responses indicative of LTBI. In accordance with current World Health Organization (WHO) predictions and previous literature reports[5,83,108], 13/27 (48.1%) HIV-1 negative individuals had Mtb-specific T cell responses (Figure 4.1A). In contrast HIV-1 positive participants had a much lower frequency, with only 3/27 (11.1%) having detectable Mtb-specific T cell responses

(Figure 4.1B). Reduced proliferative capacity and IFN-γ production in HIV-1 positive donors has previously been suggested to account for discrepancies in LTBI rates between HIV-1 seropositive and negative individuals[111,458,473,474]. Indeed observations such as these, showing pronounced differences in the detection of LTBI rates amongst HIV-1 positive and negative individuals is the basis for concerns surrounding the application of IGRA to immunocompromised individuals[111,458,473,474]. We reasoned that perhaps Mtb specific T cell responses amongst immunocompromised individuals were falling below the limits of detection for a conventional ELISpot assay.

This chapter aimed to assess whether the cultured IFN-γ ELISpot assay could overcome some of the sensitivity issues observed in the detection of LTBI in HIV-1 positive individuals. *Ex vivo* tuberculosis specific T cell responses detected in a subset of participants (26 HIV-1 negative and 22 HIV-1 positive) were compared to those observed following the cultured IFN-γ ELISpot (Figure 4.2). Consistent with previous studies the magnitude of Mtb specific responses increased greater than 20 fold following cultured IFN-γ ELISpot[81,391,479-481]. The frequency at which Mtb specific T cell responses were detectable amongst HIV-1 seronegative individuals increased from 48.1% to 65% (Figure 4.2A). More strikingly, a greater than 6 fold increase in the detection of Mtb specific T cell responses was observed in HIV-1 seropositive individuals (1/22 *ex vivo* to 7/22 following culture (Figure 4.2B)). The improved sensitivity afforded by the cultured IFN-γ ELISpot is believed to be the result of the brief, *in vitro*, peptide stimulated culture period. During culture PBMC are supplemented with anti-apoptotic and proliferative cytokines (IL-7 and IL-2 respectively) enabling not only T cells present to divide, but also permitting resting central memory T cells to differentiate into effector cells[475,479,485].

In the absence of a gold standard by which to compare detection of Mtb specific T cell responses it is difficult to assess whether the cultured IFN-γ ELISpot technique is capable of achieving 100% detection. However, data presented herein would suggest 100% detection to be highly unlikely. Whilst the cultured IFN-γ ELISpot was shown to improve the sensitivity with which Mtb specific T cell responses were detected amongst HIV-1 seropositive donors, the frequency was still considerably lower than that for seronegative donors (31.8% HIV-1 positive vs 68% HIV-1 seronegative). This

observation suggests that some Mtb specific T cell responses are still likely to fall below the limits of detection. Moreover, given the profound depletion of $CD4^+$ T cells that occurs during HIV-1 infection, and the observation that $CD4^+$ T cells from HIV-1 infected individuals have reduced proliferative capacity and an impaired ability to secrete IFN-γ[111,458,473,474], it is likely many Mtb specific responses were "missed" as a result of the chronic immune dysregulation that accompanies HIV-1 infection[114,118,119,368,375,378].

The clinical relevance of Mtb specific T cell responses detected by cultured IFN-γ ELIspot remains unknown. Moreover, it is unclear whether the improved sensitivity achieved by the cultured IFN-γ ELISpot is measuring true LTBI or markers of Mtb exposure. Evidence from malaria research suggests that whilst T cell responses detected by cultured IFN-γ ELISpot assay are of extremely low frequency and magnitude, they can be biologically relevant[479,480]. Heterologous challenge, of vaccine recipients, receiving a prime boost anti-malaria vaccine, showed that the magnitude of cultured and not *ex vivo*, IFN-γ responses correlated with protection against malaria infection[479]. Furthermore, the detection of $CD4^+$ T cell responses to a conserved epitope within the circumsporozite protein, by cultured IFN-γ ELISpot was shown to correlate with protection against *P. Falciparum* infection[480]. In both of these studies, potential correlates of protection would have been missed by conventional *ex vivo* analysis[479,480]. The authors attribute the improved sensitivity afforded to the cultured IFN-γ ELISpot due to the detection of durable, central memory T cell rather than the shorter lived effector cells measured by *ex vivo* ELISpot analysis[479,480].

To ensure that the cultured IFN-γ ELISpot was specifically improving sensitivity by expanding pre-existing immune cells, cultured Mtb-specific T cell responses were compared between a population in which Mtb infection is rare (UK – incidence 3%) and a population in which Mtb is endemic (Malawi – 68%)[5,83] (Figure 4.3). Should the brief culture period be sufficient to *in vitro* prime Mtb specific T cell responses we would hypothesize that detection rates in UK subjects would be greatly elevated following culture. However, we demonstrate this was not the case (Figure 4.3). Whilst Mtb specific T cell responses following cultured IFN-γ ELISpot were detectable in 63% of Malawian donors, they were only detected in 7% of UK subjects (Figure 4.3).

This is consistent with UK wide estimates of LTBI[5,83] and suggests that specificity was retained over the culture period of the assay. Importantly, T cell responses to FEC or 85A, which is incorporated within BCG vaccination and abundant in other mycobacteria, did not vary between UK and Malawian participant's, indicating this result was not due to an overall proliferative defect amongst UK subjects.

To examine intra-assay variability IFN-γ production to peptides spanning CFP-10, ESAT-6 and 85A were assessed from multiple cultured cell lines ascertained from the same donor (Figure 4.4A). Whilst minor fluctuations in the number of IFN-γ producing cells, were detected in all four donors studied, these did not achieve statistical significance (p=>0.1). Furthermore, antigen specific short-term cell lines could be cryopreserved and revived without significant loss of sensitivity (p=>0.05; Figure 4.6). This is particularly useful for large, high through-put studies where cell number is often limiting, since following initial screening, short-term cell lines can be stored enabling more detailed mapping and phenotype studies to be performed at a later date.

In addition to providing a reproducible means by which to specifically improve detection of low magnitude T cell responses, the cultured IFN-γ ELISpot, together with a three dimensional matrix mapping approach[396], can also be used to delineate antigen specific T cell responses, down to the peptide level (Figure 4.5). When combined with ICS, the cultured IFN-γ ELISpot technique can be used to generate both functional and phenotypic data (Figure 4.5C). The ability to map antigen specific T cell responses to the peptide level provides insight into the immune correlates of protection that could be harnessed for the design of novel epitope-based vaccine candidates[380,391,486].

In summary, this chapter has identified the cultured IFN-γ ELISpot assay as a highly sensitive, reproducible method by which to specifically improve detection of low frequency and magnitude T cell responses. Its novel application to the detection of LTBI suggests that current diagnostic tests are likely to be underestimating the true prevalence of LTBI, particularly amongst HIV-1 positive individuals. This observation is potentially of significant importance clinically, given the extremely

high morbidity rates amongst HIV-1/TB co-infected individuals[5,99,108]. The cultured IFN-γ ELISpot assay improved the sensitivity of detection of Mtb specific T cell responses 6 fold, compared to standard *ex vivo* tests in HIV-1 positive individuals. Future studies are now required to determine the clinical relevance of cultured IFN-γ ELISpot responses and LTBI rates in other cohorts from both TB endemic and non-endemic regions. However, the low cell number requirements, specificity and sensitivity of the cultured IFN-γ ELISpot, coupled with the ability to perform detailed functional and phenotypic studies, suggest it should prove a valuable tool in future research of Mtb-specific responses as well as in the detection of HIV-1 specific T cell responses in seronegative individuals.

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[i] Data generated in this chapter was accepted for publication in the European Journal of Immunology:

Campion, S., Cohen, M.S., McMichael, A.J., Galvin, S. & Goonetilleke, N. Improved detection of latent Mycobacterium tuberculosis infection in HIV-1 seropositive individuals using cultured cellular assays. *Eur J Immunol* **41**, 255-257 (2011).

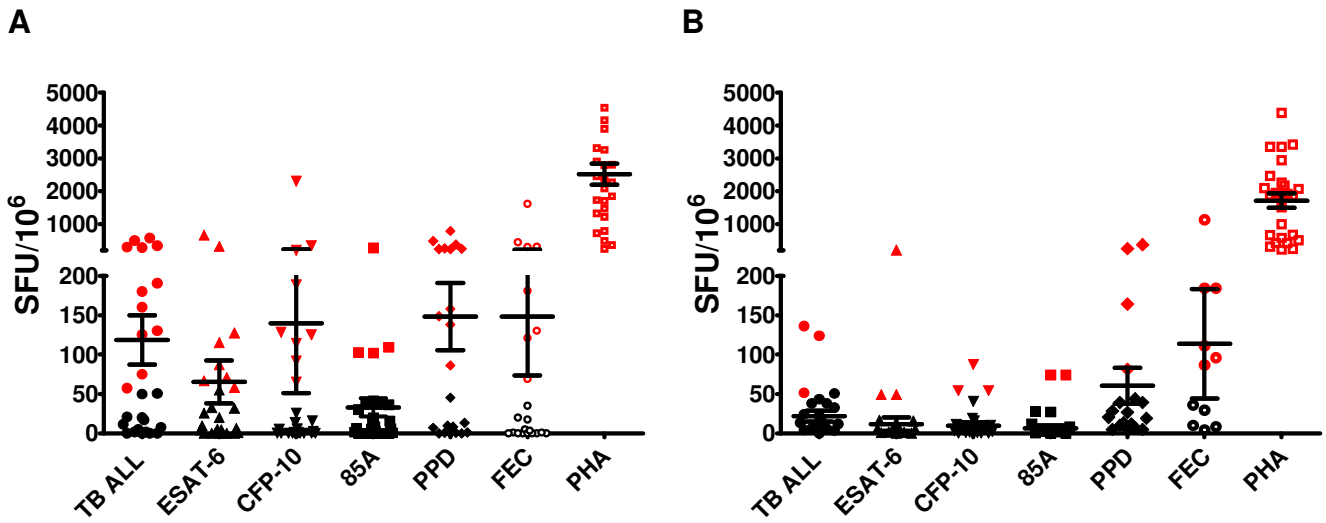


Figure 4:1

Ex vivo IFN-γ ELISpot analysis of Mtb-specific responses in (A) 27 HIV-1 seronegative individuals and (B) 27 HIV-1 positive individuals from Malawi. Data presented as number of spot forming units per million PBMC (SFU/10^6) following subtraction of mock stimulated wells (background). All assays were performed in quadruplicate using Mtb-specific antigens (ESAT-6 and CFP-10), in addition to positive control antigens (85A, PPD, FEC and PHA). Positive T cell responses (shown in red) were defined as greater than or equal to 50 SFU/10^6, and greater than 4x mock stimulation. Error bars represent standard error of mean.

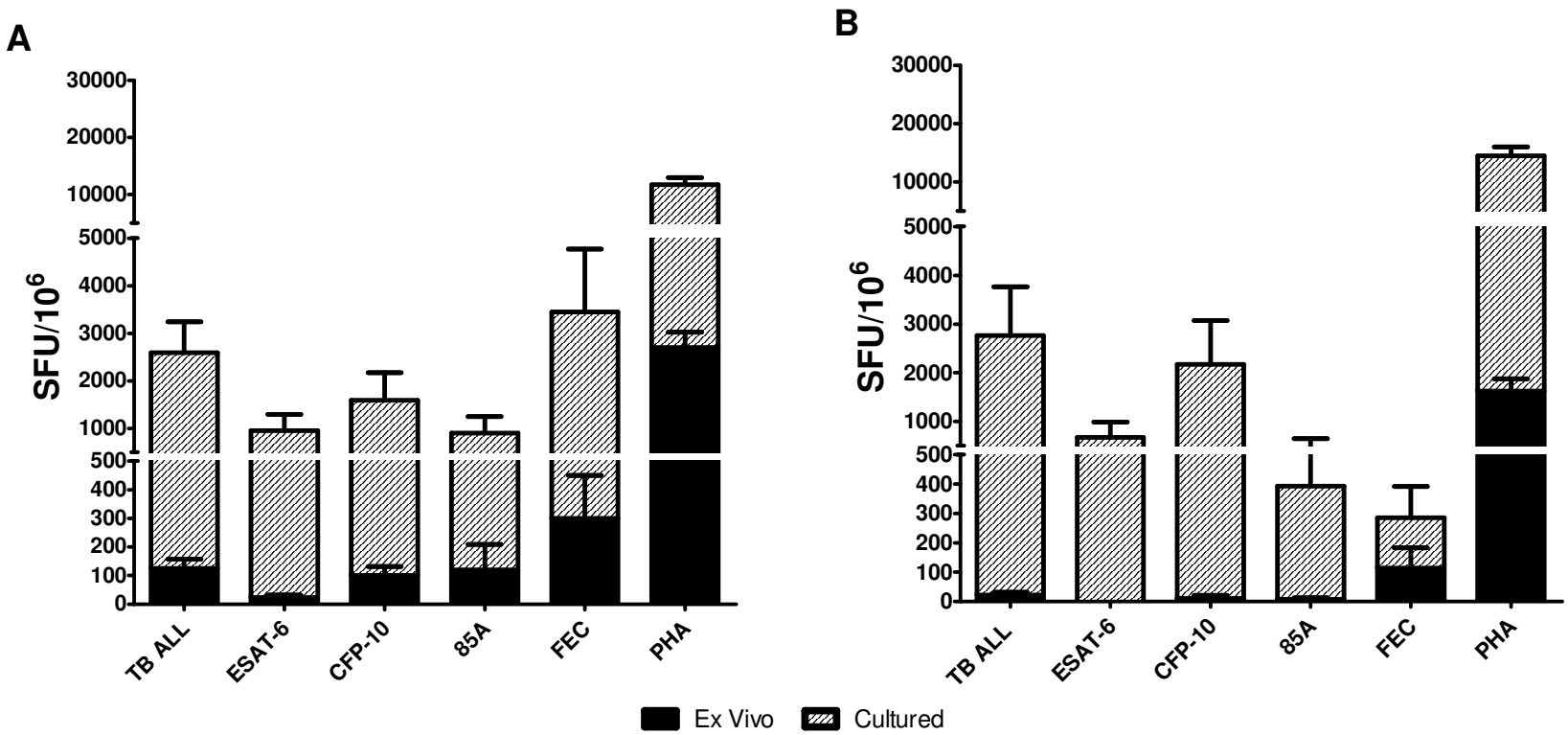


Figure 4:2

The cultured IFN-γ ELISpot technique increased sensitivity and detection of low frequency IFN-γ T-cell responses to a given antigen. Mtb-specific IFN-γ responses detected using *ex vivo* ELISpot analysis (solid bars) were compared to responses detected following cultured IFN-γ ELISpot (hatched bars) in (A) 26 HIV-1 seronegative and (B) 22 seropositive individuals. All data presented as average number of SFU/10^6 following background subtraction. The criteria used to define a positive T cell response was greater than 50 SFU/10^6 (*ex vivo* assays) or 300 SFU/10^6 (cultured assay) and 4x background. Error bars shown represent standard error of mean and all data presented is background subtracted.

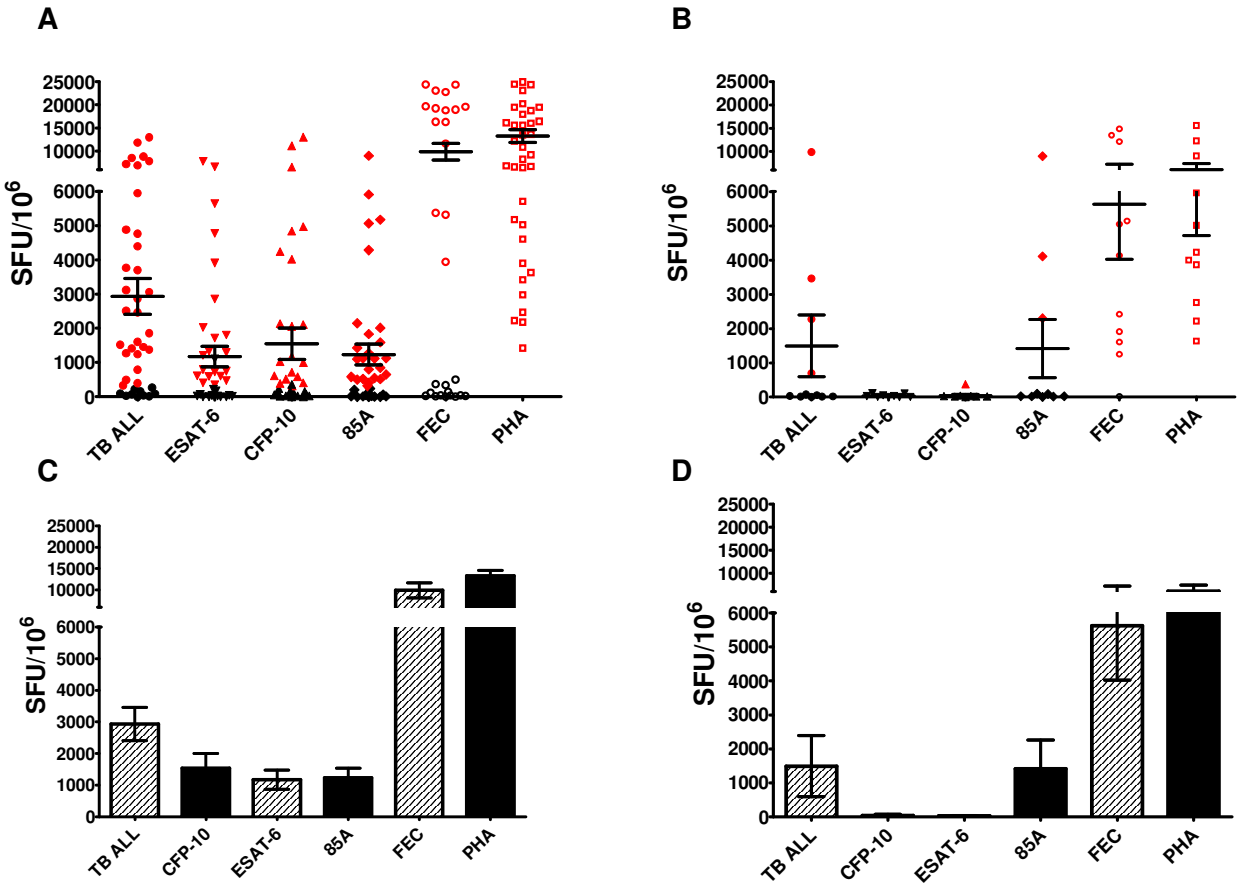


Figure 4:3

The specificity with which the cultured ELISpot technique allows for increased sensitivity and detection of low frequency IFN-γ T-cell responses was assessed by comparing Mtb specific T cell responses amongst a population where Mtb is endemic (Malawi; A, C) to an area where Mtb is rare (UK; B, D). A total of 41 Malawian and 14 UK subjects were screened for Mtb specific T cell responses using the cultured ELISpot technique. Notably, whilst T cell responses to Mtb specific proteins ESAT-6 and CFP-10 were absent from UK subjects, no significant difference in response to peptide control pools, 85A or FEC, was detected (P=>0.5). Figures A and B represent individual data points whilst C and D show the average number of SFU per million PBMC. Criteria for a positive responses (illustrated in red for A-B) was greater than 300 SFU/10^6 and >4x background. Error bars represent standard error of mean and all data presented is background subtracted.

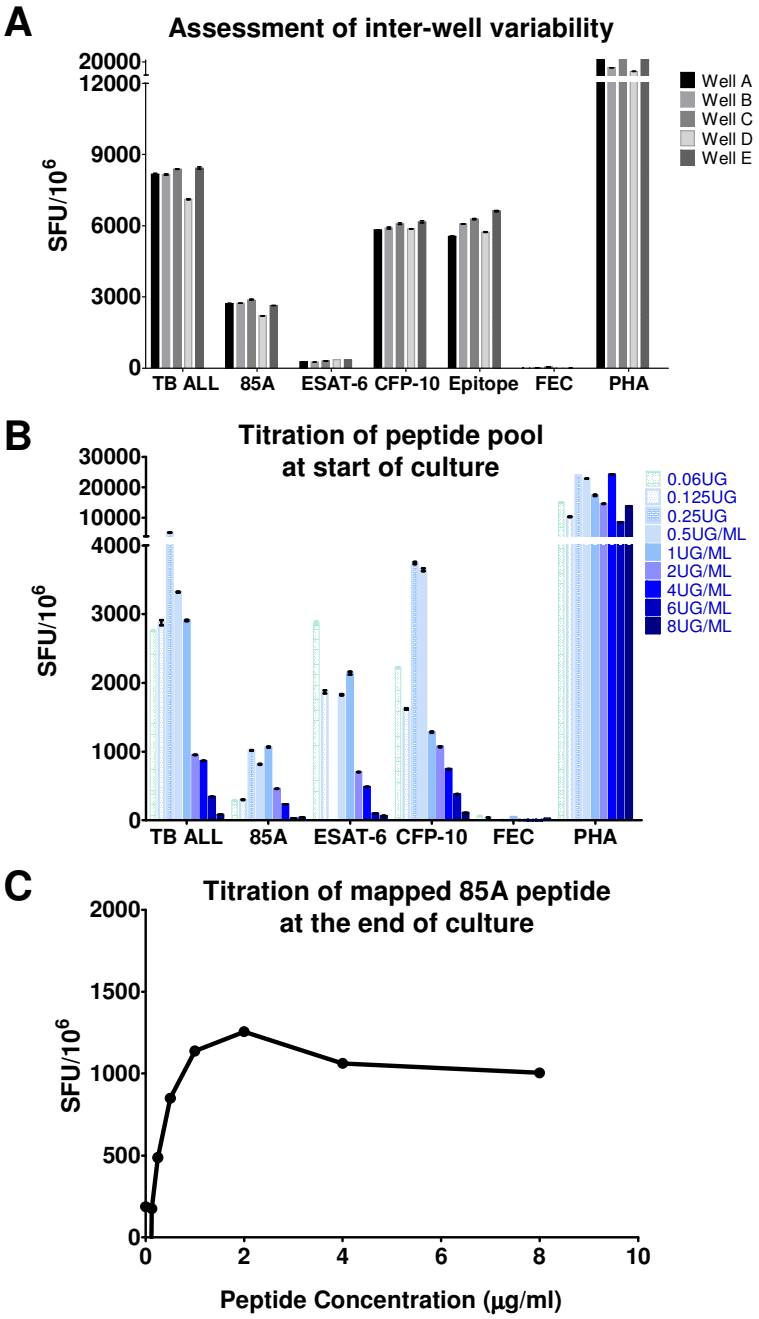
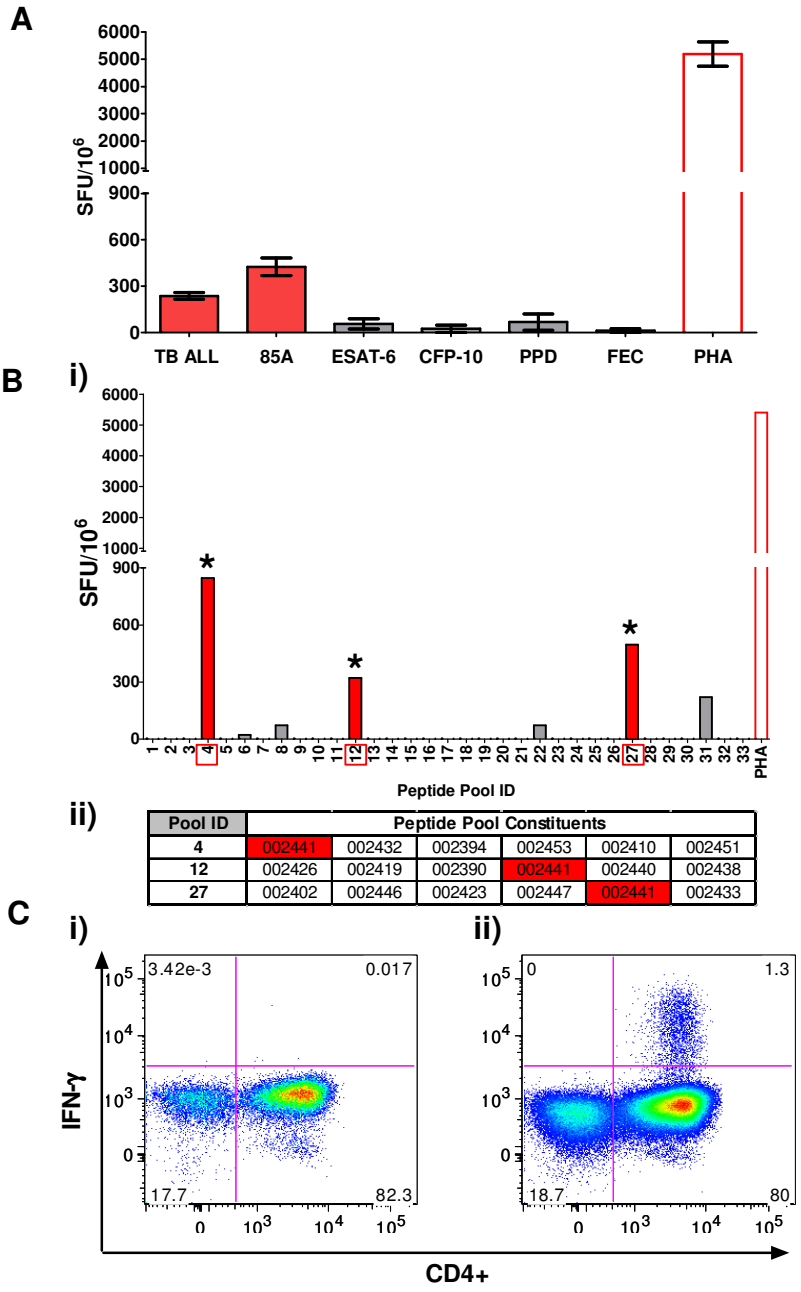


Figure 4:4

Intra-assay variability was determined to be low (p=0.2074) following 2 way ANOVA analysis of data generated from individual short-term cell lines generated from the same donor (A). Antigen specific T cell responses could be titrated at both the start (B) and end of culture (C). Data presented is representative of 4 HIV-1 seronegative subjects studied. Positivity criteria for cultured IFN-γ ELISpot were >300 SFU/10^6 and >4x background. All data presented is background subtracted, with error bars representing standard error of mean.



Pool ID	Peptide Pool Constituents					
4	002441	002432	002394	002453	002410	002451
12	002426	002419	002390	002441	002440	002438
27	002402	002446	002423	002447	002441	002433

Figure 4.5

Antigen specific T cell responses detected by cultured IFN-γ ELISpot can be mapped to the peptide level. Responses are first screened against pools of peptides (A) to identify the target protein. Using a 3 dimensional matrix mapping approach (Bi) potential epitopes are identified (Bii). Responses are then confirmed using intracellular cytokine staining (C). (Ci) mock stimulated control, (Cii) stimulation with peptide of interest at 2µg/ml). Positivity criteria for cultured IFN-γ ELISpot were >300 SFU/10^6 and >4x background. (All data presented is background subtracted, with error bars representing standard error of mean). Criteria for a positive response by intracellular cytokine staining was >0.05% and 2x background

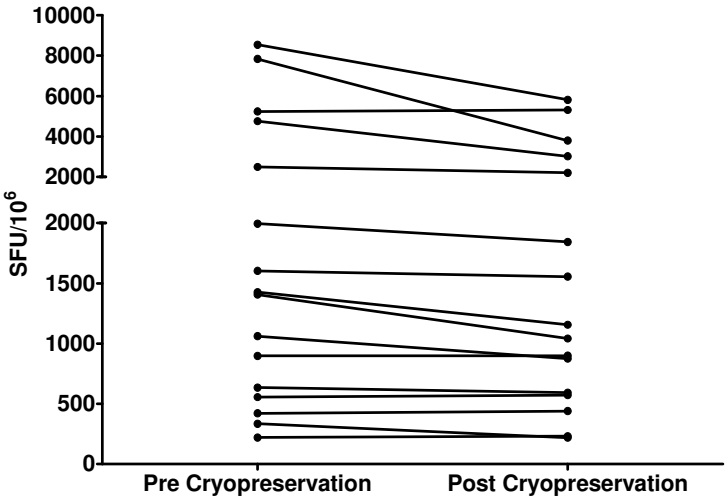


Figure 4.6

Short-term cell lines generated for cultured IFN-γ ELISpot analysis can be cryopreserved and revived without significant loss of sensitivity (two tailed, paired T test p=0.0554) Data illustrated is the total IFN-γ production in response to stimulation with a pool of Mtb specific peptides measured pre-and post- cryopreservation by cultured IFN-γ ELISpot. The final concentration of each peptide in the pool is 2μg/ml. Positivity criteria for cultured IFN-γ ELISpot were >300 SFU/10^6 and >4x background. All data presented is background subtracted.

CHAPTER 5:

DETECTION OF HIV-1 SPECIFIC T CELL RESPONSES IN SERONEGATIVE INDIVIDUALS

Chapter 5: Detection of HIV-1 specific T cell responses in seronegative individuals.

5.1 Introduction:

Exposure to HIV-1 does not always result in seroconversion. There are rare populations of individuals who, despite repeated high risk exposure to HIV-1, remain seronegative. Referred to as HIV-1 exposed seronegative (HESN)[140] these "high risk" individuals can be found in a number of cohorts, including; sero-discordant couples[136,145,296,487], commercial sex workers from areas with high prevalence of HIV-1[135,147,150], men who have sex with men (MSM)[137,156,157,488,489], infants born to HIV-1 infected mothers[158,161,162,490], health care workers exposed to contaminated blood products[138,163,164] and haemophiliacs who received HIV-1 infected blood clotting factor concentrates prior to wide scale HIV-1 screening[166,491].

The probability of transmission following a single exposure to HIV-1 is estimated to be low[30,32]. A commonly cited value of approximately 0.001 for heterosexual infectivity, is likely to represent a lower bound, obscuring important differences associated with transmission cofactors such as direction of transmission, type of sexual act, viral load, male circumcision, vaginal flora, age and sexually transmitted infections (reviewed in[31]). However, it raises the possibility that some HESN individuals may simply have been fortunate, escaping HIV-1 infection by chance. Yet "chance" alone cannot explain all instances of HESN and there is now accumulating evidence to suggest both genetic[46-48,142] and host immune responses[135,136,145,148,150,151,154,156,158,295,382-384,489,492, 149,493,494] are capable of conferring protection against the establishment of HIV-1.

To date, the only reproducibly confirmed correlate, of high level protection from HIV-1 is a homozygous 32 base pair deletion in the HIV-1 co-receptor CCR5 (CCR5Δ32)[46-48,493]. With a low allele frequency and an all but absence from non-Caucasian populations[47,495], this mutation cannot fully explain the HESN phenomena. There are examples of individuals whom, in the absence of CCR5Δ32 mutation, and in the presence of high risk HIV-1 exposure remain HIV-1 seronegative. The best example of this comes from the study of haemophiliac patients, known to have

received HIV-1 contaminated blood products. Whilst there is an enrichment of CCR5Δ32 homozygosity amongst these individuals, up to 85% lack the CCR5Δ32 genotype and appear "protected" by, as yet, unknown factors (reviewed in[167]). A greater understanding of the immune correlates of "protection" afforded in these individuals could prove beneficial to the design of novel efficacious vaccines.

It is perhaps unsurprising that a number of potential "correlates of protection" against the establishment of HIV-1 have been proposed. These include innate factors such as increased natural killer (NK) cell activity[142,189,191] and increased expression and/or sensitivity to CC chemokines such as RANTES, MIP-1α/β[149,493,494]. Interestingly, HIV-1 specific T cell responses have been detected in a proportion of HESN individuals, implicating the adaptive immunity as a potential correlate of protection[135,136,145,148,150,151,154,156,158,295,382-384,489,492].

The importance of adaptive immune responses during HIV-1 infection has been well documented, and is believed to correlate with control of viremia[38,74,76,77,79,132,133,206,252,358,359,407,496,497]. Early evidence for this came from the observation of a temporal association between the emergence of cytotoxic T lymphocytes (CTL) and the decline in peak viremia[206]. Later, primate models demonstrated that depletion of $CD8^+$ T cells results in rapid viral rebound and progression to acquired immunodeficiency disease (AIDS)[358]. Further evidence comes from the study of individuals with non-progressive disease, referred to as elite controllers or long term non-progressors (LTNP). These individuals exhibit stable, normal $CD4^+$ T cell counts in the periphery and durably low to undetectable viral loads in the absence of anti-retroviral treatment. LTNP have maintained HIV-1 specific T cell responses[76,79] and a higher prevalence of specific, human leukocyte antigen (HLA)-class I alleles, such as B57, which are now commonly referred to as "protective" alleles[74,250,252].

The study of HIV-1 itself has also highlighted a pivotal role of HIV-1 specific T cell responses. Combined viral sequencing and T cell assays have shown that HIV-1 undergoes rapid mutation to escape the proposed "pressure of CTL" responses to immunodominant epitopes[38,70,406,498]. Taken together these observations provide

strong evidence that HIV-1 specific T cell responses are important in the control of HIV-1 viremia. Whether HIV-1 specific T cell responses can protect against the establishment of HIV-1 however, remains unclear. Interestingly, both $CD4^{+}$[137,138,145,148,150,295,296,382] and $CD8^{+}$[135,147,151,156-158,163,203,296,383,384] HIV-1 specific, T cell responses have been detected in a proportion of HESN individuals, leading to speculation that they may confer a degree of protection against the establishment of HIV-1[145,147,160,386,492]. Typically of low magnitude and often transient[135,152,155], HIV-1 specific T cell responses have been mapped to the epitope level [135,157,487] and their detection correlated with HIV-1 exposure[150,152]. However, the detection and role of pre-existing HIV-1 specific T cell responses in HESN individuals remains controversial. The controversy surrounding HIV-1 specific T cell responses is multifaceted, but is owed in part to the fact that not all studies have been able to detect HIV-1 specific T cell responses in HESN individuals [134,155,499]. Whether the failure to detect HIV-1 specific T cell responses is the result of poorly recruited/defined HESN, a lack of sensitivity in detection methods or simply that pre-existing T cell responses are not present in all HESN remains unclear.

5.2 Chapter Aims

For a minority of individuals, repeated, high risk exposure to HIV-1 does not lead to seroconversion. It is likely that the apparent "protection" afforded to these individuals is multifactorial. However, the relevance of HIV-1 specific T cell responses, detected in a proportion of highly exposed, seronegative (HESN) individuals, remains poorly understood, and controversial. The relatively low infectivity of HIV-1[29-31], suggests some of the discrepancies in HESN data could stem from the criteria used to define a HESN. Furthermore, where detectable HIV-1 specific T cell responses have tended to be weak and often transient, it is possible that many responses fall below the limits of detection of standard *ex vivo* assays.

This chapter aims to address some of the discrepancies surrounding the detection of HIV-1 specific T cell responses in HESN individuals. The use of a highly sensitive, cultured IFN-γ ELISpot technique (detailed in Chapter 3), stringent criteria to define HESN subjects and the inclusion of HIV-1 unexposed, seronegative (HUSN) control subjects, should set this study apart from previous literature reports. In addition to providing a detailed examination of the frequency and maintenance of HIV-1 specific T cell responses, correlations between risk of exposure and detection of HIV-1 specific T cell responses will also be made. It is hoped that greater understanding of the potential immune correlates of protection will assist in the future development of therapeutic vaccines. We hypothesize that HIV-1 specific T cell responses will be detectable in a proportion of HESN individuals. That exposure to HIV-1 will be a pre-requisite for detection of pre-existing HIV-1 specific T cell responses and that the use of stringent criteria to define HESN subjects will improve the likelihood of frequency with which pre-existing HIV-1 specific T cell responses are detected.

5.3 Cohorts studied

Within this chapter two HESN cohorts were studied. The first is a retrospective cohort of Malawian STI clinic attendees, which was co-ordinated by Professor S. Galvin and is referred to herein as the GALVIN cohort. Thirty one HESN subjects were identified based upon a negative HIV-1 nucleic acid test (NAT) and having ≥1 STI in a clinical setting where HIV-1 has 47% prevalence. 10 low risk controls were also enrolled from Malawi, with an additional 14 low risk HUSN control samples obtained

from pre-vaccination time points of the IAVI 016 vaccine trial (Access granted by Dr N Goonetilleke)[391]. Criteria for inclusion as a low risk control were, being in a monogamous relationship with a HIV-1 negative partner, and having self-reported low risk behaviour. All work presented from these cohorts was solely performed by myself.

Observations made within the retrospective GALVIN cohort, directed studies in a much larger HESN cohort, referred to as CHAVI002. This was a specifically designed, blinded study, powered to detect differences in the frequency and magnitude at which HIV-1 specific T cell responses were observed in both HESN and low risk HUSN subjects. Twenty four serodiscordent couples and 14 HIV-1 negative-concordant couples were enrolled at St. Mary's hospital, London. A detailed sexual behaviour questionnaire was employed to define HESN as individuals who were HIV-1 antibody and NAT/p24 antigen negative, who have had unprotected intercourse with HIV-1 infected partner on $\geq$25 occasions in the preceding 12 months, with the first sexual exposure with HIV-1 positive partner occurring at least 10 months prior to the first day of screening. The CHAVI002 cohort was overseen by Dr N Goonetilleke and two post docs within the laboratory (Dr A Ritchie and Dr J Kopycinski). I was involved in assay development (specifically, intracellular cytokine staining of short-term cell lines and epitope mapping) and T cell assays performed at early study visits. Independent statistical analysis was performed on data generated from the CHAVI002 cohort, by Dr Z Moodie at the statistical centre for HIV-1 and AIDS research and prevention (SCHARP). In addition genetic analysis of CCR5Δ32 genotype for CHAVI002 subjects was performed by Dr S Brackenridge.

Full details on the cohorts presented in this chapter can be found in Chapter 2, Material and Methods Section 2.1.

5.4 Results:

5.4.1 Detection of HIV-1 specific T cell responses in seronegative donors by cultured IFN-γ ELISpot

Clade C consensus peptides, spanning the entire HIV-1 proteome were pooled according to protein, referred to as Gag, Env, Pol and Nef/Acc. The Nef/Acc pool included peptides spanning Nef and accessory proteins- Vpr, Vpu, Vif, Tat and Rev. An additional two pools labelled CTLA and CTLB were included to contain optimal CTL epitopes as defined by the LANL database[21]. Together with the cultured IFN-γ ELISpot, HIV-1 specific peptides were used screen 13 chronic HIV-1 positive, 31 HESN, and 24 low risk control individuals recruited from the GLAVIN and IAVI 016 cohorts (Figure 5.1). All 13/13 HIV-1 positive donors, 12/31 HESN and 5/24 HUSN subjects had detectable HIV-1 specific T cell responses. T cell responses detected amongst HIV-1 seropositive individuals, tended to target 2 or more proteins. Whilst, those detected amongst HESN and HUSN donors were typically restricted to a single protein (Figure 5.3A), with envelope the most commonly targeted (Figure 5.3Ci). Notably, HIV-1 specific T cell responses were not detectable by *ex vivo* ELISpot in any of the HESN (n=13) or HUSN (n=10) subjects tested (Figure 5.2A-B).

Subsequent analysis of subjects enrolled in the CHAVI002 cohorts using clade B consensus peptide sets showed similar findings. All 24 HIV-1 positive, 13/24 HESN and 9/27 HUSN subjects (Table 5.1) had detectable HIV-1 specific T cell responses. The difference in frequency of detection of HIV-1 specific T cell responses over time, amongst HESN and HUSN donors, achieved statistical significance (independent analysis, performed by SCHARP). Using generalised estimating equations (GEE) with Wald analysis, there is a 2.5 fold increased likelihood of detecting HIV-1 specific T cell responses amongst HESN individuals compared to HUSN. Similar to the findings made in the GALVIN cohort, HIV-1 specific T cell responses detected in CHAVI002 HESN and HUSN (Figure 5.3B) typically targeted a single protein. However, envelope was shown to be rarely targeted amongst HESN donors (Figure 5.3Cii) with the greatest magnitude of response directed against gag (Figure 5.3Dii).

5.4.2 Magnitude of HIV-1 specific T cell responses in HESN and HUSN

HIV-1 specific T cell responses detected in both HESN and HUSN from both the GALVIN and CHAVI002 cohorts were significantly lower (p=<0.01) in magnitude than those detected during chronic HIV-1 infection (Figure 5.4A). Despite failing to achieve statistical significance in the GALVIN cohort (p=>0.05 Figure 5.4A), within the CHAVI002 cohort, T cell responses were shown to be significantly greater in magnitude amongst HESN donors compared to HUSN controls (Wald p=0.002, Figure 5.4B).

5.4.3 No overall proliferative defect

Proliferative capacity of both $CD4^+$ and $CD8^+$ T cells was examined between HESN and HUSN donors using peptide pools spanning tuberculosis proteins (ESAT-6, CFP-10 and 85A) and commonly presented influenza, EBV and CMV epitopes (FEC)[484]. No significant difference in the magnitude of T cell responses to FEC (Figure 5.5A) or TB (Figure 5.5C) peptides (p=>0.05) was observed amongst HESN and HUSN donors from the GALVIN cohort. Whilst TB peptides were not included in the CHAVI002 cohort, no significant difference in the magnitude of response to FEC peptides was detected between HESN and HUSN donors in this cohort either (Figure 5.5B).

For each short-term cell line generated non-specific/background IFN-γ production was assessed in quadruplicate using "mock wells", which contained complete media with 0.05% DMSO. Non-specific IFN-γ production may be indicative of generalized immune activation and we did not expect to observe a difference between HESN and HUSN donors. Mann Whitney U test showed no significant (p=>0.9) difference in the background IFN-γ production made by HESN/HUSN subjects from the GALVIN cohort (Figure 5.5D). In contrast HESN subjects enrolled in the CHAVI002 cohort had significantly higher background IFN-γ production (p=<0.0001) compared to HUSN subjects (Figure 5.5E).

5.4.4 Maintenance of HIV-1 specific T cell responses

Longitudinal analysis of 4 HESN (GALVIN cohort) and 2 HUSN subjects (IAVI 016) across multiple study visits spaced 4 weeks apart suggested HIV-1 specific T cell responses tended to be better maintained in HESN compared to HUSN subjects (Figure 5.6).

Access to the CHAVI002 cohort allowed for more detailed analysis of maintenance of responses (Figure 5.7). Within this cohort, 4 study visits spanning a 12 month period were available to study. Analysis of 10 HUSN and 13 HESN subjects with detectable HIV-1 specific T cell responses demonstrated that response to HIV-1 proteins were better maintained in HESN compared to low risk controls (Figure 5.7). Whilst the study was not statistically powered to detect differences in maintenance of response between HESN and low risk controls, it is of note that the odds of detecting HIV-1 specific T cell responses amongst HESN and low risk controls did not vary over time (Wald p=0.78) nor was there a significant effect of time on T cell responses in the HESN groups (Wald p=0.78).

5.4.5 Absence of low titre virus

Blinded, low titre virus detection was performed by Professor E Delwart's laboratory (University of California) using the highly specific and sensitive isothermal transcription mediated amplification assay (TMA). FDA approved for clinical use in early diagnosis of HIV-1 infection, TMA has a 50% detection limit of 3.6 to 14 copies of RNA/ml when performed in singlicate[401,402]. To further improve sensitivity, TMA assays were performed, at minimum, in duplicate, across several study visits including those assessed for T cell responses. The output is a signal-to-cut off (S/Co) ratio (range, 0 to 30), with S/Co ratios of <1.0 considered HIV RNA "negative" and S/Co ratios of ≥1.0 considered HIV RNA "positive". Plasma samples obtained from 6 low risk subjects and 24 HESN individuals from the GALVIN cohort, together with 18 HUSN subjects from CHAVI002 (Data shown in appendix 1) were all negative for HIV-1 RNA by TMA. Of note all HIV-1 seropositive subjects from CHAVI002 cohort with plasma viral loads >50 copies/ml were positive by TMA (Data shown in appendix 1).

5.4.6 CCR5Δ32 mutation

A standard PCR based assay, visualized on a 2% agarose gel, was used to identify the 142 base pair, Δ32 mutation (wild type CCR5 fragment visualized as 174 base pair band) (Figure 5.8A). CCR5Δ32 mutation was not observed in Malawian participants however, 2/14 HUSN subjects recruited within the UK based, IAVI 016 cohort, were heterozygous for the allele (Figure 5.8B). Within the CHAVI002 cohort two HESN individuals with detectable HIV-1 specific T cell responses were shown to be homozygous for the CCR5Δ32 allele (Figure 5.8C).

5.4.7 HIV-1 specific T cell responses are typically mediated by $CD4^+$ T cells

Intracellular cytokine staining (ICS) was performed on short-term cell lines with detectable HIV-1 specific T cell responses (Figure 5.9). HIV-1 specific T cell responses detected in 6/7 HESN and 7/8 HUSN subjects enrolled in the GALVIN cohort were shown to be mediated by $CD4^+$ T cells. For 1/7 HESN and 1/8 HUSN HIV-1 specific T cell responses were mediated by both $CD4^+$ and $CD8^+$ T cells (Figure 5.9C). These observations were supported in the CHAVI002 cohort, where 20/21 HESN and 11/13 HUSN subjects had HIV-1 specific responses mediated solely by $CD4^+$ T cells. One of 21 HESN and 2/13 HUSN were shown to mediate HIV-1 specific T cell responses by both $CD4^+$ and $CD8^+$ T cells (Figure 5.9D). Notably none of the HIV specific T cell responses detected in either the GALVIN / CHAVI002 cohort were shown to be mediated exclusively by $CD8^+$ T cells (Figure 5.9C-D).

5.4.8 HIV-1 specific T cell responses typically map to previously defined, promiscuous HLA binding epitopes

A three dimensional mapping approach[396] was employed to delineate HIV-1 specific T cell responses to the peptide level. Briefly, HIV-1 specific T cell responses were defined to the protein level by initial screening performed using the cultured IFN-γ ELISpot (Figure 5.10A). A three-dimensional matrix, containing peptides spanning the protein of interest, was used to obtain a list of "probable" responding peptides (Figure 5.10B). The "probable" peptides were then confirmed by cultured IFN-γ ELISpot (Figure 5.10C) and ICS (Figure 5.10D). In total 4 Malawian HESN (Table 5.2) and 4 HUSN (3/4 IAVI 016 1/4 GALVIN) participants (Table 5.3) were mapped to the peptide level. Seven of 8 mapped responses were to epitopes previously

identified from HIV-1 positive individuals, which have been described as "promiscuous" based on their ability to bind multiple HLA DR (Table 5. 2-5.3).

Similar observations were made in the CHAVI002 cohort where available samples permitted a total of 24 participants to be mapped (13 HESN and 11 HUSN) (Data shown in Appendix 2). Of the 24 epitopes mapped, 19 had previously been defined within HIV-1 seropositive individuals as promiscuous $CD4^+$ epitopes (Data shown in Appendix 2).

5.4.9 Exclusion of peptide impurities and titration of mapped responses

The IFN-γ responses to HIV-1 specific peptides were titrated for five participants (3 HESN and 2 HUSN) (Figure 5.11A) and shown to be comparable to low avidity $CD4^+$ clones[364]. To ensure peptide purity and specificity, HIV-1 specific T cell responses were independently confirmed in two participants with *de novo* synthesized, high-performance liquid chromatography (HPLC) purified peptides (Figure 5.11C-D). The IFN-γ response to both commercially (SIGMA) synthesized and in house synthesized peptides were comparable, with the greatest IFN-γ response made to the in house synthesized peptide.

5.4.10 Design and validation of a novel "promiscuous peptide" pool

Based on the observation that HIV-1 specific T cell responses, detectable in seronegative donors, tend to map to previously defined, promiscuous epitopes, a novel "promiscuous peptide pool" was designed. Following an extensive literature search of computationally predicted and previously described promiscuous epitopes, a list of previously published, promiscuous peptides was identified. Where multiple sequences from the same region of HIV-1 were cited in the literature (Figure 5.12A), Shannon entropy scores based on >200 sequences were used to design novel 18mer consensus epitopes (Figure 5.12B) encompassing the most commonly cited amino acids at each position. These novel consensus sequences were then compared to clade specific consensus and ancestral sequences using the LANL database[20,21] for homology (Data shown in appendix 3) and where a common variant existed, this variant was also synthesized. Of the 101 promiscuous peptides identified, 20 were

novel consensus sequences (Table 5.4). Interestingly several sequences contained within the promiscuous epitope pool, spanned $CD8^+$ epitope rich sites.

The novel promiscuous epitope pool was tested for immunogenicity using ICS in 4 acutely infected HIV-1 positive subjects obtained from the CHAVI 001 cohort (Chapter 2, 2.12) and 11 chronically infected HIV-1 positive subjects from the GALVIN cohort (Figure 5.13). *Ex vivo* analysis showed 1/4 acute and 4/11 chronic HIV-1 positive subjects had detectable $CD4^+$ T cell responses to the promiscuous epitope pool (Figure 5.13). Further testing using the cultured IFN-γ ELISpot assay was performed on a subset of 10 chronically infected subjects. All subjects (10/10) had positive T cell responses to the promiscuous epitope pool by IFN-γ ELISpot. ICS, showed 10/10 had $CD8^+$ T cell responses and 6/10 subjects had $CD4^+$ mediated T cell responses to the promiscuous pool (Figure 5.13).

5.4.11 Detection of HIV-1 specific T cell responses amongst HESN and HUSN donors was not improved by inclusion of promiscuous epitope pool

A total of 63 HESN subjects recruited in the GALVIN and VAXGEN (Chapter 2 2.111, 2.114 respectively) cohorts, together with 24 HUSN controls comprised of healthy volunteers and IAVI 016 cohort participants were screened using cultured IFN-γ ELISpot assay for HIV-1 specific T cell responses to the novel promiscuous epitope pool (Figure 5.14). Four of 24 low risk HUSN subjects had detectable HIV-1 specific T cell responses to the promiscuous pool, whilst only 4/63 HESN subjects responded to the pool. 30% of HESN subjects with detectable HIV-1 specific T cell responses responded to both the promiscuous pool and consensus peptide set (Figure 5.14A), none showed an exclusive response to the promiscuous pool. In contrast, 50% of HIV-1 specific T cell responses detected in HUSN were only targeted against the promiscuous epitope pool and would not have been captured using consensus peptide sets alone (Figure 5.14B). Overall these data suggest that the promiscuous epitope pool modestly improves sensitivity of detection of HIV-1 specific T cell responses.

5.5 Discussion

With current estimates suggesting that there are 1.8 million AIDS related deaths and 2.6 million new infections per year, the HIV-1 pandemic remains a global health concern[84]. There is, however, a certain degree of heterogeneity in susceptibility to HIV-1 infection, with some individuals remaining persistently seronegative despite multiple high-risk exposures[46,134,135,144,145,147-149,155,157,158,163,166,189,191,200,296,384,493,494,500-502]. Understanding the immune correlates of protection that afford these individuals apparent resistance to HIV-1 is likely to be of benefit to the development of a novel prophylactic or therapeutic vaccine.

The detection of HIV-1 specific T cell responses in a proportion of HIV-1 exposed seronegative individuals (HESN) sparked speculation that adaptive immunity may play a role in conferring protection against the establishment of HIV-1. Indeed, evidence from both human[16,76,503] and primate studies[358,504,505] suggests HIV-1 specific T cell responses play a critical role in controlling viremia during HIV-1 infection. However, the role of HIV-1 specific T cell responses in conferring protective immunity has been met with some scepticism. Notably, not all studies have been able to detect HIV-1 specific T cell responses in HESN[134,155,499,501], and where detectable HIV-1 specific T cell responses have tended to be weak and often transient[135,152,155].

The present study utilized a highly sensitive, cultured, IFN-γ ELISpot approach, together with pools of clade B/C consensus peptides spanning the entire HIV-1 proteome. This enabled a full dissection of the breadth and magnitude of HIV-1 specific T cell responses to be made and expanded upon previous studies, which have tended to focus upon HIV-1 specific T cell responses directed against a single protein such as gag or envelope[135,145,382,499]. In addition to HESN samples available from STI clinic attendees in Malawi (GALVIN cohort) access was also granted to a UK based serodiscordent couples cohort (CHAVI002). This statistically powered, UK study was specifically designed to enable a blinded examination of the frequency and magnitude with which HIV-1 specific T cell responses could be detected amongst both HESN and HUSN individuals. The strict criteria used to define HESN subjects enrolled in

the CHAVI002 cohort and inclusion of several low risk/HUSN subjects sets this study apart from previous literature reports.

To exclude the possibility that HESN subjects enrolled in either the GALVIN or CHAVI002 cohorts were highly protected against HIV-1 infection as a result of a 32 base pair deletion in the HIV-1 co receptor CCR5[46-48,493], 15 Malawian and 66 UK participants from the GALVIN, IAVI 016 and CHAVI002 cohorts were screened for CCR5Δ32 mutation (Figure 5.8). In accordance with previously reported allele frequencies[47,495] CCRΔ32 mutation was absent from all Malawian subjects screened (Figure 5.8B), with just 4.5% of UK participants homozygous and 25.7% heterozygous for the CCR5Δ32 mutation (Figure 5.8B-C). Whilst there have been reports that CCR5/CCR5Δ32 genotype correlates with protection against HIV-1 infection, within the CHAVI002 cohort, there did not appear to be an enrichment of CCR5Δ32 homozygous/heterozygous alleles within the HESN group as compared to the HUSN group (Figure 5.8C). These data suggest that the "protection" afforded to HESN individuals from this study was not attributable CCR5Δ32 mutation.

A recent paper by Zhu *et al* suggested that a proportion of HESN subjects may actually be infected with HIV-1, at levels 10^4-10^6 fold lower than that typically detected amongst HIV-1 infected donors, and therefore undetectable using conventional diagnostic tests[506]. In a collaborative study with Professor E Delwart (University of California) plasma samples from HESN and HUSN donors enrolled in both GALVIN and CHAVI002 cohorts were screened for low titre viral RNA. Whilst in the absence of mucosal samples it was not possible to exclude occult/localized infection, highly sensitive TMA assay, which has 50% detection limit of 3.6-14 copies of RNA/ml[402,503,507], showed no evidence of viral RNA in either the GALVIN or CHAVI002 subjects (Data shown in appendix 1), suggesting abortive infection was unlikely. This observation was supported by a recent paper by Delwart *et al,* where >300 HESN subjects, followed longitudinally, were all shown to be negative by TMA, thus suggesting transient low level viremia is rarely detectable in HESN subjects[139].

Next, using the cultured IFN-γ ELISpot technique, HESN subjects were screened for the detection of pre-existing HIV-1 specific T cell responses. Within the GALVIN cohort 38.7% of HESN subjects were shown to have detectable HIV-1 specific T cell responses (Figure 5.1B). This observation was supported in the larger CHAVI002 cohort, where 53-61% of HESN had detectable HIV-1 specific T cell responses (Table 5.1). HIV-1 specific T cell responses detected amongst HESN subjects from both the GALVIN and CHAVI002 cohorts spanned the entire proteome but were typically focused to a single protein (Figure 5.3). These findings are in keeping with previous literature reports, which have often shown pre-existing T cell responses detected amongst HESN donors to be narrowly focused[136,137,162]. The discrepancy in the frequency with which HIV-1 specific T cell responses were detected amongst HESN donors from the GALVIN and CHAVI002 cohorts is most likely to reflect the stringent criteria used to define CHAVI002 HESN subjects and the availability of samples from multiple study visits.

Surprisingly, screening of HUSN subjects enrolled in the GALVIN and IAVI 016 cohorts by cultured IFN-γ ELISpot showed 20.8% had detectable HIV-1 specific T cell responses (Figure 5.1C-D). This observation was mirrored in the larger CHAVI002 cohort where 26-44% of HUSN individuals were shown to have detectable, HIV-1 specific, T cell responses (Table 5.1). Similar to HIV-1 specific T cell responses detected in HESN donors, T cell responses in HUSN spanned the entire HIV-1 proteome but were typically targeted to a single protein (Figure 5.3B), with envelope the most frequently targeted (Figure 5.3C). Very few studies of HESN individuals published to date have included low risk control subjects. However, there have been previous reports of low risk individuals with detectable HIV-1 specific T cell responses[138,391].

As may be predicted based upon antigen load and frequency of exposure, HIV-1 specific T cell responses detected amongst both HESN and HUSN subjects were of significantly lower magnitude than responses detected during chronic HIV-1 infection (Figure 5.4A). Whilst it did not achieve statistical significance within the GALVIN and IAVI 016 cohorts, HIV-1 specific T cell responses amongst HESN were found to be significantly greater in magnitude (Figure 5.4B) than those detected in HUSN

enrolled in the CHAVI002 cohort. Notably the difference in magnitude could not be attributed to an overall difference in proliferative capacity between HESN and HUSN subjects, as evidenced by a lack of significant difference (p=>0.05) in the magnitude response to cultures stimulated with common flu, EBV and CMV peptides (FEC) or tuberculosis peptides spanning ESAT-6, CFP-10 and 85A (Figure 5.5). One hypothesis would be that HIV-1 specific T cell responses are markers of exposure and that the difference in magnitude of response, between HESN and HUSN, is solely attributable to on-going exposure to HIV-1. Evidence for this hypothesis comes from studies of HIV-1 infected individuals, who actively seek HIV-1 seropositive partners through a practice known as "serosorting" and thereby persist in high risk sexual exposure to HIV-1[508]. In the absence of super-infection, HIV-1 specific T cell responses detected in these individuals are of greater magnitude and frequency to those detected in HIV-1 positive individuals who are not exposed to exogenous virus[508]. Furthermore in a study of serodiscordent couples detection and magnitude of HIV-1 specific T cell responses could be correlated with the HIV-1 infected partners' viral load[150,152].

Of note spontaneous IFN-γ production by short term cell lines (STCL) generated from HESN subjects enrolled in the CHAVI002 cohort, was significantly higher (p=<0.01) than that observed amongst HUSN subjects (Figure 5.5E). These data may be indicative of an increased immune activation amongst HESN donors. There have been mixed reports as to whether HESN show greater generalized immune activation[200,296] or a diminished activation profile[295,509] that affords them a degree of protection against HIV-1. The interpretation of systemic immune activation data is often complicated by the fact that, by definition, HESN are engaging in risky behaviours. Therefore, differences in immune activation status between HESN and HUSN donors may be associated with other infections (particularly STIs), gender differences, or intravenous drug use and not necessarily exposure to HIV-1. HESN donors with CHAVI002 were known to be negative for STIs and were not engaged in intravenous drug use. Further *ex vivo* assessment of immune activation status using flow cytometric markers such as CD38 and HLADR is therefore warranted.

To address the maintenance of HIV-1 specific T cell responses detected amongst HESN and HUSN subjects, longitudinal analysis of HIV-1 specific T cell responses detected within both the GALVIN and CHAVI002 cohorts (Figure 5.6-5.7) was performed. The results suggest that HIV-1 specific T cell responses were better maintained amongst HESN subjects than HUSN (Figure 5.6-5.7). Several literature reports have suggested that persistent antigen exposure is required to maintain HIV-1 specific T cell responses[138,148,152]. Indeed early work with commercial sex workers in Kenya showed that after a period of reduced sexual exposure to HIV-1 T cell responses waned and HESN became susceptible to HIV-1[152]. The requirement for on-going exposure to HIV-1 in order to maintain HIV-1 specific T cell responses, and the observation that seroconversion can occur despite detection of pre-existing HIV-1 specific T cell responses[152], again suggests pre-existing HIV-1 specific T cell responses are most likely to act as markers of exposure rather than correlates of protection.

We next questioned whether HIV-1 specific T cell responses detected amongst HESN and HUSN donors would vary in $CD4^+$/$CD8^+$ T cell specificity. Previous literature reports suggest both $CD4^+$[137,138,145,148,150,295,296,382] and $CD8^+$[135,147,151,156-158,163,203,296,383,384] HIV-1 specific T cell responses can be detected amongst HESN donors. However, the majority of HIV-1 specific T cell responses detected amongst HESN and HUSN donors within this study were exclusively mediated by $CD4^+$ T cells, with very few $CD8^+$ responses detected (Figure 5.9). It is likely the preferential induction of $CD4^+$ T cell responses was a result of the level of antigen exposure. Whilst the CHAVI002 cohort was extremely well-defined, the use of highly active anti-retroviral therapy (HAART), meant that viral loads in infected partners were low (80% <50copies/ml). Coupled with on-going counselling and education regarding the risks of unprotected sexual exposure to HIV-1, viral exposure within this cohort was unlikely to be as high as early studies in commercial sex workers who received almost daily exposure to high titre HIV-1[135,147,309]. $CD4^+$ T cell responses have been shown to require lower activation thresholds than $CD8^+$ T cell responses[247,309], as shown by the preferential induction of $CD4^+$ T cell responses, in attenuated viral vector vaccine studies[381,391], compared to the robust $CD8^+$ T cell responses induced by more immunogenic adenovirus vectors or live attenuated vaccines[389,510].

HIV-1 specific T cell responses were mapped to the peptide level in a total of 17 HESN and 15 HUSN individuals (Figure 5.10, Tables 5.2-5.3, Appendix 2). Strikingly, the majority (98.7%) of mapped responses corresponded to peptide responses detected during active HIV-1 infection, which have previously been defined as "promiscuous" based on their ability to bind multiple HLA class II molecules. Degeneracy is inherent within the immune system[247,511,512]. The bias towards "promiscuous" epitopes described in this study may be reflective of the structural properties of HLA-class II antigen binding and presentation which have been shown to tolerate a greater degree of amino acid substitution compared to $CD8^+$ T cells[247]. In order to try and improve the detection of HIV-1 specific T cell responses amongst HESN and HUSN subjects a novel pool of promiscuous epitopes was designed (Figure 5.12, Tables 5.4). Interestingly, a number of the $CD4^+$ promiscuous epitopes described, contain well characterized, $CD8^+$ T cell epitopes, such as SLYNTVATL, and KIRLRPGGK. Immunogenicity testing of the pool in HIV-1 positive individuals reassuringly showed 100% had dominant $CD8^+$ T cell responses to the pool (Figure 5.13). $CD4^+$ T cell responses were observed in 40% of HIV-1 positive subjects tested *ex vivo* and 50% of those tested using cultured assays (Figure 5.13). However, when tested in HESN and HUSN donors, detection of HIV-1 specific T cell responses was only modestly improved by inclusion of the promiscuous epitope pool, compared to conventional screening with consensus peptides (Figure 5.14). Whilst it could be that the peptide designs incorporated in this study simply didn't work, it is worth mentioning that not all epitopes to HIV-1 have been characterized yet and fewer still have been examined for HLA promiscuity. It is therefore possible that a number of promiscuous epitopes were omitted from this study.

The detection of HIV-1 specific T cell responses amongst uninfected donors is surprising. However, it is unlikely to be the result of poor recruitment of HUSN subjects, particularly for the CHAVI002 subjects, where thorough sexual behaviour questionnaires and persistent HIV-seronegative status of both HUSN partners was well documented. Whilst no HIV-1 specific T cell responses were detectable *ex vivo* in HUSN subjects, it is highly unlikely that the results of this study are an artefact of *in vitro* priming. Typically *in vitro* priming requires multiple rounds of antigen stimulation and the use of exogenous antigen presenting cells (APC)[370,513,514]. The cultured ELISpot has a relatively short culture period and does not include the

enrichment/exogenous addition of professional APC. Based on previous studies this would appear to exclude the possibility of inducing primed T cell responses[370,513,514]. Furthermore, concordance of epitope specificity with findings from HIV-1 infected patients (Figure 5.10; Tables 5.2-5.3; Appendix 2) strongly reinforces the validity of T cell responses observed in this study. Additionally, mapped responses could be titrated (Figure 5.11) and were consistently detected in multiple cultured assays. Although low avidity, the titration data obtained from HESN and HUSN samples was consistent with low avidity $CD4^+$ T cell clones[364]. Whilst these observations do not fully exclude the possibility of *in vitro* priming, they suggest it's highly unlikely.

Detection of antigen specific T cell responses following presumed exposure to virus is not a unique phenomena to HIV-1[515-522]. A proportion of intravenous drug users at high risk of Hepatitis C virus (HCV) exposure have been reported to have HCV specific T cell responses in the absence of infection[515,517]. Similar to HESN[152,163], HCV specific T cell responses in HCV exposed uninfected individuals declined following cessation of intravenous drug use[515,516]. Suggesting, that like HESN donors, sustained antigen exposure is required to maintain antigen specific T cell responses[515,516]. Interestingly HCV specific T cell responses could also be detected in control, unexposed HCV negative individuals[516,518]. Like HUSN, HCV specific T cell responses, detected in HCV unexposed uninfected individuals were lower in magnitude than those detected in HCV exposed individuals[516,518]. However, they were still detectable by conventional *ex vivo* assays[516,518].

Understanding why antigen specific T cell responses can be detected in individuals who are presumed not to have encountered the virus is an intriguing phenomena and one that will be explored in more detail in Chapter 6. The bigger question is whether pre-existing HIV-1 specific T cells can confer protection against establishment of infection. This question remains unanswered and will be addressed further in Chapter 7.

In summary this chapter has demonstrated that HIV-1 specific T cell responses are detectable in a proportion (20-68%) of HIV-1 uninfected donors. HIV-1 specific T cell responses detected in HESN individuals are significantly greater in magnitude and better maintained than those detectable in HUSN. However, where detectable,

both HESN and HUSN tend to have $CD4^+$ T cell responses that can be titrated and mapped to previously defined, promiscuous epitopes. Whilst the higher magnitude HIV-1 specific T cell responses HESN individuals gives weight to the notion that they are the result of true exposure to HIV-1, detection of low magnitude and frequency HIV-1 specific T cell responses in HUSN opens debate as to whether these responses could be the result of cross reactivity within the immune system.

Footnotes:

[i] Data presented within this chapter was accepted for publication in the Journal of Virology:

Ritchie, A.J., Campion, S. L., Kopycinski, J.,Moodie, Z.,Wang, Z. M., Pandya, K., Moore, S.,Liu, M. K., Brackenridge, S., Kuldanek, K., Legg, K., Cohen, M.S., Delwart, E. L., Haynes, B. F., Fidler, S., McMichael, A. J., Goonetilleke, N. Differences in HIV-specific T cell responses between HIV-exposed and -unexposed HIV-seronegative individuals. *J Virol* **85**, 3507-3516 (2011).

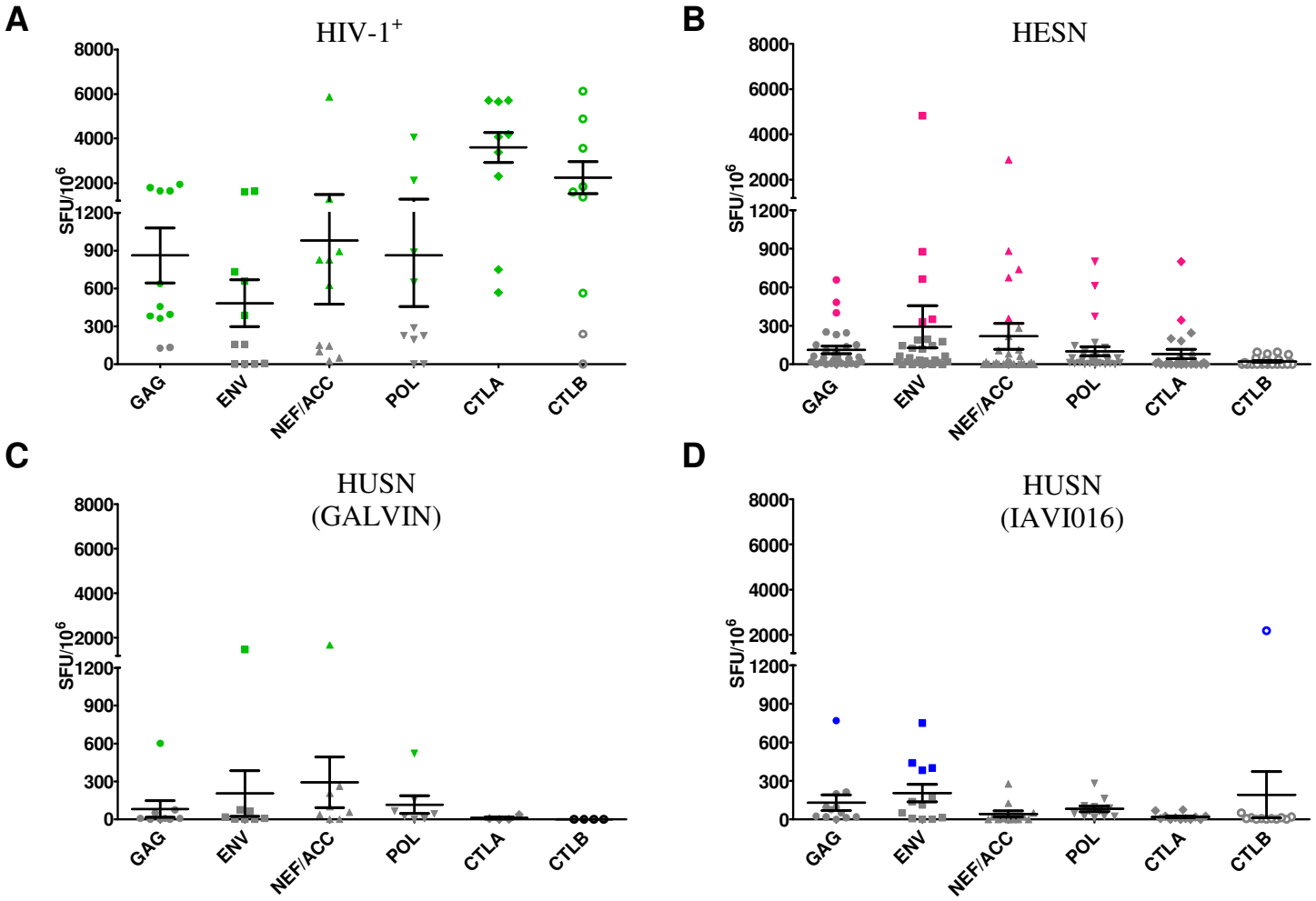


Figure 5.1

The cultured IFN-γ ELISpot technique was used to screen 13 HIV-1 positive (A), 31 HIV-1 exposed, uninfected (B) and 10 HIV-1 unexposed seronegative Malawian individuals (C) enrolled in the GALVIN cohort for HIV-1 specific T cell responses. An additional 14 unexposed uninfected subjects were included from a UK based (IAVI016) cohort (D). Criteria for a positive response (illustrated in colour) was 4x mock stimulated well and greater than 300 SFU/million PBMC. Error bars shown represent standard error of mean and all data presented is background subtracted.

	Visit 1	Visit 2	Visit 3	Visit 4
HESN	13/24 (54%)	10/19 (53%)	11/18 (61%)	10/18 (56%)
HUSN	9/27 (33%)	7/23 (30%)	10/23 (44%)	6/23 (26%)

Table 5.1

Cultured IFN-γ ELISpot assay was used to screen HIV-1 exposed and unexposed seronegative (HESN/HUSN) individuals from the CHAVI002 cohort with pools of clade B consensus peptides spanning, gag, env, pol and nef and accessory proteins (NEF/ACC) proteins. Additional pools containing optimal CTL epitopes as defined by LANL database were also tested. With a cut off criteria of >300SFU and 4x background generalised estimating equations showed a significant difference in the frequency with which HIV-1 specific T cell responses were detected amongst HESN compared to HUSN (Wald p=0.01). Data is presented as the fraction (and percentage) of participants with HIV-1 specific T cell responses detectable following cultured ELISpot analysis at a given study visit.

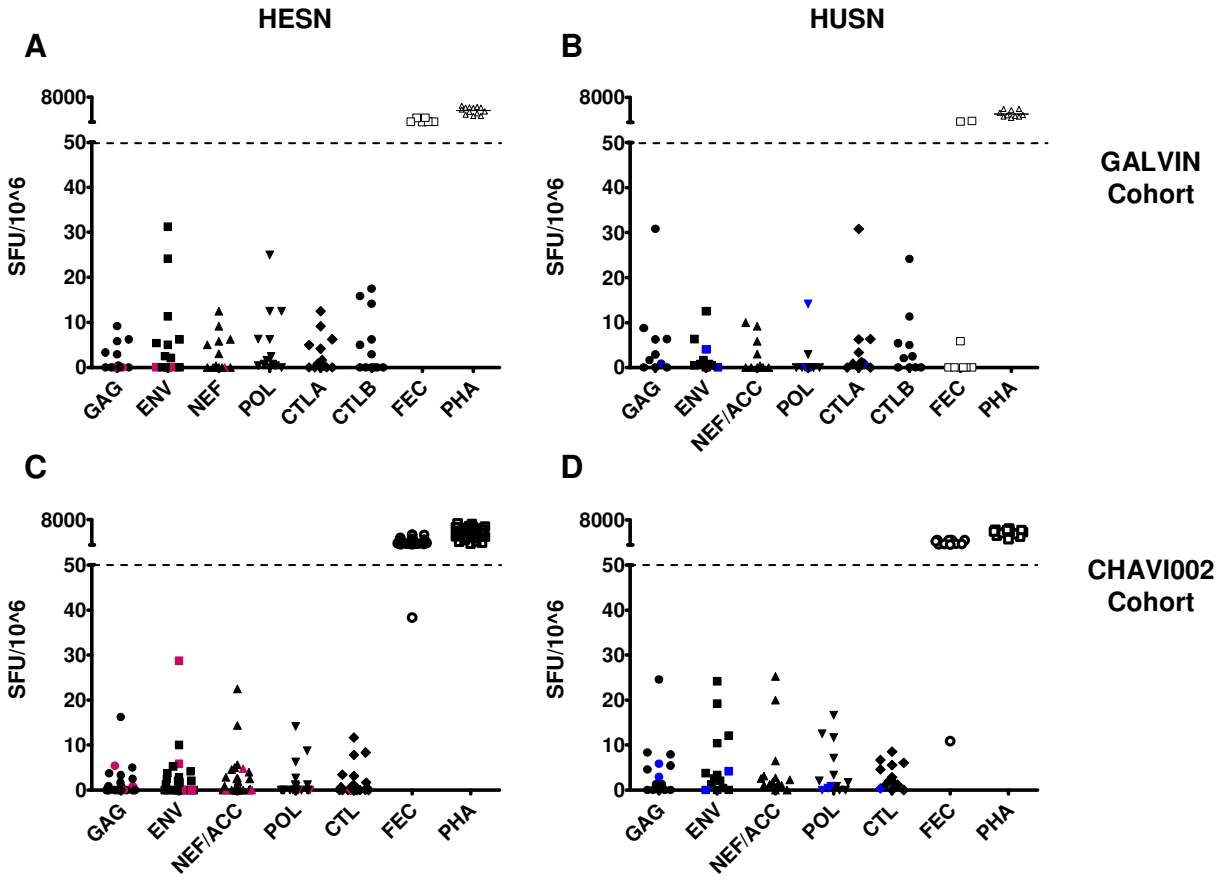


Figure 5.2

HIV-1 specific T cell responses were not detectable in either HESN (A,C) or HUSN (B,D) donors by *ex vivo* IFN-γ ELISpot assay. In total, 13 GALVIN (A) and 16 CHAVI002 (C) HESN subjects were screened. In addition 10 GALVIN (B) and 21 CHAVI002 (D) HUSN were also screened for *ex vivo* responses. Criteria used to define a positive response was >50SFU/10^6 and >4x background. All data presented is background subjected. Coloured symbols indicate subjects whom following cultured IFN-γ ELISpot had positive HIV-1 specific T cell responses targeting the protein indicated.

GALVIN Cohort

A

Group	% Subjects responding to one protein	% Subjects responding to >2 proteins
HESN	58.82%	41.7%
HUSN	66.67%	33.33%

CHAVI002

B

Group	% Subjects responding to one protein	% Subjects responding to >2 proteins
HESN	45.5-76.9%	54.5-23.1%
HUSN	50-85.7%	50-14.3%

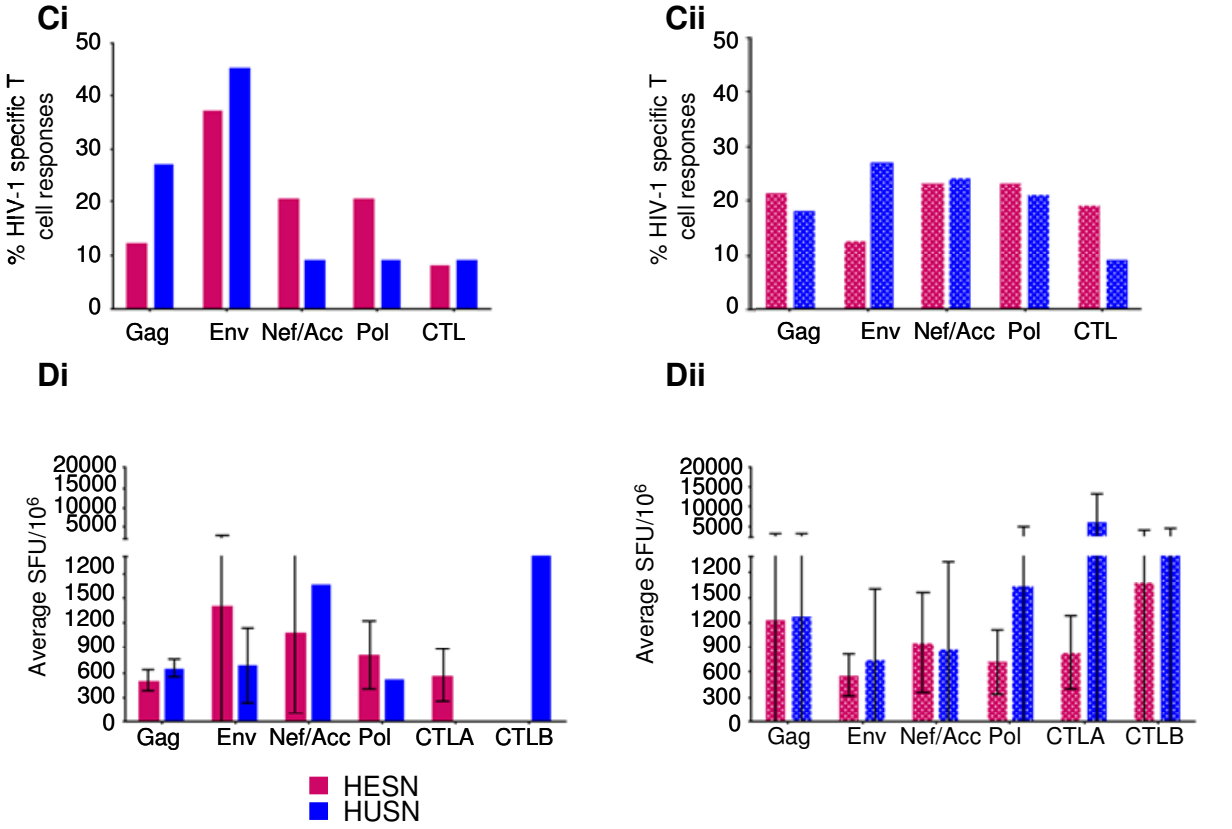


Figure 5.3

HIV-1 specific T cell responses detected by cultured IFN-γ ELISpot amongst both HIV-1 exposed seronegative (HESN) and HIV-1 unexposed seronegative individuals (HUSN) from the GALVIN (A) and 002 COHORTS (B) typically targeted a single protein. Amongst HESN (shown with pink bars) and HUSN donors (shown in blue bars) enrolled in the GALVIN cohort, env was the most frequently targeted protein (Ci). Within the CHAVI002 cohort, responses were more evenly distributed across the HIV-1 proteins (Cii). Similarly, no clear patterns in magnitude of response were detected amongst HESN and HUSN donors from either the GALVIN (Dii) or CHAVI002 cohorts (Dii). Data presented A-B as the percentage of participants with detectable HIV-1 specific T cell responses who responded to 1 or more proteins, range shown for CHAVI002 subjects is indicative of data generated across 4 study visits. The magnitude of HIV-1 specific T cell response is expressed as the number of spot forming units per million cells (SFU/10^6). Criteria used to define a positive was >300SFU/10^6 and 4x background. All data presented is background subtracted.

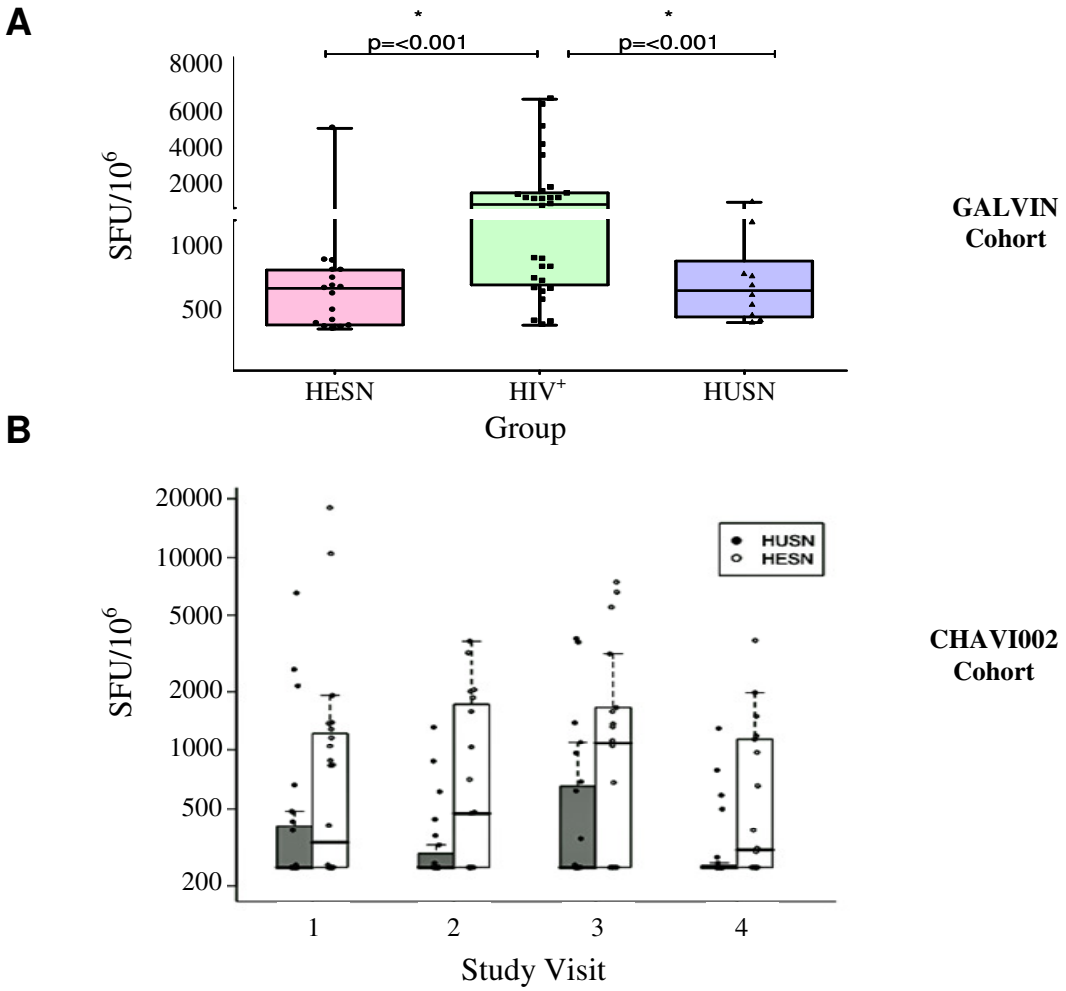


Figure 5.4

The magnitude of HIV-1 specific T cell responses detectable following cultured IFN-γ ELISpot were compared between 13 HIV-1 seropositive, 12 HIV-1 exposed seronegative (HESN) from an STI clinic attendees cohort in Malawi (GALVIN Cohort) and 5 HIV-1 unexposed seronegative (HUSN) from the GALVIN and placebo arm of 016 trial (A). HIV-1 specific T cell responses were significantly lower in magnitude in both HESN and HUSN individuals compared to seropositive individuals (p=<0.01). However there was no significant difference in magnitude between HESN and HUSN (p=0.5467) (A). Data obtained from a larger, UK based cohort (CHAVI002 Cohort) with strict criteria to define HESN and HUSN showed HIV-1 specific T cell responses were of significantly greater magnitude in HESN compared to HUSN (Wald p=0.002) and that this difference in magnitude did not vary over time (B).

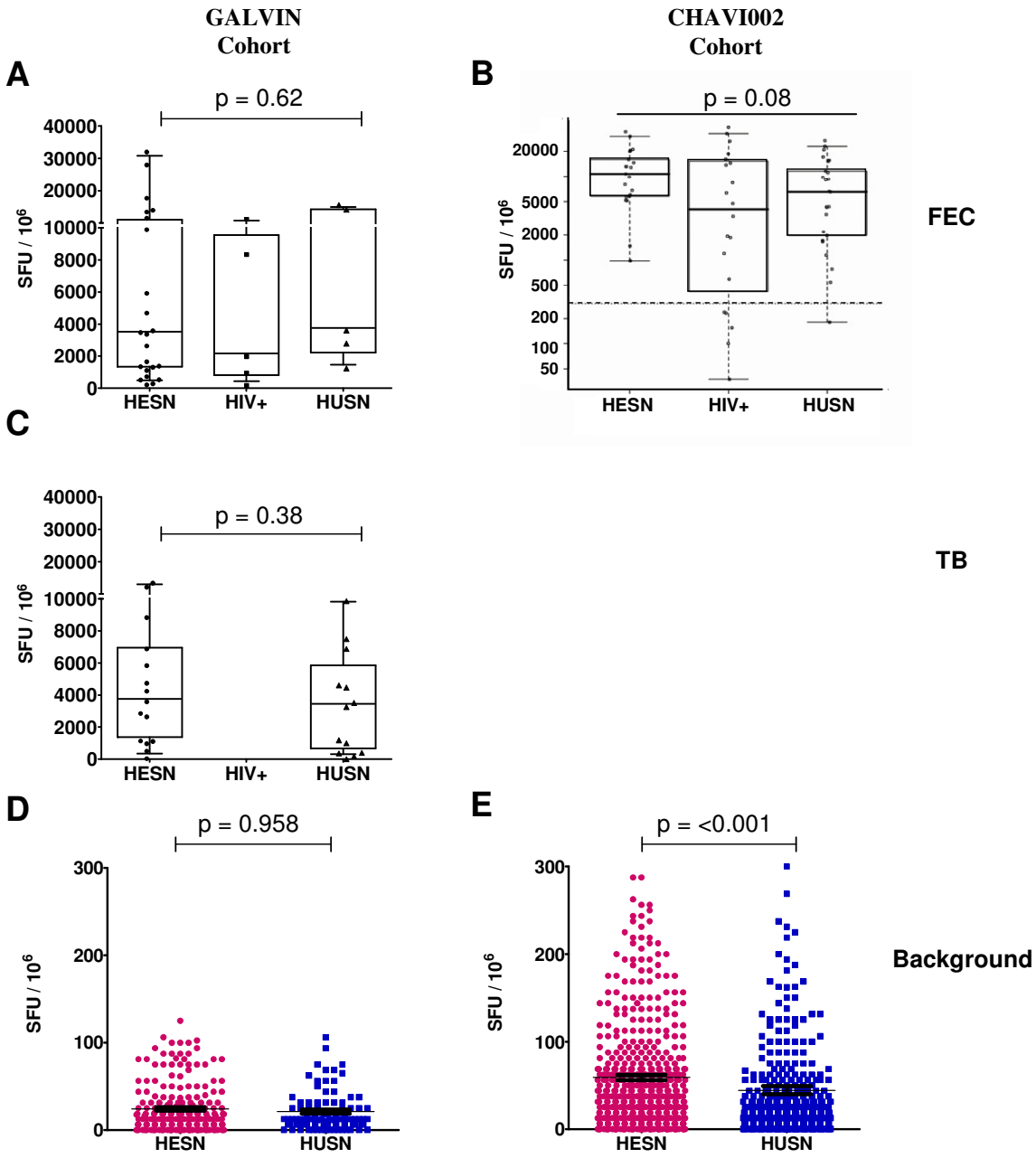
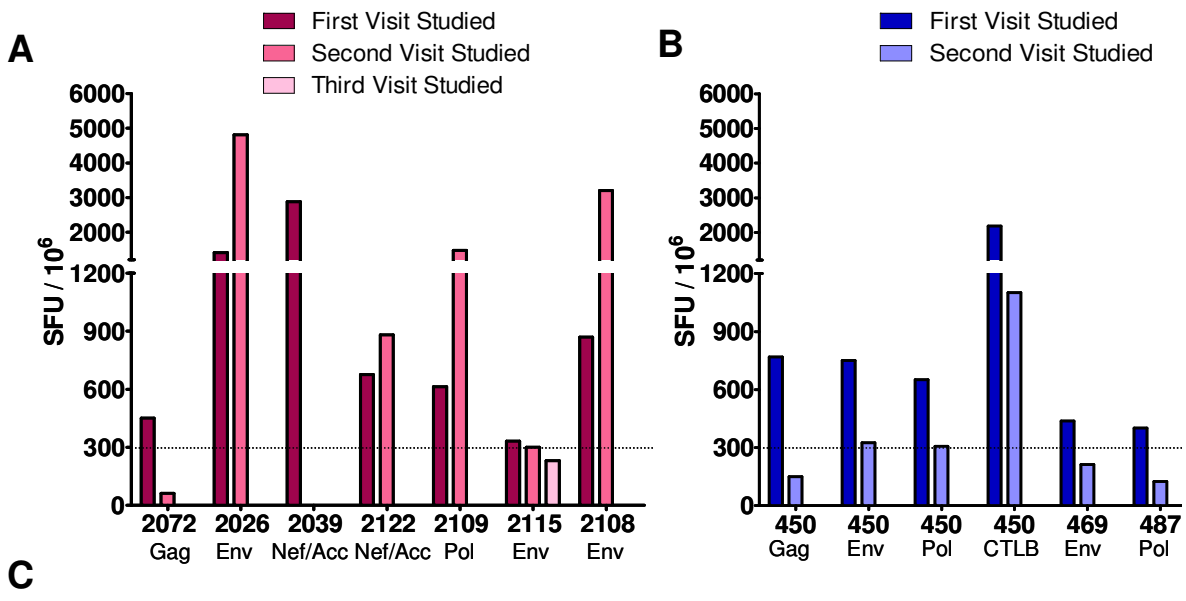


Figure 5.5

Proliferative capacity of $CD4^+$ and $CD8^+$ T cells from HESN and HUSN donors was assessed using peptide control pools, FEC (A-B) and TB (C). No significant difference in the magnitude of T cell responses to FEC peptides (A) or TB (C) peptides was observed amongst HESN and HUSN donors from the GALVIN cohort. Whilst TB responses were not assessed in CHAVI002, no significant difference in the magnitude of T cell response to FEC peptides was detected (Wilcoxon rank sum test p=0.62, p= 0.08 and p=0.38 respectively). No significant difference between background IFN-γ production between HESN and HUSN subjects enrolled in the GALVIN/IAVI 016 cohorts (D) was observed. However, HESN subjects enrolled in CHAVI002 had significantly higher background IFN-γ production compared to HUSN subjects (E). Magnitude of response represented as mean number of spot forming units per million cells (SFU/10^6) and for A-C is background subtracted. Box plots are superimposed on the raw data for each individual with midline and box used to represent the median ± the 25th and 75th percentiles; whiskers extend to the extreme data points.

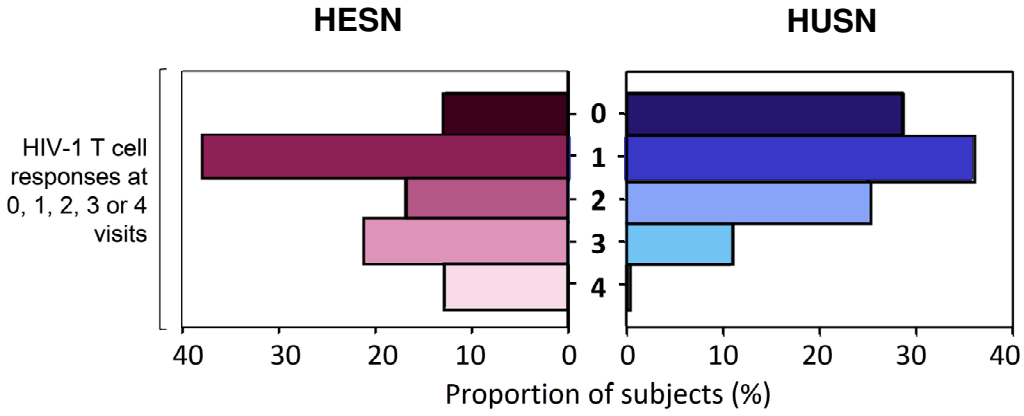


Patient ID	Responding Pool	Participant Group	Magnitude Of Cultured IFN-γ ELISpot SFU /10^6					
			Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
2072	Gag	HESN		450			62.5	
2026	Env	HESN				1406.25	4806	
2039	Nef/Acc	HESN		2875	0			
2122	Nef/Acc	HESN	675		881.25			
2109	Pol	HESN			612.5			1475
2115	Env	HESN	331.25	300	231			
2108	Env	HESN		868.75	3200			
450	Gag	HUSN				768	150	
	Env	HUSN				750	325	
	Pol	HUSN				650	306	
	CTLB	HUSN				2181	1100	
469	Env	HUSN	437.5			212.12		
487	Pol	HUSN	400			125		
523	Env	HUSN	381	337				

Figure 5.6

A subset of HIV-1 exposed (A) / unexposed (B) seronegative (HESN/HUSN) donors enrolled in the GALVIN cohort were screened across two or more study visits (≥3 months apart (C)) to establish whether HIV-1 specific T cell responses were transient or sustained. Five of the 7 HESN participants (A) and 3/4 HUSN participants (B), maintained HIV-1 specific T cell responses across two or more study visits. Data presented as number of spot forming units per million cells (SFU/10^6) following background subtraction. Positive response (indicated with dashed line) ≥300 SFU/million or 4x background. Nef/Acc = pool of peptides spanning Nef and accessory proteins (vif, vpu, vpr, rev, tat). CTLB = pool of previously defined optimal cytotoxic T lymphocyte epitopes.

A



B

HESN	Subpool	Responsive visits
S021	POL1	2, 3
S027	ENV3	1, 2, 3, 4
S048	NEF	1, 2, 3, 4
S050	GAG2	2, 3, 4
S050	CTLB3	3, 4
S051	POL2	2, 3
S052	ACC2	1, 3*
S052	CTLB2	3, 4*
S052	POL1	3, 4*
S058	GAG2	1, 4
S063	GAG2	1, 2*
S073	ENV3	1, 2, 3
S073	ENV2	2, 3
S074	CTLB3	1, 4
S074	CTLA3	2, 3
S074	POL2	2, 3

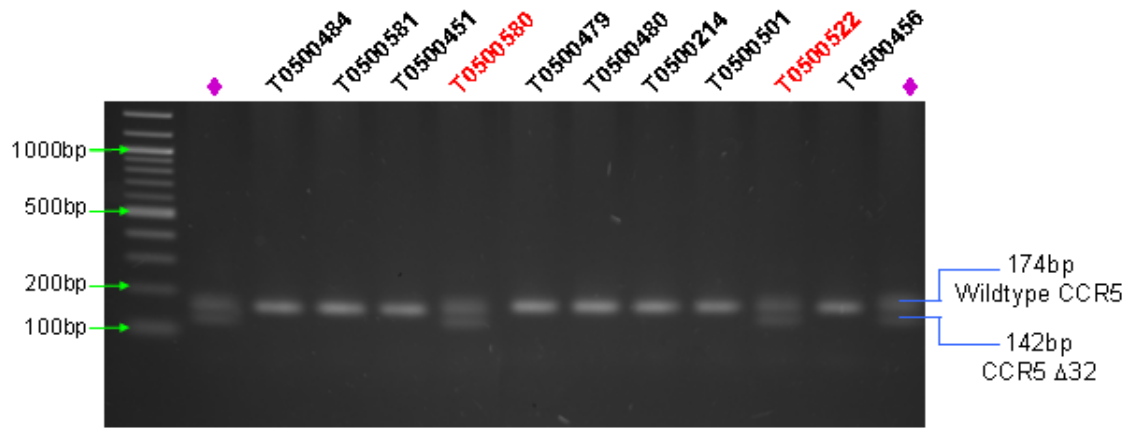
HUSN	Subpool	Responsive Visits
S012	ENV2	2, 3
S017	ACC2	1, 2, 3
S017	NEF	2, 3
S019	ACC1	2, 3
S042	ENV3	1, 4
S042	ENV2	3, 4
S072	ACC2	2, 4
S081	GAG2	2, 3
S084	ACC2	1, 3

* Other study visits not attended

Figure 5.7

HIV-1 specific T cell responses detected amongst HIV-1 exposed seronegative (HESN) individuals and HIV-1 unexposed seronegative (HUSN) individuals from the CHAVI002 cohort were examined longitudinally across 4 study visits (3 months apart). Positive HIV-1 specific T cell responses were defined as greater than 4x background and >300 SFU/10^6 for the pool and >250 SFU/10^6 for the sub pool. HIV-1 specific T cell responses were assessed at the peptide pool level (A) and subpool level (B). Data presented as the percentage of HESN (left panel) and HUSN (right panel) participants having detectable HIV-1 specific T cell responses at 0 to 4 of the 4 study visits. No significant difference between HESN and HUSN was observed, however there was a trend towards responses being better maintained amongst HESN than HUSN.

A



B

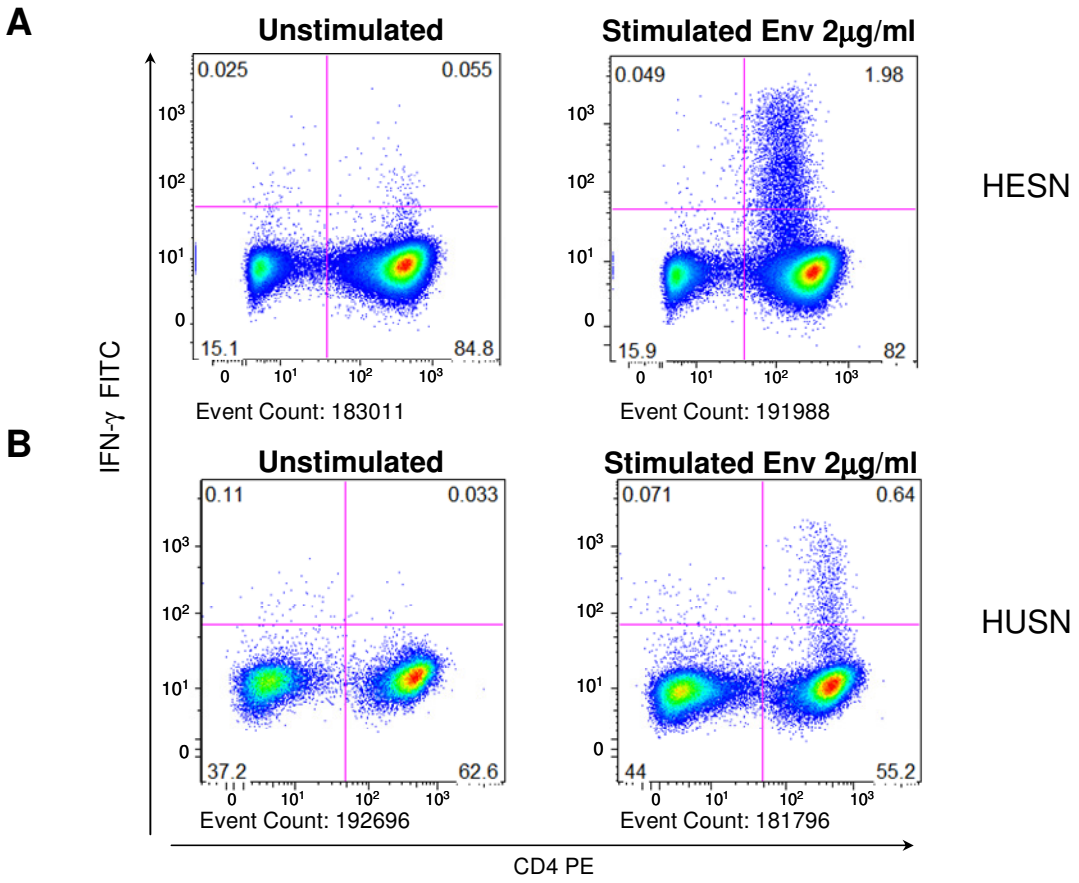
	HESN	HUSN
Wild type CCR5	13/13	17/24
Heterozygous Δ32	0/13	7/24
Homozygous Δ32	0/13	0/24

C

	HESN	HUSN
Wild type CCR5	16/24	23/28
Heterozygous Δ32	6/24	4/28
Homozygous Δ32	2/24	1/28

Figure 5.8:

A standard PCR visualized on 2% agarose gel was used to screen UK and Malawian participants for the CCR5Δ32 mutation. Wildtype CCR5 resolved as a band of 174bp whilst the Δ32 mutation resolved at 142bp (representative image of gel shown A). 13 Malawian HIV-1 exposed seronegative (HESN) and 10 Malawian HIV-1 unexposed seronegative (HUSN) were screened together with 14 HUSN subjects enrolled in IAVI 016 cohort (B). All Malawian subjects expressed wildtype CCR5 whilst 7/14 UK HUSN subjects were heterozygous for the Δ32 mutation 8.3% HESN and 3.57% HUSN enrolled in CHAVI002 were homozygous for CCR5Δ32 mutation (C). 25% HESN and 14.28% HUSN were heterozygous for the mutation (C).



C

	Total	CD4 Only	CD8 only	CD4+CD8
HESN	7	6	0	1
HUSN	8	7	0	1

D

	Total	CD4 only	CD8 only	CD4+CD8
HESN	21	20	0	1
HUSN	13	11	0	2

Figure 5.9

Representative intracellular cytokine staining (ICS) plots demonstrating $CD4^+/CD8^+$ specificity of HIV-1 specific T cell responses detected in HIV-1 exposed (A) and HIV-1 unexposed (B) seronegative individuals (HESN/HUSN). For the GALVIN/IAVI 016 (C) and CHAVI002 (D) cohorts, HIV-1 specific T cell responses detected in both HESN and HUSN subjects were predominately mediated by $CD4^+$ T cells. Interestingly no $CD8^+$ T cell responses were detectable in either the CHAVI002 or GALVIN/IAVI 016 cohorts (C-D). Positivity criteria was >0.05% and 2xbackground. All ICS assays performed on short-term cell lines, stimulation with pools of overlapping peptides (2µg/ml) spanning protein identified by ELISpot assay was for 6hours in presence of brefeldin A. Unstimulated samples received complete media + 0.05% DMSO.

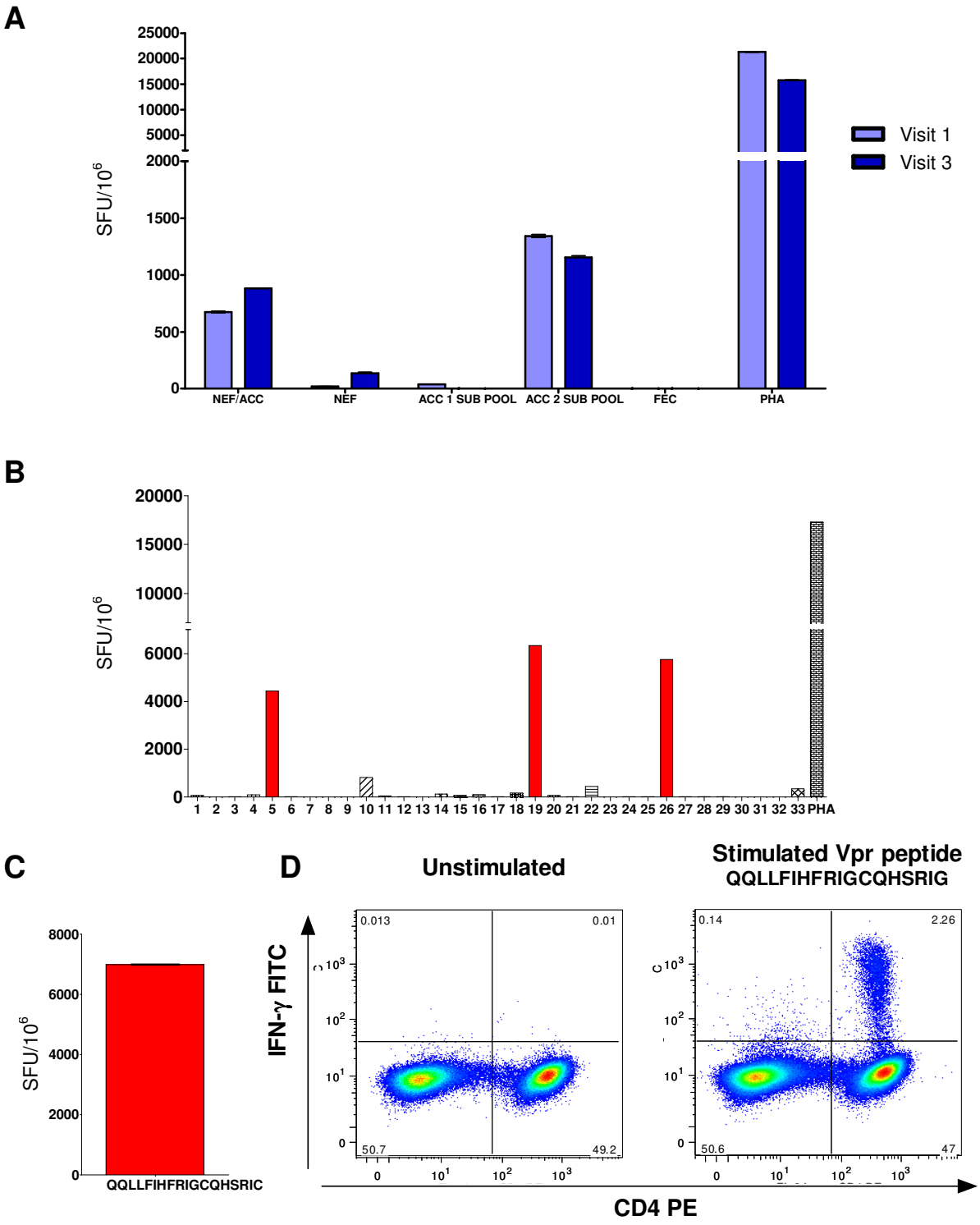


Figure 5.10

HIV-1 specific T cell responses detectable by cultured IFN-γ ELISpot (A) from both HIV-1 exposed and Unexposed individuals were mapped to a single peptide using a three dimensional matrix mapping approach (B), Mapped responses were confirmed by IFN-γ ELISpot (C) and intracellular cytokine staining(ICS) (D). Criteria for a positive ELISpot response was >300SFU/10^6 and 4x background. Criteria for positive ICS response was >0.05% and 2xbackground.

Group/ PTID	Cohort	*Ex Vivo* IFN-γ ELISpot	Cultured IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide Information
HESN 2122	GALVIN	Negative	Vpr 65-82 >2000 SFU/10^6	**$CD4^+$ Magnitude:** 2.26%	**QQLLFIHFRIGCQHSRIG** Identified as HLA-DR promiscuous epitope amongst HIV-1, clade B, infected patients[523].
HESN 2109	GALVIN	Negative	Integrase 809-826 >1000 SFU/10^6	**$CD4^+$ Magnitude:** 1.16%	**GQETAYYILKLAGRWPVK** -------ILKLAGRWP-- Computational epitope prediction defined ILKLAGRWP as a core epitope conserved across different clades. Recognized by multiple murine MHC and 23% US HIV-1 infected subjects[524].
HESN 2115	GALVIN	Negative	Gp41 822-839 >300 SFU/10^6	**$CD4^+$ Magnitude:** 0.83%	**VAEGTDRIIELIQRICRA** Multideterminant region within Env that is recognized by multiple murine MHC haplotypes and multiple HLA types. Shown to stimulate responses in 50-67% of HIV-1^+ patients who still respond to recall antigens[237].
HESN 2066	GALVIN	Not Done	Gp120 269-291 >300 SFU/10^6	**$CD4^+$ Magnitude:** 0.21%	**EVVIRSENFTNNAKTIIVQLNES** Computationally predicted HLA DR promiscuous epitope. 1/34 HIV-1 positive subjects had a $CD4^+$ T cell response to this epitope[525]

Table 5.2

HIV-1 specific T cell responses detected in HIV-1 exposed seronegative were mapped to the peptide level using a 3 dimensional ELISpot mapping technique that allowed both the peptide to be identified and the magnitude of the response to be defined. Intracellular cytokine staining (ICS) was used to characterize responses as either mediated by $CD4^+$/$CD8^+$ T cells. Magnitude of HIV-1 specific T cell responses detected by cultured ELISpot are expressed as spot forming units per million PMBC (SFU/10^6), and as a percentage of $CD3^+CD56^-CD14^-CD19^-$. Epitope responses were identified using LANL sequence database online tools pepMap and T helper cell epitope database. Positive ELISpot response was defined as >300 SFU/10^6 and 4xbackground. Criteria for a positive ICS response was >0.05% and 2x background. All data presented is background subtracted.

Group	Cohort	*Ex Vivo* IFN-γ ELISpot	Cultured IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide Information
HUSN 2240	GALVIN	Negative	Nef 104-121 >2000	**$CD4^+$ Magnitude:** 0.83% **CD8+ Magnitude:** 1.18%	**QKRQDILDLWVYHTQGYF** **----------** Recognized by 11% HIV-1^+ individuals in a study of promiscuous $CD4^+$ T cell responses.
HUSN T0500450	IAVI 016	Not Done	Gp120 269-286 >600 SFU/10^6	**$CD4^+$ Magnitude:** 0.98%	**EIIIRSENLTNNAKTIIV** EVV-----F---------QLNES Bioinformatics predicted this as a highly conserved, promiscuous CD4 epitope present in 1588 strains of HIV-1. 1 / 34 chronically infected, healthy, long term non-progressor participants tested had detectable $CD4^+$ T cell responses to this peptide[525].
HUSN T0500450	IAVI 016	Not Done	P24 240-249 >1000 SFU / 10^6	**$CD4^+$ Magnitude:** 1.87%	**---------TSTLQEQIGW-** PRGSDIAGT----------M 3/9 antiretroviral therapy-naïve adults with chronic HIV-1 infection, had detectable $CD4^+$ T cell responses to a 20mer peptide containing the TW10, p24 gag epitope following expanded ELISpot assay[526,527].
HUSN T0500469	IAVI 016	Not Done	Gp120 342-360 >400 SFU/10^6	**$CD4^+$ Magnitude:** 0.8%	**LQKVSKKLKEHFPNKTIK** Not previously defined.

Table 5.3

HIV-1 specific T cell responses detected in HIV-1 unexposed seronegative were mapped to the peptide level using a 3 dimensional ELISpot mapping technique that allowed both the peptide to be identified and the magnitude of the response to be defined. Intracellular cytokine staining (ICS) was used to characterize responses as either mediated by $CD4^+$/$CD8^+$ T cells. Magnitude of HIV-1 specific T cell responses detected by cultured ELISpot are expressed as spot forming units per million PMBC (SFU/10^6), and as a percentage of $CD3^+CD56^-CD14^-CD19^-$. Epitope responses were identified using LANL sequence database online tools pepMap and T helper cell epitope database. Positive ELISpot response was defined as >300 SFU/10^6 and 4xbackground. Criteria for a positive ICS response was >0.05% and 2xbackground. All data presented is background subtracted.

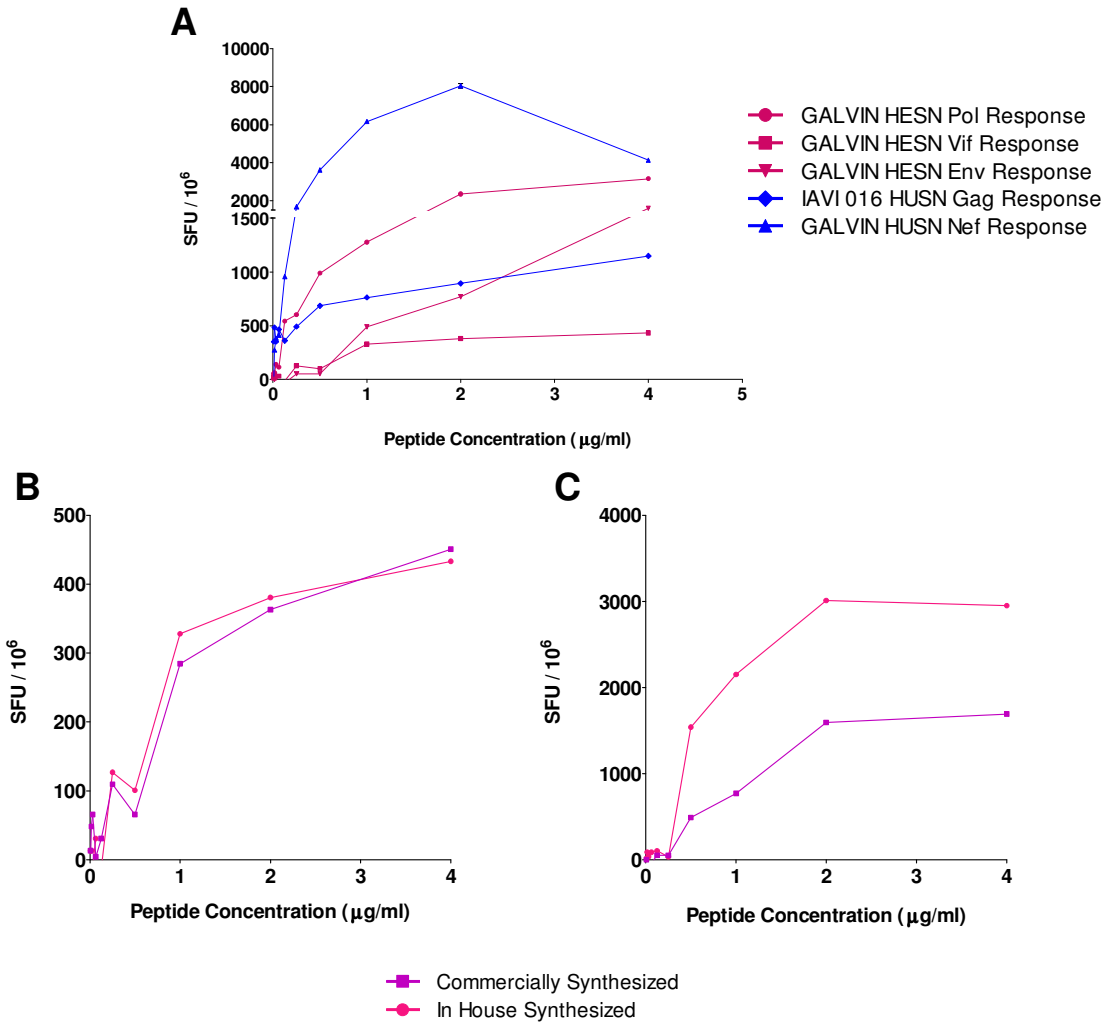


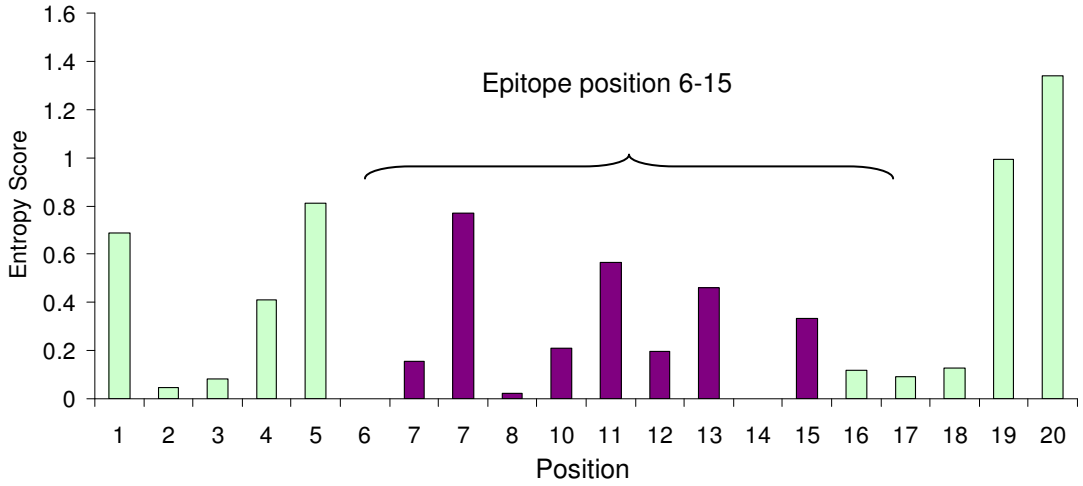
Figure 5.11

Mapped HIV-1 specific T cell responses detected in 3 HIV-1 exposed seronegative (HESN) and 2 HIV-1 unexposed seronegative (HUSN) individuals were titrated using cultured IFN-γ ELISpot (A). Mass spectrometry was used to confirm both the identity and purity of peptides. Mapped and peptide responses detected in 4 HESN subjects, were independently confirmed and titrated using both commercially synthesized and *de novo*, in house synthesized HPLC purified peptide. Representative data shown from two of four subjects tested (B-C).Magnitude of HIV-1 specific T cell IFN-γ responses was expressed as the number of spot forming units per million peripheral blood mononuclear cells (SFU/10^6) following background subtraction. Positive IFN-γ response defined as greater than 300 spot forming units per million cells (SFU/10^6) and 4x background. All titrations were performed in triplicate. Error bars represent standard error of mean and all data presented is background subtracted.

A

Peptide Sequence	Literature Report
-------SLYNTVATLYCVHQRIEV	Kaufman et al.[373]
TGSEELRSLYNTVATLY--------	Kaufman et al. [373]
---EELRSLYNTVATLYCVH-----	Fonesca et al. [523]

B



C

	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	187	121	0	0	0
2	0	0	0	307	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
3	0	0	1	305	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	2	0	0	24	0	278	1	0	0	0	2	0	0	0	0	0
5	0	0	0	0	0	1	0	0	154	0	0	1	0	1	149	1	1	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**309**	0	0	0	0
7	0	0	0	0	0	0	0	0	0	**298**	0	0	0	0	0	0	0	**11**	0	0
8	0	0	0	0	**137**	0	2	0	0	0	0	0	0	0	0	0	0	0	0	**167**
9	0	0	0	0	0	0	0	0	0	0	0	**308**	0	0	0	0	0	0	0	0
10	9	0	0	0	0	0	0	0	0	3	0	0	0	0	0	1	**296**	0	0	0
11	1	0	0	0	0	0	0	**51**	0	**1**	**1**	0	0	0	0	0	0	**252**	0	0
12	**295**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	**13**	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**256**	**53**	0	0
14	0	0	0	0	0	0	0	0	0	**309**	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**26**	**281**
16	0	303	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3
17	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	304	0	0
18	0	0	0	0	0	0	302	0	0	0	0	0	1	5	1	0	0	0	0	0
19	24	0	0	18	0	0	1	0	20	0	0	5	0	230	7	0	3	0	0	0
20	0	0	5	5	0	28	1	0	95	0	0	13	0	6	153	1	0	0	0	0

Position	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Sequence	**E**	**E**	**L**	**K**	**S**	**L**	**Y**	**N**	**T**	**V**	**A**	**T**	**L**	**Y**	**A**	**V**	**H**	**Q**
A.A. No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

Figure 5.12

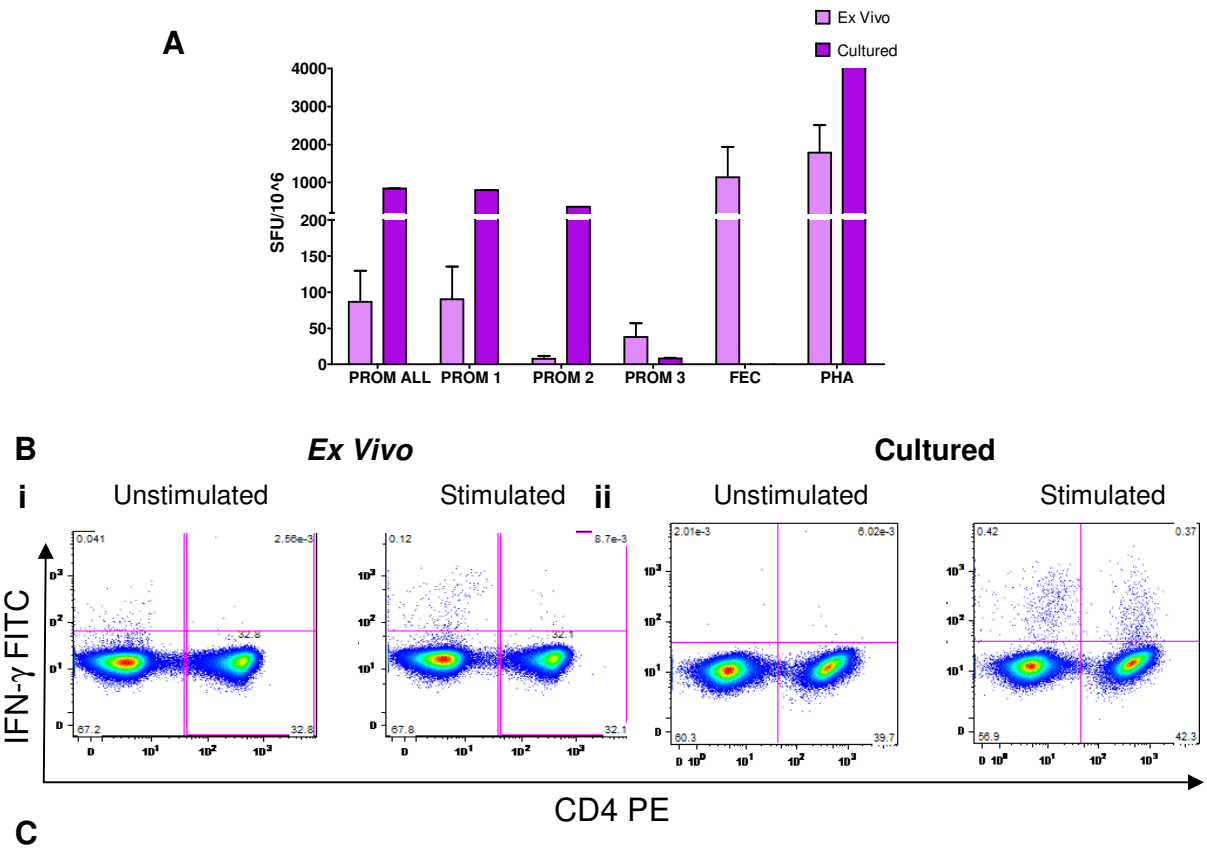
HIV-1 specific epitopes shown to bind multiple HLADR were identified following an extensive literature search. Where the same sequence occurred in multiple studies (A) novel consensus sequences were designed based on entropy scores (B) and compared to consensus sequences and ancestral sequences (C) prior to inclusion in the promiscuous pool. Example given is representative of the process used to identify 20 novel sequences.

Sequence	Protein
VWASRELERFALNPGLLE	P17
EELRSLYNTVATLYCVHQ	P17
EELKSLYNTVATLYAVHQ	P17
YKRWIILGLNKIVRMYSP	P24
LGLNKIVRMYSPVSILDI	P24
PFRDYVDRFFKTLRAEQA	P24
ANFLGKIQPSHKGRPHMF	P15
ANFLGKIWPSSKGRPGNF	P15
SRLAFHHMARELHPEYYK	NEF
SVLSGGKLDRWEKIRLRP	P17
SVLSGGKLDAWEKIRLRP	P17
KAFSPEVIPMFSALSEGA	P24
LLKTVRLIKFLYQSNPPP	REV
LLKAVRIIKILYQSNPYP	REV
LRPMTYKAAVDLSHFLKE	NEF
LRPMTYKGAFDLSFFLKE	NEF
HGIRPVVSTQLLLNGSLA	GP120
HGIKPVVSTQLLLNGSLA	GP120
PTPVNIIGRNLLTQIGCT	POL
PTPVNIIGRNMLTQIGCT	POL
NPEIVIYQYMDDLYVGSDLEIG	POL
SRELERFAV	p17
SLYNTVATLY	p17
WIILGLNKIVRMYSP	p24
IVRMYSPTSILDIRQGP	p24
YVDRFYKTLR	p24
GKIWPSHKGR	p15
AFHHMARELH	Nef
SGGELDRWEK	p17
SPEVIPMFSALSE	POL
QKQEPIDKELYPLASLR	p15
KTVRLIKFLYQSNPP	Rev
YKAAVDLS	Nef
GIRPVVSTQLLLNGSLA	GP160
SYRLISCNTSVI	GP160
TPVNIIGRNLLTQIG	Pol
IIRILQQLLFIHF	Vpr
ERFAVNPGLLETSEGCR	GAG
PEKEVLVWKFDSRLAFHH	NEF
EKIRLRPGGKKKYKLKHI	GAG
QMVHQAISPRTLNAWVKV	GAG
DRVIEVVQGAYRAIRHIPR	GP160
AYRAIRHIPRRIRQGLER	GP160
AYFLLKLAGRWPVKTIH	POL
ELKKIIGQVRDQAEHLK	POL
IVQNLQGQMVHQAISPR	GAG
LEKIEEEQNKSKKKAQQA	GAG
LELDKWASLWNFDITN	GP160
LKTAVQMAVFIHNFKRK	POL
GQETAYYILKLAGRWPVK	POL
VAEGTDRIIELIQRICRA	GP160

Sequence	Protein
WVKVVEEKAFSPEVIPMF	P24
MTETLLVQNANPDCKTIL	P24
HIAKNCRAPRKKGCWK	P15
TAPPEESFRFGEETTTPSQK	P15
VIQDNSDIKVVPRRKAKI	POL
QKRQDILDLWVYHTQGYF	NEF
LWVYHTQGYFPDWQNY	NEF
NSLLHPMSLHGMDDPEK	NEF
IRGQIRCSSNITGLLLTRDGGNN	GP160
SENFTNNAKIIIVQLNESVVINC	GP160
EVVIRSENFTNNAKTIIVQLNES	GP160
ESVVINCTRPNNNTRRSIHIGPG	GP160
TQLLLNGSLAEEEVVIRSANFSDN	GP160
PQDLNMMLNIVGGHQAAMQMLKD	GAG
GQETAYFILKLAGRWPVKVIHTD	POL
GIGGFIKVRQYDQILIEICGKKAI	POL
LWKGEGAWIQDNSDIKWPRRK	POL
WYQLEKEPIVGAETFYVDGAANR	POL
RWQVMIVWQVDRMRIRTWNSLVK	VIF
CYCKHCSYHCLVCFQTKGLGISY	TAT
AIWWSIVFIEYRKILRQRKIDR	VPU
WLWYIKIFIMIVGGLIGLRIIFA	GP160
IVGGLIGLRIVFAVLSIVN RVRQ	GP160
RSLCLFSYHRLRDLLLIVTRIVE	GP160
AVGIGAVFLGFLGAAGSTMGAAS	GP160
RIVFAVLSIVNRVRQGYSPLSFQ	GP160
QRPLVTIKIGGQLKE	POL
GKIILVAVHVASGYI	POL
RDLLLIVTRIVELLGR	GP41
TMLLGMLMICSAA	GP160
ALFYKLDVVPID	GP160
SELYLYKVVKIEPLGVAP	VPR
QQLLFIHFRIGCRHSRIG	VPR
SLQYLALVALVAPKK	VIF
VLAIVALVVATIIAI	VPU
VLEWRFDSRLAFHHV	NEF
EQMHEDIISLWDQSLKPCVK	GP160
FVTIGKIGNMRQAHC	GP160
RAKWNNTLKQIDSKL	GP160
KQIINMWQEVGKAMYAPPISGQIR	GP160
RDNWRSELYKYKVVKIEPLGVAPT	GP160
RIVELLGRRGWEALKYWW	GP160
RGWEALKYWWNLLQYWSQ	GP160
WWNLLQYWSQELKNSAVS	GP160
AVAEGTDRVIEVVQGAYR	GP160
MTKILEPFRRKQNPDIVIY	POL
RNQRKIVKCFNCGKEGHT	p15
TKELQKQITKIQNFRVYY	POL
YELHPDKWTVQPIVLPEK	POL
QQLLFIHFRIGCQHSRIG	VPR

Table 5.4

Complete list of peptide sequences included in promiscuous epitope pool and their corresponding proteins. All sequences shaded in grey are novel consensus sequences designed based on Shannon entropy scores and comparisons with ancestral/consensus sequences.



C

Ex Vivo:

	IFN-γ ELISpot	$CD4^+$ $IFN\text{-}\gamma^+$ ICS	$CD8^+$ $IFN\text{-}\gamma^+$ ICS
Acute $HIV\text{-}1^+$	Not Done	**1 / 4** Average Magnitude: 0.11%	**3 / 4** Average Magnitude:
Chronic $HIV\text{-}1^+$	10/10	**5 / 11** Average Magnitude: 0.078%	**11 / 11** Average Magnitude: 0.76%

D ***Following 10 day culture:***

	IFN-γ ELISpot	$CD4^+$ $IFN\text{-}\gamma^+$ ICS	$CD8^+$ $IFN\text{-}\gamma^+$ ICS
Chronic $HIV\text{-}1^+$	10/10	**5 / 10** Average Magnitude: 0.732%	**10 / 10** Average Magnitude: 4.43%

Figure 5.13

Representative data from a Malawian subject chronically infected with HIV-1 who was tested for detectable HIV-1 specific T cell responses using *ex vivo* and cultured IFN-γ ELISpot (A) and intracellular cytokine staining (Bi-ii) targeted towards a novel pool of previously defined HLADR promiscuous epitopes. In total immunogenicity against the novel pool of promiscuous epitopes was assessed in a total of 15 $HIV\text{-}1^+$ subjects enrolled in CHAVI 001 (acute) and GALVIN (chronic) cohorts using both *ex vivo* (C) and cultured (D) assays. Positive criteria for *ex vivo*/cultured IFN-γ ELISpot were >50/>300 $SFU/10^6$ and greater than 4x background. Intracellular cytokine responses were noted as positive if $IFN\text{-}\gamma^+$ cells were >0.05% and 2x background.

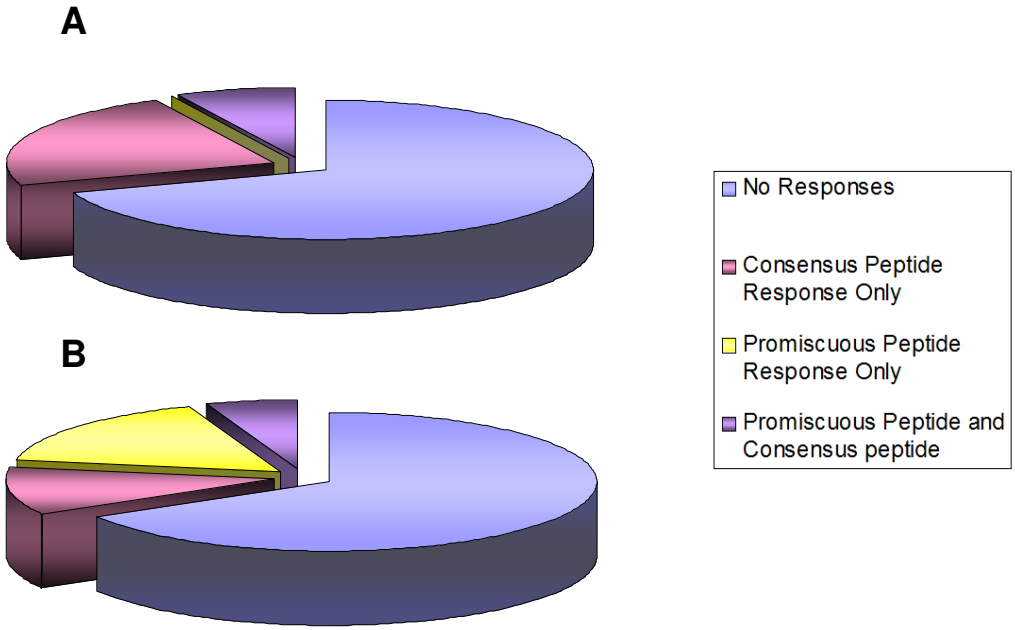


Figure 5.14

A novel set of HLADR promiscuous peptides were screened in (A) 63 HIV-1 exposed seronegateive (HESN) and (B) 24 HIV-1 unexposed seronegative subjects (HUSN). 6% of HESN (4/63) and 16% HUSN (4/24) subjects screened had detectable T cell responses targeting the promiscuous epitope pool. However there did not appear to be any benefit to including this peptide set over the use of conventional consensus peptide sets spanning the HIV-1 proteome. Samples were screened using cultured IFN-γ ELISpot with a positive response defined as >300 SFU/10^6 cells and/or 4x background.

CHAPTER 6:

HIV-1 SPECIFIC T CELL RESPONSES IN SERONEGATIVE DONORS, THE RESULT OF CROSS REACTIVITY?

Chapter 6: HIV-1 specific T cell responses in seronegative donors, the result of cross reactivity?

6.1 Introduction

Chapter 5 demonstrated that HIV-1 specific T cell responses can be detected in the absence of infection or even exposure to HIV-1. Whilst reliance on self-reported "low risk" behaviour obviously holds some caveats and past exposure can not be fully excluded, the detection of HIV-1[138,391] and HCV[516,518] specific T cell responses in a range of low risk cohorts, at similar frequencies is suggestive of a low level of cross reactivity within the immune system.

T cells display a high level of specificity toward their target antigen[231,528-530], achieved through the interaction of short peptide epitopes, presented by human leukocyte antigens (HLA), to the T cell receptor (TCR)[233,293]. Specificity is controlled not only by the amino acid sequence of the peptide but also its associated HLA allele[225,531]. The TCR itself is a heterodimeric, membrane integral protein, consisting of 2 polypeptide chains (typically α and β). Each α/β chain is composed of a variable and a constant region, derived from the random splicing and subsequent recombination of variable (V), joining (J) and diversity (D) segments. Antigen specificity is conferred by the three hypervariable complementarity-determining regions (CDRs) encoded within the V gene segments of both α and β chains (termed *TRAV* and *TRBV* genes, respectively). Whilst CDR1 and 2 regions are germline-encoded, the CDR3 region is only generated after V(D)J recombination, and therefore contains substantial non-germline variability (Reviewed in [227,246]). The V(D)J recombination system combined with the random addition of non-template nucleotides by terminal deoxynucleotidyl transferase and random α/β chain pairing, enables a theoretical 10^{15}-10^{20} unique TCR to be generated[233,261,532].

Due to physical constraints of the human lymphatic system the full recombination potential of the TCR repertoire cannot be realised. Thymic selection, is believed to reduce the peripheral TCR repertoire to around 2.5×10^7 unique TCR through the elimination of autoreactive T cells[265]. Despite this high level of diversity, the potential number of antigenic epitopes that may be encountered far exceeds the estimated

number of TCR[262]. This suggests that whilst T cells are capable of displaying a high degree of specificity, it is highly unlikely that a TCR is specific to a single epitope[262].

Degeneracy, or the ability to cross recognize multiple epitopes, is an inherent feature of T cell recognition[366,533-539]. Indeed during positive thymic selection, naïve T cells must have bound to at least one combination of self-peptide and HLA molecule prior to reaching the periphery[536-539]. Moreover, on-going recognition of self-peptides is thought to be required for the homeostatic proliferation and survival of peripheral T cells[276,540,541]. There is even evidence from murine studies using altered peptide ligands and artificially loaded antigen presenting cells (APC), to suggest that binding of self-peptides, together with agonist peptide, as a heterodimeric complex, drives T cell activation and can alter peripheral T cell responses[542,543].

In an attempt to reconcile the opposing requirements of effective negative selection, the ability to recognize almost any given peptide and the physical constraints of the lymphatic system, Mason calculated that each TCR must be capable of binding 10^6 different epitopes[262]. This prediction has been corroborated by experimental evidence, generated from synthetic peptide libraries and point mutation analysis[544,545]. Mason argues that not only is cross reactivity inherent, but that it is a necessary characteristic of T cell recognition, without which the host immune system would be vastly inadequate[262].

Examples of cross reactive T cell responses have been reported between genetically related viruses and within virus groups such as, flaviviruses (e.g. Dengue)[546-549] and orthomyxoviruses (e.g. Influenza A)[550-552]. More surprisingly, cross reactive T cell responses have also been described for unrelated viruses. There are well characterized examples of cross recognition of peptides from hepatitis C virus (HCV)/human immunodeficiency virus (HIV)[62,511], Epstein-Barr (EBV)/Influenza A virus[553], Influenza A/ HCV[554] and lymphocytic choriomeningitis virus (LCMV)/vaccinia virus (VV)[555]. Although many of these studies have relied upon T cell clones and prolonged periods of *in vitro* culture. Cross reactive T cell responses are not limited to viruses and examples can be seen between bacteria and viruses (HIV-1/Burkolderia cepacia[556], and Mycobacteria/poxviruses[557]) and even between bacteria/viruses and host proteins (EBV/myelin basic protein (MBP)[558] and MBP/E.coli peptide[559]). Whilst

many instances of degenerate recognition of cross reactive epitopes can be accounted for by high sequence homology[234,545,560,561], there are reports of cross recognition of completely unrelated sequences[558,559,562,563].

X-ray crystallography and mutational studies have revealed that only a few contact residues of the peptide-HLA-complex interact with the TCR directly[233,240,241,564]. Through point mutational studies the TCR has been shown to permit a degree of tolerance in the cross recognition of peptide sequences with shared sequence homology[547,565-569]. Detection of more structurally diverse peptides may be explained by the "induced fit" hypothesis, which suggests that the TCR can undergo large conformational changes to permit binding of the peptide-HLA-complex[570]. Structural studies have supported this hypothesis, showing that TCRs are capable of undergoing significant structural rearrangement to adopt more than one conformation and that these conformational isomers (conformers), can accommodate multiple ligands[293,571-574]. Moreover, peptides can bind HLA in multiple registers, and adapt their conformation so that, the surface presented for TCR recognition, shares a common tertiary structure with its cross reactive homologue, thereby permitting binding[566,574-576]. Typically referred to as "molecular mimicry", a number of elegant examples of the recognition of disparate peptide sequences based solely upon a shared tertiary structure have been shown[512,558,563,567]. In humanized mouse models crystallography studies have shown that both E.*coli* and EBV peptides in complex with DR2b and DRB5*0101 respectively, can stimulate T cell clones, specific to myelin basic protein (MBP), and importantly were capable of inducing experimental autoimmune encephalomyelitis (EAE). These peptides do not have a shared sequence homology, however, they were found to share a high degree of structural equivalence to MBP[558,559]. More recently, Borbulevych *et al.* showed that the T cells recognizing the Tax antigen from human T cell leukemia virus 1 were also capable of binding the neuronal protein HUD in the context of HLA-A2, again these peptides shared little sequence homology but interestingly were close, but imperfect structural mimics[574].

It is predicted that 15-900 different TCRs respond to any given epitope producing what is typically referred to as a polyclonal TCR response[257,577,578]. However, restrictions in TCRBV usage and so called "oligoclonal" T cell responses have been described in several human disease states including, viral infection[554,579-584] and

autoimmune disease[582,585,586]. It is poorly understood, as to why narrow, oligoclonal repertoires are generated, particularly in the case of viral infection, where they are often correlated with viral escape[227,587]. Both the intrinsic structure of the epitope[256] and repeated antigenic exposure have been proposed to play a role in the development of an oligoclonal TCR response[588]. However, clonotyping experiments have shown that cross reactive T cell responses are often characterized by a narrow/oligoclonal TCR repertoire[554,583,586,587]. It is hypothesized that the higher frequency and lower activation threshold of memory T cells[284,307,310,589] will result in preferential expansion of a limited number of cross reactive T cell clones rather than the typical, polyclonal recruitment of naïve T cells, thus resulting in an oligoclonal TCR response[284,307,310,587,589,590]. Support for this prediction has come from the study of a number of well-defined, cross reactive T cell responses in both mouse models[587,591] and human studies[554,592]. Although, it is not supported in all cases, and there are examples of polyclonal TCR responses to known cross reactive eptiopes[522,553].

Although primarily detected following prolonged periods of i*n vitro* expansion, T cells capable of recognizing HIV-1 have been shown to cross react with Influenza A virus[593], HCV[511], cytomegalovirus (CMV)[62] and bacterial antigens[556,594]. It is therefore conceivable, that not all HIV-1 specific T cell responses have arisen from direct exposure to HIV-1. A direct example of this came from a study by Acierno *et al*, in which tetramer staining of *in vitro* peptide stimulated cultures from HIV-1 seropositive and seronegative donors detected cross reactive T cells capable of degenerate recognition of both a HIV-1 gag peptide ($Gag_{77\text{-}85}$) and Influenza A peptide ($Flu_{58\text{-}66}$)[593]. Following detection of cytolytic T cell responses to the gag peptide amongst 3/5 HIV-1 seronegative donors, the authors conclude that influenza A was the primary antigen, that had enabled the expansion of cross reactive T cells capable of degenerate recognition of HIV-1[593].

With an infinite number of environmental, endogenous and exogenous potential antigens, and the observation that sequence homology is not required in all instances of heterologous immunity[558,559], identification of the cross reactive pathogen is unlikely to be a trivial matter. However, the observations made in Chapter 5, that HIV-1 reactive T cells from HUSN were typically mono-specific (Figure 5.3A-B) and mediated by $CD4^+$ T cells (Figure 5.9) would fit with a hypothesis of cross reactivity.

Particularly given amino acid substitution studies, with cognate $CD4^+$ and $CD8^+$ T cell ligands, suggest that $CD4^+$ T cells may be inherently more degenerate than $CD8^+$ T cells[247,263].

6.2 Chapter Aims:

In agreement with previous studies[135-138,147,150,157,160,382,487], Chapter 5 showed that HIV-1 specific T cell responses can be detected in a proportion of HIV-1 exposed, seronegative (HESN) donors. Surprisingly, HIV-1 specific T cell responses were also detected amongst a proportion of HIV-1 unexposed, seronegative (HUSN) donors. Whilst exposure to virus appears to impact upon characteristics such as magnitude and maintenance of response, the detection of HIV-1 specific T cell responses in individuals with no known prior exposure to HIV-1 is puzzling.

We hypothesize that HIV-1 specific T cell responses, detected in the absence of viral exposure, are the result of inherent cross reactivity within the immune system. Cross reactivity is known to be both intrinsic to and essential for, T cell recognition[262]. However, given the plethora of possible cross reactive pathogens and the observation that shared sequence homology is not always required, predicting or describing cross reactive epitopes is inherently difficult.

This chapter aims to further characterize HIV-1 specific T cell responses detected amongst both HESN and HUSN individuals. Using a range of techniques, it will both directly and indirectly assess whether the combination of a highly sensitive cultured ELISpot methodology and the use of large arrays of synthetic peptides, could have detected degenerate recognition of HIV-1 peptides by cross reactive T cells.

6.3 Results:

6.3.1 Attempts to directly assess T cell cross reactivity:

6.3.1.1 Sequence homology to other pathogens

Basic Local Alignment Search Tool (BLAST) was used to search for sequence homology between HIV-1 specific T cell responses detected amongst HESN and HUSN subjects identified in Chapter 5 (Table 5.6-5.7) and other known pathogens. No homology to pathogens, other than HIV-1, was detectable for any of the 28 mapped sequences searched (data not shown). The absence of known sequence homology precluded further attempts to directly assess cross reactivity.

6.3.2 Indirect methods to look for T cell cross reactivity:

6.3.2.1 Examination of TCR clonality:

6.3.2.1.1 Flow cytometric analysis

TCR clonality was assessed using a commercially available kit of pre-conjugated, mixed TCRVβ antibodies (Figure 6.1). These antibodies cover 70% of the normal human TCRVβ repertoire of $CD3^+$ lymphocytes (Figure 6.1A)[595,596]. Surface staining of TCRVβ on either $CD4^+$ or $CD8^+$ T cells was un-affected by the inclusion of a cell fixation and permeabilization step, required for examination of intracellular cytokine staining (ICS) (Figure 6.1B-C). Full details of the staining procedure and gating strategy can be found in (Chapter 2 Section 2.5.1.4). In accordance with the manufacturers recommendations Wei *et al,* nomenclature was used to describe TCRVβ identified by flow cytometry[264].

6.3.2.1.1.1 Defining polyclonal and oligoclonal TCR responses

Within the literature terms such as “polyclonal” and “oligoclonal” are frequently used however, there does not appear to be a consensus on the criteria required to be define a response as polyclonal/oligoclonal. Following extensive literature review, and personal communication with Professor D Price, the criteria used to define a polyclonal TCRVβ response identified by flow cytometry in this study, was less than 50% of $CD4^+IFN\text{-}\gamma^+$ expressing a single TCRVβ and greater than 5 TCRVβ clonotypes mediating the peptide response. By contrast an oligoclonal TCR response,

was defined as >50% $CD4^+IFN\text{-}\gamma^+$ T cells expressing a single TCRVβ, with the overall $CD4^+IFN\text{-}\gamma^+$ T cell response being mediated by <5 different TCRVβ.

6.3.2.1.1.2 Polyclonal TCR response to *Mycobacterium tuberculosis* (Mtb) peptides

To ensure that the brief *in vitro* culture period included within the cultured IFN-γ ELISpot methodology did not overtly bias T cell responses toward an oligoclonal TCRVβ clonotype, short-term cell lines specific for known recall antigen Mtb, were assessed. Mtb specific T cell responses from 3 Malawian donors were mapped to the peptide level using cultured IFN-γ ELISpot (Figure 6.2). Two out of three donors were shown to have $CD4^+$ Mtb specific T cell responses with a single donor showing a $CD8^+$ T cell response. For all three donors studied, mapped Mtb specific T cell responses were shown to be polyclonal in TCRVβ usage (Figure 6.3A-C). Exclusion of potential sources of non-specific IFN-γ production by the inclusion of a "dump gate" ensured that background IFN-γ production was low (Figure 6.2Bi) adding validity to the results.

6.3.2.1.1.3 HESN and HUSN predominately oligoclonal

Having demonstrated that the cultured $IFN\text{-}\gamma^+$ ELISpot technique permitted detection of polyclonal TCRVβ responses, mapped HIV-1 specific T cell responses detected in 4 HESN and 3 HUSN subjects (Chapter 5 Table 5.6-5.7) enrolled in the CHAVI002 cohort (Chapter 2 Section 2.1.1.4) were screened for TCRVβ usage (Table 6.1). TCRVβ usage was shown to be unique to each subject studied, however, all HIV-1 specific T cell responses, detected amongst HUSN subjects displayed an oligoclonal TCR repertoire (Table 6.1). HESN subjects were more varied in their TCRVβ usage (Table 6.1) with 2/4 subjects showing clear polyclonal responses (highlighted in purple, Table 6.1). As with Mtb specific T cell responses, non-specific IFN-γ production was low amongst both HESN and HUSN subjects (Table 6.1). Importantly the TCRVβ repertoire of total $CD4^+$ T cells remained polyclonal following culture, with no obvious skewing towards the dominant responding TCRVβ in either HESN (n=4) or HUSN (n=3) donors (Figure 6.4).

Two HESN subjects assessed for TCRVβ usage responded to the same envelope peptide (Env 830-847) (Mapping data presented appendix 2). TCRVβ usage did not appear to be shared amongst these subjects and could therefore be referred to as a private TCR specificity (Table 6.1). However, greater study numbers would be required to show this definitively.

6.3.2.1.2 TCR sequencing

6.3.2.1.2.1 Isolation of antigen specific T cells for TCR sequencing

Conventionally, live, antigen specific T cells are isolated either by tetramer sorting, or the use of activation markers. The design of novel tetramers for use in this study was outside of the scope of this thesis and implausible owing to limited sample availability. Moreover the use of activation markers such as CD154, CD69 and CD25 was precluded by the requirement for cell culture. To overcome these issues a range cytokine capture assays were assessed for the successful isolation of live, antigen specific, T cells that could subsequently be used for sensitive mRNA based sequencing.

6.3.2.1.2.1.1 IFN-γ capture assays lack specificity

Bivalent antibodies that capture cytokine-producing cells were tested for use on short-term cell lines generated against Mtb specific peptides. Compared to conventional ICS staining (Figure 6.5A), the use of bivalent, IFN-γ capture antibodies in the isolation of IFN-γ^+ cells, lacked both specificity and sensitivity (Figure 6.5). Specificity could not be improved by the titration of the capture/detection antibody, or by including additional wash steps (Figure 6.5B).

6.3.2.1.2.1.2 Background TNF-α production high

A recent publication by Haney *et al* [597], described a methodology by which to prevent surface cleavage of membrane bound TNF-α . The authors propose this methodology as a means by which to overcome the sensitivity issues associated with the use of bivalent IFN-γ capture antibodies[597]. To assess whether the TNFα methodology could be applied to the detection and successful isolation of HIV-1 specific T cell responses, 3 HESN and 2 HUSN subjects were screened for TNF-α production using ICS. Whilst 70-100% of IFN-γ positive cells were shown to co-express TNF-α (Figure

6.6), only 45-75% of TNF-α^+ cells co-expressed IFN-γ. Moreover, non-specific TNF-α production was significantly higher than IFN-γ (Figure 6.6C) precluding its use as a sensitive means by which to isolate antigen-specific T cells.

6.3.2.1.2.2 Multiplex analysis of TCR clonality using fixed cells

In the absence of a suitable method by which to isolate, live antigen specific T cells, sequence based TCR clonotyping experiments were restricted to DNA based methodologies that permitted the use of ICS. In a collaborative project with Professor D Price, IFN-γ producing, HIV-1 specific T cell responses from 4 HESN and 1 HUSN donor enrolled within the CHAVI002 cohort, were identified using ICS and sorted to 98% purity using BD FACS ARIA (Cell sorting kindly performed by Dr K Ladell, Figure 6.7A-B). Greater than 2000 antigen specific T cells were sorted from each donor and notably non-specific IFN-γ production was shown to be low (Figure 6.7C). Three pools of primers spanning TCRBV and BJ domains were utilized in a multiplex PCR approach to sequence antigen specific TCR (Chapter 2 Section 2.61) and sequence data from all subjects analysed is listed using IMGT nomenclature[400] (Figures 6.8-6.9). Two out of four HESN and 1/1 HUSN donors had oligoclonal TCR responses (Figure 6.8). Notably all three seronegative subjects were shown to share 2-3 TCR clonotypes that were almost identical at the nucleotide level (Figure 6.8). Despite being processed on different days and with different primer mastermix stocks, this observation is suggestive of DNA cross over or contamination. HIV-1 specific T cell responses detected amongst the remaining two HESN subjects, 709020154 and 709020198, were shown to have a polyclonal TCR repertoire (Figure 6.9). These subjects responded to the same envelope peptide and shared a single common TCR clonotype which differed at the nucleotide level (Figure 6.9). In light of the potential contamination of 3/5 samples all TCR sequence data must be interpreted with caution.

6.3.2.2 Ontogeny of pre-existing HIV-1 specific T cell responses

In a collaborative study* with Professor F Sallusto the ontogeny of HIV-1 specific T cell responses was examined using the T cell library technique[308].

* All assays were performed by myself under the supervision of Professor F Sallusto.

6.3.2.2.1 Establishment of T cell libraries

$CD4^+$ T cells from five healthy, HIV-1 seronegative, leukapharesis donors were sorted to 99% purity, into naïve, central memory and effector memory subsets, based on surface expression of CD45RA, CCR7 and CD25 (Figure 6.10 Cell sorting kindly performed by Dr. D. Jarrossay). 192 individual cultures were set up for each subset, with seeding densities of 2000 cells per well for naïve T cells and 1000 cells per well for the memory subsets. All cultures underwent uniform, polyclonal expansion of >2000 fold in the presence of PHA, IL-2 and irradiated (45Gy) allogeneic feeder cells (data not shown). All donors had previously been vaccinated for tetanus toxin (TT).

6.3.2.2.2 T cell library screening

All 192 individual cultures from each of the three $CD4^+$ T cell subsets were screened simultaneously for proliferative responses to HIV-1 specific peptides (final concentration 2μg/ml) and control, recall, antigen TT (final concentration 5μg/ml) using tritiated thymidine incorporation (Figure 6.11-13). On average non-specific proliferation, measured in cultures stimulated with un-pulsed autologous monocytes, was low (average 208 counts per million (cpm); Figure 6.14). All 5 donors showed clear and predominantly strong proliferative responses (delta cpm ranging from 3038-47835) to HIV-1 specific peptides that originated from both the naïve (Figure 6.11) and memory $CD4^+$ T cell subsets (Figure 6.12-6.13). One-way ANOVA showed no significant difference in proliferative response to HIV-1 peptides between the different subsets ($p=>0.05$) (Figure 6.15). T cell responses spanned the entire HIV-1 proteome, with envelope being the most frequently targeted protein (Figure 6.11-6.13). Whilst it did not achieve statistical significance ($p=>0.05$), memory T cell proliferative responses to envelope tended to be higher in magnitude than the proliferative response to other HIV-1 proteins (Figure 6.15Ci-Di).

Similar to HIV-1 specific T cell responses, recall, TT-specific T cells could be readily detected amongst both naïve and memory T cell subsets (Figure 6.11-6.13). The magnitude of TT-specific T cell responses ranged from 3018-93215 delta cpm. Overall recall, TT-specific, T cell responses were significantly greater in magnitude ($p=<0.0001$) than HIV-1 specific T cell responses (Figure 6.15A). This difference

was attributable to central and effector memory T cell subsets which showed significantly greater proliferative responses to TT rather than to HIV-1 ($p=<0.05$) (Figure 6.15Cii-Dii). The proliferative response of naïve $CD4^+$ T cells to HIV-1 peptides and TT did not significantly vary ($p=>0.05$) (Figure 6.15Bii).

6.3.2.2.3 What are the precursor frequencies of HIV-1 specific T cell responses in memory and naïve subsets?

Based on Poisson distribution, precursor frequencies for HIV-1 specific responses detected by the T cell library technique were calculated for each donor (Figure 6.16). The highest precursor frequencies of HIV-1 specific T cell responses were detected in the memory $CD4^+$ T cell subsets (5–54 HIV-1 specific T cells per million) (Figure 6.16B-C). The precursor frequency of HIV-1 specific T cell responses was lowest amongst naïve T cell subsets, typically ranging from 2-7 HIV-1 specific cells per million (Figure 6.16A). Precursor frequencies for control antigen, TT-specific T cells, were much higher than those for HIV-1 specific T cells (Figure 6.16), ranging from 10-170 cells per million naïve $CD4^+$ T cells (Figure 6.16A) to >10,000 per million memory T Cells (Figure 6.16B-C).

6.3.2.2.4 Can responses detected by cultured IFN-γ ELISpot be correlated with T cell library data?

In parallel to T cell library analysis, cultured IFN-γ ELISpot assays were performed for each subject. Consistent with findings made in Chapter 5, 1/5 donors had a detectable HIV-1 specific T cell response, targeted against envelope (Figure 6.17). Interestingly, the donor with HIV-1 specific T cell responses detectable by cultured IFN-γ ELISpot was also shown to have the highest central memory precursor frequency of envelope specific T cells by the T cell library technique (Figure 6.16B). Whilst the number of subjects studied is very limited, these data suggest that HIV-1 specific T cell responses detected by cultured IFN-γ ELISpot originate from $CD4^+$ central memory T cells.

6.4 Discussion

The use of large pools, of synthetic peptides spanning different antigens is becoming the standard means by which to study the pathogen specific, T cell repertoire. In Chapter 5, a highly sensitive, cultured, IFN-γ ELISpot assay was combined with peptides spanning the entire HIV-1 proteome to evaluate HIV-1 specific T cell responses in both HIV-1 exposed seronegative (HESN) and HIV-1 unexposed seronegative individuals (HUSN). Similar to studies with hepatitis C virus (HCV)[515-519,598,599] and malarial antigens[521,522,600,601], HIV-1 specific T cell responses were detectable in the absence of infection and surprisingly even in the absence, of known previous exposure to the virus. We questioned whether HIV-1 peptide reactive T cells, in uninfected donors, could be the result of inherent cross reactivity and sought to establish the ontogeny of HIV-1 specific T cells.

Direct assessment of T cell cross reactivity has typically focused upon epitopes that share a high degree of sequence homology[234,545,560,561]. Using a BLAST search, mapped HIV-1 specific T cell responses detected amongst both HESN and HUSN individuals (Chapter 5 Table 5.2-5.3 and appendix 2), were screened for homology to other known pathogens. No sequence homology to pathogens other than HIV-1 was detected. However, lack of sequence homology does not exclude the possibility that HIV-1 specific T cell responses detected in seronegative donors are the result of cross reactivity. Indeed, predictions of cross reactivity based solely on the detection of sequence-based homology, are likely to underestimate the true extent of cross reactivity. Not only is it constrained to the search of known sequences, but there have also been examples where completely unrelated peptides can be recognized by the same TCR[512,555,558,559,563,574,576,602].

Based on the lack of sequence homology to other known pathogens, indirect approaches had to be taken to assess whether HIV-1 specific T cell responses detected in seronegative donors could be the result of cross reactivity. A number of studies have suggested that cross reactive T cell responses are typically characterized by an oligoclonal TCR response[554,583,586,587]. Intracellular cytokine staining (ICS), together with a panel of 24Vβ antibodies (Figure 6.1) spanning 70% of the human TCRVβ

repertoire was used to assess whether the HIV-1 specific T cell responses detected amongst seronegative donors (both HESN and HUSN) were polyclonal or oligoclonal in their TCR usage (Table 6.1). Flow cytometric analysis enabled both qualitative and quantitative information to be obtained for TCRVβ usage. Despite a reliance on cultured cell lines for detection of antigen specific T cell responses a previous study of cross reactive EBV and influenza A epitopes has shown that TCR repertoire analysis of cultured T cell lines, accurately reflects the antigen specific repertoire response present *in vivo*[553]. Due to limited sample availability we were unable to assess the TCRVβ repertoire of $CD4^+$ T cells prior to culture. However, we were able to show that the TCRVβ repertoire of total $CD4^+$ T cells, post culture, was not overtly skewed towards the immune dominant Vβ (Figure 6.4). Unlike, recall *Mycobacterium tuberculosis* responses, which showed a highly polyclonal TCR response (Figure 6.3), HIV-1 specific T cell responses detected amongst seronegative donors were predominately oligoclonal (Table 6.1). The only examples of polyclonal response were from HIV-1 exposed seronegative donors (Table 6.1 highlighted in purple), suggestive of true exposure to HIV-1. The number of subjects studied within this chapter is low and further study, is required to establish whether this observation is maintained across larger cohorts.

Within the 4 HESN donors studied, two donors responded to the same envelope peptide (Env 830-847) (Table 6.1). These two individuals showed distinct patterns of TCRVβ usage, indicative of a private TCR specificity (Table 6.1). This finding is analogous to other reports that show a high degree of heterogeneity in the TCRs that cross react to the same epitope[554,587,603]. The random convergent recombination of V(D)J gene segments responsible for the generation of an individual's TCR repertoire, ensures each individual has a highly diverse and unique TCR repertoire. Together with the heterogeneity of past antigen exposure, the chances of multiple individuals responding to a secondary, cross reactive, epitope with the same TCR is extremely small, although not impossible[255,258].

Flow cytometric analysis is limited to the study of families of TCRVβ and whilst the data suggests responses are oligoclonal, there is still the possibility for variation in TCRBJ and TCRA. This is an important limitation, since the CDR3 loop is critical for

antigen specificity and needs to be fully characterized before a precise definition of an oligoclonal T cell response can be made. Moreover, the coverage of the TCRVβ antibodies is incomplete, meaning that potentially important T cell populations could have been missed. The gold standard for TCR clonotype analysis is therefore a sequence based approach. A broad array of methodologies exists for the study of TCR diversity[259,399,604-607], the majority of which require the isolation of live cells and extraction of high quality RNA[259,604,607]. We were therefore faced with the challenge of being able to isolate, live, HIV-1 specific T cells from short-term cell lines.

To date the most sensitive means by which to isolate viable, antigen specific, T cells is the use of tetramers. However, the development of novel class II tetramers, capable of recognizing the mapped, HIV-1 specific eptiopes identified in this study, was outside of the scope of this thesis and implausible owing to the limited sample availability. A number of alternative approaches were assessed based on surface detection of cytokine production and the expression of T cell activation markers. Commercially available, bivalent antibodies, which bind to the pan lymphocyte marker, CD45, and capture IFN-γ as it is produced[608] have been used successfully in the *ex vivo* detection of antigen specific T cells[608,609]. However, there has not been any published data on their application to cultured T cell lines. In the present study, the use of bivalent antibodies with short-term cultures was shown to lack the specificity and sensitivity required for isolation of antigen specific T cells (Figure 6.5). A recent publication, suggested a means by which to overcome specificity concerns of IFN-γ capture assays was to assess surface TNF-α production[597]. In this protocol TACE protease inhibitors were used to prevent cleavage of surface TNF-α, enabling viable, antigen specific, T cells to be identified[597]. To assess whether this methodology could be applied to the present study, 3 HESN and 2HUSN donors were screened for TNF-α production using conventional ICS (Figure 6.6). Whilst the majority (70-100%) of IFN-γ producing, HIV-1 specific, $CD4^+$T cells were shown to co-produce TNF-α (Figure 6.6A), the background TNF-α production was significantly higher than IFN-γ (Figure 6.6B). As a result of the high background seen by ICS, the TNF-α detection method was not pursued.

In the absence of an appropriate methodology to isolate viable, HIV-1 specific T cells, a DNA based, TCR sequencing approach was chosen[399]. Multiplex TCR sequencing has been shown to have a high degree of concordance with mRNA based sequencing approaches[399]. Due to its reliance on DNA it has successfully been used for the clonotypic characterization of cells that have undergone fixation and permeabilization[399]. In a collaborative study with Professor D Price (Cardiff University) the TCRBV and TCRBJ repertoire of 5 HESN donors, with mapped HIV-1 specific T cell responses were assessed (Figure 6.8-6.9). HIV-1 specific T cells were sorted by Dr K Ladell based on ICS for IFN-γ production. Importantly, non-specific IFN-γ production was low and the sort purity was high (89.9-96%; Figure 6.7). The TCR multiplex approach has a sensitivity limit of ≥1000 cells[399], for all 5 subjects >2000 $CD4^+$ IFN-γ^+ T cells were sorted. Using primers spanning TCRBV and TCRBJ designed to cover all functional gene segments (Chapter 2 Section 2.61). This protocol confirmed the overall observations made by flow cytometry, suggesting that pre-existing HIV-1 specific T cell responses in seronegative individuals have a predominately oligoclonal TCR repertoire (Figure 6.8). However, not all TCRVβ detected by flow cytometry were identified by the sequencing approach (Figure 6.8-6.9 and Table 6.1). There are a number of potential explanations for the discrepancies observed between these two techniques. The first is that they may reflect differences in precursor frequencies of the antigen specific T cells, which are likely to vary between sample vials. To really address this issue, future study is required to analyze multiple vials of the same sample to look for consistency. Repeated analysis would also help to define a more stringent cut off criteria, used to define a positive Vβ by the flow based approach. The discrepancies indicate that the current cut off criteria is likely to be too low. Finally there is potential bias in the sequence technique, introduced not only during the selection of positive bacteria, but also at stages such as ligation, where it is difficult to control for the successful transfection of all clonotypes. The most concerning result obtained from the TCR clonotyping, was the observation that 3 subjects, despite responding to unique peptides, shared common TCR clonotypes (Figure 6.8A-C). These clonotypes, showed near 100% homology at the nucleotide level suggestive that these data are the result of single template amplification from cross over DNA. The potential for contamination/DNA cross over was minimized by processing samples on different days, and notably all water

controls were negative. Whilst it is theoretically possible, that the peptide specificity afforded by these "shared" TCR clonotypes, was the result of marked sequence variation amongst the alpha chain, there is no evidence to support this in the literature and DNA contamination would appear the most likely explanation. To address this issue, repeat sequencing was performed on genetic material stored after proteinase K treatrement. However, the results were comparable and in the absence of duplicate samples to repeat the cell sorts contamination could not be excluded.

Whilst all sequencing data must be interpreted with caution owing to the potential crossover of DNA between 3/5 samples, interestingly the two HESN subjects, known to respond to the same peptide (709020154/709020198), were shown to have polyclonal TCR repertoires (Figure 6.9). A single, unique, clonotype was conserved between each subject. Unlike the conserved clonotypes, detected in the 3 oligoclonal TCR responses, these clonotypes varied at the nucleotide level, suggesting they were amplified from unique templates. The detection of public clonotypes in the recognition of identical peptide sequences is perhaps easier to understand than public recognition of disparate sequences. Public TCR clonotypes have been previously described in a range of antigen specific T cell responses[255] and are believed to be the result of enriched precursor frequency (D Price personal communication). Further study is now required to see if the detection of public clonotypes across multiple seronegative individuals who recognize common peptide sequences can be reproduced.

All of the data presented to date, has been based upon observations made following short-term culture. Whilst we believe *in vitro* priming is highly unlikely (Chapter 4), it cannot be fully excluded. Geiger *et al*, recently demonstrated a novel T cell library technique, which enables naïve and memory $CD4^+$ T cell populations, to be screened for antigen specificity[308]. Within their publication specific T cell responses to primary antigens, keyhole limpet hemocyanin (KLH) and cytomegalovirus (CMV), were shown to be exclusively located within the naïve T cell repertoire[308]. In a collaborative project with Professor Federica Sallusto (IRB, Bellinzona, Switzerland) the T cell library technique was used to screen 5 healthy, HIV-1 seronegative donors to help elucidate the ontogeny of HIV-1 specific T cell responses. In a method identical to that previously published[308], $CD4^+$ T cells were isolated from

leukopharesis samples, and cell sorted into naïve, central memory and effector memory populations based on surface expression of CD45RA, CD25 and CCR7 (Figure 6.10 Cell sorting performed by Dr D Jarrossay). The classification of T cells as memory/naïve based upon $CD45RA^-/CD45RA^+$ expression has recently been brought into question, when a small subset of antigen experienced cells was shown to re-express CD45RA. These cells have been shown to have a highly differentiated, effector memory-like phenotype and are typically referred to by the acronym EMRA[301,323,610]. Whilst typically accumulated under conditions of persistent viral infection[330,611,612] the inclusion of EMRA cells within the naïve subset could not be excluded. The impact, if any, that this minor subset had upon subsequent findings is not believed to be significant, since EMRA have been shown to have reduced proliferative capacity[429]. However, for future experiments inclusion of CD57 would enable these cells to be differentiated, and a more accurate gating strategy for naïve T cells achieved[330,429,612].

All donors tested, had demonstrable proliferative responses to HIV-1 specific peptides (Figure 6.11-6.13). These responses were detected amongst both the central memory and effector memory subsets (Figure 6.12-6.13), suggesting that low frequency cross reactive T cell responses to HIV-1 are relatively common. Not only does this finding concur with previous estimations of cross reactivity[262], but it adds further validity to data generated using the cultured IFN-γ ELISpot. The cultured IFN-γ ELISpot assay is hypothesized to enable detection of central memory T cell responses[479,481,485]. Interestingly, the one donor shown to have an envelope response, detectable by cultured IFN-γ ELISpot (Figure 6.17), was also shown to have the highest precursor frequency of envelope responsive T cells in the central memory compartment (Figure 6.14).

For the $CD4^+$ subset, central memory (TCM) is the dominant memory phenotype found in peripherally circulating blood[316]. It is unsurprising therefore, that the HIV-1 specific T cell responses detected in this study were predominately confined to the TCM compartment. The classification of TCM and TEM subsets was first defined by Sallusto *et al*. in 1999 and is based on two distinct criteria: the absence or presence of immediate effector function and the expression of homing receptors, which allow for

migration to secondary lymphoid tissues[316]. TEM are typically referred to as terminally differentiated, they have lost the constitutive expression of CCR7 and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues. TEM are capable of rapid cytokine production following antigenic stimulation, but have poor proliferative capacity and an increased propensity to undergo apoptosis[316]. By contrast TCM retain CCR7 surface expression, and are therefore capable of migration to secondary lymphoid organs[613]. Following TCR stimulation, TCM produce mainly IL-2 but after proliferation they efficiently differentiate into effector cells and produce large amounts of IFN-γ or IL-4[316]. In the quest for a successful HIV-1 vaccine, it is likely that induction of both TEM and TCM subsets will be required to ensure both rapid and sustained protective effects[380,437,614].

Preservation of the $CD4^+$ TCM subset has been correlated with protection in non-human primate studies, and long-term non-progression in HIV-1 infected humans[318,329,415,437,438]. It is worth stressing that the precursor frequency of HIV-1 specific T cells detected amongst seronegative donors was low, ranging from 5-53/million TCM and 1-45/million TEM (Figure 6.16). Whilst memory T cells are known to be preferentially expanded following encounter with antigen[284,307,310,589], whether such a low precursor frequency could confer a degree of protection against the establishment of HIV-1 remains unknown, and will be addressed further in Chapter 7.

All of the donors assessed by the T cell library technique had been vaccinated against tetanus toxin (TT). Therefore, a direct assessment of HIV-1 specific T cell responses to a known recall antigen could be made. Overall TT specific responses showed a greater proliferative response (Figure 6.15A) and higher precursor frequency amongst memory subsets (Figure 6.16C-D) than HIV-1 specific T cells. Although notably, no significant difference was detected between HIV-1 and TT specific naïve $CD4^+$ T cells (Figure 6.15Bii).

Given the tremendous diversity of the naïve T cell repertoire[265], the detection of HIV-1 specific T cell responses within this compartment was not surprising (Figure 6.11).

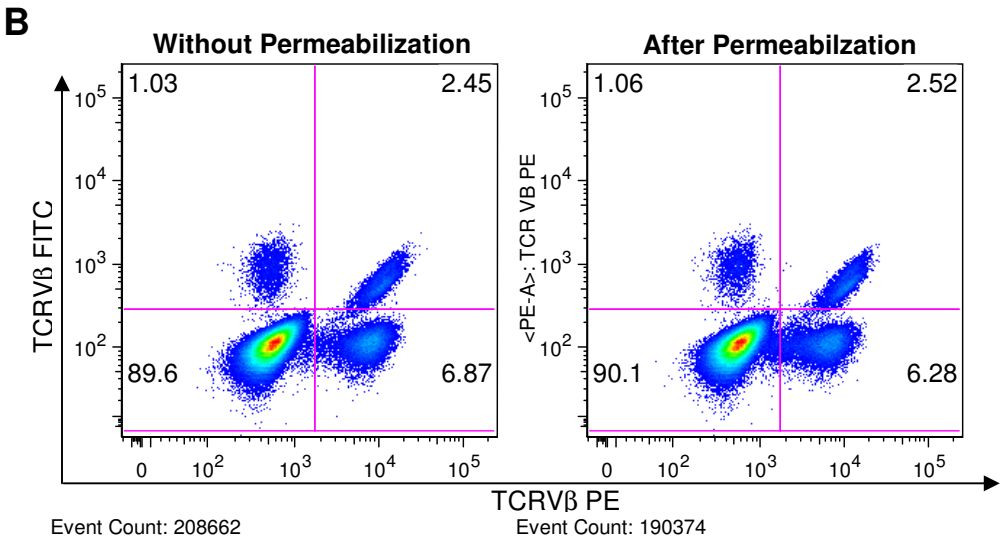
The precursor frequency of HIV-1 specific T cells ranged from 2-29/million naïve $CD4^+$ T cells with gag the most frequently targeted protein. The precursor frequency of TT specific cells was slightly higher ranging from 2-52/million naïve $CD4^+$ T cells. Whilst low, the precursor frequencies calculated in this study are in keeping with those of previous studies[308,615,616]. Indeed, Geiger *et al,* showed that cytomegalovirus, PPD and TT specific responses were found at precursor frequencies ranging between 10-170/million naïve $CD4^+$ T cells[308]. The precursor frequency of TT specific T cells was also slightly lower than published[308], ranging from 2-52/million naïve $CD4^+$ T cells (Figure 6.16), compared to 10-170. The recent advent of new technologies, such as HLA class II tetramers[615,616] and the T cell library technique[308], mean that the number of naïve T cells for any given peptide-HLA complex, is only just starting to be elucidated. However, there is already compelling evidence to suggest that the precursor frequency of naïve antigen specific T cells affects both the size and TCR diversity of subsequent T cell response following vaccination or infection[615-617]. A greater understanding of the naïve precursor frequency of HIV-1 specific T cells could therefore, be beneficial not only in understanding the patterns of immunodominance in natural HIV-1 infection, but also for vaccine development. Specific targeting of high precursor frequency epitopes could be used to ensure a highly robust and diverse T cell response is elicited upon vaccination. In primate models at least, robust T cell responses have been correlated with protective immunity[380,614].

In summary, despite a lack of sequence homology between mapped HIV-1 specific T cell responses and other known pathogens, there is strong evidence to suggest that HIV-1 specific T cell responses, detected in a proportion of seronegative donors, are the result of cross reactivity. We show that HIV-1 specific T cells can be readily detected in the memory subsets of healthy HUSN donors, albeit at a lower frequency than known recall antigens. In the absence of known HIV-1 exposure, the origin of these memory T cells remains unknown. Furthermore, in all HUSN and the majority of HESN donors tested, HIV-1 specific T cell responses are predominately oligoclonal in TCRVβ usage. Since oligoclonal T cell responses have previously been associated with cross reactive T cell responses this again supports a theory of cross reactivity. It is important to stress that the data presented herein, does not exclude the

possibility of exposure driven HIV-1 specific T cell responses. Coupled with the observations made in Chapter 5, that HIV-1 specific T cell responses are better maintained and greater in magnitude amongst HESN compared to HUSN donors, it is worth mentioning that the only observed polyclonal TCR responses were found in HESN donors. Whether the result of direct exposure to HIV-1 or degenerate recognition by cross reactive T cell clones, the biggest question surrounding pre-existing HIV-1 specific T cell responses is, the impact, if any, they have upon the acquisition of HIV-1. This question will be addressed in Chapter 7.

A

KEY	PE	PE+FITC	FITC
TUBE A	TCR Vβ 5.3	TCR Vβ 7.1	TCR Vβ 3
TUBE B	TCR Vβ 9	TCR Vβ 17	TCR Vβ 16
TUBE C	TCR Vβ 18	TCR Vβ 5.1	TCR Vβ 20
TUBE D	TCR Vβ13.1	TCR Vβ 13.6	TCR Vβ 8
TUBE E	TCR Vβ 5.2	TCR Vβ 2	TCR Vβ 12
TUBE F	TCR Vβ 23	TCR Vβ 1	TCR Vβ 21.3
TUBE G	TCR Vβ 11	TCR Vβ 22	TCR Vβ 14
TUBE H	TCR Vβ 13.2	TCR Vβ 4	TCR Vβ 7.2



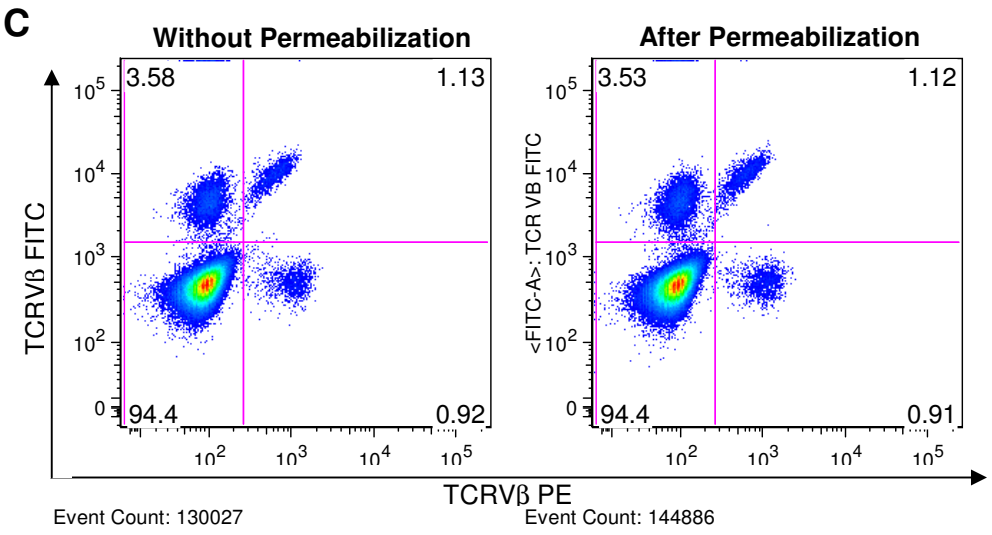


Figure 6.1

TCRVβ analysis was assessed using a panel of pre-labelled Vβ antibodies covering 70% of the human TCRVβ repertoire (A). The staining pattern of either $CD4^+$ (B) or $CD8^+$ T (C) cells was unaffected following fixation and permeabilization (B), enabling the use of the TCRVβ panel with intracellular cytokine staining. Nomenclature from Wei *et al*[264].

A

PTID	Protein	Mapped peptide	Magnitude ELISpot	Magnitude ICS
2020 v2	85A	PKEDPAWQRNDPLLNVGK	558 SFU/10^6	$CD4^+$ 1.22%
2030 v2	ESAT-6	AAWGGSGEAYGVGVQQKW	687 SFU/10^6	$CD8^+$ 1.22%
2032 v2	CFP-10	TAAQAAVVRFQEAANKQK	2500 SFU/10^6	$CD4^+$ 4.12%

B

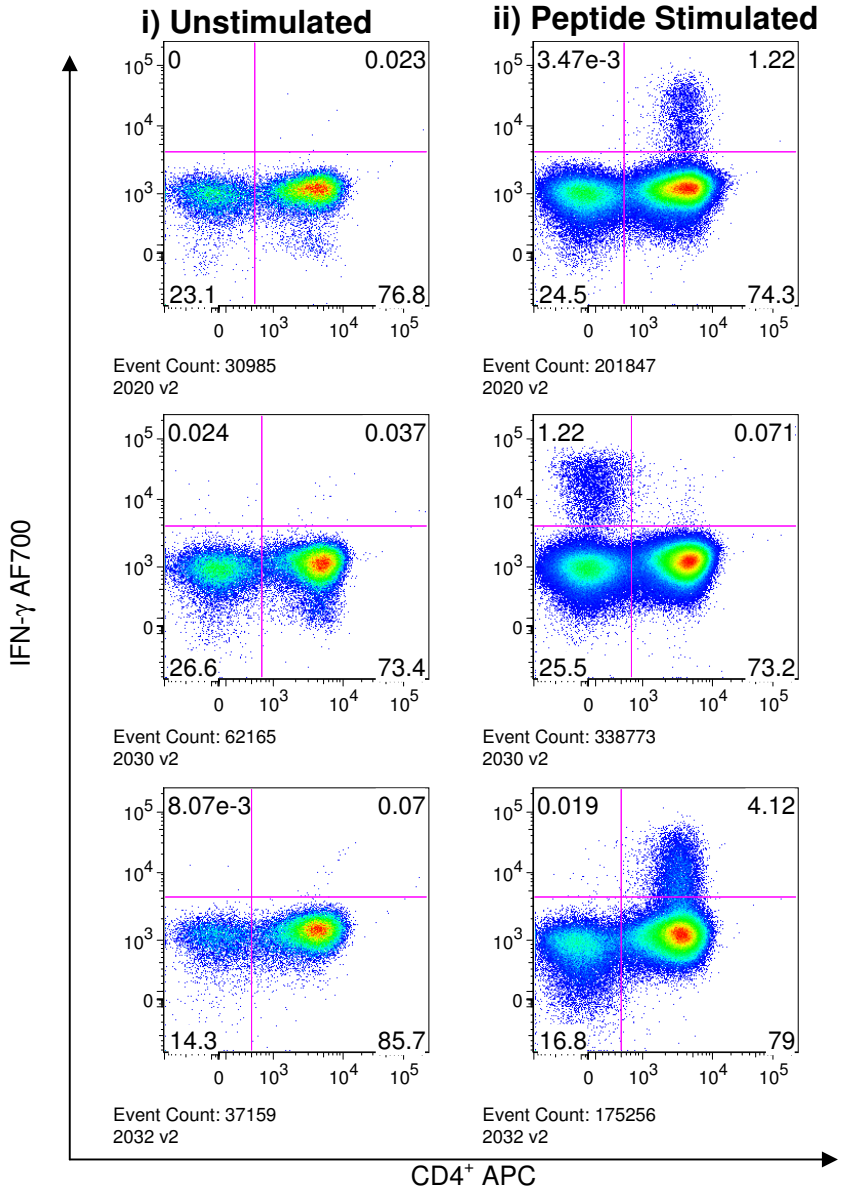


Figure 6.2

Mycobacterium tuberculosis specific T cell responses were mapped in peptide level in three Malawian, HIV-1 serongegative donors, using cultured IFN-γ ELISpot (A). The $CD4^+$/$CD8^+$ T cell specificity was assessed by intracellular cytokine staining (B). Cultured short-term cell lines were stimulated for 6 hours in the presence of either the mapped peptide (ii) or a DMSO control (i). All data presented in (A) is background subtracted. Criteria for a positive culture IFN-γ ELISpot response is >300 SFU/10^6 and 4x background. Criteria for a positive intracellular cytokine staining response was >0.05% and 2x background.

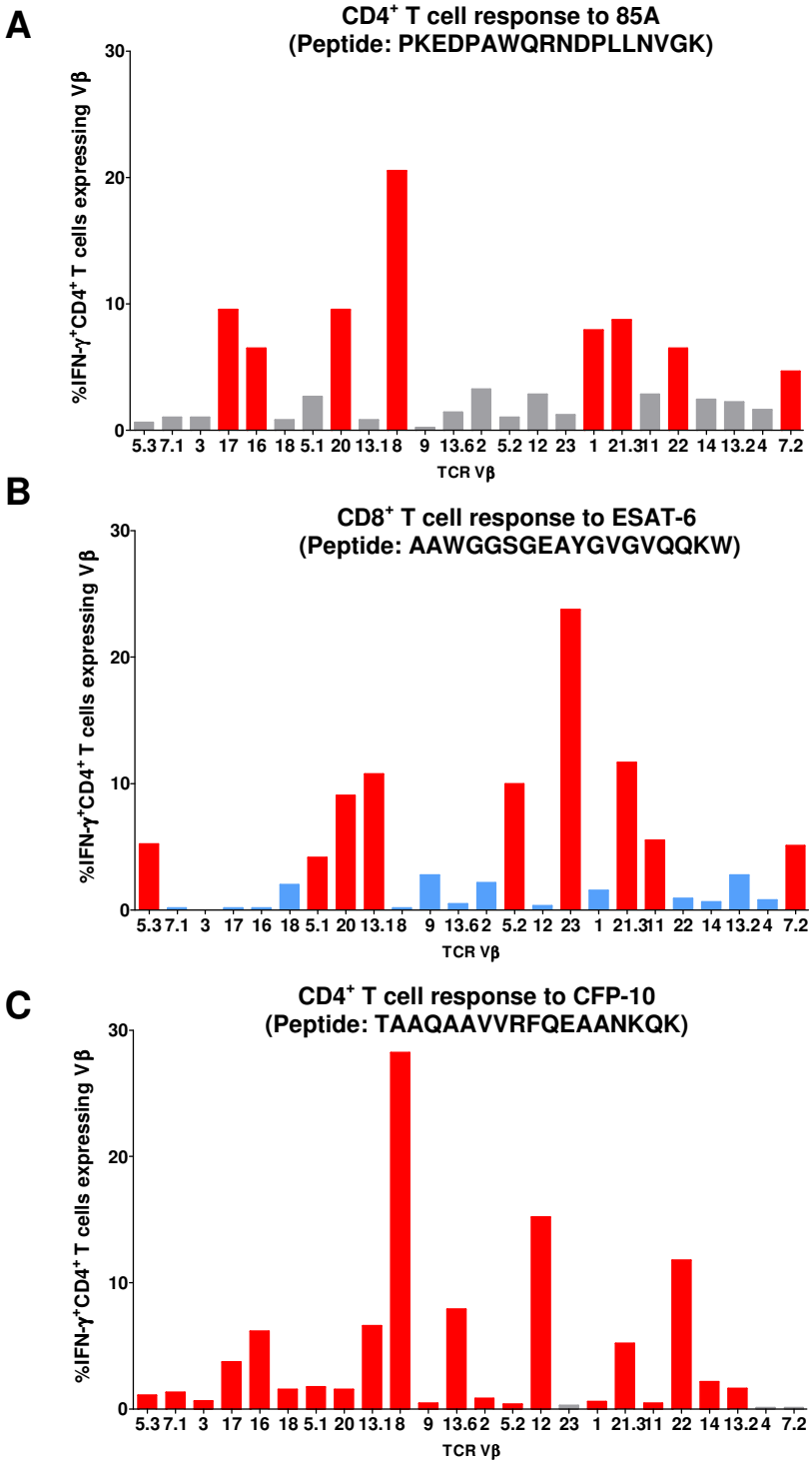


Figure 6.3

The TCRVβ usage of Mtb specific T cell responses was assessed in 3 Malawian HIV-1 seronegative subjects (A-C). All staining was performed using cultured short-term cell lines, expanded in the presence of peptides spanning 85A, ESAT-6 and CFP-10 proteins. TCRVβ usage assessed for IFN-γ^+ T cells, following 6 hour stimulation with mapped peptide (2µg/ml). Criteria for a positive TCRVβ (shown in red) was >20 IFN-γ^+ T cells expressing Vβ. For all assays background IFN-γ production was <0.03%. Criteria for an oligoclonal response was >50% $CD4^+$IFN-γ^+ T cells expressing single Vβ, and response comprised of <5 TCRVβ. Nomenclature from Wei *et al*[264].

PTID / Group	Mapped Peptide	Bkgd IFN-γ	% $CD4^+$ $IFN\text{-}\gamma^+$	Total Number. $CD4^+$ $IFN\text{-}\gamma^+$ cells	Vβ Wei *et al*[264]	% $CD4^+$ $IFN\text{-}\gamma^+$ $V\beta^+$	IMGT[400]
709020154 HESN *	ENV 830-847	0.047%	4.2%	2662	3 13.2 13.6 8 21.3	54.1% 20.9% 10.6% 8% 3.4%	TRBV 28 TRBV 6-2/6-3 TCRBV 6-6 TRBV 12-3/12-4 TRBV 11.2
709020198 HESN *	ENV 830-847	0.014%	2.15%	894	2 13.2 13.6 12 8 20 5.2	23.3% 22.8% 18.1% 6.9% 5.1% 5% 3.8%	TRBV 20-1 TRBV 6-2/6-3 TCRBV 6-6 TRBV 10 TRBV 12-3/12-4 TRBV 30 TRBV 5-6
709090435 HESN	NEF 73-90	0.019%	2.65%	3532	17 8 16 9 18 5.1	65.3% 14.4% 11.8% 5% 0.96% 0.57%	TRBV 19 TRBV 12-3/12-4 TRBV 14 TRBV 3-1 TRBV 18 TRBV 5-1
709090078 HESN	POL 969-986	0.016%	0.3%	705	8 3 5.1 20	34% 32.3% 16.9% 7.9%	TRBV 12-3/12-4 TRBV 28 TRBV 5-1 TRBV 30
709020264 HUSN	VIF 129-146	0.033%	0.6%	203	8	85.7%	TRBV 12-3/12-4
709020366 HUSN	ENV 485-502	0.049%	0.3%	274	20 5.1 8	55.83% 17.8% 8%	TRBV 30 TRBV 5-1 TRBV 12-3/12-4
709090463 HUSN	VPR 33-50	0.073%	0.5%	638	2 8 3	63.6% 12.7% 5.5%	TRBV 20-1 TRBV 12-3/12-4 TRBV 28

Table 6.1
The TCR clonotype of HIV-1 specific T cell responses was assessed in 4 HIV-1 exposed seronegative (HESN) and 3 HIV-1, unexposed seronegative (HUSN) individuals using a flow based assay. Following 10 day culture, short-term cell lines were stimulated for 6 hours with mapped peptide (final concentration 2μg/ml) or DMSO control (background). The magnitude of IFN-γ response is expressed as % $CD4^+$ T cells producing IFN-γ. The dominant TCRVβ, mediating the response is shown in red font, and TCRVβ listed according to dominance. Two subjects (highlighted in purple) had a polyclonal TCRVβ response, notably both were HESN. Two HESN donors, 709020154 and 709020198, indicated with an asterisk, responded to the same envelope peptide, however TCRVβ usage was unique to each individual. Criteria for a positive TCRVβ was >20 $IFN\text{-}\gamma^+$ T cells expressing Vβ. Criteria for an oligoclonal response was >50% $CD4^+IFN\text{-}\gamma^+$ T cells expressing single Vβ, and response comprised of <5 TCRVβ. For comparison with sequencing data IMGT[400] conversion for Wei[264] nomenclature is shown.

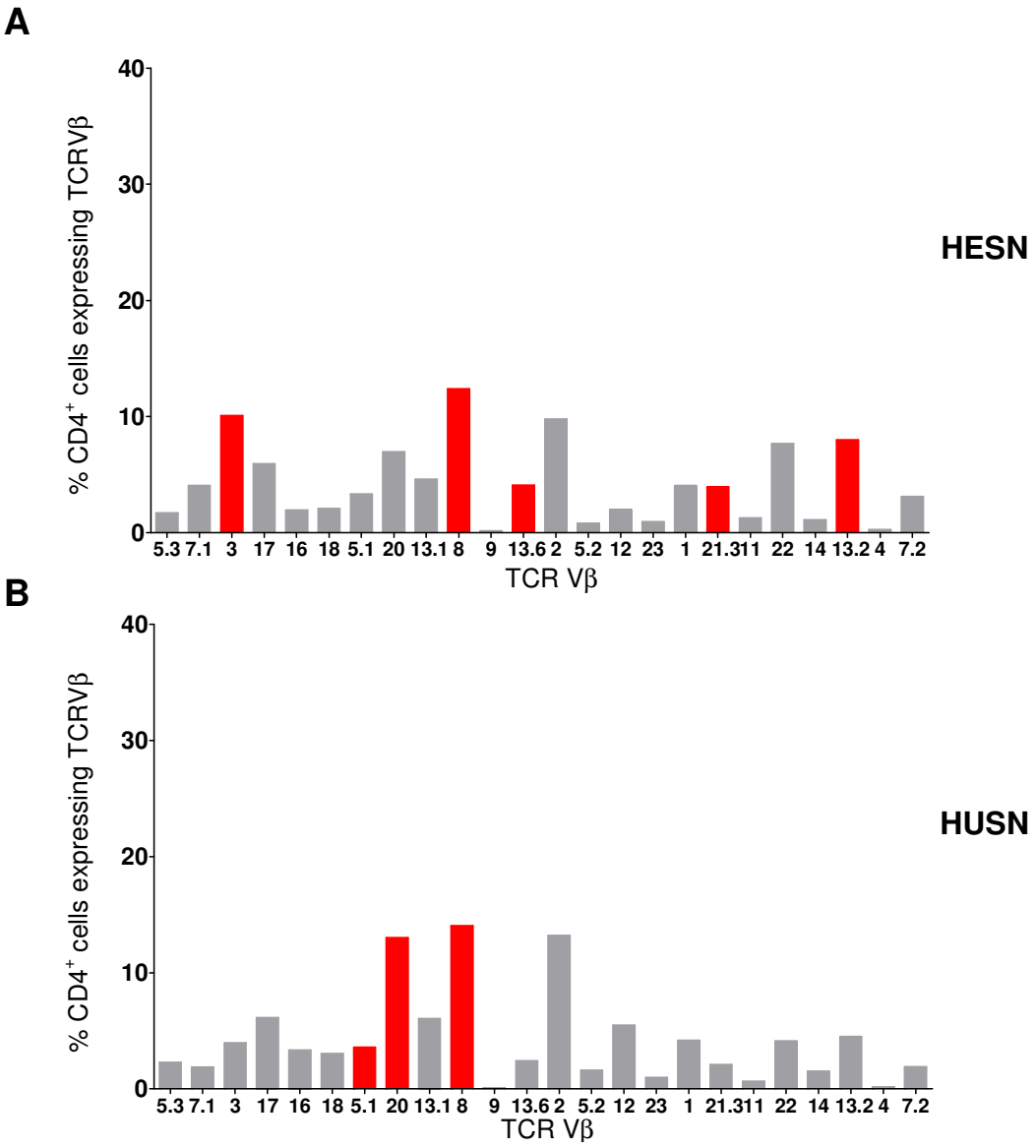


Figure 6.4

The TCRVβ repertoire of $CD4^+$ T cells was assessed for 4 HIV-1 exposed seronegative (HESN) and 3 HIV-1 unexposed seronegative (HUSN) subjects following 10 day culture. Due to the addition of exogenous cytokines IL-7 and IL-2 the short-term cell line was not overtly skewed towards the cells expressing TCRVβ involved in the IFN-γ response to HIV-1 specific peptide (shown in red). The figures shown are representative of 4 HESN individuals (A) studied and 3 HUSN individuals studied (B). Criteria for a positive TCRVβ response was >20 $CD4^+$ IFN-γ^+ cells expressing TCRVβ. All data presented as % $CD4^+$ T cells expressing TCRVβ. Nomenclature from Wei *et al*[264].

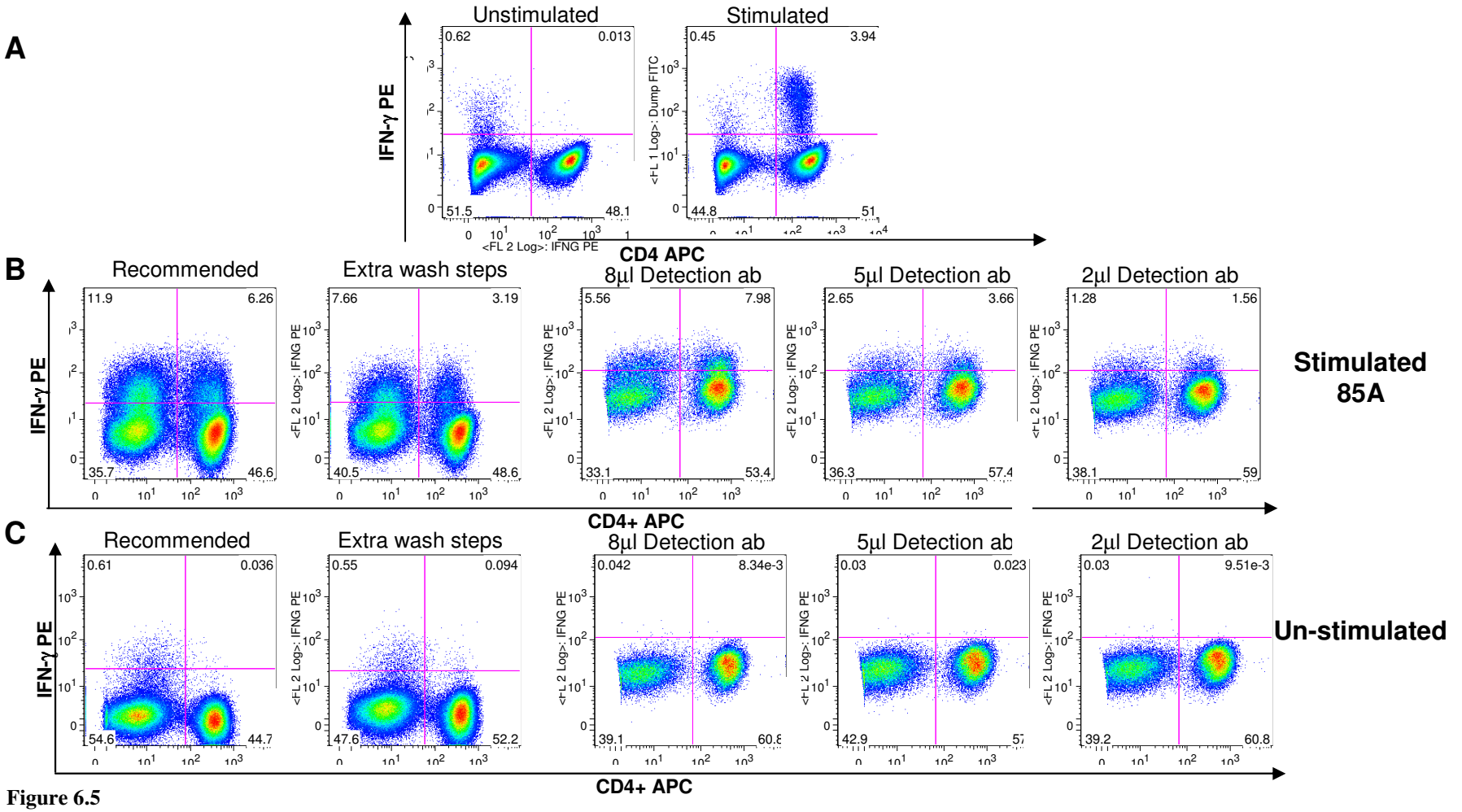


Figure 6.5

Antigen specific T cell responses detected by intracellular cytokine staining (ICS)(A), were compared to those observed using a commercially available IFN-γ capture assay (B). The capture assay was shown to lack the sensitivity and specificity required to be used for the successful cell sorting of IFN-γ^+ T cells. A number of modifications to the recommended protocol were tested (B) to try and improve specificity. All stimulations were made using a pool of peptides spanning 85A antigen. "Unstimulated" tubes received a DMSO control in complete media (C). (Recommended detection antibody (ab) volume was 10µl).

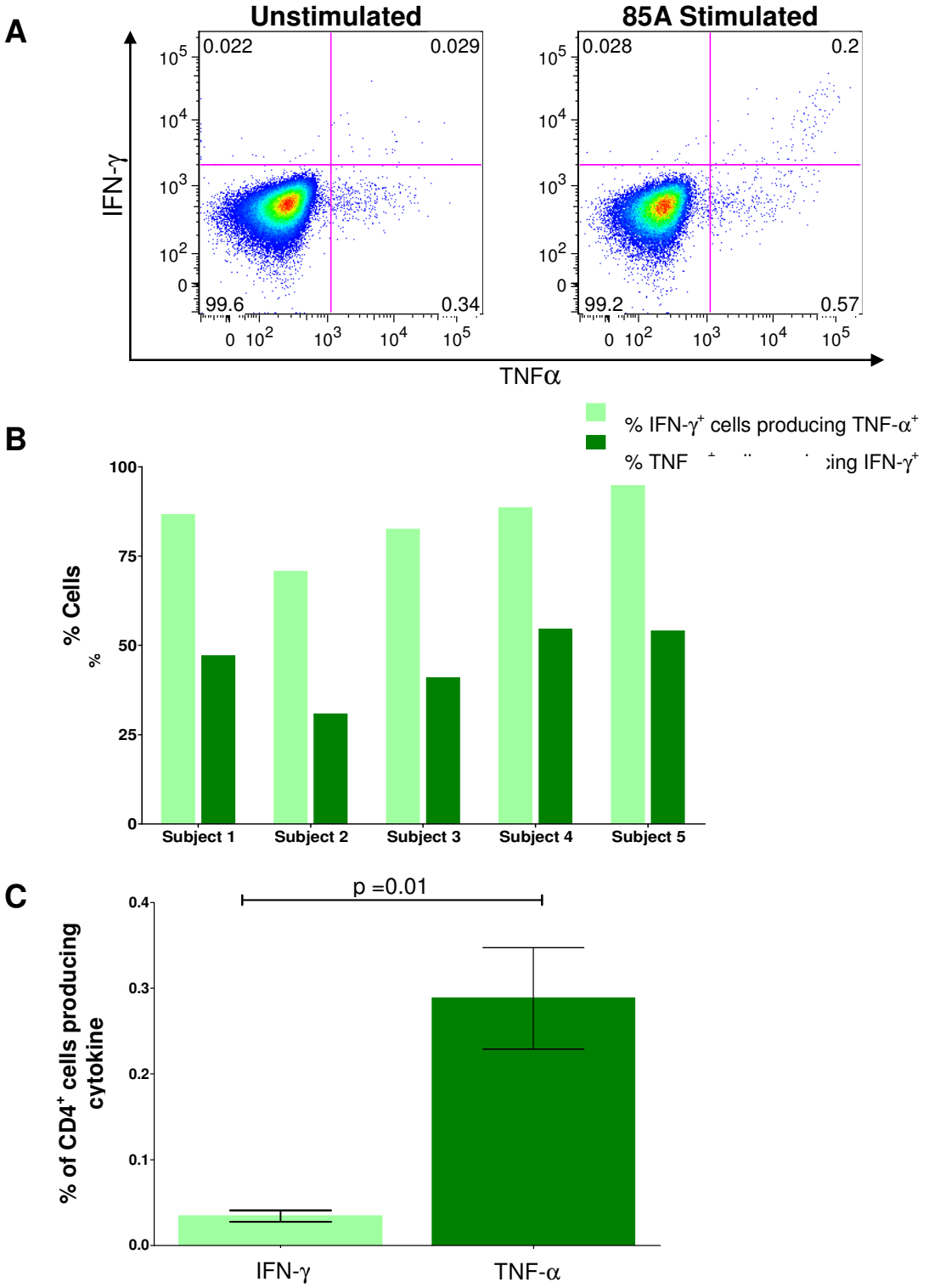
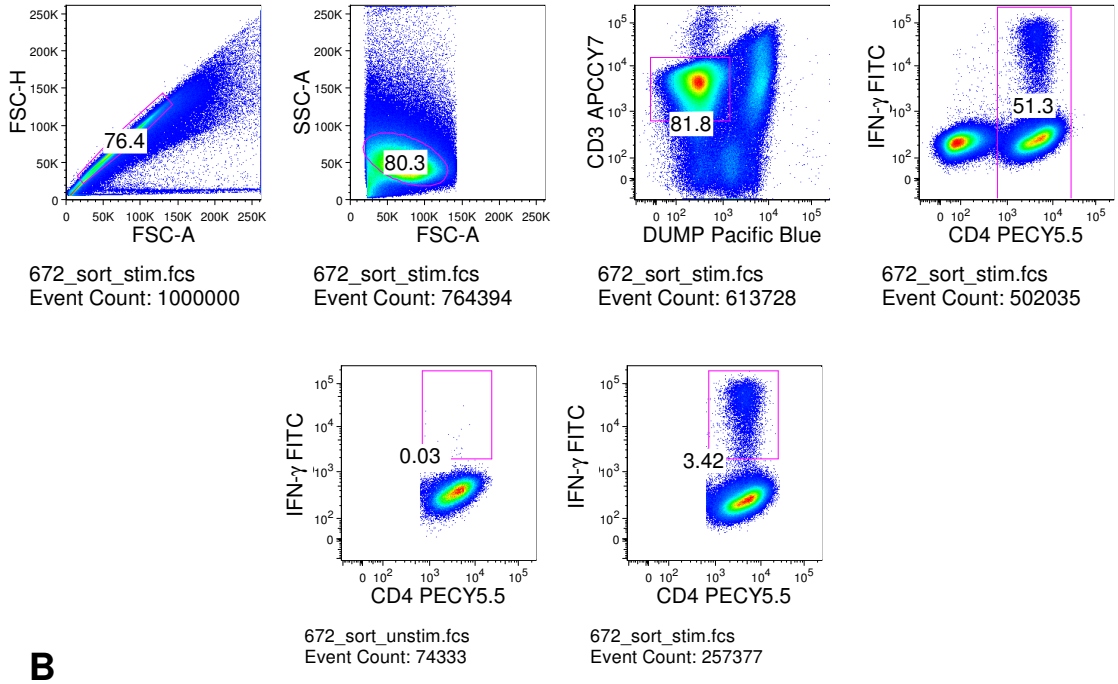


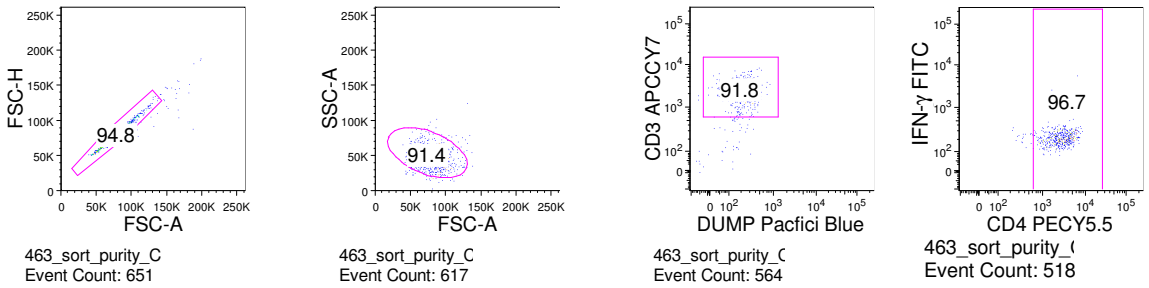
Figure 6.6

Intracellular cytokine staining was used to examine HIV-1 specific, $CD4^+$ T cell responses from 3 HIV-1 exposed, seronegative and 2 HIV-1 unexposed seronegative donors for their ability to co-produce TNF-α and IFN-γ (A). Between 70-100% of IFN-γ producing cells co-produced TNF-α (A-B). However, 45-75% of TNF-α^+ $CD4^+$ T cells were shown to solely produce TNF-α (B). Moreover, background TNF-α production was significantly higher than IFN-γ production (p=0.01) (C). Criteria for a positive response by intracellular cytokine staining was defined as >0.05% and >2xbackground. Peptides were used at a final concentration of 2μg/ml with DMSO control used to assess background.

A



B



C

PTID	Group	% $CD4^+IFN\text{-}\gamma^+$ T cells Unstimulated	% $CD4^+IFN\text{-}\gamma^+$ T cells Stimulated	Total number HIV-1 specific $CD4^+$ $IFN\text{-}\gamma^+$ T cells sorted
709020154	HESN	0.66	1.73	21,978
709020198	HESN	0.10	1.22	8,576
709020435	HESN	0.037	4.16	86,103
709020463	HESN	0.10	0.36	2,415
709020672	HUSN	0.039	3.39	26,367

Figure 6.7

Short-term cell lines, generated from 4 HIV-1 exposed seronegative individuals, and 1 HIV-1 unexposed seronegative individual were stimulated for 6 hours in the presence of mapped HIV-1 peptide or DMSO control. HIV-1 specific $CD4^+$ $IFN\text{-}\gamma^+$ T cells were sorted based upon intracellular IFN-γ staining (A). Sorted $CD4^+$ T cells were re-analysed to assess the purity of the cell sort (B). Sort purity was shown to be high ranging from 89.9%-96% in the two samples tested. For all subjects >2000 $CD4^+$ $IFN\text{-}\gamma^+$ T cells were sorted (C).

A

HESN
709020435 –Nef$_{73-90}$

TRBV	CDR3	TRBJ
7-3	CARTGGTNINYGYT	1-2
19*	CPSGENGNTIY	1-3
19*	CASGVNGNTIY	1-3

B

HESN
709020672 –Gag$_{268-285}$

TRBV	CDR3	TRBJ
20-1	CSAPGSSERYNEQF	2-1
7-3	CARTGGTNINYGYT	1-2
18	CASSPEGGSYEQY	2-7
7-9	CASSPKDWINEQF	2-1
19	CASGVNGNTIY	1-3
2	CASNLVAYEQY	2-7

C

HUSN
709020463 – Vpr $_{33-50}$

TRBV	CDR3	TRBJ
7-3	CARTGGTNINYGYT	1-2
18	CASSPEGGSYEQY	2-7
12-3/12-4*	CASSWTEEYEQY	2-7
19	CASGVNGNTIY	1-3

Figure 6.8

DNA multiplex TCR clonotyping was performed on HIV-1 exposed/unexposed seronegative individuals (HESN/HUSN). Two of the 4 HESN donors (A-B) and 1/1 HUSN donors (C) tested showed an oligoclonal TCR repertoire. The TCRVβ identified by flow cytometry are indicated by *, with the dominant TCRVβ identified by flow cytometry shown in red font. Notably subject 709020672 (B) was not tested using flow cytometry. Tables list sequencing data for TRBV, TRBJ and CD3 regions using standardised IMGT^{400} nomenclature, ordered according to CDR3 length.

A

HESN
709020154 Env$_{830-847}$

TRBV	CDR3	TRBJ
19	CASSMVGSPQGHSPLH	1-6
19	CASSMVGSPRGHSPLH	1-6
19	CASSMAGSPQGHSPLH	1-6
19	CASSIWTSGRGNEQF	2-1
7-9	CASSYGQGFYNSPLH	1-6
7-9	CASSRGQGFYNSPLH	1-6
7-9	CASSNGQGFFNSPLH	1-6
7-9	CASSLGTGFYNSPLH	1-6
7-2	CASSPRGGATGSPLH	1-6
19	CASSFPETGGHEQY	2-7
19	CASSFPGTGGHEQY	2-7
14	CASSLWAGGSYEQY	2-7
6-1	CASSSSASYTNTHY	2-3
6-1	CASSEGGGPDTQY	2-3
19	CASRDRDYQPQH	1-5
12-3/12-4*	CASSLGQGLAG	2-7
28*	CASNRYNEQF	2-1
6-6*	CASSSYYEQY	2-7
6-2/6-3*	CASSSYNEQF	2-1
6-2/6-3*	CASSIYNEQF	2-1
6-1	CASSIYNEQF	2-1

Vβ detected by flow cytometry not seen with sequencing:
TRBV11.2 (Vβ 21.3) expressed by 3.4% CD4$^+$IFN-γ$^+$ T cells

B

HESN
709020198 Env$_{830-847}$

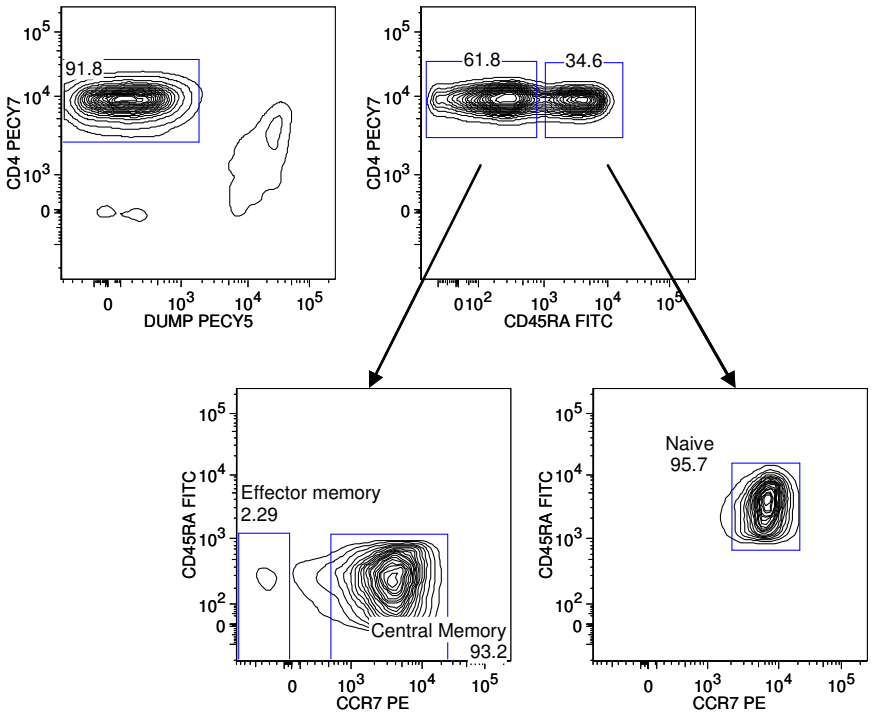
TRBV	CDR3	TRBJ
7-2	CASSLGGPRQGGYT	1-2
7-2	CASSLGGPGQGGYT	1-2
7-2	CASSIGGPGQGGYT	1-2
7-2	CASRLWGRGQGGYT	1-2
7-2	CASSSRGEGYEQY	2-7
6-6*	CASSEGGGPDTQY	2-3
6-1	CASSEGGGPDTQY	2-3
6-6*	CATHTYNEQF	2-1
6-6*	CASSSYNEQF	2-1
6-2/6-3*	CASSSYYEQY	2-7
6-2/6-3*	CATSSYTEAF	1-1
6-2/6-3*	CASSQYTEAF	1-1
6-2/6-3*	CASLSYTEAF	1-1

Vβ detected by flow cytometry not seen with sequencing:
TRBV20-1 (Vβ 2) expressed by 23.3% CD4$^+$IFN-γ$^+$ T cells
TRBV10 (Vβ 12) expressed by 6.9% CD4$^+$IFN-γ$^+$ T cells
TRBV 12-3/12-4 (Vβ 8) expressed by 5.1% CD4$^+$IFN-γ$^+$ T cells
TRBV 30 (Vβ 20) expressed by 5% CD4$^+$IFN-γ$^+$ T cells
TRBV 5-6 (Vβ 5.2) expressed by 3.8% CD4$^+$IFN-γ$^+$ T cells

Figure 6.9

Two HESN donors (A-B) known to respond to the same envelope peptide were sequenced to ascertain the TCR clonality of antigen specific T cells. Both donors were shown to have a polyclonal T cell response however they shared a common clonotype (Highlighted in pink). Notably the dominant TCRVβ detected by flow cytometry (shown in red font) was also detected using the sequencing approach. Vβ identified by both flow cytometry and sequencing are depicted by* and all responses not shown by sequencing listed below the tables with Wei *et al* nomenclature[264] given in brackets. IMGT nomenclature used for all sequence data[400].

A



B

DONOR	NAÏVE	CENTRAL MEMORY	EFFECTOR MEMORY
BC AR 20	$0.53x10^6$	$0.59x10^6$	$0.22x10^6$
BC AR 21	$0.78x10^6$	$0.71x10^6$	$0.37x10^6$
BC AR 22	$2.04x10^6$	$2.18x10^6$	$0.61x10^6$
BC AR 23	$1.75x10^6$	$1.98x10^6$	$0.73x10^6$
BC AR 24	$1.95x10^6$	$1.98x10^6$	$0.62x10^6$

Figure 6.10

Healthy, HIV-1 seronegative leukapheresis samples were sorted into $CD4^+$ naïve, central memory and effector memory subsets based on cell surface expression of CD45RA, CD25 and CCR7 (A). Following cell sorts manual cell counts of viable cells were made using trypan blue exclusion (B). Cell sorting performed by D. Jarrossay (IRB, Bellinzona).

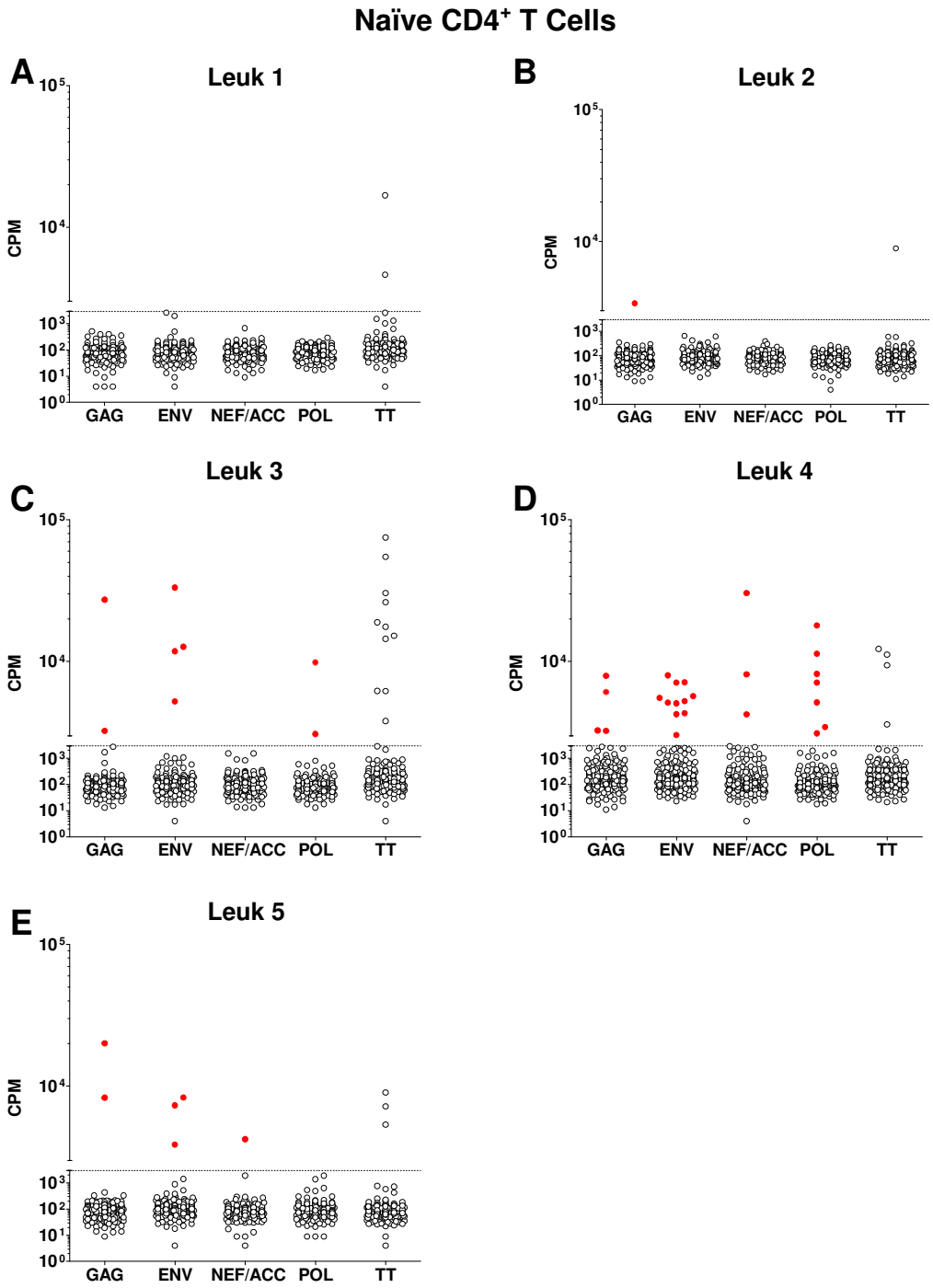


Figure 6.11

T cell libraries established from naïve ($CD45RA^+$ $CCR7^+$ $CD25^-$) $CD4^+$ T cells of 5 healthy (A-E), HIV-1 seronegative, leukapharesis donors were screened for proliferative responses to HIV-1 peptides and recall antigen tetanus toxin. Proliferative responses were assessed on day 4 following 16 hour tritiated thymidine incorporation and all data presented is the delta cpm (cpm in response to antigen pulsed monocytes–cpm in response to un-pulsed monocytes). HIV-1 peptides spanned the entire proteome, and were pooled by protein gag, env, nef and accessory proteins vpr, vpu, tat, rev, vif (Nef/Acc), and pol. Peptides were used at a final concentration of 2µg/ml. TT was used as a whole protein at a final concentration of 5µg/ml. Dotted lines represent the cut of value for a positive response (delta cpm $>3x10^3$cpm). All positive responses to HIV-1 peptides are depicted in red.

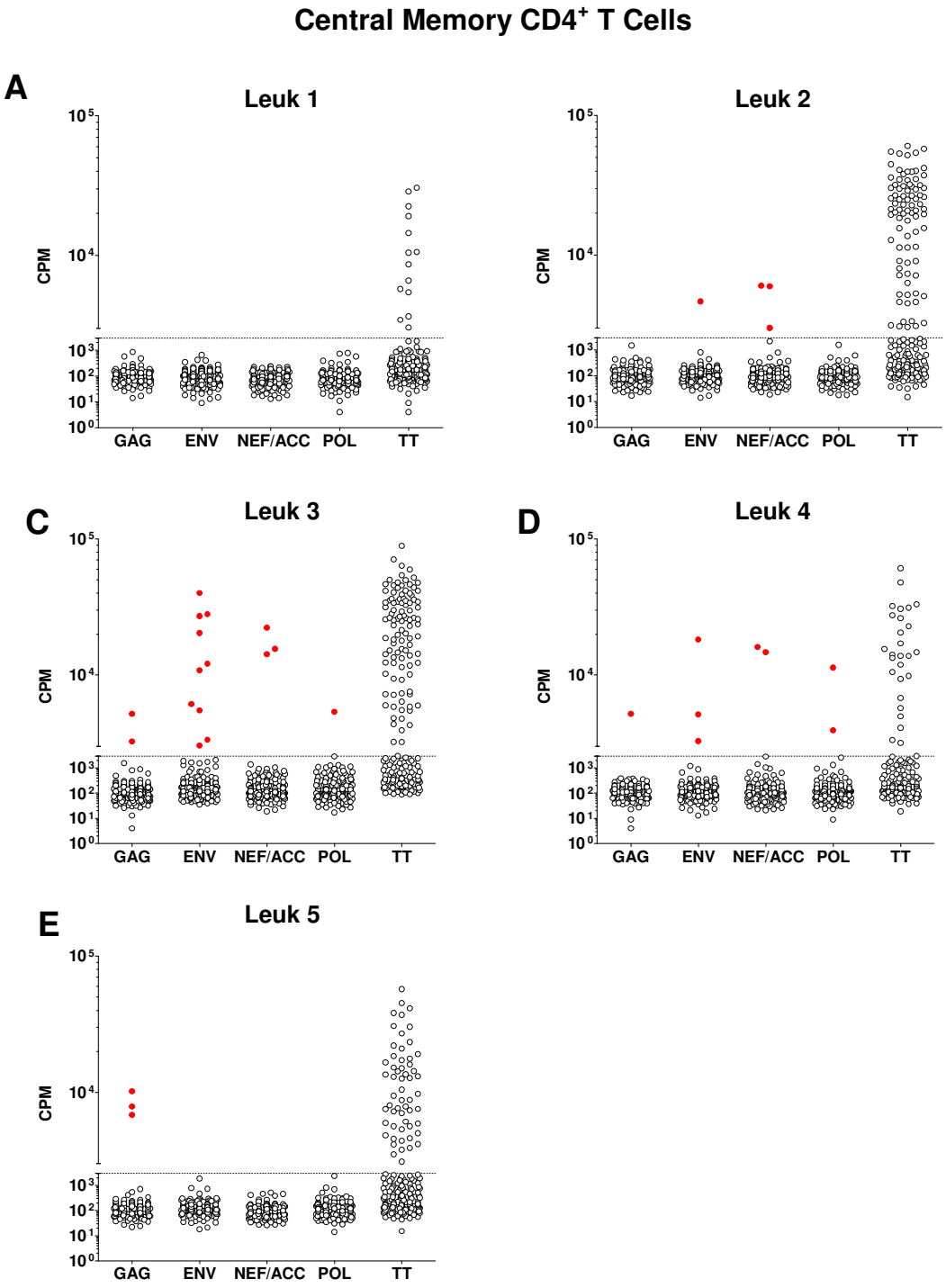


Figure 6.12

T cell libraries established from central memory ($CD45RA^{-}CCR7^{+}CD25^{-}$) $CD4^{+}$ T cells of 5 healthy (A-E), HIV-1 seronegative, leukapharesis donors were screened for proliferative responses to HIV-1 peptides and recall antigen tetanus toxin. Proliferative responses were assessed on day 4 following 16 hour tritiated thymidine incorporation and all data presented is the delta cpm (cpm in response to antigen pulsed monocytes – cpm in response to un-pulsed monocytes). HIV-1 peptides spanned the entire proteome, and were pooled by protein gag, env, nef and accessory proteins vpr, vpu, tat, rev, vif (Nef/Acc), and pol. Peptides were used at a final concentration of 2µg/ml. TT was used as a whole protein at a final concentration of 5µg/ml. Dotted lines represent the cut of value for a positive response (delta cpm $>3x10^{3}$cpm). All positive responses to HIV-1 peptides are depicted in red.

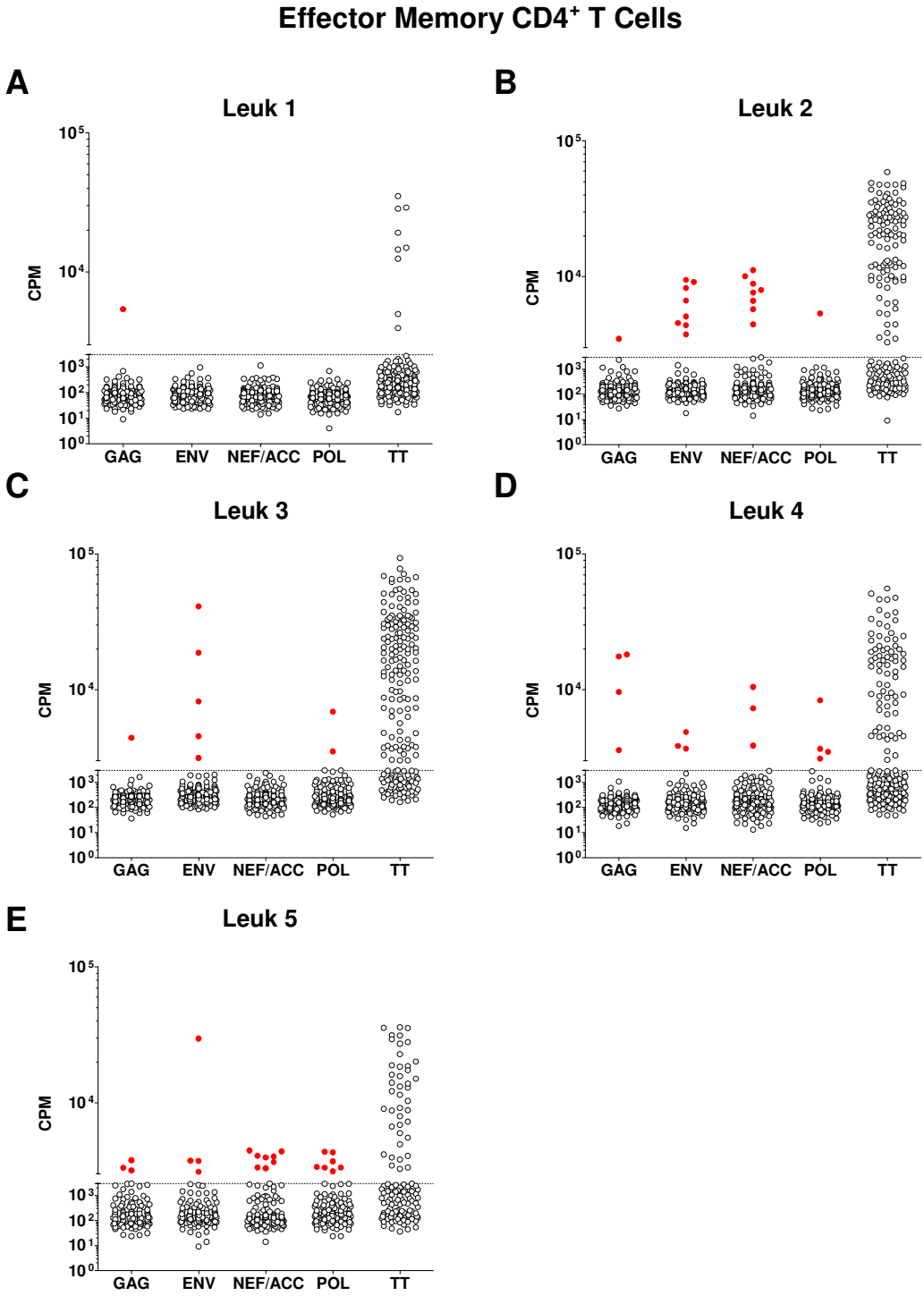


Figure 6.13

T cell libraries established from effector memory ($CD45RA^{-}CCR7^{-}CD25^{-}$) $CD4^{+}$ T cells of 5 healthy (A-E), HIV-1 seronegative, leukapharesis donors were screened for proliferative responses to HIV-1 peptides and recall antigen tetanus toxin. Proliferative responses were assessed on day 4 following 16 hour tritiated thymidine incorporation and all data presented is the delta cpm (cpm in response to antigen pulsed monocytes – cpm in response to un-pulsed monocytes). HIV-1 peptides spanned the entire proteome, and were pooled by protein gag, env, nef and accessory proteins vpr, vpu, tat, rev, vif (Nef/Acc), and pol. Peptides were used at a final concentration of 2µg/ml. TT was used as a whole protein at a final concentration of 5µg/ml. Dotted lines represent the cut of value for a positive response (delta cpm $>3x10^{3}$cpm). All positive responses to HIV-1 peptides are depicted in red.

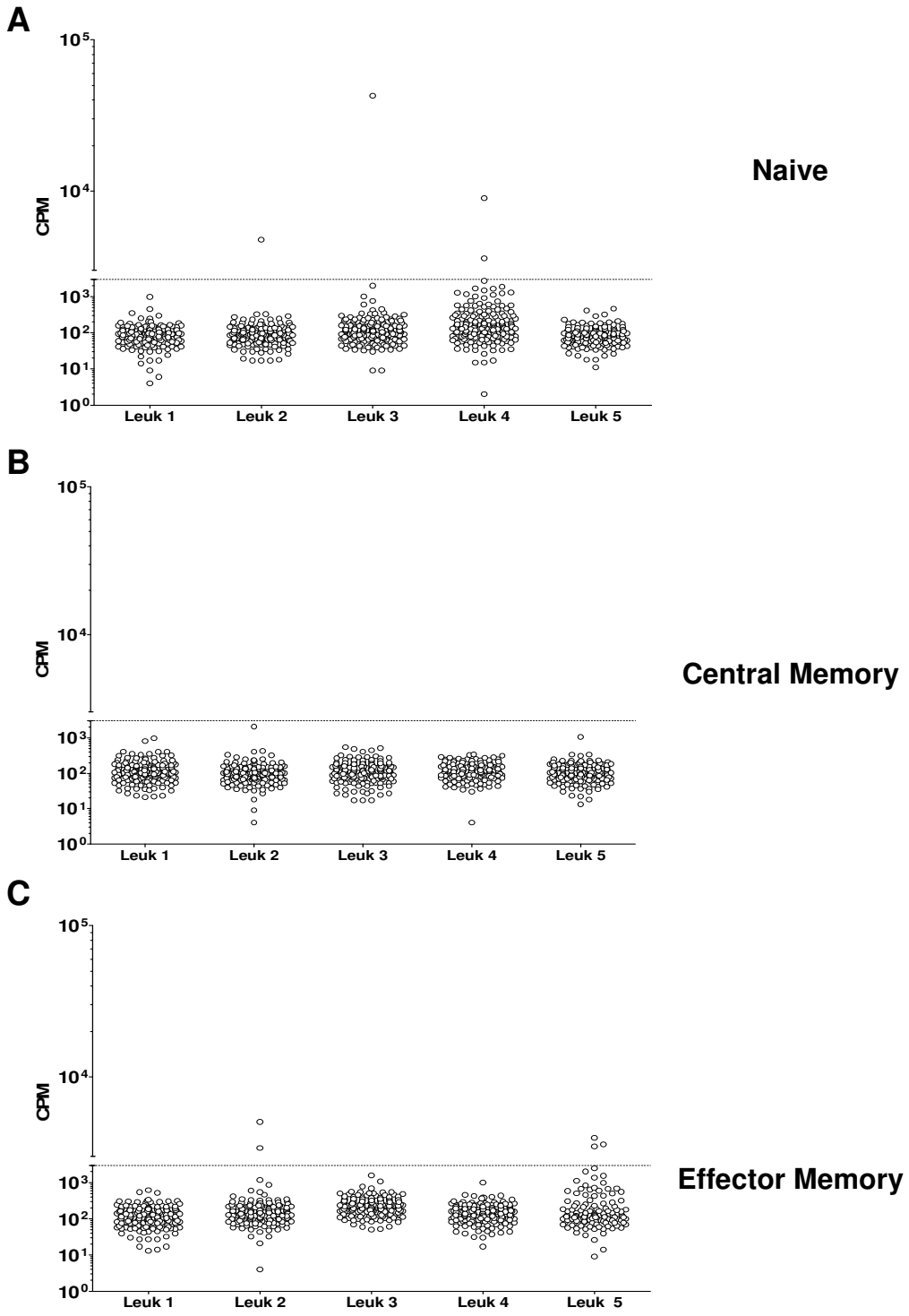


Figure 6.14

Non-specific proliferation, was assessed in naïve (A), central memory (B) and effector memory (C) $CD4^+$ T cell libraries generated from 5 healthy leukapharesis donors. Cultures with background proliferation of >3000 counts per million (cpm) were excluded from subsequent analysis.

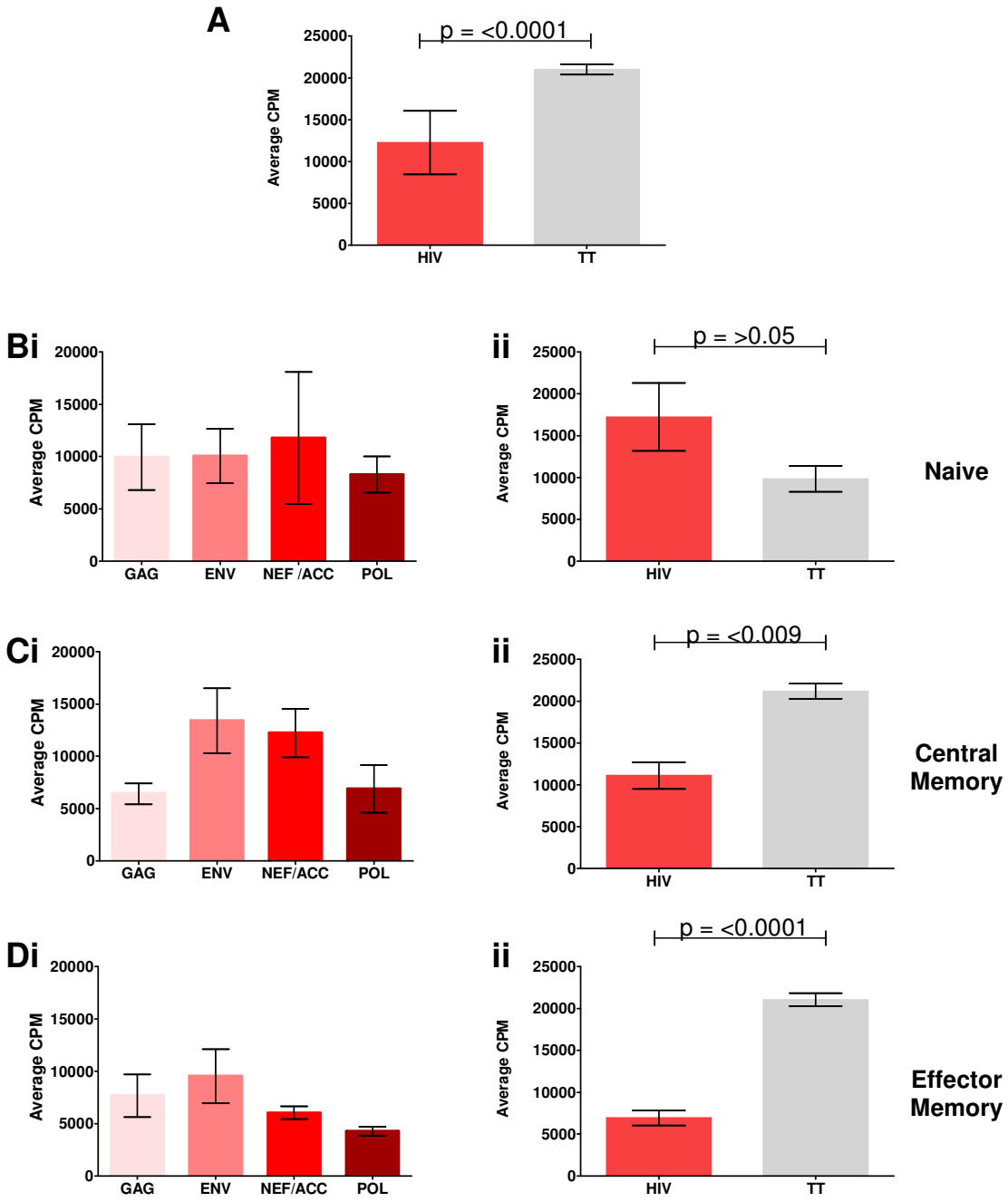
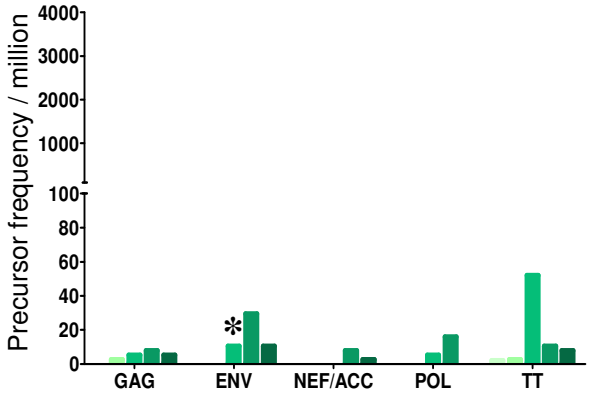


Figure 6.15

The overall proliferative response of $CD4^+$ T cells to peptides spanning the entire HIV-1 proteome was significantly lower than the proliferative response to known recall antigen tetanus toxin (TT) (A). Examination of the naïve (Bii), central memory (Cii) and effector memory (Dii) $CD4^+$ T cell subsets showed that this overall proliferative difference was primarily attributed to the memory $CD4^+$ T cell subsets (Cii-Dii, Paired T test p=<0.01). The proliferative response of naïve $CD4^+$ T cells to HIV-1 and TT was not significantly different (Paired T test p=>0.05, Bii). No significant difference (One way ANOVA p=>0.05) in the proliferative response to individual HIV-1 proteins was detected across either the naïve (Bi) or memory (Ci-Di). Data presented as the average, delta counts per minute (cpm) obtained from 5 leukapharesis donors. Error bars represent standard error of mean. For these analyses only positive responses of >3000delta cpm were included.

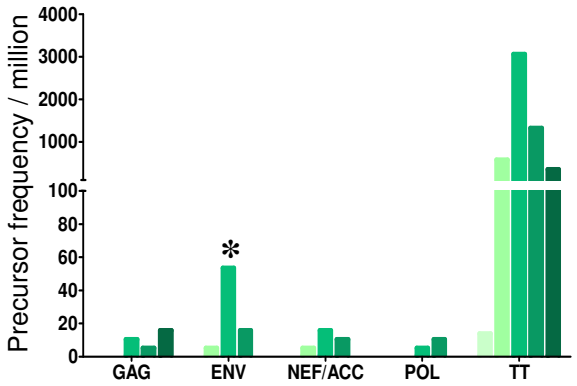
A



Precursor frequency / 10^6 Naive $CD4^+$ T Cells

	Gag	ENV	NEF/ ACC	POL	TT
Leuk 1					2
Leuk 2	2				2
Leuk 3	5	10		5	52
Leuk 4	7	29	7	15	10
Leuk 5	5	10	2		7

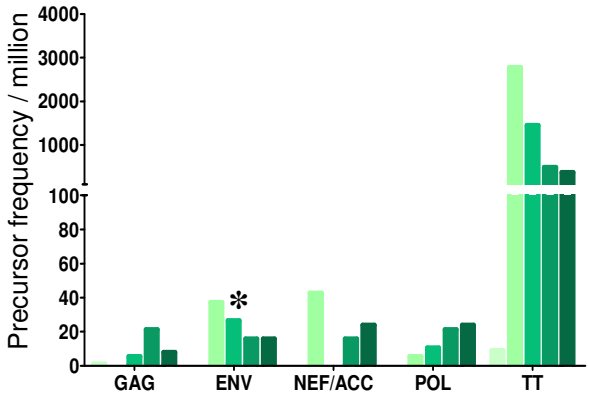
C



Precursor frequency / 10^6 CM $CD4^+$ T Cells

	Gag	ENV	NEF/ ACC	POL	TT
Leuk 1					14
Leuk 2		5	5		575
Leuk 3	10	53	15	5	3060
Leuk 4	5	15	10	10	1325
Leuk 5	15				352

E



Precursor frequency / 10^6 EM $CD4^+$ T Cells

	Gag	ENV	NEF/ ACC	POL	TT
Leuk 1	1				9
Leuk 2		37	45	5	2772
Leuk 3	5	26		10	1450
Leuk 4	21	15	15	21	486
Leuk 5	7	15	23	23	374



Figure 6.16

The precursor frequency of HIV-1 specific and TT specific T cells was assessed for the $CD4^+$ naïve (A) central memory (B) and effector memory subsets. Precursor frequency was calculated based on Poisson distribution and is expressed as number of antigen specific T cells per million. Responses to pools of peptides spanning HIV-1 proteins gag, env, nef and accessory (Nef/Acc) proteins (vpr, vpu, vif, tat, rev) and pol were compared to recall antigen to tetanus toxin (TT). Asterix / red font indicates leukapharesis donor (Leuk 3) with positive Env response by cultured IFN-γ ELISpot.

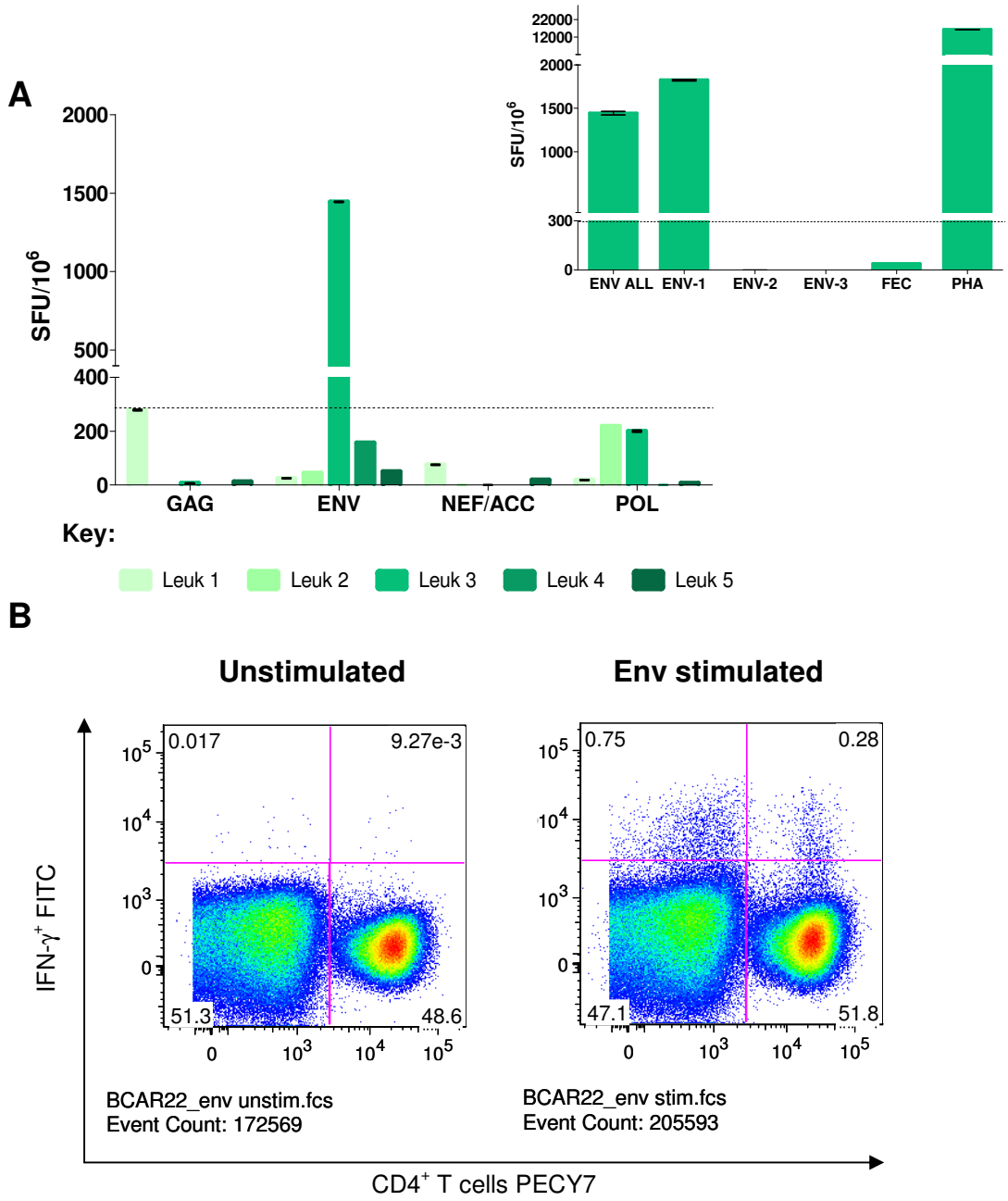


Figure 6.17

In parallel to T cell library assays, cultured IFN-γ ELISpot assays were performed for each of the 5 leukapharesis donors (A). Overlapping pools of HIV-1 specific peptides spanning gag, env, pol and nef and accessory proteins (vpr, vpu, vif, tat, rev) (Nef/Acc) were used to screen for HIV-1 specific T cell responses. Donor Leuk 3 was the only subject to have a positive HIV-1 specific T cell response (inset A), which targeted env with a magnitude of >1000 spot forming units (SFU) per million cells. Flow cytometric analysis confirmed the response was mediated by $CD4^+$ T cells (B). Data presented is the average of quadruplicate wells and background subtracted. Error bars represent standard error of mean. Criteria for a positive response was >300SFU/10^6 and >4x background for cultured IFN-γ ELISpot and >0.05% and >2x background for flow cytometric analysis. All peptides used at a final concentration of 2μg/ml.

CHAPTER 7:

DO PRE-EXISITING HIV-1 SPECIFIC T CELLS PROTECT?

Chapter 7: Do pre-existing HIV-1 specific T cells protect?

7.1 Introduction

Immune correlates of protection against the establishment of HIV-1 remain poorly understood. The principal reason for this is that there are no known examples of individuals capable of spontaneously clearing HIV-1 infection. Therefore, studies aimed at improving our understanding of immune correlates of protection have focused primarily upon a minority of individuals, who are either capable of sustaining normal $CD4^+$ counts and low viral loads in the absence of anti-retroviral therapy (defined as long-term non-progresser; LTNP), or who appear protected against the establishment of HIV-1 (HIV-1 exposed seronegative; HESN). LTNPs are rare, typically comprising just 2-5% of all HIV-1 infected individuals[78]. A number of factors have been associated with the apparent viral control exhibited by LTNPs including; viral attenuation[131,618], host genetic characteristics[619,620], innate immune responses[195] as well as adaptive immune responses[76,77,79,414]. Particular focus has been paid to the maintenance of broad, robust and polyfunctional T cell responses, mediated by both $CD4^+$ and $CD8^+$ T cells, amongst LTNPs[76,79,128,132,329,379,414,621]. T cell responses have consistently been shown to correlate with reduced viral load, and delayed progression to AIDS defining illness, a correlation corroborated by primate studies[318,358,622] and studies of less pathogenic HIV-2 virus[16]. Moreover, depletion of $CD8^+$ T cells during chronic stages of HIV-1 infection leads to rapid viral rebound, signifying the importance of these cells in the control of viremia[358].

Similar to LTNPs, the study of rare individuals who, despite repeated high risk exposure to HIV-1, remain seronegative (HESN) has also generated a number of potential correlates of protection. Indeed, the only reproducibly demonstrated correlate of protection came from early studies of HESN who were shown to possess a homozygous 32base pair deletion in the HIV-1 co-receptor, C-C chemokine receptor 5 (CCR5Δ32)[46-48,493]. In light of observations made in LTNPs[76,79,128,329,379,621] and primate models[318,358,622], the detection of HIV-1 specific T cell responses amongst a proportion of HESN individuals[135,136,145,148,150,151,154,156,158,295,382-384,489,492] (Chapter 5) was taken to be indicative of protective immunity. However, whether HIV-1 specific

T cell responses are truly capable of conferring protection or whether they are merely remnants indicating past exposure to HIV-1 remains unknown. Even for LTNPs the causal relationship between the detection of broad polyfunctional T cell responses and low viral load is yet to be fully established.

The search for immune correlates of protection is inherently difficult, not only due to a lack of individuals who are capable of clearing the virus, but also because the infectivity of HIV-1 is estimated to be low, at around 1 infection per 1000 contacts following heterosexual exposure[29,623]. Given the fact that more than 33 million people are infected with HIV-1 and that each year 2.5 million new infections are reported, an infectivity estimate of 0.001 is likely to be a conservative one. Indeed, infectiousness and susceptibility to HIV-1 has been shown to be highly influenced by a number of co-factors, not least, the presence of sexually transmitted infections (STIs) which disrupt the mucosal surfaces[29,33]. In addition the transmission route, type of sexual act and viral load of the HIV-1 infected partner have also been shown to affect infectivity[29-31,34,37,624]. In instances where one or more of these co-factors are present, infectivity can exceed 1 infection per 3 contacts for penile-anal exposure or 1 infection per 10 contacts for penile-vaginal exposure[31]. Given the variability in these infectivity estimates it would seem essential that large numbers of individuals be recruited in the study of HESN, and that strict criteria used to define HESN. This should ensure any potential "correlates of protection" are not obscured by the inclusion of individuals who are at lower risk of HIV-1.

Whilst it is likely that a number of the HESN studied to date have merely been fortunate, avoiding HIV-1 infection due to stochastic events, chance alone does not account for all cases of HESN. Indeed, the HESN phenotype has now been modelled in a number of primate studies[388,390,499,625]. Clerici *et al* showed that macaques who received a single low-dose intravenous inoculation of simian immunodeficiency virus (SIV) were protected against subsequent high dose rectal challenge[625]. Whilst the HESN phenotype can be mimicked in primate studies, the role and even detection of HIV-1 specific T cell responses remains controversial[388,390,499,625]. The most convincing evidence for T cell mediated protection against the establishment of HIV-1 comes from primate vaccine studies. Vaccines capable of inducing broad, polyfunctional T cell responses have been correlated with protective and even

sterilizing immunity[389,504,614]. Unfortunately, the only HIV-1 vaccine based solely on the assumption that "T cell responses protect", failed to induce protective immunity during phase 2B clinical trials, despite eliciting potent, polyfunctional T cell responses[626,627].

Further debate as to whether pre-existing HIV-1 specific T cell responses are capable of conferring protection, has focused upon the fact that not all studies have been able to detect HIV-1 specific T cell responses in HESN[134,155]. In contrast to the broad robust T cell responses evoked by successful primate vaccine models[389,504,614], where detected, HIV-1 specific T cell responses tend to be weak, transient and only detectable in a proportion of HESN[135,152,155]. Furthermore, detection of HIV-1 specific T cell responses is not limited to HESN, they can also be detected amongst HIV-1 unexposed seronegative (HUSN) individuals[138,391] (Chapter 6). This raises the possibility that not all HIV-1 specific T cell responses have primed by HIV-1[511,593,594] (Chapter 6). The current inability to distinguish between HIV-1 primed and HIV-1 cross reactive T cells adds further debate as to whether detection of HIV-1 specific T cell responses in HESN really are correlates of protection. The most persuasive evidence to suggest pre-existing HIV-1 specific T cell responses do not provide protection comes from the observation that previously defined HESN, have subsequently seroconverted despite having detectable HIV-1 specific T cell responses[152].

The magnitude, maintenance and frequency of detection, of HIV-1 specific T cell responses have been correlated with exposure to HIV-1[150,152] (Chapter 5). It would appear more likely, therefore, that pre-existing HIV-1 specific T cell responses are "markers of exposure" rather than "correlates of protection''. In a seminal paper by Douek *et al,* HIV-1 was shown to preferentially infect HIV-1 specific T cells. Although the mechanism behind this observation remains poorly understood[368] it could be argued that pre-existing HIV-1 specific T cell responses, rather than conferring a degree of protection against HIV-1, may actually serve as a reservoir of potential targets for HIV-1 infection.

7.2 Chapter Aims

Detection of HIV-1 specific T cell responses, whether primed by exposure to HIV-1 or an unrelated pathogen, has long been considered indicative of protective immunity. However, the evidence supporting a role in protection appears limited. HIV-1 specific T cell responses are typically weak, transient, and not detected in all HESN[134,135,152,155]. Moreover, there is evidence that seroconversion can occur despite detection of pre-existing HIV-1 specific T cell responses[152]. Whilst pre-existing HIV-1 specific T cell responses may simply be markers of exposure, there is also a concern that they may have a detrimental effect upon either the acquisition of HIV-1 or subsequent immunopathology[28,62,368,554,628,629].

The aim of this chapter is to focus specifically on the effect, if any, that pre-existing, HIV-1 specific, T cell responses have on the relative risk of HIV-1 infection. Given that HIV-1 is known to preferentially infect HIV-1 specific $CD4^+$ T lymphocytes[28,62,368], we hypothesize, that participants, who harbour $CD4^+$ HIV-1 specific T cell responses, may have increased risk of susceptibility to HIV-1 infection. To address this hypothesis samples were obtained from the placebo arm of the VAX004 trial[220-222,392] (Chapter 2 Section 2.1.1.5). With prior knowledge of which subjects subsequently became HIV-1 infected, a blinded, retrospective, cross-sectional study was undertaken. The study was powered to detect a 3 fold increased risk of HIV-1 infection in subjects with detectable HIV-1 specific T cell responses (Power calculations performed by Dr P. Gilbert SCHARP).

7.3 Results

7.3.1 Study power

We predicted that the impact, if any, that pre-existing HIV-1 specific T cell responses would have upon acquisition of HIV-1 would be subtle. Power calculations (performed by Dr P. Gilbert, SCHARP) determined that over 200 individuals (100 infected, 100 uninfected) would be required to detect a 2 fold increase in HIV-1 infection rates due to pre-existing, $CD4^+$ HIV-1 specific T cells (Table 7.1). Allowing for constraints of sample availability, the study was powered to detect a 3-4 fold increase in risk of HIV-1 infection. To achieve this 80 subjects, 40 whom subsequently became HIV-1 infected and 40 who remained seronegative for the duration of study were studied (Table 7.1).

7.3.2 Detection Of HIV-1 Specific T Cell Responses

In a blinded, cross sectional analysis, 87 high risk men who have sex with men (MSM), enrolled in the placebo arm of VAX004 were screened for HIV-1 specific T cell responses using cultured IFN-γ ELISpot. Pools of peptides spanning the entire HIV-1 proteome were grouped according to protein (gag, env, nef and accessory proteins vif, vpr, vpu, tat, rev (Nef/Acc) and pol. In addition two pools of previously defined optimal cytotoxic T lymphocyte (CTL) epitopes were included (CTLA and CTLB) to ensure the assay was not biased towards $CD4^+$ T cell responses. Of the 87 subjects screened, 38 subsequently became HIV-1 seropositive and 49 remained HIV-1 seronegative for the duration of study (3 years)[392]. HIV-1 specific T cell responses were detected in 10/49 subjects who did not become HIV-1 positive during the course of the study (Figure 7.1A) and 14/38 subjects whom subsequently became HIV-1 positive (Figure 7.1B). The responses were typically directed against one protein with env the most frequently targeted (Figure 7.1A-B). No HIV-1 specific T cell responses were detected by *ex vivo* IFN-γ ELISpot analysis. Background, non-specific IFN-γ production was low (Figure 7.2B) and did not significantly vary between groups ($p=>0.05$).

7.3.3 Magnitude of HIV-1 specific T cell responses

HIV-1 specific T cell responses detected in subjects whom subsequently became HIV-1 seropositive were significantly greater in magnitude than those found in subjects who remained seronegative (Figure 7.2A). Unfortunately PBMC samples from each group arrived in two separate shipments. To ensure that the difference in magnitude was not the result of a difference in cell quality, following shipment, the magnitude of response to positive control antigens PHA and peptide control "FEC", (commonly targeted, and HLA class I presented epitopes from flu, Epstein Barr Virus, and Cytomegalovirus), were examined *ex vivo*. No significant difference (p=>0.05) in either PHA or FEC responses was detected (Figure 7.3A-B). The cell viability was also assessed using trypan blue exclusion upon freshly revived cells. Similar to PHA and FEC responses, no significant difference in PBMC viability (Figure 7.3C) was observed between the groups (p=>0.05). Together these data suggest sample quality was not compromised during shipment.

7.3.4 Maintenance of responses

The maintenance of HIV-1 specific T cell responses was assessed by screening PBMC samples taken one year after the first study visit by cultured IFN-γ ELISpot (Figure 7.4). The time between study visits ranged between 215-501 days, and did not vary significantly for either group (data not shown). HIV-1 specific T cell responses were maintained in 4/7 subjects who subsequently became HIV-1 seropositive, as compared to 2/10 subjects who remained seronegative (Figure 7.4A). All maintained responses were targeted against pools of peptides spanning the envelope protein (Figure 7.4B). The magnitude of response detected at visit one did not indicate which responses would be maintained and which would be lost at visit 2 (Figure 7.4B).

7.3.5 Epitope mapping and $CD4^+$/$CD8^+$ specificity

Where sample was available, HIV-1 specific T cell responses were mapped to the peptide level using a three dimension matrix mapping approach as previously described (Chapter 2 Section 2.2.2.3). In addition, intracellular cytokine staining (ICS) was performed to examine whether responses were mediated by $CD4^+$ or $CD8^+$ T cells. In total 6 subjects were mapped, 4 of whom subsequently seroconverted (Table 7.2 B) and 2 who remained HIV-1 seronegative for the duration of the study

(Table 7.2 A). Interestingly, and in contrast to previous findings, 4/6 responses mapped to a single peptide (Env 253-269) (Table 7.2) and were found to be mediated by $CD8^+$ T cells. Two of the six subjects studied had $CD4^+$ T cell responses, which mapped to previously defined, promiscuous, $CD4^+$ epitopes, (Table 7.2). The numbers of subjects studied by ICS was too low to perform any form of statistical correlation between the detection of either $CD4^+/CD8^+$ T cell responses and risk of HIV-1 seroconversion.

7.3.6 Impact of HIV-1 infection

Post infection samples, from within the first year of infection were available for 3 subjects with detectable HIV-1 specific T cell responses. To establish whether pre-existing, HIV-1 specific T cell responses were maintained or lost, *ex vivo* and cultured ICS assays were performed. Interestingly, HIV-1 specific T cell responses were maintained in 2/3 subjects tested, subject 5153123 (Figure 7.5A) had polyfunctional IL-2^+ IFN-γ^+ response to the peptide, whilst subject 1744107 had a monofunctional IL-2^+ response (Figure 7.5C). There was insufficient sample to perform detailed profiling of immunodominance. However, *ex vivo* analysis of IFN-γ response to peptide pools spanning each HIV-1 protein, showed that the mapped, pre-existing T cell responses were not immunodominant (Figure 7.5).

7.4 Discussion

The detection of HIV-1 specific T cell responses has long been considered as indicative of protective immunity. However, not all seronegative individuals at high risk of HIV-1 infection have detectable HIV-1 specific T cell responses[134,155]. Primate vaccine studies have shown broad, robust, polyfunctional T cell responses are required to induce protective immunity[389,504,614]. Yet, where detected, HIV-1 specific T cell responses in HESN tend to be weak and often transient[135,152,155]. Moreover, seroconversion has been observed in individuals with pre-existing HIV-1 specific T cell responses[152]. The hypothesized role as correlates of protection is therefore somewhat perplexing. The correlation of magnitude and maintenance of response with exposure to HIV-1[150,152] would suggest that where detected, HIV-1 specific T cell responses are more likely to represent markers of exposure.

Determining the impact, if any, that pre-existing HIV-1 specific T cell responses have upon the acquisition of HIV-1 is of particular relevance to vaccine design. Whilst the mechanism is not clearly defined, HIV-1 has been shown to preferentially infect HIV-1 specific $CD4^+$ T cells[368]. Therefore, detection of pre-existing HIV-1 specific T cell responses may actually serve as a reservoir of potential HIV-1 targets which increase an individual's risk of acquiring HIV-1 infection. Furthermore, we showed in Chapter 6 that not all HIV-1 specific T cell responses may have been primed by HIV-1. Cross reactive T cell responses have predominately been associated with negative clinical outcomes, whether that be more severe immunopathology[554,628,629] or even autoimmunity[512,558,561,563,567,586,630]. It is difficult to discern whether the predominance of reports of negative associations between cross reactive T cell responses and immunopathology are the result of research bias. Given Mason's original calculations[262] and observations made in Chapter 6, that low level cross reactivity is a relatively common, it would seem unlikely that cross reactivity is almost exclusively associated with negative clinical outcome. Furthermore, many vaccine strategies have induced HIV-1 specific T cell responses without increasing the risk of HIV-1 acquisition[220-222,224,381,631,632].

This study was designed to directly assess the role, if any, that pre-existing HIV-1 specific T cell responses play in conferring protection against HIV-1. The study was

powered (Table 7.1) to detect a 3 fold increased risk of HIV-1 infection in the presence of pre-existing HIV-1 specific T cell responses. In a blinded, cross sectional study 87 high risk, MSM subjects enrolled in the placebo arm of VAX004 were screened for HIV-1 specific T cell responses using the cultured IFN-γ ELISpot. HIV-1 specific T cell responses could be detected in 36.8% of donors whom subsequently became HIV-1 infected compared to 20.4% of subjects who remained seronegative (Figure 7.1). These data suggest a trend, towards increased frequency of detection of HIV-1 specific T cell responses in individuals whom subsequently become HIV-1 positive yet failed to reach statistical significance.

HIV-1 specific T cell responses detected amongst participants whom subsequently seroconverted were of significantly higher magnitude than those detected in individuals who remained seronegative for the duration of study (Figure 7.2). Furthermore, in a subset of subjects studied across two study visits (215-501 days apart) HIV-1 specific T cell responses appeared better maintained amongst subjects who subsequently seroconverted than those who remained seronegative for the duration of study (Table 7.2), although study number was low for these analyses and further study is required to validate this observation.

Unfortunately the receipt of samples from each group was made in two independent shipments. To exclude the possibility that observed differences between the two groups were a result of compromised sample integrity during shipment, *ex vivo* assessments of viability, cell recovery and response to positive control antigen were made. Reassuringly, no significant differences were observed between the two groups, suggesting that sample integrity had not been compromised during shipment (Figure 7.3).

For a limited number of subjects (n=6) mapping studies were performed. Surprisingly, and in contrast to observations made in Chapter 5, we found that 4/6 subjects responded to a single peptide (Table 7.2). Moreover, the response to this peptide was shown to be mediated by $CD8^+$ T cells (Table 7.2). The mapped peptide had been reported previously in natural cases of HIV-1 infection, but did not contain epitopes known to be immunodominant in natural HIV-1 infection[633]. Blast searches did not

reveal any sequence homology to pathogens other than HIV-1 despite, a previous study suggesting similarity to the human lymphatic endothelium-specific hyaluronan receptor LYVE-1 fragment (Table 7.2)[633]. LYVE-1 is an integral membrane polypeptide that is capable of binding both soluble and immobilized extracellular matrix glycosaminoglycan hyaluronan (HA)[634]. Unlike CD44, which is the main HA receptor, LYVE-1 is believed to have a role in the immobilization of HA in the vessel lumen rather than acting as a positional cue for cell migration[634,635]. Further study is required to confirm whether or not HIV-1 specific T cells responding to the Env 253-269 epitope are capable of cross reacting with the LYVE-1 receptor. It is most likely that the detection of 4/6 subjects responding to a single envelope peptide is a statistical anomaly, highlighting the requirement for large, statistically powered studies for the analysis of HIV-1 specific T cell responses amongst HESN.

Although, primarily designed to induce neutralizing antibody protection against the establishment of HIV-1, the VAX004 trial showed that vaccination induced HIV-1 specific T cell responses[221]. HIV-1 specific T cell proliferative responses were detected in 17.2% of vaccine recipients, as compared to just 1.6% of placebo volunteers[221]. However, in agreement with the findings made here, HIV-1 specific T cell responses were detected at higher frequencies amongst individuals whom subsequently seroconverted as compared to those who remained seronegative (34.8% compared to 10.9%), and were primarily mediated by $CD8^+$ T cells[221]. It is interesting to speculate whether, vaccination, in addition to stimulating *de novo* T cell responses, may have also expanded pre-existing HIV-1 specific T cell responses. Detailed mapping experiments, and examination of pre-vaccination samples by cultured IFN-γ ELISpot, would provide greater insight as to whether pre-existing T cell responses were "boosted" upon vaccination.

The higher frequency with which $CD8^+$ T cell responses were detected amongst this cohort, compared to the cohorts studied in Chapters 5 and 6, is likely to reflect the relative risk of HIV-1 infection. The VAX004 cohort had a seroconversion rate of 7% amongst placebo subjects, suggesting that donors were at extremely high risk of HIV-1[220]. Unlike subjects enrolled in the GALVIN cohort (Chapter 5), who were at risk from heterosexual exposure, penile to anal transmission of HIV-1 is known to have a

higher risk of infectivity than penile to vaginal transmission[30,31,34]. Furthermore, whilst the MSM serodiscordant couples enrolled in the CHAVI002 cohort were extremely well-defined, the risk of exposure to HIV-1 was relatively low, owing to the widespread use of anti-retroviral therapy. Vaccination and murine studies have suggested that $CD4^+$ T cell responses require lower activation thresholds than $CD8^+$ T cell responses[247,309]. Therefore, detection of $CD4^+$ T cell responses may be indicative of lower risk encounters with HIV-1 whilst $CD8^+$ T cell responses depict higher risk exposures. Arguably the findings presented herein, highlight the necessity to study large numbers of well-defined HESN subjects in order to gain a more comprehensive understanding of the frequencies/associated risk behaviours leading to detection of $CD4^+$/$CD8^+$ HIV-1 specific T cell responses.

The availability of sample at post HIV-1 infection time points for a limited number of subjects enabled the fate of pre-existing, HIV-1 specific T cell responses to be examined. We predicted the rapid evolution of viral escape mutations[38,72,587] would mean that pre-existing HIV-1 specific T cell responses would be rapidly lost upon HIV-1 seroconversion. However, mapped pre-existing $CD8^+$ HIV-1 specific T cell responses were still detectable one year post infection in 2/3 subjects tested (Figure 7.5). Unlike murine studies, where cross reactive T cell responses have been shown to preferentially expand upon heterologous challenge[590], mapped pre-existing, HIV-1 specific T cell responses did not appear to be immunodominant. The number of subjects studied is low and further study is required to draw accurate conclusions as to the maintenance of pre-existing HIV-1 specific T cell responses in light of subsequent infection.

Clinical data regarding the viral set point, or progression status of HIV-1 infected donors was sadly unavailable. Whilst pre-existing T cell responses appear unable to confer protection against establishment of HIV-1, it remains to be seen what impact they have upon clinical progression. It is possible that heterologous immunity and HIV-1 primed T cell responses, may account for some of the heterogeneity within HIV-1 infection.

In summary, this chapter presents a novel, statistically powered study aimed at directly assessing the impact, if any pre-existing HIV-1 specific T cell responses have

upon acquisition of HIV-1 infection. The results suggest that whether the result of exposure or cross reactivity, pre-existing HIV-1 specific T cell responses do not appear to confer an increased risk of HIV-1 infection. In a smaller, hypothesis generating study of a subset of participants we show, pre-existing HIV-1 specific T cell responses persisted in 2/3 subjects following seroconversion. The impact, if any they have upon clinical progression of HIV-1 infection remains unknown and is an area that warrants further study.

No. of HIV-1 infected Subjects	p0	Fold Increase in Relative Risk
20	0.05 0.1 0.2 0.3	12.0 8.5 6.8 6.5
30	0.05 0.1 0.2 0.3	8.5 6.0 4.8 4.5
40	0.05 0.1 0.2 **0.3**	6.8 4.9 3.9 **3.7**
50	0.05 0.1 0.2 0.3	5.8 4.3 3.4 3.2
100	0.05 0.1 0.2 0.3	3.8 2.9 2.4 2.3

Table 7.1

Power calculations performed by Dr P Gilbert (Statistical centre for HIV/AIDS research centre (SCHARP)) showed that the study of 200 individuals (100 infected, 100 uninfected) are required to detect a 2 fold increased risk of HIV-1 infection, due to pre-existing, $CD4^+$, HIV-1 specific T cell responses (blue text). Due to sample constraints, and a belief that it is likely multiple factors contribute to protection against HIV-1 acquisition, 40 individuals who became HIV-1 infected were compared with equal numbers of subjects who remained seronegative. The screening of a total of 80 subjects, will achieve 80% power to detect a 3-4 fold increase in risk of HIV-1 infection, amongst participants with detectable HIV-1 specific T cell responses (red text). Column one of this table, lists the number of subjects per group to be studied. Column 2 gives the percentage of never infected placebo recipients that have a detectable HIV-1 specific T cell response (p0), and the third column gives the smallest, detectable, relative risk of HIV acquisition.

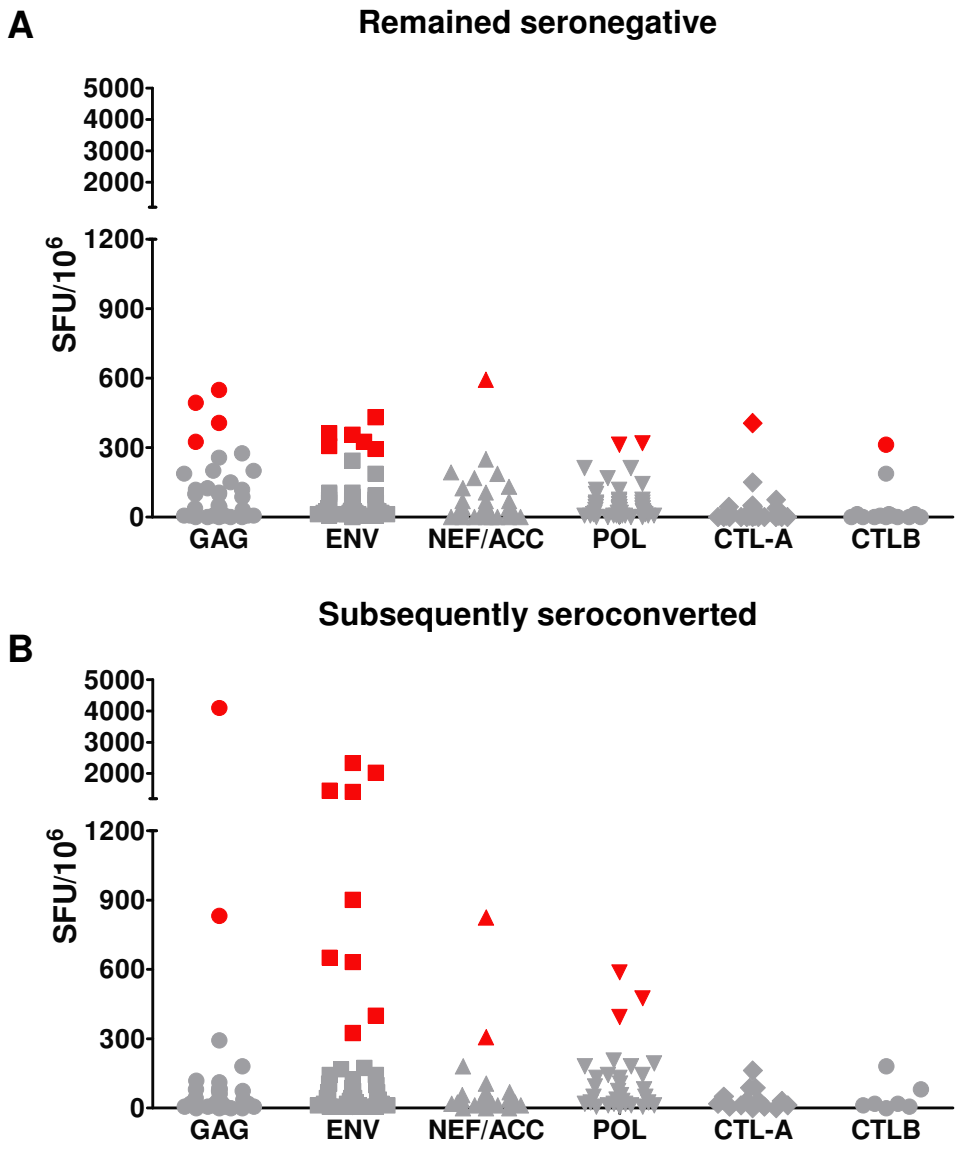


Figure 7.1

Cultured IFN-γ ELISpot was used to screen 87 HIV-1 seronegative, MSM, subjects at high risk of HIV-1 infection enrolled in the placebo arm of VAX004 trial. Of the 87 subjects studied, 49 remained HIV-1 seronegative for the duration of study (A) and 38 subsequently seronconverted (B). Overlapping 18mer, clade C consensus peptides were pooled according to protein with nef/acc pool comprising nef and accessory proteins, (vif, vpr, vpu, tat and rev). Two additional pools containing previously defined, optimal, CTL epitopes (CTLA and CTLB) were included to ensure that the assay was not biased towards detection of $CD4^+$ responses. HIV-1 specific T cell responses were detected in 1/5 subjects who remained seronegative (10/49) (A) and 1 in 3 subjects who subsequently seroconverted (14/38) (B). Data presented as the average number of spot forming units per million cells ($SFU/10^6$). Positive responses, shown in red, were defined as greater than 300 $SFU/10^6$ and >4x background. All peptides were used at a final concentration of 2μg/ml.

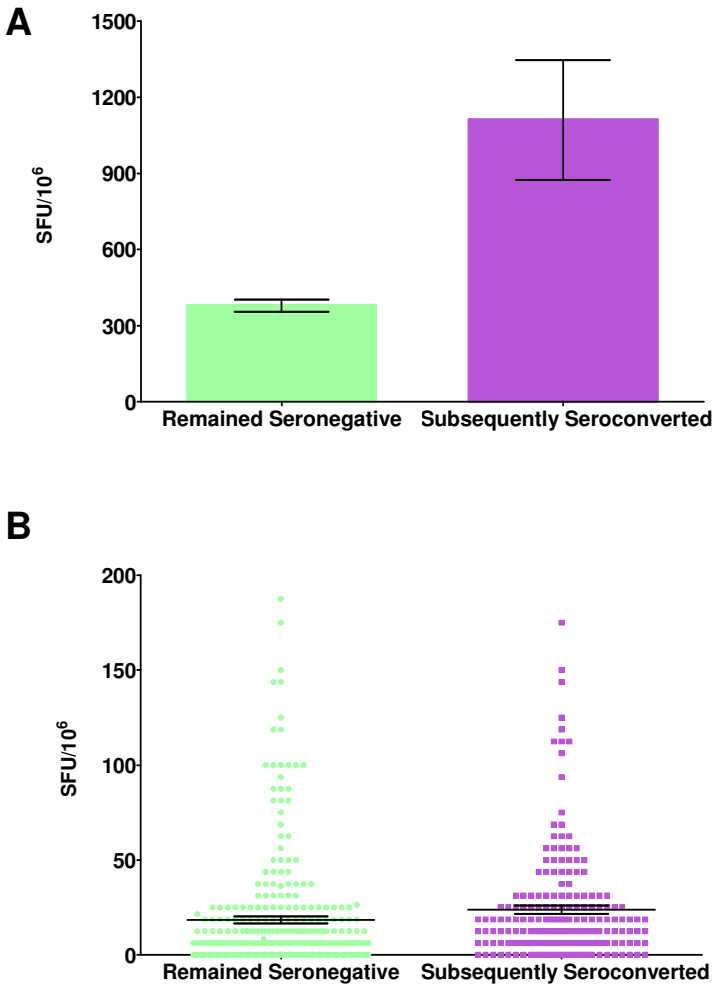


Figure 7.2

The magnitude of HIV-1 specific T cell responses detected amongst seronegative donors, were significantly higher in subjects whom subsequently became HIV-1 positive (p=0.002;A). Data presented as the average number of spot forming units per million cells (SFU/106) following background subtraction. Only subjects with detectable HIV-1 specific T cell responses were included in the analysis. Criteria for a positive response was >300 SFU/10^6 and greater than 4x background. Notably background IFN-γ production (B) did not significantly vary between groups (p=>0.05).

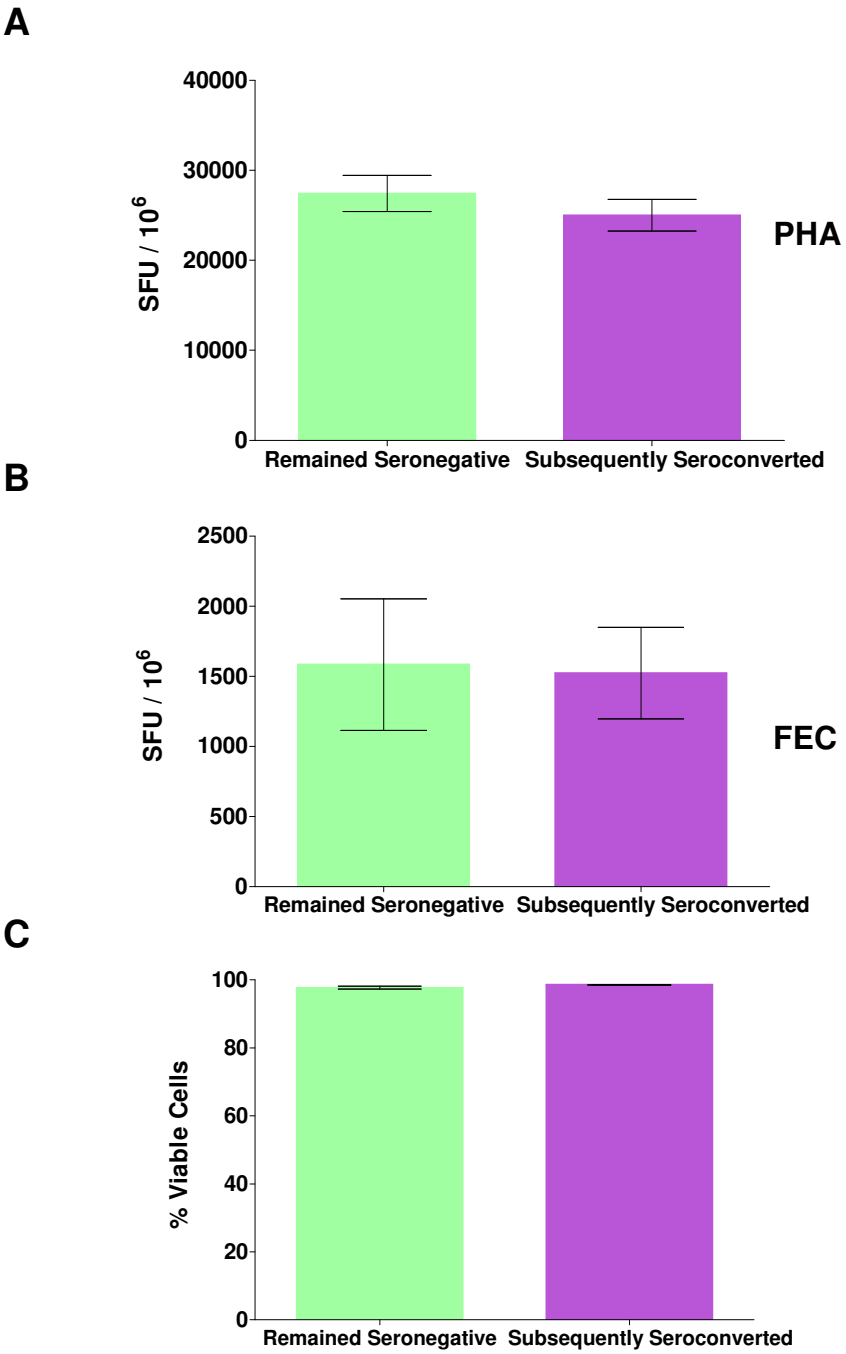
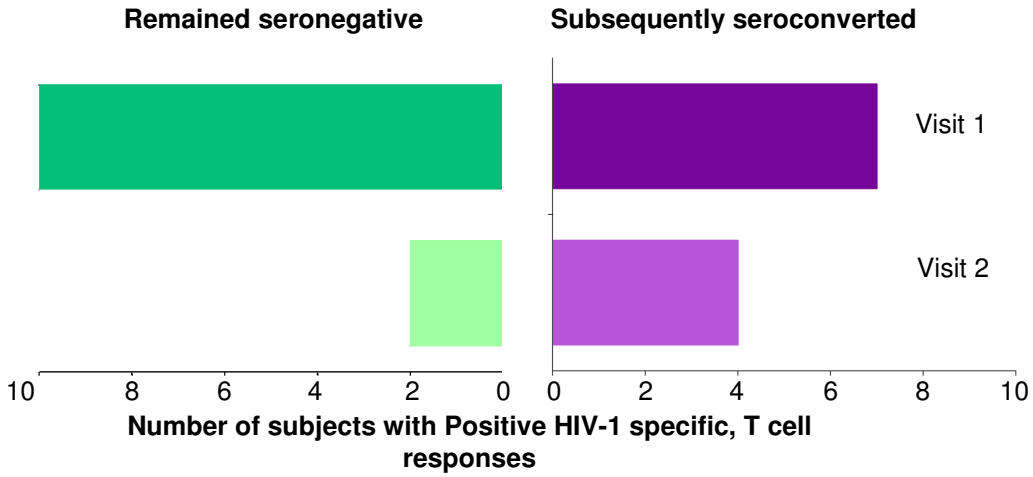


Figure 7.3

Samples from each group were received in independent shipments. To ensure the differences observed between each group were not a result of compromised sample quality during shipment, *ex vivo* assessment of mitogenic positive control antigen PHA (A) and peptide control of common flu, Epstein Barr Virus and cytomegalovirus virus epitopes (FEC) were made (B). No significant difference in the magnitude of positive control antigens (A-B) or viability was detected between each group. Furthermore, Trypan blue exclusion was used to assess sample viability upon revival of cryopreserved cells for each shipment (C). Again no significant difference was detected in sample viability. Together these observations suggest sample quality had not been compromised during shipment. FEC peptide set was used at a final concentration of 2µg/ml, and PHA at 5µg/ml.

A



B

PTID	Positive Pool	Magnitude (SFU/10^6)	Maintained Visit 2?
122232	GAG	>500	NO
1420249	GAG	>300	NO
4627200	GAG	>400	NO
838382	NEF/ACC	>500	NO
3209665	POL	>300	NO
5647112	ENV	>300	NO
318501	ENV	>300	YES
2622614	ENV	>300	NO
341834	ENV	>400	YES
	CTLB	>300	NO
5930992	GAG	>400	NO
	ENV	>300	NO
	POL	>300	NO
	CTLA	>400	NO

PTID	Positive Pool	Magnitude (SFU/10^6)	Maintained at visit 2
304243	ENV	>900	YES
550329	ENV	>2000	NO
851794	ENV	>600	NO
1744107	ENV	>300	YES
749448	ENV	>2000	YES
1914236	POL	>400	NO
5153123	ENV	>1400	YES

Figure 7.4

The maintenance of HIV-1 specific T cell responses was assessed between subjects whom subsequently became HIV-1 infected and those who remained seronegative. Of the subjects who remained seronegative 2/10 maintained HIV-1 specific T cell responses across the two study visits tested (A, left hand panel). This was in contrast to subjects whom subsequently seroconverted, where 4/7 maintained T cell responses (A, right hand panel). Notably all maintained responses were targeted towards envelope, and the magnitude of response at visit one did not predict which responses were maintained (B). Positive T cell responses were defined as greater than 300 spot forming units per million cells (SFU/10^6) and greater than 4x background. All peptides were used at a final concentration of 2µg/ml.

A

PTID	*Ex Vivo* IFN-γ ELISpot	Culture IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide information Published sequences aligned to mapped sequence	Details of previously published sequences
318501	Not Done	**Env 838 – 855** >400 SFU/10^6	**$CD4^+$** **Magnitude:** 3.74%	**RAIRNIPRRIRQGFEAAL** ----------QGLER H-------LERILL AVAEGTDRVIEVVQGAY----------LER	Shown to be a multiderminant region of HIV-1. In murine study mice of 3 different MHC haplotypes[636]. Show to induce a proliferative response amongst HIV-1 seropositive individuals[637]. This epitope was shown to elicit IL-2 responses in 1 / 12 asymptomatic HIV-1 seropositive individuals[237]
341834	Not Done	**Env 253-269** >500 SFU/10^6	**$CD8^+$** **Magnitude:** 2.89%	**PVVSTQLLLNGSLAEEEI** ---------- K--------L R--------L	Epitope suggested to have similarity with the lymphatic endothelium-specific hyaluronan receptor (LYVE-1) fragment TTRLLVQGSLRAEE[633]. Bioinformatics predict this epitope has 46% binding probability for promiscuous presentation by B08 and B07[638]. Conserved sequence promiscuous recognition by BO8 and B07 individuals[638]

B

PTID	*Ex Vivo* IFN-γ ELISpot	Culture IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide information	
				Published sequences aligned to mapped peptide	**Details of previously published sequences**
304243	Negative	**Env 34-51** >300 SFU/10^6	**$CD4^+$ Magnitude:** 3.49%	**LWVTVYYGVPVWKEAKTT** ATEK---------- TEK----------	2/15 asymptomatic HIV-1^+ subjects responded to this peptide[376] Epitope shown to bind HLADR04 and HLADR02 with IC50 values of 4 and 22nM respectively. T cell stimulatory and detected in 2 of 15 asymptomatic HIV-1 seropositive subjects[376]
1744107	Negative	**Env 253-269** 300 SFU/10^6	**$CD8^+$ Magnitude:** 0.11%	**PVVSTQLLLNGSLAEEEI** ----------	Epitope suggested to have similarity with the lymphatic endothelium-specific hyaluronan receptor (LYVE-1) fragment TTRLLVQGSLRAEE[633]. Bioinformatics predict this epitope has 46% binding probability for promiscuous presentation by B08 and B07[638]. Conserved sequence promiscuous recognition by BO8 and B07 individuals[638]
749448	Negative	**Env 253-269** >1300 SFU/10^6	**$CD8^+$ Magnitude:** 2.39%	K--------L	
5153123	Negative	**Env 253-269** >400 SFU/10^6	**$CD8^+$ Magnitude:** 1.84%	R--------L	

Table 7.2

Maintained, HIV-1 specific, T cell responses from 6 HIV-1 seronegative subjects (2 who remained seronegative for the duration of study (A) and 4 who subsequently seroconverted (B)), were mapped to the peptide level and characterized as $CD4^+$ or $CD8^+$. 4/6 subjects mapped had a $CD8^+$ T cell response to the same peptide (Env 253-269). ELISpot responses presented as the average number of spot forming units per million cells (SFU/10^6). Criteria for a positive response was >50 SFU/10^6 (*ex vivo*) or >300SFU/10^6 (cultured) and >4x background. The magnitude of IFN-γ response detected by ICS is given as percentage of $CD4^+$/$CD8^+$ cells that were IFN-γ^+ criteria for a positive ICS response was >0.05% and 2x background. All data presented is background subtracted. Peptides used at a final concentration of 2μg/ml. LANL, HIV-1 immunology database, was used to search for previously published sequences contained within the mapped peptide[404].

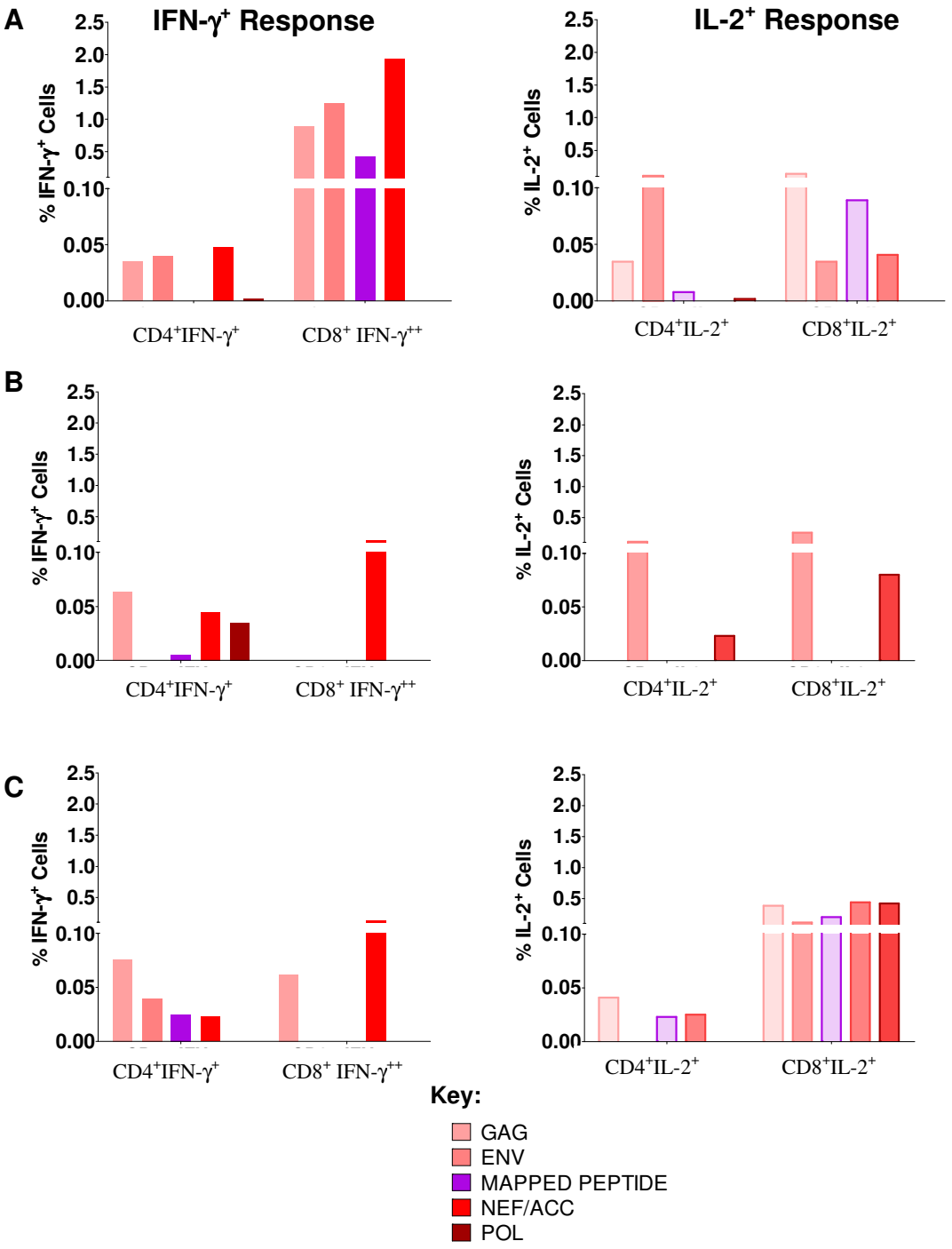


Figure 7.5

Following 6 hour peptide/mock stimulation, post infection samples from 3 VAX004 subjects (A-C) were screened using ICS to assess whether mapped, pre-existing HIV-1 specific T cell responses (represented with purple bar) persisted post HIV-1 infection. One of 3 subjects maintained a $CD8^+$ $IFN\text{-}\gamma^+IL\text{-}2^+$ response to the mapped peptide and a second showed a $CD8^+IL\text{-}2^+$ response. The pre-existing $CD8^+$ T cell response was not shown to be immunodominant in either of the 2 subjects who maintained responses post infection. Criteria for a positive response was >0.05% and 2x background. All data presented is background subtracted.

CHAPTER 8:
DISCUSSION

Chapter 8: Discussion

Human Immunodeficiency virus-1 (HIV-1) remains a global pandemic, with 2.6 million new cases diagnosed each year[84]. Despite more than 20 years research a prophylactic vaccine remains elusive. Intriguingly there are a minority of individuals who, despite multiple high-risk exposures to HIV-1 remain seronegative[46,134,135,144,147-149,155,157,158,163,166,189,191,200,296,384,493,494,500-502]. Previous reports have shown that a proportion of HIV-1 exposed seronegative (HESN) individuals harbour pre-existing HIV-1 specific T cell responses[135,136,145,147,148,150-152,156,158,295,382-384,492] leading to speculation that adaptive immunity may play a role in conferring protection against the establishment of HIV-1.

There is considerable evidence from both human[16,76,503] and primate studies[358,504,505] to suggest that HIV-1 specific T cell responses play a critical role in controlling viremia following establishment of productive HIV-1 infection. However, the extension of these observations to correlates of protection against the establishment of HIV-1 has been met with some scepticism. The controversy surrounding this topic has arisen from the fact that not all studies have been able to detect pre-existing HIV-1 specific T cell responses amongst HESN[134,155,499,501]. Moreover, where detected pre-existing HIV-1 specific T cell responses have tended to be weak and often transient[135,152,155] and seroconversion has been shown to occur despite detection of pre-existing T cell responses[152]. It is often difficult to justify the clinical relevance of pre-existing T cell responses amongst HESN owing to the fact that many studies have failed to include appropriate controls[134,135,137,145,150,155,157].

The primary focus of this thesis was to address some of the controversy surrounding the detection of HIV-1 specific T cell responses amongst HESN. Based on the body of literature surrounding this topic, three main points were identified. Firstly, the absolute requirement for accurately defined HESN subjects; secondly, the methods used to screen for pre-existing HIV-1 specific T cell responses needed to be robust, specific and importantly, highly sensitive; Thirdly, the pre-requisite for carefully controlled studies. This final chapter will provide discussion on how each of these

three points were addressed, and presents the key findings of this thesis, together with a broader discussion as to how these findings may help in the design of a novel therapeutic vaccine.

8.1 Addressing the three main limitations of previous HESN studies

8.1.1 Defining a HESN

It is unlikely that all individuals enrolled as "HESN" will have truly been exposed to HIV-1. HIV-1 is predicted to have a relatively low transmission rate, estimated at just 1 infection per 1000 contacts for heterosexual transmission[29,623]. A number of risk factors can associate to increase this to 1 infection per 3 contacts depending on the type of sex act, route of transmission and the presence of other sexually transmitted diseases[29-31,34,37,624]. Given these transmission rates, it is possible that some HESN subjects may have simply been "lucky", avoiding productive HIV-1 infection due to stochastic events. However, in order to accurately detect factors associated with "resistance" to HIV-1 it would seem it would seem critical to have an extremely well-defined, large cohort of HESN subjects where the risk of HIV-1 can be quantified.

To tackle this issue, two independent cohorts of HESN subjects were examined. Preliminary observations were made in a retrospective Malawian cohort (GALVIN Cohort) of sexually transmitted infection (STI) clinic attendees. HESN subjects were loosely defined as STI clinic attendees harbouring 1 or more STI in a clinical setting where HIV-1 has a prevalence of 47% (Chapter 2 Section 2.1.1.1). This cohort represents many of those previously studied, in that risk of exposure to HIV-1 is merely implied rather than the sero-status of sexual partners being known. Observations made within the GALVIN cohort directed studies in a much larger, HESN cohort, referred to as CHAVI002. This was a specifically designed, powered study, blinded at the laboratory level. The recruitment of serodiscordant couples, meant exposure to HIV-1 and therefore risk of HIV-1 infection could be quantified through detailed sexual behaviour questionnaires. Inclusion criteria for HESN subjects were individuals who self-reported unprotected sex with a HIV-1 positive partner on at least 25 occasions in the last 12 months (Chapter 2 Section 2.1.1.4). Whilst extremely well-defined, this cohort was not without its limitations. Firstly its reliance on self-reported high risk behaviours, and secondly the inability to quantify risk from sexual exposures occurring outside of the primary relationship. The main

critique of this cohort is the relative risk of exposure to HIV-1. The widespread use of antiretroviral therapy (ART) amongst HIV-1 infected partners meant that 88% had viral loads of <50 copies per ml. Large, community based studies of heterosexual couples, in Uganda have shown viral load to be a chief determinant in the risk of transmission of HIV-1[29,36], with transmission probability increased from 0.0001 per act, at viral loads of less than 1700copies/ml to 0.0023 per act at viral loads of greater than 38,500 copies per ml[29,36]. It is highly unlikely the transmission rate within CHAVI002 HESN was as low as the 0.0001 calculated within the Ugandan studies[29,36], since the route of transmission within CHAVI002 was penile to anal, which has significantly higher risk than penile to vaginal exposure[30,32]. However, the extremely low viral load of HIV-1 infected partners suggests that whilst extremely well-defined, the actual risk of exposure to HIV-1 amongst CHAVI002 HESN was relatively low. The relatively "low risk" of exposure to HIV-1 amongst HESN is not restricted to the CHAVI002 cohort. With ethical guidelines now in place as to initiation of ART and counselling to educate study participants in ways to minimize their risk of HIV-1 exposure, the true "risk" amongst many HESN cohorts is likely to be considerably lower than it was in original HESN cohorts where ART use was less widespread[154,386,489]. The detailed sexual behaviour questionnaires and strict enrolment criteria for CHAVI002 HESN subjects, therefore represents the most robust means available by which to quantifiably study HESN subjects.

A number of individuals have been shown to be capable of controlling HIV-1 viremia to near undetectable levels for several months prior to seronconversion[506,639]. A lack of sensitivity, particularly in early clinical diagnostics, meant that these subjects were often falsely included in HESN cohorts[489,639]. A recent study by Zhu *et al*, found that 2/10 high risk, homosexual male subjects, with detectable $CD8^+$ HIV-1 specific T cell responses, had very low levels of HIV-1 DNA (mean, 0.05 ± 0.01 per million cells) in resting $CD4^+$ T cells, isolated across multiple time points yet maintained negative serology[506]. Transient or persistent low level HIV-1 replication in the absence of seroconversion could be considered to reflect particularly effective cellular immune responses, with the host seemingly restricting viral dissemination. The identification of transient or low level HIV-1 infection in HESN donors is therefore potentially of benefit in the search for "correlates of protection" as well as ensuring an accurate description/enrolment of HESN subjects. Whilst the study by Zhu *et al*[506], is very

interesting, a more recent publication by Delwart *et al* suggests that detection of low level viral RNA/DNA is extremely rare amongst HESN cohorts[139]. To address whether low level RNA/DNA could be detected amongst HESN subjects enrolled in this study, a highly sensitive isothermal transcription mediated amplification (TMA) assay, capable of detecting viral loads of just 3.6 to 14 copies of RNA/ml[401,402], was used to screen all CHAVI002 and a subset of GALVIN subjects. As may have been predicted from the Delwart *et al,* study[139], all subjects were negative for low titre viral RNA (Appendix 1). The obvious caveat here is that the study relied upon plasma samples, whilst typically the primary site of exposure to HIV-1 is the genital mucosa (Reviewed in [218]). Indeed, the transmission of HIV-1 at the mucosal surface precedes primary HIV-1 infection and offers a potential "window of opportunity" for preventing primary infection[218]. Therefore the failure of this study to detect, HIV-1 RNA (Chapter 5 Section 5.4.5 and appendix 1) in the plasma of HESN subjects, does not exclude occult/abortive infection at the mucosa and further study is required to examine this possibility.

Given that this study was specifically aimed at addressing the role, if any, pre-existing HIV-1 specific T cell responses had upon the acquisition of HIV-1, it seemed important to identify HESN subjects for whom, "protection" could not be explained by genetic factors. All subjects enrolled in the CHAVI002 cohort and a subset of donors from the GALVIN cohort were screened for the presence of a 32base pair deletion in the HIV-1 co-receptor CCR5, using standard PCR visualised on a 2% agarose gel (Figure 5.8). The homozygous 32base pair deletion in CCR5 was first described as a correlate of protection in 1996[45-47] and has reproducibly been shown to afford HESN subjects almost complete resistance from HIV-1 infection[46-48,493]. In accordance with published allele frequencies[47,495], only 2/38 Caucasian subjects were homozygous for the CCR5Δ32 allele, whilst none on the non-Caucasian subjects tested had the mutation. Of note the two HESN subjects found to be homozygous for CCR5Δ32, 1 had detectable HIV-1 specific T cell responses and one did not. The apparent resistance to HIV-1 infection, demonstrated amongst the majority of HESN donors studied herein could not therefore be attributed to CCR5Δ32 mutation.

8.1.2 A robust, reproducible and sensitive detection method

Satisfied that the HESN subjects were as accurately defined as possible, the next point to address was the detection of HIV-1 specific T cell responses amongst HESN. Where detected, HIV-1 specific T cell responses in HESN donors have tended to be weak and transient[135,152,155], suggesting that some T cell responses may fall below the limits of detection by conventional assays. This may help explain why not all studies of HESN have been able to detect pre-existing HIV-1 specific T cell responses[134,155,499,501]. To address this issue, a modified version of the *ex vivo* ELISpot was standardized for use as a highly sensitive, reproducible method by which to specifically improve the detection of low frequency and magnitude T cell responses (Chapter 3). Referred to as the cultured IFN-γ ELISpot assay, this technique combines a brief, *in vitro*, peptide stimulated, culture period prior to the conventional ELISpot protocol. During culture, peripheral blood mononuclear cells (PBMC) are supplemented with anti-apoptotic and proliferative cytokines (Interleukin (IL)-7 and IL-2 respectively)[391]. The culture period enables not only T cells present to divide but also permits the differentiation of resting central memory T cells into effector cells[475,479,485]. The cultured ELISpot technique has been applied to malaria research[479,481,485] and to past phase 1 clinical HIV-1 vaccine trials[81,391], but has yet to be applied to HESN studies.

The detection of latent *Mycobacterium tuberculosis* (LTBI) infection was used as a setting in which to standardize the cultured ELISpot for the detection of low frequency and magnitude T cell responses (Chapter 4). Amongst both HIV-1 seronegative and seropositive individuals, the cultured IFN-γ ELISpot was shown to reproducibly (Figure 4.4) increase the magnitude of antigen specific T cell responses >20fold (Figure 4.2) compared to *ex vivo* ELISpot. Importantly the improved sensitivity did not appear to compromise the specificity of the assay (Figure 4.3) and the cultured ELISpot technique could be successfully combined with matrix mapping approaches and intracellular cytokine staining to provide detailed functional and phenotypic data (Figure 4.5).

To overcome limitations associated with previous HESN studies, which have tended to focus upon the restricted examination of T cell responses to a single HIV-1 protein,

typically gag or env[137,138,150,163,382], the present study used a comprehensive approach, combining the cultured IFN-γ ELISpot with overlapping pools of peptides spanning the entire HIV-1 proteome. This enabled a highly sensitive and thorough assessment of the breadth and magnitude of HIV-1 specific T cell responses to be made. Peptides were synthesized as 18 amino acids (aa) overlapping by 11aa, using consensus sequences obtained from the Los Alamos National Database[20,21]. In order to try and minimize the bias of results towards $CD4^+$ epitopes, which typically recognize longer peptides than $CD8^+$ T cells, two pools of previously defined[20,21], optimal $CD8^+$ T cell epitopes were included in each assay.

The low cell number requirements, ability to generate functional and phenotypic data, together with the relative ease of screening large cohorts against multiple peptide pools, suggests that the cultured IFN-γ ELISpot could prove a useful and attractive tool for large scale clinical and/or vaccine trials. However, the clinical relevance of antigen specific T cell responses detected by cultured IFN-γ ELISpot remains to be determined. Some studies with malaria vaccines suggest that low frequency T cell responses detected by cultured IFN-γ ELISpot at the day of challenge correlate with vaccine protection more accurately than *ex vivo* responses[479]. Further study, ideally using primate challenge models or viral inhibition assays, is now required to ascertain whether detection of HIV-1 specific T cell responses by cultured IFN-γ ELISpot can be correlated with any form of immune protection.

Whilst undoubtedly, the cultured IFN-γ ELISpot improved the sensitivity of detection of low frequency and magnitude T cell responses, it is not without its limitations. Perhaps the biggest criticism is the reliance on *in vitro* culture, which makes it difficult to fully exclude *in vitro* priming. However, *in vitro* priming typically requires repeated rounds of antigen stimulation and the presence of exogenous antigen presenting cells (APCs), neither of which were included in the cultured IFN-γ ELISpot protocol. Although this alone does not exclude *in vitro* priming since, there is evidence to suggest, particularly under *in vitro* culture conditions, that T cells themselves are capable of antigen presentation[239,513]. Further evidence to suggest *in vitro* priming is unlikely, comes from the fact that antigen specific T cell responses could be mapped to the peptide level, and reproducibly demonstrated across multiple

study visits and multiple vials from the same study visit. Should antigen specific T cell responses have been the result of *in vitro* priming, it might have been suspected that detection of antigen specific T cell responses would be more sporadic and inconsistent. Moreover, HIV-1 specific T cell responses detected amongst seronegative donors mapped to epitopes previously defined within natural infection (Table 5.2-5.3, Table 7.2 and appendix 2), again adding validity to the technique. The most robust demonstration that antigen specific T cell responses detected by cultured ELISpot were not the result of *in vitro* priming comes from work presented in Chapter 7. *In vitro* primed responses would have originated from the naïve subsets. However, cell sorting of bulk $CD4^+$ T cells into naïve and memory subsets prior to culture revealed HIV-1 specific T cell responses could be detected within the memory compartment of $CD4^+$ T cells (Figure 6.12-6.13). Future study is now planned to examine the ontogeny of antigen specific T cell responses detected by cultured IFN-γ ELISpot in more detail, using a combination of cultured IFN-γ ELISpot epitope mapping and the T cell library technique[308].

The studies presented in this thesis, exemplify how the detection of low frequency and magnitude T cell responses can be significantly improved with the aid of cultured IFN-γ ELISpot. However, it is unlikely that the cultured IFN-γ ELISpot is capable of achieving 100% detection. Perhaps this is best exemplified by the novel application of the cultured IFN-γ ELISpot, to the detection of latent tuberculosis infection (LTBI) (Chapter 4). The absence of a gold standard for the detection of LTBI makes it difficult to assess whether the cultured ELISpot was able to accurately detect all individuals with LTBI. However, whilst cultured IFN-γ ELISpot improved the sensitivity of LTBI detection from 4-31.8% amongst HIV-1 seropositive individuals, this was still somewhat lower than the frequency of detection (65%) amongst seronegative individuals from the same region, suggesting that there is still scope for improvement (Figure 4.2). Furthermore, the T cell library technique[308], which relied solely upon proliferative response to peptide, was able to detect considerably more HIV-1 specific T cell responses (Figure 6.9-6.11) than the cultured IFN-γ ELISpot (Figure 6.15). These data suggest that there are likely to be a number of antigen specific T cell responses that fall below the limits of detection afforded by cultured IFN-γ ELISpot. However, the T cell library technique itself is laborious, costly and

requires high input cell numbers. It is also more at risk of inducing *in vitro* priming owing to the inclusion of exogenous APC, and prolonged culture period (Chapter 2 Section 2.3.6.2).

The final potential criticism of the cultured IFN-γ ELISpot, is the restriction imposed by screening for antigen specific T cell responses based solely upon their ability to secrete IFN-γ and the inherent assumption, that all clinically relevant, antigen specific T cells produce IFN-γ. In natural HIV-1 infection neither the magnitude nor breadth of IFN-γ response by HIV-1 specific T cells, has been shown to correlate with viral load[373,640,641]. More recently it has been argued that rather than the quantity of HIV-1 specific T cell responses, it is the quality of the response that confers protection[75,354,364,504]. In particular, the ability of individual T cells to secrete multiple cytokines has been correlated with reduced viral loads and improved survival times[75,354,364,504]. Therefore, a more comprehensive screening method, including multiple effector functions is potentially desirable to improve detection of responses that may have been missed by IFN-γ release[75]. The advent of the "dual colour ELISpot" technique has gone some way to address this issue, enabling two effector functions to be detected simultaneously[642-646]. However, this approach requires higher input cell numbers, and has been shown to decrease the magnitude of IFN-γ responses[645]. Another option would be to use multiplex-based approach, such as Luminex. However, whilst Luminex provides a methodology by which to quantify multiple analytes from the same sample, this technology does not provide information as to whether individual cells are capable of secreting multiple cytokines. Perhaps the most comprehensive means by which to address polyfunctionality is using a flow cytometric approach, such as that employed in Chapter 3. However, flow cytometic assays again require large numbers of cells and are currently not financially viable for large scale screening. Furthermore, in the absence of a known correlate of protection, determining which effector functions to study is inherently difficult.

In summary, whilst there are some limitations to the cultured IFN-γ ELISpot technique, it currently offers the most robust, reliable and cost effective means by which to conduct large throughput studies, of low frequency and magnitude T cell responses, with relatively limited cell numbers. The ability to combine it with flow

cytometric and mapping approaches enables detailed functional and phenotypic data to be generated. Moreover, the ability to cryopreserve short-term cell lines without significant loss of sensitivity (Figure 4.6) permits a degree of flexibility in settings where sample is limited.

8.1.3 Controlled studies

With a well-defined cohort of HESN subjects and the means by which to sensitively detect low magnitude and frequency T cell responses, the final area to address was the inclusion of appropriate controls. Several HESN studies have failed to recruit low risk, control individuals[134,135,137,145,150,155,157]. Not only has this fuelled some of the scepticism surrounding detection of HIV-1 specific T cell responses amongst HESN, but it also detracts from the clinical relevance of pre-existing HIV-1 specific T cell responses. To address this issue, great care was taken in the recruitment of individuals, who self-reported low risk behaviour, were in a monogamous relationship with a concordantly seronegative partner. The stipulation of strict enrolment criteria and HIV-1 testing for HIV-1 unexposed, seronegative (HUSN) subjects, avoided potentially misguided, assumptions that laboratory workers are appropriate "low risk" controls. To further control these studies, all screening performed using HESN cohorts (Chapter 5 and 7) were blinded at the laboratory level. This helped to ensure that results were not unintentionally biased, due to pre-conceptions on how the data "should look". Together, these precautions added both validity and confidence to the dataset.

8.2 Detection of HIV-1 specific T cell responses amongst HESN

With sensitive detection methods in place and recruitment of well-defined HESN and HUSN subjects, 31 HESN and 14 HUSN subjects from the GALVIN/IAVI 016 cohorts, together with 24 HESN and 48 HUSN from the CHAVI002 cohort, were screened for pre-existing HIV-1 specific T cell responses (Chapter 5). Using the sensitive cultured IFN-γ ELISpot approach, HIV-1 specific T cell responses were detectable in a proportion of HESN donors (38.7-61% Figure 5.1, Table 5.1). Consistent with previous studies[135,152,155] HIV-1 specific T cell responses were relatively weak and often transient (Figure 5.2, 5.6-5.7, 7.4). Moreover, the frequency at which HIV-1 specific T cell responses were detected amongst HESN was in

keeping with previous literature reports[135,147,148,157,159,384]. The higher frequency at which HIV-1 specific T cell responses were detected amongst CHAVI002 (Table 5.1) subjects, as compared to GALVIN subjects (Figure 5.1) is likely to be a direct reflection upon the stringent criteria used to define HESN subjects in CHAVI002. This again highlights the importance of having well-defined cohorts of HESN. HIV-1 specific T cell responses were shown to target the entire HIV-1 proteome, with no individual protein showing immunodominance (Figure 5.3). This is perhaps surprising since it may have been predicted that the immunodominant targets of acute HIV-1 infection, such as nef or gag[373,443-445], would emerge as the immunodominant targets of HESN subjects. However, immunodominance is a complex issue, and may require immune activation for optimal antigen processing and peptide-HLA presentation.

No HIV-1 specific T cell responses were detectable using *ex vivo* ELISpot analysis (Figure 5.2). The requirement for culture underscored the low magnitude and frequency of HIV-1 specific T cell responses detected in this cohort, and perhaps goes some way to explaining why not all studies of HESN have detected HIV-1 specific T cell responses [134,155,499,501]. The requirement for culture in order to detect HIV-1 specific T cell responses amongst HESN has not been a prerequisite for all studies of HESN, and indeed many have been detected using conventional *ex vivo* ELISpot analysis[135,147,158,159,163]. The most likely explanation for why culture was required in these studies is a reflection on the relatively low risk HIV-1 exposure. Whilst extremely well-defined, the risk of HIV-1 exposure within the CHAVI002 cohort was likely to be low, owing to the use of ART by the HIV-1 seropositive partner. Moreover, the loose criteria used to define HESN recruited within the GALVIN cohort meant that exposure to HIV-1 could not be confirmed.

Importantly, HIV-1 specific T cell responses could be mapped to the peptide level (Figure 5.10, Table 5.2-5.3, Table 7.2 and Appendix 2) and titrated (Figure 5.11). The majority of pre-existing HIV-1 specific T cell responses amongst HESN donors were shown to be mediated by $CD4^+$ T cells (Figure 5.9) and mapped to epitopes previously defined in progressive HIV-1 infection (Table 5.2-5.3, Table 7.2 and Appendix 2). Interestingly, the majority of epitopes identified, had previously been defined as "promiscuous", that is to say they are capable of binding multiple HLA alleles. Whether the detection of "promiscuous" epitopes reflects a general increased

level of degeneracy within the $CD4^+$ compartment compared to the $CD8^+$ T cell compartment remains to be determined[247]. However, the inclusion of a novel pool of "promiscuous" peptides was not shown to improve detection of HIV-1 specific T cell responses (Figure 5.14).

8.3 Why were pre-existing HIV-1 specific T cell responses predominately mediated by $CD4^+$ T cells?

Previous HESN studies have detected both $CD4^{+}$[137,138,145,148,150,295,296,382] and $CD8^{+}$[135,147,151,156-158,163,203,296,383,384] T cell responses. It is therefore surprising that HIV-1 specific T cell responses detected in these studies were almost exclusively mediated by $CD4^+$ T cells. It is unlikely the culture technique or use of 18aa peptides inadvertently biased results toward $CD4^+$ T cell responses, since within the VAX004 cohort (Chapter 7), $CD8^+$ T cell responses were readily detectable. Despite the profound depletion of $CD4^+$ T cells that occurs during the acute stages of HIV-1 infection, $CD4^+$ T cell responses are readily detected during chronic HIV-1 infection (Figure 5.13)[66,133,303,363,373,414,526,647-654]. Indeed, findings presented within Chapter 3, confirm previous literature reports, showing that $CD4^+$ T cell responses can be detected in the majority of HIV-1 infected subjects, albeit at lower magnitude than $CD8^+$ T cell responses[303,327,373,374,377,640]. These data imply that successful HLA class II presentation of HIV-1 peptides does occur in productive HIV-1 infection. Furthermore, whilst the number of subjects studied was low, data obtained from 4/6 subjects with acute HIV-1 infection suggest gag specific $CD4^+$ T cell responses may precede gag-specific $CD8^+$ T cell responses (Figure 3.8,). This study was limited to gag-specific T cell responses and further study is required to see whether the patterns of divergent kinetics hold true for other HIV-1 proteins. Should $CD4^+$ T cell responses be shown to routinely precede $CD8^+$ T cell responses this may account for why $CD4^+$ T cell responses were preferentially detected amongst HESN.

The most likely hypothesis as to why $CD4^+$ T cells were preferentially detected amongst HESN, lies in the risk of HIV-1 exposure and likely innoculum dose. Although no *ex vivo* studies have directly compared the minimal peptide required for $CD4^+$ vs. $CD8^+$ T cell activation, evidence from vaccine studies, suggests $CD4^+$ T cells have a lower activation threshold compared to $CD8^+$ T cells[247,309]. In attenuated viral vector vaccine studies, $CD4^+$ T cell responses were preferentially induced[381,391]

compared to the robust $CD8^+$ T cell responses induced by more immunogenic adenovirus vectors or live attenuated vaccines[389,510]. Furthermore, in murine studies, the adoptive transfer of $CD4^+$ and $CD8^+$ T cells into neonatal, severe combined immunodeficiency mice revealed discrepancies in the stimulation threshold of memory $CD4^+$ and $CD8^+$ T cells, with memory $CD4^+$ T cells being sensitive to lower concentrations of anti-TCR-β than $CD8^+$ T cells[309]. Although once activated, $CD8^+$ T cells were shown to proliferate and produce IFN-γ at much higher levels than $CD4^+$ T cells[309]. Given the primary role of $CD8^+$ T cells as cytotoxic lymphocytes, the authors suggest that the discrepancy in activation threshold is a defence mechanism protecting the host from damage[309]. In light of the extremely low viral loads present within HIV-1 infected partners of HESN enrolled in CHAVI002, the low activation threshold of $CD4^+$ T cells provides one possible explanation for the preferential detection of $CD4^+$ T cell responses in these studies.

A further explanation may lie in the structure of the HLA class II molecule itself. Unlike HLA class 1 molecules, the peptide binding groove of HLA class II molecules is open at both ends allowing longer (15-20 amino acid) peptides to bind in multiple registers[238,655]. The lack of rigid allele-specific binding motifs permits broader binding specificity and may explain the higher frequency with which promiscuous and cross reactive $CD4^+$ responses were detected compared to $CD8^+$ [238,247].

In summary, the predominance of $CD4^+$ T cell responses amongst HESN subjects studied within this thesis cannot be fully explained. The preponderance of $CD4^+$ T cells toward degenerate epitope recognition coupled with the potential low levels of HIV-1 exposure may have colluded to bias initiation of a $CD4^+$ T cell mediated response.

8.4 What role, if any, could pre-existing $CD4^+$ HIV-1 specific T cell responses play in the acquisition of HIV-1 infection?

Whilst profound depletion of $CD4^+$ T cells is the hallmark of HIV-1 infection, there is growing evidence to suggest early $CD4^+$ T cell responses may play a critical role in viral control[16,76,414,415]. Indeed the preservation of robust, $CD4^+$ T cell responses differentiates non-pathogenic HIV-2 infection from the more pathogenic HIV-1[16].

Moreover, HIV-1 seropositive individuals who sustain low viral loads and normal $CD4^+$ T cell counts, in the absence of antiretroviral therapy (ART), have been shown to maintain robust $CD4^+$ T cell responses[76,130,379]. Discerning whether these correlations are cause or effect of reduced viral loads remains to be determined.

8.4.1 Direct role in protection?

There is limited evidence to suggest $CD4^+$ T cells are capable of playing a direct role in HIV-1 control. Within Chapter 3, $CD4^+$ T cell responses detected during acute HIV-1 infection were capable of secreting a range of effector functions, such as IFN-γ, TNF-α and IL-2 (Figure 3.9). The detection of $CD4^+$ T cell responses in HESN subjects, has in the past, been identified as a "correlate of protection". In agreement with previous studies[135,152,155], where detectable, HIV-1 specific T cell responses amongst HESN subjects were shown to be weak and often transient (Figure 5.2, 5.6-5.7, 7.4). The absolute requirement for culture demonstrated in the studies presented herein, underscores the low magnitude and frequency of HIV-1 specific T cell responses detected in HESN and brings into question their role as immune correlates of protection. Vaccine challenge models set up in primate studies have shown "protection" against establishment of HIV-1 to be accompanied with broad and robust HIV-1 specific T cell responses[389,504,614]. Moreover, $CD4^+$ T cell responses thought to aid in viral control amongst long term non progressors (LTNP) or individuals infected with HIV-2 have been shown to be of high magnitude and polyfunctional[75,76,128,414]. Together these observations would suggest that the magnitude of $CD4^+$ T cell responses detected amongst HESN donors was insufficient to act as a correlate of protection.

There is a paucity of more generalized data supporting a direct role for $CD4^+$ T cell responses in control of HIV-1. Cytotoxic $CD4^+$ T cells have been detected in a range of viral infections including CMV[653], hepatitis B/C[336] and HIV-1[173,337,363,377]. Typically believed to be a by-product of chronic viral infection, cytotoxic $CD4^+$ T cells from elite HIV-1 controllers, have recently been shown to suppress viral infection in both macrophages and $CD4^+$ T cells[337]. However, a lot of these data have been generated following prolonged *in vitro* culture and there is little evidence to suggest cytotoxic $CD4^+$ T cell responses occur *in vivo*.

Similar to the associations between "protective" HLA-class I alleles and control of viral loads, a limited number of HLA class II alleles, in particular several *DRB1*13* alleles, have been correlated with reduced viral load, non-progressive HIV-1 infection[129,133,236,434]. Interestingly, DRB1*13 has also been associated with the viral clearance and protection from vertical transmission of Hepatitis C and B[253,656-658]. However, no study to date has assessed whether HESN subjects are enriched for HLA DRB1*13 alleles. Genetic material is available for study from HESN donors enrolled in CHAVI002 and therefore this is one area that could be addressed in future studies.

The best evidence to support a role for $CD4^+$ T cells, in exerting immune pressure upon HIV-1, is the detection of viral escape mutations in $CD4^+$ epitope regions[421,422]. However, very few studies have detected viral escape mutation in $CD4^+$ epitope regions, casting doubt as to their clinical relevance.

Taken together the observations made in this thesis together with previous literature reports paint an unlikely picture for direct, $CD4^+$ T cell mediated control of HIV-1 viremia.

8.4.2 Indirect role in protection?

It would seem that the role of $CD4^+$ T cells as "helper cells" is most likely to play a role in conferring protection against the establishment of HIV-1. Seminal work by Matloubian *et al* in 2003, showed using mouse model of LCMV infection, that for acute infections that are rapidly resolved, $CD4^+$ depletion has little effect upon effector control of infection[340]. However, in chronic infection where $CD8^+$ T cell responses are required to persist for several months or more, $CD4^+$ T cells were found to play a critical role in sustaining $CD8^+$ T cell responses[340]. Shedlock *et al* confirmed this observation, showing that depletion of $CD4^+$ T cells during primary phase of infection leads to impaired $CD8^+$ T cell memory response upon secondary challenge, a deficiency that could be restored by the transfer of memory $CD8^+$ T cells generated from $CD4^+$ competent mice[341]. Although circumstantial, within Chapter 3 gag specific $CD4^+$ T cell responses were shown to typically precede $CD8^+$ T cell responses (Figure 3.8). It could be suggested that the kinetics of these responses are indicative of $CD4^+$ T cells providing cognate "help" to the $CD8^+$ T cells. However, the number of

subjects studied within this thesis is not sufficient to fully endorse this statement, and it is yet to be assessed for other proteins.

Whilst the majority of literature surrounding the importance of $CD4^+$ T cell "help" has been primarily demonstrated in mouse models, there is a considerable body of literature to support a critical role of $CD4^+$ T cells in the generation of an efficient $CD8^+$ T cell response[332,333,340-343,367,411,413]. These observations may help explain why $CD8^+$ T cells appear to exert such a profound effect upon HIV-1 viral load in the acute stages of infection, yet despite an increased breadth of response in chronic infection, no further reduction in viral load is detected. The direct study of $CD4^+$ T cell "help" in HIV-1 infection using depletion methods such as those outlined in mouse studies, is complicated by the requirement of sufficient $CD4^+$ target cells to sustain HIV-1 infection.

In addition to providing "help" to $CD8^+$ T cells, specialised subsets of $CD4^+$ T cells referred to as T follicular helper cells (TFH)[346,347], have been shown to provide vital "help" to antibody producing B cells. Identifiable by the cell surface phenotype, $CD4^+CD57^+CXCR5^+$ (circulating phenotype $CD4^+ICOS^{high}$ $PD1^{high}$) and the cytokine profile IL-21, IL-6 and IL-27 (Reviewed in[344]), TFH cells are believed to act as "tolerance keepers" in germinal centres. Their primary effector functions include the induction of germinal centre (GC) formation, B cell differentiation, somatic hypermutation and immunoglobulin class-switch recombination[659,660](Reviewed in[344]). A key player mediating these effector functions is CD40L (CD154), which is expressed at high levels of the surface of TFH[346]. Murine studies have shown that an absence of CD154 signalling typically results in abortive B cell responses and a failure to generate germinal centres and long term $CD8^+$ T cell memory[283,345,348].

The principle cytokine secreted by TFH, IL-21, has also been shown to play an important role in stimulating GC B cells and antibody production[339,659-661]. Whilst a role for TFH in HIV-1 infection remains to be fully defined or demonstrated, IL-21 has recently been correlated with control of HIV-1 replication, with LTNPs sustaining higher levels of $CD4^+$IL-21^+ T cells compared to progressors[650]. During chronic HIV-1 infection IL-21 has been shown to enhance the *in vitro* production of perforin and granzyme B by $CD8^+$ T cells and therefore implicated to play an important role in

enhancing $CD8^+$ cytotoxic effects[650]. Within murine studies, IL-21 has also been shown to play a vital role in the control of chronic LCMV, though sustained polyfunctional $CD8^+$ T cell responses[662-664]. Furthermore, IL-21 production during the acute stages of chronic viral infections has been shown to be significantly lower than that detected in rapidly cleared viral infections[662,665]. Together these data would suggest the early induction of IL-21^+ TFH cells could prove advantageous in the development of an efficacious HIV-1 vaccine. Given the low frequency and magnitude of HIV-1 specific T cell responses detected within HESN subjects studied within this thesis, it would have been impractical to subtype responses beyond $CD4^+$/$CD8^+$ lineages. However, future studies are now planned to assess whether TFH expand following vaccination and whether enhancing these T cell responses can improve clinical outcome by reducing viral set point.

8.4.3 Detrimental effect upon HIV-1 acquisition?

Thus far, the assumption has been that detection of HIV-1 specific T cell responses amongst HESN subjects is likely to reflect a "correlate of protection", against the establishment of HIV-1. However, there is an argument that pre-existing $CD4^+$ T cell responses could be detrimental to the host. In a seminal paper by Douek *et al*, HIV-1 was shown to preferentially infect HIV-1 specific $CD4^+$ T cells[368]. Whilst poorly defined mechanistically, this observation has led to fears that incorporation of vaccine constructs that boost $CD4^+$ specific immunity may have a detrimental effect, both in terms of susceptibility to HIV-1 and disease progression following seroconversion. This would seem unlikely, since several vaccines tested both in primate models and clinical trials have been shown to induce $CD4^+$ T cell responses, without demonstrably increasing risk of susceptibility to HIV-1[221,380,381,504]. Moreover the recent RV144 trial has proved the most promising vaccine candidate to date, conferring modest protection against the establishment of HIV-1 following induction of a combination of $CD4^+$ T cell responses, weak antibody responses and limited $CD8^+$ T cell responses.

8.4.4 Merely markers of exposure?

Classical HLA class 1 signalling is achieved through the processing of intracellular pathogens. The predominance of $CD4^+$ T cell responses detected amongst HESN subjects in these studies, suggests that viral integration and infection of host cells did

not occur. Rather, it could be argued, that HESN subjects were exposed to defective/replication deficient virions or to abortive infection, which meant that non-infectious virus was taken up by pinocytosis/phagocytosis by APC and processed through the endosomal pathway for classical HLA class II presentation. There is evidence from the literature, to suggest that the detection of HIV-1 specific T cell responses can be directly correlated with the viral load of an infected partner[150,152]. Whilst risk was not formally assessed for subjects studied in Chapter 7, it could be inferred that the individuals who subsequently seroconverted were engaged in higher risk exposures to HIV-1 than those who remained seronegative for the duration of study. Notably individuals who subsequently seroconverted had higher magnitude T cell responses than those who remained seronegative (Figure 7.2). There is an argument, therefore, that pre-existing HIV-1 specific T cell responses are merely markers of exposure playing little to no role in the acquisition of HIV-1.

8.4.5 Direct assessment of pre-existing HIV-1 specific T cells and acquisition of HIV-1

Access to a retrospective cohort of high risk MSM provided a unique opportunity in which to formally address the role, if any, pre-existing HIV-1 specific T cell responses play in the acquisition of HIV-1 (Chapter 7). In a blinded study, statistically powered to detect a 3-4 fold increased risk of HIV-1 acquisition, 87 MSM subjects were screened for pre-existing HIV-1 specific T cell responses using the cultured IFN-γ ELISpot technique. Of the 87 subjects, 38 were known to have subsequently seroconverted during the course of study, whilst 49 remained HIV-1 seronegative for the duration of study (3 years)[392]. Initial screening showed that HIV-1 specific T cell responses were detected in 36.8% of donors whom subsequently became HIV-1 infected, compared to 20.4% of subjects who remained seronegative (Figure 7.1). Whilst indicative of a trend, towards increased frequency of detection amongst individuals whom subsequently became HIV-1 positive, this difference failed to reach statistical significance. Interestingly, there was a significant difference in the magnitude of HIV-1 specific T cell responses between the two groups (Figure 5.2) with those who subsequently seroconverted showing significantly higher magnitude responses than those who remained seronegative. It is likely that this difference was a result of "risk of exposure", with an assumption that individuals who subsequently

seroncoverted were engaging in higher risk activities than those who remained seronegative. An approach, such as the detailed sexual behaviour questionnaire employed in the CHAVI002 cohort, would be required to formally address this hypothesis. Surprisingly, within the limited number of subjects available to study using mapping experiments, 4/6 were found to have $CD8^+$ T cell responses (Table 7.2). Notably, this study was not sufficiently powered to differentiate between detection of $CD4^+$ and $CD8^+$ HIV-1 specific T cell responses. The detection of $CD8^+$ T cell responses amongst this cohort was likely to reflect higher risk exposure to HIV-1. Based on the hypothesis that $CD8^+$ T cell responses are detectable following increased risk of exposure to HIV-1 or exposure to replication competent virions, further study, of pre-exisiting $CD8^+$ HIV-1 specific T cell responses is warrented. All 4 donors with $CD8^+$ T cell responses could be mapped to a single peptide (Table 7.2). This peptide has previously been detected in subjects with chronic HIV-1 infection following identification as a "potential epitope" using a bioinformatics approach[638]. T cell responses targeting this epitope have not been shown to be immunodominant in HIV-1 infection, nor were responses detected to this epitope in the 52 Malawian, and 14 UK seronegative subjects screened in these studies. We conclude, that whilst interesting, this observation perhaps highlights the requirement for large study numbers, and future study is required to ascertain whether HIV-1 specific T cell responses to this peptide are common amongst Caucasian individuals.

For a very limited number of subjects, the fate of pre-existing HIV-1 specific T cell responses, following HIV-1 infection was examined. Responses were maintained in the first year of HIV-1 infection in 2/3 subjects studied (Figure 7.2). This observation raises the possibility, that whilst pre-existing HIV-1 specific T cell responses fail to protect against the acquisition of HIV-1, their role post infection remains unknown. Further study, of much larger cohorts is required to substantiate the observation that pre-existing HIV-1 specific T cell responses can be maintained post seroconversion. Furthermore, it would be interesting to compare factors such as viral set-point amongst subjects with maintained pre-existing HIV-1 specific T cell responses to those without. Such studies would help to ascertain whether pre-existing T cell responses have an impact upon clinical progression of HIV-1.

8.5 Correlates of protection: If not T cells then what?

The data presented in Chapter 7 suggests pre-existing HIV-1 specific T cell responses are unlikely to confer protection against the establishment of HIV-1. However, the host immune response to pathogens is not restricted to compartmentalised responses, and it is highly probable that the correlates of protection afforded to HESN individuals are multifactorial. Indeed a number of factors have been associated with the apparent viral control exhibited by long term non progressors, including viral attenuation[131,618], host genetic characteristics[619,620], innate Immune responses[195] as well as adaptive immune responses[76,77,79,414]. Whilst this thesis has focused exclusively upon the role of pre-existing IFN-γ^+ T cell responses, the detection of IgA responses within HESN subjects has been reported as a correlate of protection, independent of host cellular responses[143,153,500]. Indeed, low level IgA has been detected in the genital tract and plasma of a large number of HESN subjects working as commercial sex workers in Nairobi[143,153,500]. IgA purified from these HESN donors has been shown to have cross clade neutralizing activity and could inhibit HIV-1 mucosal transcytosis in an *in vitro* trans-well model of the human mucosal epithelium[143]. Together these data suggest that IgA may play an important functional role in the protection against establishment of HIV-1. However, as for pre-existing HIV-1 specific T cell responses, the data is not clear cut. Recent findings from the Rv144 vaccine trial have shown that detection of high plasma IgA concentrations amongst vaccinees conferred at 1.54 ($p=0.027$) increased relative risk of HIV-1 infection. Notably, detection of IgA alone did not enhance the risk of HIV-1 acquisition, vaccinees with high IgA had similar HIV-1 infection rates as placebo controls (presented by B Haynes AIDS Vaccine 2011 conference 12-15 September 2011)[631,632]. Further research is now required to ascertain the true role of IgA in HIV-1 infection, and to establish why high levels of IgA would confer reduced vaccine efficacy.

Systemic immune activation has also been shown to play a role in the susceptibility to HIV-1. Indeed, the surface expression of activation markers, such as CD38 and HLADR, has proven to be a reliable, $CD4^+$ T cell count and viral load independent, means by which to predict disease progression following productive infection with HIV-1[298-300,304-306,417-419]. There have been a "mixed bag" of reports suggesting both hyper and hypo active immune states to be beneficial in protection against HIV-1

infection. This area is particularly difficult to study, since by their very definition HESN subjects are typically engaged in high risk activities such as intravenous drug use, which impact upon immune activation status. Whilst not directly assessed in this study, it was noted that background IFN-γ production was significantly higher amongst HESN donors from CHAVI002 as compared to HUSN donors (Figure 5.5D). Notably HESN recruited in CHAVI002 were negative for STI and not taking intravenous drugs, perhaps suggesting increased activation was due to HIV-1 exposure and potentially affording a degree of protection. Further study would need to be made to directly address this question, preferably using *ex vivo* flow cytometric analysis combining markers of immune activation and lineage markers such as those used in Chapter 3.

In addition to adaptive immune responses, components of the innate immune system have been hypothesized to play a role in "protection". Indeed primary interactions at the mucosal surface form the "first line of defence" against HIV-1 infection (Reviewed in [26]). A large body of work has focused upon the role of natural killer (NK) cell responses in HIV-1 infection[142,189-192,195-197]. Increased NK cell activity, both in terms of cell lysis and production of cytokines, has been noted in several HESN cohorts[189-191]. However, the precise mechanisms behind this activation remain unknown, and the role of NK cells in infection appears to be a delicate balancing act between ligand interactions with inhibitory and activating KIR receptors[142,188,192,193]. HESN cohorts appear to be enriched for individuals homozygous for the activating NK receptor KIR3DS1[142]. Notably, KIR3DS1$^+$ NK cells have been shown to be particularly potent inhibitors of HIV-1 replication in HLA-Bw480I-positive target cells *in vitro*[192]. The relevance of these observations remain controversial, since although homozygous expression of KIR3DS1 and HLA-B4801 has been shown to correlate with delayed progression to AIDS, homozygosity for KIRDS1 in the absence of HLA-B4801 has been correlated with rapid disease progression[188,193]. A role for inhibitory KIR receptors was recently suggested following a recent genome wide association study which identified a single nucleotide polymorphism upstream of HLA-C (-35C/T) as being a major determinant in determining viral load set point. Notably HLA-C allotypes dominate over HLA-A and HLA-B as a source of ligands for KIR, and preferentially interact with inhibitory KIR receptors[196]. This is a very

complicated field and further study is required to fully elucidate the specific requirements of NK activity for "protection" against the acquisition of HIV-1.

Finally it is possible that there are as yet unidentified host genetic factors that are contributing toward the protection afforded by HESN. Large genome wide sequencing studies are currently underway in collaborators laboratories to examine this possibility, using samples obtained from HIV-1 negative haemophiliac subjects known to have been exposed to contaminated blood products in the early 1980s.

8.6 Detection of HIV-1 specific T cell responses amongst HUSN donors

One of the key, and most surprising, observations made in this thesis was the detection of HIV-1 specific T cell responses amongst HIV-1 seronegative individuals, with no known exposure to HIV-1 (Figure 5.1, Table 5.1). Notably, HIV-1 specific T cell responses detected amongst HUSN donors could be mapped to the peptide level (Table 5.3 and appendix 2) and titrated (Figure 5.11). This observation is somewhat perplexing, and opens up a much broader debate as to the ontogeny of pre-existing HIV-1 specific T cell responses, and the propensity for cross reactivity within the immune system.

Original calculations performed by mathematical modellers such as D. Mason, suggest that cross reactivity is an inherent and necessary characteristic of T cell recognition, without which the host immune system would be vastly inadequate[262]. In equations set to reconcile the opposing constraints of the size of the lymphatic system and the requirement to be able to respond to a vast array of epitopes, Mason predicted that each T cell receptor (TCR) must be capable of binding ~10^6 different peptides[262]. Structural studies have shown that peptide binding to TCR occurs at key anchor residues[233,240,241,564], and that a degree of "tolerance" to the substitution of non-anchor residues will be permitted without ablation of the IFN-γ response[544,545]. However, sequence homology is not a pre-requisite, and cross reactive T cell responses have been detected against completely unrelated sequences[558,559,562,563]. Within this study, no sequence homology between the HIV-1 specific peptides recognized by seronegative individuals from this study and other pathogens was detected.

It is hypothesized that cross recognition of disparate sequences occurs either due to a shared structural homology between epitopes[512,558,563,567], or significant structural rearrangement of the TCR to permit binding[566,574-576]. A large body of work surrounding the detection and indeed existence of cross reactive T cells, capable of recognizing highly disparate sequences, has been performed in mouse studies, and often using T cell clones. Whilst hypothesized to play an important role in the immunopathology of certain autoimmune diseases[558,559,574] and the exacerbation of viral infections[546,553,587,590,598,628] direct *ex vivo* evidence of human T cells capable of cross recognition of epitopes from different pathogens, is limited. This does not mean it doesn't occur, but is more likely to reflect the fact that unless implicated in immunopathology there is no cause to look for it. Indeed, where reported, detection of cross reactive T cell responses is often a fortuitous or incidental observation made during an unrelated study[368].

Given the infinite number of possible nonamers and the lack of requirement for sequence homology, detection and characterization of cross reactive T cells is an extremely difficult task. In a hypothesis generating study, the work presented within this thesis took a number of indirect approaches, to explore the possibility HIV-1 specific T cell responses detected in HUSN were the result of cross reactivity (Chapter 6). The clonality of TCR response to peptide-HLA complex has been suggested to confer an indication of cross reactivity[554,583,586,587], with highly skewed, oligoclonal TCR responses believed to be indicative of preferential expansion of rare cross reactive T cell clones[284,307,310,587,589,590]. We examined a subset of responses from both HESN and HUSN donors for TCR clonality using both flow cytometric and sequence based approaches. Whilst flow cytometry is restricted to the study of families of Vβ antibodies and does not provide an indication as to the diversity of VJ or Vα chains the application of this approach to cultured short-term cell lines proved more productive, and arguably more reliable than the sequence based approach (Figure 6.3, Table 6.1 and Figure 6.8-6.9 respectively). There is not a clear consensus on the definition of an "oligoclonal" or "polyclonal" TCR response within the literature. For the purposes of this study, and oligoclonal TCR response was defined as response in which >50% of IFN-γ^+ T cells expressed a single TCRVβ, with the overall response being mediated by <5 TCRVβ. There is a clear need, within this field

for a more standardized means by which to define TCR responses, and clearly study of much larger cohorts would prove beneficial in determining reliable "cut off criteria" particularly for the flow based approach. Interestingly, HIV-1 specific T cell responses observed amongst HUSN subjects were shown to be oligoclonal in TCR usage, whilst T cell responses detected within HESN were more mixed, with both poly and oligloclonal responses detected. This finding suggests that HUSN responses were likely to be the result of expansion of a limited number of T cell clones, however further study would be required to substantiate whether they are truly cross reactive or not.

The ontogeny of HIV-1 specific T cell responses was next examined using a novel method referred to as the T cell library technique[308] (Figure 6.11-6.16). Originally described as a means by which to interrogate naïve $CD4^+$ T cell repertoires, this approach is based on *ex vivo* cell sorts of $CD4^+$ T cells into naïve, central memory and effector memory populations based upon the expression of various surface markers (Figure 6.10)[308]. After sorting, cells are seeded at a known concentration and expanded in the presence of phytohemagglutinin (PHA), IL-2 and allogeneic feeder cells[308]. Following *in vitro* culture the T cell libraries are interrogated for proliferative response to antigen measured by tritiated thymidine incorporation[308]. This approach not only enables the detection and ontogeny of antigen specific T cells to be determined but moreover allows precursor frequency to be calculated based on Poisson distribution, thereby forming a powerful research tool. In collaboration with Professor F Sallusto 5 leukapharesis donors, presumed HUSN, were screened using for HIV-1 specific T cell responses using the T cell library technique. Surprisingly, HIV-1 specific, proliferative responses were detected amongst the memory $CD4^+$ T cell subsets of all 5 donors, albeit at low frequency (Figure 6.10-6.11). These data suggest, in agreement with original calculations[262], that low level cross reactivity is a relatively common occurrence within the adaptive immune system. Clearly the number of individuals studied here was low, and to gain confidence in these data future studies are planned to screen both $CD4^+$ and $CD8^+$ T cell subsets in additional leukapharesis subjects as well as in cord blood samples to gain a better understanding of the levels of cross reactivity within the immune system. In addition to peptide screening, these followup experiments will test whole protein/recombinant virus to confirm that detection of HIV-1 specific T cell responses amongst HUSN is not an

artefact of the use of synthetic peptide which may not be processed and presented *in vivo*.

In addition to the enormous potential of the T cell library to dissect HIV-1 specific T cell responses into different subsets[308,322] the ability to calculate precursor frequency also holds tremendous opportunity for future study. Typically, the primary T cell responses in HIV-1 infection have been shown to be narrowly targeted[38,362,666,667]. Whether this is due to preferential expansion of pre-existing, HIV-1 specific T cell responses, or a higher precursor frequency of these cells remains to be seen. Serial sampling across pre-infection and acute stages of HIV-1 infection would be required to fully address this question. Notably individuals shown to mount one of the immunodominant T cell responses during acute infection have, on average, lower viral load set points than those who don't[497]. Moreover, preservation of these early immune responses has been linked to slower disease progression and preserved $CD4^+$ T cell count[497]. The precursor frequency of antigen specific T cells has been correlated with immunodominance patterns during infection, at least in murine studies[615,616]. Therefore, the ability to generate quantitative data regarding precursor frequencies of HIV-1 specific T cell responses, using the T cell library technique holds potentially important implications for future vaccine development. By preferentially targeting the expansion of high precursor frequency, HIV-1 specific, T cell responses the efficiency of HIV-1 vaccination could be improved. In addition this approach should help improve our understanding as to why particular HLA associated epitopes dominate HIV-1 infection.

8.7 Are all pre-existing HIV-1 specific T cell responses the result of cross reactivity?

There are number of differences between HIV-1 specific T cell responses detected amongst HESN and HUSN donors. These differences are evidenced by the fact that HIV-1 specific T cell responses detected amongst HESN were significantly higher in magnitude (Figure 5.4) and better maintained than those observed in HUSN (Figure 5.6-5.7). Furthermore, whilst study number is limited, the only polyclonal TCR responses detected against HIV-1 specific peptides were shown in HESN donors with HUSN demonstrating a more restricted oligoclonal TCR response. Together these observations suggest that there are clear examples of individuals, for whom exposure

to HIV-1 has primed HIV-1 specific T cell responses. However, there is an underlying, low level of cross reactivity that suggests that not all HIV-1 specific T cell responses have been primed by HIV-1. Our inability to easily distinguish HIV-1 primed T cells from cross reactive T cells perhaps highlights the critical requirement for appropriate controls in study of HESN.

8.8 Designing the next HIV-1 vaccine

The results presented in this thesis suggest there is considerable work still to be done to elucidate the correlates of protection afforded to HESN individuals. Clearly the holy grail of HIV-1 vaccine development would be the induction of sterilizing immunity. However, perhaps a more realistic approach would be one that provides partial immune control against viral replication, conferring lower viral set point and delayed progression to AIDS defining illness.

As mentioned previously most current HIV-1 vaccine strategies have shied away from constructs aimed at inducing potent and robust $CD4^+$ T cell responses, in favour of preferential expansion of $CD8^+$ T cell responses. However, the absence of cognate help, provided by $CD4^+$ T cells is well known to affect both the generation and reactivation of memory $CD8^+$ T cells[332,341,343,413], suggesting vaccines that fail to induce $CD4^+$ T cell responses are likely to induce suboptimal $CD8^+$ T cell responses. Together, these data suggest an ideal vaccine would utilize a combination of immunological responses, incorporating the induction of broad and robust $CD4^+$ and $CD8^+$ T cell responses, perhaps ensuring the induction of TFH cells, to provide B cell "help" for the generation of effective antibodies. Delivery to the mucosal surface would be advantageous, where the "window of opportunity" afforded by the genetic bottle-neck effect of a single founder virus[26,38], could allow for direct targeting of HIV-1 prior to viral dissemination[26]. Furthermore, an improved understanding of the precursor frequency, at a population based level, of immunodominant epitopes correlated with control of viremia in LTNPs, should enable a focused vaccine to be developed ensuring early emergence of immunodominant, protective T cell responses. The combination of these approaches should ensure the vaccine is capable of inducing broad, robust, polyfunctional T cell responses capable of conferring lower viral set point and delayed progression to AIDS.

8.9 Conclusion

This thesis set out to provide a detailed examination of HIV-1 specific T cell responses, detectable amongst seronegative individuals. A carefully controlled cohort of extremely well-defined HESN was screened, using highly sensitive detection methods, for pre-existing HIV-1 specific T cell responses. In the absence of protective CCR5Δ32 mutation or low titre viral RNA, HIV-1 specific T cell responses were shown to be detectable in a proportion of HESN individuals. These responses spanned the entire HIV-1 proteome, although tended to be narrowly focused to a single protein. HIV-1 specific T cell responses could be titrated, were typically mediated by $CD4^+$ T cells and tended to map to previously defined, promiscuous epitopes.

No evidence was found to support a role for pre-existing HIV-1 specific T cell responses in protection against the establishment of HIV-1 infection. Interestingly we show in acute HIV-1 infection, gag specific $CD4^+$ T cell responses typically precede $CD8^+$ T cell response, indicative perhaps, of a role in providing cognate help. In a limited number of subjects studied, pre-existing HIV-1 specific T cell responses were shown to be maintained post seroconversion. A role for pre-existing $CD4^+$ T cell responses in providing cognate "help", and conferring protection during productive HIV-1 infection could therefore not be excluded.

Exposure to HIV-1 was shown to have a clear impact upon detection of pre-existing HIV-1 specific T cell responses, both in terms of magnitude and maintenance of response, as well the clonality of TCRVβ response, suggesting, at least for some individuals, exposure to HIV-1 was truly priming HIV-1 specific T cell responses. However, surprisingly exposure to HIV-1 was not found to be a prerequisite for the detection of HIV-1 specific T cell responses, responses were detectable amongst HUSN donors. Similar to HESN, HIV-1 specific T cell responses detected amongst HUSN, could be mapped to the peptide level, titrated and were predominately mediated by $CD4^+$ T cells. Moreover, they were shown to be detectable amongst memory $CD4^+$ T cell subsets, suggesting they were not the result of *in vitro* priming. Whilst sample size was low, interestingly, HIV-1 specific T cell responses, detected amongst HUSN donors were shown to be oligoclonal in TCRVβ usage.

The detection of memory $CD4^+$ HIV-1 specific T cell responses amongst HUSN opens a much broader debate as to the ontogeny of HIV-1 specific T cell responses, the inherent properties of the adaptive immune system and its preponderance toward degeneracy and cross reactivity. Greater understanding of these fundamental immunology questions should, hopefully, provide greater insight into the development of a more tailored and successful therapeutic vaccine.

CHAPTER 9:

REFERENCES

Chapter 9: References

1 Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. *MMWR Morb Mortal Wkly Rep* **30**, 305-308, (1981).

2 Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A. & Saxon, A. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305**, 1425-1431, (1981).

3 Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871, (1983).

4 Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J. & Popovic, M. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**, 865-867, (1983).

5 World Health Organization Global Health Atlas, http://apps.who.int/globalatlas/data/Query/default.asp

6 Baltimore, D. The enigma of HIV infection. *Cell* **82**, 175-176, (1995).

7 Cohen, M. S., Hellmann, N., Levy, J. A., DeCock, K. & Lange, J. The spread, treatment, and prevention of HIV-1: evolution of a global pandemic. *The Journal of clinical investigation* **118**, 1244-1254, (2008).

8 Costin, J. M. Cytopathic mechanisms of HIV-1. *Virology journal* **4**, 100, (2007).

9 Drake, J. W. Rates of spontaneous mutation among RNA viruses. *Proc Natl Acad Sci U S A* **90**, 4171-4175, (1993).

10 Korber, B., Gaschen, B., Yusim, K., Thakallapally, R., Kesmir, C. & Detours, V. Evolutionary and immunological implications of contemporary HIV-1 variation. *Br Med Bull* **58**, 19-42, (2001).

11 Hemelaar, J., Gouws, E., Ghys, P. D. & Osmanov, S. Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* **25**, 679-689, (2011).

12 Sodora, D. L., Gettie, A., Miller, C. J. & Marx, P. A. Vaginal transmission of SIV: assessing infectivity and hormonal influences in macaques inoculated with cell-free and cell-associated viral stocks. *AIDS Res Hum Retroviruses* **14 Suppl 1**, S119-123, (1998).

13 Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**, 389-392, (1989).

14 Campbell-Yesufu, O. T. & Gandhi, R. T. Update on human immunodeficiency virus (HIV)-2 infection. *Clin Infect Dis* **52**, 780-787, (2011).

15 da Silva, Z. J., Oliveira, I., Andersen, A., Dias, F., Rodrigues, A., Holmgren, B., Andersson, S. & Aaby, P. Changes in prevalence and incidence of HIV-1, HIV-2 and dual infections in urban areas of Bissau, Guinea-Bissau: is HIV-2 disappearing? *AIDS* **22**, 1195-1202, (2008).

16 Duvall, M. G., Jaye, A., Dong, T., Brenchley, J. M., Alabi, A. S., Jeffries, D. J., van der Sande, M., Togun, T. O., McConkey, S. J., Douek, D. C., McMichael, A. J., Whittle, H. C., Koup, R. A. & Rowland-Jones, S. L. Maintenance of HIV-specific CD4+ T cell help distinguishes HIV-2 from HIV-1 infection. *J Immunol* **176**, 6973-6981, (2006).

17 Ho, D. D. & Bieniasz, P. D. HIV-1 at 25. *Cell* **133**, 561-565, (2008).

18 Rowland-Jones, S. L. & Whittle, H. C. Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1? *Nat Immunol* **8**, 329-331, (2007).

19 Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M. & Hahn, B. H. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436-441, (1999).

20 Kuiken, C., Korber, B. & Shafer, R. W. HIV sequence databases. *AIDS Rev* **5**, 52-61, (2003).

21 Los Alamos National Laboratory Sequence Database, http://www.hiv.lanl.gov/

22 Robertson, D. L., Anderson, J. P., Bradac, J. A., Carr, J. K., Foley, B., Funkhouser, R. K., Gao, F., Hahn, B. H., Kalish, M. L., Kuiken, C., Learn, G. H., Leitner, T., McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Salminen, M., Sharp, P. M., Wolinsky, S. & Korber, B. HIV-1 nomenclature proposal. *Science* **288**, 55-56, (2000).

23 Gupta, P., Collins, K. B., Ratner, D., Watkins, S., Naus, G. J., Landers, D. V. & Patterson, B. K. Memory CD4(+) T cells are the earliest detectable human immunodeficiency virus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system. *J Virol* **76**, 9868-9876, (2002).

24 Bomsel, M. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. *Nat Med* **3**, 42-47, (1997).

25 Hladik, F., Sakchalathorn, P., Ballweber, L., Lentz, G., Fialkow, M., Eschenbach, D. & McElrath, M. J. Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. *Immunity* **26**, 257-270, (2007).

26 Keele, B. F. & Estes, J. D. Barriers to mucosal transmission of immunodeficiency viruses. *Blood* **118**, 839-846, (2011).

27 Lehner, T., Wang, Y., Whittall, T. & Seidl, T. Innate immunity and HIV-1 infection. *Adv Dent Res* **23**, 19-22, (2011).

28 Sugaya, M., Lore, K., Koup, R. A., Douek, D. C. & Blauvelt, A. HIV-infected Langerhans cells preferentially transmit virus to proliferating autologous CD4+ memory T cells located within Langerhans cell-T cell clusters. *J Immunol* **172**, 2219-2224, (2004).

29 Gray, R. H., Wawer, M. J., Brookmeyer, R., Sewankambo, N. K., Serwadda, D., Wabwire-Mangen, F., Lutalo, T., Li, X., vanCott, T. & Quinn, T. C. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet* **357**, 1149-1153, (2001).

30 Fox, J. & Fidler, S. Sexual transmission of HIV-1. *Antiviral Res* **85**, 276-285, (2010).

31 Powers, K. A., Poole, C., Pettifor, A. E. & Cohen, M. S. Rethinking the heterosexual infectivity of HIV-1: a systematic review and meta-analysis. *Lancet Infect Dis* **8**, 553-563, (2008).

32 Royce, R. A., Sena, A., Cates, W., Jr. & Cohen, M. S. Sexual transmission of HIV. *N Engl J Med* **336**, 1072-1078, (1997).

33 Sheffield, J. S., Wendel, G. D., Jr., McIntire, D. D. & Norgard, M. V. Effect of genital ulcer disease on HIV-1 coreceptor expression in the female genital tract. *J Infect Dis* **196**, 1509-1516, (2007).

34 Levy, J. A. The transmission of HIV and factors influencing progression to AIDS. *Am J Med* **95**, 86-100, (1993).

35 Haaland, R. E., Hawkins, P. A., Salazar-Gonzalez, J., Johnson, A., Tichacek, A., Karita, E., Manigart, O., Mulenga, J., Keele, B. F., Shaw, G. M., Hahn, B. H., Allen, S. A., Derdeyn, C. A. & Hunter, E. Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. *PLoS Pathog* **5**, e1000274, (2009).

36 Quinn, T. C., Wawer, M. J., Sewankambo, N., Serwadda, D., Li, C., Wabwire-Mangen, F., Meehan, M. O., Lutalo, T. & Gray, R. H. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med* **342**, 921-929, (2000).

37 Campo, J., Perea, M. A., del Romero, J., Cano, J., Hernando, V. & Bascones, A. Oral transmission of HIV, reality or fiction? An update. *Oral Dis* **12**, 219-228, (2006).

38 Goonetilleke, N., Liu, M. K., Salazar-Gonzalez, J. F., Ferrari, G., Giorgi, E., Ganusov, V. V., Keele, B. F., Learn, G. H., Turnbull, E. L., Salazar, M. G., Weinhold, K. J., Moore, S., Letvin, N., Haynes, B. F., Cohen, M. S., Hraber, P., Bhattacharya, T., Borrow, P., Perelson, A. S., Hahn, B. H., Shaw, G. M., Korber, B. T. & McMichael, A. J. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med* **206**, 1253-1272, (2009).

39 Keele, B. F., Giorgi, E. E., Salazar-Gonzalez, J. F., Decker, J. M., Pham, K. T., Salazar, M. G., Sun, C., Grayson, T., Wang, S., Li, H., Wei, X., Jiang, C., Kirchherr, J. L., Gao, F., Anderson, J. A., Ping, L. H., Swanstrom, R., Tomaras, G. D., Blattner, W. A., Goepfert, P. A., Kilby, J. M., Saag, M. S., Delwart, E. L., Busch, M. P., Cohen, M. S., Montefiori, D. C., Haynes, B. F., Gaschen, B., Athreya, G. S., Lee, H. Y., Wood, N., Seoighe, C., Perelson, A. S., Bhattacharya, T., Korber, B. T., Hahn, B. H. & Shaw, G. M. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* **105**, 7552-7557, (2008).

40 Miller, C. J., Li, Q., Abel, K., Kim, E. Y., Ma, Z. M., Wietgrefe, S., La Franco-Scheuch, L., Compton, L., Duan, L., Shore, M. D., Zupancic, M., Busch, M., Carlis, J., Wolinsky, S. & Haase, A. T. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol* **79**, 9217-9227, (2005).

41 Nawaz, F., Cicala, C., Van Ryk, D., Block, K. E., Jelicic, K., McNally, J. P., Ogundare, O., Pascuccio, M., Patel, N., Wei, D., Fauci, A. S. & Arthos, J. The genotype of early-transmitting HIV gp120s promotes alpha (4) beta(7)-reactivity, revealing alpha (4) beta(7) +/CD4+ T cells as key targets in mucosal transmission. *PLoS Pathog* **7**, e1001301, (2011).

42 Salazar-Gonzalez, J. F., Bailes, E., Pham, K. T., Salazar, M. G., Guffey, M. B., Keele, B. F., Derdeyn, C. A., Farmer, P., Hunter, E., Allen, S., Manigart, O., Mulenga, J., Anderson, J. A., Swanstrom, R., Haynes, B. F., Athreya, G. S., Korber, B. T., Sharp, P. M., Shaw, G. M. & Hahn, B. H. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. *J Virol* **82**, 3952-3970, (2008).

43 Salazar-Gonzalez, J. F., Salazar, M. G., Keele, B. F., Learn, G. H., Giorgi, E. E., Li, H., Decker, J. M., Wang, S., Baalwa, J., Kraus, M. H., Parrish, N. F., Shaw, K. S., Guffey, M. B., Bar, K. J., Davis, K. L., Ochsenbauer-Jambor, C., Kappes, J. C., Saag, M. S., Cohen, M. S., Mulenga, J., Derdeyn, C. A., Allen, S., Hunter, E., Markowitz, M., Hraber, P., Perelson, A. S., Bhattacharya, T., Haynes, B. F., Korber, B. T., Hahn, B. H. & Shaw, G. M. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med* **206**, 1273-1289, (2009).

44 Bar, K. J., Li, H., Chamberland, A., Tremblay, C., Routy, J. P., Grayson, T., Sun, C., Wang, S., Learn, G. H., Morgan, C. J., Schumacher, J. E., Haynes, B. F., Keele, B. F., Hahn, B. H. & Shaw, G. M. Wide variation in the multiplicity of HIV-1 infection among injection drug users. *J Virol* **84**, 6241-6247, (2010).

45 Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. & Paxton, W. A. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673, (1996).

46 Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A. & Landau, N. R. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367-377, (1996).

47 Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., Kaslow, R., Saah, A., Rinaldo, C., Detels, R. & O'Brien, S. J. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**, 1856-1862, (1996).

48 Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G. & Parmentier, M. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722-725, (1996).

49 McMichael, A. J., Borrow, P., Tomaras, G. D., Goonetilleke, N. & Haynes, B. F. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol* **10**, 11-23, (2010).

50 Cohen, M. S., Shaw, G. M., McMichael, A. J. & Haynes, B. F. Acute HIV-1 Infection. *The New England journal of medicine* **364**, 1943-1954, (2011).

51 Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J. & Haase, A. T. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* **434**, 1148-1152, (2005).

52 Brenchley, J. M. & Douek, D. C. HIV infection and the gastrointestinal immune system. *Mucosal Immunol* **1**, 23-30, (2008).

53 Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M. & Roederer, M. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093-1097, (2005).

54 Arthos, J., Cicala, C., Martinelli, E., Macleod, K., Van Ryk, D., Wei, D., Xiao, Z., Veenstra, T. D., Conrad, T. P., Lempicki, R. A., McLaughlin, S.,

Pascuccio, M., Gopaul, R., McNally, J., Cruz, C. C., Censoplano, N., Chung, E., Reitano, K. N., Kottilil, S., Goode, D. J. & Fauci, A. S. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol* **9**, 301-309, (2008).

55 Cicala, C., Martinelli, E., McNally, J. P., Goode, D. J., Gopaul, R., Hiatt, J., Jelicic, K., Kottilil, S., Macleod, K., O'Shea, A., Patel, N., Van Ryk, D., Wei, D., Pascuccio, M., Yi, L., McKinnon, L., Izulla, P., Kimani, J., Kaul, R., Fauci, A. S. & Arthos, J. The integrin alpha4beta7 forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1. *Proc Natl Acad Sci U S A* **106**, 20877-20882, (2009).

56 McMichael, A. J. HIV vaccines. *Annu Rev Immunol* **24**, 227-255, (2006).

57 Cohen, M. S., Gay, C. L., Busch, M. P. & Hecht, F. M. The detection of acute HIV infection. *J Infect Dis* **202 Suppl 2**, S270-277, (2010).

58 Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G. & van Kooyk, Y. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587-597, (2000).

59 Moris, A., Nobile, C., Buseyne, F., Porrot, F., Abastado, J. P. & Schwartz, O. DC-SIGN promotes exogenous MHC-I-restricted HIV-1 antigen presentation. *Blood* **103**, 2648-2654, (2004).

60 Moris, A., Pajot, A., Blanchet, F., Guivel-Benhassine, F., Salcedo, M. & Schwartz, O. Dendritic cells and HIV-specific CD4+ T cells: HIV antigen presentation, T-cell activation, and viral transfer. *Blood* **108**, 1643-1651, (2006).

61 Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T. & Douek, D. C. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200**, 749-759, (2004).

62 Brenchley, J. M., Ruff, L. E., Casazza, J. P., Koup, R. A., Price, D. A. & Douek, D. C. Preferential infection shortens the life span of human immunodeficiency virus-specific CD4+ T cells in vivo. *J Virol* **80**, 6801-6809, (2006).

63 Walker, C. M., Moody, D. J., Stites, D. P. & Levy, J. A. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234**, 1563-1566, (1986).

64 Chun, T. W., Nickle, D. C., Justement, J. S., Meyers, J. H., Roby, G., Hallahan, C. W., Kottilil, S., Moir, S., Mican, J. M., Mullins, J. I., Ward, D. J., Kovacs, J. A., Mannon, P. J. & Fauci, A. S. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. *J Infect Dis* **197**, 714-720, (2008).

65 Lerner, P., Guadalupe, M., Donovan, R., Hung, J., Flamm, J., Prindiville, T., Sankaran-Walters, S., Syvanen, M., Wong, J. K., George, M. D. & Dandekar, S. The gut mucosal viral reservoir in HIV-infected patients is not the major source of rebound plasma viremia following interruption of highly active antiretroviral therapy. *J Virol* **85**, 4772-4782, (2011).

66 Appay, V., Papagno, L., Spina, C. A., Hansasuta, P., King, A., Jones, L., Ogg, G. S., Little, S., McMichael, A. J., Richman, D. D. & Rowland-Jones, S. L. Dynamics of T cell responses in HIV infection. *J Immunol* **168**, 3660-3666, (2002).

67 Fraser, C., Hollingsworth, T. D., Chapman, R., de Wolf, F. & Hanage, W. P. Variation in HIV-1 set-point viral load: epidemiological analysis and an evolutionary hypothesis. *Proc Natl Acad Sci U S A* **104**, 17441-17446, (2007).

68 Abrahams, M. R., Anderson, J. A., Giorgi, E. E., Seoighe, C., Mlisana, K., Ping, L. H., Athreya, G. S., Treurnicht, F. K., Keele, B. F., Wood, N., Salazar-Gonzalez, J. F., Bhattacharya, T., Chu, H., Hoffman, I., Galvin, S., Mapanje, C., Kazembe, P., Thebus, R., Fiscus, S., Hide, W., Cohen, M. S., Karim, S. A., Haynes, B. F., Shaw, G. M., Hahn, B. H., Korber, B. T., Swanstrom, R. & Williamson, C. Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. *J Virol* **83**, 3556-3567, (2009).

69 Davenport, M. P., Loh, L., Petravic, J. & Kent, S. J. Rates of HIV immune escape and reversion: implications for vaccination. *Trends Microbiol* **16**, 561-566, (2008).

70 Feeney, M. E., Tang, Y., Pfafferott, K., Roosevelt, K. A., Draenert, R., Trocha, A., Yu, X. G., Verrill, C., Allen, T., Moore, C., Mallal, S., Burchett, S., McIntosh, K., Pelton, S. I., St John, M. A., Hazra, R., Klenerman, P., Altfeld, M., Walker, B. D. & Goulder, P. J. HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. *J Immunol* **174**, 7524-7530, (2005).

71 Feeney, M. E., Tang, Y., Roosevelt, K. A., Leslie, A. J., McIntosh, K., Karthas, N., Walker, B. D. & Goulder, P. J. Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child. *J Virol* **78**, 8927-8930, (2004).

72 Ferrari, G., Korber, B., Goonetilleke, N., Liu, M. K., Turnbull, E. L., Salazar-Gonzalez, J. F., Hawkins, N., Self, S., Watson, S., Betts, M. R., Gay, C., McGhee, K., Pellegrino, P., Williams, I., Tomaras, G. D., Haynes, B. F., Gray, C. M., Borrow, P., Roederer, M., McMichael, A. J. & Weinhold, K. J. Relationship between functional profile of HIV-1 specific CD8 T cells and epitope variability with the selection of escape mutants in acute HIV-1 infection. *PLoS Pathog* **7**, e1001273, (2011).

73 Geels, M. J., Cornelissen, M., Schuitemaker, H., Anderson, K., Kwa, D., Maas, J., Dekker, J. T., Baan, E., Zorgdrager, F., van den Burg, R., van Beelen, M., Lukashov, V. V., Fu, T. M., Paxton, W. A., van der Hoek, L., Dubey, S. A., Shiver, J. W. & Goudsmit, J. Identification of sequential viral escape mutants associated with altered T-cell responses in a human immunodeficiency virus type 1-infected individual. *J Virol* **77**, 12430-12440, (2003).

74 Zhang, Y., Peng, Y., Yan, H., Xu, K., Saito, M., Wu, H., Chen, X., Ranasinghe, S., Kuse, N., Powell, T., Zhao, Y., Li, W., Zhang, X., Feng, X., Li, N., Leligdowicz, A., Xu, X., John, M., Takiguchi, M., McMichael, A., Rowland-Jones, S. & Dong, T. Multilayered Defense in HLA-B51-Associated HIV Viral Control. *J Immunol*, (2011).

75 Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A., Connors, M., Roederer, M. & Koup, R. A. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**, 4781-4789, (2006).

76 Dyer, W. B., Zaunders, J. J., Yuan, F. F., Wang, B., Learmont, J. C., Geczy, A. F., Saksena, N. K., McPhee, D. A., Gorry, P. R. & Sullivan, J. S.

Mechanisms of HIV non-progression; robust and sustained CD4+ T-cell proliferative responses to p24 antigen correlate with control of viraemia and lack of disease progression after long-term transfusion-acquired HIV-1 infection. *Retrovirology* **5**, 112, (2008).

77 Miura, T., Brockman, M. A., Schneidewind, A., Lobritz, M., Pereyra, F., Rathod, A., Block, B. L., Brumme, Z. L., Brumme, C. J., Baker, B., Rothchild, A. C., Li, B., Trocha, A., Cutrell, E., Frahm, N., Brander, C., Toth, I., Arts, E. J., Allen, T. M. & Walker, B. D. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. *J Virol* **83**, 2743-2755, (2009).

78 Okulicz, J. F., Marconi, V. C., Landrum, M. L., Wegner, S., Weintrob, A., Ganesan, A., Hale, B., Crum-Cianflone, N., Delmar, J., Barthel, V., Quinnan, G., Agan, B. K. & Dolan, M. J. Clinical outcomes of elite controllers, viremic controllers, and long-term nonprogressors in the US Department of Defense HIV natural history study. *J Infect Dis* **200**, 1714-1723, (2009).

79 Owen, R. E., Heitman, J. W., Hirschkorn, D. F., Lanteri, M. C., Biswas, H. H., Martin, J. N., Krone, M. R., Deeks, S. G. & Norris, P. J. HIV+ elite controllers have low HIV-specific T-cell activation yet maintain strong, polyfunctional T-cell responses. *AIDS* **24**, 1095-1105, (2010).

80 Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S. & Schooley, R. T. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* **328**, 345-348, (1987).

81 Winstone, N., Guimaraes-Walker, A., Roberts, J., Brown, D., Loach, V., Goonetilleke, N., Hanke, T. & McMichael, A. J. Increased detection of proliferating, polyfunctional, HIV-1-specific T cells in DNA-modified vaccinia virus Ankara-vaccinated human volunteers by cultured IFN-gamma ELISPOT assay. *Eur J Immunol* **39**, 975-985, (2009).

82 Yang, O. O., Kalams, S. A., Trocha, A., Cao, H., Luster, A., Johnson, R. P. & Walker, B. D. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol* **71**, 3120-3128, (1997).

83 Dye, C., Scheele, S., Dolin, P., Pathania, V. & Raviglione, M. C. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *Jama* **282**, 677-686, (1999).

84 UNAIDS. UNAIDS Report on the global AIDS epidemic 2010. *http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf*, (2010).

85 Breen, R. A., Janossy, G., Barry, S. M., Cropley, I., Johnson, M. A. & Lipman, M. C. Detection of mycobacterial antigen responses in lung but not blood in HIV-tuberculosis co-infected subjects. *Aids* **20**, 1330-1332, (2006).

86 Carvalho, A. C., Matteelli, A., Airo, P., Tedoldi, S., Casalini, C., Imberti, L., Cadeo, G. P., Beltrame, A. & Carosi, G. gammadelta T lymphocytes in the peripheral blood of patients with tuberculosis with and without HIV co-infection. *Thorax* **57**, 357-360, (2002).

87 Harries, A. D. & Dye, C. Tuberculosis. *Ann Trop Med Parasitol* **100**, 415-431, (2006).

88 Liebeschuetz, S., Bamber, S., Ewer, K., Deeks, J., Pathan, A. A. & Lalvani, A. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet* **364**, 2196-2203, (2004).

89 Mallet, V., Vallet-Pichard, A. & Pol, S. The impact of human immunodeficiency virus on viral hepatitis. *Liver Int* **31 Suppl 1**, 135-139, (2011).

90 Mutsvangwa, J., Millington, K. A., Chaka, K., Mavhudzi, T., Cheung, Y. B., Mason, P. R., Butterworth, A. E., Corbett, E. L. & Lalvani, A. Identifying recent Mycobacterium tuberculosis transmission in the setting of high HIV and TB burden. *Thorax* **65**, 315-320, (2010).

91 Nahid, P. & Daley, C. L. Prevention of tuberculosis in HIV-infected patients. *Curr Opin Infect Dis* **19**, 189-193, (2006).

92 Ranjbar, S., Boshoff, H. I., Mulder, A., Siddiqi, N., Rubin, E. J. & Goldfeld, A. E. HIV-1 replication is differentially regulated by distinct clinical strains of Mycobacterium tuberculosis. *PLoS ONE* **4**, e6116, (2009).

93 Sharma, S. K., Mohan, A. & Kadhiravan, T. HIV-TB co-infection: epidemiology, diagnosis & management. *Indian J Med Res* **121**, 550-567, (2005).

94 Villacian, J. S., Tan, G. B., Teo, L. F. & Paton, N. I. The effect of infection with Mycobacterium tuberculosis on T-cell activation and proliferation in patients with and without HIV co-infection. *J Infect* **51**, 408-412, (2005).

95 Danta, M., Semmo, N., Fabris, P., Brown, D., Pybus, O. G., Sabin, C. A., Bhagani, S., Emery, V. C., Dusheiko, G. M. & Klenerman, P. Impact of HIV on host-virus interactions during early hepatitis C virus infection. *J Infect Dis* **197**, 1558-1566, (2008).

96 Falconer, K., Gonzalez, V. D., Reichard, O., Sandberg, J. K. & Alaeus, A. Spontaneous HCV clearance in HCV/HIV-1 coinfection associated with normalized CD4 counts, low level of chronic immune activation and high level of T cell function. *J Clin Virol* **41**, 160-163, (2008).

97 Thein, H. H., Yi, Q., Dore, G. J. & Krahn, M. D. Natural history of hepatitis C virus infection in HIV-infected individuals and the impact of HIV in the era of highly active antiretroviral therapy: a meta-analysis. *AIDS* **22**, 1979-1991, (2008).

98 Salgame, P. Host innate and Th1 responses and the bacterial factors that control Mycobacterium tuberculosis infection. *Curr Opin Immunol* **17**, 374-380, (2005).

99 Whalen, C., Horsburgh, C. R., Hom, D., Lahart, C., Simberkoff, M. & Ellner, J. Accelerated course of human immunodeficiency virus infection after tuberculosis. *Am J Respir Crit Care Med* **151**, 129-135, (1995).

100 Whalen, C., Horsburgh, C. R., Jr., Hom, D., Lahart, C., Simberkoff, M. & Ellner, J. Site of disease and opportunistic infection predict survival in HIV-associated tuberculosis. *AIDS* **11**, 455-460, (1997).

101 Whalen, C. C., Nsubuga, P., Okwera, A., Johnson, J. L., Hom, D. L., Michael, N. L., Mugerwa, R. D. & Ellner, J. J. Impact of pulmonary tuberculosis on survival of HIV-infected adults: a prospective epidemiologic study in Uganda. *AIDS* **14**, 1219-1228, (2000).

102 Goletti, D., Weissman, D., Jackson, R. W., Graham, N. M., Vlahov, D., Klein, R. S., Munsiff, S. S., Ortona, L., Cauda, R. & Fauci, A. S. Effect of Mycobacterium tuberculosis on HIV replication. Role of immune activation. *J Immunol* **157**, 1271-1278, (1996).

103 Sakula, A. Robert Koch: centenary of the discovery of the tubercle bacillus, 1882. *Thorax* **37**, 246-251, (1982).
104 Andersen, P., Doherty, T. M., Pai, M. & Weldingh, K. The prognosis of latent tuberculosis: can disease be predicted? *Trends Mol Med* **13**, 175-182, (2007).
105 Cardona, P. J. New insights on the nature of latent tuberculosis infection and its treatment. *Inflamm Allergy Drug Targets* **6**, 27-39, (2007).
106 Tufariello, J. M., Chan, J. & Flynn, J. L. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect Dis* **3**, 578-590, (2003).
107 Cahn, P., Perez, H., Ben, G. & Ochoa, C. Tuberculosis and HIV: a partnership against the most vulnerable. *J Int Assoc Physicians AIDS Care (Chic Ill)* **2**, 106-123, (2003).
108 Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C. & Dye, C. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med* **163**, 1009-1021, (2003).
109 Rangaka, M. X., Diwakar, L., Seldon, R., van Cutsem, G., Meintjes, G. A., Morroni, C., Mouton, P., Shey, M. S., Maartens, G., Wilkinson, K. A. & Wilkinson, R. J. Clinical, immunological, and epidemiological importance of antituberculosis T cell responses in HIV-infected Africans. *Clin Infect Dis* **44**, 1639-1646, (2007).
110 Mogues, T., Goodrich, M. E., Ryan, L., LaCourse, R. & North, R. J. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *J Exp Med* **193**, 271-280, (2001).
111 Aabye, M. G., Ravn, P., PrayGod, G., Jeremiah, K., Mugomela, A., Jepsen, M., Faurholt, D., Range, N., Friis, H., Changalucha, J. & Andersen, A. B. The impact of HIV infection and CD4 cell count on the performance of an interferon gamma release assay in patients with pulmonary tuberculosis. *PLoS ONE* **4**, e4220, (2009).
112 Bonnet, M. M., Pinoges, L. L., Varaine, F. F., Oberhauser, B. B., O'Brien, D. D., Kebede, Y. Y., Hewison, C. C., Zachariah, R. R. & Ferradini, L. L. Tuberculosis after HAART initiation in HIV-positive patients from five countries with a high tuberculosis burden. *Aids* **20**, 1275-1279, (2006).
113 Pathan, A. A., Wilkinson, K. A., Klenerman, P., McShane, H., Davidson, R. N., Pasvol, G., Hill, A. V. & Lalvani, A. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in Mycobacterium tuberculosis-infected individuals: associations with clinical disease state and effect of treatment. *J Immunol* **167**, 5217-5225, (2001).
114 Sutherland, R., Yang, H., Scriba, T. J., Ondondo, B., Robinson, N., Conlon, C., Suttill, A., McShane, H., Fidler, S., McMichael, A. & Dorrell, L. Impaired IFN-gamma-secreting capacity in mycobacterial antigen-specific CD4 T cells during chronic HIV-1 infection despite long-term HAART. *Aids* **20**, 821-829, (2006).
115 Ellner, J. J., Hirsch, C. S. & Whalen, C. C. Correlates of protective immunity to Mycobacterium tuberculosis in humans. *Clin Infect Dis* **30 Suppl 3**, S279-282, (2000).
116 Hashemi, M., Sharifi-Mood, B., Nezamdoost, M., Moazeni-Roodi, A., Naderi, M., Kouhpayeh, H., Taheri, M. & Ghavami, S. Functional polymorphism of interferon-gamma (IFN-gamma) gene +874T/A polymorphism is associated

with pulmonary tuberculosis in Zahedan, Southeast Iran. *Prague Med Rep* **112**, 38-43, (2011).

117 Qu, H. Q., Fisher-Hoch, S. P. & McCormick, J. B. Molecular immunity to mycobacteria: knowledge from the mutation and phenotype spectrum analysis of Mendelian susceptibility to mycobacterial diseases. *Int J Infect Dis* **15**, e305-313, (2011).

118 Karam, F., Mbow, F., Fletcher, H., Senghor, C. S., Coulibaly, K. D., LeFevre, A. M., Ngom Gueye, N. F., Dieye, T., Sow, P. S., Mboup, S. & Lienhardt, C. Sensitivity of IFN-gamma release assay to detect latent tuberculosis infection is retained in HIV-infected patients but dependent on HIV/AIDS progression. *PLoS ONE* **3**, e1441, (2008).

119 Lalvani, A. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest* **131**, 1898-1906, (2007).

120 Leidl, L., Mayanja-Kizza, H., Sotgiu, G., Baseke, J., Ernst, M., Hirsch, C., Goletti, D., Toossi, Z. & Lange, C. Relationship of immunodiagnostic assays for tuberculosis and numbers of circulating CD4+ T-cells in HIV infection. *Eur Respir J* **35**, 619-626, (2010).

121 Geldmacher, C., Ngwenyama, N., Schuetz, A., Petrovas, C., Reither, K., Heeregrave, E. J., Casazza, J. P., Ambrozak, D. R., Louder, M., Ampofo, W., Pollakis, G., Hill, B., Sanga, E., Saathoff, E., Maboko, L., Roederer, M., Paxton, W. A., Hoelscher, M. & Koup, R. A. Preferential infection and depletion of Mycobacterium tuberculosis-specific CD4 T cells after HIV-1 infection. *J Exp Med* **207**, 2869-2881, (2010).

122 Geldmacher, C., Schuetz, A., Ngwenyama, N., Casazza, J. P., Sanga, E., Saathoff, E., Boehme, C., Geis, S., Maboko, L., Singh, M., Minja, F., Meyerhans, A., Koup, R. A. & Hoelscher, M. Early depletion of Mycobacterium tuberculosis-specific T helper 1 cell responses after HIV-1 infection. *J Infect Dis* **198**, 1590-1598, (2008).

123 Hanna, L. E., Bose, J. C., Nayak, K., Subramanyam, S. & Swaminathan, S. Short communication: Influence of active tuberculosis on chemokine and chemokine receptor expression in HIV-infected persons. *AIDS Res Hum Retroviruses* **21**, 997-1002, (2005).

124 Hoshino, Y., Tse, D. B., Rochford, G., Prabhakar, S., Hoshino, S., Chitkara, N., Kuwabara, K., Ching, E., Raju, B., Gold, J. A., Borkowsky, W., Rom, W. N., Pine, R. & Weiden, M. Mycobacterium tuberculosis-induced CXCR4 and chemokine expression leads to preferential X4 HIV-1 replication in human macrophages. *J Immunol* **172**, 6251-6258, (2004).

125 Ranjbar, S., Ly, N., Thim, S., Reynes, J. M. & Goldfeld, A. E. Mycobacterium tuberculosis recall antigens suppress HIV-1 replication in anergic donor cells via CD8+ T cell expansion and increased IL-10 levels. *J Immunol* **172**, 1953-1959, (2004).

126 Toossi, Z. Virological and immunological impact of tuberculosis on human immunodeficiency virus type 1 disease. *J Infect Dis* **188**, 1146-1155, (2003).

127 Ma, Z. M., Stone, M., Piatak, M., Jr., Schweighardt, B., Haigwood, N. L., Montefiori, D., Lifson, J. D., Busch, M. P. & Miller, C. J. High specific infectivity of plasma virus from the pre-ramp-up and ramp-up stages of acute simian immunodeficiency virus infection. *J Virol* **83**, 3288-3297, (2009).

128 Betts, M. R., Krowka, J. F., Kepler, T. B., Davidian, M., Christopherson, C., Kwok, S., Louie, L., Eron, J., Sheppard, H. & Frelinger, J. A. Human immunodeficiency virus type 1-specific cytotoxic T lymphocyte activity is

inversely correlated with HIV type 1 viral load in HIV type 1-infected long-term survivors. *AIDS Res Hum Retroviruses* **15**, 1219-1228, (1999).

129 Chen, Y., Winchester, R., Korber, B., Gagliano, J., Bryson, Y., Hutto, C., Martin, N., McSherry, G., Petru, A., Wara, D. & Ammann, A. Influence of HLA alleles on the rate of progression of vertically transmitted HIV infection in children: association of several HLA-DR13 alleles with long-term survivorship and the potential association of HLA-A*2301 with rapid progression to AIDS. Long-Term Survivor Study. *Hum Immunol* **55**, 154-162, (1997).

130 Ciccone, E. J., Greenwald, J. H., Lee, P. I., Biancotto, A., Read, S. W., Yao, M. A., Hodge, J. N., Thompson, W. L., Kovacs, S. B., Chairez, C. L., Migueles, S. A., Kovacs, J. A., Margolis, L. B. & Sereti, I. CD4+ T cells, including Th17 and cycling subsets, are intact in the gut mucosa of HIV-1-infected long-term nonprogressors. *J Virol* **85**, 5880-5888, (2011).

131 Crotti, A., Neri, F., Corti, D., Ghezzi, S., Heltai, S., Baur, A., Poli, G., Santagostino, E. & Vicenzi, E. Nef alleles from human immunodeficiency virus type 1-infected long-term-nonprogressor hemophiliacs with or without late disease progression are defective in enhancing virus replication and CD4 down-regulation. *J Virol* **80**, 10663-10674, (2006).

132 Ferre, A. L., Hunt, P. W., Critchfield, J. W., Young, D. H., Morris, M. M., Garcia, J. C., Pollard, R. B., Yee, H. F., Jr., Martin, J. N., Deeks, S. G. & Shacklett, B. L. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. *Blood* **113**, 3978-3989, (2009).

133 Ferre, A. L., Hunt, P. W., McConnell, D. H., Morris, M. M., Garcia, J. C., Pollard, R. B., Yee, H. F., Jr., Martin, J. N., Deeks, S. G. & Shacklett, B. L. HIV controllers with HLA-DRB1*13 and HLA-DQB1*06 alleles have strong, polyfunctional mucosal CD4+ T-cell responses. *J Virol* **84**, 11020-11029, (2010).

134 Addo, M. M., Altfeld, M., Brainard, D. M., Rathod, A., Piechocka-Trocha, A., Fideli, U., Mulenga, J., Shutes, E., Alvino, D. M., Hunter, E., Allen, S. A. & Walker, B. D. Lack of detectable HIV-1-specific CD8(+) T cell responses in Zambian HIV-1-exposed seronegative partners of HIV-1-positive individuals. *J Infect Dis* **203**, 258-262, (2011).

135 Alimonti, J. B., Kimani, J., Matu, L., Wachihi, C., Kaul, R., Plummer, F. A. & Fowke, K. R. Characterization of CD8 T-cell responses in HIV-1-exposed seronegative commercial sex workers from Nairobi, Kenya. *Immunol Cell Biol* **84**, 482-485, (2006).

136 Beretta, A., Furci, L., Burastero, S., Cosma, A., Dinelli, M. E., Lopalco, L., DeSantis, C., Tambussi, G., Carrow, E., Sabbatani, S., Clerici, M., Lazzarin, A. & Siccardi, A. G. HIV-1-specific immunity in persistently seronegative individuals at high risk for HIV infection. *Immunol Lett* **51**, 39-43, (1996).

137 Clerici, M., Giorgi, J. V., Chou, C. C., Gudeman, V. K., Zack, J. A., Gupta, P., Ho, H. N., Nishanian, P. G., Berzofsky, J. A. & Shearer, G. M. Cell-mediated immune response to human immunodeficiency virus (HIV) type 1 in seronegative homosexual men with recent sexual exposure to HIV-1. *J Infect Dis* **165**, 1012-1019, (1992).

138 Clerici, M., Levin, J. M., Kessler, H. A., Harris, A., Berzofsky, J. A., Landay, A. L. & Shearer, G. M. HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood. *JAMA* **271**, 42-46, (1994).

139 Delwart, E., Bernardin, F., Lee, T. H., Winkelman, V., Liu, C., Sheppard, H., Liu, A., Greenblatt, R., Anastos, K., DeHovitz, J., Nowicki, M., Cohen, M., Golub, E. T., Barbour, J., Buchbinder, S. & Busch, M. P. Absence of reproducibly detectable low-level HIV viremia in highly exposed seronegative men and women. *AIDS* **25**, 619-623, (2011).

140 Young, J. M., Turpin, J. A., Musib, R. & Sharma, O. K. Outcomes of a National Institute of Allergy and Infectious Diseases Workshop on Understanding HIV-Exposed but Seronegative Individuals. *AIDS Res Hum Retroviruses*, (2011).

141 Zalenskaya, I. A., Cerocchi, O. G., Joseph, T., Donaghay, M. A., Schriver, S. D. & Doncel, G. F. Increased COX-2 expression in human vaginal epithelial cells exposed to nonoxynol-9, a vaginal contraceptive microbicide that failed to protect women from HIV-1 infection. *Am J Reprod Immunol* **65**, 569-577, (2011).

142 Boulet, S., Sharafi, S., Simic, N., Bruneau, J., Routy, J. P., Tsoukas, C. M. & Bernard, N. F. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. *AIDS* **22**, 595-599, (2008).

143 Broliden, K., Hinkula, J., Devito, C., Kiama, P., Kimani, J., Trabbatoni, D., Bwayo, J. J., Clerici, M., Plummer, F. & Kaul, R. Functional HIV-1 specific IgA antibodies in HIV-1 exposed, persistently IgG seronegative female sex workers. *Immunol Lett* **79**, 29-36, (2001).

144 Card, C. M., McLaren, P. J., Wachihi, C., Kimani, J., Plummer, F. A. & Fowke, K. R. Decreased Immune Activation in Resistance to HIV-1 Infection Is Associated with an Elevated Frequency of CD4(+)CD25(+)FOXP3(+) Regulatory T Cells. *J Infect Dis*, (2009).

145 Kebba, A., Kaleebu, P., Serwanga, J., Rowland, S., Yirrell, D., Downing, R., Gilmour, J., Imami, N., Gotch, F. & Whitworth, J. HIV type 1 antigen-responsive CD4+ T-lymphocytes in exposed yet HIV Type 1 seronegative Ugandans. *AIDS Res Hum Retroviruses* **20**, 67-75, (2004).

146 Phillips, R. E., Rowland-Jones, S., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R. & et al. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **354**, 453-459, (1991).

147 Rowland-Jones, S., Sutton, J., Ariyoshi, K., Dong, T., Gotch, F., McAdam, S., Whitby, D., Sabally, S., Gallimore, A., Corrah, T. & et al. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nat Med* **1**, 59-64, (1995).

148 Perez, C. L., Hasselrot, K., Bratt, G., Broliden, K. & Karlsson, A. C. Induction of systemic HIV-1-specific cellular immune responses by oral exposure in the uninfected partner of discordant couples. *AIDS* **24**, 969-974, (2010).

149 Iqbal, S. M., Ball, T. B., Kimani, J., Kiama, P., Thottingal, P., Embree, J. E., Fowke, K. R. & Plummer, F. A. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. *J Infect Dis* **192**, 728-738, (2005).

150 Jennes, W., Vuylsteke, B., Borget, M. Y., Traore-Ettiegne, V., Maurice, C., Nolan, M., Nkengasong, J. N. & Kestens, L. HIV-specific T helper responses and frequency of exposure among HIV-exposed seronegative female sex workers in Abidjan, Cote d'Ivoire. *J Infect Dis* **189**, 602-610, (2004).

151 Kaul, R., Dong, T., Plummer, F. A., Kimani, J., Rostron, T., Kiama, P., Njagi, E., Irungu, E., Farah, B., Oyugi, J., Chakraborty, R., MacDonald, K. S.,

Bwayo, J. J., McMichael, A. & Rowland-Jones, S. L. CD8(+) lymphocytes respond to different HIV epitopes in seronegative and infected subjects. *J Clin Invest* **107**, 1303-1310, (2001).

152 Kaul, R., Rowland-Jones, S. L., Kimani, J., Dong, T., Yang, H. B., Kiama, P., Rostron, T., Njagi, E., Bwayo, J. J., MacDonald, K. S., McMichael, A. J. & Plummer, F. A. Late seroconversion in HIV-resistant Nairobi prostitutes despite pre-existing HIV-specific CD8+ responses. *J Clin Invest* **107**, 341-349, (2001).

153 Kaul, R., Trabattoni, D., Bwayo, J. J., Arienti, D., Zagliani, A., Mwangi, F. M., Kariuki, C., Ngugi, E. N., MacDonald, K. S., Ball, T. B., Clerici, M. & Plummer, F. A. HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. *AIDS* **13**, 23-29, (1999).

154 Ranki, A., Mattinen, S., Yarchoan, R., Broder, S., Ghrayeb, J., Lahdevirta, J. & Krohn, K. T-cell response towards HIV in infected individuals with and without zidovudine therapy, and in HIV-exposed sexual partners. *AIDS* **3**, 63-69, (1989).

155 Hladik, F., Desbien, A., Lang, J., Wang, L., Ding, Y., Holte, S., Wilson, A., Xu, Y., Moerbe, M., Schmechel, S. & McElrath, M. J. Most highly exposed seronegative men lack HIV-1-specific, IFN-gamma-secreting T cells. *J Immunol* **171**, 2671-2683, (2003).

156 Schmechel, S. C., Russell, N., Hladik, F., Lang, J., Wilson, A., Ha, R., Desbien, A. & McElrath, M. J. Immune defence against HIV-1 infection in HIV-1-exposed seronegative persons. *Immunol Lett* **79**, 21-27, (2001).

157 Erickson, A. L., Willberg, C. B., McMahan, V., Liu, A., Buchbinder, S. P., Grohskopf, L. A., Grant, R. M. & Nixon, D. F. Potentially exposed but uninfected individuals produce cytotoxic and polyfunctional human immunodeficiency virus type 1-specific CD8(+) T-cell responses which can be defined to the epitope level. *Clin Vaccine Immunol* **15**, 1745-1748, (2008).

158 Rowland-Jones, S. L., Nixon, D. F., Aldhous, M. C., Gotch, F., Ariyoshi, K., Hallam, N., Kroll, J. S., Froebel, K. & McMichael, A. HIV-specific cytotoxic T-cell activity in an HIV-exposed but uninfected infant. *Lancet* **341**, 860-861, (1993).

159 Kuhn, L., Coutsoudis, A., Moodley, D., Trabattoni, D., Mngqundaniso, N., Shearer, G. M., Clerici, M., Coovadia, H. M. & Stein, Z. T-helper cell responses to HIV envelope peptides in cord blood: protection against intrapartum and breast-feeding transmission. *AIDS* **15**, 1-9, (2001).

160 John-Stewart, G. C., Mbori-Ngacha, D., Payne, B. L., Farquhar, C., Richardson, B. A., Emery, S., Otieno, P., Obimbo, E., Dong, T., Slyker, J., Nduati, R., Overbaugh, J. & Rowland-Jones, S. HV-1-specific cytotoxic T lymphocytes and breast milk HIV-1 transmission. *J Infect Dis* **199**, 889-898, (2009).

161 Lynch, J. B., Nduati, R., Blish, C. A., Richardson, B. A., Mabuka, J. M., Jalalian-Lechak, Z., John-Stewart, G. & Overbaugh, J. The breadth and potency of passively acquired human immunodeficiency virus type 1-specific neutralizing antibodies do not correlate with the risk of infant infection. *J Virol* **85**, 5252-5261, (2011).

162 Clerici, M., Sison, A. V., Berzofsky, J. A., Rakusan, T. A., Brandt, C. D., Ellaurie, M., Villa, M., Colie, C., Venzon, D. J., Sever, J. L. & et al. Cellular immune factors associated with mother-to-infant transmission of HIV. *AIDS* **7**, 1427-1433, (1993).

163 Pinto, L. A., Sullivan, J., Berzofsky, J. A., Clerici, M., Kessler, H. A., Landay, A. L. & Shearer, G. M. ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *J Clin Invest* **96**, 867-876, (1995).

164 Pinto, L. A., Landay, A. L., Berzofsky, J. A., Kessler, H. A. & Shearer, G. M. Immune response to human immunodeficiency virus (HIV) in healthcare workers occupationally exposed to HIV-contaminated blood. *Am J Med* **102**, 21-24, (1997).

165 Yasukawa, M., Hato, T., Matsumoto, M., Takada, K., Fujita, S. & Kobayashi, Y. Proliferation of double-negative (CD4-CD8-) T cells bearing T-cell receptor-alpha beta in a haemophiliac with human immunodeficiency virus type 1 infection and factor VIII inhibitor: functional properties of double-negative T-cell receptor-alpha beta+ T cells. *Br J Haematol* **79**, 372-376, (1991).

166 Tenenbaum, S. A., Morris, C. A., Alexander, S. S., McFerrin, H. E., Garry, R. F. & Leissinger, C. A. Evidence of HIV exposure and transient seroreactivity in archived HIV-negative severe hemophiliac sera. *Virol J* **2**, 65, (2005).

167 Lederman, M. M. Haemophilia, human immunodeficiency virus and human immunodeficiency virus pathogenesis. *Thromb Haemost* **104**, 911-914, (2010).

168 Kramer, H. B., Lavender, K. J., Qin, L., Stacey, A. R., Liu, M. K., di Gleria, K., Simmons, A., Gasper-Smith, N., Haynes, B. F., McMichael, A. J., Borrow, P. & Kessler, B. M. Elevation of intact and proteolytic fragments of acute phase proteins constitutes the earliest systemic antiviral response in HIV-1 infection. *PLoS Pathog* **6**, e1000893, (2010).

169 Daar, E. S., Moudgil, T., Meyer, R. D. & Ho, D. D. Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N Engl J Med* **324**, 961-964, (1991).

170 Clark, S. J., Saag, M. S., Decker, W. D., Campbell-Hill, S., Roberson, J. L., Veldkamp, P. J., Kappes, J. C., Hahn, B. H. & Shaw, G. M. High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N Engl J Med* **324**, 954-960, (1991).

171 Misse, D., Yssel, H., Trabattoni, D., Oblet, C., Lo Caputo, S., Mazzotta, F., Pene, J., Gonzalez, J. P., Clerici, M. & Veas, F. IL-22 participates in an innate anti-HIV-1 host-resistance network through acute-phase protein induction. *J Immunol* **178**, 407-415, (2007).

172 Stacey, A. R., Norris, P. J., Qin, L., Haygreen, E. A., Taylor, E., Heitman, J., Lebedeva, M., DeCamp, A., Li, D., Grove, D., Self, S. G. & Borrow, P. Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. *J Virol* **83**, 3719-3733, (2009).

173 Nemes, E., Bertoncelli, L., Lugli, E., Pinti, M., Nasi, M., Manzini, L., Manzini, S., Prati, F., Borghi, V., Cossarizza, A. & Mussini, C. Cytotoxic granule release dominates gag-specific CD4+ T-cell response in different phases of HIV infection. *AIDS* **24**, 947-957, (2010).

174 Ruhwald, M. & Ravn, P. Immune reconstitution syndrome in tuberculosis and HIV-co-infected patients: Th1 explosion or cytokine storm? *Aids* **21**, 882-884, (2007).

175 Cheney, K. M. & McKnight, A. Interferon-alpha mediates restriction of human immunodeficiency virus type-1 replication in primary human macrophages at an early stage of replication. *PLoS ONE* **5**, e13521, (2010).
176 Klein, S. A., Dobmeyer, J. M., Dobmeyer, T. S., Falke, D., Kabelitz, D., Friese, K., Helm, E. B., Hoelzer, D. & Rossol-Voth, R. TNF-alpha mediated apoptosis of CD4 positive T-lymphocytes. A model of T-cell depletion in HIV infected individuals. *Eur J Med Res* **1**, 249-258, (1996).
177 Marrack, P., Kappler, J. & Mitchell, T. Type I interferons keep activated T cells alive. *J Exp Med* **189**, 521-530, (1999).
178 Rodriguez, B., Lederman, M. M., Jiang, W., Bazdar, D. A., Garate, K., Harding, C. V. & Sieg, S. F. Interferon-alpha differentially rescues CD4 and CD8 T cells from apoptosis in HIV infection. *AIDS* **20**, 1379-1389, (2006).
179 Herbeuval, J. P. & Shearer, G. M. HIV-1 immunopathogenesis: how good interferon turns bad. *Clin Immunol* **123**, 121-128, (2007).
180 Cho, S. S., Bacon, C. M., Sudarshan, C., Rees, R. C., Finbloom, D., Pine, R. & O'Shea, J. J. Activation of STAT4 by IL-12 and IFN-alpha: evidence for the involvement of ligand-induced tyrosine and serine phosphorylation. *J Immunol* **157**, 4781-4789, (1996).
181 Biasin, M., Piacentini, L., Lo Caputo, S., Kanari, Y., Magri, G., Trabattoni, D., Naddeo, V., Lopalco, L., Clivio, A., Cesana, E., Fasano, F., Bergamaschi, C., Mazzotta, F., Miyazawa, M. & Clerici, M. Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals. *J Infect Dis* **195**, 960-964, (2007).
182 Dang, Y., Siew, L. M. & Zheng, Y. H. APOBEC3G is degraded by the proteasomal pathway in a Vif-dependent manner without being polyubiquitylated. *J Biol Chem* **283**, 13124-13131, (2008).
183 Vazquez-Perez, J. A., Ormsby, C. E., Hernandez-Juan, R., Torres, K. J. & Reyes-Teran, G. APOBEC3G mRNA expression in exposed seronegative and early stage HIV infected individuals decreases with removal of exposure and with disease progression. *Retrovirology* **6**, 23, (2009).
184 Casartelli, N., Guivel-Benhassine, F., Bouziat, R., Brandler, S., Schwartz, O. & Moris, A. The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells. *J Exp Med* **207**, 39-49, (2010).
185 Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D. J., Sundquist, W. I. & Sodroski, J. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* **103**, 5514-5519, (2006).
186 Letvin, N. L., Rao, S. S., Montefiori, D. C., Seaman, M. S., Sun, Y., Lim, S. Y., Yeh, W. W., Asmal, M., Gelman, R. S., Shen, L., Whitney, J. B., Seoighe, C., Lacerda, M., Keating, S., Norris, P. J., Hudgens, M. G., Gilbert, P. B., Buzby, A. P., Mach, L. V., Zhang, J., Balachandran, H., Shaw, G. M., Schmidt, S. D., Todd, J. P., Dodson, A., Mascola, J. R. & Nabel, G. J. Immune and Genetic Correlates of Vaccine Protection Against Mucosal Infection by SIV in Monkeys. *Sci Transl Med* **3**, 81ra36, (2011).
187 Casartelli, N., Sourisseau, M., Feldmann, J., Guivel-Benhassine, F., Mallet, A., Marcelin, A. G., Guatelli, J. & Schwartz, O. Tetherin restricts productive HIV-1 cell-to-cell transmission. *PLoS Pathog* **6**, e1000955, (2010).
188 Martin, M. P., Gao, X., Lee, J. H., Nelson, G. W., Detels, R., Goedert, J. J., Buchbinder, S., Hoots, K., Vlahov, D., Trowsdale, J., Wilson, M., O'Brien, S.

J. & Carrington, M. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* **31**, 429-434, (2002).

189 Scott-Algara, D., Truong, L. X., Versmisse, P., David, A., Luong, T. T., Nguyen, N. V., Theodorou, I., Barre-Sinoussi, F. & Pancino, G. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. *J Immunol* **171**, 5663-5667, (2003).

190 Montoya, C. J., Velilla, P. A., Chougnet, C., Landay, A. L. & Rugeles, M. T. Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. *Clin Immunol* **120**, 138-146, (2006).

191 Ravet, S., Scott-Algara, D., Bonnet, E., Tran, H. K., Tran, T., Nguyen, N., Truong, L. X., Theodorou, I., Barre-Sinoussi, F., Pancino, G. & Paul, P. Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals. *Blood* **109**, 4296-4305, (2007).

192 Alter, G., Martin, M. P., Teigen, N., Carr, W. H., Suscovich, T. J., Schneidewind, A., Streeck, H., Waring, M., Meier, A., Brander, C., Lifson, J. D., Allen, T. M., Carrington, M. & Altfeld, M. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* **204**, 3027-3036, (2007).

193 Martin, M. P., Qi, Y., Gao, X., Yamada, E., Martin, J. N., Pereyra, F., Colombo, S., Brown, E. E., Shupert, W. L., Phair, J., Goedert, J. J., Buchbinder, S., Kirk, G. D., Telenti, A., Connors, M., O'Brien, S. J., Walker, B. D., Parham, P., Deeks, S. G., McVicar, D. W. & Carrington, M. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet* **39**, 733-740, (2007).

194 Alter, G., Teigen, N., Ahern, R., Streeck, H., Meier, A., Rosenberg, E. S. & Altfeld, M. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J Infect Dis* **195**, 1452-1460, (2007).

195 Vieillard, V., Fausther-Bovendo, H., Samri, A. & Debre, P. Specific phenotypic and functional features of natural killer cells from HIV-infected long-term nonprogressors and HIV controllers. *J Acquir Immune Defic Syndr* **53**, 564-573, (2010).

196 Older Aguilar, A. M., Guethlein, L. A., Adams, E. J., Abi-Rached, L., Moesta, A. K. & Parham, P. Coevolution of killer cell Ig-like receptors with HLA-C to become the major variable regulators of human NK cells. *J Immunol* **185**, 4238-4251, (2010).

197 Gumperz, J. E., Barber, L. D., Valiante, N. M., Percival, L., Phillips, J. H., Lanier, L. L. & Parham, P. Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol* **158**, 5237-5241, (1997).

198 Fellay, J., Shianna, K. V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A., Cossarizza, A., Cozzi-Lepri, A., De Luca, A., Easterbrook, P., Francioli, P., Mallal, S., Martinez-Picado, J., Miro, J. M., Obel, N., Smith, J. P., Wyniger, J., Descombes, P., Antonarakis, S. E., Letvin, N. L., McMichael, A. J., Haynes, B. F., Telenti, A. & Goldstein, D. B. A whole-genome association study of major determinants for host control of HIV-1. *Science* **317**, 944-947, (2007).

199 Kulpa, D. A. & Collins, K. L. The emerging role of HLA-C in HIV-1 infection. *Immunology* **134**, 116-122, (2011).

200 Tran, H. K., Chartier, L., Troung, L. X., Nguyen, N. N., Fontanet, A., Barre-Sinoussi, F. E., Pancino, G. & Scott-Algara, D. Systemic immune activation in HIV-1-exposed uninfected Vietnamese intravascular drug users. *AIDS Res Hum Retroviruses* **22**, 255-261, (2006).

201 Iwasaki, A. Mucosal dendritic cells. *Annu Rev Immunol* **25**, 381-418, (2007).

202 Guermonprez, P. & Amigorena, S. Pathways for antigen cross presentation. *Springer Semin Immunopathol* **26**, 257-271, (2005).

203 Larsson, M., Fonteneau, J. F., Lirvall, M., Haslett, P., Lifson, J. D. & Bhardwaj, N. Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells: roles for cross-presentation and non-infectious HIV-1 virus. *AIDS* **16**, 1319-1329, (2002).

204 Wijewardana, V., Soloff, A. C., Liu, X., Brown, K. N. & Barratt-Boyes, S. M. Early myeloid dendritic cell dysregulation is predictive of disease progression in simian immunodeficiency virus infection. *PLoS Pathog* **6**, e1001235, (2010).

205 Lichtner, M., Rossi, R., Rizza, M. C., Mengoni, F., Sauzullo, I., Massetti, A. P., Luzi, G., Hosmalin, A., Mastroianni, C. M. & Vullo, V. Plasmacytoid dendritic cells count in antiretroviral-treated patients is predictive of HIV load control independent of CD4+ T-cell count. *Curr HIV Res* **6**, 19-27, (2008).

206 Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. & Ho, D. D. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68**, 4650-4655, (1994).

207 Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D. & Shaw, G. M. Antibody neutralization and escape by HIV-1. *Nature* **422**, 307-312, (2003).

208 Hoxie, J. A. Toward an antibody-based HIV-1 vaccine. *Annu Rev Med* **61**, 135-152, (2010).

209 Alter, G. & Moody, M. A. The humoral response to HIV-1: new insights, renewed focus. *J Infect Dis* **202 Suppl 2**, S315-322, (2010).

210 Richman, D. D., Wrin, T., Little, S. J. & Petropoulos, C. J. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* **100**, 4144-4149, (2003).

211 Gilbert, P. B., Peterson, M. L., Follmann, D., Hudgens, M. G., Francis, D. P., Gurwith, M., Heyward, W. L., Jobes, D. V., Popovic, V., Self, S. G., Sinangil, F., Burke, D. & Berman, P. W. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J Infect Dis* **191**, 666-677, (2005).

212 Sagar, M., Wu, X., Lee, S. & Overbaugh, J. Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. *J Virol* **80**, 9586-9598, (2006).

213 Deeks, S. G., Schweighardt, B., Wrin, T., Galovich, J., Hoh, R., Sinclair, E., Hunt, P., McCune, J. M., Martin, J. N., Petropoulos, C. J. & Hecht, F. M. Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol* **80**, 6155-6164, (2006).

214 Rusert, P., Krarup, A., Magnus, C., Brandenberg, O. F., Weber, J., Ehlert, A. K., Regoes, R. R., Gunthard, H. F. & Trkola, A. Interaction of the gp120 V1V2 loop with a neighboring gp120 unit shields the HIV envelope trimer against cross-neutralizing antibodies. *J Exp Med* **208**, 1419-1433, (2011).

215 Burton, D. R., Hessell, A. J., Keele, B. F., Klasse, P. J., Ketas, T. A., Moldt, B., Dunlop, D. C., Poignard, P., Doyle, L. A., Cavacini, L., Veazey, R. S. & Moore, J. P. Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody. *Proc Natl Acad Sci U S A* **108**, 11181-11186, (2011).

216 Moore, P. L., Ranchobe, N., Lambson, B. E., Gray, E. S., Cave, E., Abrahams, M. R., Bandawe, G., Mlisana, K., Abdool Karim, S. S., Williamson, C. & Morris, L. Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. *PLoS Pathog* **5**, e1000598, (2009).

217 Moir, S., Ho, J., Malaspina, A., Wang, W., DiPoto, A. C., O'Shea, M. A., Roby, G., Kottilil, S., Arthos, J., Proschan, M. A., Chun, T. W. & Fauci, A. S. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med* **205**, 1797-1805, (2008).

218 Shacklett, B. L. Immune responses to HIV and SIV in mucosal tissues: 'location, location, location'. *Curr Opin HIV AIDS* **5**, 128-134, (2010).

219 Shen, X. & Tomaras, G. D. Alterations of the B-cell response by HIV-1 replication. *Curr HIV/AIDS Rep* **8**, 23-30, (2011).

220 Harro, C. D., Judson, F. N., Gorse, G. J., Mayer, K. H., Kostman, J. R., Brown, S. J., Koblin, B., Marmor, M., Bartholow, B. N. & Popovic, V. Recruitment and baseline epidemiologic profile of participants in the first phase 3 HIV vaccine efficacy trial. *J Acquir Immune Defic Syndr* **37**, 1385-1392, (2004).

221 Jones, N. G., DeCamp, A., Gilbert, P., Peterson, M. L., Gurwith, M. & Cao, H. AIDSVAX immunization induces HIV-specific CD8+ T-cell responses in high-risk, HIV-negative volunteers who subsequently acquire HIV infection. *Vaccine* **27**, 1136-1140, (2009).

222 Gilbert, P., Wang, M., Wrin, T., Petropoulos, C., Gurwith, M., Sinangil, F., D'Souza, P., Rodriguez-Chavez, I. R., DeCamp, A., Giganti, M., Berman, P. W., Self, S. G. & Montefiori, D. C. Magnitude and breadth of a nonprotective neutralizing antibody response in an efficacy trial of a candidate HIV-1 gp120 vaccine. *J Infect Dis* **202**, 595-605, (2010).

223 Moir, S. & Fauci, A. S. B cells in HIV infection and disease. *Nat Rev Immunol* **9**, 235-245, (2009).

224 Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de Souza, M., Adams, E., Benenson, M., Gurunathan, S., Tartaglia, J., McNeil, J. G., Francis, D. P., Stablein, D., Birx, D. L., Chunsuttiwat, S., Khamboonruang, C., Thongcharoen, P., Robb, M. L., Michael, N. L., Kunasol, P. & Kim, J. H. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* **361**, 2209-2220, (2009).

225 Zinkernagel, R. M. & Doherty, P. C. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-702, (1974).

226 Zinkernagel, R. M. & Doherty, P. C. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* **251**, 547-548, (1974).
227 Miles, J. J., Douek, D. C. & Price, D. A. Bias in the alphabeta T-cell repertoire: implications for disease pathogenesis and vaccination. *Immunol Cell Biol* **89**, 375-387, (2011).
228 Collins, K. L. & Baltimore, D. HIV's evasion of the cellular immune response. *Immunol Rev* **168**, 65-74, (1999).
229 McMichael, A. J., Ogg, G., Wilson, J., Callan, M., Hambleton, S., Appay, V., Kelleher, T. & Rowland-Jones, S. Memory CD8+ T cells in HIV infection. *Philos Trans R Soc Lond B Biol Sci* **355**, 363-367, (2000).
230 Poropatich, K. & Sullivan, D. J., Jr. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. *J Gen Virol* **92**, 247-268, (2011).
231 Hedrick, S. M., Matis, L. A., Hecht, T. T., Samelson, L. E., Longo, D. L., Heber-Katz, E. & Schwartz, R. H. The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome c. *Cell* **30**, 141-152, (1982).
232 Jenkins, M. K. & Schwartz, R. H. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* **165**, 302-319, (1987).
233 Davis, M. M. & Bjorkman, P. J. T-cell antigen receptor genes and T-cell recognition. *Nature* **334**, 395-402, (1988).
234 Fujinami, R. S., Nelson, J. A., Walker, L. & Oldstone, M. B. Sequence homology and immunologic cross-reactivity of human cytomegalovirus with HLA-DR beta chain: a means for graft rejection and immunosuppression. *J Virol* **62**, 100-105, (1988).
235 Golding, H., Shearer, G. M., Hillman, K., Lucas, P., Manischewitz, J., Zajac, R. A., Clerici, M., Gress, R. E., Boswell, R. N. & Golding, B. Common epitope in human immunodeficiency virus (HIV) I-GP41 and HLA class II elicits immunosuppressive autoantibodies capable of contributing to immune dysfunction in HIV I-infected individuals. *J Clin Invest* **83**, 1430-1435, (1989).
236 Julg, B., Moodley, E. S., Qi, Y., Ramduth, D., Reddy, S., Mncube, Z., Gao, X., Goulder, P. J., Detels, R., Ndung'u, T., Walker, B. D. & Carrington, M. Possession of HLA class II DRB1*1303 associates with reduced viral loads in chronic HIV-1 clade C and B infection. *J Infect Dis* **203**, 803-809, (2011).
237 Berzofsky, J. A., Pendleton, C. D., Clerici, M., Ahlers, J., Lucey, D. R., Putney, S. D. & Shearer, G. M. Construction of peptides encompassing multideterminant clusters of human immunodeficiency virus envelope to induce in vitro T cell responses in mice and humans of multiple MHC types. *J Clin Invest* **88**, 876-884, (1991).
238 Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A., Lane, W. S. & Strominger, J. L. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* **178**, 27-47, (1993).
239 Barnaba, V., Watts, C., de Boer, M., Lane, P. & Lanzavecchia, A. Professional presentation of antigen by activated human T cells. *Eur J Immunol* **24**, 71-75, (1994).

240 Garrett, T. P., Saper, M. A., Bjorkman, P. J., Strominger, J. L. & Wiley, D. C. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* **342**, 692-696, (1989).
241 Saper, M. A., Bjorkman, P. J. & Wiley, D. C. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. *J Mol Biol* **219**, 277-319, (1991).
242 York, I. A. & Rock, K. L. Antigen processing and presentation by the class I major histocompatibility complex. *Annu Rev Immunol* **14**, 369-396, (1996).
243 Stevanovic, S. & Schild, H. Quantitative aspects of T cell activation--peptide generation and editing by MHC class I molecules. *Semin Immunol* **11**, 375-384, (1999).
244 Lanzavecchia, A., Lezzi, G. & Viola, A. From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell* **96**, 1-4, (1999).
245 Davis, M. M., Boniface, J. J., Reich, Z., Lyons, D., Hampl, J., Arden, B. & Chien, Y. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* **16**, 523-544, (1998).
246 Rudolph, M. G., Stanfield, R. L. & Wilson, I. A. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* **24**, 419-466, (2006).
247 Wilson, D. B., Wilson, D. H., Schroder, K., Pinilla, C., Blondelle, S., Houghten, R. A. & Garcia, K. C. Specificity and degeneracy of T cells. *Mol Immunol* **40**, 1047-1055, (2004).
248 Selin, L. K., Brehm, M. A., Naumov, Y. N., Cornberg, M., Kim, S. K., Clute, S. C. & Welsh, R. M. Memory of mice and men: CD8+ T-cell cross-reactivity and heterologous immunity. *Immunol Rev* **211**, 164-181, (2006).
249 Baumgartner, C. K., Ferrante, A., Nagaoka, M., Gorski, J. & Malherbe, L. P. Peptide-MHC class II complex stability governs CD4 T cell clonal selection. *J Immunol* **184**, 573-581, (2010).
250 Pereyra, F., Jia, X., McLaren, P. J., Telenti, A., de Bakker, P. I., Walker, B. D., Ripke, S., Brumme, C. J., Pulit, S. L., Carrington, M., Kadie, C. M., Carlson, J. M., Heckerman, D., Graham, R. R., Plenge, R. M., Deeks, S. G., Gianniny, L., Crawford, G., Sullivan, J., Gonzalez, E., Davies, L., Camargo, A., Moore, J. M., Beattie, N., Gupta, S., Crenshaw, A., Burtt, N. P., Guiducci, C., Gupta, N., Gao, X., Qi, Y., Yuki, Y., Piechocka-Trocha, A., Cutrell, E., Rosenberg, R., Moss, K. L., Lemay, P., O'Leary, J., Schaefer, T., Verma, P., Toth, I., Block, B., Baker, B., Rothchild, A., Lian, J., Proudfoot, J., Alvino, D. M., Vine, S., Addo, M. M., Allen, T. M., Altfeld, M., Henn, M. R., Le Gall, S., Streeck, H., Haas, D. W., Kuritzkes, D. R., Robbins, G. K., Shafer, R. W., Gulick, R. M., Shikuma, C. M., Haubrich, R., Riddler, S., Sax, P. E., Daar, E. S., Ribaudo, H. J., Agan, B., Agarwal, S., Ahern, R. L., Allen, B. L., Altidor, S., Altschuler, E. L., Ambardar, S., Anastos, K., Anderson, B., Anderson, V., Andrady, U., Antoniskis, D., Bangsberg, D., Barbaro, D., Barrie, W., Bartczak, J., Barton, S., Basden, P., Basgoz, N., Bazner, S., Bellos, N. C., Benson, A. M., Berger, J., Bernard, N. F., Bernard, A. M., Birch, C., Bodner, S. J., Bolan, R. K., Boudreaux, E. T., Bradley, M., Braun, J. F., Brndjar, J. E., Brown, S. J., Brown, K., Brown, S. T., Burack, J., Bush, L. M., Cafaro, V., Campbell, O., Campbell, J., Carlson, R. H., Carmichael, J. K., Casey, K. K., Cavacuiti, C., Celestin, G., Chambers, S. T., Chez, N., Chirch, L. M., Cimoch, P. J., Cohen, D., Cohn, L. E., Conway, B., Cooper, D. A., Cornelson, B., Cox, D. T., Cristofano, M. V., Cuchural, G., Jr., Czartoski, J. L., Dahman, J. M., Daly, J. S., Davis, B. T., Davis, K., Davod, S. M., DeJesus, E., Dietz, C. A.,

Dunham, E., Dunn, M. E., Ellerin, T. B., Eron, J. J., Fangman, J. J., Farel, C. E., Ferlazzo, H., Fidler, S., Fleenor-Ford, A., Frankel, R., Freedberg, K. A., French, N. K., Fuchs, J. D., Fuller, J. D., Gaberman, J., Gallant, J. E., Gandhi, R. T., Garcia, E., Garmon, D., Gathe, J. C., Jr., Gaultier, C. R., Gebre, W., Gilman, F. D., Gilson, I., Goepfert, P. A., Gottlieb, M. S., Goulston, C., Groger, R. K., Gurley, T. D., Haber, S., Hardwicke, R., Hardy, W. D., Harrigan, P. R., Hawkins, T. N., Heath, S., Hecht, F. M., Henry, W. K., Hladek, M., Hoffman, R. P., Horton, J. M., Hsu, R. K., Huhn, G. D., Hunt, P., Hupert, M. J., Illeman, M. L., Jaeger, H., Jellinger, R. M., John, M., Johnson, J. A., Johnson, K. L., Johnson, H., Johnson, K., Joly, J., Jordan, W. C., Kauffman, C. A., Khanlou, H., Killian, R. K., Kim, A. Y., Kim, D. D., Kinder, C. A., Kirchner, J. T., Kogelman, L., Kojic, E. M., Korthuis, P. T., Kurisu, W., Kwon, D. S., LaMar, M., Lampiris, H., Lanzafame, M., Lederman, M. M., Lee, D. M., Lee, J. M., Lee, M. J., Lee, E. T., Lemoine, J., Levy, J. A., Llibre, J. M., Liguori, M. A., Little, S. J., Liu, A. Y., Lopez, A. J., Loutfy, M. R., Loy, D., Mohammed, D. Y., Man, A., Mansour, M. K., Marconi, V. C., Markowitz, M., Marques, R., Martin, J. N., Martin, H. L., Jr., Mayer, K. H., McElrath, M. J., McGhee, T. A., McGovern, B. H., McGowan, K., McIntyre, D., McLeod, G. X., Menezes, P., Mesa, G., Metroka, C. E., Meyer-Olson, D., Miller, A. O., Montgomery, K., Mounzer, K. C., Nagami, E. H., Nagin, I., Nahass, R. G., Nelson, M. O., Nielsen, C., Norene, D. L., O'Connor, D. H., Ojikutu, B. O., Okulicz, J., Oladehin, O. O., Oldfield, E. C., 3rd, Olender, S. A., Ostrowski, M., Owen, W. F., Jr., Pae, E., Parsonnet, J., Pavlatos, A. M., Perlmutter, A. M., Pierce, M. N., Pincus, J. M., Pisani, L., Price, L. J., Proia, L., Prokesch, R. C., Pujet, H. C., Ramgopal, M., Rathod, A., Rausch, M., Ravishankar, J., Rhame, F. S., Richards, C. S., Richman, D. D., Rodes, B., Rodriguez, M., Rose, R. C., 3rd, Rosenberg, E. S., Rosenthal, D., Ross, P. E., Rubin, D. S., Rumbaugh, E., Saenz, L., Salvaggio, M. R., Sanchez, W. C., Sanjana, V. M., Santiago, S., Schmidt, W., Schuitemaker, H., Sestak, P. M., Shalit, P., Shay, W., Shirvani, V. N., Silebi, V. I., Sizemore, J. M., Jr., Skolnik, P. R., Sokol-Anderson, M., Sosman, J. M., Stabile, P., Stapleton, J. T., Starrett, S., Stein, F., Stellbrink, H. J., Sterman, F. L., Stone, V. E., Stone, D. R., Tambussi, G., Taplitz, R. A., Tedaldi, E. M., Theisen, W., Torres, R., Tosiello, L., Tremblay, C., Tribble, M. A., Trinh, P. D., Tsao, A., Ueda, P., Vaccaro, A., Valadas, E., Vanig, T. J., Vecino, I., Vega, V. M., Veikley, W., Wade, B. H., Walworth, C., Wanidworanun, C., Ward, D. J., Warner, D. A., Weber, R. D., Webster, D., Weis, S., Wheeler, D. A., White, D. J., Wilkins, E., Winston, A., Wlodaver, C. G., van't Wout, A., Wright, D. P., Yang, O. O., Yurdin, D. L., Zabukovic, B. W., Zachary, K. C., Zeeman, B. & Zhao, M. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science* **330**, 1551-1557, (2010).

251 Kaslow, R. A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A. J., Goedert, J. J., Winkler, C., O'Brien, S. J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, H. & Mann, D. L. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med* **2**, 405-411, (1996).

252 Hendel, H., Caillat-Zucman, S., Lebuanec, H., Carrington, M., O'Brien, S., Andrieu, J. M., Schachter, F., Zagury, D., Rappaport, J., Winkler, C., Nelson, G. W. & Zagury, J. F. New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. *J Immunol* **162**, 6942-6946, (1999).

253 Kummee, P., Tangkijvanich, P., Poovorawan, Y. & Hirankarn, N. Association of HLA-DRB1*13 and TNF-alpha gene polymorphisms with clearance of chronic hepatitis B infection and risk of hepatocellular carcinoma in Thai population. *J Viral Hepat* **14**, 841-848, (2007).

254 Davenport, M. P., Price, D. A. & McMichael, A. J. The T cell repertoire in infection and vaccination: implications for control of persistent viruses. *Curr Opin Immunol* **19**, 294-300, (2007).

255 Venturi, V., Price, D. A., Douek, D. C. & Davenport, M. P. The molecular basis for public T-cell responses? *Nat Rev Immunol* **8**, 231-238, (2008).

256 Turner, S. J., Kedzierska, K., Komodromou, H., La Gruta, N. L., Dunstone, M. A., Webb, A. I., Webby, R., Walden, H., Xie, W., McCluskey, J., Purcell, A. W., Rossjohn, J. & Doherty, P. C. Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations. *Nat Immunol* **6**, 382-389, (2005).

257 Kedzierska, K., Day, E. B., Pi, J., Heard, S. B., Doherty, P. C., Turner, S. J. & Perlman, S. Quantification of repertoire diversity of influenza-specific epitopes with predominant public or private TCR usage. *J Immunol* **177**, 6705-6712, (2006).

258 Venturi, V., Chin, H. Y., Asher, T. E., Ladell, K., Scheinberg, P., Bornstein, E., van Bockel, D., Kelleher, A. D., Douek, D. C., Price, D. A. & Davenport, M. P. TCR beta-chain sharing in human CD8+ T cell responses to cytomegalovirus and EBV. *J Immunol* **181**, 7853-7862, (2008).

259 Venturi, V., Quigley, M. F., Greenaway, H. Y., Ng, P. C., Ende, Z. S., McIntosh, T., Asher, T. E., Almeida, J. R., Levy, S., Price, D. A., Davenport, M. P. & Douek, D. C. A mechanism for TCR sharing between T cell subsets and individuals revealed by pyrosequencing. *J Immunol* **186**, 4285-4294, (2011).

260 Manfras, B. J., Terjung, D. & Boehm, B. O. Non-productive human TCR beta chain genes represent V-D-J diversity before selection upon function: insight into biased usage of TCRBD and TCRBJ genes and diversity of CDR3 region length. *Hum Immunol* **60**, 1090-1100, (1999).

261 Shortman, K., Egerton, M., Spangrude, G. J. & Scollay, R. The generation and fate of thymocytes. *Semin Immunol* **2**, 3-12, (1990).

262 Mason, D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol Today* **19**, 395-404, (1998).

263 Hemmer, B., Vergelli, M., Pinilla, C., Houghten, R. & Martin, R. Probing degeneracy in T-cell recognition using peptide combinatorial libraries. *Immunol Today* **19**, 163-168, (1998).

264 Wei, S., Charmley, P., Robinson, M. A. & Concannon, P. The extent of the human germline T-cell receptor V beta gene segment repertoire. *Immunogenetics* **40**, 27-36, (1994).

265 Arstila, T. P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J. & Kourilsky, P. A direct estimate of the human alphabeta T cell receptor diversity. *Science* **286**, 958-961, (1999).

266 Schwarz, B. A., Sambandam, A., Maillard, I., Harman, B. C., Love, P. E. & Bhandoola, A. Selective thymus settling regulated by cytokine and chemokine receptors. *J Immunol* **178**, 2008-2017, (2007).

267 Love, P. E. & Bhandoola, A. Signal integration and crosstalk during thymocyte migration and emigration. *Nat Rev Immunol* **11**, 469-477, (2011).

268 Chao, D. L., Davenport, M. P., Forrest, S. & Perelson, A. S. The effects of thymic selection on the range of T cell cross-reactivity. *Eur J Immunol* **35**, 3452-3459, (2005).
269 Anderson, M. S., Venanzi, E. S., Chen, Z., Berzins, S. P., Benoist, C. & Mathis, D. The cellular mechanism of Aire control of T cell tolerance. *Immunity* **23**, 227-239, (2005).
270 Hernandez-Hoyos, G., Sohn, S. J., Rothenberg, E. V. & Alberola-Ila, J. Lck activity controls CD4/CD8 T cell lineage commitment. *Immunity* **12**, 313-322, (2000).
271 Yui, M. A., Feng, N. & Rothenberg, E. V. Fine-scale staging of T cell lineage commitment in adult mouse thymus. *J Immunol* **185**, 284-293, (2010).
272 Saini, M., Sinclair, C., Marshall, D., Tolaini, M., Sakaguchi, S. & Seddon, B. Regulation of Zap70 expression during thymocyte development enables temporal separation of CD4 and CD8 repertoire selection at different signaling thresholds. *Sci Signal* **3**, ra23, (2010).
273 He, X., Dave, V. P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B. A. & Kappes, D. J. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* **433**, 826-833, (2005).
274 Gimferrer, I., Hu, T., Simmons, A., Wang, C., Souabni, A., Busslinger, M., Bender, T. P., Hernandez-Hoyos, G. & Alberola-Ila, J. Regulation of GATA-3 expression during CD4 lineage differentiation. *J Immunol* **186**, 3892-3898, (2011).
275 Tough, D. F. & Sprent, J. Turnover of naive- and memory-phenotype T cells. *J Exp Med* **179**, 1127-1135, (1994).
276 Goldrath, A. W. & Bevan, M. J. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity* **11**, 183-190, (1999).
277 Clarke, S. R. & Rudensky, A. Y. Survival and homeostatic proliferation of naive peripheral CD4+ T cells in the absence of self peptide:MHC complexes. *J Immunol* **165**, 2458-2464, (2000).
278 Jacobs, S. R., Michalek, R. D. & Rathmell, J. C. IL-7 is essential for homeostatic control of T cell metabolism in vivo. *J Immunol* **184**, 3461-3469, (2010).
279 Palmer, M. J., Mahajan, V. S., Chen, J., Irvine, D. J. & Lauffenburger, D. A. Signaling thresholds govern heterogeneity in IL-7-receptor-mediated responses of naive CD8(+) T cells. *Immunol Cell Biol* **89**, 581-594, (2011).
280 Hathcock, K. S., Laszlo, G., Pucillo, C., Linsley, P. & Hodes, R. J. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med* **180**, 631-640, (1994).
281 Kosmaczewska, A., Ciszak, L., Bocko, D. & Frydecka, I. Expression and functional significance of CTLA-4, a negative regulator of T cell activation. *Arch Immunol Ther Exp (Warsz)* **49**, 39-46, (2001).
282 Viola, A. & Lanzavecchia, A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273**, 104-106, (1996).
283 Borrow, P., Tishon, A., Lee, S., Xu, J., Grewal, I. S., Oldstone, M. B. & Flavell, R. A. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *J Exp Med* **183**, 2129-2142, (1996).
284 Croft, M., Bradley, L. M. & Swain, S. L. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell

costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol* **152**, 2675-2685, (1994).

285 Riha, P. & Rudd, C. E. CD28 co-signaling in the adaptive immune response. *Self Nonself* **1**, 231-240, (2010).

286 Ferguson, S. E., Han, S., Kelsoe, G. & Thompson, C. B. CD28 is required for germinal center formation. *J Immunol* **156**, 4576-4581, (1996).

287 Alarcon, B., Mestre, D. & Martinez-Martin, N. The immunological synapse: a cause or consequence of T-cell receptor triggering? *Immunology* **133**, 420-425, (2011).

288 Janes, P. W., Ley, S. C., Magee, A. I. & Kabouridis, P. S. The role of lipid rafts in T cell antigen receptor (TCR) signalling. *Semin Immunol* **12**, 23-34, (2000).

289 Labrecque, N., Whitfield, L. S., Obst, R., Waltzinger, C., Benoist, C. & Mathis, D. How much TCR does a T cell need? *Immunity* **15**, 71-82, (2001).

290 Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**, 148-151, (1995).

291 Harding, C. V. & Unanue, E. R. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* **346**, 574-576, (1990).

292 Valitutti, S., Muller, S., Dessing, M. & Lanzavecchia, A. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J Exp Med* **183**, 1917-1921, (1996).

293 Garcia, K. C., Degano, M., Speir, J. A. & Wilson, I. A. Emerging principles for T cell receptor recognition of antigen in cellular immunity. *Rev Immunogenet* **1**, 75-90, (1999).

294 Grandjean, I., Duban, L., Bonney, E. A., Corcuff, E., Di Santo, J. P., Matzinger, P. & Lantz, O. Are major histocompatibility complex molecules involved in the survival of naive CD4+ T cells? *J Exp Med* **198**, 1089-1102, (2003).

295 Koning, F. A., Otto, S. A., Hazenberg, M. D., Dekker, L., Prins, M., Miedema, F. & Schuitemaker, H. Low-level CD4+ T cell activation is associated with low susceptibility to HIV-1 infection. *J Immunol* **175**, 6117-6122, (2005).

296 Suy, A., Castro, P., Nomdedeu, M., Garcia, F., Lopez, A., Fumero, E., Gallart, T., Lopalco, L., Coll, O., Gatell, J. M. & Plana, M. Immunological profile of heterosexual highly HIV-exposed uninfected individuals: predominant role of CD4 and CD8 T-cell activation. *J Infect Dis* **196**, 1191-1201, (2007).

297 Liu, Z., Cumberland, W. G., Hultin, L. E., Prince, H. E., Detels, R. & Giorgi, J. V. Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol* **16**, 83-92, (1997).

298 Mocroft, A., Bofill, M., Lipman, M., Medina, E., Borthwick, N., Timms, A., Batista, L., Winter, M., Sabin, C. A., Johnson, M., Lee, C. A., Phillips, A. & Janossy, G. CD8+,CD38+ lymphocyte percent: a useful immunological marker for monitoring HIV-1-infected patients. *J Acquir Immune Defic Syndr Hum Retrovirol* **14**, 158-162, (1997).

299 Ganesan, A., Chattopadhyay, P. K., Brodie, T. M., Qin, J., Gu, W., Mascola, J. R., Michael, N. L., Follmann, D. A. & Roederer, M. Immunologic and

virologic events in early HIV infection predict subsequent rate of progression. *J Infect Dis* **201**, 272-284, (2010).

300 Nakanjako, D., Ssewanyana, I., Mayanja-Kizza, H., Kiragga, A., Colebunders, R., Manabe, Y. C., Nabatanzi, R., Kamya, M. R. & Cao, H. High T-cell immune activation and immune exhaustion among individuals with suboptimal CD4 recovery after 4 years of antiretroviral therapy in an African cohort. *BMC Infect Dis* **11**, 43, (2011).

301 Appay, V., van Lier, R. A., Sallusto, F. & Roederer, M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* **73**, 975-983, (2008).

302 Zaunders, J. J., Dyer, W. B., Munier, M. L., Ip, S., Liu, J., Amyes, E., Rawlinson, W., De Rose, R., Kent, S. J., Sullivan, J. S., Cooper, D. A. & Kelleher, A. D. CD127+CCR5+CD38+++ CD4+ Th1 effector cells are an early component of the primary immune response to vaccinia virus and precede development of interleukin-2+ memory CD4+ T cells. *J Virol* **80**, 10151-10161, (2006).

303 Zaunders, J. J., Munier, M. L., Kaufmann, D. E., Ip, S., Grey, P., Smith, D., Ramacciotti, T., Quan, D., Finlayson, R., Kaldor, J., Rosenberg, E. S., Walker, B. D., Cooper, D. A. & Kelleher, A. D. Early proliferation of CCR5(+) CD38(+++) antigen-specific CD4(+) Th1 effector cells during primary HIV-1 infection. *Blood* **106**, 1660-1667, (2005).

304 Benito, J. M., Lopez, M., Lozano, S., Ballesteros, C., Martinez, P., Gonzalez-Lahoz, J. & Soriano, V. Differential upregulation of CD38 on different T-cell subsets may influence the ability to reconstitute CD4+ T cells under successful highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **38**, 373-381, (2005).

305 Deeks, S. G., Kitchen, C. M., Liu, L., Guo, H., Gascon, R., Narvaez, A. B., Hunt, P., Martin, J. N., Kahn, J. O., Levy, J., McGrath, M. S. & Hecht, F. M. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* **104**, 942-947, (2004).

306 Giorgi, J. V., Ho, H. N., Hirji, K., Chou, C. C., Hultin, L. E., O'Rourke, S., Park, L., Margolick, J. B., Ferbas, J. & Phair, J. P. CD8+ lymphocyte activation at human immunodeficiency virus type 1 seroconversion: development of HLA-DR+ CD38- CD8+ cells is associated with subsequent stable CD4+ cell levels. The Multicenter AIDS Cohort Study Group. *J Infect Dis* **170**, 775-781, (1994).

307 Tanchot, C., Lemonnier, F. A., Perarnau, B., Freitas, A. A. & Rocha, B. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* **276**, 2057-2062, (1997).

308 Geiger, R., Duhen, T., Lanzavecchia, A. & Sallusto, F. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. *J Exp Med* **206**, 1525-1534, (2009).

309 Pettersson, F. E. & Gronvik, K. O. Long-term CD4+ and CD8+ memory T cells developed in severe combined immunodeficiency mice during homoeostasis exhibit differences in sensitivity to antigen. *Scand J Immunol* **57**, 311-318, (2003).

310 Rogers, P. R., Dubey, C. & Swain, S. L. Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. *J Immunol* **164**, 2338-2346, (2000).

311 Lanzavecchia, A. & Sallusto, F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* **290**, 92-97, (2000).
312 Reiner, S. L., Sallusto, F. & Lanzavecchia, A. Division of labor with a workforce of one: challenges in specifying effector and memory T cell fate. *Science* **317**, 622-625, (2007).
313 Kaech, S. M., Wherry, E. J. & Ahmed, R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* **2**, 251-262, (2002).
314 van Leeuwen, E. M., Sprent, J. & Surh, C. D. Generation and maintenance of memory CD4(+) T Cells. *Curr Opin Immunol* **21**, 167-172, (2009).
315 Amara, R. R., Nigam, P., Sharma, S., Liu, J. & Bostik, V. Long-lived poxvirus immunity, robust CD4 help, and better persistence of CD4 than CD8 T cells. *J Virol* **78**, 3811-3816, (2004).
316 Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-712, (1999).
317 Younes, S. A., Yassine-Diab, B., Dumont, A. R., Boulassel, M. R., Grossman, Z., Routy, J. P. & Sekaly, R. P. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med* **198**, 1909-1922, (2003).
318 Bostik, P., Noble, E. S., Mayne, A. E., Gargano, L., Villinger, F. & Ansari, A. A. Central memory CD4 T cells are the predominant cell subset resistant to anergy in SIV disease resistant sooty mangabeys. *Aids* **20**, 181-188, (2006).
319 Catron, D. M., Rusch, L. K., Hataye, J., Itano, A. A. & Jenkins, M. K. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. *J Exp Med* **203**, 1045-1054, (2006).
320 Song, K., Rabin, R. L., Hill, B. J., De Rosa, S. C., Perfetto, S. P., Zhang, H. H., Foley, J. F., Reiner, J. S., Liu, J., Mattapallil, J. J., Douek, D. C., Roederer, M. & Farber, J. M. Characterization of subsets of CD4+ memory T cells reveals early branched pathways of T cell differentiation in humans. *Proc Natl Acad Sci U S A* **102**, 7916-7921, (2005).
321 Huster, K. M., Koffler, M., Stemberger, C., Schiemann, M., Wagner, H. & Busch, D. H. Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. *Eur J Immunol* **36**, 1453-1464, (2006).
322 Zielinski, C. E., Corti, D., Mele, F., Pinto, D., Lanzavecchia, A. & Sallusto, F. Dissecting the human immunologic memory for pathogens. *Immunol Rev* **240**, 40-51, (2011).
323 Geginat, J., Lanzavecchia, A. & Sallusto, F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* **101**, 4260-4266, (2003).
324 Teixeiro, E., Daniels, M. A., Hamilton, S. E., Schrum, A. G., Bragado, R., Jameson, S. C. & Palmer, E. Different T cell receptor signals determine CD8+ memory versus effector development. *Science* **323**, 502-505, (2009).
325 Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S., Lipp, M., Forster, R., Rowland-Jones, S., Sekaly, R. P., McMichael, A. J. & Pantaleo, G. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* **410**, 106-111, (2001).

326 Dai, J., Agosto, L. M., Baytop, C., Yu, J. J., Pace, M. J., Liszewski, M. K. & O'Doherty, U. Human immunodeficiency virus integrates directly into naive resting CD4+ T cells but enters naive cells less efficiently than memory cells. *J Virol* **83**, 4528-4537, (2009).

327 Harari, A., Rizzardi, G. P., Ellefsen, K., Ciuffreda, D., Champagne, P., Bart, P. A., Kaufmann, D., Telenti, A., Sahli, R., Tambussi, G., Kaiser, L., Lazzarin, A., Perrin, L. & Pantaleo, G. Analysis of HIV-1- and CMV-specific memory CD4 T-cell responses during primary and chronic infection. *Blood* **100**, 1381-1387, (2002).

328 Maenetje, P., Riou, C., Casazza, J. P., Ambrozak, D., Hill, B., Gray, G., Koup, R. A., de Bruyn, G. & Gray, C. M. A steady state of CD4+ T cell memory maturation and activation is established during primary subtype C HIV-1 infection. *J Immunol* **184**, 4926-4935, (2010).

329 Potter, S. J., Lacabaratz, C., Lambotte, O., Perez-Patrigeon, S., Vingert, B., Sinet, M., Colle, J. H., Urrutia, A., Scott-Algara, D., Boufassa, F., Delfraissy, J. F., Theze, J., Venet, A. & Chakrabarti, L. A. Preserved central memory and activated effector memory CD4+ T-cell subsets in human immunodeficiency virus controllers: an ANRS EP36 study. *J Virol* **81**, 13904-13915, (2007).

330 Ladell, K., Hellerstein, M. K., Cesar, D., Busch, R., Boban, D. & McCune, J. M. Central memory CD8+ T cells appear to have a shorter lifespan and reduced abundance as a function of HIV disease progression. *J Immunol* **180**, 7907-7918, (2008).

331 Sachsenberg, N., Perelson, A. S., Yerly, S., Schockmel, G. A., Leduc, D., Hirschel, B. & Perrin, L. Turnover of CD4+ and CD8+ T lymphocytes in HIV-1 infection as measured by Ki-67 antigen. *J Exp Med* **187**, 1295-1303, (1998).

332 Gao, F. G., Khammanivong, V., Liu, W. J., Leggatt, G. R., Frazer, I. H. & Fernando, G. J. Antigen-specific CD4+ T-cell help is required to activate a memory CD8+ T cell to a fully functional tumor killer cell. *Cancer Res* **62**, 6438-6441, (2002).

333 Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G. & Schoenberger, S. P. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* **421**, 852-856, (2003).

334 Crawley, A. M. & Angel, J. B. Expression of gamma-chain cytokine receptors on CD8(+) T cells in HIV infection with a focus on IL-7Ralpha (CD127). *Immunol Cell Biol*, (2011).

335 Sallusto, F. & Lanzavecchia, A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *Eur J Immunol* **39**, 2076-2082, (2009).

336 Aslan, N., Yurdaydin, C., Wiegand, J., Greten, T., Ciner, A., Meyer, M. F., Heiken, H., Kuhlmann, B., Kaiser, T., Bozkaya, H., Tillmann, H. L., Bozdayi, A. M., Manns, M. P. & Wedemeyer, H. Cytotoxic CD4 T cells in viral hepatitis. *J Viral Hepat* **13**, 505-514, (2006).

337 Zheng, N., Fujiwara, M., Ueno, T., Oka, S. & Takiguchi, M. Strong ability of Nef-specific CD4+ cytotoxic T cells to suppress human immunodeficiency virus type 1 (HIV-1) replication in HIV-1-infected CD4+ T cells and macrophages. *J Virol* **83**, 7668-7677, (2009).

338 Doherty, P. C., Topham, D. J., Tripp, R. A., Cardin, R. D., Brooks, J. W. & Stevenson, P. G. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. *Immunol Rev* **159**, 105-117, (1997).

339 Eto, D., Lao, C., DiToro, D., Barnett, B., Escobar, T. C., Kageyama, R., Yusuf, I. & Crotty, S. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS ONE* **6**, e17739, (2011).

340 Matloubian, M., Concepcion, R. J. & Ahmed, R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* **68**, 8056-8063, (1994).

341 Shedlock, D. J. & Shen, H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**, 337-339, (2003).

342 Ramsburg, E. A., Publicover, J. M., Coppock, D. & Rose, J. K. Requirement for CD4 T cell help in maintenance of memory CD8 T cell responses is epitope dependent. *J Immunol* **178**, 6350-6358, (2007).

343 Sun, J. C. & Bevan, M. J. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300**, 339-342, (2003).

344 Crotty, S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* **29**, 621-663, (2011).

345 Deenick, E. K., Ma, C. S., Brink, R. & Tangye, S. G. Regulation of T follicular helper cell formation and function by antigen presenting cells. *Curr Opin Immunol* **23**, 111-118, (2011).

346 Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M. & Forster, R. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* **192**, 1545-1552, (2000).

347 Kim, C. H., Rott, L. S., Clark-Lewis, I., Campbell, D. J., Wu, L. & Butcher, E. C. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* **193**, 1373-1381, (2001).

348 Xu, J., Foy, T. M., Laman, J. D., Elliott, E. A., Dunn, J. J., Waldschmidt, T. J., Elsemore, J., Noelle, R. J. & Flavell, R. A. Mice deficient for the CD40 ligand. *Immunity* **1**, 423-431, (1994).

349 Ely, K. H., Kasper, L. H. & Khan, I. A. Augmentation of the CD8+ T cell response by IFN-gamma in IL-12-deficient mice during Toxoplasma gondii infection. *J Immunol* **162**, 5449-5454, (1999).

350 Curtsinger, J. M. & Mescher, M. F. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* **22**, 333-340, (2010).

351 Mosmann, T. R., Li, L. & Sad, S. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin Immunol* **9**, 87-92, (1997).

352 Akinsiku, O. T., Bansal, A., Sabbaj, S., Heath, S. L. & Goepfert, P. A. Interleukin-2 Production by Polyfunctional HIV-1-Specific CD8 T-Cells is Associated with Enhanced Viral Suppression. *J Acquir Immune Defic Syndr*, (2011).

353 Critchfield, J. W., Young, D. H., Hayes, T. L., Braun, J. V., Garcia, J. C., Pollard, R. B. & Shacklett, B. L. Magnitude and complexity of rectal mucosa HIV-1-specific CD8+ T-cell responses during chronic infection reflect clinical status. *PLoS ONE* **3**, e3577, (2008).

354 Makedonas, G. & Betts, M. R. Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. *Springer Semin Immunopathol* **28**, 209-219, (2006).

355 Precopio, M. L., Betts, M. R., Parrino, J., Price, D. A., Gostick, E., Ambrozak, D. R., Asher, T. E., Douek, D. C., Harari, A., Pantaleo, G., Bailer, R., Graham,

B. S., Roederer, M. & Koup, R. A. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8+ T cell responses. *J Exp Med* **204**, 1405-1416, (2007).

356 de Jong, S., Timmer, T., Heijenbrok, F. J. & de Vries, E. G. Death receptor ligands, in particular TRAIL, to overcome drug resistance. *Cancer Metastasis Rev* **20**, 51-56, (2001).

357 Migueles, S. A., Osborne, C. M., Royce, C., Compton, A. A., Joshi, R. P., Weeks, K. A., Rood, J. E., Berkley, A. M., Sacha, J. B., Cogliano-Shutta, N. A., Lloyd, M., Roby, G., Kwan, R., McLaughlin, M., Stallings, S., Rehm, C., O'Shea, M. A., Mican, J., Packard, B. Z., Komoriya, A., Palmer, S., Wiegand, A. P., Maldarelli, F., Coffin, J. M., Mellors, J. W., Hallahan, C. W., Follman, D. A. & Connors, M. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* **29**, 1009-1021, (2008).

358 Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L. & Reimann, K. A. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**, 857-860, (1999).

359 Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M. & Oldstone, M. B. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* **68**, 6103-6110, (1994).

360 Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., Perelson, A. S. & Ho, D. D. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **189**, 991-998, (1999).

361 Papagno, L., Appay, V., Sutton, J., Rostron, T., Gillespie, G. M., Ogg, G. S., King, A., Makadzanhge, A. T., Waters, A., Balotta, C., Vyakarnam, A., Easterbrook, P. J. & Rowland-Jones, S. L. Comparison between HIV- and CMV-specific T cell responses in long-term HIV infected donors. *Clin Exp Immunol* **130**, 509-517, (2002).

362 Yu, X. G., Addo, M. M., Rosenberg, E. S., Rodriguez, W. R., Lee, P. K., Fitzpatrick, C. A., Johnston, M. N., Strick, D., Goulder, P. J., Walker, B. D. & Altfeld, M. Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T-cell responses following acute HIV-1 infection. *J Virol* **76**, 8690-8701, (2002).

363 Appay, V., Zaunders, J. J., Papagno, L., Sutton, J., Jaramillo, A., Waters, A., Easterbrook, P., Grey, P., Smith, D., McMichael, A. J., Cooper, D. A., Rowland-Jones, S. L. & Kelleher, A. D. Characterization of CD4(+) CTLs ex vivo. *J Immunol* **168**, 5954-5958, (2002).

364 Almeida, J. R., Sauce, D., Price, D. A., Papagno, L., Shin, S. Y., Moris, A., Larsen, M., Pancino, G., Douek, D. C., Autran, B., Saez-Cirion, A. & Appay, V. Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV suppressive activity. *Blood*, (2009).

365 Hersperger, A. R., Pereyra, F., Nason, M., Demers, K., Sheth, P., Shin, L. Y., Kovacs, C. M., Rodriguez, B., Sieg, S. F., Teixeira-Johnson, L., Gudonis, D., Goepfert, P. A., Lederman, M. M., Frank, I., Makedonas, G., Kaul, R., Walker, B. D. & Betts, M. R. Perforin expression directly ex vivo by HIV-

specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog* **6**, e1000917, (2010).

366 Douek, D. C., Betts, M. R., Brenchley, J. M., Hill, B. J., Ambrozak, D. R., Ngai, K. L., Karandikar, N. J., Casazza, J. P. & Koup, R. A. A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape. *J Immunol* **168**, 3099-3104, (2002).

367 Grakoui, A., Shoukry, N. H., Woollard, D. J., Han, J. H., Hanson, H. L., Ghrayeb, J., Murthy, K. K., Rice, C. M. & Walker, C. M. HCV persistence and immune evasion in the absence of memory T cell help. *Science* **302**, 659-662, (2003).

368 Douek, D. C., Brenchley, J. M., Betts, M. R., Ambrozak, D. R., Hill, B. J., Okamoto, Y., Casazza, J. P., Kuruppu, J., Kunstman, K., Wolinsky, S., Grossman, Z., Dybul, M., Oxenius, A., Price, D. A., Connors, M. & Koup, R. A. HIV preferentially infects HIV-specific CD4+ T cells. *Nature* **417**, 95-98, (2002).

369 Li, D., Chen, J., Jia, M., Hong, K., Ruan, Y., Liang, H., Liu, S., Zhang, X., Zhao, H., Peng, H., Ma, P. & Shao, Y. Loss of balance between T helper type 17 and regulatory T cells in chronic human immunodeficiency virus infection. *Clin Exp Immunol* **165**, 363-371, (2011).

370 Lubong Sabado, R., Kavanagh, D. G., Kaufmann, D. E., Fru, K., Babcock, E., Rosenberg, E., Walker, B., Lifson, J., Bhardwaj, N. & Larsson, M. In vitro priming recapitulates in vivo HIV-1 specific T cell responses, revealing rapid loss of virus reactive CD4 T cells in acute HIV-1 infection. *PLoS ONE* **4**, e4256, (2009).

371 Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., Boden, D., Racz, P. & Markowitz, M. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* **200**, 761-770, (2004).

372 Mukadi, Y., Perriens, J. H., St Louis, M. E., Brown, C., Prignot, J., Willame, J. C., Pouthier, F., Kaboto, M., Ryder, R. W., Portaels, F. & et al. Spectrum of immunodeficiency in HIV-1-infected patients with pulmonary tuberculosis in Zaire. *Lancet* **342**, 143-146, (1993).

373 Kaufmann, D. E., Bailey, P. M., Sidney, J., Wagner, B., Norris, P. J., Johnston, M. N., Cosimi, L. A., Addo, M. M., Lichterfeld, M., Altfeld, M., Frahm, N., Brander, C., Sette, A., Walker, B. D. & Rosenberg, E. S. Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. *J Virol* **78**, 4463-4477, (2004).

374 Ramduth, D., Day, C. L., Thobakgale, C. F., Mkhwanazi, N. P., de Pierres, C., Reddy, S., van der Stok, M., Mncube, Z., Nair, K., Moodley, E. S., Kaufmann, D. E., Streeck, H., Coovadia, H. M., Kiepiela, P., Goulder, P. J. & Walker, B. D. Immunodominant HIV-1 Cd4+ T cell epitopes in chronic untreated clade C HIV-1 infection. *PLoS ONE* **4**, e5013, (2009).

375 Clark, S., Page, E., Ford, T., Metcalf, R., Pozniak, A., Nelson, M., Henderson, D. C., Asboe, D., Gotch, F., Gazzard, B. G. & Kelleher, P. Reduced T(H)1/T(H)17 CD4 T cell numbers are associated with impaired purified protein derivative-specific cytokine responses in patients with HIV-1 infection. *J Allergy Clin Immunol*, (2011).

376 Geretti, A. M., Van Baalen, C. A., Borleffs, J. C., Van Els, C. A. & Osterhaus, A. D. Kinetics and specificities of the T helper-cell response to gp120 in the asymptomatic stage of HIV-1 infection. *Scand J Immunol* **39**, 355-362, (1994).

377 Norris, P. J., Moffett, H. F., Yang, O. O., Kaufmann, D. E., Clark, M. J., Addo, M. M. & Rosenberg, E. S. Beyond help: direct effector functions of human immunodeficiency virus type 1-specific CD4(+) T cells. *J Virol* **78**, 8844-8851, (2004).

378 Munier, M. L. & Kelleher, A. D. Acutely dysregulated, chronically disabled by the enemy within: T-cell responses to HIV-1 infection. *Immunol Cell Biol* **85**, 6-15, (2007).

379 Hunt, P. W., Hatano, H., Sinclair, E., Lee, T. H., Busch, M. P., Martin, J. N., McCune, J. M. & Deeks, S. G. HIV-specific CD4+ T cells may contribute to viral persistence in HIV controllers. *Clin Infect Dis* **52**, 681-687, (2011).

380 Rosa, D. S., Ribeiro, S. P., Almeida, R. R., Mairena, E. C., Postol, E., Kalil, J. & Cunha-Neto, E. A DNA vaccine encoding multiple HIV CD4 epitopes elicits vigorous polyfunctional, long-lived CD4+ and CD8+ T cell responses. *PLoS ONE* **6**, e16921, (2011).

381 Sun, Y., Santra, S., Buzby, A. P., Mascola, J. R., Nabel, G. J. & Letvin, N. L. Recombinant vector-induced HIV/SIV-specific CD4+ T lymphocyte responses in rhesus monkeys. *Virology* **406**, 48-55, (2010).

382 Eyeson, J., King, D., Boaz, M. J., Sefia, E., Tomkins, S., Waters, A., Easterbrook, P. J. & Vyakarnam, A. Evidence for Gag p24-specific CD4 T cells with reduced susceptibility to R5 HIV-1 infection in a UK cohort of HIV-exposed-seronegative subjects. *Aids* **17**, 2299-2311, (2003).

383 Liu, H. W., Hong, K. X., Ma, J., Yuan, L., Liu, S., Chen, J. P., Zhang, Y. Z., Ruan, Y. H., Xu, J. Q. & Shao, Y. M. Identification of HIV-1 specific T lymphocyte responses in highly exposed persistently seronegative Chinese. *Chin Med J (Engl)* **119**, 1616-1621, (2006).

384 Pallikkuth, S., Wanchu, A., Bhatnagar, A., Sachdeva, R. K. & Sharma, M. Human immunodeficiency virus (HIV) gag antigen-specific T-helper and granule-dependent CD8 T-cell activities in exposed but uninfected heterosexual partners of HIV type 1-infected individuals in North India. *Clin Vaccine Immunol* **14**, 1196-1202, (2007).

385 Rowland-Jones, S. L. & McMichael, A. Immune responses in HIV-exposed seronegatives: have they repelled the virus? *Curr Opin Immunol* **7**, 448-455, (1995).

386 Fowke, K. R., Nagelkerke, N. J., Kimani, J., Simonsen, J. N., Anzala, A. O., Bwayo, J. J., MacDonald, K. S., Ngugi, E. N. & Plummer, F. A. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet* **348**, 1347-1351, (1996).

387 Miyazawa, M., Lopalco, L., Mazzotta, F., Lo Caputo, S., Veas, F. & Clerici, M. The 'immunologic advantage' of HIV-exposed seronegative individuals. *AIDS* **23**, 161-175, (2009).

388 Dittmer, U., Spring, M., Petry, H., Nisslein, T., Rieckmann, P., Luke, W., Stahl-Hennig, C., Hunsmann, G. & Bodemer, W. Cell-mediated immune response of macaques immunized with low doses of simian immunodeficiency virus (SIV). *J Biotechnol* **44**, 105-110, (1996).

389 Lakhashe, S. K., Velu, V., Sciaranghella, G., Siddappa, N. B., Dipasquale, J. M., Hemashettar, G., Yoon, J. K., Rasmussen, R. A., Yang, F., Lee, S. J., Montefiori, D. C., Novembre, F. J., Villinger, F., Amara, R. R., Kahn, M., Hu,

S. L., Li, S., Li, Z., Frankel, F. R., Robert-Guroff, M., Johnson, W. E., Lieberman, J. & Ruprecht, R. M. Prime-boost vaccination with heterologous live vectors encoding SIV gag and multimeric HIV-1 gp160 protein: Efficacy against repeated mucosal R5 clade C SHIV challenges. *Vaccine*, (2011).

390 Shacklett, B. L., Ling, B., Veazey, R. S., Luckay, A., Moretto, W. J., Wilkens, D. T., Hu, J., Israel, Z. R., Nixon, D. F. & Marx, P. A. Boosting of SIV-specific T cell responses in rhesus macaques that resist repeated intravaginal challenge with SIVmac251. *AIDS Res Hum Retroviruses* **18**, 1081-1088, (2002).

391 Goonetilleke, N., Moore, S., Dally, L., Winstone, N., Cebere, I., Mahmoud, A., Pinheiro, S., Gillespie, G., Brown, D., Loach, V., Roberts, J., Guimaraes-Walker, A., Hayes, P., Loughran, K., Smith, C., De Bont, J., Verlinde, C., Vooijs, D., Schmidt, C., Boaz, M., Gilmour, J., Fast, P., Dorrell, L., Hanke, T. & McMichael, A. J. Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. *J Virol* **80**, 4717-4728, (2006).

392 Flynn, N. M., Forthal, D. N., Harro, C. D., Judson, F. N., Mayer, K. H. & Para, M. F. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* **191**, 654-665, (2005).

393 Fiebig, E. W., Wright, D. J., Rawal, B. D., Garrett, P. E., Schumacher, R. T., Peddada, L., Heldebrant, C., Smith, R., Conrad, A., Kleinman, S. H. & Busch, M. P. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *Aids* **17**, 1871-1879, (2003).

394 Smith, S. M., Brookes, R., Klein, M. R., Malin, A. S., Lukey, P. T., King, A. S., Ogg, G. S., Hill, A. V. & Dockrell, H. M. Human CD8+ CTL specific for the mycobacterial major secreted antigen 85A. *J Immunol* **165**, 7088-7095, (2000).

395 Wiker, H. G. & Harboe, M. The antigen 85 complex: a major secretion product of Mycobacterium tuberculosis. *Microbiol Rev* **56**, 648-661, (1992).

396 Roederer, M. & Koup, R. A. Optimized determination of T cell epitope responses. *J Immunol Methods* **274**, 221-228, (2003).

397 Lamoreaux, L., Roederer, M. & Koup, R. Intracellular cytokine optimization and standard operating procedure. *Nat Protoc* **1**, 1507-1516, (2006).

398 Roederer, M. Multiparameter FACS analysis. *Curr Protoc Immunol* **Chapter 5**, Unit 5 8, (2002).

399 Scheinberg, P., Melenhorst, J. J., Hill, B. J., Keyvanfar, K., Barrett, A. J., Price, D. A. & Douek, D. C. The clonal composition of human CD4+CD25+Foxp3+ cells determined by a comprehensive DNA-based multiplex PCR for TCRB gene rearrangements. *J Immunol Methods* **321**, 107-120, (2007).

400 Folch, G. & Lefranc, M. P. The human T cell receptor beta variable (TRBV) genes. *Exp Clin Immunogenet* **17**, 42-54, (2000).

401 Lelie, P. N., van Drimmelen, H. A., Cuypers, H. T., Best, S. J., Stramer, S. L., Hyland, C., Allain, J. P., Moncharmont, P., Defer, C., Nubling, M., Glauser, A., da Silva Cardoso, M., Viret, J. F., Lankinen, M. H., Grillner, L., Wirthmuller, U., Coste, J., Schottstedt, V., Masecar, B. & Dax, E. M.

Sensitivity of HCV RNA and HIV RNA blood screening assays. *Transfusion* **42**, 527-536, (2002).

402 Busch, M. P., Glynn, S. A., Wright, D. J., Hirschkorn, D., Laycock, M. E., McAuley, J., Tu, Y., Giachetti, C., Gallarda, J., Heitman, J. & Kleinman, S. H. Relative sensitivities of licensed nucleic acid amplification tests for detection of viremia in early human immunodeficiency virus and hepatitis C virus infection. *Transfusion* **45**, 1853-1863, (2005).

403 Lefkovits. I., W. H. Limiting Dilution Analysis of Cells of the Immune System. *Cambridge* **Cambridge university Press**, 304, (1979).

404 Los Alamos National Laboratory Database, http://www.hiv.lanl.gov/content/immunology

405 Piatak, M., Jr., Saag, M. S., Yang, L. C., Clark, S. J., Kappes, J. C., Luk, K. C., Hahn, B. H., Shaw, G. M. & Lifson, J. D. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259**, 1749-1754, (1993).

406 Turnbull, E. L., Baalwa, J., Conrod, K. E., Wang, S., Wei, X., Wong, M., Turner, J., Pellegrino, P., Williams, I., Shaw, G. M. & Borrow, P. Escape is a more common mechanism than avidity reduction for evasion of CD8+ T cell responses in primary human immunodeficiency virus type 1 infection. *Retrovirology* **8**, 41, (2011).

407 Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B. & Shaw, G. M. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* **3**, 205-211, (1997).

408 Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R. & Phillips, R. E. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci U S A* **94**, 1890-1895, (1997).

409 Scherer, A., Frater, J., Oxenius, A., Agudelo, J., Price, D. A., Gunthard, H. F., Barnardo, M., Perrin, L., Hirschel, B., Phillips, R. E. & McLean, A. R. Quantifiable cytotoxic T lymphocyte responses and HLA-related risk of progression to AIDS. *Proc Natl Acad Sci U S A* **101**, 12266-12270, (2004).

410 Zhong, W., Roberts, A. D. & Woodland, D. L. Antibody-independent antiviral function of memory CD4+ T cells in vivo requires regulatory signals from CD8+ effector T cells. *J Immunol* **167**, 1379-1386, (2001).

411 Snyder, C. M., Loewendorf, A., Bonnett, E. L., Croft, M., Benedict, C. A. & Hill, A. B. CD4+ T cell help has an epitope-dependent impact on CD8+ T cell memory inflation during murine cytomegalovirus infection. *J Immunol* **183**, 3932-3941, (2009).

412 Kaur, A., Hale, C. L., Noren, B., Kassis, N., Simon, M. A. & Johnson, R. P. Decreased frequency of cytomegalovirus (CMV)-specific CD4+ T lymphocytes in simian immunodeficiency virus-infected rhesus macaques: inverse relationship with CMV viremia. *J Virol* **76**, 3646-3658, (2002).

413 Salkowitz, J. R., Sieg, S. F., Harding, C. V. & Lederman, M. M. In vitro human memory CD8 T cell expansion in response to cytomegalovirus requires CD4+ T cell help. *J Infect Dis* **189**, 971-983, (2004).

414 Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A. & Walker, B. D. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**, 1447-1450, (1997).

415 Letvin, N. L., Mascola, J. R., Sun, Y., Gorgone, D. A., Buzby, A. P., Xu, L., Yang, Z. Y., Chakrabarti, B., Rao, S. S., Schmitz, J. E., Montefiori, D. C., Barker, B. R., Bookstein, F. L. & Nabel, G. J. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* **312**, 1530-1533, (2006).

416 Zhang, Z. Q., Wietgrefe, S. W., Li, Q., Shore, M. D., Duan, L., Reilly, C., Lifson, J. D. & Haase, A. T. Roles of substrate availability and infection of resting and activated CD4+ T cells in transmission and acute simian immunodeficiency virus infection. *Proc Natl Acad Sci U S A* **101**, 5640-5645, (2004).

417 Bofill, M., Mocroft, A., Lipman, M., Medina, E., Borthwick, N. J., Sabin, C. A., Timms, A., Winter, M., Baptista, L., Johnson, M. A., Lee, C. A., Phillips, A. N. & Janossy, G. Increased numbers of primed activated CD8+CD38+CD45RO+ T cells predict the decline of CD4+ T cells in HIV-1-infected patients. *AIDS* **10**, 827-834, (1996).

418 Kitchen, C. M., Yeghiazarian, L., Hoh, R., McCune, J. M., Sinclair, E., Martin, J. N. & Deeks, S. G. Immune activation, CD4+ T cell counts, and viremia exhibit oscillatory patterns over time in patients with highly resistant HIV infection. *PLoS ONE* **6**, e21190, (2011).

419 Carbone, J., Gil, J., Benito, J. M., Navarro, J., Munoz-Fernandez, A., Bartolome, J., Zabay, J. M., Lopez, F. & Fernandez-Cruz, E. Increased levels of activated subsets of CD4 T cells add to the prognostic value of low CD4 T cell counts in a cohort of HIV-infected drug users. *AIDS* **14**, 2823-2829, (2000).

420 Grossman, Z., Meier-Schellersheim, M., Paul, W. E. & Picker, L. J. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med* **12**, 289-295, (2006).

421 Jones, R. B., Yue, F. Y., Gu, X. X., Hunter, D. V., Mujib, S., Gyenes, G., Mason, R. D., Mohamed, R., MacDonald, K. S., Kovacs, C. & Ostrowski, M. A. Human immunodeficiency virus type 1 escapes from interleukin-2-producing CD4+ T-cell responses without high-frequency fixation of mutations. *J Virol* **83**, 8722-8732, (2009).

422 Rychert, J., Saindon, S., Placek, S., Daskalakis, D. & Rosenberg, E. Sequence variation occurs in CD4 epitopes during early HIV infection. *J Acquir Immune Defic Syndr* **46**, 261-267, (2007).

423 Topham, D. J. & Doherty, P. C. Longitudinal analysis of the acute Sendai virus-specific CD4+ T cell response and memory. *J Immunol* **161**, 4530-4535, (1998).

424 Christensen, J. P. & Doherty, P. C. Quantitative analysis of the acute and long-term CD4(+) T-cell response to a persistent gammaherpesvirus. *J Virol* **73**, 4279-4283, (1999).

425 Rentenaar, R. J., Gamadia, L. E., van DerHoek, N., van Diepen, F. N., Boom, R., Weel, J. F., Wertheim-van Dillen, P. M., van Lier, R. A. & ten Berge, I. J. Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection. *J Clin Invest* **105**, 541-548, (2000).

426 Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., Mackey, E. W., Miller, J. D., Leslie, A. J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E. J., Coovadia, H. M., Goulder, P. J., Klenerman, P., Ahmed, R., Freeman, G. J. &

Walker, B. D. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* **443**, 350-354, (2006).

427 D'Souza, M., Fontenot, A. P., Mack, D. G., Lozupone, C., Dillon, S., Meditz, A., Wilson, C. C., Connick, E. & Palmer, B. E. Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. *J Immunol* **179**, 1979-1987, (2007).

428 Brenchley, J. M., Karandikar, N. J., Betts, M. R., Ambrozak, D. R., Hill, B. J., Crotty, L. E., Casazza, J. P., Kuruppu, J., Migueles, S. A., Connors, M., Roederer, M., Douek, D. C. & Koup, R. A. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* **101**, 2711-2720, (2003).

429 Di Mitri, D., Azevedo, R. I., Henson, S. M., Libri, V., Riddell, N. E., Macaulay, R., Kipling, D., Soares, M. V., Battistini, L. & Akbar, A. N. Reversible Senescence in Human CD4+CD45RA+CD27- Memory T Cells. *J Immunol*, (2011).

430 Petrovas, C., Casazza, J. P., Brenchley, J. M., Price, D. A., Gostick, E., Adams, W. C., Precopio, M. L., Schacker, T., Roederer, M., Douek, D. C. & Koup, R. A. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* **203**, 2281-2292, (2006).

431 Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., Vanderford, T. H., Chennareddi, L., Silvestri, G., Freeman, G. J., Ahmed, R. & Amara, R. R. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* **458**, 206-210, (2009).

432 Leen, A., Meij, P., Redchenko, I., Middeldorp, J., Bloemena, E., Rickinson, A. & Blake, N. Differential immunogenicity of Epstein-Barr virus latent-cycle proteins for human CD4(+) T-helper 1 responses. *J Virol* **75**, 8649-8659, (2001).

433 Oxenius, A., Fidler, S., Brady, M., Dawson, S. J., Ruth, K., Easterbrook, P. J., Weber, J. N., Phillips, R. E. & Price, D. A. Variable fate of virus-specific CD4(+) T cells during primary HIV-1 infection. *Eur J Immunol* **31**, 3782-3788, (2001).

434 Zaunders, J., Dyer, W. B. & Churchill, M. The Sydney Blood Bank Cohort: implications for viral fitness as a cause of elite control. *Curr Opin HIV AIDS* **6**, 151-156, (2011).

435 Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C. & Picker, L. J. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med* **5**, 518-525, (1999).

436 Harari, A., Petitpierre, S., Vallelian, F. & Pantaleo, G. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* **103**, 966-972, (2004).

437 Sun, Y., Schmitz, J. E., Acierno, P. M., Santra, S., Subbramanian, R. A., Barouch, D. H., Gorgone, D. A., Lifton, M. A., Beaudry, K. R., Manson, K., Philippon, V., Xu, L., Maecker, H. T., Mascola, J. R., Panicali, D., Nabel, G. J. & Letvin, N. L. Dysfunction of simian immunodeficiency virus/simian human immunodeficiency virus-induced IL-2 expression by central memory CD4+ T lymphocytes. *J Immunol* **174**, 4753-4760, (2005).

438 van Grevenynghe, J., Procopio, F. A., He, Z., Chomont, N., Riou, C., Zhang, Y., Gimmig, S., Boucher, G., Wilkinson, P., Shi, Y., Yassine-Diab, B., Said,

E. A., Trautmann, L., El Far, M., Balderas, R. S., Boulassel, M. R., Routy, J. P., Haddad, E. K. & Sekaly, R. P. Transcription factor FOXO3a controls the persistence of memory CD4(+) T cells during HIV infection. *Nat Med* **14**, 266-274, (2008).

439 Duvall, M. G., Precopio, M. L., Ambrozak, D. A., Jaye, A., McMichael, A. J., Whittle, H. C., Roederer, M., Rowland-Jones, S. L. & Koup, R. A. Polyfunctional T cell responses are a hallmark of HIV-2 infection. *European journal of immunology* **38**, 350-363, (2008).

440 Sutherland, J. S., Young, J. M., Peterson, K. L., Sanneh, B., Whittle, H. C., Rowland-Jones, S. L., Adegbola, R. A., Jaye, A. & Ota, M. O. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. *J Immunol* **184**, 6537-6544.

441 Yue, F. Y., Kovacs, C. M., Dimayuga, R. C., Gu, X. X., Parks, P., Kaul, R. & Ostrowski, M. A. Preferential apoptosis of HIV-1-specific CD4+ T cells. *J Immunol* **174**, 2196-2204, (2005).

442 Gamadia, L. E., Rentenaar, R. J., van Lier, R. A. & ten Berge, I. J. Properties of CD4(+) T cells in human cytomegalovirus infection. *Hum Immunol* **65**, 486-492, (2004).

443 Lichterfeld, M., Yu, X. G., Cohen, D., Addo, M. M., Malenfant, J., Perkins, B., Pae, E., Johnston, M. N., Strick, D., Allen, T. M., Rosenberg, E. S., Korber, B., Walker, B. D. & Altfeld, M. HIV-1 Nef is preferentially recognized by CD8 T cells in primary HIV-1 infection despite a relatively high degree of genetic diversity. *AIDS* **18**, 1383-1392, (2004).

444 Cao, J., McNevin, J., Holte, S., Fink, L., Corey, L. & McElrath, M. J. Comprehensive analysis of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-secreting CD8+ T cells in primary HIV-1 infection. *J Virol* **77**, 6867-6878, (2003).

445 Mlotshwa, M., Riou, C., Chopera, D., de Assis Rosa, D., Ntale, R., Treunicht, F., Woodman, Z., Werner, L., van Loggerenberg, F., Mlisana, K., Abdool Karim, S., Williamson, C. & Gray, C. M. Fluidity of HIV-1-specific T-cell responses during acute and early subtype C HIV-1 infection and associations with early disease progression. *J Virol* **84**, 12018-12029, (2010).

446 Gruener, N. H., Lechner, F., Jung, M. C., Diepolder, H., Gerlach, T., Lauer, G., Walker, B., Sullivan, J., Phillips, R., Pape, G. R. & Klenerman, P. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *Journal of virology* **75**, 5550-5558, (2001).

447 Lechner, F., Wong, D. K., Dunbar, P. R., Chapman, R., Chung, R. T., Dohrenwend, P., Robbins, G., Phillips, R., Klenerman, P. & Walker, B. D. Analysis of successful immune responses in persons infected with hepatitis C virus. *The Journal of experimental medicine* **191**, 1499-1512, (2000).

448 Thimme, R., Oldach, D., Chang, K. M., Steiger, C., Ray, S. C. & Chisari, F. V. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *The Journal of experimental medicine* **194**, 1395-1406, (2001).

449 Gallimore, A., Glithero, A., Godkin, A., Tissot, A. C., Pluckthun, A., Elliott, T., Hengartner, H. & Zinkernagel, R. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* **187**, 1383-1393, (1998).

450 Aukrust, P., Muller, F., Lien, E., Nordoy, I., Liabakk, N. B., Kvale, D., Espevik, T. & Froland, S. S. Tumor necrosis factor (TNF) system levels in human immunodeficiency virus-infected patients during highly active antiretroviral therapy: persistent TNF activation is associated with virologic and immunologic treatment failure. *J Infect Dis* **179**, 74-82, (1999).

451 Duh, E. J., Maury, W. J., Folks, T. M., Fauci, A. S. & Rabson, A. B. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proc Natl Acad Sci U S A* **86**, 5974-5978, (1989).

452 Garg, R., Barat, C., Ouellet, M., Lodge, R. & Tremblay, M. J. Leishmania infantum amastigotes enhance HIV-1 production in cocultures of human dendritic cells and CD4 T cells by inducing secretion of IL-6 and TNF-alpha. *PLoS Negl Trop Dis* **3**, e441, (2009).

453 Czerkinsky, C. C., Nilsson, L. A., Nygren, H., Ouchterlony, O. & Tarkowski, A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* **65**, 109-121, (1983).

454 Cox, J. H., Ferrari, G., Kalams, S. A., Lopaczynski, W., Oden, N. & D'Souza M, P. Results of an ELISPOT proficiency panel conducted in 11 laboratories participating in international human immunodeficiency virus type 1 vaccine trials. *AIDS Res Hum Retroviruses* **21**, 68-81, (2005).

455 Streeck, H., Frahm, N. & Walker, B. D. The role of IFN-gamma Elispot assay in HIV vaccine research. *Nat Protoc* **4**, 461-469, (2009).

456 Xu, Y., Theobald, V., Sung, C., DePalma, K., Atwater, L., Seiger, K., Perricone, M. A. & Richards, S. M. Validation of a HLA-A2 tetramer flow cytometric method, IFNgamma real time RT-PCR, and IFNgamma ELISPOT for detection of immunologic response to gp100 and MelanA/MART-1 in melanoma patients. *J Transl Med* **6**, 61, (2008).

457 Richeldi, L., Losi, M., D'Amico, R., Luppi, M., Ferrari, A., Mussini, C., Codeluppi, M., Cocchi, S., Prati, F., Paci, V., Meacci, M., Meccugni, B., Rumpianesi, F., Roversi, P., Cerri, S., Luppi, F., Ferrara, G., Latorre, I., Gerunda, G. E., Torelli, G., Esposito, R. & Fabbri, L. M. Performance of tests for latent tuberculosis in different groups of immunocompromised patients. *Chest* **136**, 198-204, (2009).

458 Talati, N. J., Seybold, U., Humphrey, B., Aina, A., Tapia, J., Weinfurter, P., Albalak, R. & Blumberg, H. M. Poor concordance between interferon-gamma release assays and tuberculin skin tests in diagnosis of latent tuberculosis infection among HIV-infected individuals. *BMC Infect Dis* **9**, 15, (2009).

459 Cohen, M. S., Hellmann, N., Levy, J. A., DeCock, K. & Lange, J. The spread, treatment, and prevention of HIV-1: evolution of a global pandemic. *J Clin Invest* **118**, 1244-1254, (2008).

460 Reid, A., Scano, F., Getahun, H., Williams, B., Dye, C., Nunn, P., De Cock, K. M., Hankins, C., Miller, B., Castro, K. G. & Raviglione, M. C. Towards universal access to HIV prevention, treatment, care, and support: the role of tuberculosis/HIV collaboration. *Lancet Infect Dis* **6**, 483-495, (2006).

461 Huebner, R. E., Schein, M. F. & Bass, J. B., Jr. The tuberculin skin test. *Clin Infect Dis* **17**, 968-975, (1993).

462 Fine, P. E., Sterne, J. A., Ponnighaus, J. M. & Rees, R. J. Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity. *Lancet* **344**, 1245-1249, (1994).

463 Rothel, J. S., Jones, S. L., Corner, L. A., Cox, J. C. & Wood, P. R. A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. *Aust Vet J* **67**, 134-137, (1990).

464 Lalvani, A., Nagvenkar, P., Udwadia, Z., Pathan, A. A., Wilkinson, K. A., Shastri, J. S., Ewer, K., Hill, A. V., Mehta, A. & Rodrigues, C. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. *J Infect Dis* **183**, 469-477, (2001).

465 Mazurek, G. H., Jereb, J., Vernon, A., LoBue, P., Goldberg, S. & Castro, K. Updated guidelines for using Interferon Gamma Release Assays to detect Mycobacterium tuberculosis infection - United States, 2010. *MMWR Recomm Rep* **59**, 1-25, (2010).

466 Stanley, S. A., Raghavan, S., Hwang, W. W. & Cox, J. S. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proc Natl Acad Sci U S A* **100**, 13001-13006, (2003).

467 Majlessi, L., Brodin, P., Brosch, R., Rojas, M. J., Khun, H., Huerre, M., Cole, S. T. & Leclerc, C. Influence of ESAT-6 secretion system 1 (RD1) of Mycobacterium tuberculosis on the interaction between mycobacteria and the host immune system. *J Immunol* **174**, 3570-3579, (2005).

468 Welin, A., Eklund, D., Stendahl, O. & Lerm, M. Human macrophages infected with a high burden of ESAT-6-expressing M. tuberculosis undergo caspase-1- and cathepsin B-independent necrosis. *PLoS ONE* **6**, e20302, (2011).

469 Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K. E., Marchal, G., Leclerc, C. & Cole, S. T. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat Med* **9**, 533-539, (2003).

470 Pym, A. S., Brodin, P., Brosch, R., Huerre, M. & Cole, S. T. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. *Mol Microbiol* **46**, 709-717, (2002).

471 Wards, B. J., de Lisle, G. W. & Collins, D. M. An esat6 knockout mutant of Mycobacterium bovis produced by homologous recombination will contribute to the development of a live tuberculosis vaccine. *Tuber Lung Dis* **80**, 185-189, (2000).

472 Zhang, M., Gong, J., Iyer, D. V., Jones, B. E., Modlin, R. L. & Barnes, P. F. T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection. *J Clin Invest* **94**, 2435-2442, (1994).

473 Taylor, Z., Nolan, C. M. & Blumberg, H. M. Controlling tuberculosis in the United States. Recommendations from the American Thoracic Society, CDC, and the Infectious Diseases Society of America. *MMWR Recomm Rep* **54**, 1-81, (2005).

474 Menzies, D., Pai, M. & Comstock, G. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. *Ann Intern Med* **146**, 340-354, (2007).

475 Feske, M., Nudelman, R. J., Medina, M., Lew, J., Singh, M., Couturier, J., Graviss, E. A. & Lewis, D. E. Enhancement of human antigen-specific memory T-cell responses by interleukin-7 may improve accuracy in diagnosing tuberculosis. *Clin Vaccine Immunol* **15**, 1616-1622, (2008).

476 Godard, B., Gazagne, A., Gey, A., Baptiste, M., Vingert, B., Pegaz-Fiornet, B., Strompf, L., Fridman, W. H., Glotz, D. & Tartour, E. Optimization of an

elispot assay to detect cytomegalovirus-specific CD8+ T lymphocytes. *Hum Immunol* **65**, 1307-1318, (2004).

477 Jennes, W., Kestens, L., Nixon, D. F. & Shacklett, B. L. Enhanced ELISPOT detection of antigen-specific T cell responses from cryopreserved specimens with addition of both IL-7 and IL-15--the Amplispot assay. *J Immunol Methods* **270**, 99-108, (2002).

478 Janetzki, S., Song, P., Gupta, V., Lewis, J. J. & Houghton, A. N. Insect cells as HLA-restricted antigen-presenting cells for the IFN-gamma elispot assay. *J Immunol Methods* **234**, 1-12, (2000).

479 Keating, S. M., Bejon, P., Berthoud, T., Vuola, J. M., Todryk, S., Webster, D. P., Dunachie, S. J., Moorthy, V. S., McConkey, S. J., Gilbert, S. C. & Hill, A. V. Durable human memory T cells quantifiable by cultured enzyme-linked immunospot assays are induced by heterologous prime boost immunization and correlate with protection against malaria. *J Immunol* **175**, 5675-5680, (2005).

480 Reece, W. H., Pinder, M., Gothard, P. K., Milligan, P., Bojang, K., Doherty, T., Plebanski, M., Akinwunmi, P., Everaere, S., Watkins, K. R., Voss, G., Tornieporth, N., Alloueche, A., Greenwood, B. M., Kester, K. E., McAdam, K. P., Cohen, J. & Hill, A. V. A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease. *Nat Med* **10**, 406-410, (2004).

481 Todryk, S. M., Walther, M., Bejon, P., Hutchings, C., Thompson, F. M., Urban, B. C., Porter, D. W. & Hill, A. V. Multiple functions of human T cells generated by experimental malaria challenge. *Eur J Immunol* **39**, 3042-3051, (2009).

482 Ravn, P., Demissie, A., Eguale, T., Wondwosson, H., Lein, D., Amoudy, H. A., Mustafa, A. S., Jensen, A. K., Holm, A., Rosenkrands, I., Oftung, F., Olobo, J., von Reyn, F. & Andersen, P. Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis. *J Infect Dis* **179**, 637-645, (1999).

483 Berthet, F. X., Rasmussen, P. B., Rosenkrands, I., Andersen, P. & Gicquel, B. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). *Microbiology* **144 (Pt 11)**, 3195-3203, (1998).

484 Currier, J. R., Kuta, E. G., Turk, E., Earhart, L. B., Loomis-Price, L., Janetzki, S., Ferrari, G., Birx, D. L. & Cox, J. H. A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. *J Immunol Methods* **260**, 157-172, (2002).

485 Todryk, S. M., Pathan, A. A., Keating, S., Porter, D. W., Berthoud, T., Thompson, F., Klenerman, P. & Hill, A. V. The relationship between human effector and memory T cells measured by ex vivo and cultured ELISPOT following recent and distal priming. *Immunology* **128**, 83-91, (2009).

486 Tan, P. T., Khan, A. M. & August, J. T. Highly conserved influenza A sequences as T cell epitopes-based vaccine targets to address the viral variability. *Hum Vaccin* **7**, (2011).

487 Ritchie, A. J., Campion, S. L., Kopycinski, J., Moodie, Z., Wang, Z. M., Pandya, K., Moore, S., Liu, M. K., Brackenridge, S., Kuldanek, K., Legg, K., Cohen, M. S., Delwart, E. L., Haynes, B. F., Fidler, S., McMichael, A. J. & Goonetilleke, N. Differences in HIV-specific T cell responses between HIV-

exposed and -unexposed HIV-seronegative individuals. *J Virol* **85**, 3507-3516, (2011).

488 Vyakarnam, A., Matear, P. M., Cranenburg, C., Michie, C., Beverley, P. C., Wahren, B., Gill, S. K. & Weller, I. T cell responses to peptides covering the gag p24 region of HIV-1 occur in HIV-1 seronegative individuals. *Int Immunol* **3**, 939-947, (1991).

489 Imagawa, D. T., Lee, M. H., Wolinsky, S. M., Sano, K., Morales, F., Kwok, S., Sninsky, J. J., Nishanian, P. G., Giorgi, J., Fahey, J. L. & et al. Human immunodeficiency virus type 1 infection in homosexual men who remain seronegative for prolonged periods. *N Engl J Med* **320**, 1458-1462, (1989).

490 Cheynier, R., Langlade-Demoyen, P., Marescot, M. R., Blanche, S., Blondin, G., Wain-Hobson, S., Griscelli, C., Vilmer, E. & Plata, F. Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1-infected mothers. *Eur J Immunol* **22**, 2211-2217, (1992).

491 Salkowitz, J. R., Purvis, S. F., Meyerson, H., Zimmerman, P., O'Brien, T. R., Aledort, L., Eyster, M. E., Hilgartner, M., Kessler, C., Konkle, B. A., White, G. C., 2nd, Goedert, J. J. & Lederman, M. M. Characterization of high-risk HIV-1 seronegative hemophiliacs. *Clin Immunol* **98**, 200-211, (2001).

492 Goh, W. C., Markee, J., Akridge, R. E., Meldorf, M., Musey, L., Karchmer, T., Krone, M., Collier, A., Corey, L., Emerman, M. & McElrath, M. J. Protection against human immunodeficiency virus type 1 infection in persons with repeated exposure: evidence for T cell immunity in the absence of inherited CCR5 coreceptor defects. *J Infect Dis* **179**, 548-557, (1999).

493 Paxton, W. A., Liu, R., Kang, S., Wu, L., Gingeras, T. R., Landau, N. R., Mackay, C. R. & Koup, R. A. Reduced HIV-1 infectability of CD4+ lymphocytes from exposed-uninfected individuals: association with low expression of CCR5 and high production of beta-chemokines. *Virology* **244**, 66-73, (1998).

494 Suresh, P., Wanchu, A., Bhatnagar, A., Sachdeva, R. K. & Sharma, M. Spontaneous and antigen-induced chemokine production in exposed but uninfected partners of HIV type 1-infected individuals in North India. *AIDS Res Hum Retroviruses* **23**, 261-268, (2007).

495 Stephens, J. C., Reich, D. E., Goldstein, D. B., Shin, H. D., Smith, M. W., Carrington, M., Winkler, C., Huttley, G. A., Allikmets, R., Schriml, L., Gerrard, B., Malasky, M., Ramos, M. D., Morlot, S., Tzetis, M., Oddoux, C., di Giovine, F. S., Nasioulas, G., Chandler, D., Aseev, M., Hanson, M., Kalaydjieva, L., Glavac, D., Gasparini, P., Kanavakis, E., Claustres, M., Kambouris, M., Ostrer, H., Duff, G., Baranov, V., Sibul, H., Metspalu, A., Goldman, D., Martin, N., Duffy, D., Schmidtke, J., Estivill, X., O'Brien, S. J. & Dean, M. Dating the origin of the CCR5-Delta32 AIDS-resistance allele by the coalescence of haplotypes. *Am J Hum Genet* **62**, 1507-1515, (1998).

496 Hartigan-O'Connor, D. J., Hirao, L. A., McCune, J. M. & Dandekar, S. Th17 cells and regulatory T cells in elite control over HIV and SIV. *Curr Opin HIV AIDS* **6**, 221-227, (2011).

497 Streeck, H., Jolin, J. S., Qi, Y., Yassine-Diab, B., Johnson, R. C., Kwon, D. S., Addo, M. M., Brumme, C., Routy, J. P., Little, S., Jessen, H. K., Kelleher, A. D., Hecht, F. M., Sekaly, R. P., Rosenberg, E. S., Walker, B. D., Carrington, M. & Altfeld, M. Human immunodeficiency virus type 1-specific CD8+ T-cell

responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. *J Virol* **83**, 7641-7648, (2009).

498 Schellens, I. M., Navis, M., van Deutekom, H. W., Boeser-Nunnink, B., Berkhout, B., Kootstra, N., Miedema, F., Kesmir, C., Schuitemaker, H., van Baarle, D. & Borghans, J. A. Loss of HIV-1 derived CTL epitopes restricted by protective HLA-B alleles during the HIV-1 epidemic. *AIDS*, (2011).

499 Letvin, N. L., Rao, S. S., Dang, V., Buzby, A. P., Korioth-Schmitz, B., Dombagoda, D., Parvani, J. G., Clarke, R. H., Bar, L., Carlson, K. R., Kozlowski, P. A., Hirsch, V. M., Mascola, J. R. & Nabel, G. J. No evidence for consistent virus-specific immunity in simian immunodeficiency virus-exposed, uninfected rhesus monkeys. *J Virol* **81**, 12368-12374, (2007).

500 Kaul, R., Plummer, F., Clerici, M., Bomsel, M., Lopalco, L. & Broliden, K. Mucosal IgA in exposed, uninfected subjects: evidence for a role in protection against HIV infection. *AIDS* **15**, 431-432, (2001).

501 Nguyen, M., Pean, P., Lopalco, L., Nouhin, J., Phoung, V., Ly, N., Vermisse, P., Henin, Y., Barre-Sinoussi, F., Burastero, S. E., Reynes, J. M., Carcelain, G. & Pancino, G. HIV-specific antibodies but not t-cell responses are associated with protection in seronegative partners of HIV-1-infected individuals in Cambodia. *J Acquir Immune Defic Syndr* **42**, 412-419, (2006).

502 Paxton, W. A., Martin, S. R., Tse, D., O'Brien, T. R., Skurnick, J., VanDevanter, N. L., Padian, N., Braun, J. F., Kotler, D. P., Wolinsky, S. M. & Koup, R. A. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. *Nat Med* **2**, 412-417, (1996).

503 Hatano, H., Delwart, E. L., Norris, P. J., Lee, T. H., Dunn-Williams, J., Hunt, P. W., Hoh, R., Stramer, S. L., Linnen, J. M., McCune, J. M., Martin, J. N., Busch, M. P. & Deeks, S. G. Evidence for Persistent Low-level Viremia in Individuals Who Control HIV in the Absence of Antiretroviral Therapy. *J Virol*, (2008).

504 Lasaro, M. O., Haut, L. H., Zhou, X., Xiang, Z., Zhou, D., Li, Y., Giles-Davis, W., Li, H., Engram, J. C., Dimenna, L. J., Bian, A., Sazanovich, M., Parzych, E. M., Kurupati, R., Small, J. C., Wu, T. L., Leskowitz, R. M., Klatt, N. R., Brenchley, J. M., Garber, D. A., Lewis, M., Ratcliffe, S. J., Betts, M. R., Silvestri, G. & Ertl, H. C. Vaccine-induced T cells provide partial protection against high-dose rectal SIVmac239 challenge of rhesus macaques. *Mol Ther* **19**, 417-426, (2011).

505 Faul, E. J., Aye, P. P., Papaneri, A. B., Pahar, B., McGettigan, J. P., Schiro, F., Chervoneva, I., Montefiori, D. C., Lackner, A. A. & Schnell, M. J. Rabies virus-based vaccines elicit neutralizing antibodies, poly-functional CD8+ T cell, and protect rhesus macaques from AIDS-like disease after SIV(mac251) challenge. *Vaccine* **28**, 299-308, (2009).

506 Zhu, T., Corey, L., Hwangbo, Y., Lee, J. M., Learn, G. H., Mullins, J. I. & McElrath, M. J. Persistence of extraordinarily low levels of genetically homogeneous human immunodeficiency virus type 1 in exposed seronegative individuals. *J Virol* **77**, 6108-6116, (2003).

507 Stekler, J., Swenson, P. D., Wood, R. W., Handsfield, H. H. & Golden, M. R. Targeted screening for primary HIV infection through pooled HIV-RNA testing in men who have sex with men. *Aids* **19**, 1323-1325, (2005).

508 Willberg, C. B., McConnell, J. J., Eriksson, E. M., Bragg, L. A., York, V. A., Liegler, T. J., Hecht, F. M., Grant, R. M. & Nixon, D. F. Immunity to HIV-1

is influenced by continued natural exposure to exogenous virus. *PLoS Pathog* **4**, e1000185, (2008).

509 Card, C. M., McLaren, P. J., Wachihi, C., Kimani, J., Plummer, F. A. & Fowke, K. R. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. *J Infect Dis* **199**, 1318-1322, (2009).

510 Pillai, V. K., Kannanganat, S., Penaloza-Macmaster, P., Chennareddi, L., Robinson, H. L., Blackwell, J. & Amara, R. R. Different patterns of expansion, contraction and memory differentiation of HIV-1 Gag-specific CD8 T cells elicited by adenovirus type 5 and modified vaccinia Ankara vaccines. *Vaccine*, (2011).

511 Vali, B., Tohn, R., Cohen, M. J., Sakhdari, A., Sheth, P. M., Yue, F. Y., Wong, D., Kovacs, C., Kaul, R. & Ostrowski, M. A. Characterization of cross-reactive CD8+ T-cell recognition of HLA-A2-restricted HIV-Gag (SLYNTVATL) and HCV-NS5b (ALYDVVSKL) epitopes in individuals infected with human immunodeficiency and hepatitis C viruses. *J Virol* **85**, 254-263, (2011).

512 Wucherpfennig, K. W. & Strominger, J. L. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* **80**, 695-705, (1995).

513 Gagliardi, M. C., De Petrillo, G., Salemi, S., Boffa, L., Longobardi, M. G., Dellabona, P., Casorati, G., Tanigaki, N., Harris, R., Lanzavecchia, A. & et al. Presentation of peptides by cultured monocytes or activated T cells allows specific priming of human cytotoxic T lymphocytes in vitro. *Int Immunol* **7**, 1741-1752, (1995).

514 Moser, J. M., Sassano, E. R., Leistritz del, C., Eatrides, J. M., Phogat, S., Koff, W. & Drake, D. R., 3rd. Optimization of a dendritic cell-based assay for the in vitro priming of naive human CD4+ T cells. *J Immunol Methods* **353**, 8-19, (2010).

515 Mizukoshi, E., Eisenbach, C., Edlin, B. R., Newton, K. P., Raghuraman, S., Weiler-Normann, C., Tobler, L. H., Busch, M. P., Carrington, M., McKeating, J. A., O'Brien, T. R. & Rehermann, B. Hepatitis C virus (HCV)-specific immune responses of long-term injection drug users frequently exposed to HCV. *J Infect Dis* **198**, 203-212, (2008).

516 Thurairajah, P. H., Hegazy, D., Demaine, A., Kaminski, E. R. & Cramp, M. E. Loss of virus-specific T-cell responses in HCV exposed uninfected injection drug users with drug rehabilitation. *J Infect Dis* **203**, 847-853, (2011).

517 Thurairajah, P. H., Hegazy, D., Chokshi, S., Shaw, S., Demaine, A., Kaminski, E. R., Naoumov, N. V. & Cramp, M. E. Hepatitis C virus (HCV)--specific T cell responses in injection drug users with apparent resistance to HCV infection. *J Infect Dis* **198**, 1749-1755, (2008).

518 Al-Sherbiny, M., Osman, A., Mohamed, N., Shata, M. T., Abdel-Aziz, F., Abdel-Hamid, M., Abdelwahab, S. F., Mikhail, N., Stoszek, S., Ruggeri, L., Folgori, A., Nicosia, A., Prince, A. M. & Strickland, G. T. Exposure to hepatitis C virus induces cellular immune responses without detectable viremia or seroconversion. *Am J Trop Med Hyg* **73**, 44-49, (2005).

519 Scognamiglio, P., Accapezzato, D., Casciaro, M. A., Cacciani, A., Artini, M., Bruno, G., Chircu, M. L., Sidney, J., Southwood, S., Abrignani, S., Sette, A. & Barnaba, V. Presence of effector CD8+ T cells in hepatitis C virus-exposed healthy seronegative donors. *J Immunol* **162**, 6681-6689, (1999).

520 Koumbi, L., Bertoletti, A., Anastasiadou, V., Machaira, M., Goh, W., Papadopoulos, N. G., Kafetzis, D. A. & Papaevangelou, V. Hepatitis B-specific T helper cell responses in uninfected infants born to HBsAg+/HBeAg- mothers. *Cell Mol Immunol* **7**, 454-458, (2010).

521 Currier, J., Beck, H. P., Currie, B. & Good, M. F. Antigens released at schizont burst stimulate Plasmodium falciparum-specific CD4+ T cells from non-exposed donors: potential for cross-reactive memory T cells to cause disease. *Int Immunol* **7**, 821-833, (1995).

522 Fell, A. H., Silins, S. L., Baumgarth, N. & Good, M. F. Plasmodium falciparum-specific T cell clones from non-exposed and exposed donors are highly diverse in TCR beta chain V segment usage. *Int Immunol* **8**, 1877-1887, (1996).

523 Fonseca, S. G., Coutinho-Silva, A., Fonseca, L. A., Segurado, A. C., Moraes, S. L., Rodrigues, H., Hammer, J., Kallas, E. G., Sidney, J., Sette, A., Kalil, J. & Cunha-Neto, E. Identification of novel consensus CD4 T-cell epitopes from clade B HIV-1 whole genome that are frequently recognized by HIV-1 infected patients. *Aids* **20**, 2263-2273, (2006).

524 De Groot, A. S., Marcon, L., Bishop, E. A., Rivera, D., Kutzler, M., Weiner, D. B. & Martin, W. HIV vaccine development by computer assisted design: the GAIA vaccine. *Vaccine* **23**, 2136-2148, (2005).

525 De Groot, A. S., Bishop, E. A., Khan, B., Lally, M., Marcon, L., Franco, J., Mayer, K. H., Carpenter, C. C. & Martin, W. Engineering immunogenic consensus T helper epitopes for a cross-clade HIV vaccine. *Methods* **34**, 476-487, (2004).

526 Boritz, E., Rapaport, E. L., Campbell, T. B., Koeppe, J. R. & Wilson, C. C. CD4+ T cell targeting of human immunodeficiency virus type 1 (HIV-1) peptide sequences present in vivo during chronic, progressive HIV-1 disease. *Virology* **361**, 34-44, (2007).

527 Picker, L. J. Immunopathogenesis of acute AIDS virus infection. *Curr Opin Immunol* **18**, 399-405, (2006).

528 Persaud, S. P., Donermeyer, D. L., Weber, K. S., Kranz, D. M. & Allen, P. M. High-affinity T cell receptor differentiates cognate peptide-MHC and altered peptide ligands with distinct kinetics and thermodynamics. *Mol Immunol* **47**, 1793-1801, (2010).

529 Sykulev, Y., Joo, M., Vturina, I., Tsomides, T. J. & Eisen, H. N. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* **4**, 565-571, (1996).

530 Reay, P. A., Kantor, R. M. & Davis, M. M. Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of moth cytochrome c (93-103). *J Immunol* **152**, 3946-3957, (1994).

531 Bevan, M. J. In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature* **269**, 417-418, (1977).

532 Lieber, M. R. Site-specific recombination in the immune system. *FASEB J* **5**, 2934-2944, (1991).

533 Cameron, T. O., Cohen, G. B., Islam, S. A. & Stern, L. J. Examination of the highly diverse CD4(+) T-cell repertoire directed against an influenza peptide: a step towards TCR proteomics. *Immunogenetics* **54**, 611-620, (2002).

534 Pewe, L. L., Netland, J. M., Heard, S. B. & Perlman, S. Very diverse CD8 T cell clonotypic responses after virus infections. *J Immunol* **172**, 3151-3156, (2004).

535 Price, D. A., West, S. M., Betts, M. R., Ruff, L. E., Brenchley, J. M., Ambrozak, D. R., Edghill-Smith, Y., Kuroda, M. J., Bogdan, D., Kunstman, K., Letvin, N. L., Franchini, G., Wolinsky, S. M., Koup, R. A. & Douek, D. C. T cell receptor recognition motifs govern immune escape patterns in acute SIV infection. *Immunity* **21**, 793-803, (2004).

536 Ignatowicz, L., Kappler, J. & Marrack, P. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* **84**, 521-529, (1996).

537 Huesmann, M., Scott, B., Kisielow, P. & von Boehmer, H. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell* **66**, 533-540, (1991).

538 Ashton-Rickardt, P. G., Van Kaer, L., Schumacher, T. N., Ploegh, H. L. & Tonegawa, S. Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. *Cell* **73**, 1041-1049, (1993).

539 Viret, C., He, X. & Janeway, C. A., Jr. On the self-referential nature of naive MHC class II-restricted T cells. *J Immunol* **165**, 6183-6192, (2000).

540 Ernst, B., Lee, D. S., Chang, J. M., Sprent, J. & Surh, C. D. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* **11**, 173-181, (1999).

541 Viret, C., Wong, F. S. & Janeway, C. A., Jr. Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. *Immunity* **10**, 559-568, (1999).

542 Vidal, K., Hsu, B. L., Williams, C. B. & Allen, P. M. Endogenous altered peptide ligands can affect peripheral T cell responses. *J Exp Med* **183**, 1311-1321, (1996).

543 Krogsgaard, M., Li, Q. J., Sumen, C., Huppa, J. B., Huse, M. & Davis, M. M. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature* **434**, 238-243, (2005).

544 Hiemstra, H. S., van Veelen, P. A., Willemen, S. J., Benckhuijsen, W. E., Geluk, A., de Vries, R. R., Roep, B. O. & Drijfhout, J. W. Quantitative determination of TCR cross-reactivity using peptide libraries and protein databases. *Eur J Immunol* **29**, 2385-2391, (1999).

545 Frankild, S., de Boer, R. J., Lund, O., Nielsen, M. & Kesmir, C. Amino acid similarity accounts for T cell cross-reactivity and for "holes" in the T cell repertoire. *PLoS ONE* **3**, e1831, (2008).

546 Beaumier, C. M. & Rothman, A. L. Cross-reactive memory CD4+ T cells alter the CD8+ T-cell response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. *Viral Immunol* **22**, 215-219, (2009).

547 Moran, E., Simmons, C., Vinh Chau, N., Luhn, K., Wills, B., Dung, N. P., Thao le, T. T., Hien, T. T., Farrar, J., Rowland-Jones, S. & Dong, T. Preservation of a critical epitope core region is associated with the high degree of flaviviral cross-reactivity exhibited by a dengue-specific CD4+ T cell clone. *Eur J Immunol* **38**, 1050-1057, (2008).

548 Aihara, H., Takasaki, T., Matsutani, T., Suzuki, R. & Kurane, I. Establishment and characterization of Japanese encephalitis virus-specific, human CD4(+) T-cell clones: flavivirus cross-reactivity, protein recognition, and cytotoxic activity. *J Virol* **72**, 8032-8036, (1998).

549 Kurane, I., Brinton, M. A., Samson, A. L. & Ennis, F. A. Dengue virus-specific, human CD4+ CD8- cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. *J Virol* **65**, 1823-1828, (1991).

550 Scheible, K., Zhang, G., Baer, J., Azadniv, M., Lambert, K., Pryhuber, G., Treanor, J. J. & Topham, D. J. CD8+ T cell immunity to 2009 pandemic and seasonal H1N1 influenza viruses. *Vaccine* **29**, 2159-2168, (2011).

551 Lee, L. Y., Ha do, L. A., Simmons, C., de Jong, M. D., Chau, N. V., Schumacher, R., Peng, Y. C., McMichael, A. J., Farrar, J. J., Smith, G. L., Townsend, A. R., Askonas, B. A., Rowland-Jones, S. & Dong, T. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J Clin Invest* **118**, 3478-3490, (2008).

552 Jameson, J., Cruz, J. & Ennis, F. A. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J Virol* **72**, 8682-8689, (1998).

553 Clute, S. C., Naumov, Y. N., Watkin, L. B., Aslan, N., Sullivan, J. L., Thorley-Lawson, D. A., Luzuriaga, K., Welsh, R. M., Puzone, R., Celada, F. & Selin, L. K. Broad cross-reactive TCR repertoires recognizing dissimilar Epstein-Barr and influenza A virus epitopes. *J Immunol* **185**, 6753-6764, (2010).

554 Urbani, S., Amadei, B., Fisicaro, P., Pilli, M., Missale, G., Bertoletti, A. & Ferrari, C. Heterologous T cell immunity in severe hepatitis C virus infection. *J Exp Med* **201**, 675-680, (2005).

555 Cornberg, M., Sheridan, B. S., Saccoccio, F. M., Brehm, M. A. & Selin, L. K. Protection against vaccinia virus challenge by CD8 memory T cells resolved by molecular mimicry. *J Virol* **81**, 934-944, (2007).

556 Venturini, S., Allicotti, G., Zhao, Y., Simon, R., Burton, D. R., Pinilla, C. & Poignard, P. Identification of peptides from human pathogens able to cross-activate an HIV-1-gag-specific CD4+ T cell clone. *Eur J Immunol* **36**, 27-36, (2006).

557 Mathurin, K. S., Martens, G. W., Kornfeld, H. & Welsh, R. M. CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses. *J Virol* **83**, 3528-3539, (2009).

558 Lang, H. L., Jacobsen, H., Ikemizu, S., Andersson, C., Harlos, K., Madsen, L., Hjorth, P., Sondergaard, L., Svejgaard, A., Wucherpfennig, K., Stuart, D. I., Bell, J. I., Jones, E. Y. & Fugger, L. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat Immunol* **3**, 940-943, (2002).

559 Harkiolaki, M., Holmes, S. L., Svendsen, P., Gregersen, J. W., Jensen, L. T., McMahon, R., Friese, M. A., van Boxel, G., Etzensperger, R., Tzartos, J. S., Kranc, K., Sainsbury, S., Harlos, K., Mellins, E. D., Palace, J., Esiri, M. M., van der Merwe, P. A., Jones, E. Y. & Fugger, L. T cell-mediated autoimmune disease due to low-affinity crossreactivity to common microbial peptides. *Immunity* **30**, 348-357, (2009).

560 Lu, G., Villalba, M., Coscia, M. R., Hoffman, D. R. & King, T. P. Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. *J Immunol* **150**, 2823-2830, (1993).

561 Fujinami, R. S. & Oldstone, M. B. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* **230**, 1043-1045, (1985).

562 Felix, N. J., Donermeyer, D. L., Horvath, S., Walters, J. J., Gross, M. L., Suri, A. & Allen, P. M. Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. *Nat Immunol* **8**, 388-397, (2007).

563 Martin, R., Gran, B., Zhao, Y., Markovic-Plese, S., Bielekova, B., Marques, A., Sung, M. H., Hemmer, B., Simon, R., McFarland, H. F. & Pinilla, C. Molecular mimicry and antigen-specific T cell responses in multiple sclerosis and chronic CNS Lyme disease. *J Autoimmun* **16**, 187-192, (2001).

564 Krieger, J. I., Karr, R. W., Grey, H. M., Yu, W. Y., O'Sullivan, D., Batovsky, L., Zheng, Z. L., Colon, S. M., Gaeta, F. C., Sidney, J. & et al. Single amino acid changes in DR and antigen define residues critical for peptide-MHC binding and T cell recognition. *J Immunol* **146**, 2331-2340, (1991).

565 Jardetzky, T. S., Gorga, J. C., Busch, R., Rothbard, J., Strominger, J. L. & Wiley, D. C. Peptide binding to HLA-DR1: a peptide with most residues substituted to alanine retains MHC binding. *EMBO J* **9**, 1797-1803, (1990).

566 Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E. & Wiley, D. C. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* **384**, 134-141, (1996).

567 Wucherpfennig, K. W., Sette, A., Southwood, S., Oseroff, C., Matsui, M., Strominger, J. L. & Hafler, D. A. Structural requirements for binding of an immunodominant myelin basic protein peptide to DR2 isotypes and for its recognition by human T cell clones. *J Exp Med* **179**, 279-290, (1994).

568 Lorenz, R. G., Tyler, A. N. & Allen, P. M. Reconstruction of the immunogenic peptide RNase(43-56) by identification and transfer of the critical residues into an unrelated peptide backbone. *J Exp Med* **170**, 203-215, (1989).

569 Takahashi, H., Merli, S., Putney, S. D., Houghten, R., Moss, B., Germain, R. N. & Berzofsky, J. A. A single amino acid interchange yields reciprocal CTL specificities for HIV-1 gp160. *Science* **246**, 118-121, (1989).

570 Boniface, J. J., Reich, Z., Lyons, D. S. & Davis, M. M. Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. *Proc Natl Acad Sci U S A* **96**, 11446-11451, (1999).

571 Reiser, J. B., Darnault, C., Gregoire, C., Mosser, T., Mazza, G., Kearney, A., van der Merwe, P. A., Fontecilla-Camps, J. C., Housset, D. & Malissen, B. CDR3 loop flexibility contributes to the degeneracy of TCR recognition. *Nat Immunol* **4**, 241-247, (2003).

572 Reiser, J. B., Gregoire, C., Darnault, C., Mosser, T., Guimezanes, A., Schmitt-Verhulst, A. M., Fontecilla-Camps, J. C., Mazza, G., Malissen, B. & Housset, D. A T cell receptor CDR3beta loop undergoes conformational changes of unprecedented magnitude upon binding to a peptide/MHC class I complex. *Immunity* **16**, 345-354, (2002).

573 Holler, P. D. & Kranz, D. M. T cell receptors: affinities, cross-reactivities, and a conformer model. *Mol Immunol* **40**, 1027-1031, (2004).

574 Borbulevych, O. Y., Piepenbrink, K. H. & Baker, B. M. Conformational melding permits a conserved binding geometry in TCR recognition of foreign and self molecular mimics. *J Immunol* **186**, 2950-2958, (2011).

575 Lee, J. K., Stewart-Jones, G., Dong, T., Harlos, K., Di Gleria, K., Dorrell, L., Douek, D. C., van der Merwe, P. A., Jones, E. Y. & McMichael, A. J. T cell cross-reactivity and conformational changes during TCR engagement. *J Exp Med* **200**, 1455-1466, (2004).

576 Quaratino, S., Thorpe, C. J., Travers, P. J. & Londei, M. Similar antigenic surfaces, rather than sequence homology, dictate T-cell epitope molecular mimicry. *Proc Natl Acad Sci U S A* **92**, 10398-10402, (1995).

577 Maryanski, J. L., Jongeneel, C. V., Bucher, P., Casanova, J. L. & Walker, P. R. Single-cell PCR analysis of TCR repertoires selected by antigen in vivo: a high magnitude CD8 response is comprised of very few clones. *Immunity* **4**, 47-55, (1996).

578 Pewe, L., Heard, S. B., Bergmann, C., Dailey, M. O. & Perlman, S. Selection of CTL escape mutants in mice infected with a neurotropic coronavirus: quantitative estimate of TCR diversity in the infected central nervous system. *J Immunol* **163**, 6106-6113, (1999).

579 Manfras, B. J., Weidenbach, H., Beckh, K. H., Kern, P., Moller, P., Adler, G., Mertens, T. & Boehm, B. O. Oligoclonal CD8+ T-cell expansion in patients with chronic hepatitis C is associated with liver pathology and poor response to interferon-alpha therapy. *J Clin Immunol* **24**, 258-271, (2004).

580 Wilson, J. D., Ogg, G. S., Allen, R. L., Goulder, P. J., Kelleher, A., Sewell, A. K., O'Callaghan, C. A., Rowland-Jones, S. L., Callan, M. F. & McMichael, A. J. Oligoclonal expansions of CD8(+) T cells in chronic HIV infection are antigen specific. *J Exp Med* **188**, 785-790, (1998).

581 Pantaleo, G., Demarest, J. F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P. & et al. Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* **370**, 463-467, (1994).

582 Fae, K., Kalil, J., Toubert, A. & Guilherme, L. Heart infiltrating T cell clones from a rheumatic heart disease patient display a common TCR usage and a degenerate antigen recognition pattern. *Mol Immunol* **40**, 1129-1135, (2004).

583 Miyazaki, E., Kawana-Tachikawa, A., Tomizawa, M., Nunoya, J., Odawara, T., Fujii, T., Shi, Y., Gao, G. F. & Iwamoto, A. Highly restricted T-cell receptor repertoire in the CD8+ T-cell response against an HIV-1 epitope with a stereotypic amino acid substitution. *AIDS* **23**, 651-660, (2009).

584 Meyer-Olson, D., Shoukry, N. H., Brady, K. W., Kim, H., Olson, D. P., Hartman, K., Shintani, A. K., Walker, C. M. & Kalams, S. A. Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape. *J Exp Med* **200**, 307-319, (2004).

585 Platsoucas, C. D. & Oleszak, E. L. Human autoimmune diseases are specific antigen-driven T-cell diseases: identification of the antigens. *Immunol Res* **38**, 359-372, (2007).

586 Boucher, A., Desforges, M., Duquette, P. & Talbot, P. J. Long-term human coronavirus-myelin cross-reactive T-cell clones derived from multiple sclerosis patients. *Clin Immunol* **123**, 258-267, (2007).

587 Cornberg, M., Chen, A. T., Wilkinson, L. A., Brehm, M. A., Kim, S. K., Calcagno, C., Ghersi, D., Puzone, R., Celada, F., Welsh, R. M. & Selin, L. K. Narrowed TCR repertoire and viral escape as a consequence of heterologous immunity. *J Clin Invest* **116**, 1443-1456, (2006).

588 McHeyzer-Williams, M. G. & Davis, M. M. Antigen-specific development of primary and memory T cells in vivo. *Science* **268**, 106-111, (1995).

589 Curtsinger, J. M., Lins, D. C. & Mescher, M. F. CD8+ memory T cells (CD44high, Ly-6C+) are more sensitive than naive cells to (CD44low, Ly-6C-) to TCR/CD8 signaling in response to antigen. *J Immunol* **160**, 3236-3243, (1998).

590 Brehm, M. A., Pinto, A. K., Daniels, K. A., Schneck, J. P., Welsh, R. M. & Selin, L. K. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat Immunol* **3**, 627-634, (2002).

591 Haanen, J. B., Wolkers, M. C., Kruisbeek, A. M. & Schumacher, T. N. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. *J Exp Med* **190**, 1319-1328, (1999).

592 Misko, I. S., Cross, S. M., Khanna, R., Elliott, S. L., Schmidt, C., Pye, S. J. & Silins, S. L. Crossreactive recognition of viral, self, and bacterial peptide ligands by human class I-restricted cytotoxic T lymphocyte clonotypes: implications for molecular mimicry in autoimmune disease. *Proc Natl Acad Sci U S A* **96**, 2279-2284, (1999).

593 Acierno, P. M., Newton, D. A., Brown, E. A., Maes, L. A., Baatz, J. E. & Gattoni-Celli, S. Cross-reactivity between HLA-A2-restricted FLU-M1:58-66 and HIV p17 GAG:77-85 epitopes in HIV-infected and uninfected individuals. *J Transl Med* **1**, 3, (2003).

594 Hohn, H., Kortsik, C., Tully, G., Nilges, K., Necker, A., Freitag, K., Neukirch, C., Galle, P., Lohr, H. & Maeurer, M. J. Longitudinal analysis of Mycobacterium tuberculosis 19-kDa antigen-specific T cells in patients with pulmonary tuberculosis: association with disease activity and cross-reactivity to a peptide from HIVenv gp120. *Eur J Immunol* **33**, 1613-1623, (2003).

595 MacIsaac, C., Curtis, N., Cade, J. & Visvanathan, K. Rapid analysis of the Vbeta repertoire of CD4 and CD8 T lymphocytes in whole blood. *J Immunol Methods* **283**, 9-15, (2003).

596 Muraro, P. A., Jacobsen, M., Necker, A., Nagle, J. W., Gaber, R., Sommer, N., Oertel, W. H., Martin, R. & Hemmer, B. Rapid identification of local T cell expansion in inflammatory organ diseases by flow cytometric T cell receptor Vbeta analysis. *J Immunol Methods* **246**, 131-143, (2000).

597 Haney, D., Quigley, M. F., Asher, T. E., Ambrozak, D. R., Gostick, E., Price, D. A., Douek, D. C. & Betts, M. R. Isolation of viable antigen-specific CD8+ T cells based on membrane-bound tumor necrosis factor (TNF)-alpha expression. *J Immunol Methods* **369**, 33-41, (2011).

598 Kennedy, P. T., Urbani, S., Moses, R. A., Amadei, B., Fisicaro, P., Lloyd, J., Maini, M. K., Dusheiko, G., Ferrari, C. & Bertoletti, A. The influence of T cell cross-reactivity on HCV-peptide specific human T cell response. *Hepatology* **43**, 602-611, (2006).

599 Stramer, S. L., Glynn, S. A., Kleinman, S. H., Strong, D. M., Caglioti, S., Wright, D. J., Dodd, R. Y. & Busch, M. P. Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid-amplification testing. *N Engl J Med* **351**, 760-768, (2004).

600 Currier, J., Sattabongkot, J. & Good, M. F. 'Natural' T cells responsive to malaria: evidence implicating immunological cross-reactivity in the maintenance of TCR alpha beta+ malaria-specific responses from non-exposed donors. *Int Immunol* **4**, 985-994, (1992).

601 Zevering, Y., Amante, F., Smillie, A., Currier, J., Smith, G., Houghten, R. A. & Good, M. F. High frequency of malaria-specific T cells in non-exposed humans. *Eur J Immunol* **22**, 689-696, (1992).

602 Hemmer, B., Vergelli, M., Gran, B., Ling, N., Conlon, P., Pinilla, C., Houghten, R., McFarland, H. F. & Martin, R. Predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *J Immunol* **160**, 3631-3636, (1998).

603 Kim, S. K., Cornberg, M., Wang, X. Z., Chen, H. D., Selin, L. K. & Welsh, R. M. Private specificities of CD8 T cell responses control patterns of heterologous immunity. *J Exp Med* **201**, 523-533, (2005).

604 Robins, H. S., Campregher, P. V., Srivastava, S. K., Wacher, A., Turtle, C. J., Kahsai, O., Riddell, S. R., Warren, E. H. & Carlson, C. S. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* **114**, 4099-4107, (2009).

605 Cook, J. E. & Beverley, P. C. Analysis of lymphocyte diversity in the elderly: heteroduplex analysis and alternative techniques. *Exp Gerontol* **36**, 583-589, (2001).

606 Freeman, J. D., Warren, R. L., Webb, J. R., Nelson, B. H. & Holt, R. A. Profiling the T-cell receptor beta-chain repertoire by massively parallel sequencing. *Genome Res* **19**, 1817-1824, (2009).

607 Quigley, M. F., Almeida, J. R., Price, D. A. & Douek, D. C. Unbiased molecular analysis of T cell receptor expression using template-switch anchored rt-PCR. *Curr Protoc Immunol* **Chapter 10**, Unit10 33, (2011).

608 Brosterhus, H., Brings, S., Leyendeckers, H., Manz, R. A., Miltenyi, S., Radbruch, A., Assenmacher, M. & Schmitz, J. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. *Eur J Immunol* **29**, 4053-4059, (1999).

609 Manz, R., Assenmacher, M., Pfluger, E., Miltenyi, S. & Radbruch, A. Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. *Proc Natl Acad Sci U S A* **92**, 1921-1925, (1995).

610 Willinger, T., Freeman, T., Hasegawa, H., McMichael, A. J. & Callan, M. F. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. *J Immunol* **175**, 5895-5903, (2005).

611 Cantisan, S., Solana, R., Lara, R., Rodriguez-Benot, A., Vaquero, J. M., Gutierrez-Aroca, J., Gayoso, I., Montejo, M., Rivero, A. & Torre-Cisneros, J. CD45RA expression on HCMV-specific effector memory CD8+ T cells is associated with the duration and intensity of HCMV replication after transplantation. *Clin Immunol* **137**, 81-88, (2010).

612 Hammond, T., Lee, S., Watson, M. W., Flexman, J. P., Cheng, W., Fernandez, S. & Price, P. Toll-like receptor (TLR) expression on CD4+ and CD8+ T-cells in patients chronically infected with hepatitis C virus. *Cell Immunol* **264**, 150-155, (2010).

613 Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E. & Lipp, M. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**, 23-33, (1999).

614 Hansen, S. G., Ford, J. C., Lewis, M. S., Ventura, A. B., Hughes, C. M., Coyne-Johnson, L., Whizin, N., Oswald, K., Shoemaker, R., Swanson, T., Legasse, A. W., Chiuchiolo, M. J., Parks, C. L., Axthelm, M. K., Nelson, J. A., Jarvis, M. A., Piatak, M., Jr., Lifson, J. D. & Picker, L. J. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* **473**, 523-527, (2011).

615 Schmidt, J., Neumann-Haefelin, C., Altay, T., Gostick, E., Price, D. A., Lohmann, V., Blum, H. E. & Thimme, R. Immunodominance of HLA-A2-restricted hepatitis C virus-specific CD8+ T cell responses is linked to naive-precursor frequency. *J Virol* **85**, 5232-5236, (2011).

616 Moon, J. J., Chu, H. H., Pepper, M., McSorley, S. J., Jameson, S. C., Kedl, R. M. & Jenkins, M. K. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* **27**, 203-213, (2007).

617 Tan, A. C., La Gruta, N. L., Zeng, W. & Jackson, D. C. Precursor Frequency and Competition Dictate the HLA-A2-Restricted CD8+ T Cell Responses to Influenza A Infection and Vaccination in HLA-A2.1 Transgenic Mice. *J Immunol* **187**, 1895-1902, (2011).

618 Sandonis, V., Casado, C., Alvaro, T., Pernas, M., Olivares, I., Garcia, S., Rodriguez, C., del Romero, J. & Lopez-Galindez, C. A combination of defective DNA and protective host factors are found in a set of HIV-1 ancestral LTNPs. *Virology* **391**, 73-82, (2009).

619 Eugen-Olsen, J., Iversen, A. K., Garred, P., Koppelhus, U., Pedersen, C., Benfield, T. L., Sorensen, A. M., Katzenstein, T., Dickmeiss, E., Gerstoft, J., Skinhoj, P., Svejgaard, A., Nielsen, J. O. & Hofmann, B. Heterozygosity for a deletion in the CKR-5 gene leads to prolonged AIDS-free survival and slower CD4 T-cell decline in a cohort of HIV-seropositive individuals. *AIDS* **11**, 305-310, (1997).

620 Jang, D. H., Choi, B. S. & Kim, S. S. The effects of RANTES/CCR5 promoter polymorphisms on HIV disease progression in HIV-infected Koreans. *Int J Immunogenet* **35**, 101-105, (2008).

621 Migueles, S. A. & Connors, M. Frequency and function of HIV-specific CD8(+) T cells. *Immunol Lett* **79**, 141-150, (2001).

622 Estes, J. D., Gordon, S. N., Zeng, M., Chahroudi, A. M., Dunham, R. M., Staprans, S. I., Reilly, C. S., Silvestri, G. & Haase, A. T. Early resolution of acute immune activation and induction of PD-1 in SIV-infected sooty mangabeys distinguishes nonpathogenic from pathogenic infection in rhesus macaques. *J Immunol* **180**, 6798-6807, (2008).

623 Mazzotta, F., Di Pietro, M., Balocchini, E., Paci, P., Galanti, C., Abeni, D. D. & Perucci, C. A. Prevalence of HIV-1 infection in 18 year old men in Tuscany. The Toscana Collaborative Group. *Int J Epidemiol* **24**, 1030-1033, (1995).

624 Saracco, A., Musicco, M., Nicolosi, A., Angarano, G., Arici, C., Gavazzeni, G., Costigliola, P., Gafa, S., Gervasoni, C., Luzzati, R. & et al. Man-to-woman sexual transmission of HIV: longitudinal study of 343 steady partners of infected men. *J Acquir Immune Defic Syndr* **6**, 497-502, (1993).

625 Clerici, M., Clark, E. A., Polacino, P., Axberg, I., Kuller, L., Casey, N. I., Morton, W. R., Shearer, G. M. & Benveniste, R. E. T-cell proliferation to subinfectious SIV correlates with lack of infection after challenge of macaques. *AIDS* **8**, 1391-1395, (1994).

626 Buchbinder, S. P., Mehrotra, D. V., Duerr, A., Fitzgerald, D. W., Mogg, R., Li, D., Gilbert, P. B., Lama, J. R., Marmor, M., Del Rio, C., McElrath, M. J., Casimiro, D. R., Gottesdiener, K. M., Chodakewitz, J. A., Corey, L. & Robertson, M. N. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* **372**, 1881-1893, (2008).

627 McElrath, M. J., De Rosa, S. C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., Defawe, O. D., Carter, D. K., Hural, J., Akondy, R., Buchbinder, S. P., Robertson, M. N., Mehrotra, D. V., Self, S. G., Corey, L., Shiver, J. W. &

Casimiro, D. R. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* **372**, 1894-1905, (2008).

628 Clute, S. C., Watkin, L. B., Cornberg, M., Naumov, Y. N., Sullivan, J. L., Luzuriaga, K., Welsh, R. M. & Selin, L. K. Cross-reactive influenza virus-specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus-associated infectious mononucleosis. *J Clin Invest* **115**, 3602-3612, (2005).

629 Mongkolsapaya, J., Dejnirattisai, W., Xu, X. N., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., Sawasdivorn, S., Duangchinda, T., Dong, T., Rowland-Jones, S., Yenchitsomanus, P. T., McMichael, A., Malasit, P. & Screaton, G. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med* **9**, 921-927, (2003).

630 Garcia, K. C., Radu, C. G., Ho, J., Ober, R. J. & Ward, E. S. Kinetics and thermodynamics of T cell receptor- autoantigen interactions in murine experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **98**, 6818-6823, (2001).

631 Haynes, B. Case Control Study of the RV144 Trial for Immune Correlates: The Analysis and Way Forward. *http://app2.capitalreach.com/esp1204/servlet/tc?c=10188&cn=aidsvac&s=20457&dp=player.jsp&e=16010&mediaType=slideVideo*, (2011).

632 Rolland, M. & Gilbert, P. Evaluating immune correlates in HIV-1 vaccine efficacy trials: what RV144 may provide. *AIDS Res Hum Retroviruses*, (2011).

633 Maksiutov, A. Z., Bachinskii, A. G. & Bazhan, S. I. [Searching for local similarities between HIV-1 and human proteins. Application to vaccines]. *Mol Biol (Mosk)* **36**, 447-459, (2002).

634 Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, J., Tammi, R., Jones, M. & Jackson, D. G. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* **144**, 789-801, (1999).

635 Laurent, T. C. & Fraser, J. R. Hyaluronan. *FASEB J* **6**, 2397-2404, (1992).

636 Hale, P. M., Cease, K. B., Houghten, R. A., Ouyang, C., Putney, S., Javaherian, K., Margalit, H., Cornette, J. L., Spouge, J. L., DeLisi, C. & et al. T cell multideterminant regions in the human immunodeficiency virus envelope: toward overcoming the problem of major histocompatibility complex restriction. *Int Immunol* **1**, 409-415, (1989).

637 Wahren, B., Rosen, J., Sandstrom, E., Mathiesen, T., Modrow, S. & Wigzell, H. HIV-1 peptides induce a proliferative response in lymphocytes from infected persons. *J Acquir Immune Defic Syndr* **2**, 448-456, (1989).

638 De Groot, A. S., Jesdale, B., Martin, W., Saint Aubin, C., Sbai, H., Bosma, A., Lieberman, J., Skowron, G., Mansourati, F. & Mayer, K. H. Mapping cross-clade HIV-1 vaccine epitopes using a bioinformatics approach. *Vaccine* **21**, 4486-4504, (2003).

639 Wolinsky, S. M., Rinaldo, C. R., Kwok, S., Sninsky, J. J., Gupta, P., Imagawa, D., Farzadegan, H., Jacobson, L. P., Grovit, K. S., Lee, M. H. & et al. Human immunodeficiency virus type 1 (HIV-1) infection a median of 18 months before a diagnostic western blot. Evidence from a cohort of homosexual men. *Ann Intern Med* **111**, 961-972, (1989).

640 Betts, M. R., Ambrozak, D. R., Douek, D. C., Bonhoeffer, S., Brenchley, J. M., Casazza, J. P., Koup, R. A. & Picker, L. J. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses:

relationship to viral load in untreated HIV infection. *J Virol* **75**, 11983-11991, (2001).

641 Addo, M. M., Yu, X. G., Rathod, A., Cohen, D., Eldridge, R. L., Strick, D., Johnston, M. N., Corcoran, C., Wurcel, A. G., Fitzpatrick, C. A., Feeney, M. E., Rodriguez, W. R., Basgoz, N., Draenert, R., Stone, D. R., Brander, C., Goulder, P. J., Rosenberg, E. S., Altfeld, M. & Walker, B. D. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol* **77**, 2081-2092, (2003).

642 Okamoto, Y., Abe, T., Niwa, T., Mizuhashi, S. & Nishida, M. Development of a dual color enzyme-linked immunospot assay for simultaneous detection of murine T helper type 1- and T helper type 2-cells. *Immunopharmacology* **39**, 107-116, (1998).

643 Gazagne, A., Claret, E., Wijdenes, J., Yssel, H., Bousquet, F., Levy, E., Vielh, P., Scotte, F., Goupil, T. L., Fridman, W. H. & Tartour, E. A Fluorospot assay to detect single T lymphocytes simultaneously producing multiple cytokines. *J Immunol Methods* **283**, 91-98, (2003).

644 Okamoto, Y., Gotoh, Y., Shiraishi, H. & Nishida, M. A human dual-color enzyme-linked immunospot assay for simultaneous detection of interleukin 2- and interleukin 4-secreting cells. *Int Immunopharmacol* **4**, 149-156, (2004).

645 Boulet, S., Ndongala, M. L., Peretz, Y., Boisvert, M. P., Boulassel, M. R., Tremblay, C., Routy, J. P., Sekaly, R. P. & Bernard, N. F. A dual color ELISPOT method for the simultaneous detection of IL-2 and IFN-gamma HIV-specific immune responses. *J Immunol Methods* **320**, 18-29, (2007).

646 Burton, C. T., Gotch, F. & Imami, N. Rapid qualitative and quantitative analysis of T-cell responses in HIV-1-infected individuals receiving successful HAART and HIV-1 sero-negative controls: concomitant assessment of perforin, IFN-gamma and IL-4 secretion. *J Immunol Methods* **308**, 216-230, (2006).

647 Adams, S. L., Biti, R. A. & Stewart, G. J. T-cell response to HIV in natural infection: optimized culture conditions for detecting responses to gag peptides. *J Acquir Immune Defic Syndr Hum Retrovirol* **15**, 257-263, (1997).

648 Castelli, F. A., Houitte, D., Munier, G., Szely, N., Lecoq, A., Briand, J. P., Muller, S. & Maillere, B. Immunoprevalence of the CD4+ T-cell response to HIV Tat and Vpr proteins is provided by clustered and disperse epitopes, respectively. *Eur J Immunol* **38**, 2821-2831, (2008).

649 Cohen, W. M., Pouvelle-Moratille, S., Wang, X. F., Farci, S., Munier, G., Charron, D., Menez, A., Busson, M. & Maillere, B. Scanning the HIV genome for CD4+ T cell epitopes restricted to HLA-DP4, the most prevalent HLA class II molecule. *J Immunol* **176**, 5401-5408, (2006).

650 Chevalier, M. F., Julg, B., Pyo, A., Flanders, M., Ranasinghe, S., Soghoian, D. Z., Kwon, D. S., Rychert, J., Lian, J., Muller, M. I., Cutler, S., McAndrew, E., Jessen, H., Pereyra, F., Rosenberg, E. S., Altfeld, M., Walker, B. D. & Streeck, H. HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. *J Virol* **85**, 733-741, (2011).

651 Draenert, R., Altfeld, M., Brander, C., Basgoz, N., Corcoran, C., Wurcel, A. G., Stone, D. R., Kalams, S. A., Trocha, A., Addo, M. M., Goulder, P. J. & Walker, B. D. Comparison of overlapping peptide sets for detection of

antiviral CD8 and CD4 T cell responses. *J Immunol Methods* **275**, 19-29, (2003).

652 Lotti, B., Wendland, T., Furrer, H., Yawalkar, N., von Greyerz, S., Schnyder, K., Brandes, M., Vernazza, P., Wagner, R., Nguyen, T., Rosenberg, E., Pichler, W. J. & Brander, C. Cytotoxic HIV-1 p55gag-specific CD4+ T cells produce HIV-inhibitory cytokines and chemokines. *J Clin Immunol* **22**, 253-262, (2002).

653 Zaunders, J. J., Dyer, W. B., Wang, B., Munier, M. L., Miranda-Saksena, M., Newton, R., Moore, J., Mackay, C. R., Cooper, D. A., Saksena, N. K. & Kelleher, A. D. Identification of circulating antigen-specific CD4+ T lymphocytes with a CCR5+, cytotoxic phenotype in an HIV-1 long-term nonprogressor and in CMV infection. *Blood* **103**, 2238-2247, (2004).

654 Muller, N. G., Alencar, R., Jamal, L., Hammer, J., Sidney, J., Sette, A., Brindeiro, R. M., Kalil, J., Cunha-Neto, E. & Moraes, S. L. CD4(+) T cells from HIV-1-infected patients recognize wild-type and mutant human immunodeficiency virus-1 protease epitopes. *Clin Exp Immunol* **164**, 90-99, (2011).

655 Southwood, S., Sidney, J., Kondo, A., del Guercio, M. F., Appella, E., Hoffman, S., Kubo, R. T., Chesnut, R. W., Grey, H. M. & Sette, A. Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol* **160**, 3363-3373, (1998).

656 Diepolder, H. M., Jung, M. C., Keller, E., Schraut, W., Gerlach, J. T., Gruner, N., Zachoval, R., Hoffmann, R. M., Schirren, C. A., Scholz, S. & Pape, G. R. A vigorous virus-specific CD4+ T cell response may contribute to the association of HLA-DR13 with viral clearance in hepatitis B. *Clin Exp Immunol* **113**, 244-251, (1998).

657 Bosi, I., Ancora, G., Mantovani, W., Miniero, R., Verucchi, G., Attard, L., Venturi, V., Papa, I., Sandri, F., Dallacasa, P. & Salvioli, G. P. HLA DR13 and HCV vertical infection. *Pediatr Res* **51**, 746-749, (2002).

658 Kuzushita, N., Hayashi, N., Katayama, K., Hiramatsu, N., Yasumaru, M., Murata, H., Shimizu, Y., Yamazaki, T., Fushimi, H., Kotoh, K., Kasahara, A., Fusamoto, H. & Kamada, T. Increased frequency of HLA DR13 in hepatitis C virus carriers with persistently normal ALT levels. *J Med Virol* **48**, 1-7, (1996).

659 Linterman, M. A., Beaton, L., Yu, D., Ramiscal, R. R., Srivastava, M., Hogan, J. J., Verma, N. K., Smyth, M. J., Rigby, R. J. & Vinuesa, C. G. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* **207**, 353-363, (2010).

660 Zotos, D., Coquet, J. M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L. M., Godfrey, D. I., Toellner, K. M., Smyth, M. J., Nutt, S. L. & Tarlinton, D. M. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* **207**, 365-378, (2010).

661 Ozaki, K., Spolski, R., Feng, C. G., Qi, C. F., Cheng, J., Sher, A., Morse, H. C., 3rd, Liu, C., Schwartzberg, P. L. & Leonard, W. J. A critical role for IL-21 in regulating immunoglobulin production. *Science* **298**, 1630-1634, (2002).

662 Yi, J. S., Du, M. & Zajac, A. J. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* **324**, 1572-1576, (2009).

663 Frohlich, A., Kisielow, J., Schmitz, I., Freigang, S., Shamshiev, A. T., Weber, J., Marsland, B. J., Oxenius, A. & Kopf, M. IL-21R on T cells is critical for

sustained functionality and control of chronic viral infection. *Science* **324**, 1576-1580, (2009).

664 Yi, J. S., Ingram, J. T. & Zajac, A. J. IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection. *J Immunol* **185**, 4835-4845, (2010).

665 Publicover, J., Goodsell, A., Nishimura, S., Vilarinho, S., Wang, Z. E., Avanesyan, L., Spolski, R., Leonard, W. J., Cooper, S. & Baron, J. L. IL-21 is pivotal in determining age-dependent effectiveness of immune responses in a mouse model of human hepatitis B. *J Clin Invest* **121**, 1154-1162, (2011).

666 Turnbull, E. L., Wong, M., Wang, S., Wei, X., Jones, N. A., Conrod, K. E., Aldam, D., Turner, J., Pellegrino, P., Keele, B. F., Williams, I., Shaw, G. M. & Borrow, P. Kinetics of expansion of epitope-specific T cell responses during primary HIV-1 infection. *J Immunol* **182**, 7131-7145, (2009).

667 Goulder, P. J., Altfeld, M. A., Rosenberg, E. S., Nguyen, T., Tang, Y., Eldridge, R. L., Addo, M. M., He, S., Mukherjee, J. S., Phillips, M. N., Bunce, M., Kalams, S. A., Sekaly, R. P., Walker, B. D. & Brander, C. Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. *J Exp Med* **193**, 181-194, (2001).

668 De Groot, A. S., Clerici, M., Hosmalin, A., Hughes, S. H., Barnd, D., Hendrix, C. W., Houghten, R., Shearer, G. M. & Berzofsky, J. A. Human immunodeficiency virus reverse transcriptase T helper epitopes identified in mice and humans: correlation with a cytotoxic T cell epitope. *J Infect Dis* **164**, 1058-1065, (1991).

669 Wilson, C. C., Palmer, B., Southwood, S., Sidney, J., Higashimoto, Y., Appella, E., Chesnut, R., Sette, A. & Livingston, B. D. Identification and antigenicity of broadly cross-reactive and conserved human immunodeficiency virus type 1-derived helper T-lymphocyte epitopes. *J Virol* **75**, 4195-4207, (2001).

670 Berzofsky, J. A., Pendleton, C. D., Clerici, M., Ahlers, J., Lucey, D. R., Putney, S. D. & Shearer, G. M. Peptides containing multideterminant clusters of human immunodeficiency virus envelope induce murine and human T-cell responses in diverse histocompatibility types. *Trans Assoc Am Physicians* **104**, 69-77, (1991).

APPENDIX I:

TMA DATA

Group	Cohort	Subject Identifier	Study Visit	TMA Assay	HIV-1 specific T cell responses by cultured IFN-γ ELISpot?
HESN	**GALVIN**	2014	3	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2017	2	0/4	Negative By cultured ELISpot
HESN	**GALVIN**	2026	4	0/6	Envelope Response >1000SFU/10^6
HESN	**GALVIN**	2026	5	0/6	Envelope Response >4000SFU/10^6
HESN	**GALVIN**	2038	2	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2039	2	0/4	Nef / Accessory Pool response >2000SFU/10^6
HESN	**GALVIN**	2040	2	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2043	3	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2053	2	0/6	Nef /Accessory Pool response >700 SFU/10^6 Pol response >300 SFU/10^6
HESN	**GALVIN**	2066	3	0/4	Negative By cultured ELISpot
HESN	**GALVIN**	2067	1	0/4	Envelope response >300SFU/10^6 Nef and Accessory pool response >300SFU/10^6
HESN	**GALVIN**	2070	1	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2071	2	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2072	2	0/4	Gag response >400SFU/10^6
HESN	**GALVIN**	2087	1	0/5	Negative By cultured ELISpot
HESN	**GALVIN**	2088	1	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2089	1	0/4	Negative By cultured ELISpot
HESN	**GALVIN**	2096	1	0/4	Pol response >800 SFU/10^6
HESN	**GALVIN**	2102	2	0/2	Negative By cultured ELISpot
HESN	**GALVIN**	2104	2	0/5	Negative By cultured ELISpot
HESN	**GALVIN**	2105	2	0/2	Negative By cultured ELISpot
HESN	**GALVIN**	2108	2	0/5	Envelope response >800SFU/10^6
HESN	**GALVIN**	2115	1	0/6	Envelope response >300 SFU/10^6
HESN	**GALVIN**	2116	1	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2119	1	0/4	Negative By cultured ELISpot
HESN	**GALVIN**	2122	1	0/5	Nef / Accessory Pool Response >800 SFU/10^6
HESN	**GALVIN**	2122	4	0/6	Negative By cultured ELISpot
HESN	**GALVIN**	2124	4	0/6	Negative By cultured ELISpot
HESN	**CHAVI002**	709020031	1	0/6	Gag response >400 SFU/10^6
HESN	**CHAVI002**	709020031	2	0/9	Negative by cultured ELISpot
HESN	**CHAVI002**	709020059	1	0/6	Negative by cultured ELISpot
HESN	**CHAVI002**	709020078	1	0/5	Gag response >300 SFU/10^6 Env response >400 SFU/10^6 Pol response > 1000 SFU/10^6
HESN	**CHAVI002**	709020078	2	0/6	Nef/Acc response >300 SFU/10^6
HESN	**CHAVI002**	709020093	1	0/7	Negative by cultured ELISpot
HESN	**CHAVI002**	709020093	2	0/9	Nef /Acc response > 1000 SFU/10^6 Pol response >400 SFU/10^6
HESN	**CHAVI002**	709020110	1	0/6	Gag response > 700 SFU/10^6 Nef/Acc response >800 SFU/10^6 Pol response >500 SFU/10^6 CTL pool response >1000 SFU/10^6
HESN	**CHAVI002**	709020110	2	0/6	Gag response >400 SFU/10^6
HESN	**CHAVI002**	709020154	1	0/6	Env response >100 SFU/10^6
HESN	**CHAVI002**	709020154	2	0/9	Env response >700 SFU/10^6
HESN	**CHAVI002**	709020173	1	0/6	Negative
HESN	**CHAVI002**	709020173	2	1/9	CTL pool response >500 SFU/10^6

Group	Cohort	Subject Identifier	Study Visit	TMA Assay	HIV-1 specific T cell responses by cultured IFN-γ ELISpot?
HESN	**CHAVI002**	709020198	1	0/6	Env response 400 SFU/10^6 Nef/Acc response >500 SFU/10^6
HESN	**CHAVI002**	709020198	2	0/9	Negative by cultured ELISpot
HESN	**CHAVI002**	709020212	1	0/9	Gag response >2000 SFU/10^6
HESN	**CHAVI002**	709020270	1	0/9	Gag response >900 SFU/10^6 Env response >400 SFU/10^6
HESN	**CHAVI002**	709020435	1	0/9	Nef/Acc Response >1000 SFU/10^6
HESN	**CHAVI002**	709020435	2	0/9	Gag response 400 SFU/10^6 Env response >400 SFU/10^6 Nef/Acc response >2000 SFU/10^6
HESN	**CHAVI002**	709020474	1	0/9	Negative by cultured ELISpot
HESN	**CHAVI002**	709020497	1	0/9	CTL pool response >900 SFU/10^6
HESN	**CHAVI002**	709020517	1	0/9	Negative by cultured ELISpot
HESN	**CHAVI002**	709020534	1	0/9	Gag response >300 SFU/10^6 Nef/Acc Response >300 SFU/10^6
HUSN	**CHAVI002**	709020026	1	0/6	Gag response >800 SFU/10^6 Env response >300 SFU/10^6 Nef/Acc response > 1000 SFU/10^6 Pol response >300 SFU/10^6 CTL pool response >700 SFU/10^6
			HUSN		
HUSN	**GALVIN**	2211	1	0/6	Negative By cultured ELISpot
HUSN	**GALVIN**	2220	1	0/6	Negative By cultured ELISpot
HUSN	**GALVIN**	2221	1	0/6	Negative By cultured ELISpot
HUSN	**GALVIN**	2231	1	0/6	Negative By cultured ELISpot
HUSN	**GALVIN**	2241	1	0/6	Negative By cultured ELISpot
HUSN	**GALVIN**	2251	1	0/6	Negative By cultured ELISpot
HUSN	**CHAVI002**	709020239	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020247	1	0/9	Pol response >900 SFU/10^6
HUSN	**CHAVI002**	709020247	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020251	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020251	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020264	1	0/9	Nef/Acc response >700 SFU/10^6
HUSN	**CHAVI002**	709020264	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020296	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020296	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020303	1	0/9	Env response >900 SFU/10^6 Pol response >600 SFU/10^6
HUSN	**CHAVI002**	709020303	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020319	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020319	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020325	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020325	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020332	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020332	2	0/9	Gag response >400 SFU/10^6 Env response >500 SFU/10^6 Pol response >300 SFU/10^6
HUSN	**CHAVI002**	709020340	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020340	2	0/9	Nef/Acc response >300 SFU/10^6
HUSN	**CHAVI002**	709020358	1	0/9	Pol response >400 SFU/10^6
HUSN	**CHAVI002**	709020358	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020366	1	0/9	Env response >3000 SFU/10^6
HUSN	**CHAVI002**	709020366	2	0/9	Env response >300 SFU/10^6
HUSN	**CHAVI002**	709020377	1	0/9	Gag response >700 SFU/10^6
HUSN	**CHAVI002**	709020377	2	0/9	Negative by cultured ELISpot

Group	Cohort	Subject Identifier	Study Visit	TMA Assay	HIV-1 specific T cell responses by cultured IFN-γ ELISpot?
HUSN	**CHAVI002**	709020381	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020381	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020394	1	0/9	Env response >400 SFU/10^6
HUSN	**CHAVI002**	709020394	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020406	1	0/9	Gag response >500 SFU/10^6 Nef/Acc response >500 SFU/10^6 Pol response >400 SFU/10^6
HUSN	**CHAVI002**	709020406	2	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020450	1	0/9	Negative by cultured ELISpot
HUSN	**CHAVI002**	709020463	1	0/9	Nef/Acc response >400 SFU/10^6
			HIV$^+$		
HIV$^+$	**CHAVI002**	709020080	2	2/3	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >7000 SFU/10^6
HIV$^+$	**CHAVI002**	709020121	2	0/3	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >1000 SFU/10^6
HIV$^+$	**CHAVI002**	709020162	2	1/9	Responses to Gag, Pol and CTL pool >900 SFU/10^6
HIV$^+$	**CHAVI002**	709020185	1	6/6	Responses to Gag and Nef/Acc >600 SFU/10^6
HIV$^+$	**CHAVI002**	709020185	2	9/9	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >700 SFU/10^6
HIV$^+$	**CHAVI002**	709020205	2	2/9	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >1000 SFU/10^6
HIV$^+$	**CHAVI002**	709020223	1	9/9	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >3000 SFU/10^6
HIV$^+$	**CHAVI002**	709020288	1	9/9	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >400 SFU/10^6
HIV$^+$	**CHAVI002**	709020422	1	9/9	Not tested
HIV$^+$	**CHAVI002**	709020448	1	8/9	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >500 SFU/10^6
HIV$^+$	**CHAVI002**	709020448	2	6/9	Not tested
HIV$^+$	**CHAVI002**	709020489	1	9/9	No responses detected by cultured ELISpot
HIV$^+$	**CHAVI002**	709020500	1	9/9	Not done
HIV$^+$	**CHAVI002**	709020528	1	9/9	Responses to Gag, Nef/Acc and CTL pools >1000 SFU/10^6
HIV$^+$	**CHAVI002**	709020542	1	1/9	Responses to Gag, Env, Nef/Acc, Pol and CTL pools >400 SFU/10^6

Appendix one:

Transcription Mediated Amplification (TMA) performed by Professor Eric Delwart's laboratory (University of California) was used to screen HIV-1 exposed seronegative (HESN) and HIV-1 unexposed seronegative (HUSN) subjects from the GALVIN and CHAVI002 cohorts for low-level HIV-1 RNA. On average 5.18 replicate TMA assays were run per GALVIN plasma sample and 8.34 replicate assays per CHAVI002 sample. Data expressed as signal/C ratio. Criteria for a positive response was defined as an S/C ratio >1 and a negative response defined as S/Co<1. Of 26 HESN and 6 HUSN subjects from the GALVIN cohort, 16 HESN and 18 HUSN from CHAVI002 cohort, all were negative for HIV-1 viral RNA by TMA. Of note all HIV-1 positive subjects, enrolled in CHAVI002, with plasma viral loads >50 copies/ml were positive by TMA.

APPENDIX II:

CHAVI002 MAPPING DATA

Group/ PTID	Cohort	*Ex Vivo* IFN-γ ELISpot	Cultured IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide Information
HESN 709020776	CHAVI002	Negative	**GAG 216-224** 600 SFU/10^6	**$CD4^+$ Magnitude:** 2.69%	**HPVHAGPIA** DRV---------PGQMREPRGS EWDRV---------PGQMRE Immunodominant $CD4^+$ T cell response detected in 1/2 long term non progressors[414] 3/5 clade B infected subjects responded to this peptide in context with multiple HLADRb1 alleles[526].
HESN 709020776	CHAVI002	Negative	**GAG 240-249** 600 SFU/10^6	**$CD4^+$ Magnitude:** 0.46%	**TSTLQEQIGW** PRGSDIAGT----------M 3/9 antiretroviral therapy-naïve adults with chronic HIV-1 infection, had detectable $CD4^+$ T cell responses to a 20mer peptide containing the TW10, p24 gag epitope following expanded ELISpot assay[526,527].
HESN 709020212 709020517 709020672	CHAVI002	Negative	**GAG 268-285** >3000 SFU/10^6	**$CD4^+$ Magnitude:** 2.5%	**LGLNKIVRMYSPTSILDI** I-----------------R I---------- WII--------------- 8/24 HIV-1 infected donors responded to this peptide[647]. Shown to be a core epitope, capable of binding 3/8 HLADR molecules tested[373]. IL-2 response to this peptide detected in 1/3 acutely infected HIV-1 donors[421]. Also shown to have high cross reactive binding potential, binding 8/8 common HLADR molecules tested[373].
HESN 709020575	CHAVI002	Negative	**POL 249-266** >400 SFU/10^6	**$CD4^+$ Magnitude:** 0.68&	**IPHPAGLKKKKSVTVLDV** WEVQLG--------- Peptide shown to stimulate murine $CD4^+$ and $CD8^+$ T cell responses. In humans 9/17 seropositive individuals had IL-2 response to this peptide[668]

Group	Cohort	*Ex Vivo* IFN-γ ELISpot	Cultured IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide Information
HUSN 709020303	CHAVI002	Negative	**POL 681-698** >3000 SFU/10^6	**$CD4^+$ Magnitude:** 1.28%	**IKKEKVYLAWVPAHKGIG** --------------- Peptide shown to be conserved in 94% clade B isolates, and capable of binding ten HLADR alleles with an IC_{50} threshold below 1000[669].
709020377	CHAVI002	Negative	**GAG 364-381** >400 SFU/10^6	**$CD8^+$ Magnitude:** 1.12%	**AEAMSQVTNSATIMMQRG** ----------2 HIV-1 controllers, showed an IL-21 response to this peptide[650].
HUSN 709020303	CHAVI002	Negative	**POL 745-762** >2000 SFU/10^6	**$CD4^+$ Magnitude:** 1.28%	**PVVAKEIVASCDKCQLKG** Not previously described
HUSN 709020377	CHAVI002	Negative	**VIF 49-66** >400 SFU/10^6	**$CD4^+$ Magnitude:** 0.98% **$CD8^+$ Magnitude:** 1%	**PRISSEVHIPLGDARLVI** Not previously described
HESN 709020264	CHAVI002	Negative	**VIF 129-146** >500SFU/10^6	**$CD4^+$ Magnitude:** 1%	**VSPRCEYQAGHNKVGSLQ** Not previously described
HUSN 709020463	CHAVI002	Negative	**VIF 137-154** >400 SFU/10^6	**$CD4^+$ Magnitude:** 0.1%	**AGHNKVGSLQYLALAALI** -------V—VAPKK Shown to bind 7/9 HLADR molecules tested. Responses detected in 29/32 HIV-1^+ subjects tested[523]
HUSN 709020463	CHAVI002	Negative	**VPR 33-50** >400 SFU/10^6	**$CD4^+$ Magnitude:** 0.75%	**HFPRIWLHGLGQHIYETY** R---------- Following in vitro stimulation 2/9 healthy subjects responded to this peptide[648].

Group/ PTID	Cohort	Ex Vivo IFN-γ ELISpot	Cultured IFN-γ ELISpot	Cultured IFN-γ ICS	Mapped Peptide Information
HUSN 709020366	CHAVI002	Negative	**ENV 485-502** >300 SFU/10^6	**$CD4^+$ Magnitude:** 0.68%	**KYKVVKIEPLGVAPTKAK** SELYL---------- ELY---------- Shown to bind 8/9 HLADR molecules tested[523]. 3/15 HIV-1+ subjects recognized this epitope with an average stimulation index of 6.0[376]
HUSN 709020394	CHAVI002	Negative	**ENV 661-678** >300 SFU/10^6	**$CD4^+$ Magnitude: 0.53%**	**LELDKWASLWNWFDITNW** -------N----LWY DCs pulsed with HIV-1 were shown to *in vitro* prime T cell responses to this peptide from naive T cells[370].
HUSN 709020608	CHAVI002	Negative	**ENV 741-758** >300 SFU/10^6	**$CD4^+$ Magnitude:** 0.81%	**DRDRSGRLVDGFLALIWD** Not previously defined
HUSN 709020394	CHAVI002	Negative	**ENV 798-815** >300SFU/10^6	**$CD4^+$ Magnitude:** 0.62%	**NLLQYWSQELKNSAVSLL** ---------- 17/29 asymptomatic HIV-1^+ individuals had an IL-2 response to this peptide[237,670]

Appendix Two:

HIV-1 specific T cell responses detectable amongst HIV-1 exposed and unexposed seronegative individuals (HESN/HUSN) enrolled in the CHAVI 002 cohort were mapped to the peptide level using a matrix mapping approach. Cultured IFN-γ ELISpot together with intracellular cytokine staining allowed mapped responses to be characterized in terms of CD4/CD8 specificity and magnitude. Data presented as the average number of spot forming units per million cells (SFU/10^6) for ELISpot data and % $CD4^+$/$CD8^+$IFN-γ^+ cells. Positivity criteria for ELISpot assays were >50SFU/10^6 (for *ex vivo* assays)/>300 SFU/10^6 (cultured assays) and/ or 4x background. ICS positivity criteria were >0.05% and 2 x background.

APPENDIX III:

DESIGN OF A NOVEL

PROMISCUOUS EPITOPE POOL

	VWASRELERFALNPGLLE	EELKSLYNTVATLYAVHQ	YKRWIILGLNKIVRMYSP	LGLNKIVRMYSPVSILDI	PFRDYVDRFFKTLRAEQA	SRLAFHHMARELHPEYYK	ANFLGKIQPSHKGRPHMF
CON_OF_CONS	----------	---R-------C---	----------	----------	----------	----LR-I-------F--	-------W--N-------
M.group.anc	----------	---R-------C---	----------	----------	----------	----LR-I-------F--	-------W--N-------
CONSENSUS_A1	----------S---	---R-------C---	----------	----------	----------	----LR-I-------F--	-------W--S-------
A1.anc	----------	---R-------C---	----------	----------	----------	----LK-R-QEL------	-------W--S-------
CONSENSUS_A2	--------K-SI------	----------C---	----------	----------	----------	----LK---------F--	-------W--N-------
CONSENSUS_B	----------V------	---R-------C---	----------	----------T-----	----------	----LR-R-------F--	-------W----------
B.anc	----------V------	---R-------C---	----------	----------I-----	----------	----------	-------W----------
CONSENSUS_C	----------	---R-------C-E	----------	----------	----------	----------	-------W----------
C.anc	----------	---R-------C-E	----------	----------	----------	-----K---------F--	-------W----------
CONSENSUS_D	----------	---R-------C-E	----------	----------	----------	-H--RR----------	-------W----------
CONSENSUS_F1	----------D-----	---R-------C---	----------	----------	----------	-H--RR----------	-------W--N-------
CONSENSUS_G	----------D---	---R--F-------C---	----------	----------	----------	-----E-K--V----F--	-------W--N-------
CONSENSUS_H	----------	---Q--F-------C---	----------	----------	----------	----LR-I---R-----Q	-------W--S-------
CONSENSUS_K	----------S---	---K--F-------C---	----------	----------	----------	-----R-----R------	-------W----------
CONSENSUS_01_AE	----------	---K--F------WC---	----------	----------	----------	-S--RR-I----------	-------W----------
CONSENSUS_02_AG	----------	---K---------WC---	-----V----------	----------	----------	-----R-I-------F--	-------W--S-------
CONSENSUS_03_AB	----------S---	---K-------C---	----------	----------	----------	-A--RK-I----------	-----R-W--S-------
CONSENSUS_04_CPX	----------	---K--F--I---WC---	----------	----------	----------C------	----LT-R-------F--	-----RMW--S-------
CONSENSUS_06_CPX	----------	---K-------C---	----------	----------	----------	----YK-I-------F--	-------W--N-------
CONSENSUS_07_BC	----------	------F-------C-T	----------	----------T-----	----------	-S--RR-I---M---F--	-------W----------
CONSENSUS_08_BC	----------	------F-------C-A	----------	----------T-----	----------	-Q---R-R-------F--	-------W----------
CONSENSUS_10_CD	----------	--I-------C-E	----------	----------	----------	-N--HK----------	-------W--N-------
CONSENSUS_11_CPX	----------S---	----------C-H	----------	----------	----------	-S--RR-I-------DF--	-------W--S-------
CONSENSUS_12_BF	----------	---------I----F---	----------	----------	----------	-R--LR-I-------F-Q	-------W--N-------
CONSENSUS_14_BG	----------D---	--I---F-------C---	----------	----------	----------	-S--RR-I-------DF--	-------W--N-------
Sequence to synthesize	**VWASRELERFALNPGLLE**	**EELKSLYNTVATLYAVHQ**	**YKRWIILGLNKIVRMYSP**	**LGLNKIVRMYSPVSILDI**	**PFRDYVDRFFKTLRAEQA**	**SRLAFHHMARELHPEYYK**	**ANFLGKIQPSHKGRPHMF**
ADDITIONAL SEQUENCES							**ANFLGKIWPSSKGRPGNF**

	SVLSGGKLDRWEKIRLRP	KAFSPEVIPMFSALSEGA	KTVRLIKFLYQSNPP	LRPMTYKAAVDLSHFLKE	GIRPVVSTQLLLNGSLA	PTPVNIIGRNLLTQIGCT	NPEIVIYQYMDDLYVGSDLEIG
CON_OF_CONS	---------A---------	-------------------	---A-I-I--------Y-	-------G-F--------	---K---------------	----------M--------	----------
M.group.anc	---------A---------	-------------------	---A--I--I------Y-	---------F--------	---K---------------	----------M--------	----------
CONSENSUS_A1	---------A---------	-------------------	---A--I-I-------Y-	-----F-G-F---F----	---K---------------	----------M--------	----------
A1.anc	---------A---------	-------------------	--RA--I-I-------Y-	-------G----------	---K---------------	----------M--------	----------
CONSENSUS_A2	-I-------A---------	-------------------	--RAI-I--I------Y-	-------G-F--------	---K---------------	----------M---L----	---M----------
CONSENSUS_B	-------------------	-------------------	-------------------	-----F-G-F--------	-------------------	-------------------	----------
B.anc	---------K---------	-------------------	---A-----------	-------G-L--------	-------------------	-------------------	----------
CONSENSUS_C	-I-R-----K---------	-------------------	--QA-I----------Y-	---------L--------	---K---------------	----------M---L----	----------
C.anc	-I-R-----T---------	-------------------	--QA--I--I------Y-	-------------------	---K---------------	----------M---L----	----------
CONSENSUS_D	---------A---------	-------------------	---A-----I--------	---------F---F----	-------------------	----------L--------	----------
CONSENSUS_F1	---------A---------	-------------------	---A--Y-I-------Y-	---------F---F----	---K---------------	----------M--------	----------
CONSENSUS_G	---------A---------	-------------------	---A--Y--I------Y-	---------L--------	-------------------	----------M--------	----------
CONSENSUS_H	---------A---------	-------------------	---A--I--I------Y-	-------G----------	---K---------------	----------M--------	----------
CONSENSUS_K	---------T---------	-------------------	--QVCKI--I------Y-	---------F--------	-------------------	----------I--------	---M----------
CONSENSUS_01_AE	---------A---------	-G-----------------	--RA--I--I------Y-	-------G-F---F----	---K---------------	----------M--------	---M----------
CONSENSUS_02_AG	---------A---------	-------------------	--RA--I-I-------Y-	-------G-F--------	---K---------------	----------M--------	----------
CONSENSUS_03_AB	---------A---------	-------------------	----I----------	-------G-F---F----	---K---------------	----------M---L----	----------
CONSENSUS_04_CPX	---------A---------	-------------------	---A-GIV-I------Y-	-------G-F--------	---K---------------	----------M---L----	----------
CONSENSUS_06_CPX	---------E---------	-------------------	--LA--I--I------Y-	-----F---L--------	---K---------------	----------M--------	----------
CONSENSUS_07_BC	-I-R-----K---------	-------------------	-------------------	-------G-F---F----	-------------------		
CONSENSUS_08_BC	-I-R-----K---------	-------------------	-------------------	-----F-G-L---F----	---K---------------	----------M---L----	----------
CONSENSUS_10_CD	---------E---------	-------------------	--QA--I-I--------	-------G-F---F----	---K---------------	-------------------	---M----------
CONSENSUS_11_CPX	---------A---------	-------------------	--TA--I--I------Y-	-------G-F---F----	---K---------------	----------M--------	----------
CONSENSUS_12_BF	-------------------	-------------------	---A--L--F------Y-	-------G-L--------	---K---------------	----------L----	----------
CONSENSUS_14_BG	---------A---------	-------------------	--R---I--I------Y-	-------G-F---F----	-------------------	----------M--------	----------
Sequence to synthesize	**SVLSGGKLDRWEKIRLRP**	**KAFSPEVIPMFSALSEGA**	**LLKTVRLIKFLYQSNPP**	**LRPMTYKAAVDLSHFLKE**	**GIRPVVSTQLLLNGSLA**	**PTPVNIIGRNLLTQIGCT**	**NPEIVIYQYMDDLYVGSDLEIG**
ADDITIONAL SEQUENCES	**SVLSGGKLDAWEKIRLRP**		LLKAVRILKILYQSNYP	**LRPMTYKGAFDLSFFLKE**	**HGIKPVVSTQLLLNGSLA**	**PTPVNIIGRNMLTQIGCT**	

Appendix Three:

Novel promiscuous epitopes designed based on entropy scores were compared against consensus and ancestral sequences. Where significant amino acid diversity occurred in the epitope chosen and consensus / ancestral sequences additional peptides were synthesized for the dominant variant epitope.