

**ACTIVATION AND MEMORY DIFFERENTIATION OF TOTAL AND HIV-SPECIFIC
T CELLS THAT ASSOCIATE WITH VIRAL CONTROL DURING SUBTYPE C HIV-1
INFECTION**

Pholo Wilson Maenetje

A thesis submitted to the School of Pathology, University of the Witwatersrand, in fulfillment of the
requirements for the degree of Doctor of Philosophy.

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of Witwatersrand, Johannesburg. It has not being submitted before for any degree or examination at this or any other University.

Pholo Wilson Maenetje

_____ day of _____, _____

DEDICATION

This thesis is dedicated to my mother, Lydia Maenetje. Her support, motivation, encouragement, and constant love have sustained me throughout my life.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

PUBLICATION FROM THIS THESIS

Maenetje P, Riou C, Casazza JP, Ambrozak A, Hill B, Gray G, Koup RA, de Bruyn G, and Gray GM. A Steady State of CD4⁺ T cell Memory Maturation and Activation is Established During Primary Subtype C HIV-1 Infection. *J. Immunol.* 2010.184, 4926-4935.

OTHER PUBLICATION

Burgers WA, Riou C, Mlotshwa M, **Maenetje P**, de Assis Rosa D, Brenchley J, Mlisana K, Douek DC, Koup R, Roederer M, de Bruyn G, Abdool Karim S, Williamson C, Gray CM and the CAPRISA 002 Acute Infection Study Team. 2009. Association of HIV-specific and Total CD8⁺ T Memory Phenotypes in Subtype C HIV-1 Infection with Viral Set Point. *J. Immunol.* 2009. 182, 4751-4761.

PRESENTATIONS AT MEETINGS

Maenetje, P., Catherine Riou, Joseph Casazza, David Ambrozak, Richard Koup, Glenda Gray, Guy de Bruyn, Clive M Gray. Functional profile of Gag-specific CD8⁺ T cells during primary HIV-1 subtype C infection. *A Poster presentation at the Atlanta AIDS Vaccine Conference 2010, Atlanta USA 2010.*

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Maenetje, P., Joseph Casazza, David Ambrozak, Richard Koup, Glenda Gray, Guy de Bruyn, Clive M Gray. In vivo susceptibility of activated memory CD4⁺ T cells to HIV during early subtype C infection. *An Oral Presentation at the South African AIDS Vaccine Conference, South Africa 2009).*

ABSTRACT

The development of an effective HIV-1 vaccine is critical in mitigating the global HIV epidemic. Understanding the interplay between host immune functions, such as cellular memory differentiation, activation, inflammatory cytokine production and the virus, may provide key insight into anti-HIV immunity that can inform vaccine development. This PhD aims at understanding and identifying T cell memory, functional profiles and the effect of immune activation on *in vivo* HIV-1 control during primary/early infection. Furthermore, this study aims to examine and understand the potential mechanisms related to immune activation during primary HIV-1 infection.

Use was made of a unique cohort of individuals recruited during primary HIV-1 infection and using a battery of assays to characterize and identify properties and mechanisms of T cell reactivity and activation. Multiparameter flow cytometry was used to measure memory differentiation (CD27 and CD45RO), activation (CD38, HLA-DR), proliferation (Ki67), and multiple cellular functions (CD107, IFN γ , IL-2, MIP-1 β and TNF α) of total and antigen-specific CD4⁺ and CD8⁺ T cells from 15 HIV-1 and CMV-coinfected individuals followed over 15 months of HIV-1 infection. Plasma samples were used to measure markers associated with intestinal permeability (LBP, sCD14, I-FABP and IgM EndoCAb) and inflammation (IL-1 β , IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1).

The differentiation profile of HIV-Gag specific memory CD4⁺ and CD8⁺ T cells was found to be mainly characterized by an early differentiated (ED) memory phenotype relative to CMV-

specific CD4⁺ and CD8⁺ T cells. Moreover, the proportion of HIV-specific ED-memory CD4⁺ T cells inversely associated with viraemia, suggesting that HIV-1 antigen burden could be shaping the differentiation of HIV-specific memory CD4⁺ T cells during primary infection. Primary HIV-1 infection was also characterized by significantly elevated levels of activated and proliferating total and HIV-specific memory CD4⁺ and CD8⁺ T cells, which positively correlated with viraemia. Furthermore, upon sorting of total activated memory CD4⁺ T cells, these cells harboured more *gag* provirus DNA than non-activated memory cells, suggesting that activated memory CD4⁺ T cells support ongoing HIV-1 replication. When examining the relationship between memory differentiation and activation markers, the level of T cell activation was equally expanded across the different memory CD4⁺ T cell subpopulations, suggesting that memory differentiation of CD4⁺ T cells was unlikely driven per se by the level of T cell activation. In addition, when teasing out events that may result in T cell activation during primary HIV-1 infection using statistical models, plasma markers of microbial translocation and inflammation were found to correlate with immune activation. The lack of these associations in HIV-uninfected controls suggests that microbial translocation and inflammation were unlikely causative.

Analysis of the polyfunctional profile of memory T cells during primary HIV-1 infection showed that HIV-specific CD4⁺ and CD8⁺ T cell responses are less polyfunctional relative to CMV-specific memory CD4⁺ and CD8⁺ T cell responses. Furthermore, the polyfunctional status of HIV-specific CD4⁺ T cells significantly correlated with viraemia at 3 months post-infection, indicating that the polyfunctionality of memory CD4⁺ T cells is likely driven by HIV-1 antigenemia. Overall, these observations suggest that HIV-1 antigenic burden appears to be a

central driver of memory differentiation, activation/inflammation and polyfunctionality of T cells. Given the impact of HIV-1 viraemia on immune activation and memory T cell dysfunction (as measured by limited polyfunctional HIV-specific responses), preventing high levels of viral replication, with a vaccine or other early interventions may serve as an important strategy for delaying HIV-1 disease progression.

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TABLE OF CONTENTS

DECLARATION	ii
DEDICATION	iii
PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY	iv
PUBLICATION FROM THIS THESIS	iv
OTHER PUBLICATION.....	iv
PRESENTATIONS AT MEETINGS	iv
ABSTRACT.....	vi
ACKNOWLEDGEMENTS.....	ix
TABLE OF CONTENTS.....	x
LIST OF FIGURES	xvii
LIST OF TABLES.....	xxiv
LIST OF APPENDIX FIGURES.....	xxvi
LIST OF APPENDIX TABLES	xxviii
ABBREVIATIONS	xxx
CHAPTER 1: LITERATURE REVIEW	1
1.1 The Immune System	2
1.1.1 Innate Immunity.....	2
1.1.2 Adaptive immunity	4
1.1.2.1 Ontogeny and function of T cells.....	5
1.1.2.2 Major Histocompatibility Complex	8
1.1.2.3 Memory	10

1.2 The HIV Epidemic and Origin.....	16
1.2.1 Origin and distribution.....	17
1.2.2 HIV structure and function	19
1.2.3 HIV life cycle.....	23
1.3 Adaptive Immunity and HIV infection	26
1.3.1 HIV transmission	26
1.3.2 T cell responses.....	30
1.3.2.1 CD8 ⁺ T cells.....	30
1.3.2.2 CD4 ⁺ T cells.....	33
1.3.2.3 T cell Polyfunction.....	35
1.3.3 T cell Memory and HIV infection	37
1.3.4 Immune Activation and HIV infection	39
1.3.4.1 Microbial Translocation and Immune Activation.....	42
1.3.4.2 TCR-independent Immune activation.....	45
1.4 Rationale and Objectives of this Study	46
CHAPTER 2: MATERIALS AND METHODS	50
2.1 Study Participants	51
2.1.1 Primary HIV-infection Cohort.....	51
2.1.2 CAPT Cohort	51
2.1.3 Cohort Characteristics.....	52
2.1.4 Plasma Viral Load and Absolute CD4 ⁺ T cell Counts.....	54
2.2 Synthetic Peptides.....	54
2.3 Sample Preparation	54
2.4 Cell Stimulation and Intracellular Staining.....	55

2.4.1 Measurement of T cell Polyfunction	55
2.4.2 Measurement of T cell Memory and Activation Status	56
2.5 Measurement of Plasma EndoCAb, I-FABP, LBP and sCD14	56
2.6 Measurement of Plasma Cytokine Levels	57
2.7 Data Analysis	58
2.7.1 Flow Cytometry Analysis	58
2.7.1.1 Cell Acquisition and Gating	58
2.7.1.2 Boolean Gating Analysis	59
2.7.2 Cell Sorting and HIV Gag-DNA quantification	59
2.7.2.1 Gag-DNA quantification using real time PCR	60
2.7.3 Plasma markers of microbial translocation and cytokine analysis	61
2.8 Statistical Analysis	61
CHAPTER 3: T CELL MEMORY DIFFERENTIATION	65
3.1 Introduction	66
3.2 Results	67
3.2.1 Antigen-specific memory T cell responses	67
3.2.2 Defining the memory differentiation profile of T cells at 3 months post-infection	67
3.2.2.1 Definition of T cell phenotypes during primary HIV infection	67
3.2.2.2 The differentiation profile of memory CD4 ⁺ T cells	71
3.2.2.3 The differentiation profile of memory CD8 ⁺ T cells	74
3.2.3 Relationship between memory differentiation and CD57 expression in primary HIV-1 infection.	78
3.2.4 Longitudinal changes in the differentiation profile of memory T cells during the course of HIV infection	80

3.2.4.1 CD4 ⁺ T cells.....	80
3.2.4.2 CD8 ⁺ T cells.....	83
3.2.5 Examination of the T cell memory differentiation steady-state	86
3.2.5.1 CD4 ⁺ T cells.....	86
3.2.5.2 CD8 ⁺ T cells.....	87
3.3 Discussion	91
CHAPTER 4: T CELL ACTIVATION	96
4.1 Introduction.....	97
4.2 Results.....	98
4.2.1 Defining the activation profiles of memory T cells during at 3 months post-infection... 98	
4.2.1.1 Activation profile of memory CD4 ⁺ T cells.....	98
4.2.1.2 Activation profile of memory CD8 ⁺ T cells.....	103
4.2.1.3 Comparison of the activation profile between memory CD4 ⁺ and CD8 ⁺ T cells ...	106
4.2.2 The relationship between CD4 ⁺ T cell activation and memory differentiation	109
4.2.2.1 Distribution of activation markers within memory CD4 ⁺ subpopulations	109
4.2.3 Susceptibility of Gag-specific and total activated memory CD4 ⁺ T cells to <i>in vivo</i> HIV-1 infection	117
4.2.4 The relationship between CD8 ⁺ T cell activation and memory differentiation	120
4.2.4.1 Distribution of activation markers within memory CD8 ⁺ subpopulations	120
4.2.3 Longitudinal changes in the activation profile of memory T cells during the course of HIV infection	125
4.2.3.1 CD4 ⁺ T cells.....	125
4.2.3.2 CD8 ⁺ T cells.....	128

4.2.4 Examination of the T cell memory activation steady-state.....	130
4.2.4.1 CD4 ⁺ T cells.....	130
4.2.4.2 CD8 ⁺ T cells.....	132
4.3 Discussion.....	134
CHAPTER 5: T CELL POLYFUNCTION	142
5.1 Introduction.....	143
5.2 Results.....	144
5.2.1 Comparison between Gag- and CMV-specific memory responses in HIV-infected and -uninfected controls.....	144
5.2.2 Comparison between the polyfunctional profile of Gag- and CMV-specific memory CD4 ⁺ T cells in HIV-infected and -uninfected controls.....	151
5.2.3 Comparison between the polyfunctional profile of Gag- and CMV-specific memory CD8 ⁺ T cells in HIV-infected and -uninfected controls.....	155
5.2.4 The relationship between T cell polyfunction and memory differentiation	160
5.2.4.1 Polyfunctional profile of antigen-specific CD4 ⁺ T cell memory subpopulations...	160
5.2.5.2 Polyfunctional profile of antigen-specific CD8 ⁺ T cell memory subpopulations...	164
5.2.5 The longitudinal changes in the functional profile of HIV-specific memory T cells during HIV-1 infection	167
5.3 Discussion.....	175
CHAPTER 6: MICROBIAL TRANSLOCATION AND INFLAMMATORY CYTOKINES .	184
6.1 Introduction.....	185
6.2 Results.....	185
6.2.1 Plasma LBP, I-FABP, sCD14 and EndoCAb levels in HIV infection	185
6.2.2 Plasma cytokines concentrations and their association with microbial translocation ...	190

6.2.3 Associations between markers of microbial translocation and plasma cytokines	192
6.2.4 Associations between microbial translocation, plasma cytokines with HIV-1 viraemia	194
6.2.5 Associations between microbial translocation, plasma cytokines with T cell activation	198
6.2.6 Multivariate associations between microbial translocation, plasma cytokines, HIV-1 viraemia and T cell activation.....	203
6.2.7 The longitudinal changes of microbial translocation markers and plasma cytokines ...	206
6.3 Discussion	209
CHAPTER 7: THE RELATIONSHIP BETWEEN T CELL RESPONSES AND DISEASE PROGRESSION	215
7.1 Introduction.....	216
7.2 Results.....	217
7.2.1 Associations between the CD4 ⁺ T cell differentiation and HIV-1 viral load.....	217
7.2.2 Associations between the CD8 ⁺ T cell differentiation and HIV-1 viral load.....	222
7.2.3 Associations between memory CD4 ⁺ T cell activation and HIV-1 viral load	226
7.2.4 Associations between the memory CD4 ⁺ T cell activation and CD4 count	229
7.2.5 Associations between the memory CD8 ⁺ T cell activation and HIV-1 viral load	233
7.2.6 Associations between the memory CD8 ⁺ T cell activation and CD4 count	236
7.2.7 Associations between T cell function and HIV-1 viral load.....	239
7.3 Discussion	246
CHAPTER 8: CONCLUDING REMARKS	254
8.1 Summarizing Discussion and Conclusions	254
APPENDICES	260
APPENDIX A: ETHICS CLEARANCE I	260

APPENDIX B: ETHICS CLEARANCE II	261
APPENDIX C: ETHICS CLEARANCE III	262
APPENDIX D: PBMC AND PLASMA ISOLATION	263
APPENDIX E: LSRII 4-LASER SYSTEM	265
APPENDIX F: T CELL ACTIVATION PANEL OPTIMIZATION.....	267
APPENDIX G: CD4 ⁺ T CELL SORTING	269
APPENDIX H: REAL TIME PCR STANDARD CURVES	271
APPENDIX I: QUANTITATIVE REAL TIME PCR TEMPLATE	272
APPENDIX J: MICROBIAL TRANSLOCATION STANDARD CURVES	273
APPENDIX K: MEMORY SUBPOPULATIONS.....	274
APPENDIX L: MEMORY ACTIVATION.....	276
APPENDIX M: MEMORY POLYFUNCTION	278
APPENDIX N: MICROBIAL TRANSLOCATION AND INFLAMMATORY MOLECULES	281
APPENDIX O: NON-ACTIVATED MEMORY CELLS.....	287
REFERENCES	288

LIST OF FIGURES

Figure 1.1: Pathways of memory differentiation	12
Figure 1.2: The global incidence of HIV infection.....	17
Figure 1.3: Schematic illustration of the HIV-1 structure	22
Figure 1.4: The HIV replicative life cycle	24
Figure 1.5: The dynamics of CD4 ⁺ T cell count and HIV viraemia over the average course of untreated HIV infection	29
Figure 1.6: Schematic representation of the microbial translocation process	43
Figure 3.1: The Gating strategy for determining the memory phenotype of CD4 ⁺ and CD8 ⁺ T cells	70
Figure 3.2: Representative contour plots showing the gating scheme employed to determine distinct differentiation profiles of memory CD4 ⁺ and CD8 ⁺ T cells at 3 months post-infection .	71
Figure 3.3: The distribution of total CD4 ⁺ T cell subpopulations in HIV-infected and -uninfected controls.....	73
Figure 3.4: Comparison between the differentiation profiles of total ED, LD and FD CD4 ⁺ T cells with Gag- and CMV-specific memory CD4 ⁺ T cells in both HIV-infected and -uninfected controls.....	74
Figure 3.5: The distribution of total CD8 ⁺ T cell subpopulations in HIV-infected and -uninfected controls.....	76
Figure 3.6: Comparison between the differentiation profiles of total ED, Int-, LD and FD CD8 ⁺ T cells with Gag- and CMV-specific memory cells in both HIV-infected and -uninfected controls	77

Figure 3.7: The phenotypic expression of CD57 within total memory T cell subpopulations.....	79
Figure 3.8: The longitudinal changes of the differentiation profile of CD4 ⁺ T cells over the first 15 months of HIV infection	81
Figure 3.9: The longitudinal changes of the differentiation profile of antigen-specific memory CD4 ⁺ T cells over the first 15 months of HIV infection.....	82
Figure 3.10: The longitudinal changes of the differentiation profile of CD8 ⁺ T cells over the first 15 months of HIV infection	84
Figure 3.11: The longitudinal changes of the differentiation profile of antigen-specific memory CD8 ⁺ T cells over the first 15 months of HIV infection.....	85
Figure 3.12: The relationship between the proportions CD4 ⁺ T cell subpopulations at 3 and 12 months post-infection.....	88
Figure 3.13: The relationship between the proportions of CD8 ⁺ T cell subpopulations at 3 and 12 months post-infection.....	90
Figure 4.1: Gating scheme for activated total and antigen-specific memory CD4 ⁺ and CD8 ⁺ T cells in HIV-infected and -uninfected controls.....	99
Figure 4.2: The activation profile of total and antigen-specific memory CD4 ⁺ T cells in HIV-infected and -uninfected controls.....	101
Figure 4.3: Comparison of the activation profiles of total with Gag- and CMV-specific memory CD4 ⁺ T cells in both HIV-infected and -uninfected controls	102
Figure 4.4: The activation profile of total and antigen-specific memory CD8 ⁺ T cells in HIV-infected and -uninfected controls.....	104
Figure 4.5: Comparison of the activation profiles of total with Gag- and CMV-specific memory CD8 ⁺ T cells in both HIV-infected and -uninfected controls	105

Figure 4.6: Comparison of the activation levels between memory CD4 ⁺ and CD8 ⁺ T cells in HIV-infected and HIV-uninfected controls	107
Figure 4.7: Comparison of the activation levels between antigen-specific memory CD4 ⁺ and CD8 ⁺ T cells in HIV-infected and -uninfected controls	109
Figure 4.8: Gating strategy of activated cells within the ED-, Int-, LD- and FD-memory T cell subpopulations	111
Figure 4.9: Activation profiles of the different memory CD4 ⁺ T cell subpopulations at 3 months post-infection	112
Figure 4.10: Activation profiles of the antigen-specific CD4 ⁺ T cells within different memory subpopulations at 3 months post-infection	114
Figure 4.11: The relationship between the activation and the memory differentiation status of CD4 ⁺ T cells at 3 months-post infection.....	116
Figure 4.12: HIV-1 infection of Gag-specific and total memory activated CD4 ⁺ T cells at 3 months post-infection.....	119
Figure 4.13: Activation profiles of the different memory CD8 ⁺ T cell subpopulations at 3 months post-infection	121
Figure 4.14: Activation profiles of the antigen-specific CD8 ⁺ T cells within different memory subpopulations at 3 months post-infection	123
Figure 4.15: The relationship between the activation and the memory differentiation status of CD8 ⁺ T cells at 3 months-post infection.....	124
Figure 4.16: The longitudinal patterns of activated memory CD4 ⁺ T cells over the first 15 months of HIV infection.....	126

Figure 4.17: The longitudinal changes on the expression of CD38, HLA-DR and Ki67 on antigen-specific CD4 ⁺ T cells during the first 15 months of HIV infection	127
Figure 4.18: The longitudinal patterns of activated memory CD8 ⁺ T cells over the first 15 months of HIV infection	128
Figure 4.19: The longitudinal changes on the expression of CD38, HLA-DR and Ki67 on antigen-specific CD8 ⁺ T cells during the first 15 months of HIV infection	129
Figure 4.20: The relationship between the proportions of activated memory CD4 ⁺ T cells at 3 and 12 months post-infection.....	131
Figure 4.21: Correlations between memory CD8 ⁺ T cell activation profiles at 3 and 12 months post-infection	133
Figure 5.1: Representative dot plots showing the cytokine gating strategy for the determination of multi-functional memory CD4 ⁺ T cell responses	145
Figure 5.2: Representative dot plots showing the cytokine gating strategy for the determination of multi-functional memory CD8 ⁺ T cell responses	146
Figure 5.3: The total frequency of HIV-1 Gag- and CMV-specific T cell responses in early, chronically HIV- infected and -uninfected controls	148
Figure 5.4: The frequency of HIV-1 Gag- and CMV-specific T cell responses in early, chronically HIV-infected and -uninfected controls	150
Figure 5.5: Comparison between the polyfunctionality of HIV-Gag-specific and CMV-specific memory CD4 ⁺ T cells in early, chronically HIV-infected and HIV-uninfected controls	153
Figure 5.6: Comparison between the polyfunctionality of HIV-Gag-specific and CMV-specific memory CD8 ⁺ T cells in early, chronically HIV-infected and HIV-uninfected controls	157

Figure 5.7: Comparison of the polyfunctional profile between antigen-specific memory CD4 ⁺ and CD8 ⁺ T cells in at 3 months post-infection.....	159
Figure 5.8: The polyfunctionality of Gag-specific ED- and LD-memory CD4 ⁺ T cells at 3 months post-infection.....	161
Figure 5.9: The polyfunctionality of CMV-specific ED- and LD-memory CD4 ⁺ T cells in chronically HIV-infected individuals	163
Figure 5.10: The polyfunctionality of Gag-specific ED-, Int-, LD- and FD-memory CD8 ⁺ T cells at 3 months post-infection.....	165
Figure 5.11: The polyfunctionality of CMV-specific ED-, Int-, LD- and FD-memory CD8 ⁺ T cells in chronically HIV-infected individuals	166
Figure 5.12: Longitudinal characterization of HIV-Gag-specific memory T cells over the first 15 months of infection	168
Figure 5.13: The functional profile of Gag-specific memory CD4 ⁺ T cells over the first 15 months of infection	170
Figure 5.14: The functional contribution of monofunctional IFN γ and TNF α to the global of Gag-specific CD4 ⁺ T cell response over the first 15 months of HIV infection.....	171
Figure 5.15: The functional profile of Gag-specific memory CD8 ⁺ T cells over the first 15 months of infection	172
Figure 5.16: The contribution of functional combinations to the global Gag-specific memory CD8 ⁺ T cell response over the first 15 months of HIV infection	174
Figure 6.1: Plasma levels of markers associated with microbial translocation at early and chronic HIV-1 infection.....	187

Figure 6.2: Plasma concentrations of markers of microbial translocation in chronically HIV-infected individuals with CD4 count less than 200 cells/mm ³	189
Figure 6.3: Comparison of plasma cytokines levels in HIV-uninfected, early and chronically HIV-infected individuals	191
Figure 6.4: The association between markers of microbial translocation and cytokines at 3 months post-infection.....	193
Figure 6.5: The relationship between plasma markers of microbial translocation and plasma cytokine concentrations in HIV-uninfected individuals	194
Figure 6.6: Correlations of plasma levels of markers associated microbial translocation and plasma cytokines with viral load at 3 months post-infection.....	195
Figure 6.7: The relationship between plasma cytokines with viral load at 3 months post-infection.	197
Figure 6.8: The relationship between plasma markers of microbial translocation with memory T cell activation at 3 month post-infection.....	200
Figure 6.9: The relationship between plasma cytokines with memory T cell activation at 3 month post-infection	202
Figure 6.10: The relationship between total memory T cell activation with plasma cytokines in HIV-uninfected individuals	203
Figure 6.11: The longitudinal changes of the markers of microbial translocation during the first 12 months of HIV infection	207
Figure 6.12: The longitudinal changes of the markers of plasma cytokines during HIV infection	208

Figure 7.1: The relationship between the total CD4 ⁺ T cell subpopulations with viral load at 3 months post-infection.....	219
Figure 7.2: The relationship between the Gag-specific memory CD4 ⁺ T cell subpopulations with viral load at 3 months post-infection	220
Figure 7.3: The relationship between the total CD8 ⁺ T cell subpopulations with viral load at 3 months post-infection.....	223
Figure 7.4: The relationship between the Gag-specific memory CD8 ⁺ T cell subpopulations with viral load at 3 months post-infection	224
Figure 7.5: Correlations between the percentage of activated memory CD4 ⁺ T cells and viral load at 3 months post-infection.....	227
Figure 7.6: The relationship between the memory CD4 ⁺ T cell activation and CD4 T cell count at 3 months post-infection.....	231
Figure 7.7: The relationship between the memory CD8 ⁺ T cell activation and viral load at 3 months post-infection.....	235
Figure 7.8: Correlations between memory T cell activation profiles with CD4 ⁺ T cell counts at 3 months post-infection.....	237
Figure 7.9: Correlations between total HIV-1 Gag-specific memory CD4 ⁺ and CD8 ⁺ T cells with viral load at 3 months post-infection	240
Figure 7.10: Relationships between HIV-1 Gag-specific T cells and viral load at 3 months post-infection	242
Figure 7.11: Relationships between the functional profile of Gag-specific memory T cells and viral load at 3 months post-infection	244

LIST OF TABLES

Table 1.1: The proposed differentiation profiles of CD8 ⁺ and CD4 ⁺ T cell subpopulations.....	15
Table 2.1: Clinical characteristics of study subjects stratified by viral load differences between 3 and 12 months post-infection.....	53
Table 3.1: Antigen-specific memory T cell responses in early HIV-infected and HIV-uninfected individuals.....	69
Table 6.1: Mixed-effects linear associations between the plasma markers of microbial translocation, cytokines and viral load over the first 12 months of HIV infection.....	198
Table 6.2: Multivariate regression analysis associating plasma sCD14 and viral load with T cell activation at 3 months post-infection.....	205
Table 6.3: Multivariate regression analysis associating plasma IL-10 and TNF α with CD4 ⁺ T cell activation at 3 months post-infection.....	205
Table 7.1: Mixed-effects linear regression associating the differentiation CD4 ⁺ T cell with viral load over time	221
Table 7.2: Mixed-effects linear regression associating the differentiation CD8 ⁺ T cell with viral load over time	225
Table 7.3: Mixed-effects linear regression associating the proportions of activated memory CD4 ⁺ T cells with viral load over time	229
Table 7.4: Mixed-effects linear regression associating the proportions of activated memory CD4 ⁺ T cells with CD4 ⁺ T cell counts over time.....	232
Table 7.5: Multivariate analysis with mixed-effects linear regression associating activated memory CD4 ⁺ T cells with covariates viral load and CD4 ⁺ T cell counts over time	233

Table 7.6: Mixed-effects linear regression associating the proportions of activated memory CD8 ⁺ T cells with viral load over time	236
Table 7.7: Mixed-effects linear regression associating the proportions of activated memory CD8 ⁺ T cells with CD4 ⁺ T cell counts over time.....	238
Table 7.8: Multivariate analysis with mixed-effects linear regression associating activated memory CD8 ⁺ T cells with covariates viral load and CD4 ⁺ T cell counts over time	239
Table 7.9: Mixed-effects linear regression associating Gag-specific memory CD4 ⁺ and CD8 ⁺ T cell responses with viral load over time.....	246

LIST OF APPENDIX FIGURES

Figure F1: The expression of activation markers on CD4 ⁺ T cells after 6h stimulation.	268
Figure G1: Gating strategy employed for sorting of Gag-specific memory CD4 ⁺ T cells	269
Figure G2: Gating strategy employed for sorting of activated total memory CD4 ⁺ T cells	270
Figure M1: Comparison of the polyfunctional profile between CMV-specific memory CD4 ⁺ and CD8 ⁺ T cells in HIV-uninfected individuals	278
Figure M2: The polyfunctionality of CMV-specific ED- and LD-memory CD4 ⁺ T cells in HIV-uninfected individuals	279
Figure M3: The polyfunctionality of CMV-specific ED-, Int-, LD- and FD-memory CD8 ⁺ T cells in HIV-uninfected individuals	280
Figure N1: The association between sCD14 and IL-12(p70) at 3 months post-infection	281
Figure N2: The relationship between plasma cytokines with viral load at 3 months post-infection	281
Figure N3: The relationship between plasma markers of microbial translocation and cytokines with total memory T cell activation in HIV-infected individuals	282
Figure N4: The relationship between plasma markers of microbial translocation with antigen-specific memory T cell activation at 3 months post-infection	283
Figure N5: The relationship between plasma cytokines with activated Gag-specific memory CD4 ⁺ T cells activation at 3 months post-infection	284
Figure N6: The relationship between plasma cytokines with activated Gag-specific memory CD8 ⁺ T cells activation at 3 months post-infection	285

Figure N7: The relationship between memory T cell activation with IL-10 in HIV-infected individuals.....	286
Figure O1: The relationship between non-activated total and Gag-specific memory T cells with viraemia at 3 months post-infection.....	287

LIST OF APPENDIX TABLES

Table E1: Detector configuration and nomenclature.	265
Table K1: Correlations between the proportion of total memory CD4 ⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.	274
Table K2: Correlations between the proportion of Gag-specific memory CD4 ⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.....	274
Table K3: Correlations between the proportion of total memory CD8 ⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.	275
Table K4: Correlations between the proportion of Gag-specific memory CD8 ⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.....	275
Table L1: Correlations between the proportions of activated total memory CD4 ⁺ T cells at 3 months post-infection with activated memory CD4 ⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection.	276
Table L2: Correlations between the proportions of activated Gag-specific memory CD4 ⁺ T cells at 3 months post-infection with activated Gag-specific memory CD4 ⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection.....	276

Table L3: Correlations between the proportions of activated total memory CD8⁺ T cells at 3 months post-infection with activated memory CD8⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection. 277

Table L4: Correlations between the proportions of activated Gag-specific memory CD8⁺ T cells at 3 months post-infection with activated Gag-specific memory CD8⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection..... 277

ABBREVIATIONS

µg:	Microgram
µl:	Microlitre
AIDS:	Acquired Immunodeficiency Virus Syndrome
APC Cy7:	Allophycocyanin Cyanin 7
APCs:	Antigen Presenting Cells
ARV:	Antiretroviral
BHQ-1:	Black Hole Quencher-1
CAPT:	Canadian-Africa Prevention Trails network
CCR4:	Chemokine (C-C motif) Receptor 4
CCR5:	Chemokine (C-C motif) Receptor 5
CCR8:	Chemokine (C-C motif) Receptor 8
CD14:	Cluster of Differentiation 14
CD19:	Cluster of Differentiation 19
CD4:	Cluster of Differentiation 4
CD8:	Cluster of Differentiation 8
cDNA:	Complementary Deoxyribonucleic Acid
CI:	Confidence Interval
CMV:	Cytomegalovirus
C _t :	Threshold Cycle
CTL:	Cytotoxic T-Lymphocyte
CXCR3:	Chemokine (C-X-C motif) Receptor 3

CXCR4:	Chemokine (C-X-C motif) Receptor 4
CXCR5:	Chemokine (C-X-C motif) Receptor 5
CXCR6:	Chemokine (C-X-C motif) Receptor 6
DC:	Dendritic cells
DMSO:	Dimethylsulphoxide
DNA:	Deoxyribonucleic Acid
dNTP:	Deoxyribonucleotide Triphosphate
EBV:	Epstein-Barr virus
ED:	Early Differentiated memory cells
ELISA:	Enzyme Linked Immunosorbent Assay
EndoCAb:	Endotoxin Core Antibody
Env:	Envelope
FACS:	Fluorescence-Activated Cell Sorting
FAM:	6-carboxyfluorescein
FBS:	Foetal Bovine Serum
FD:	Fully Differentiated memory cells
FITC:	Fluorescein isothiocyanate
FSC:	Forward scatter
Gag:	Group Specific Antigen
GATA-3:	GATA-binding protein 3
gp120:	Glycoprotein 120Kda
gp41:	Glycoprotein 41Kda
HBSS:	Hanks-Balanced Salt Solution

HCV:	Hepatitis C virus
HIV-1:	Human Immunodeficiency virus type 1
HLA:	Human Leukocyte Antigen
HLA-DR:	Human Leukocyte Antigen-DR
HSV:	Herpes Simplex virus
ICS:	Intracellular Cytokine Staining
I-FABP:	Intestinal Fatty Acid Binding Protein
IFN γ :	Interferon gamma
IgM:	Immunoglobulin M
IL-1 α :	Interleukin-1alpha
IL-2:	Interleukin-2
IL-4:	Interleukin-4
IL-6:	Interleukin-6
IL-7:	Interleukin-7
IN:	Integrase
Int:	Intermediate memory cells
IQR:	Interquartile Range
LBP:	Lipopolysaccharide Binding Protein
LD:	Late Differentiated memory cells
LPS:	Lipopolysaccharide
LTNPs:	Long-Term non-progressors
LTR:	Long-Term Repeats
mAbs:	Monoclonal Antibodies

MCP-1:	Monocyte Chemotactic Protein 1
MD-2:	Myeloid Differentiation-2
MFI:	Mean Fluorescence Intensity
mg:	Milligram
MHC:	Major Histocompatibility Complex
MIP-1 β :	Macrophage Inflammatory Protein-1 beta
ml:	Millilitre
MTCT:	Mother-To-Child Transmission
Nef:	Negative Regulatory Factor
NIAID:	National Institute of Allergy and Infectious Diseases
NIH:	National Institutes of Health
NK:	Natural Killer cells
$^{\circ}\text{C}$:	Degrees Celsius
Pac Blue:	Pacific Blue
PAMPS:	Pathogen-Associated Molecular Patterns
PBMC:	Peripheral Blood Mononuclear Cells
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PE Cy7:	Phycoerythrin Cyanin 7
PE:	Phycoerythrin
PI:	Post-Infection
PMT:	Photomultiplier Tube
Pol:	Polymerase

PRR:	Pattern-Recognition Receptor
p-value:	Probability Value
qPCR:	quantitative Polymerase Chain Reaction
R10:	RPMI media with 10% FBS
Rev:	Regulatory of Viron
RNA:	Ribonucleic Acid
RPM:	Revolutions per Minute
RPMI:	Roswell Park Memorial Institute Media
RT:	Reverse Transcriptase
SAAVI:	South African AIDS Vaccine Initiative
sCD14:	Soluble Cluster of Differentiation 14
SIV:	Simian Immunodeficiency Virus
SSC:	Side scatter
TCR:	T cell Receptor
TNF α :	Tumour Necrosis Factor-alpha
UNAIDS:	United Nations programme on HIV/AIDS
Vif:	Viral infectivity factor
Vpr:	Viral protein R
Vpu:	Viral protein U
VRC:	Vaccine Research Center

CHAPTER 1: LITERATURE REVIEW

TABLE OF CONTENTS

1.1 The Immune System	2
1.1.1 Innate Immunity	2
1.1.2 Adaptive immunity	4
1.1.2.1 Ontogeny and function of T cells.....	5
1.1.2.2 Major Histocompatibility Complex	8
1.1.2.3 Memory	10
1.2 The HIV Epidemic and Origin.....	16
1.2.1 Origin and distribution.....	17
1.2.2 HIV structure and function	19
1.2.3 HIV life cycle.....	23
1.3 Adaptive Immunity and HIV infection	26
1.3.1 HIV transmission	26
1.3.2 T cell responses.....	30
1.3.2.1 CD8 ⁺ T cells.....	30
1.3.2.2 CD4 ⁺ T cells.....	33
1.3.2.3 T cell Polyfunction.....	35
1.3.3 T cell Memory and HIV infection	37
1.3.4 Immune Activation and HIV infection	39
1.3.4.1 Microbial Translocation and Immune Activation.....	42
1.3.4.2 TCR-independent Immune activation.....	45
1.4 Rationale and Objectives of this Study	46

1.1 The Immune System

The immune system is a defense mechanism found in most vertebrates and provides means to mount rapid, highly specific, and often very protective responses against the myriad pathogenic microorganisms. The immune system can be considered as a multilayered system comprising of two major defense mechanisms; the innate and adaptive (or acquired) immunity (Parkin and Cohen, 2001). Innate immunity includes physical (skin), chemical (stomach acids) and microbiological immune barriers (macrophages or dendritic cells), which provide immediate host defense. Adaptive immunity consists of antigen-specific reactions through T lymphocytes (cellular immunity) and B lymphocytes (humoral immunity). The adaptive response has memory, so that subsequent re-exposure to pathogen leads to a more vigorous and rapid response (Parkin and Cohen, 2001).

1.1.1 Innate Immunity

Innate immunity represents the first line of host defense against pathogenic microorganisms (Janeway and Medzhitov, 2002). Cells participating in innate immunity include APCs such as macrophages and dendritic cells (mononuclear phagocytes), neutrophils, eosinophils and basophils (polymorphonuclear phagocytes), mast and NK cells (Beutler, 2004). In addition, innate immune responses form part of the skin and the epithelial cells lining the respiratory, gastrointestinal, and genitourinary tracts. These identify and eliminate pathogens, by either binding and/or opsonizing them for phagocytosis (Beutler, 2004). Innate immunity also has a humoral component that includes complement proteins, LPS binding proteins, C-reactive protein and other pentraxins, collectins and antimicrobial peptides, including defensins (Beutler, 2004). Innate immunity also plays an important role as a mediator in the priming/activation of adaptive

immune responses (Janeway, 1989). The essential link between innate and adaptive immunity is provided by APCs, among which dendritic cells can induce a primary immune response (Fearon and Locksley, 1996, Palucka and Banchereau, 1999).

Distributed as sentinels throughout the body, immature DC are poised to capture antigen, migrate to lymphoid organs, and after a process of differentiation, mature DC select antigen-specific T lymphocytes to which they present processed antigen, thereby initiating clonal expansion and differentiation of antigen-specific T lymphocytes (Watts, 1997, Cella *et al.*, 1997). The process of antigen capturing and antigen processing involves opsonizing and phagocytosis of microbial particles into large endocytic vesicles called phagosomes. Endocytosed antigens are transferred to late endosomes, which have a degradative microenvironment with an acidic pH and active proteases, and antigens are degraded into short sequence peptides. These are then translocated from the cytosol to the endoplasmic reticulum where they bind to MHC molecules (Watts, 1997). Peptides/epitopes are then presented at the cell surface of APC with MHC molecules, which are then scanned and recognized by circulating T lymphocytes via their antigen-specific cell surface TCRs.

Innate immune cells also function by recognizing highly conserved sets of molecular structures specific to microbes (PAMPs) through a limited set of germ-line encoded receptors, often referred to as pattern-recognition receptors (PRRs) (Janeway, 1989). Among PRRs, TLRs have emerged as pivotal components of innate immunity, and are capable of sensing a wide spectrum of microorganisms ranging from viruses to parasites. TLR have the ability to recognize pathogens or pathogen-derived products and initiate signaling events leading to activation of innate host

defenses. Signaling by TLR initiates acute inflammatory responses by induction of antimicrobial genes and inflammatory cytokines and chemokines (Janeway and Medzhitov, 2002). Furthermore, there is evidence that TLR also contribute significantly to activation of adaptive immune responses (Schnare *et al.*, 2001). Thus, studies by Schanare *et al.* (2001) showed that efficient priming of adaptive immune responses requires not only the presentation of antigens in the context MHC, but recognition of pathogens by TLR on APC leading to the induction of accessory signals (co-stimulators and cytokines) that are necessary for the activation of adaptive immune responses (Schnare *et al.*, 2001). Moreover, this mechanism would ensure that only pathogen-specific immune responses would get activated (Pasare and Medzhitov, 2004).

1.1.2 Adaptive immunity

Adaptive immunity consists of antibody-mediated (humoral immunity) and cell-mediated (cellular immunity) responses, which are carried out by different B lymphocytes and T lymphocytes, respectively. A key feature of the adaptive immune response is the development of immunological memory, which is the ability to recall previously encountered antigen and mount rapid immune response in the event of re-exposure. Both B and T lymphocytes develop from progenitor stem cells within the bone marrow, where B cells remain within the bone marrow for duration of their development, whereas T cells migrate to the thymus as thymocytes for development. Both cell types undergo similar genomic rearrangements leading production of antigen-specific receptors. In T lymphocytes, gene rearrangement leads to a creation of functional genes encoding α and β or γ and δ chains of the TCR, whereas in B cells gene product is a membrane-bound form of IgM, which acts as an antigen receptor (Parkin and Cohen, 2001, Bonilla and Oettgen, 2010).

1.1.2.1 Ontogeny and function of T cells

The earliest T cell precursors are CD4⁻CD8⁻ double negative in phenotype, comprising about 3-5% of the total thymocyte numbers. The sequential productive rearrangements of TCR genes leading to surface expression of $\alpha\beta$ or $\gamma\delta$ TCR, marks the transition from precursors to double-positive thymocytes cells (Bonilla and Oettgen, 2010). Thus, these early precursors proliferate extensively and become CD4⁺CD8⁺ double positive, which are still immature and are target cells for TCR-based selection events (Jameson *et al.*, 1995). The selection process involves the ability to discriminate self-antigens from foreign antigens, where positive and negative selection events involve antigen receptors, antigens and molecules of the MHC. For positive selection of CD4⁺CD8⁺ thymocytes, cells bearing a TCR that recognizes and binds self-MHC with sufficiently low affinity are positively selected, whereas double positive cells expressing TCR with high affinity to self-MHC are eliminated by apoptosis (negative selection) (van Meerwijk *et al.*, 1997, van Meerwijk and MacDonald, 1999). Low-affinity recognition of self-MHC not only promotes survival, but also determines further differentiation of double positive thymocytes to either a CD4⁺ or CD8⁺ lineage (Robey and Fowlkes, 1994, Kisielow and von Boehmer, 1995). Thus, engagement of MHC class I molecules results in commitment to the CD8⁺ T cell lineage, while engagement of MHC class II molecules results to commitment to CD4⁺ T cells. These two cell lineages differ fundamentally in how they recognize antigen, but also mediate different types of regulatory and effector functions.

Cells that emerge from the thymus and/or bone marrow after having undergone selection and differentiation are known as naïve cells, which have not yet encountered antigen. Naïve cells enter the periphery and populate secondary lymphoid tissues, lymph nodes, spleen, tonsils and

mucosa associated lymphoid tissues (Parkin and Cohen, 2001). Lymphoid tissues express adhesion molecules in an orderly array, allowing cells to move through tissues, thereby providing the environment for antigen encounter via APCs. Thus, once antigen is brought to the lymphoid tissues by dendritic cells (or other APCs), activation of naïve T cells is initiated when the TCR recognize the peptide-MHC complex on APCs. This leads to rapid clustering of TCR-associated molecules at the physical interface between T cells and APCs, and the formation of an immunologic synapse and subsequent activation of either CD4⁺ or CD8⁺ T cells (Dustin, 2009).

CD4⁺ T cells serve a helper function and have been designated Th cells, where studies in mice originally demonstrated two main types of helper T cell clones, Th1 and Th2 (Mosmann *et al.*, 1986). Th1 cells are characterized based on their capacity to produce IFN γ and IL-2 cytokines and have been shown to differentiate from naïve Th0 cells under the influence of IFN γ and transcription factor T-bet, whereas Th2 mainly produce IL-4, IL-10 and IL-13, and their development is driven/polarized by IL-4 and the transcription factor GATA-3 (Murphy and Reiner, 2002, Cao *et al.*, 2005). Th1 cells are mainly involved in autoimmune diseases and immunity against intracellular pathogens, including the activation of mononuclear phagocytes, NK cells and CTL, while Th2 cytokines mediate antibody production against extracellular pathogens. More recently, a third Th cell lineage, termed Th17 has been identified and defined based on the ability to produce IL-17, but not IFN γ or IL4 (Harrington *et al.*, 2005, Dong, 2008). The differentiation of Th17 has been shown to develop under the influence of transforming growth factor (TFG)- β , IL-1 β , IL-6 and transcription factor Ror- γ t (Acosta-Rodriguez *et al.*, 2007a). These cells are thought to be critical in the defense against bacteria and fungi, particularly at mucosal surfaces, and also contribute to the homeostasis of enterocytes (Cecchinato *et al.*,

2008). Moreover, Th17 cells have been proposed to play an essential role during HIV pathogenesis (Klatt and Brenchley, 2010, Singh *et al.*, 2012). In addition, CD4⁺ Th cells can also develop into a variety of regulatory subsets (T_{reg} cells) as defined by the expression of forkhead box P3 (FoxP3) and/or their ability to produce cytokines such as TFG- β , IL-10 and IL-35 (Vignali *et al.*, 2008, Allan *et al.*, 2008). T_{reg} cells have been shown to exert anti-inflammatory functions and control self-reactive T cells including Th1, Th2 and Th17 cells (Sakaguchi and Powrie, 2007).

CD8⁺ T cells recognize and destroy cells harbouring intracellular pathogens, including viruses and pathogens. These cells are often termed CTL, referring to use of the granule exocytosis pathway, which results in apoptosis of infected target cells. CTL can induce target cell apoptosis through two distinct pathways; secretion of cytolytic granules and through Fas/FasL interactions (Shresta *et al.*, 1998). Thus, through contact-dependent mechanisms, CTL recognition of foreign particles via the MHC class I, leading to the formation tight junction synapse that leads to apoptotic cell death of a target cell. This process is mediated by a release of cytotoxic molecules, perforin, granzymes and/or granulysin to the membrane of target cells. Perforin results in pore formation on target cells, allowing passage of cytotoxins such as serine proteases, granzyme A or B. Upon entrance into target cells, granzymes target a number of host cell proteins leading to activation of apoptosis. For the pro-apoptotic pathway, TCR activation of the immune synapse drives the expression of Fas ligand on CTL, which engages Fas (CD95) on the target cell membrane and thereby triggers apoptosis (Shresta *et al.*, 1998, Trapani and Smyth, 2002).

1.1.2.2 Major Histocompatibility Complex

As mentioned above, proteolytic degradation of antigenic proteins result in peptides of different fragments that are subsequently displayed by MHC molecules on the surface of antigen presenting cells. In humans, the MHC encodes the HLA, which contains a number of genes that reside on chromosome 6. The two major pathways in which antigens are presented include HLA class I molecules (HLA-A, B and C), which activate CD8⁺ CTLs to kill infected cells, and HLA class II molecules (HLA-DR, DQ and DP), which activate CD4⁺ helper T lymphocytes to perform their key roles in controlling humoral, CTL-mediated and inflammatory responses (Watts, 1997, Palucka and Banchereau, 1999, Bonilla and Oettgen, 2010). Peptides or epitopes presented by HLA class I molecules are predominantly derived from intracellular proteins, and all proteins present in the cytosol are potential sources of peptides for this pathway (Pamer and Cresswell, 1998). In contrast to HLA class I-mediated processing and presentation, the HLA class II pathway is restricted to specialized APCs that present peptides derived from the extracellular environment of the APCs (Chapman, 1998).

For both HLA class I and II molecules, the polymorphic amino acid residues within the antigen-peptide binding groove interact with specific residues of the TCR. The highly polymorphic/variable region of the binding groove allows the HLA molecules to have broad specificity, and can bind a variety of antigenic peptides with differing affinities, thereby influencing the efficiency of immune protection by both specificity and affinity of peptide binding and recognition by T cells (Gotch *et al.*, 1996). Three models have been proposed to explain the maintenance of HLA polymorphism: (i) balancing selection, where alleles that confer resistance to one disease and susceptibility to another; (ii) heterozygous advantage, where

increasing the number of unique HLA type increases the breadth of peptide recognition and immune protection against invading pathogen; and (iii) frequency-dependent selection, where a pathogen has evolved to escape an efficient immune response mediated by common alleles in the population, but remains susceptible to responses mediated low-frequency alleles (Carrington and O'Brien, 2003). Therefore, the nature of HLA allele diversity is likely to play a significant role in the regulation of immunity and disease progression to rapidly evolving viral infections such as HIV (Gotch *et al.*, 1996). While there is potentially vast numbers of epitopes that could be generated and displayed at the surface of APCs, it has been demonstrated that only a few, and in some cases a single antigenic HLA-epitope complexes elicit immune responses, resulting in a rather restricted immune response (Yewdell and Bennink, 1999). These few epitopes that dominate an immune response are known as immunodominant, whereas those that are infrequently targeted by the immune system are referred to as subdominant.

Of the three HLA class I loci, HLA-B is the most polymorphic, with most HLA-B molecules described compared to HLA-A and C (Goulder and Watkins, 2008). Several HLA-B alleles such as HLA-B*27 (HLA-B*27:05), B*57 (HLA-B*57:01, B*57:02 and B57*03) and HLA-B*58:01 alleles have been consistently associated with delayed HIV disease progression (Carrington and O'Brien, 2003, O'Brien and Nelson, 2004, Goulder and Watkins, 2008). The mechanism behind the protective association with B*27 is suggested to involve an immunodominant CTL response to a conserved HIV epitope (KK10) in the p24 Gag (Goulder *et al.*, 1997, Kelleher *et al.*, 2001). CTL responses restricted by B*57 molecules have been shown to target multiple HIV Gag epitopes and reverse transcriptase motif (Goulder *et al.*, 1996, Klein *et al.*, 1998). Thus, the broad peptide recognition specificity of B*57 may account for compromised viral fitness leading

to its protective nature. Furthermore, a number of studies have indicated that CD8⁺ T cell responses restricted by HLA-B*27 and/or B*57 possess superior functional properties, which may render them more effective (Migueles *et al.*, 2002, Betts *et al.*, 2006). Other HLA class I alleles associated with delayed progression to AIDS include HLA-A25, A26 and A*68 (Kaslow *et al.*, 1996). In contrast, other allelic groups (HLA-B*35:02, HLA-B*35:03, B*53:01 and HLA-Cw*04) have shown to be associated with accelerated disease progression (Itescu *et al.*, 1992, Carrington *et al.*, 1999, Gao *et al.*, 2001).

1.1.2.3 Memory

Memory is one the core features of the adaptive immune system. Early reports of memory identity were through studies conducted by the British physician Edward Jenner in 1796, with the initial injection of an 8 year old boy with harmless cowpox resulting in immunity and protection against smallpox. This not only showed the existence of immunological memory, but also showed benefits of vaccine immunization against potential pathogens. According to Janeway (2001), memory is the ability of the immune system to respond more rapidly and effectively to pathogens encountered previously and reflects the pre-existence of clonally expanded populations of antigen-specific lymphocytes. However, it is still debatable whether specific memory is maintained by distinct populations of long lived memory cells that can persist without antigen, or by lymphocytes that are under constant stimulation by residual antigen or antigen re-exposure (Janeway, 2001). The ability to develop memory confers lifelong immunity to many infectious agents after an initial encounter, and it is the basis of all active immunization strategies.

Priming of naïve T cells leads to formation of long-lived memory cells that are able to self-renew by undergoing homeostatic proliferation (Dutton *et al.*, 1998, Swain, 2000). Memory T cells have been shown to be heterogeneous and memory populations can be differentiated based on a variety of migratory and functional attributes (Hamann *et al.*, 1997, Sallusto *et al.*, 1999, Sallusto and Lanzavecchia, 2001, Sallusto *et al.*, 2004). For example, central memory T cells are known to localize in lymphoid tissues, have a higher proliferative capacity following TCR triggering, express high levels IL-2 (Iezzi *et al.*, 1998, Sallusto *et al.*, 2004) and exhibit reduced immediate effector function and cytotoxicity (Seder *et al.*, 2008). Effector memory cells on the other hand localize in non-lymphoid peripheral tissues, have poor proliferative capacity, produce low levels of IL-2, express high Fas levels and thus prone to undergo apoptosis, but rapidly produce elevated levels of IFN γ and cytolytic molecules such as perforin and granzymes (Hamann *et al.*, 1997, Bachmann *et al.*, 1999, Kaech *et al.*, 2002, Romero *et al.*, 2007, Decrion *et al.*, 2007).

There has been a growing interest with regard to understanding the differentiation pathways of memory T cells following antigen exposure. However, it is still unclear whether the pathway of T cell memory differentiation follows a linear or a branched profile. Thus, several reports appear to support the linear model, whereby the priming of naïve cells by antigen results in their differentiation into effector T cells, most of which die by apoptosis after antigen clearance, and the remaining antigen-experienced T cells eventually populate a pool of long-lived memory T cells (Wherry and Ahmed, 2004). In contrast, other studies suggest that upon antigen engagement, naïve cells branch into a heterogeneous pool of both effector and memory subpopulations that are able to revert from one phenotype to a previous phenotype (Moulton and Farber, 2006, Stemberger *et al.*, 2007) (Figure 1.1).

Linear pathway of effector and memory T cells



Branched pathway of effector and memory T cells

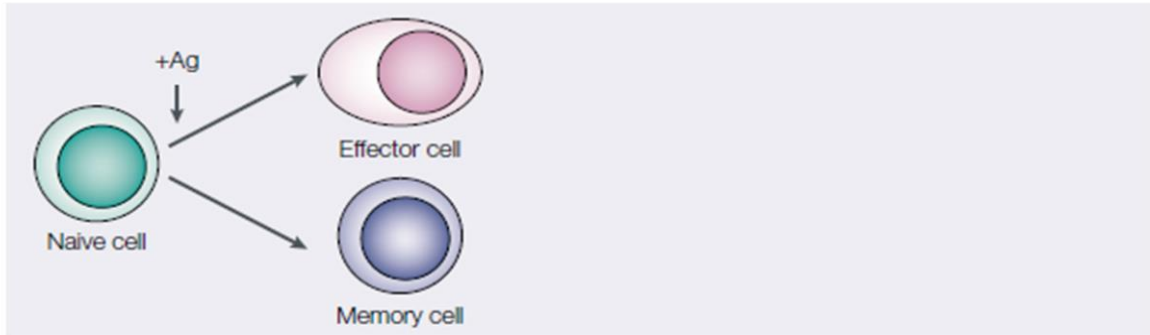


Figure 1.1: Pathways of memory differentiation. Linear pathway: upon antigenic stimulation, naïve T cells proliferate and differentiate and acquire effector functions. After antigen (Ag) clearance a proportion of antigen-primed T cells (effector cells) further differentiate and give rise to memory T cells that continue in the absence of antigen. Branched pathway: activated naïve T cells give rise to a pool of both effector and memory T cell subpopulations. Figure adapted and modified from Kaech et al. (2002)

Each of the differentiated memory subpopulations display different surface glycoproteins (markers), which provide a mean to examine the differentiation phenotype of each cell subset (De Rosa *et al.*, 2001, Chattopadhyay and Roederer, 2005, Appay *et al.*, 2008) (Table 1.1). Surface markers used to characterize T cell differentiation include lymphoid organ-homing receptors (CCR7 and CD62L), co-stimulatory (CD27 and CD28), memory markers (CD45RA or CD45RO isoforms) and markers of replicative senescence (CD57), where almost every possible combination of these markers can be used to discern different memory subpopulations (Sallusto

et al., 2004, Radziejewicz *et al.*, 2007). T cell lymphocytes that express the combination of markers such as, CD27, CD28, CCR7, CD45RA, CD62L and down-regulate CD45RO and CD57 simultaneously are referred to as naive cells whereas these that down-regulate CD45RA and/or up-regulate CD45RO are referred to as central memory/early differentiated cells (Chattopadhyay and Roederer, 2005, Appay *et al.*, 2008). Memory T cells that lack the expression of CCR7 and CD45RA are termed effector memory/late differentiated cells (Sallusto *et al.*, 1999, Sallusto *et al.*, 2004), whereas those that express high levels of CD57 and down-regulate CCR7, CD27 and CD45RO are referred to as terminally/ fully differentiated T cells (Appay *et al.*, 2008).

Using surface expression of co-stimulatory molecules CD27 and CD28, studies conducted by Appay *et al.* (2002a) showed different memory phenotypes of antigen-specific CD8⁺ T cells and were defined as early (CD27⁺CD28⁺), intermediate (CD27⁺CD28⁻) and late memory (CD27⁻CD28⁻) cells. Furthermore, late differentiated cells were characterized by increased expression of CD57, a marker of replicative senescence. Other studies have shown the use of additional markers such as CD45RA and CCR7, which could lead to further discrimination of more memory CD8⁺ T cell subpopulations (Tomiyama *et al.*, 2002b, Decrion *et al.*, 2007). These additional memory subpopulations are not only phenotypically different, but are able to mediate distinct effector functions. For instance, the stepwise loss of CCR7, CD27 and CD28 was paralleled with increased up-regulation of cytolytic activity (ability to produce perforin and granzymes). Similarly, the expression of these surface markers have also been used to characterize memory CD4⁺ T cell subpopulations (Amyes *et al.*, 2005, Fritsch *et al.*, 2005, Okada *et al.*, 2008), where 5-6 distinct memory CD4⁺ phenotypes were identified. Although, the intermediate

subpopulation of memory CD4⁺ T cells were identified in these studies, this subpopulation was found to make up <5% of the total CD4⁺ T cells (Okada *et al.*, 2008).

CD4⁺ memory T cells can also be subdivided based on the surface expression of cytokine receptors (Rivino *et al.*, 2004). Thus, expression of CCR5, CXCR3, and CXCR6 and the capacity produce IFN γ characterizes Th1 cells, while cells expressing CCR3, CCR4, CCR8 and CCR2 and have the capacity to produce IL-4 identifies CD4⁺ T cells with a Th2 profile (Sallusto *et al.*, 1998). Studies conducted by Rivino *et al.* (2004) showed that central memory CD4⁺ T cells are composed of heterogeneous pool of non-polarized (CXCR5⁺), pre-Th1 (CXCR3⁺) and pre-Th2 (CCR4⁺), where further differentiation of pre-Th1 and -Th2 cells into effector memory cells is accompanied by increased capacity to produce IFN γ and IL-4, respectively (Fritsch *et al.*, 2005, Okada *et al.*, 2008). Furthermore, other studies have also shown that the expression of CCR6 and CCR4 could further discriminate Th17 cells (Acosta-Rodriguez *et al.*, 2007b), while the expression of CD4⁺CD25^{high}FoxP3⁺ could be used to discriminate T_{reg} cells (Li *et al.*, 2011).

Table 1.1: The proposed differentiation profiles of CD8⁺ and CD4⁺ T cell subpopulations.

Naïve	Central Memory		Transitory		Intermediate	Effector memory		Effector cells	
CD8/CD4	CD8 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺
CCR7 ⁺ CD62L ⁺ CD45RA ⁺ CD45RO ⁻ CD27 ⁺ CD28 ⁺	CCR7 ⁺ CD62L ⁺ CD45RA ⁻ CD45RO ⁺ CD27 ⁺ CD28 ⁺	CCR7 ⁺ CD62L ⁺ CD45RA ⁻ CD45RO ⁺ CD27 ⁺ CD28 ⁺	CCR7 ⁻ CD62L ⁺ CD45RA ⁻ CD45RO ⁺ CD27 ⁺ CD28 ⁺	CCR7 ⁻ CD62L ⁺ CD45RA ⁻ CD45RO ⁺ CD27 ⁺ CD28 ⁺	CCR7 ⁻ CD62L ^{int} CD45RA ⁺ CD45RO ⁻ CD27 ^{dim} CD28 ⁺	CCR7 ⁻ CD62L ^{low} CD45RA ⁻ CD45RO ⁺ CD27 ⁻ CD28 ⁺	CCR7 ⁻ CD62L ^{low} CD45RA ⁻ CD45RO ⁺ CD27 ⁻ CD28 ⁺	CCR7 ⁻ CD62L ⁻ CD45RA ⁺ CD45RO ⁻ CD27 ⁻ CD28 ⁻	CCR7 ⁻ CD62L ⁻ CD45RA ⁺ CD45RO ⁻ CD27 ⁻ CD28 ⁻
Functional Attributes									
	TNF ⁺⁺ IFN γ ⁺⁺ Perforin ⁻	IFN γ ⁺ IL-2 ⁺⁺⁺ IL-4 ⁺ Perforin ⁻	TNF ⁺⁺ IFN γ ⁺⁺ Perforin ⁺	IFN γ ⁺⁺ IL-2 ⁺ IL-4 ⁺ Perforin ⁻	TNF ⁺⁺ IFN γ ⁺⁺ Perforin ⁺⁺	TNF ⁺⁺ IFN γ ⁺⁺ Perforin ⁺⁺⁺	IFN γ ⁺⁺⁺ IL-2 ⁺ IL-4 ⁺⁺ Perforin ⁻	TNF ⁺⁺⁺ IFN γ ⁺⁺⁺ Perforin ⁺⁺⁺⁺	IFN γ ⁺ IL2 ⁺ IL-4 ⁺ Perforin ⁺⁺

The phenotypic lineage of CD8⁺ and CD4⁺ T cells is based according to previously described differentiation models (Tomiyama *et al.*, 2002b, Decrion *et al.*, 2007, Appay *et al.*, 2008, Fritsch *et al.*, 2005, Okada *et al.*, 2008).

1.2 The HIV Epidemic and Origin

Whilst innate and adaptive immunity has evolved in humans to effectively clear antigen and prevent pathology, the emergence of HIV, as a persistent chronic pathogen, results in multiple levels of immune dysfunction. Infection with the HIV results in a complex clinical disease known as AIDS (Barre-Sinoussi *et al.*, 1983), of which the disease often takes years to develop. Infection with HIV is a crisis worldwide, with over 33.3 million people with estimated 2.5 million of children living with HIV, of which 2.6 million new cases and 1.8 million deaths occurring per year according to a report of the UNAIDS, 2010 (UNAIDS, 2010) (Figure 1.2). The largest epidemics have been found in sub-Saharan African countries (South Africa, Ethiopia, Zambia, Zimbabwe, Ethiopia and Nigeria), with over 22.5 million (68%) of the global population living with HIV. Of these countries, South Africa has been severely affected with the HIV epidemic, and currently has the highest number of people living with the virus in the world. In 2009, the national antenatal sentinel HIV and syphilis prevalence in South Africa was estimated at 17.9% (SADH, 2011). This is estimated to be 5.63 million people, including 3.3 million women and 334000 children. Moreover, the estimate among antenatal women living with HIV was 29.4% (28.5-30.2, 95% confidence interval). The burden of HIV disease has further been exacerbated by co-infection with other pathogens, particularly tuberculosis (Corbett *et al.*, 2003, Groenewald *et al.*, 2005). Although, much effort has been devoted to reduce HIV transmission rates, by initiating national ARV treatment programs (SADH, 2007), the emergence of anti-retroviral drug-resistance has been shown to be a leading cause of therapeutic failure in treated individuals (Barth *et al.*, 2008, Marconi *et al.*, 2008). This may highlight an area of concern regarding long-term ARV treatment success, and suggests a need for developing additional strategies in circumventing and/or preventing HIV infection. The possibility of

developing an effective vaccine that is capable of generating a protective and effective antiviral cell-mediated immunity appears to be an important strategy and a cost-effective public intervention in establishing control of the HIV/AIDS epidemic (Virgin and Walker, 2010).

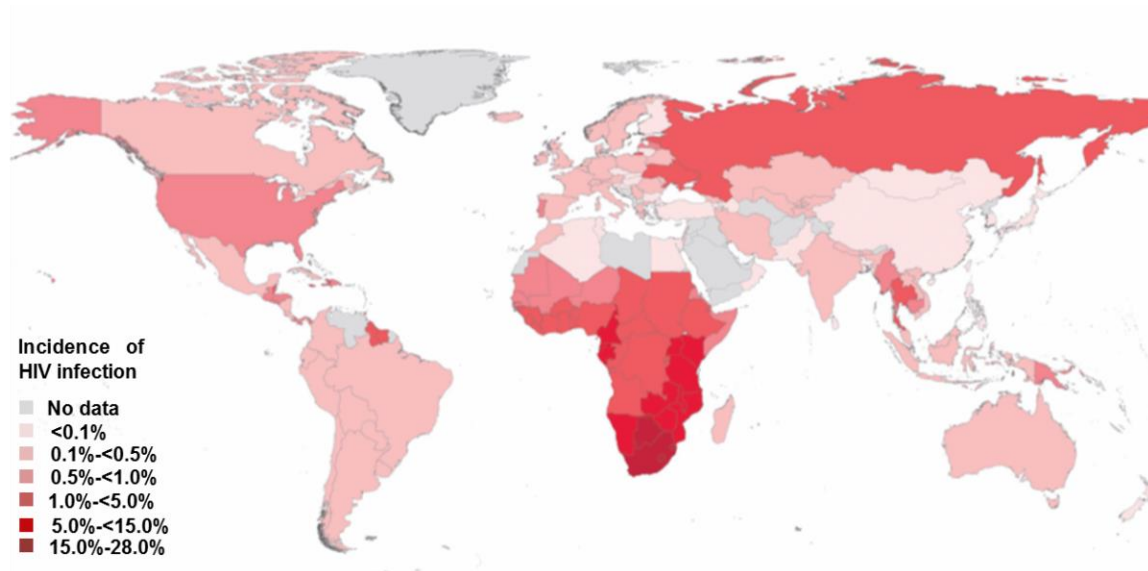


Figure 1.2: The global incidence of HIV infection. The highest prevalence rates are found in sub-Saharan Africa, where up to a quarter of the population is infected with HIV. High rates of transmission are also observed in Asia and Eastern Europe. Figure adapted from the UNAIDS 2010 Report on the global AIDS epidemic (UNAIDS, 2010).

1.2.1 Origin and distribution

One of the major characteristics of HIV relevant to vaccine development is its extensive and constant changing genetic diversity, which results in differing variants that can be categorized into distinct genetic subtypes as well as recombinant forms. The HIV is classified under the genus *Lentivirus* and is a member of the *Retroviridae* family of viruses (commonly known as retroviruses) (Montagnier, 2002). HIV is closely related to viruses that cause similar

immunodeficiency diseases in a range of animal species. Its origin is widely accepted to have resulted from cross-species transfer from the SIV group, which is widely distributed in African primates; African green monkeys (SIV_{agm}) (Kraus *et al.*, 1989), sooty mangabeys (SIV_{sm}) (Marx *et al.*, 1991), and chimpanzee (SIV_{cpz}) (Sharp *et al.*, 1995, Chen *et al.*, 1997). Based on the genetic variations, there are two main types of HIV, type 1 and type 2 (Barre-Sinoussi *et al.*, 1983, Clavel *et al.*, 1986, Dietrich *et al.*, 1989), of which HIV-1 is more pathogenic, broadly distributed and responsible for the majority of infections. The natural hosts of HIV-1 are the chimpanzee subspecies which harbour a closely related SIV_{cpz} virus (Gao *et al.*, 1999, Corbet *et al.*, 2000), while HIV-2 closely resembles SIV_{sm} found in West African sooty mangabey (Hirsch *et al.*, 1989, Chen *et al.*, 1996). HIV-1 has been further classified into several clades, whose geographical distribution varies from country to country. The HIV-1 type viruses fall into four distinct phylogenic clusters, which have been termed groups M, O, N (Simon *et al.*, 1998, Peeters and Sharp, 2000) and a recently identified group P (Vallari *et al.*, 2011). The M group viruses are the most numerous and responsible for the vast majority of AIDS cases worldwide, and are further divided into ten phylogenetic subtypes (designated by letters: A to K) that are classified based on phylogenetic clustering and subgenomic regions such as Gag, Nef, Pol and Env of individual genes from same isolates. The O and N group viruses are scarce and are commonly found in western equatorial Africa (De Leys *et al.*, 1990, Simon *et al.*, 1998).

HIV-1 subtypes A, B and C have been shown to be the most prevalent HIV-1 subtypes globally, with subtype C accounting for approximately 50% of infections (Hemelaar *et al.*, 2011), while subtypes B and C account for approximately 12% and 11% for infections globally. The HIV-1 subtype A is commonly found in countries of west, central and eastern Africa (e.g. Uganda,

Kenya and Rwanda), whilst subtype B is mainly found in western and central Europe, north and south America and Australia. HIV-1 subtype C viruses are predominant in sub-Saharan countries such as South Africa, Zambia and Zimbabwe, and this subtypes has also been shown to be prevalent in India (Hemelaar *et al.*, 2006, Hemelaar *et al.*, 2011). The subtypes D and G account for 2% and 5% of the global HIV infections respectively and are commonly found in central Africa, whereas subtypes F, H, J and K account for 1% of the global HIV-1 infections.

1.2.2 HIV structure and function

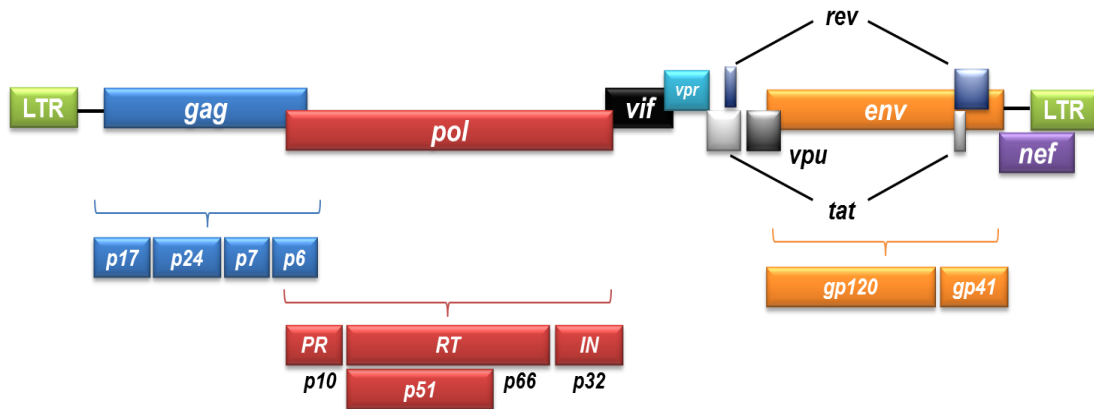
The HIV genome is 9200 bases in length (Sierra *et al.*, 2005), and is composed of two copies of the single-stranded RNA that codes for the virus's nine genes. These genes are flanked on both sides by repetitive LTR (Frankel and Young, 1998) (Figure 1.3A). The 5'-LTR functions as promoter and directs HIV transcription, whereas the 3'-LTR is required for cleavage and polyadenylation of the viral mRNA (Klaver and Berkhout, 1994). HIV genome encodes for three main structural proteins (Gag, Pol and Env), regulatory proteins (Tat, Rev) and accessory proteins (Vpu, Vif, Vpr and Nef) (Hirsch and Curran, 1990, Frankel and Young, 1998). The *gag* gene region, encodes for structural proteins such as the matrix (p17), capsid (p24), nucleocapsid (p7) and the p6 proteins (Gallo *et al.*, 1988, Gelderblom *et al.*, 1989). The matrix forms the scaffold between the core and the outer envelope of the capsid, and contains a nuclear localization domain that facilitates nuclear transport of the viral genome and allows HIV to infect non-dividing cells (Fassati, 2006). The capsid forms the conical core of the viral particles (Gottlinger, 2001), and is important for proper folding of the HIV virion particle (Gottlinger, 2001). Although these Gag regions are vital for the fitness of HIV, they are also rich in CTL epitopes, and are hence targeted by the immune system.

The viral enzymes, protease (p10), reverse transcriptase (p66/p55) and integrase (p32), which are encoded by Pol, are essential for transcription of viral proteins into DNA, integration of viral DNA into cell genome and cleavage of HIV-1 proteins, respectively (Sherman and Greene, 2002, Kaplan *et al.*, 1994, Miller and Bushman, 1995). The viral envelope (Env) is composed of a lipid bilayer, formed from the host cellular membrane when a newly formed virus particle buds from the cell (Wyatt and Sodroski, 1998). The envelope consists of two non-covalently linked glycoproteins (gp):gp120, the outer envelope protein and gp41, transmembrane protein that anchors the structure into the viral envelope (Chan *et al.*, 1997). This glycoprotein complex enables the virus to attach and fuse to the CD4 and secondary receptors of the target cells to initiate the infectious cycle (Stevenson, 2003).

Regulatory proteins, Tat and Rev also play a critical role in the viral life cycle. The Tat protein is a transcriptional transactivator, which up-regulates viral transcription through the interaction with the TAR element (Tat-responsive element) and while Rev facilitates the transport of singly-spliced and unspliced viral mRNAs from the nucleus into the cytoplasm (Pollard and Malim, 1998). The accessory proteins, Vif, Vpr, Vpu, and Nef are involved in mediating viral infectivity, targeting the nuclear localization of pre-integration complexes, enhancing virion release from the host cell and downregulation of immune surveillance molecules (CD4, MHC class I and II molecules) on the host cell surface, respectively (Ott, 2002). The mature virions are spherical in morphology and are 100-120nm in diameter (Hirsch and Curran, 1990) (Figure 1.3B). The outermost host-derived lipid-bilayer is anchored by 72 trimeric knobs of the gp120, which are connected to the inner virion matrix via the gp41 transmembrane protein (Berger, 1998, Gelderblom, 1991). The matrix lines the inner surface of the viral envelope surrounding

the inner nucleocapsid complex, which houses the viral genome consisting of two copies of single stranded RNAs, and other viral proteins, such as reverse transcriptase, integrase and accessory proteins.

A



B

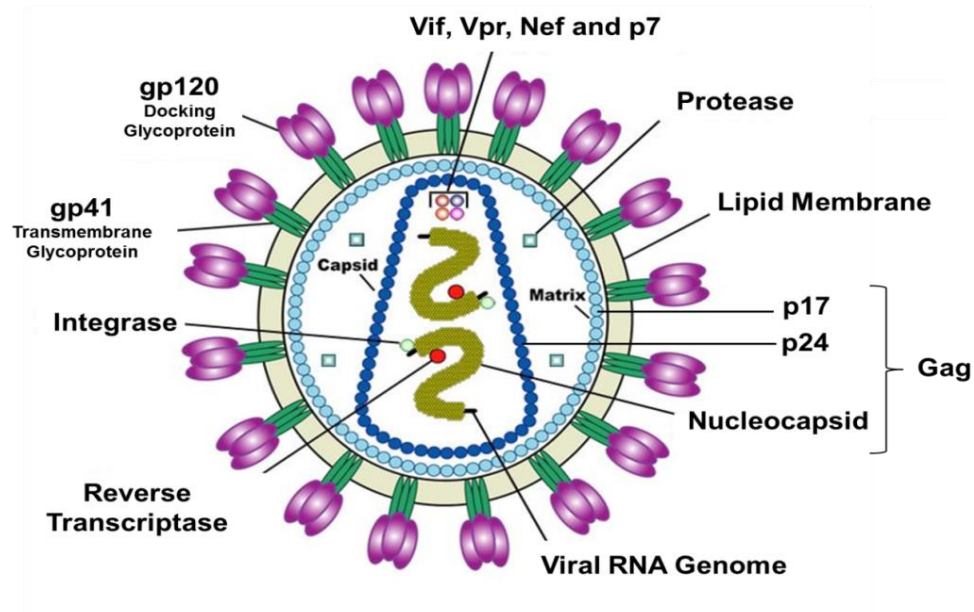


Figure 1.3: Schematic illustration of the HIV-1 structure. (A) The genomic organization of HIV-1. The *gag* gene encodes for structural proteins, p17 (matrix), p24 (capsid), p7 (nucleocapsid) and p6, while the *pol* gene encodes for enzymes, p10 (protease; PR), p66/p55 (reverse transcriptase; RT), and the p32 (integrase; IN). The *env* gene encodes for the envelope glycoproteins (gp120 and gp41). The *tat* and the *rev* genes encode for regulatory proteins, while *vif*, *vpr*, *vpu* and *nef* genes encode for accessory proteins. The schematic representation of HIV genes was adapted and modified from MWCHO (MWCHO, 2005). (B) The schematic overview of the HIV-1 virion.

1.2.3 HIV life cycle

HIV infection of a cell is mediated through the interactions between the viral surface gp120 and the CD4 receptor on the host cell membrane (Chan and Kim, 1998) (Figure 1.4). When these glycoproteins bind to the CD4 receptor on the T cell surface, the gp120 undergoes conformational changes, leading to the exposure of gp120-buried domains that interact with the β -chemokine receptors (CCR5 or CXCR4) and HIV co-receptors (Clapham and McKnight, 2002). The CXCR4 receptors allows infection with T-tropic (syncytium-inducing) HIV virions and is usually expressed on many T cells, but usually on macrophages and does not allow fusion by macrophage-tropic (M-tropic) which is non-syncytium (Deng *et al.*, 1996). The CCR5 co-receptor, which is mainly expressed on macrophages and some populations of T cells, allows M-tropic HIV membrane fusion (Deng *et al.*, 1996, Dragic *et al.*, 1996). HIV strains that use CCR5 as a co-receptor for entry are termed R5 and those that use CXCR4 only are termed X4 (Berger, 1998). Some viral strains however, are able to use both co-receptors alternatively and are reported as dual-tropic or R5X4. HIV has been shown to infect immune cells such as developing thymocytes (Brooks *et al.*, 2001, Keir *et al.*, 2002), dendritic cells (de Silva *et al.*, 2012), macrophages (Peters *et al.*, 2006), microglial cells and any cells that expresses both CD4 and CCR5 receptors (activated, resting memory, effector memory and terminally differentiated CD4⁺ T cells) (Brenchley *et al.*, 2004a).

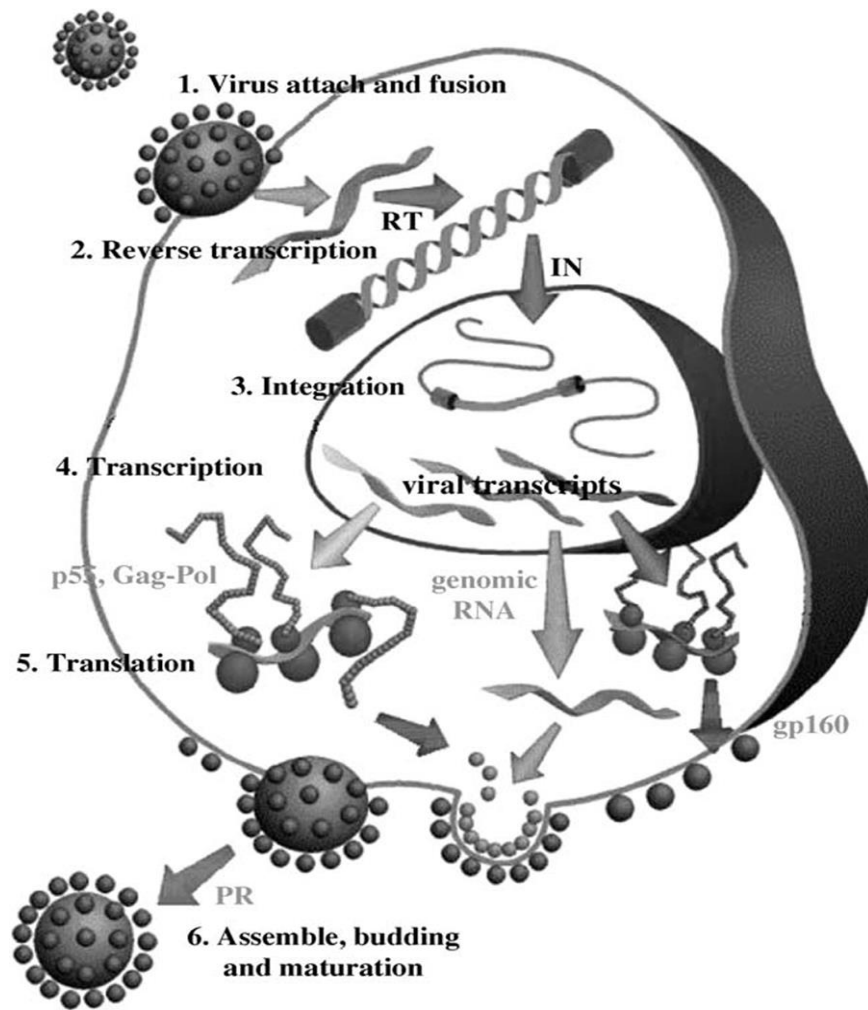


Figure 1.4: The HIV replicative life cycle. The main steps of HIV replication are number from 1 to 6. (1.) The binding of virus with CD4 and co-receptors results in fusion of the viral envelope and cellular membrane and release of viral nucleocapsid into the host cell cytoplasm. (2.) This is followed by reverse transcription of the viral RNA by the enzyme reverse transcriptase. (3.) The resulting complementary proviral DNA migrates into the cell nucleus and integrated into the cell genome by the integrase. The proviral DNA is then transcribed by the cellular DNA polymerase II. (5.) This is followed by translation of the mRNAs by cellular polysomes. (6.) The viral proteins and genomic RNA are then transported to the cellular membrane and assembly. The immature virions are release and polyproteins processed the viral protease to produce mature viral particles. Figure adapted from Sierra et al. (2005).

After fusion between the virus and the chemokine receptors, the nucleocapsid is integrated into the cell cytoplasm, where the RNA genome is transcribed into DNA by reverse transcriptase (Sherman and Greene, 2002). The process of reverse transcription is highly error-prone, which is more likely due to the lack of proof reading capacity of the reverse transcriptase enzyme (Roberts *et al.*, 1988). As a consequence, the virus is highly mutagenic, allowing it to evade immune responses and also develop resistance to the antiretroviral agents (Preston *et al.*, 1988, Coffin, 1995). Following complementary DNA synthesis, viral DNA migrates to the cell nucleus in a form of a pre-integration complex and integrates into the host cell genome using viral integrase (Miller and Bushman, 1995). Following integration, proviral DNA is transcribed by cellular RNA polymerase II and the resulting messenger RNA (mRNA) is then spliced into smaller pieces that are transported into the cytoplasm. Within the host cytoplasm, RNA is translated into regulatory proteins, Tat and Rev. Accumulation of Rev in the nucleus allows the transportation of unspliced mRNA from the nucleus into the cytoplasm. Structural proteins, Gag and Env are then produced from the full-length mRNA. These proteins result in the production of viral proteins, which are then transported to the cellular membrane for assembly. At the host surface membrane, the viral core proteins assemble beneath the membrane acquiring the modified host plasma membrane during the budding process (Nguyen and Hildreth, 2000). The viral Gag and Gag-Pol polyproteins are then cleaved by the protease shortly after budding, and generation of mature virions (Kaplan *et al.*, 1994).

During the HIV replication cycle, translocated proteins are cleaved by proteosomal enzymes and are presented on the surface of infected cells by MHC molecules (Schwartz *et al.*, 1998). It has been suggested that the effectiveness of CTL responses against HIV may be related to the

kinetics and timing of HIV protein expression during the viral replication cycle (van Baalen *et al.*, 2002, Ali *et al.*, 2004). Thus, early recognition of infected cells could result in rapid elimination of infected target cells before production of infectious viral particles, thereby resulting in control of viral dissemination (van Baalen *et al.*, 2002). Studies have also suggested that CTLs recognizing epitopes derived from early expressed proteins Nef, Tat and Rev might be more effective than CTL directed against epitopes located in Gag, Pol or Env proteins, which are expressed later during the HIV life cycle (Van Baalen *et al.*, 1998, Gruters *et al.*, 2002). In support of this notion, the targeting of Tat and Rev proteins by CTL have been associated with control of HIV replication (van Baalen *et al.*, 1997, Addo *et al.*, 2001, van Baalen *et al.*, 2002). In contrast, studies conducted by van Baalen (2002) showed no associations between Nef and control of viraemia, which may be related to Nef-mediated down-regulation of MHC class I molecules resulting in reduced ability of CTLs to recognize and eliminate infected cells (Tomiya *et al.*, 2002a, Ali *et al.*, 2004, Adnan *et al.*, 2006). Nonetheless, Nef has been shown to be highly immunodominant during the early stages of HIV infection (Gray *et al.*, 2009), and this may in part be explained by its early expression during the HIV replication cycle.

1.3 Adaptive Immunity and HIV infection

1.3.1 HIV transmission

Infection with HIV occurs through several routes such as intravenous drug use (Des Jarlais and Friedman, 1987, Des Jarlais *et al.*, 1987, Crofts and Hay, 1991, Peng *et al.*, 2011) or MTCT, either in utero, at birth or via breastfeeding (Newell *et al.*, 1997, Tess *et al.*, 1998, Dunn *et al.*, 1998). Most new HIV infections globally (80%) occur via sexual interaction through the genital tract or rectal mucosa (UNAIDS, 2010). Although the mechanisms or pathways by which HIV

transverses the genital mucosal epithelial cells is less clear, studies suggest that the virus crosses through the mucosal barrier by transcytosis (Bomsel *et al.*, 1998) or by making direct contact with the dendrites of the intraepithelial DC, which then transmit virus to adjacent cells in the mucosal stroma and the local lymphatics (Geijtenbeek *et al.*, 2000, Gurney *et al.*, 2005). In addition, other studies have shown that HIV could also directly infect intraepithelial vaginal Langerhans cells and CD4⁺ T cells through endocytosis and CCR5-mediated direct fusion, respectively (Hladik *et al.*, 2007). While HIV/SIV transmission occurs mainly at vaginal mucosal surfaces, recent studies using single genome amplification and ultra-deep sequencing suggest that 60-80% of mucosal infections originate from a single viral variant transmission (Haaland *et al.*, 2009, Stone *et al.*, 2010, Shen *et al.*, 2012). Although, the reasons for single variant selection across the cervical/vaginal mucosa are currently unclear, factors such as selective immune pressure on the transmitted/founder virus at the mucosal site, competitive selection of viral variants at the regional draining lymph nodes after crossing the cervical mucosa and transmission of single or limited variants from the mixture of quasispecies, may be responsible for this phenomenon (Shen *et al.*, 2012). The less frequent multivariant transmissions could in turn increase viral genetic diversity, thereby providing the virus with greater opportunity to escape acute/early immune selective pressures (Abrahams *et al.*, 2009).

In the 2-3 week incubation period following infection, the virus becomes well established in the lymphatic tissues (Haase, 1999). This period allows a burst of HIV replication and an increase in plasma viraemia, which reaches peak levels between 21-28 days after infection (Ribeiro *et al.*, 2010) (Figure 1.5). Enhanced viral replication during acute infection is often associated with a rapid decline of peripheral (Clark *et al.*, 1991, Daar *et al.*, 1991, Pantaleo *et al.*, 1993) and

gastrointestinal CD4⁺ T cells (Mehandru *et al.*, 2007, Brenchley and Douek, 2008). The decline in CD4⁺ T cells in acute infection can be attributed to both HIV-mediated killing and re-trafficking of cells to the lymphoid tissues and other organs (Fauci, 1993). The initial peak viraemia during acute or primary HIV infection has also been associated with emergence of clinical symptoms ranging from fever, fatigue, sore throat, headache, lymphadenopathy and rash (Tindall and Cooper, 1991, Schacker *et al.*, 1996, Hecht *et al.*, 2002, Kelley *et al.*, 2007). Following peak viraemia, the onset of HIV-specific T cell responses associates with the decline of plasma viraemia (Borrow *et al.*, 1994, Koup *et al.*, 1994), which reaches a steady-state or set-point, where the level of plasma viraemia could remain stable for months to years (Schacker *et al.*, 1998, Lindback *et al.*, 2000). Although, viral set-point varies significantly among individuals, a number of studies have shown viral set-point to be predictive of disease progression, where a high set-point is associated with a more rapid loss of CD4⁺ T cells and progression to AIDS (Pantaleo *et al.*, 1997, Rodriguez *et al.*, 2006, Mellors *et al.*, 2007). A steady-state of viraemia is also associated with the achievement of a period of HIV clinical “latency”, which is characterized by persistent viral replication and gradual loss of CD4⁺ T-cells, loss of the immune functions, susceptibility to opportunistic infections and eventual progression to AIDS (Pantaleo *et al.*, 1993, Ho *et al.*, 1995).

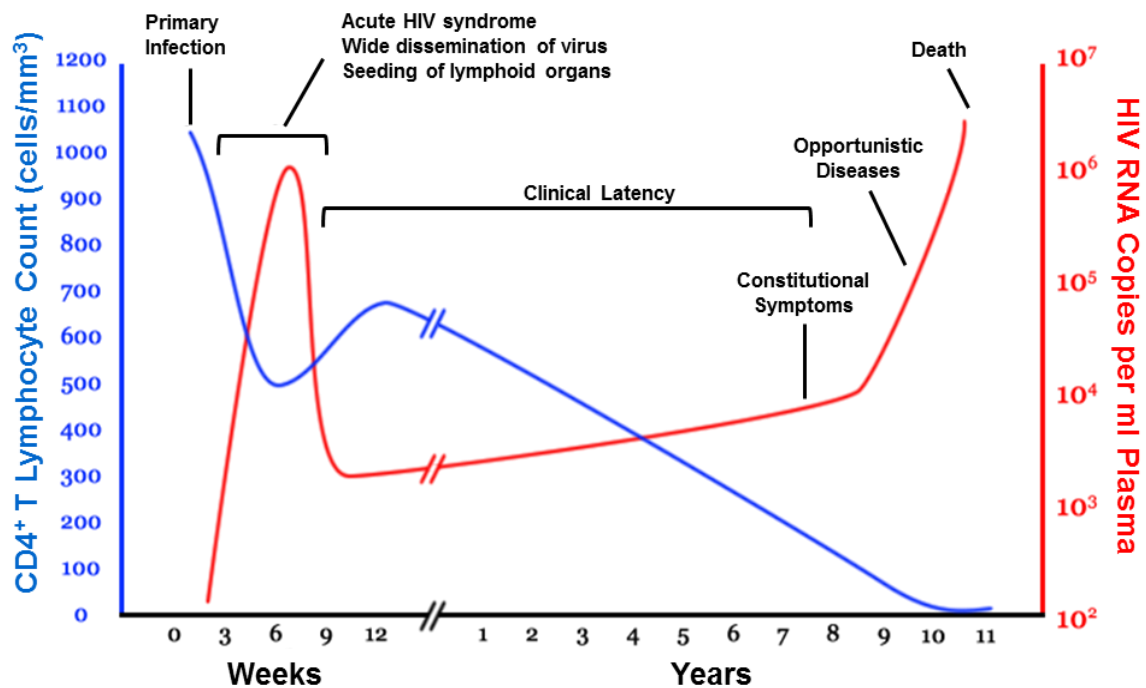


Figure 1.5: The dynamics of CD4⁺ T cell count and HIV viraemia over the average course of untreated HIV infection. During acute infection the virus disseminates and continues to replicate exponentially, peaking at 2-6 weeks after infection. This stage is often associated with rapid loss of peripheral and gastrointestinal CD4⁺ T cells. At peak viraemia the majority of individuals develop clinical symptoms (Flu-like symptoms) and the reservoirs of the latent virus are established in cells, where the virus can persist for a longer time in non-dividing resting lymphocytes. After peak viraemia, level of viraemia begins to decrease, stabilizes and reaches a steady level known as viral set-point. At this stage a period of clinical latency is also established, whereby CD4⁺ T cell count continue to decline, until they fall to critical levels below which there is substantial risk of infection with opportunistic pathogens. Figure adapted and modified from Fauci et al. (1996).

1.3.2 T cell responses

1.3.2.1 CD8⁺ T cells

Virus-specific T cell-mediated immune responses are believed to be crucial in the control of a number of viral infections such as CMV, EBV and HSV (Pantaleo and Koup, 2004). Therefore ways to induce potentially “protective” CD8⁺ T cell responses in vaccine development strategies has been a good goal for HIV vaccine research. Although infection by HIV and/or SIV is persistent and progressive in the vast majority of untreated individuals, HIV/SIV-specific CD8⁺ T cell may constitute a critical component of controlling viral replication because their emergence is paralleled with the decline of acute-phase viraemia in HIV/SIV infection (Borrow *et al.*, 1994, Koup *et al.*, 1994, Yang *et al.*, 1997, Ogg *et al.*, 1999, Yang and Walker, 1997, Kiepiela *et al.*, 2007). Cytotoxic CD8⁺ T cell responses against HIV have also been observed in individuals that are able to control HIV viraemia (Musey *et al.*, 1997), and specific HLA alleles and breadth of HIV-Gag specific T cell responses have been associated with the control of HIV replication (Kiepiela *et al.*, 2004, Kiepiela *et al.*, 2007). The pivotal role of virus-specific CD8⁺ T cells in the control of viral replication has further been demonstrated in the SIV model, where the depletion of CD8⁺ T cells is associated with enhanced viral replication (Schmitz *et al.*, 1999, Jin *et al.*, 1999). Furthermore, cytotoxic CD8⁺ T cell lymphocytes have also been shown to exert pressure on the viral genome, resulting in escape mutations (Price *et al.*, 1997, Altfeld *et al.*, 2001, Jones *et al.*, 2004). Pressure exerted by HIV-specific CD8⁺ T cell responses can result in escape mutations that may be detrimental to viral fitness and subsequent reduction in viral replicative capacity (Altfeld and Allen, 2006). However, mutations in epitopes have been found to negate binding of the peptide to the MHC class I molecule, thereby altering the recognition by the TCR (Chen *et al.*, 2000). Thus, generation of escape mutations could also result in the

selection of viral variants than can evade recognition by the immune system leading to loss of viral control (Goulder and Watkins, 2004). It is therefore imperative to understand the protective correlates of immunity versus the deleterious consequences of generating immune responses that could potentially benefit viral persistence and damage the host.

Multiple studies have shown that immunodominant HIV-specific T cells responses toward certain HIV-1 proteins (i.e. Gag proteins) are associated with control of HIV replication (Pontesilli *et al.*, 1998, Edwards *et al.*, 2002, Novitsky *et al.*, 2003, Masemola *et al.*, 2004, Ramduth *et al.*, 2005, Zuniga *et al.*, 2006, Kiepiela *et al.*, 2007, Geldmacher *et al.*, 2007a). Moreover, Gag-specific CD8⁺ T cell responses have been associated with control of viral replication in HIV controllers (Saez-Cirion *et al.*, 2009). Thus, targeting Gag epitopes such as TW10 (restricted by HLA-B*57/B58*01) and KK10 (restricted by HLA-B*27:05) have been previously correlated with slower disease progression (Altfeld *et al.*, 2003, Altfeld *et al.*, 2006, Miura *et al.*, 2009, Dinges *et al.*, 2010). In contrast, broadly directed CD8⁺ T cell responses against the Nef and Env proteins have had no effect on viral replication or have mostly been associated with elevated viraemia (Betts *et al.*, 2001, Geldmacher *et al.*, 2007a, Kiepiela *et al.*, 2007). Furthermore, Gag-specific CD8⁺ T cells have also been shown to associate with *in vivo* viral control, relative to Env-specific CD8⁺ T cell responses (Chen *et al.*, 2009). Overall these observations suggest that CD8⁺ T cells recognizing different HIV proteins may vary in their respective antiviral efficiencies. Although the mechanisms underlying protective immunity between different HIV antigens are not fully defined, the enhanced antiviral pressure elicited by Gag-specific CD8⁺ T cell responses compared to other virus-specific CD8⁺ T cells, could likely be related to the selection of viral escape mutations within the highly conserved Gag regions, and

thereby resulting in the impairment of viral fitness and subsequent outgrowth of viral quasispecies with reduced replicative capacity (Martinez-Picado *et al.*, 2006, Crawford *et al.*, 2007, Goepfert *et al.*, 2008, Brumme *et al.*, 2008).

The qualitative attributes of CD8⁺ T cell responses have recently become the focus of attempts in order to identify correlates of immune protection against HIV. Key indicators of the antiviral efficiency of CD8⁺ T cells may be related to the ability to mediate antiviral effector cytokines such as IFN γ , which suppresses HIV replication (Pitha, 1994, Bailer *et al.*, 1999) and chemokines such as MIP-1 β , which are capable of inhibiting infection of CD4⁺ T cells by R5 HIV-1 strains (Cocchi *et al.*, 1995). Proliferation of HIV-specific CD8⁺ T cell responses has also been suggested to be an important correlate of immune protection against HIV (Almeida *et al.*, 2007, Day *et al.*, 2007). Moreover, the secretion of IL-2 or the simultaneous expression of IFN γ and IL-2 from either CD4⁺ or CD8⁺ T cells have been associated with the support of CD8⁺ T cell proliferative capacity (Boaz *et al.*, 2002, Lichterfeld *et al.*, 2004a, Zimmerli *et al.*, 2005). As previously mentioned (Section 1.1.2.1, page 7), other attributes that may also be considered to be important in controlling HIV replication may be related to the destruction of HIV infected cells through the release of cytolytic enzymes such as perforin and/or granzymes by CTLs (Migueles *et al.*, 2002, Saez-Cirion *et al.*, 2007, Hersperger *et al.*, 2010). On the other hand, the antiviral efficiency of CD8⁺ T cells for successfully clearing an antigen may most likely depend on the support of helper CD4⁺ T cells (Kalams and Walker, 1998, Musey *et al.*, 1999, Sun *et al.*, 2004, Kumaraguru *et al.*, 2005). Therefore, understanding the role of helper CD4⁺ T cell responses during HIV infection may be essential in efforts to modulate effective antiviral CD8⁺ T cell responses by future vaccines.

1.3.2.2 CD4⁺ T cells

Although CD4⁺ helper T cells play an essential role in mediating immunity against myriad pathogens (Section 1.1.2.1, page 6), CD4⁺ T cells are also major targets for HIV infection and are progressively depleted during the course of HIV infection (Fauci, 1988, Fauci, 1993, Schacker *et al.*, 2001, Mattapallil *et al.*, 2005). However, there is increasing evidence to suggest that the preservation of virus-specific CD4⁺ T cell responses could play an important role in the control of HIV replication (Rosenberg *et al.*, 1997, Kalams and Walker, 1998, Boaz *et al.*, 2002, Boritz *et al.*, 2004). The importance of effective CD4⁺ T cell responses and long term control of persistent infections has been highlighted in murine (Battegay *et al.*, 1994) and human (Gerlach *et al.*, 1999, Gamadia *et al.*, 2003) viral infections. In HIV infection, individuals with long-term non-progressive HIV infection have been shown to exhibit increased frequencies of HIV-specific CD4⁺ proliferative responses than in individuals with progressive infection (Rosenberg *et al.*, 1997, Dyer *et al.*, 2008, Porichis and Kaufmann, 2011). Moreover, individuals infected with HIV-2, which is characterized by a less aggressive disease course, have also been shown to mediate strong polyfunctional HIV-specific CD4⁺ T cell responses (Duvall *et al.*, 2006). However, the mechanisms through which HIV-specific CD4⁺ T cells exert their antiviral effects are not well understood.

CD4⁺ T cells may contribute to the control of HIV replication through a variety of mechanisms that include; 1) help for maintenance of HIV-specific CD8⁺ T cell responses (Kalams and Walker, 1998, Altfeld and Rosenberg, 2000) and memory CD8⁺ T cell generation (Shedlock and Shen, 2003, Janssen *et al.*, 2003); 2) produce cytokines that facilitate the induction of neutralizing antibodies by B cells, and formation of memory B cells (McHeyzer-Williams and

Ahmed, 1999); and 3) through the production of effector cytokines (Boaz *et al.*, 2002) and cytotoxic molecules (Appay *et al.*, 2002c, Zaunders *et al.*, 2004, Soghoian *et al.*, 2012). Preferential targeting of immunodominant Gag epitopes by HIV-specific CD4⁺ T cells has previously associated with low viraemia and higher CD4⁺ T cell numbers (Ramduth *et al.*, 2009). Furthermore, the ability to produce IL-2 and/or co-express both IL-2 and IFN γ has also been associated with immunologic memory and protective immunity from HIV disease progression (Sousa *et al.*, 2001, Boaz *et al.*, 2002, Palmer *et al.*, 2004). The early differentiated state of HIV-specific CD4⁺ T cells has also been associated with improved control of HIV replication (Younes *et al.*, 2003). Studies conducted by Younes *et al.* (2003) have previously demonstrated that viraemic individuals are characterized mainly by HIV-specific CD4⁺ T cells with an effector memory phenotype, whereas aviraemic individuals exhibit HIV-specific CD4⁺ T cells with both central memory (which mainly produce IL-2) and effector memory phenotypes. The diminished IL-2-producing capacity of HIV-specific CD4⁺ T cells, may probably limit the ability to mediate T cell helper functions, and may in particular affect the anti-HIV CD8⁺ T cell responses. Therefore, the skewing of HIV-specific CD4⁺ T cells toward a memory phenotype with limited proliferative capacity and reduced T cell helper function may be a mechanism associated with HIV pathogenesis.

While the induction of HIV-specific CD4⁺ T cell could potentially play a major role in the control of HIV via direct antiviral effects or maintenance of anti-HIV CD8⁺ T cell-mediated responses, there is growing uncertainty concerning the role of HIV-specific CD4⁺ T cells in the control versus the promotion of HIV replication. Thus, there is evidence suggesting that infected activated CD4⁺ T cells amplify HIV replication that propagates infection, by producing more

virions that infect neighboring cells (Zhang *et al.*, 2004). Moreover, it has also been demonstrated that HIV preferentially infects HIV-specific CD4⁺ T cells (Douek *et al.*, 2002). In addition, the high frequencies of activated and HIV-specific CD4⁺ T cells in HIV controllers have been associated with continuous replenishment of a reservoir of latently infected cells, thereby preventing the complete eradication of HIV (Hunt *et al.*, 2011a). Together, these observations suggest that both activated and HIV-specific CD4⁺ T cells may also play a major role in HIV persistence, and may further support concerns that induction of a large population of HIV-specific CD4⁺ T cells that may enhance rather than control HIV infection. As it is not clear on the kind of HIV-specific CD4⁺ T cells required for a protective immune response, further studies are therefore required to fully understand the possible protective roles of HIV-specific CD4⁺ T cell responses during HIV infection.

1.3.2.3 T cell Polyfunction

In efforts to better understand correlates of immune protection, recent studies have focused on cohorts of elite HIV controllers (Saksena *et al.*, 2007, Deeks and Walker, 2007) and/or LTNP. These are asymptomatic HIV infected individuals who are able to maintain low to undetectable levels of viraemia and maintain normal CD4⁺ T cell counts, without ARV treatment, for years after infection (Pantaleo *et al.*, 1995, Cao *et al.*, 1995). HIV controllers are known to be enriched with protective HLA alleles such as HLA-B*57:01, HLA-B*57:02, HLA-B*58:01 or HLA-B*27:05 molecules (Kaslow *et al.*, 1996, Migueles *et al.*, 2000, Gao *et al.*, 2005, Miura *et al.*, 2009). These individuals can maintain high frequencies of CD4⁺ T cells that are able to express IL-2 and proliferate in response HIV (Rosenberg *et al.*, 1997, Emu *et al.*, 2005). Additionally, high frequencies of HIV-specific CD8⁺ T cells have also been observed in LTNPs

(Cao *et al.*, 1995, Harrer *et al.*, 1996a, Harrer *et al.*, 1996b, Hay *et al.*, 1999). However, the increased numbers of HIV-specific CD8⁺ T cells in LTNP, does not fully explain the reduced HIV disease given that these responses are also detected in normal progressors (Gea-Banacloche *et al.*, 2000, Lopez *et al.*, 2008). Studies have also shown that the efficiency of cytotoxic HIV-specific CD8⁺ T cell responses in killing autologous HIV infected CD4⁺ T cells is much greater in LTNP than in progressors (Migueles *et al.*, 2008). This may suggest that the measurements of the quality rather than the quantity of responses are essential in ascertaining the correlates of a protective immune response against HIV (Seder *et al.*, 2008).

Recent advances in the field of viral and HIV immunology has allowed the measurement of cells that are capable of mediating multiple effector functions simultaneously, using multicolour flow cytometry (Betts *et al.*, 2006). Studies conducted by Betts *et al.* (2006) demonstrated that HIV-specific CD8⁺ T cells that concurrently express IFN γ , IL-2, MIP-1 β , TNF α and the degranulation marker CD107a, associate with long-term nonprogressive HIV infection. Indeed, some of these functions may have direct antiviral effects (i.e. degranulation, MIP-1 β and TNF α), while others (IL-2) might promote viral replication (Geldmacher *et al.*, 2010). Nonetheless, the protective role of polyfunctional cells has been demonstrated in other infections such as HCV (Ciuffreda *et al.*, 2008), *Leishmania major* in mouse models (Darrah *et al.*, 2007) and *Mycobacterium tuberculosis* (Forbes *et al.*, 2008). Furthermore, polyfunctional CD8⁺ T cell responses have also been detected in the highly efficacious smallpox virus vaccine (Precopio *et al.*, 2007) further suggesting that the protective effect of polyfunctional T cells may well extend beyond HIV. Conversely, the increased frequencies of monofunctional virus-specific CD4⁺ and/or CD8⁺ T cell responses

characterized by the production of IFN γ have been previously associated with progressive HIV infection (Harari *et al.*, 2004a, Kannanganat *et al.*, 2007b).

Although polyfunctional cells have been associated with viral control, other studies have suggested that the functional profile of T cells is largely a consequence of antigen load and the duration of antigen exposure, rather than a mediator of viral control (Streeck *et al.*, 2008, Rehr *et al.*, 2008). However, studies conducted using murine models showed that vaccinated mice generated more polyfunctional CD4⁺ T cell responses when exposed to *Leishmania major* and subsequent reduced disease burden (Darrah *et al.*, 2007). In addition, recent studies have shown that polyfunctional HIV-specific CD8⁺ T cell responses have a greater capacity to suppress viral replication *in vitro* than monofunctional T cell responses (Julg *et al.*, 2010). Consistent with these studies, Akinsiku *et al.* (2011) demonstrated that polyfunctional IL-2⁺ HIV-specific CD8⁺ T cell lines from HIV controllers exhibit enhanced viral suppression *in vitro*. These observations may highlight the importance of understanding the role of polyfunctional T cell responses in the rational development of an effective T cell-based vaccine.

1.3.3 T cell Memory and HIV infection

The skewing of memory T cells towards late or highly differentiated phenotype has been directly associated with increased HIV viral load (Palmer *et al.*, 2004, Papagno *et al.*, 2004), whilst other studies have demonstrated that fully or highly differentiated memory T cells may be more beneficial with regard to the control of viraemia (van Baarle *et al.*, 2002, Addo *et al.*, 2007, Barbour *et al.*, 2009). These reports reflect uncertainty regarding the ideal phenotypic profile of a protective immune response against HIV. Different conclusions regarding the role of central and

effector memory phenotypes in protection against HIV may in part be explained by heterogeneous pool of memory cells that are functionally diverse, and different study designs. Nevertheless, improved understanding of functional memory T cell development and identity of unique phenotypic markers of memory T cells that associate with HIV control may be crucial toward expanding current vaccine strategies to enhance antiviral memory (Ahlers and Belyakov, 2010).

While many infections are cleared by an acute immune response, infection with persistent (HIV, EBV or CMV) pathogen has been associated with altered differentiation memory cells (Radziejewicz *et al.*, 2007, Shin and Wherry, 2007, Desai and Landay, 2010). For instance, antigen persistence has been associated with the reduced ability of CTL to eliminate virally infected target cells (Zajac *et al.*, 1998), where constant antigen exposure leads to exhaustion of memory T cell function and increased depletion of memory cells (Wherry *et al.*, 2003). Similarly, HIV infection is characterized by rapid accumulation of highly differentiated or senescent CD8⁺ T cells (CD27⁻CD28⁻) (Appay and Rowland-Jones, 2002, Papagno *et al.*, 2004, Appay *et al.*, 2007, Cao *et al.*, 2009), that have limited lifespan, reduced proliferative capacity (Appay *et al.*, 2007) and are highly prone to apoptosis. Furthermore, persistent HIV infection appears to drive HIV-specific CD4⁺ T cells toward an effector memory phenotype characterized by diminished proliferative capacity and reduced IL-2 production (Palmer *et al.*, 2004). Although HIV persistence appears to play role on the impairment of functional memory profiles of both CD4⁺ and CD8⁺ T cells, precise pathways contributing to altered memory differentiation during HIV infection remains poorly understood. Thus, it is unclear whether properties of memory T cell subpopulations are dynamic or remain stable over time. Therefore, further examination into

mechanisms governing T cell differentiation during HIV infection may further aid in development of HIV control strategies.

1.3.4 Immune Activation and HIV infection

Activation of the immune system is an intrinsic and natural process of an immune response to foreign pathogens. In general, after the immune response has adequately cleared foreign antigen, the immune system returns to a state of relative quiescence until the next stimulus is introduced. However, this is not the case with HIV infection. HIV infection results in an enhanced state of T cell (Hazenbergh *et al.*, 2003) and B cell activation (Moir and Fauci, 2009), T cell turnover (Hellerstein *et al.*, 1999) and increased serum levels of proinflammatory cytokines and chemokines (Valdez and Lederman, 1997). In untreated chronic HIV infection, the enhanced state of immune activation has been shown to be an independent predictor of disease progression as defined by the progressive loss CD4⁺ T cell lymphocytes and development of AIDS (Sousa *et al.*, 2002, Deeks *et al.*, 2004, Vajpayee *et al.*, 2009).

T cell hyperactivation leads to rapid and high turnover of T cells that are destined to proliferate and undergo increased rates of apoptosis (McCune *et al.*, 2000). Moreover, given the limited regenerative ability of T cells in HIV infection (Hazenbergh *et al.*, 2000a, Grossman *et al.*, 2002), the continuous recruitment of cells to a pool of rapidly and dying T cells, could further contribute to the gradual loss of CD4⁺ T cell numbers (Grossman *et al.*, 2006). Other mechanisms that link immune activation and HIV pathogenesis may involve the continuous supply of susceptible target cells to HIV and/or SIV infection (Zhang *et al.*, 2004, Grossman *et al.*, 2006), and the loss

of HIV-specific host defenses as a result of anergy and replicative senescence (Papagno *et al.*, 2004).

There have been a number of markers (CD38, HLA-DR and CD69) that have been used to measure T cell activation in HIV infection. Of these, the best characterized marker of immune activation is the expression of CD38. The multifunctional transmembrane glycoprotein, CD38 is up-regulated on neonatal T cells, mature thymocytes and activated T cells (Savarino *et al.*, 2000), and has been shown to be a stronger predictor of HIV disease progression (Bofill *et al.*, 1996, Liu *et al.*, 1997, Mocroft *et al.*, 1997, Giorgi *et al.*, 2002, Deeks *et al.*, 2004). While CD38 has been shown to be involved in number of functions that include enzymatic activity, cell-to-cell adhesion and signaling (Savarino *et al.*, 2000), its expression on CD8⁺ T cells has been shown to increase with HIV disease progression (Giorgi *et al.*, 1993). Moreover, the measurement of the mean fluorescent intensity (MFI) or the number of CD38 molecules on CD8⁺ T cells has also been strongly associated with CD4⁺ T cell depletion (Roederer and Herzenberg, 1996) and AIDS (Liu *et al.*, 1997, Liu *et al.*, 1998). HLA-DR is the MHC class II antigen, which is constitutively expressed by antigen-presenting cells and is involved in presentation of peptide antigens to CD4⁺ T cells. Although not expressed on resting T cells, MHC class II is rapidly up-regulated upon activation and during an active T cell immune response. The surface glycoprotein CD69 is another marker used to measure T cell activation. Though its role is also unclear, it has been shown to be one of the earliest markers to be up-regulated upon acute T cell activation. In addition, markers of cellular proliferation (Ki67) can also be used as surrogates for T cell activation (since activation often results in cell proliferation). Ki67 is the nuclear antigen that is expressed exclusively by cells that are in the G1, S, G2 and M phases of cell division, but not

during the resting G0 phase (Gerdes *et al.*, 1984). Although Ki67 has an important advantage in that it provides a measure of T cell turnover, this marker does not reveal cells that are arrested in cell cycle (Sieg *et al.*, 2001). Nonetheless, the combination of Ki67 and labeling of dividing cells with 5-bromo-2-deoxyuridine (BrdU) confirms the use of Ki67 as a surrogate marker for T cell proliferation (Kovacs *et al.*, 2001).

The underlying causes and sources of enhanced immune activation upon HIV infection are not fully understood (Grossman *et al.*, 2006). However, increased immune activation in chronic HIV infection has been associated with immune responses to HIV itself (Cohen Stuart *et al.*, 2000, Deeks *et al.*, 2004), together with indirect mechanisms that could also be related to HIV replication. The attenuation of immune activation as result of ARV treatment (Giorgi *et al.*, 1998, Hazenberg *et al.*, 2000b, Barbour *et al.*, 2009) highlights the link between viral replication and immune activation. *In vitro* studies have also shown that HIV viral proteins such as the gp120 could directly induce cellular activation even in the absence of direct infection (Rieckmann *et al.*, 1991, Oravecz and Norcross, 1993). Furthermore, the accessory protein Nef has been shown to promote the elaboration of paracrine factors by infected macrophages and activating bystander T cells (Swingler *et al.*, 1999), and has also been associated with proliferation and activation of resting CD4⁺ T cells (St Gelais *et al.*, 2012). Infection with HIV could also result in the activation of the innate immune system, and studies have demonstrated that HIV-1 could directly induce the activation of DC (Fonteneau *et al.*, Beignon *et al.*, 2005, Boasso and Shearer, 2008), macrophages (Heil *et al.*, 2004) and NK cells (Alter *et al.*, 2007) via TLR-mediated pathways. Altogether, these studies show the direct role of HIV-driven immune activation, however other studies have demonstrated that the majority of activated cells are

neither HIV-specific nor HIV-infected (Douek *et al.*, 2002, Doisne *et al.*, 2004), suggesting the involvement of indirect and/or non-HIV antigen specific mechanisms in driving increased immune activation during HIV infection.

Previous studies have shown that HIV-infected individuals who are capable of controlling viraemia, still exhibit increased numbers of activated T cells (Hunt *et al.*, 2008), suggesting that viraemia is not a sole contributor for the increased immune activation during HIV-1 infection. Other causes of immune activation that may directly or indirectly be related to HIV-1 replication range from: 1) co-infection with pathogens other than HIV (CMV and HCV) (Lenkei and Andersson, 1995, Valdez *et al.*, 2000, Kovacs and Masur, 2008). In addition, co-infection with opportunistic infections such as parasites and mycobacteria have also been shown to exacerbate T cell activation (Eggena *et al.*, 2005b) during HIV infection; 2) depletion of regulatory T cells that are normally responsible for suppressing T cell activation during HIV infection (Eggena *et al.*, 2005a, Hunt *et al.*, 2011b); 3) translocation of microbial products, such as LPS that have breached the lumen of the intestine into the system circulation (Brenchley *et al.*, 2006b); and 4) homeostatic proliferation in response to progressive loss of CD4⁺ T cell lymphocytes during HIV infection (Catalfamo *et al.*, 2008).

1.3.4.1 Microbial Translocation and Immune Activation

Numerous studies have suggested that the massive loss of CD4⁺ T cells from the gut-associated lymphoid tissue in early HIV infection could result in the disruption of the mucosal barrier, allowing the translocation of bacterial products into the lymphatic and blood circulation (Figure 1.6) (Brenchley *et al.*, 2006a, Hofer and Speck, 2009, Gordon *et al.*, 2010, Mavigner *et al.*,

2012). These bacterial products can stimulate immune cells directly via PRRs such as the TLR (Medzhitov and Janeway, 2000). The TLRs are known to trigger immediate innate anti-pathogen responses, and also guide the development of adaptive immunity (Schnare *et al.*, 2001, Iwasaki and Medzhitov, 2004). Numerous cell types (such as lymphocytes, monocytes and dendritic cells) express various TLRs and respond broadly to TLR ligands (Hornung *et al.*, 2002). For example, the interactions between TLR ligands, such as LPS (a component of the cell wall of gram negative bacteria) and TLRs results in enhanced activation of monocytes (Miller *et al.*, 2005, Miyake, 2006, Gioannini and Weiss, 2007, Lester *et al.*, 2009b).

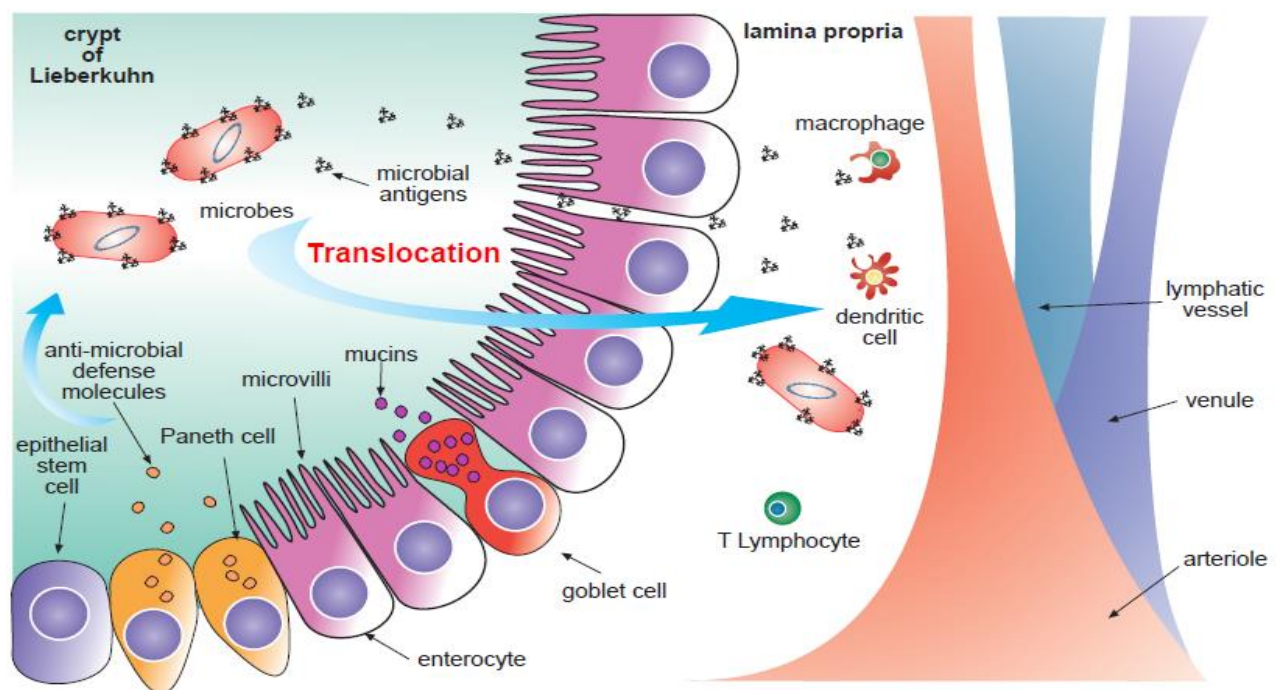


Figure 1.6: Schematic representation of the microbial translocation process. Depletion of CD4⁺ helper T cells in the lamina propria during to HIV infection results in damage/disruption of the gastrointestinal barrier leading to infiltration of bacteria and bacterial products from the gut lumen into the blood circulation. The translocation of microbes and microbial antigens into the periphery results in pro-inflammatory cytokine production further leading activation of immune cells. Figure adapted from www.immunopaedia.org.

The increased levels of plasma LPS and bacterial DNA, which are indicators of microbial translocation, have been mainly observed in the chronic stages of HIV infection (Ancuta *et al.*, 2008, Jiang *et al.*, 2009, Redd *et al.*, 2009). Microbial translocation in chronic HIV infection is also accompanied by an increase in plasma levels of sCD14, LBP (Brenchley *et al.*, 2006b) and the I-FABP (Pelsers *et al.*, 2003). Thus, various endotoxins such as LPS elicit the synthesis of LBP from the liver, which binds to LPS and triggers CD14-dependant monocyte activation (Wright *et al.*, 1990, Kitchens and Thompson, 2005). Increased plasma levels of LBP have been shown to have prognostic significance in patients with HIV-associated dementia (Ancuta *et al.*, 2008), sepsis (Opal *et al.*, 1999), and in HIV patients with or without HCV related-liver cirrhosis (de Oca Arjona *et al.*, 2011). Thus, determination of LBP serves as a proxy to LPS and is more reliable because of its extended half-life (Schumann and Latz, 2000, Redd *et al.*, 2009, de Oca Arjona *et al.*, 2011). Moreover, plasma LBP levels have been shown to reflect long-term exposure to circulating endotoxins more accurately than plasma LPS levels (Opal *et al.*, 1999) (Albillos *et al.*, 2003). Gastrointestinal permeability can also be assessed by measurement of I-FABP, a marker for enterocyte damage (Pelsers *et al.*, 2003). This cytosolic protein is expressed in epithelial cells of the small intestine, and is released into the circulation upon intestinal cell damage (Lieberman *et al.*, 1997), such as in patients with celiac disease (Derikx *et al.*, 2009) and abdominal injury (Relja *et al.*, 2010). Furthermore, increased plasma I-FABP levels have been demonstrated in HIV infected individuals, and also found to associate with lower baseline CD4⁺ T cell numbers (Sandler *et al.*, 2011).

There is a debate whether microbial translocation causes progression to AIDS or whether it is consequence of HIV itself (Redd *et al.*, 2009). The translocation of microbial products from the

gut may result in activated immunity which may enhance viral replication. Furthermore, microbial translocation has been implicated in number of pathological conditions such as AIDS dementia (Ancuta *et al.*, 2008), sepsis (Yu and Martin, 2000, Opal, 2007), arthritis (Scuderi *et al.*, 2003), inflammatory bowel disease (Caradonna *et al.*, 2000, Kamat *et al.*, 2010), liver cirrhosis (de Oca Arjona *et al.*, 2011) and idiopathic CD4 lymphocytopenia (Lee *et al.*, 2009), which may consequently lead to increased risk of mortality in HIV-1 infection (Sandler *et al.*, 2011). The association between microbial translocation and HIV disease progression in African populations remains contentious (Redd and Quinn, 2009, Lester *et al.*, 2009a, Cassol *et al.*, 2011). Therefore, further studies are needed to reveal the implications of microbial translocation in African HIV-infected populations.

1.3.4.2 TCR-independent Immune activation

Infection with HIV has also been associated with the increased expression of pro-inflammatory cytokines (Norris *et al.*, 2006, Barqasho *et al.*, 2009, Stacey *et al.*, 2009). In addition, increased microbial translocation during HIV infection has been shown to trigger inflammatory responses and subsequent production of pro-inflammatory cytokines such as IFN γ , IFN α , IL-1 β , IL-6 and TNF α (Kitchens and Thompson, 2005, Lu *et al.*, 2008). Although cytokines can induce or accelerate T cell anti-viral immune response (Goonetilleke *et al.*, 2009), the excessive production of cytokines could have negative effects by increasing the pool of activated cells through TCR-independent mechanisms (Tough *et al.*, 1996, Boasso *et al.*, 2008). Studies conducted by Tough *et al.* (1996), showed that IFN α could induce marked proliferation and activation of mouse memory CD8⁺ CD44⁺ T cells in the absence of antigenic stimulation. Exposure to type I interferon cytokines in the absence of antigen stimulation has also been shown to up-regulate T

cell activation markers CD38 and CD69 independent of antigen (Boasso *et al.*, 2008). In line with these reports, other cytokines such as IL-15 have been implicated in the activation and proliferation of bystander T cells (Lodolce *et al.*, 2001), while cytokines such as IL-6, IL-10, IL-12 and TNF α were shown to result in the proliferation and differentiation of naïve and memory CD4⁺ T cell subpopulations (Unutmaz *et al.*, 1994, Geginat *et al.*, 2001). Altogether, these studies provide additional mechanisms that could result in pronounced immune activation during HIV infection.

1.4 Rationale and Objectives of this Study

Understanding the nature of T cell immunity in natural HIV infection remains a valuable foundation for understanding what protective immune responses may need to be elicited with a vaccine. Examination of the interplay between host immune responses (quantity and/or quality) and the virus will provide important clues in determining correlates of protection against HIV. Furthermore, given that the initial emergence of HIV-specific T cells is closely associated with the decrease in plasma viraemia during acute infection (Borrow *et al.*, 1994, Koup *et al.*, 1994), it is likely that defining and understanding immune responses during acute and/or early infection may provide valuable information on the possible mechanisms of viral control.

Until recently, there has been little focus on unraveling the relationship between memory differentiation, activation and the functional profile of T cells with viral control during the early stages of HIV infection. Firstly, the differentiation profiles of total and HIV-specific T cells in early HIV infection are poorly understood. Moreover, it is also unclear as to the functional memory phenotype required for effective control of HIV. Secondly, the level of T cell activation

in early infection may also prove to be critical to HIV pathogenesis and therefore identifying the factors that associate with activation may be important for understating the mechanisms of immune activation during early stages HIV infection. Thirdly, the identity of early and functionally diverse (polyfunctional) HIV-specific T cells in total and various memory subpopulations may be an important signature for protective immunity.

Overall characterizing the function and activation status of T cells that associate with viral control or disease progression may shed light on potential immune correlates of protection. **It is therefore hypothesized that polyfunctional, early differentiated memory T cells, with low activation status associate with control of *in vivo* HIV-1 replication.** The basis of this study is to explore the quality of virus-specific memory CD4⁺ and CD8⁺ T cell by direct engagement with antigen and to select markers or immune profiles that associate with *in vivo* viral control.

Specific Aims and Objectives:

To test the hypothesis that polyfunctional, early differentiated memory T cells, with low activation status associate with control of *in vivo* HIV-1 replication, the following objectives are explored in this thesis.

Objective 1: To examine and compare the phenotype of total and antigen-specific (HIV and CMV) CD4⁺ and CD8⁺ T cells in early HIV-infected and HIV-uninfected controls. The T cell differentiation phenotypes will be identified based on the surface expression of CD27 and CD45RO. This objective is aimed at addressing the functional heterogeneity between total, HIV and CMV-specific memory T cell phenotypes during primary HIV infection (Chapter 3).

Objective 2a: To compare the activation level of total and antigen-specific (Gag and CMV) memory CD4⁺ and CD8⁺ T cells in HIV-infected and HIV-uninfected controls. The level of T cell activation will be measured by the surface expression of CD38 and HLA-DR, while dividing cells will be assessed through the expression of the nuclear antigen Ki67. The goal of this analysis is to examine the impact of HIV on memory T cell activation and proliferation during primary infection (Chapter 4).

Objective 2b: To assess the levels of proviral-*gag* DNA within activated/non-activated and antigen-specific memory CD4⁺ T-cells. The will address whether activated cells are susceptible to HIV-1 *in vivo* (Chapter 4).

Objective 2c: To investigate and understand the relationship between total and antigen-specific CD4⁺ and CD8⁺ T cell activation with memory differentiation during primary HIV-1 infection. This analysis aims to explore whether activation in early HIV infection drives the differentiation of memory T cells (Chapter 4).

Objective 3: To identify and compare the polyfunctional profile of HIV- and CMV-specific memory CD4⁺ and CD8⁺ T cell responses in HIV-1 infected individuals. The functional profile of antigen-specific T cells will be measured based on the simultaneous expression of five functions consisting of degranulation (CD107), IFN γ , IL2, MIP-1 β and TNF α . This analysis is aimed at exploring the polyfunctional nature of HIV-specific memory T cells during early stages of HIV infection (Chapter 5).

Objective 4: To identify the mechanisms of memory T cell activation by examining the levels and relationship between the levels of gut microbial by-products (LBP, sCD14, I-FABP and EndoCAb), inflammatory molecules (IL-1 β , IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1) in plasma with immune activation. This analysis is aimed at addressing whether immune activation during primary HIV-1 infection is associated with bacterial products translocated from the gut causing increased levels of systemic inflammatory cytokines (Chapter 6).

Objective 5: To examine and understand the relationship between total and HIV-specific memory CD4⁺ and CD8⁺ T cell differentiation (CD27, CD45RO), activation (CD38, HLA-DR), proliferation (Ki67) and function (CD107, IFN γ , IL2, MIP-1 β and TNF α) with HIV-1 disease progression over the first year of infection. This objective is aimed at exploring the functional memory phenotype and the activation status that associates with control of HIV viraemia in early infection. (Chapter 7).

CHAPTER 2: MATERIALS AND METHODS

TABLE OF CONTENTS

2.1 Study Participants	51
2.1.1 Primary HIV-infection Cohort.....	51
2.1.2 CAPT Cohort	51
2.1.3 Cohort Characteristics.....	52
2.1.4 Plasma Viral Load and Absolute CD4 ⁺ T cell Counts.....	54
2.2 Synthetic Peptides.....	54
2.3 Sample Preparation	54
2.4 Cell Stimulation and Intracellular Staining	55
2.4.1 Measurement of T cell Polyfunction	55
2.4.2 Measurement of T cell Memory and Activation Status.....	56
2.5 Measurement of Plasma EndoCAb, I-FABP, LBP and sCD14.....	56
2.6 Measurement of Plasma Cytokine Levels	57
2.7 Data Analysis.....	58
2.7.1 Flow Cytometry Analysis	58
2.7.1.1 Cell Acquisition and Gating.....	58
2.7.1.2 Boolean Gating Analysis	59
2.7.2 Cell Sorting and HIV Gag-DNA quantification	59
2.7.2.1 Gag-DNA quantification using real time PCR	60
2.7.3 Plasma markers of microbial translocation and cytokine analysis	61
2.8 Statistical Analysis.....	61

2.1 Study Participants

2.1.1 Primary HIV-infection Cohort

A cohort of persons with early HIV-1 infection were recruited over 18 months from individuals who were a part of an HIV-negative cohort and who seroconverted, with dates of seroconversion known to be within a 90 day window (Table 2.1). Upon enrollment, participants were followed up at four weekly intervals for the first 12 weeks and quarterly (i.e., at 12 week intervals) thereafter, for a total of 48 weeks and seven visits. At enrolment, a total of 100-110ml of blood was drawn and for subsequent visits approximately 50ml of blood was obtained. Thus, approximately 650ml of blood was drawn for individuals who completed all seven visits. The time of HIV-1 infection was estimated by prospective RNA-positive/Ab negative measurements or as the midpoint between the last antibody-negative and the first antibody-positive ELISA test. Data reported herein, was measured within the first 12 and/or 15 months of HIV-1 infection. Study participants were antiretroviral naïve and yet to reach CD4 levels that would qualify for South African's national guidelines for management and treatment of people with HIV (www.sanac.org.za/resources/art-guidelines). All participants provided written informed consent for participation of the study. The Human Research Ethics Committee (Medical) of the University of the Witwatersrand approved the clinical protocol and all participants provided written informed consent for participation in this study (Appendix A and C, page 260 and 262).

2.1.2 CAPT Cohort

A cohort of HIV-infected and -uninfected individuals involved in a sero-discordant relationship were recruited as part of the CAPT study at the Perinatal HIV Research Unit, Soweto, South Africa (Table 2.1). At enrollment visit, a total of 110-120ml of blood draw was obtained and a

similar volume of blood was obtained on second visit (60 days after enrollment). Participation in this study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Appendix B and C, page 261 and 262).

2.1.3 Cohort Characteristics

Table 2.1 shows clinical characteristics of the early HIV-infected participants, the majority of whom were women, stratified by change in viral load between 3 and 12 months post-infection. Participant's estimated days post-infection were also categorized into Fiebig stages based on measuring the presence/absence of plasma HIV-specific antibodies using ELISA (Fiebig *et al.*, 2003). Seven individuals were classified as Fiebig stage V, while 8 individuals were considered Fiebig stage VI at visit 1 (enrolment). Two participants (PHR006 and 9) were lost to follow-up and there was no 12-month viral load ($\square$). The median reduction of plasma viraemia in the group over the first nine months of follow-up was $-0.27 \log_{10}$ RNA copies/ml and median rate of absolute CD4 cell loss was -15 cells/month. Viral loads at baseline ranged from 2.6 to $5.88 \log_{10}$ RNA copies/ml, providing a variance of $3.28 \log_{10}$ to correlate with cell measurements. One individual (PHR0012) showed an increase in viraemia in the first year ($\triangle$); six individuals showed a change of $\pm 0.5 \log_{10}$ RNA copies/ml and were considered as having reached a set point ($\bullet$) and six individuals showed reduced RNA copies/ml below $0.5 \log_{10}$ change between 3 and 12 months ($\circ$). Of the 64 chronic HIV infected individuals, 62 were CMV infected and of the 24 HIV-uninfected controls 22 were CMV infected.

Table 2.1: Clinical characteristics of study subjects stratified by viral load differences between 3 and 12 months post-infection

Early HIV-infected participants			EDI	Fiebig Stage	pVL ₁₂₋₃ (Log10 copies/ml)		pVL ₁₂₋₃	Symbol	CD4 cells/mm ³		CD4 ₁₂₋₃ (Slope)
PID	Age	Female (%)			3 Mo.	12 Mo.			3 Mo.	12 Mo.	
PHR009	24	F	105	VI	2.60	-	-	□	617	NS	-
PHR006	40	F	48	V	4.90	-	-	□	530	NS	-
PHR012	34	M	95	V	4.09	4.87	0.78	△	551	391	-20 ± 10
PHR014	38	M	78	V	5.34	5.59	0.25	●	338	291	-5 ± 2
PHR008	27	F	76	V	3.67	3.88	0.21	●	1003	1034	-2.0 ± 2
PHR010	32	M	100	VI	4.15	4.28	0.12	●	438	365	-15 ± 13
PHR007	20	F	73	V	2.60	2.60	0.00	●	1176	684	-19 ± 36
PHR005	32	F	77	V	4.75	4.55	-0.20	●	693	614	-19 ± 13
PHR004	46	F	122	VI	5.52	5.26	-0.27	●	625	453	-23 ± 9
PHR001	21	F	134	VI	4.56	3.99	-0.57	○	504	497	-3.0 ± 11
PHR011	38	M	101	VI	4.76	4.09	-0.67	○	477	427	-5.0 ± 6
PHR003	21	F	137	VI	5.23	4.42	-0.81	○	1066	593	-24 ± 27
PHR013	26	F	98	V	3.43	2.60	-0.82	○	851	800	-14 ± 13
PHR002	34	F	138	VI	3.48	2.60	-0.88	○	368	250	-19 ± 11
PHR015	43	F	111	VI	5.88	3.95	-1.93	○	385	408	10 ± 13
Median	30	73%			4.56	4.09	-0.27		551	453	-15
IQR		-			3.58-5.07	3.88-4.55	-0.81-0.12		457-772	391-614	-19- -5
CAPT Cohort (Chronic HIV-infected participants; n=42)											
Median	35	67%						◆	396		
IQR									286-606		
CAPT Cohort (HIV-uninfected participants; n=24)											
Median	36	38%						■	1024		
IQR									709-1297		

EDI; Estimated Days Post-Infection, F, female; IQR, interquartile range; M, male; NS, no sample; Mo., months; PID, participant identification number; pVL, plasma viral load.

2.1.4 Plasma Viral Load and Absolute CD4⁺ T cell Counts

Plasma HIV-1 RNA levels were quantified using the COBAS AMPLICOR HIV-1 monitor test version 1.5 (Roche Diagnostic Systems, Somerville, NJ). Absolute blood CD4⁺ and CD8⁺ T cell counts were measured using pan-leukocyte count and expressed as cells/mm³.

2.2 Synthetic Peptides

A panel of 66 peptides (15-18 mers) overlapping by 10 amino acid residues and corresponding to gene products of consensus subtype C HIV-Gag sequence were synthesized using 9-fluorenylmethoxy carbonyl chemistry and standard based solid-phase techniques (Natural and Medical Science Institute, University of Tuebingen, Reutlingen, Germany). All peptides were checked for the correct molecular weight by Elektrospray QTOF-mass spectrometry, and estimated purities of the peptides were >80% as measured by high-performance liquid chromatography and mass spectrometry. Peptides were dissolved in 100% DMSO (Sigma-Aldrich, St. Louis, MO) at an initial concentration of 10mg/ml, pooled and used at a final concentration of 2µg/ml (<1% DMSO). A set of 138 peptides (15 mers) overlapping by 11 amino acid residues corresponding to the human CMV pp65 were obtained from the NIH AIDS Research and Reference Reagent Programs (Bethesda, MD) were pooled and dissolved in DMSO. All prepared peptides were stored at -80°C prior to use.

2.3 Sample Preparation

Peripheral blood mononuclear cells (PBMC) and plasma were isolated from blood using a standard Ficoll-Hypaque density gradient centrifugation method (Amershan Pharmacia, Uppsala, Sweden) (Appendix D, page 263). Plasma was then separated and stored in a -80°C freezer,

whereas PBMC were cryopreserved in 90% heat-activated FBS (Invitrogen, Paisely, UK) plus 10% DMSO, and stored in liquid nitrogen until needed. Thawed PBMC were washed twice with RPMI 1640 supplemented with 10% heat inactivated FBS, 100U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 1.7mM sodium glutamate (R10). The cells were then rested in R10 at 37°C and 5% CO₂ for 2 hours in the presence of 10U/ml Dnase I (Roche Diagnostic Systems) prior to use in intracellular cytokine staining assays.

2.4 Cell Stimulation and Intracellular Staining

2.4.1 Measurement of T cell Polyfunction

After a 2 hour rest, PBMC were washed and resuspended at 2×10^6 cells/ml with R10 and stimulated for 6h at 37°C and 5% CO₂ with a pool of HIV-1 C Gag and/or human CMV pp65 peptides (2µg/ml) in the presence of CD107 Alexa680 (kindly provided by Dr. Mario Roederer, VRC, NIH), 1µg/ml each of αCD28 and αCD49d co-stimulatory antibodies (BD Bioscience), 0.7 µg/ml monensin plus 10 µg/ml brefeldin A (Sigma-Aldrich). A negative control (unstimulated) containing PBMC and co-stimulatory antibodies from the same subject, but without the peptide mix was also included for each assay. Following stimulation, cells were washed with PBS 1% FBS and 0.1% sodium azide (wash buffer) and further surface stained with violet reactive dye (Vivid; Molecular Probes, Eugene, OR) and a cocktail of mAbs containing CD14 Pacific blue, CD19 Pacific blue, CD57 QD565, CD8 QD655 (provided by DR. Roederer, VRC), CD27 PE-Cy5 and CD45RO Texas Red PE (Beckman Coulter, Fullerton, CA) for 20 min in the dark at 4°C. Cells were then washed with wash buffer and permeabilized according to the manufacturer's instructions using a Cytofix/Cytoperm buffer kit (BD Biosciences) and then stained intracellularly with CD3 APC-Cy7, IFN-γ FITC, IL-2 APC, MIP-1β PE, TNFα PE-Cy7

(BD Pharmingen), and CD4 PE-Cy5.5 (Caltag laboratories, Burlingame, CA) for 20 min in the dark at 4°C. After labeling, cells were washed and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich) and stored at 4°C prior to flow cytometry acquisition within 24 hours.

2.4.2 Measurement of T cell Memory and Activation Status

Under the same conditions as explained above, thawed PBMC were stimulated for 6h with or without HIV-Gag C and/or human CMV pp65 peptides (2µg/ml) in the presence of 1µg/ml αCD28 and αCD49d and 10µg/ml brefeldin A. After washing, cells were stained with a panel consisting of HLADR-Alexa680, CD38-APC, CD14-Pacific blue, CD19-Pacific blue, CD57-QD565 and CD8-QD655, CD27-PE-Cy5, CD45RO-Texas-Red PE and the viability violet reactive dye. Following incubation, cells were washed and permeabilized with Cytofix/Cytoperm buffer kit and stained intracellularly with IFNγ- and IL-2-PE (BD Bioscience), CD3-APC-Cy7, Ki67-FITC (BD Pharmingen) and CD4-PE-Cy5.5. After labeling, cells were washed and fixed in PBS containing 1% paraformaldehyde and stored at 4°C prior to flow cytometry acquisition within 24 hours. To ensure that short-term stimulation and the use of brefeldin A did not affect surface expression of the activation markers used, the activation panel was optimized and kinetic experiments were run to investigate this (Appendix F, page 267). The clones for all antibody reagents are given in Table E1, Appendix E, page 265.

2.5 Measurement of Plasma EndoCAb, I-FABP, LBP and sCD14

Plasma EndoCAb, I-FABP, LBP and sCD14 (Hycult Biotechnology, Netherlands) were quantified using ELISA kits according to the manufacture's recommendations. Briefly, standards and plasma samples were allowed to thaw at room temperature (20-25°C) diluted and directly

transferred to microtitre wells pre-coated with antibodies recognizing human EndoCAb, I-FABP, LBP or sCD14. After incubation, the wells were washed and followed by the addition of the biotinylated tracer antibody against the captured EndoCAb, I-FABP, LBP or sCD14 and incubated. Following incubation, the wells were washed and streptavidin-peroxidase conjugate was added. Following a further wash, tetramethylbenzidine substrate was added to the wells for 25min and the enzyme-substrate reaction colour was stopped by an addition of a stop solution (oxalic acid).

2.6 Measurement of Plasma Cytokine Levels

Plasma IL-1 β , IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1 were measured simultaneously using the customized/premixed Bio-Rad multiplex plates according to the manufacture's recommendations. Briefly, plasma samples were filtered through 0.22 μ l filter and diluted at 1:4 with Bio-Plex sample diluent (Bio-Rad Laboratories, Hercules, California, USA). The 96 well filter plate was pre-wet with 100 μ l of Bio-plex assay buffer and vacuum filtered at 2 inches Hg and blotted (blotting of bottom plate was done after every vacuum filtration). 50 μ l of reconstituted anti-cytokine beads were added to each well and filter-washed twice with Bio-plex wash buffer. Standards and samples (50 μ l) were added to appropriate wells, covered with adhesive sealer and incubated at room temperature for 30 min at 300rpm (shaker speed was always maintained at 1100rpm for the first 30 seconds of incubation and thereafter reduced and maintained at 300rpm). Following incubation, the plate was further filter-washed 3 times with 100 μ l of wash buffer and 25 μ l of 1 X biotinylated detection antibody was then added to the wells and incubated for 30min at 300rpm. Following a further 3 times filter-wash, 50 μ l of 1X streptavidin-PE was added to the wells and incubated for 10min at 300rpm. After filter-washing

the plate 3 times, beads were then resuspended with 125µl Bio-plex assay buffer at 1100rpm for 30 seconds and plate read with high PMT setting using the Bio-Plex Reader, Luminex Technology (Bio-Rad, USA).

2.7 Data Analysis

2.7.1 Flow Cytometry Analysis

2.7.1.1 Cell Acquisition and Gating

Approximately 500 000-1 000 000 total events were collected per sample on a customized 4-laser LSRII flow cytometer (BD Bioscience) (Appendix E, page 265). Electronic compensation was conducted with antibody capture beads (BD Bioscience) stained separately with individual mAbs used in test samples. Data was analysed with FlowJo Version 8.8.6 (Tree Star, Ashland, OR). Single function gates were set based on unstimulated samples and were placed consistently across each sample. For the initial gating of each sample set, forward scatter area (FSC-A) versus a forward scatter height plot (FSC-H) was employed to gate out cell aggregates. T cell lymphocytes were then gated from populations derived from a FSC-A versus a side scatter area plot (SSC-A). Cells that were stained with Pacific blue: monocytes (CD14⁺) and B cells (CD19⁺), and including those stained with a live/dead marker: violet amine dye, were removed from the analysis. The exclusion of these cells was done by gating on live CD3⁺ cells on a CD3 versus a pacific blue/violet amine plot. Further debris was removed by gating on a CD8 versus a CD57 plot. This was followed by gating on CD4⁺ and CD8⁺, memory T cell subpopulations (based on expression of CD27⁺ and CD45RO⁺) (See Chapter 3, Figure 3.1, page 70), activation (CD38⁺, HLA-DR⁺) and proliferation (Ki67⁺) (See Chapter 4, Figure 4.1, page 99) and polyfunction (CD107⁺, IFNγ⁺, IL-2, MIP-1β⁺ and TNFα⁺) (See Chapter 5, Figure 5.1, page 145).

2.7.1.2 Boolean Gating Analysis

After the gates of each function were created (CD107, IFN γ , IL-2, MIP-1 β and TNF α), Boolean gating analysis as described by Betts et al. (2006), was employed to identify a full array of the possible response combinations, equating to 32 response patterns when testing all five individual cytokine response parameters. A positive cytokine response was defined as at least twice background (no Ag, only co-stimulatory Abs), >0.05% after subtraction of background, and at least 40 events (Burgers *et al.*, 2009). The latter criterion was introduced so as to minimize the possibility of error due to a low number of events when further subdividing these cells into the four memory subpopulations. Similarly, for the Boolean gating analysis to detect multiple cytokine responses, values greater than twice the background were considered as positive after background subtraction. Analysis and graphical representation of polyfunctional cells were performed by Pestle, version 1.5 and Spice, version 4.3 (provided by DR. Roederer, VRC). Boolean gating analysis was also used to measure the activation profile of memory T cells based on the expression of CD38, HLA-DR and Ki67, where a total of eight activated T cell phenotype combinations were discerned.

2.7.2 Cell Sorting and HIV Gag-DNA quantification

Cell sorting was explored to quantify cell-associated HIV-Gag proviral DNA and was accomplished by using the FACS Aria cell sorter (BDIS) at 70Ib/in². Electronic compensation was conducted with antibody capture beads (BD Biosciences) stained separately with the individual mAbs used in the test samples. The above mentioned gating strategy was employed to gate for CD4⁺ T cell populations. Antigen-specific CD4⁺ memory T cells were then sorted based on the expression of CD107, IFN- γ , IL-2, MIP-1 β and TNF α . Activated memory T cells were

sorted based on cells expressing CD38, Ki67 or HLA-DR (Appendix G, page 269 and 270 for the sorting gating strategies). At least 40 million PBMC were sorted in each experiment and populations were consistently >98% pure. The level of HIV-1 infection of these cells was then determined using real time PCR to quantify the amount of HIV-*gag* DNA per cell.

2.7.2.1 Gag-DNA quantification using real time PCR

Immediately after cell sorting, cells were spun down in 1.5ml polypropylene conical tubes, supernatant removed and frozen at -20°C prior to use. Cells were then lysed in 25-100 µl of 10mM Tris buffer containing proteinase K (Qiagen, Valencia, CA). Supernatant (5µl) was used as input DNA for the quantification of HIV *gag*-DNA using the 5' nuclease (Taqman) assay with an ABI7500 system (Applied Biosystems, Foster City, CA) (Douek *et al.*, 2002, Brenchley *et al.*, 2004a). HIV *gag*-DNA degenerate primers and probes were designed spanning conserved regions of subtype C *gag* genes identified in the Los Alamos HIV sequence database (www.hiv.lanl.gov/). Gag degenerate primer sequences were: gag-forward; 5'-GGGGAAGTGAYATAGCAGGA, gag-reverse: 5'-GGYCCTTGTYTTATGTCCAA and probe: 5'-Fam-CTACTAGTAVCCTTCARGAACARATARCATGGATGA-BHQ1 (Inqaba biotec, Pretoria, South Africa). For determining the cell number per reaction, quantitative PCR (qPCR) was performed simultaneously for albumin copy numbers using primers and probe sequences (Douek *et al.*, 2002); human albumin-forward: 5'-TGCATGAGAAAACGCCAGTAA, human albumin-reverse: 5'-ATGGTCGCCTGTTACCAA and probe 5'-TGACAGAGTCACCAAATGCTGCACAGAA-BHQ1. Standards were constructed for absolute quantitation of *gag* C and human albumin copy number and were validated with 10-fold serial dilutions of 8E5 and Ach2 cell lysates, which contain one copy of *gag* per cell (Appendix H,

page 271). Duplicate reactions were run and template copies calculated using ABI7500 software (Applied Biosystems) (Appendix I, page 272).

2.7.3 Plasma markers of microbial translocation and cytokine analysis

The intensity of the colour produced (absorbance) was measured with a microplate reader at 450nm, with the correction wavelength set at 540nm. Plasma samples were diluted at 1:100 for EndoCAb, 1:1000 LBP, and 1:600 sCD14 whereas I-FABP was used undiluted. Standard curves were generated and used to determine the plasma concentrations of LBP, I-FABP, sCD14 and EndoCAb (Appendix J, page 273). For diluted samples, concentrations read from the standard curve were multiplied by the dilution factor. Using the Bio-Plex Manager software version 5.0, a range of standards were also used to establish standard curves that enabled the quantitation of individual plasma cytokines. Blank values (background) were subtracted from all readings. The lower limits of detection were 1.27pg/ml for IL-1 β , 1.54pg/ml for IL-6, 1.69pg/ml for IL-7, 1.48pg/ml IL-10, 1.19pg/ml for IL-12(p70), 3.7 for TNF α and 1.24pg/ml for MCP-1. Plasma cytokine concentrations that fell below the range of the standard curve were reported as the mid-point between the lowest limit of quantification and zero (Roberts *et al.*, 2010).

2.8 Statistical Analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software (GraphPad, San Diego, CA). Data were expressed as median values and compared by the use of Mann-Whitney *U* nonparametric test and values of $p < 0.05$ were considered statistically significant. Univariate relationships between plasma markers of microbial translocation, plasma cytokines/chemokine, memory T cell activation, T cell function and plasma

viral load were assessed by non-parametric Spearman rank correlations. For multiple comparisons Bonferroni correction was applied (Bland and Altman, 1995). Multivariate associations were performed according to linear regression models using STATA version 10 (StataCorp., College Station, Texas, USA). For the inclusion in the multivariate analyses, variables were chosen with an observed $p < 0.05$ in the univariate analysis.

Linear mixed-effects models for longitudinal data (Laird and Ware, 1982) were also performed using STATA. To examine this, the proportion of memory subpopulations from 15 participants was collected at seven different time points (in months); 3, 4, 5, 6, 9, 12 and 15, and analyzed by a linear mixed-effects model across all time points. To minimize bias as a result of skewness, all variables, except time (visit), were log-transformed. Linear mixed-effects models were used because they take into account the inter- and intra-patient sources of variability and they can handle longitudinal data that is unbalanced and incomplete (Diggle *et al.*, 2000, Pinheiro and Bates, 2000, Fitzmaurice *et al.*, 2004). These models were applied to the following three analyses:

Model 1: Longitudinal changes of outcome over time

In order to test the significance of time effect on markers of memory differentiation (naïve, Int ED, LD and FD cells), activation (CD38, HLA-DR and Ki67), polyfunction, microbial translocation (LBP, I-FABP, sCD14 and EndoCAb), and inflammation (IL-1 β , IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1), the following model was used:

$$y_{ij} = \beta_0 + \beta_1 \times \text{time}_{ij} + u_i + e_{ij}$$

where $i = 1, \dots, 15$ participants and $j = 1, \dots, 7$ time points (visits). y_{ij} = response or outcome observation/variable (memory differentiation or activation or polyfunction or microbial translocation or inflammation), $time_{ij}$ = visit j for participant i , u_i is the participant-specific random effect for participant i and e_{ij} is the residual for participant i at time j . Since the participants themselves are independent of each other, the covariance structure for the participant-specific random effects u_i was modeled as being independent. The slopes were then evaluated to assess significant changes in the outcome variable across the different time points.

Model 2: Longitudinal associations between viral load and CD4⁺ T cell count with memory differentiation, activation, polyfunction, markers of microbial translocation and inflammation

In order to test the significance of the longitudinal association between memory differentiation, activation, polyfunction, markers of microbial translocation, and inflammation with viral load or CD4⁺ T cell counts, the following model was used:

$$y_{ij} = \beta_0 + \beta_1 \times x_{ij} + u_i + e_{ij}$$

where $i = 1, \dots, 15$ participants and $j = 1, 2, \dots, 7$ time points (visits). y_{ij} = viral load or CD4⁺ T cell count, x_{ij} = memory differentiation or activation or polyfunction or microbial translocation or inflammation, u_i is the participant-specific random effect for participant i and e_{ij} is the residual for participant i at time j . Because the successive measurements within each participant are auto-correlated, the within-participant residuals were modeled as being autoregressive of order 1. Since the participants themselves are independent of each other, the covariance structure for the subject-specific random effects u_i was modeled as being independent.

Model 3: Multivariate longitudinal associations between viral load and CD4⁺ T cell count with memory activation

Multivariate analysis using mixed-effects linear regression was employed to assess the relationship between memory activation with viral load and CD4⁺ T cell counts over the first 15 months of HIV-1 infection. The multivariate model fitted to the data across all time points was performed with the following equation:

$$\text{Activation}_{ij} = \beta_0 + \beta_1 \times \text{viral load}_{ij} + \beta_2 \times \text{cd4count}_{ij} + u_i + e_{ij}$$

where $i = 1, \dots, 15$ participants and $j = 1, 2, \dots, 7$ time points (visits). u_i is the participant-specific random effect for subject i and e_{ij} is the residual for participant i at time j . Because the successive measurements within each participant are auto-correlated, the within-participant residuals were modeled as being autoregressive of order 1. Since the participants themselves are independent of each other, the covariance structure for the participant-specific random effects u_i was modeled as being independent. In all the three models, no imputation of missing data was carried out as there were minimal missing data.

CHAPTER 3: T CELL MEMORY DIFFERENTIATION

TABLE OF CONTENTS

3.1 Introduction.....	66
3.2 Results.....	67
3.2.1 Antigen-specific memory T cell responses.....	67
3.2.2 Defining the memory differentiation profile of T cells at 3 months post-infection	67
3.2.2.1 Definition of T cell phenotypes during primary HIV infection	67
3.2.2.2 The differentiation profile of memory CD4 ⁺ T cells	71
3.2.2.3 The differentiation profile of memory CD8 ⁺ T cells	74
3.2.3 Relationship between memory differentiation and CD57 expression in primary HIV-1 infection.	78
3.2.4 Longitudinal changes in the differentiation profile of memory T cells during the course of HIV infection.....	80
3.2.4.1 CD4 ⁺ T cells.....	80
3.2.4.2 CD8 ⁺ T cells.....	83
3.2.5 Examination of the T cell memory differentiation steady-state	86
3.2.5.1 CD4 ⁺ T cells.....	86
3.2.5.2 CD8 ⁺ T cells.....	87
3.3 Discussion.....	91

3.1 Introduction

Memory T cells play an important role in viral eradication through their ability to respond quickly to virus re-encounter and mediate effector functions that directly inhibit viral replication and/or eliminate virus-infected cells. Improved understanding of functional memory development and identification of unique phenotypic markers of memory T cells could be critical in vaccine development for viral infections where adaptive immune responses play an important role in clearance and/or control (Ahlers and Belyakov, 2010). Although most viral infections may be cleared or controlled by immune responses, others result in persistent infection that could lead to altered memory differentiation of host T cells (Appay and Rowland-Jones, 2002, Appay *et al.*, 2007). There is evidence showing that HIV-1 results in alteration of memory T differentiation during the chronic stages of infection (Appay *et al.*, 2007), but less is known regarding the impact of intense viral replication on memory differentiation during early/primary HIV-1 infection. It was therefore hypothesized **that primary HIV-1 infection is characterized by skewing of the differentiation pathway toward an accumulation of highly differentiated and senescent T cells.** To address the hypothesis, this chapter aims to; (i) characterize the distribution of memory CD4⁺ and CD8⁺ T cell subpopulations between early HIV-infected and HIV-uninfected controls, (ii) compare the frequencies of memory subsets of different antigen specific T cells (Gag- and CMV-specific) in HIV-infected and -uninfected controls, (iii) examine and compare T cell immunosenescence between early HIV-infected and -uninfected individuals, and (iv) determine the longitudinal changes in the differentiation profile during the first 15 months of HIV infection. Given that the targeting of HIV-Gag by HIV-specific T cells has mainly been associated with control of viraemia, pooled Gag peptides

sequences were used to characterize the differentiation of HIV-specific memory CD4⁺ and CD8⁺ T cells.

3.2 Results

3.2.1 Antigen-specific memory T cell responses

The immunophenotyping of PBMCs was performed by using multi-parameter flow cytometry and HIV-Gag and CMV-specific responses at 3 months post-infection (IQR: 2.5-3.9) are shown in Table 3.1. Two patients showed no CD4⁺ response to Gag peptide pools (PHR009 and PHR011), although one of these showed a positive response to CMV. The median frequency of CMV-specific memory CD4⁺ T cell responses was 0.16%, while the median frequency of Gag-specific memory CD4⁺ T cells was found to be 0.19%. The median frequencies of antigen-specific memory CD8⁺ T cell responses to CMV and Gag were 0.25% and 0.12%, respectively. Fifteen HIV-uninfected individuals were used as control subjects, and they all responded to CMV peptides (median: 0.15% for CD4⁺ and 0.31% for CD8⁺ T cell responses), none responded to Gag peptides.

3.2.2 Defining the memory differentiation profile of T cells at 3 months post-infection

3.2.2.1 Definition of T cell phenotypes during primary HIV infection

Using the memory and activation panel (Chapter 2, Section 2.4.2, page 56), Figure 3.1 shows the gating strategy employed to gate and identify CD4⁺ and CD8⁺ T cell populations, while Figure 3.2 shows representative contour and dot plots of total and antigen-specific memory CD4⁺ and CD8⁺ T cell subpopulations, based on the cell surface expression of CD27 and CD45RO. Although there is broad consensus on how to classify naïve T cells, there is still not yet a unified

model that clearly defines and describes different memory T cell subpopulations (Appay *et al.*, 2008, Chattopadhyay and Roederer, 2010). The use of differentiation markers, CD27 and CD45RO has previously allowed several studies to discriminate T cells into distinct naïve and memory subpopulations (Casazza *et al.*, 2006, Duvall *et al.*, 2008). In this study, using CD27 and CD45RO, T cell subpopulations were classified as naive, early differentiated (ED)-memory, intermediate memory (Int), late differentiated (LD)- memory and fully differentiated (FD)-memory cell and were gated respectively on CD45RO⁺CD27⁺, CD45RO⁻CD27^{dim}, CD45RO⁺CD27⁻ and CD45RO⁻CD27⁻ populations. Naive cells were gated on the CD45RO⁻CD27^{bright} T cells. A conservative gating strategy was purposely employed (Song *et al.*, 2005, Chattopadhyay *et al.*, 2006, Petrovas *et al.*, 2009) to avoid misclassifying cells bearing dim expression of CD27 or CD45RO in the ED or LD compartment. In addition, within the CD8⁺ T cell compartment a unique subpopulation (Intermediate; CD45RO⁻CD27^{dim}) of memory T cells was identified. This population appeared to be distinct from naive cells and fully differentiated cells, as they showed slightly lower surface expression of CD27 than naive cells and slightly higher expression of CD27 compared to full differentiated cells. In addition, this T cell memory subpopulation has been previously noted in other studies (Appay *et al.*, 2002a, Burgers *et al.*, 2009, Barbour *et al.*, 2009, Mojumdar *et al.*, 2012).

Table 3.1: Antigen-specific memory T cell responses in early HIV-infected and HIV-uninfected individuals

Early HIV infected participants	Symbol	Ag-specific CD4 ⁺ responses (%)		Ag-specific CD8 ⁺ responses (%)	
PID		CMV	GAG	CMV	GAG
PHR009	□	0.09	NR	NR	0.33
PHR006	□	0.33	0.19	0.37	0.59
PHR012	△	0.16	0.15	0.25	1.10
PHR014	●	0.13	0.39	0.29	0.12
PHR008	●	0.13	0.13	0.06	NR
PHR010	●	NR	0.12	NR	0.10
PHR007	●	0.09	0.20	0.26	0.12
PHR005	●	0.26	0.13	0.57	NR
PHR004	●	0.20	0.14	0.21	2.07
PHR001	○	0.16	0.20	0.38	0.08
PHR011	○	NR	NR	NR	NR
PHR003	○	0.18	0.27	0.37	0.05
PHR013	○	0.51	0.21	0.02	2.32
PHR002	○	0.16	0.18	0.22	0.10
PHR015	○	0.26	0.21	0.22	0.11
Median		0.16	0.19	0.25	0.12
IQR		0.13-0.26	0.14-0.21	0.22-0.37	0.1-0.72
HIV- participants (n=15)					
Median	■	0.15	-	0.31	-
IQR		0.08-0.17	-	0.23-1.29	-

NR, no response;

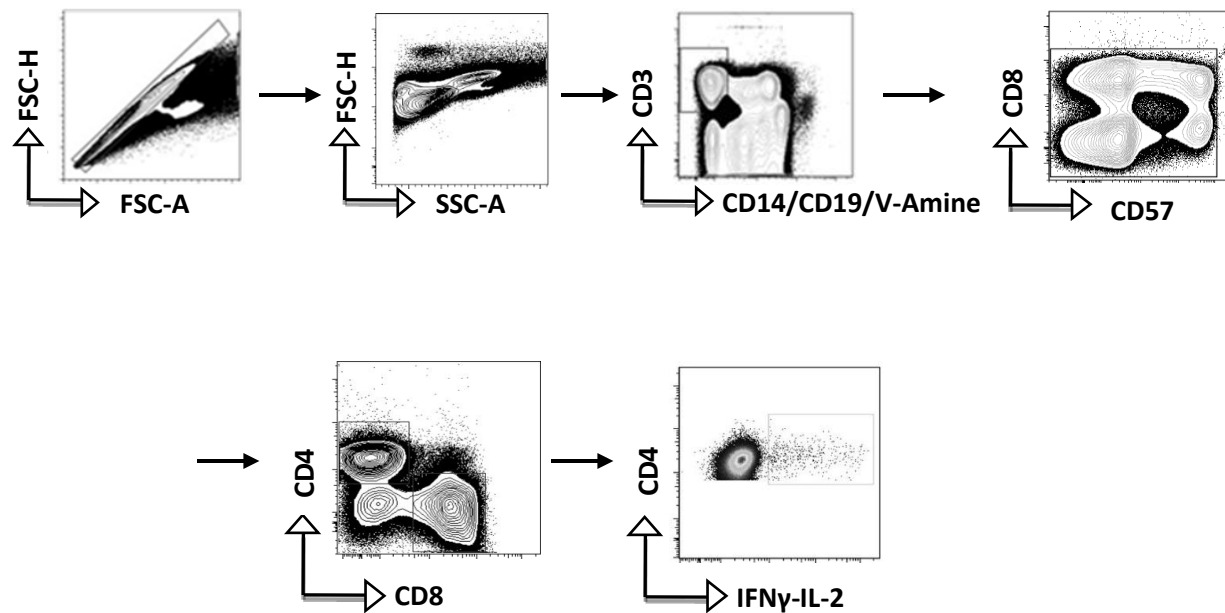


Figure 3.1: The Gating strategy for determining the memory phenotype of CD4⁺ and CD8⁺ T cells. For the initial gating of each sample set, forward scatter area versus a forward scatter height contour plot was employed to gate out cell aggregates. T cell lymphocytes were then gated from populations derived from a forward scatter area versus a side scatter area plot. Cells that were stained with Pacific blue: monocytes (CD14⁺), B cells (CD19⁺), and dead cells stained with violet amine dye, were removed from the analysis. The exclusion of these cells was done by gating on live CD3⁺ cells on a CD3 versus a pacific blue/violet amine plot. Further debris was removed by gating on a CD8 versus a CD57 plot. This was followed by gating on either CD4⁺ or CD8⁺ T cell populations, and the frequency of antigen-specific memory T cell responses were quantified from the combined expression of IFN γ and/or IL-2.

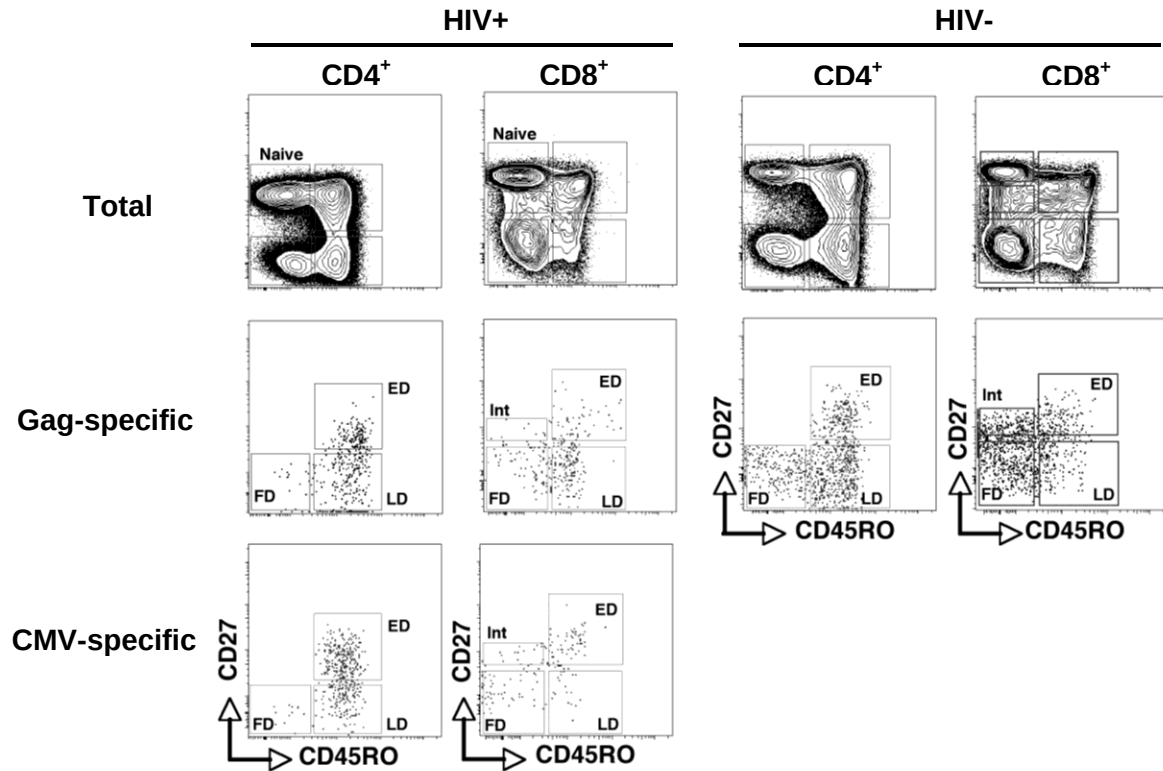


Figure 3.2: Representative contour plots showing the gating scheme employed to determine distinct differentiation profiles of memory $CD4^+$ and $CD8^+$ T cells at 3 months post-infection. Using differentiation markers CD45RO and CD27, different memory T cell subsets: naïve ($CD45RO^-CD27^{bright}$), early differentiated memory (ED, $CD45RO^+CD27^+$), intermediate (Int, $CD45RO^-CD27^{dim}$) for $CD8^+$ T cells, late differentiated memory (LD, $CD45RO^+CD27^-$) and fully differentiated effector memory cells (FD, $CD45RO^-CD27^-$) were identified in both total and antigen-specific (Gag and CMV) memory T cells. Memory T cells subpopulations were analysed from parent $CD4^+$ and $CD8^+$ populations.

3.2.2.2 The differentiation profile of memory $CD4^+$ T cells

For the first level of analysis, the differentiation phenotype of total $CD4^+$ T cells was compared between the proportions of naïve, ED-, LD- and FD-memory $CD4^+$ T cells between early HIV-infected individuals and HIV-uninfected controls, where no differences were identified (Figure

3.3A). Using a read-out of the combined IL-2/IFN- γ expression, the phenotype of antigen-specific (Gag- and CMV-specific) CD4⁺ T cells were also compared (Figure 3.3B). There was a significantly higher proportion of Gag-specific memory cells relative to CMV-specific cells within the ED compartment ($p=0.0014$), and significantly lower proportion of Gag-specific memory cells than CMV-specific memory cells within the LD compartment ($p<0.0001$). This finding is in line with previous studies describing the skewing of HIV-specific CD4⁺ T cells toward an early central memory phenotype relative to CMV-specific CD4⁺ T cells (Yue *et al.*, 2004). Analysis on CMV-specific T cell populations showed no significant differences in the proportions of ED, LD or FD memory cells between HIV-infected and HIV-uninfected controls (Figure 3.3B).

Given that the differentiation profile of either total or antigen-specific T cells may distinctly associate with HIV-1 disease outcome, this chapter compared the memory differentiation profile between total and antigen-specific CD4⁺ T cells within the same individuals (Figure 3.4). In HIV-infected individuals, the proportions of total ED- and LD-memory CD4⁺ T cells were found to be significantly lower compared to that of Gag-specific CD4⁺ T cells, ($p=0.0005$ and $p<0.05$, respectively) (Figure 3.4A). The proportion of LD CMV-specific memory CD4⁺ T cells were also significantly higher than total LD-memory CD4⁺ T cells ($p<0.0001$). With regard to HIV-uninfected controls, phenotypic differences were noted within the LD compartment, whereby the proportion CMV-specific cells were found to be significantly higher than total CD4⁺ T cells ($p<0.0001$) (Figure 3.4B). The FD-memory T cell populations made up the smallest proportions of both total and antigen-specific CD4⁺ T cell populations in HIV-infected and -uninfected individuals, and no differences were observed when comparing Gag- and/or CMV-specific cells

to total CD4⁺ T cells. Together, these data show distinct phenotypic characteristics between total and antigen-specific T cells, whereby Gag-specific memory CD4⁺ T cells appear to be skewed towards an early differentiated phenotype compared to CMV-specific T cells.

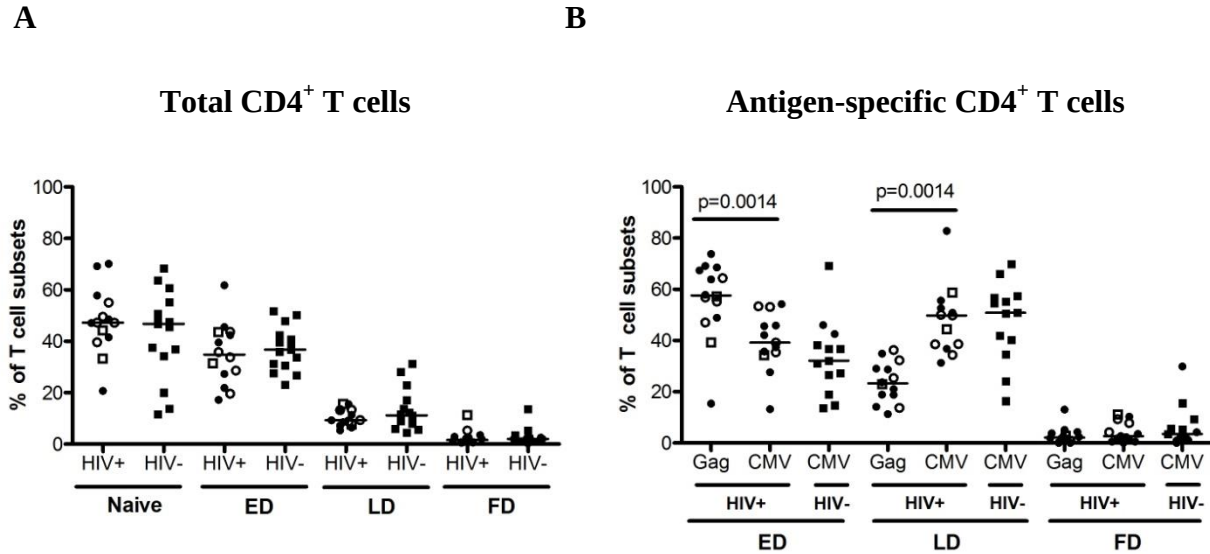


Figure 3.3: The distribution of total CD4⁺ T cell subpopulations in HIV-infected and -uninfected controls. (A) Comparison between the proportion of Naïve, ED-, LD- and FD-memory in early HIV-infected individuals (n=14) and -uninfected controls (n=15, ■). **(B)** Comparison between the proportion of Gag- and CMV-specific memory CD4⁺ T cell differentiation profiles in HIV-infected and -uninfected controls. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by a Mann-Whitney *U* non-parametric *t* test.

A

B

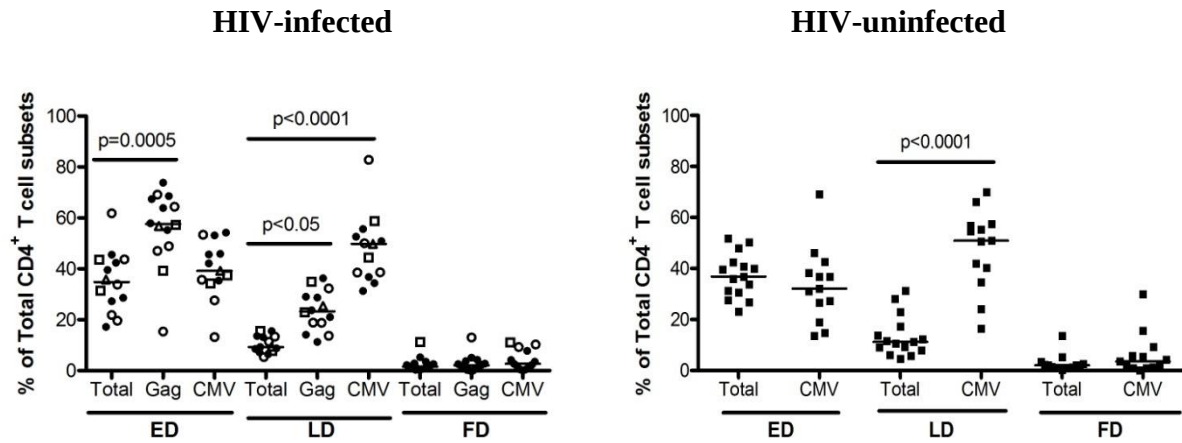


Figure 3.4: Comparison between the differentiation profiles of total ED, LD and FD CD4⁺ T cells with Gag- and CMV-specific memory CD4⁺ T cells in both HIV-infected and -uninfected controls. (A) Comparison between the proportion of total CD4⁺ T cells with Gag- and CMV-specific memory cells in HIV-infected individuals (n=14) at 3 months post-infection. (B) Comparison between the proportion of total CD4⁺ T cells and CMV-specific memory cells in HIV-uninfected controls (n=15, ■). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5log₁₀ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within ±0.5log₁₀ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase >0.5log₁₀ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by non-parametric One-way ANOVA.

3.2.2.3 The differentiation profile of memory CD8⁺ T cells

Distinct memory subpopulations within the CD8⁺ T cell compartment were also observed between total, Gag- and CMV-specific cells. Although no proportional differences were observed between total CD4⁺ T cell naïve populations of HIV-infected and -uninfected controls, significant differences were observed within the total CD8⁺ T cell compartment, whereby the frequencies of

naïve CD8⁺ T cells in HIV-infected individuals were found to be lower compared to those in HIV-uninfected controls ($p=0.0073$, Figure 3.5A). Additionally, the proportions of ED- and Int-memory CD8⁺ T cells were significantly higher in HIV-infected individuals relative to uninfected controls, $p=0.0032$ and $p=0.004$ respectively. Although no significant differences were observed in the proportion FD-memory cells between HIV-infected individuals and HIV-uninfected controls, a trend toward a relative increase of FD-memory cells in HIV-infected individuals was observed (median: 22.3% vs. 13.7%, respectively). When comparing the proportion of antigen-specific CD8⁺ T cells within HIV-infected individuals, significant differences between Gag- and CMV-specific cells were reflected in the Int and LD compartments. Thus, the proportions of Gag-specific CD8⁺ T cells within the Int compartment were found to be greater than CMV-specific CD8⁺ T cells ($p=0.0013$, Figure 3.5B), whilst increased proportions of CMV-specific CD8⁺ T cells were observed within the LD compartment relative to Gag-specific CD8⁺ T cells ($p<0.0001$). The differentiation profiles of CMV-specific CD8⁺ T cells were of comparable frequencies between HIV-infected and HIV-uninfected controls (Figure 3.5B).

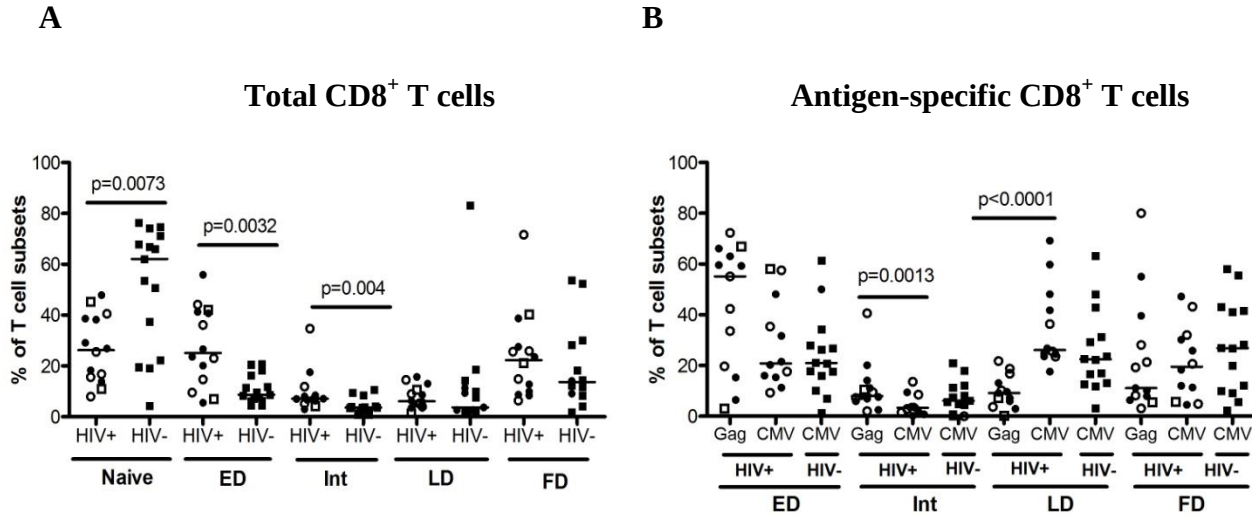


Figure 3.5: The distribution of total CD8⁺ T cell subpopulations in HIV-infected and -uninfected controls. (A) Comparison between the proportion of Naïve, ED-, LD- and FD-memory in HIV-infected (n=14) and -uninfected individuals (n=15, ■). (B) Comparison between the proportion of Gag- and CMV-specific memory CD8⁺ T cell differentiation profiles in HIV-infected and -uninfected controls. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by a Mann-Whitney *U* non-parametric *t* test.

Similarly, the proportions of total ED-, Int-, LD- and FD-memory T cells were also compared to the proportions of Gag- and/or CMV-specific cells within the CD8⁺ compartment (Figure 3.6). No differences were noted between the proportions of both Gag- and CMV-specific CD8⁺ T cells with total ED-, Int- and FD-memory CD8⁺ T cells, except within the LD compartment whereby the proportion of CMV-specific cells were higher than total CD8⁺ LD-memory cells ($p<0.0001$) (Figure 3.6A). A trend towards higher proportions of Gag-specific ED cells than total ED cells

was also observed (median: 55.1% vs. 25.1%, respectively). In HIV-uninfected individuals, higher proportions of CMV-specific CD8⁺ T cells compared total CD8⁺ T cells were observed within the ED- and LD-memory cells ($p=0.0064$ and $p<0.001$, respectively) (Figure 3.6B). Collectively, these data indicate that in early HIV infection, HIV- and CMV-specific CD4⁺ and CD8⁺ T cells possess different memory differentiation profiles where Gag-specific T cells are predominantly less mature when compared with CMV-specific cells.

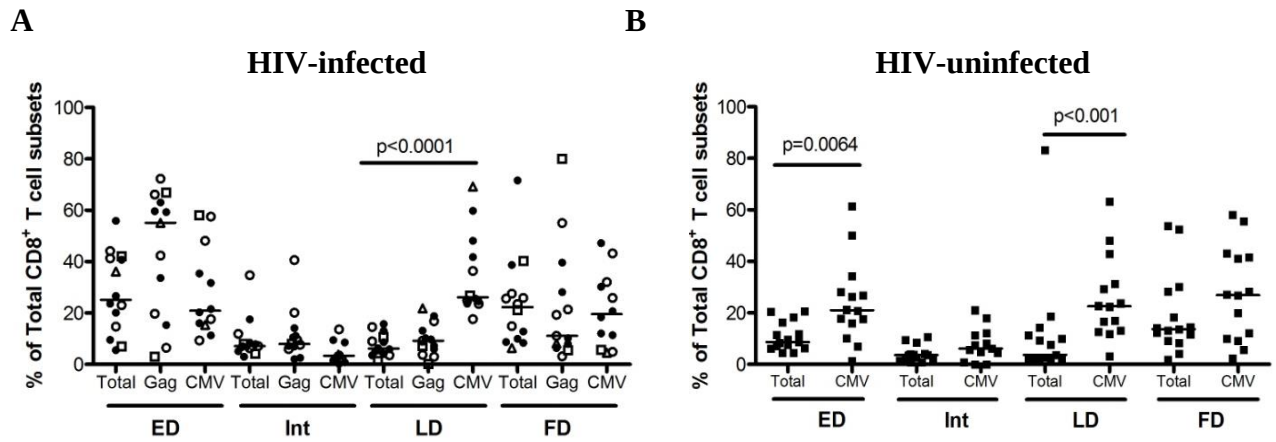


Figure 3.6: Comparison between the differentiation profiles of total ED, Int-, LD and FD CD8⁺ T cells with Gag- and CMV-specific memory cells in both HIV-infected and -uninfected controls. (A) Comparison between the proportion of total CD8⁺ T cells with Gag- and CMV-specific memory cells in HIV-infected individuals ($n=14$) at 3 months post-infection. (B) Comparison between the proportion of total CD8⁺ T cells and CMV-specific memory cells in HIV-uninfected controls ($n=15$, ■). Open circles (O) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by non-parametric One-way ANOVA.

3.2.3 Relationship between memory differentiation and CD57 expression in primary HIV-1 infection.

Progressive chronic HIV infection has been associated with increased CD57 expression (Appay and Rowland-Jones, 2002). However, memory lineage of T cells based on the expression of CD57 in primary HIV-1 infection is not fully elucidated. Figure 3.7A shows the gating strategy employed for CD57 expression on memory CD4⁺ and CD8⁺ T cell subpopulations. There was an overall low expression level of CD57 within the ED CD4⁺ T cell compartment, whereby a median of 0.88% of cells expressed CD57 during early HIV-1 infection (Figure 3.7B). No significant differences were observed in the expression of CD57 on ED CD4⁺ T cells between HIV-infected and uninfected controls. The expression of CD57 was found to be higher on LD-memory CD4⁺ T cells relative to ED-memory CD4⁺ T cells, with a median of 13.8% of cells for HIV-infected individuals. The expression levels of CD57 within the LD compartment were also found to be comparable between HIV-infected and uninfected individuals. The FD CD4⁺ T cell compartment displayed the highest CD57 expression, with a median of 36.7% for early HIV-infected individuals, and similarly, no significant differences were noted in the expression CD57 between HIV-infected and HIV-uninfected controls. A similar pattern was observed on memory CD8⁺ T cell subpopulations, where increased expression of CD57 was most apparent within the FD compartment. The proportion of cells expressing CD57 were found to be significantly elevated within the Int and FD subpopulations of HIV-infected individuals relative to HIV-uninfected controls ($p=0.0072$ and $p=0.0094$, respectively). Collectively, these data show that fully differentiated memory CD4⁺ and CD8⁺ T cell subpopulations are characterized by an increased expression of CD57 associated with a senescent phenotype.

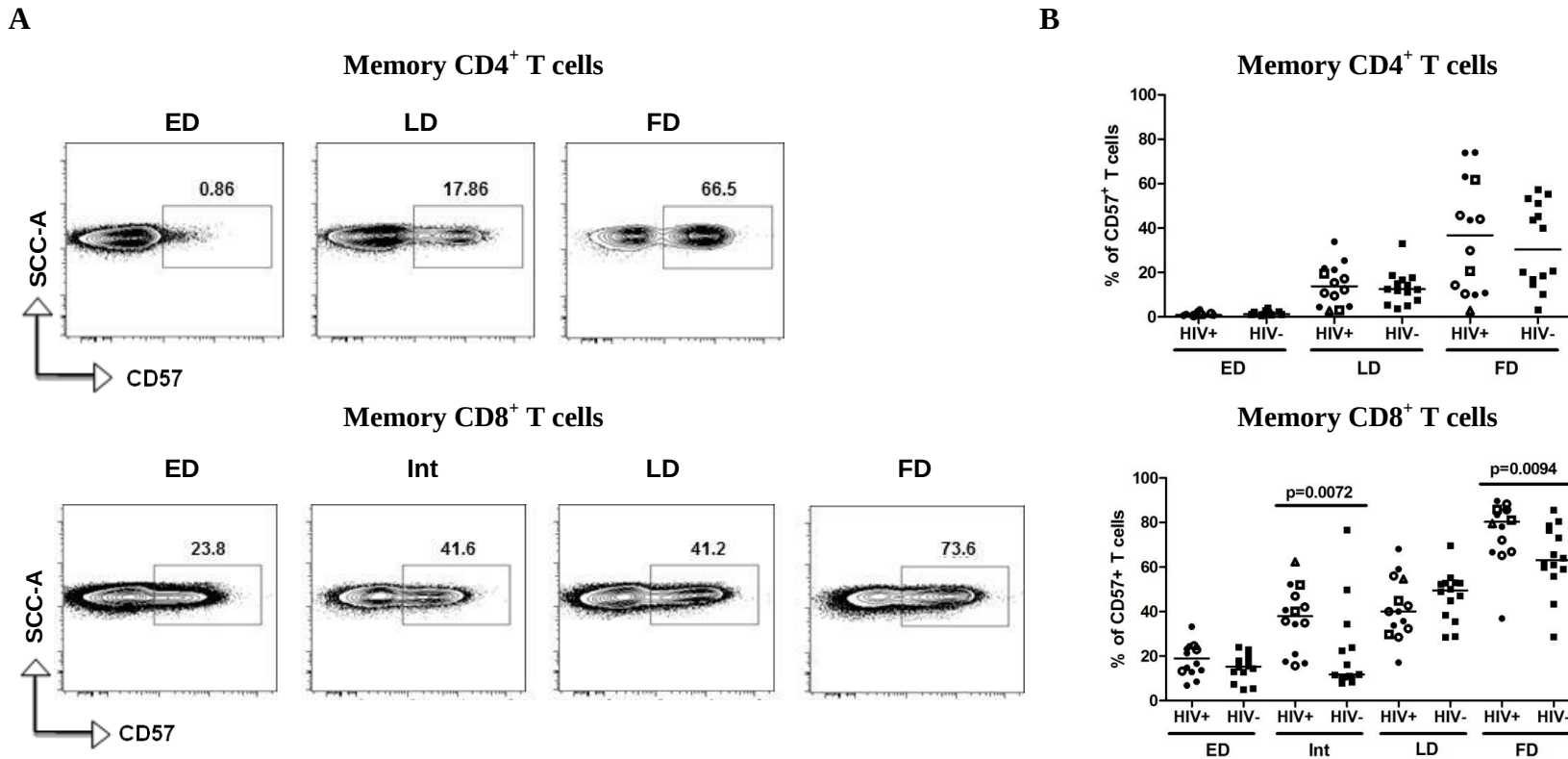


Figure 3.7: The phenotypic expression of CD57 within total memory T cell subpopulations. (A) Representative contour plots showing the expression of CD57 within the total memory CD4⁺ and CD8⁺ T cell subpopulations. (B) Comparison between the proportions of CD57⁺ cells between HIV-infected (n=14) and -uninfected controls (n=15, ■). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5log₁₀ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within ±0.5log₁₀ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase >0.5log₁₀ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by a Mann-Whitney *U* non-parametric *t* test.

3.2.4 Longitudinal changes in the differentiation profile of memory T cells during the course of HIV infection

3.2.4.1 CD4⁺ T cells

The differentiation profile of T cell subpopulations was also analysed longitudinally over the first 15 months of HIV-1 infection. The proportion of memory subpopulations from the 15 HIV-infected participants was analysed at 3, 4, 5, 6, 9, 12 and 15 weeks post seroconversion and a linear mixed-effects model across all time points was used to assess changes in cell subsets (Chapter 2, Model 1, page 62). Slopes were evaluated to assess significant changes in memory differentiation across the different time points. There were no significant changes over time in the level of total naïve, ED- and FD-memory CD4⁺ T cells in HIV-infected individuals (Figure 3.8). A significant increase was however noted in the proportions LD CD4⁺ T cells over the first 15 months of infection (slope=0.007, p=0.008). Care was taken when measuring antigen-specific cells to gate out and remove naïve cells from the analysis so that the frequency of antigen-specific T cells were not diluted by the naïve cells. Although there were no significant changes in the proportions of ED, LD and FD Gag-specific memory CD4⁺ T cells over time, a trend towards a decrease of ED cells relative to an increase in FD cell was observed (Figure 3.9A). There was a significant decrease in the proportions of ED CMV-specific memory CD4⁺ T cells (slope=-0.012, p=0.016), and a significant accumulation of LD CMV-specific memory CD4⁺ T cells over the first 15 months of infection (slope=0.007, p=0.046) (Figure 3.9B). The significant accumulation of LD CMV-specific memory cells over time may be related to persistent antigen stimulation to CMV antigens resulting in the differentiation of memory cells toward a more differentiated phenotype.

Total CD4⁺ T cells

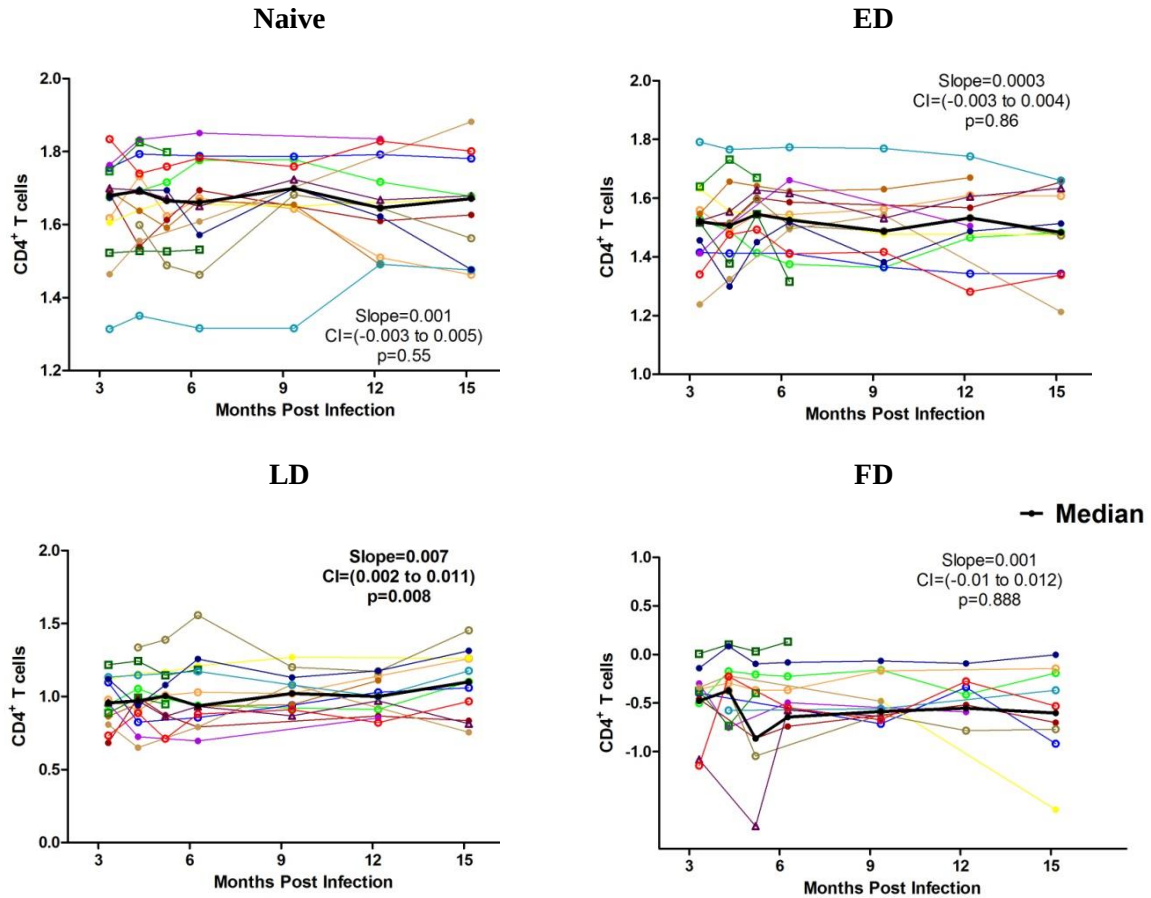
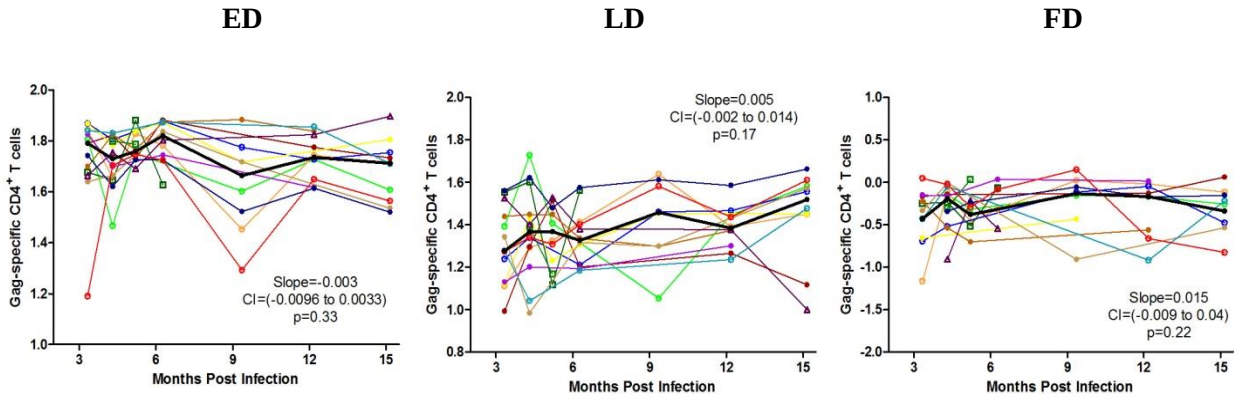


Figure 3.8: The longitudinal changes of the differentiation profile of CD4⁺ T cells over the first 15 months of HIV infection. The frequency of log naïve, ED, LD and FD T cells were assessed with a linear mixed-effects model to determine the phenotypic changes over time (n=15). The median change across time is represented by a bold solid line. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coded lines show proportions of memory subpopulations at each time point, and coloured lines are consistent for patients in each represented graph.

A

Gag-specific memory CD4⁺ T cells



B

CMV-specific memory CD4⁺ T cells

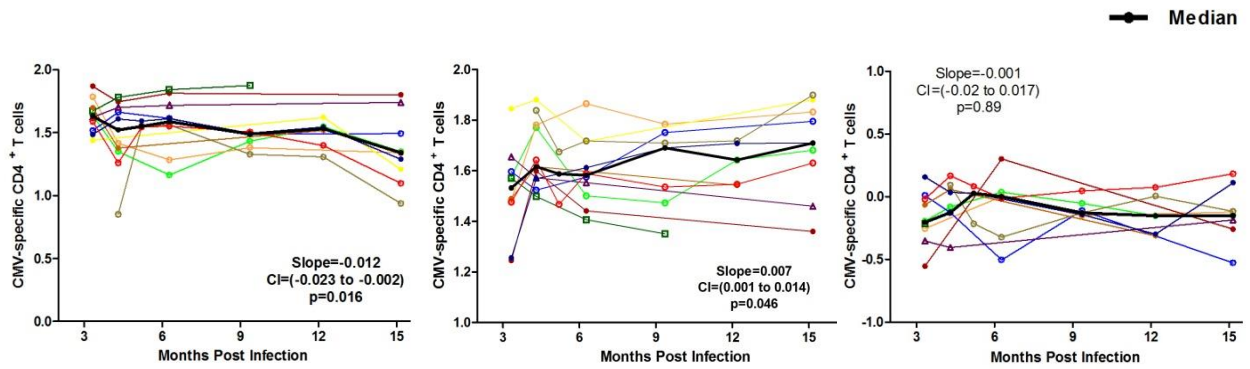


Figure 3.9: The longitudinal changes of the differentiation profile of antigen-specific memory CD4⁺ T cells over the first 15 months of HIV infection. The longitudinal changes in the differentiation profile of log (A) Gag-specific, and (B) CMV-specific memory CD4⁺ T cells between 3 and 15 months PI (n=15). The proportions of ED, LD and FD T cells were assessed with a linear mixed-effects model to determine the phenotypic changes over time. The median change across time is represented by a bold solid line. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coded lines show proportions of memory T cell subpopulations at each time point, and coloured lines are consistent for patients in each represented graph.

3.2.4.2 CD8⁺ T cells

Within the CD8⁺ compartment, there were no significant changes in the proportions of total naïve, ED-, LD-memory T cells over time, between 3 and 15 months post-infection (Figure 3.10). There was however a decreasing trend in the proportion of total ED-memory over time (slope=-0.006, p=0.07). There was a significant decrease in the proportion of Int memory CD8⁺ T cells (slope=-0.007, p=0.014), and significant increase in the proportion of FD-memory CD8⁺ T cells (slope=0.011, p<0.0001) over time. These data suggest that there is an accumulation of fully differentiated memory CD4⁺ and CD8⁺ T cells over the first year of HIV infection of probably many differing antigen specificities.

Longitudinal analysis of Gag-specific CD8⁺ T cells showed no significant changes in memory differentiation profile of ED, LD, Int and FD over time (Figure 3.11A). Although proportions of CMV-specific ED-, LD- and Int-memory T cells remained relatively stable over time, there was a significant increase in the proportion FD-memory cells (slope=0.016, p=0.001; Figure 3.11B). In summary, there was a significant accumulation of fully differentiated CD4⁺ and CD8⁺ total memory T cells over the first 15 months of infection, while Gag-specific CD4⁺ and CD8⁺ T cell memory status remained relatively stable. These results infer that a steady-state of Gag-specific T cell memory subpopulations operate during the first year of HIV infection, but not for total memory, where there is a gradual increase in proportions of more fully differentiated memory cells.

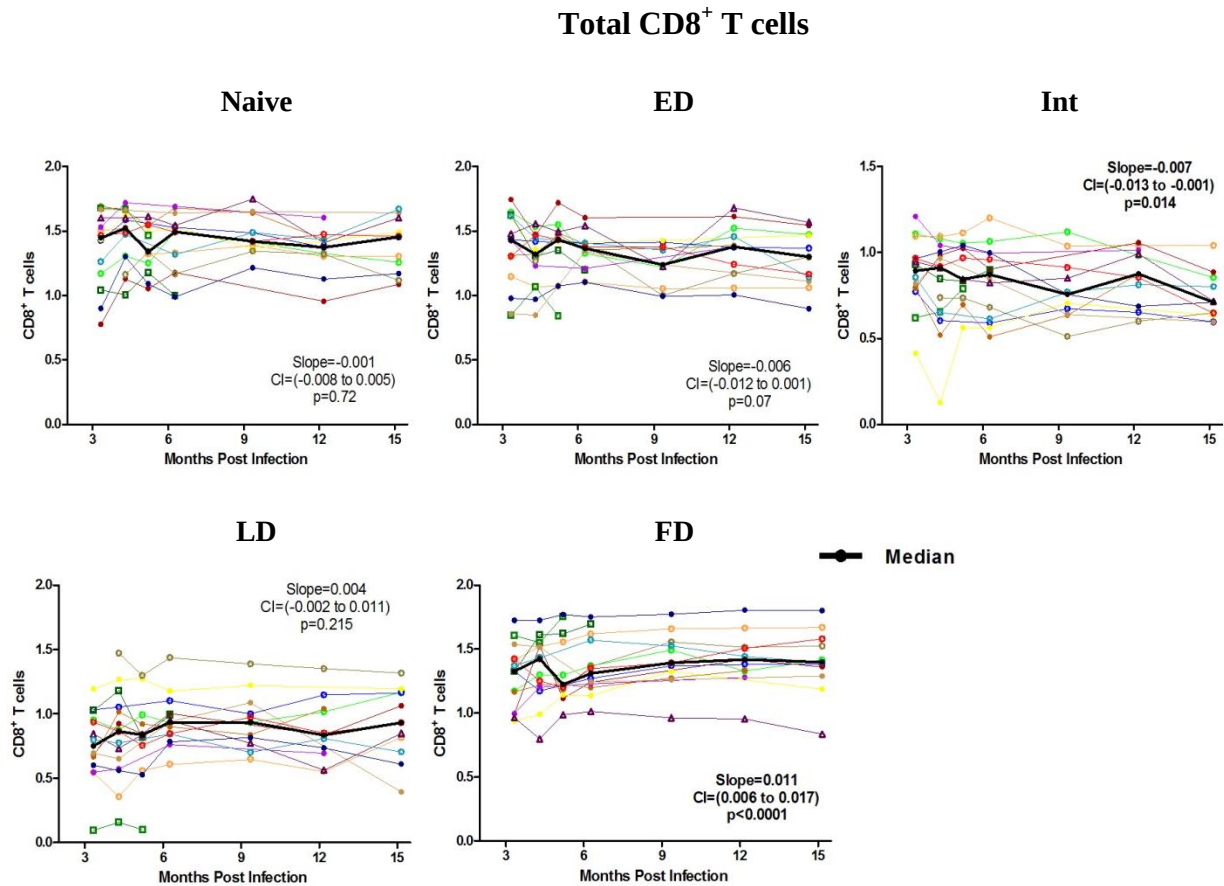


Figure 3.10: The longitudinal changes of the differentiation profile of CD8⁺ T cells over the first 15 months of HIV infection. The proportions of log naïve, ED, Int, LD and FD T cell subpopulations were assessed with a linear mixed-effects model to determine the phenotypic changes over time (n=15). The median change across time is represented by a bold solid line. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coded lines show proportions of T cell subpopulations at each time point, and coloured lines are consistent for patients in each represented graph.

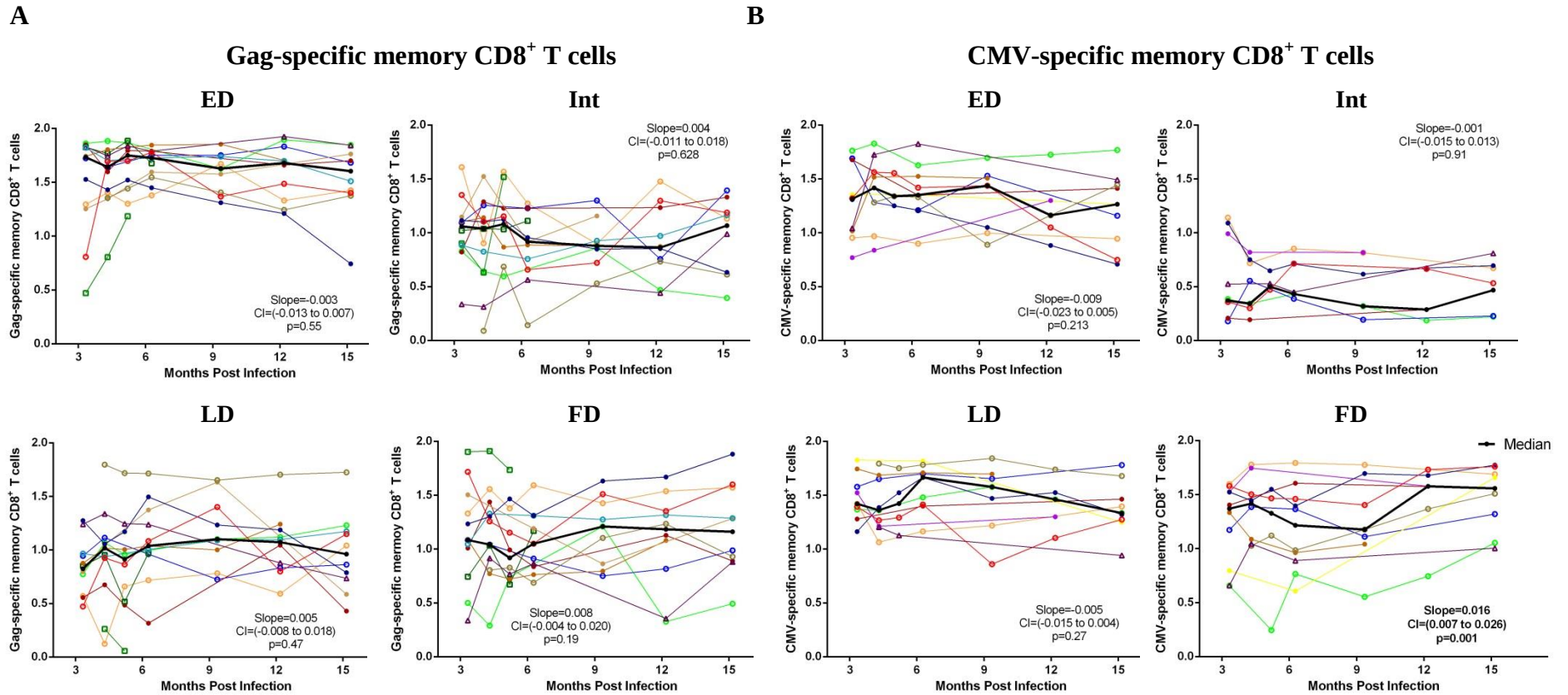


Figure 3.11: The longitudinal changes of the differentiation profile of antigen-specific memory CD8⁺ T cells over the first 15 months of HIV infection. The longitudinal changes in the differentiation profile of log (A) Gag-specific, and (B) CMV-specific memory CD8⁺ T cells, between 3 and 15 months PI in early HIV-infected individuals (n=15). The proportions of ED, Int, LD and FD T cell subpopulations were assessed with a linear mixed-effects model to determine the phenotypic changes over time. The median change across time is represented by a bold sold line. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coded lines show proportions of memory T cell subpopulations at each time point, and coloured lines are consistent for patients in each represented graph.

3.2.5 Examination of the T cell memory differentiation steady-state

3.2.5.1 CD4⁺ T cells

Given that events that occur during the early stages of infection may have a strong impact on HIV-1 disease progression, this section examines whether memory differentiation reaches a steady-state during primary HIV-1 infection. To further investigate whether the differentiation status of total and Gag-specific CD4⁺ T cells reached a set-point or a steady-state during the early stages of HIV-1 infection, the proportions of naïve, ED-, LD- and FD-memory cells at 3 months post-infection were correlated with those at 4, 5, 6, 9, 12, and 15 months post-infection. Given that the same data for month 3 was used for multiple comparisons (six hypothesis tests), a Bonferroni-adjusted significance level of 0.0083 was calculated to account for the increased possibility of type-I error (Bland and Altman, 1995). Thus, *p*-values above 0.0083 were therefore considered not statistically significant. A comprehensive Table K1 of data showing all the associations between 3 months with later time points is shown in Appendix K, page 274. A selection of significant correlations between the proportions of CD4⁺ T cell subpopulations at 3 and 12 months post-infection are shown in Figure 3.12A and B. Significant associations were found between the proportions of naïve, ED-, and FD-memory CD4⁺ T cells between 3 and 12 months post-infection ($r=0.87$, $p=0.001$; $r=0.89$, $p<0.0001$ and $r=0.96$, $p<0.0001$ respectively; Figure 3.13A).

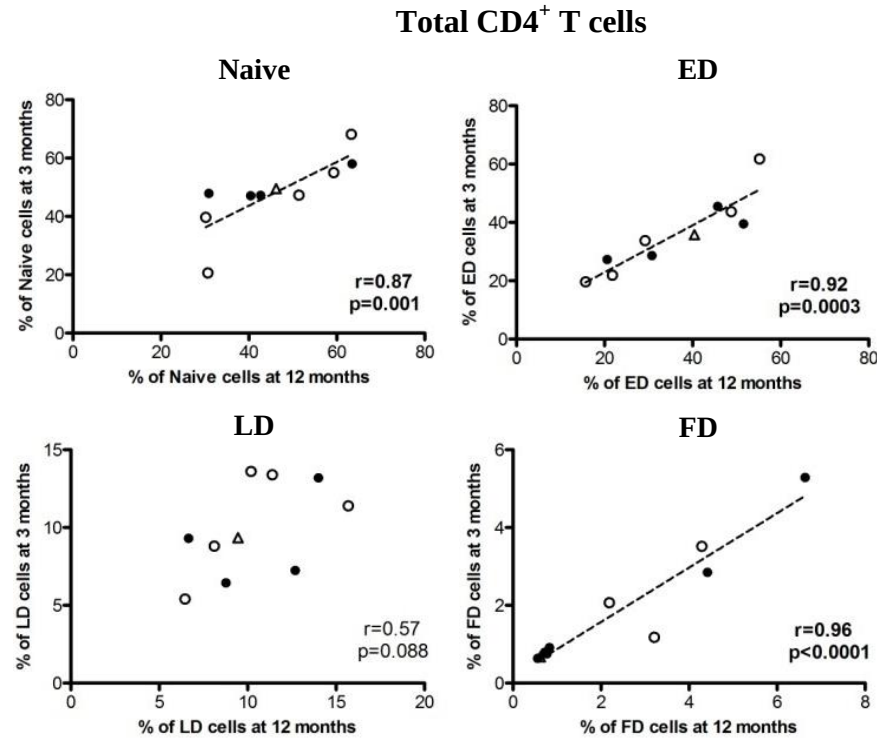
A positive trend was observed between the proportions of LD-memory cells at 3 and 12 months. Although no significant associations were observed in the differentiation status of Gag-specific memory T cells between 3 and 12 months, positive trends were however noted within the ED and LD compartments (Figure 3.12B). Such trends were also observed between the

differentiation status between 3 months and other later time points (Appendix K, Table K2, page 274). While total FD CD4⁺ T cells showed strong associations between 3 and 12 months, no significant associations were observed in Gag-specific FD cells. This could be due to the low or undetectable frequencies of Gag-specific FD cells within the CD4⁺ compartment. In summary, the significant associations in the differentiation phenotype at 3 and 12 months post-infection may suggest that the differentiation profile of total CD4⁺ T cells could have reached a steady-state of differentiation during primary HIV infection and that this appeared to be independent of the course of viraemia over the first year of infection (Figure 3.12A and B, open and closed symbols).

3.2.5.2 CD8⁺ T cells

The differentiation status of total CD8⁺ T cells at 3 months post-infection was also correlated with events at 4, 5, 6, 9, 12 and 15 months post-infection. When associating the differentiation profile of CD8⁺ T cells between 3 and 12 months PI, positive and significant associations were observed in the proportions of naïve, ED-, LD- and FD-memory cells ($r=0.79$, $p=0.001$; $r=0.89$, $p=0.0011$; $r=0.8$, $p=0.0072$ and $r=0.9$, $p=0.0005$, respectively; Figure 3.13A). No significant associations were found between total memory Int cells at 3 and 12 months post-infection. It is worth noting that the proportion of Int-memory cells at 3 months post-infection significantly associated with the proportion of Int-memory cells at 4, 5, 6 and 9 months PI (Appendix K, Table K3, page 275). Although the proportions of Int- and to some extent ED-memory CD8⁺ were found to have significantly decreased over time, while FD-memory CD8⁺ T cells increased, the significant associations between 3 and 12 months suggests that a steady-state of total CD8⁺ T cell differentiation was achieved during the early stages of infection.

A



B

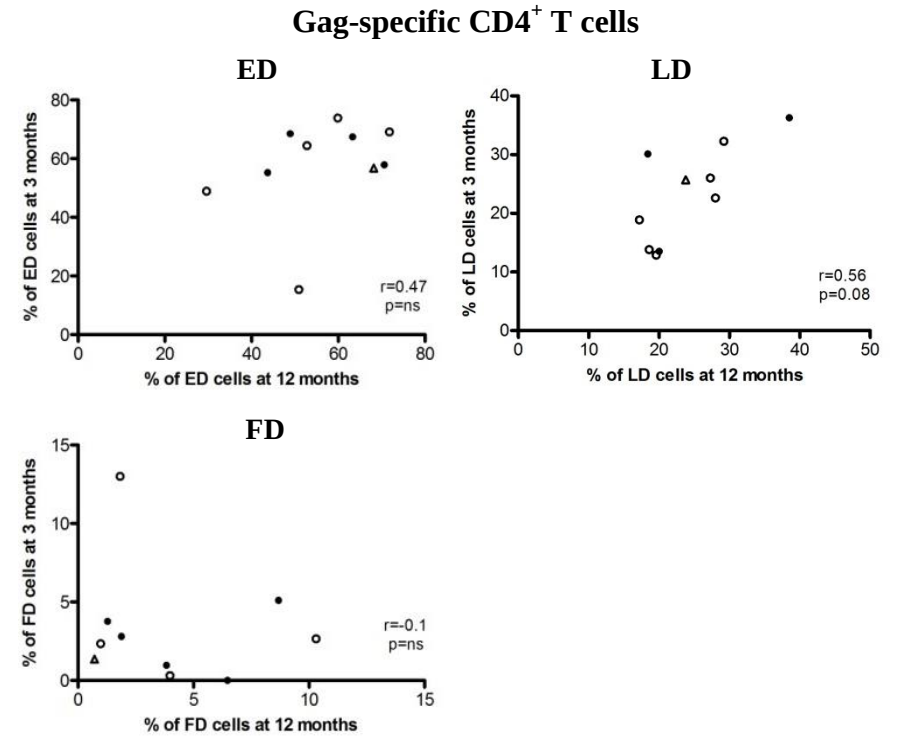


Figure 3.12: The relationship between the proportions CD4⁺ T cell subpopulations at 3 and 12 months post-infection. (A) Correlation between the proportions of total naïve, ED, LD and FD CD4⁺ T cells at 3 and 12 months post-infection. (B) Correlation between the proportion of Gag-specific memory ED, LD and LD CD4⁺ T cells at 3 and 12 months post-infection. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

With regard to Gag-specific memory cells, although the proportions of Int and FD cells at 3 months correlated with those at 12 months post-infection using unadjusted correlations ($r=0.75$, $p=0.025$ and $r=0.78$, $p=0.017$, respectively; Figure 3.13B), no associations were observed when adjusted with Bonferroni correction (adjusted p -value below α). No associations were found between the proportions of ED cells between 3 and 12 months ($r=0.66$, $p=0.058$). Similarly, no associations were found for the proportions of LD cells between 3 and 12 months post-infection. Significant associations however were observed between the proportions of LD cells between 3 months and 4 and 5 months PI using unadjusted correlation, however the significance was lost after adjusting with Bonferroni correction (Appendix K, Table K4, page 275). The loss of statistical significance after correction for multiple comparisons may be related to the small sample size. Although the positive associations between events at 3 months with later time-points suggests that a steady-state of memory status exists from 3 to 15 months post-infection, it can only be speculated that these associations reflect pre-existing and established events earlier than 3 months post-infection.

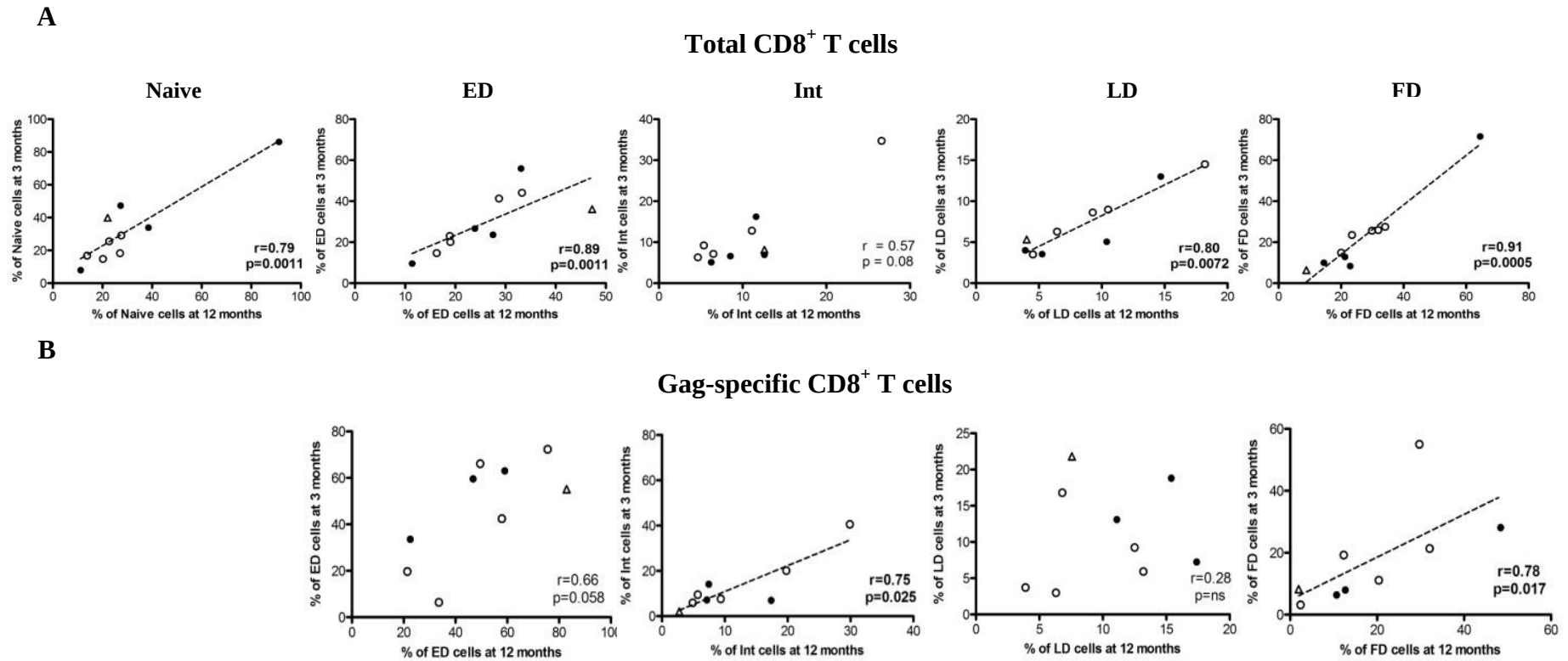


Figure 3.13: The relationship between the proportions of CD8⁺ T cell subpopulations at 3 and 12 months post-infection. (A) Correlation between the proportions of total naïve, ED, Int, LD and FD CD8⁺ T cells at 3 and 12 months post-infection. (B) Correlation between the proportion of Gag-specific ED, LD and LD memory CD8⁺ T cells at 3 and 12 months post-infection. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation

3.3 Discussion

An increasing body of evidence suggests that qualitative (e.g. polyfunction, activation and differentiation) rather than quantitative characteristics of T cells may be associated with the differences in the control of viraemia and HIV disease outcome (Papagno *et al.*, 2004, Betts *et al.*, 2006, Addo *et al.*, 2007, Almeida *et al.*, 2007). This chapter focuses on defining and understanding the differentiation profile of memory CD4⁺ and CD8⁺ T cells during primary HIV-1 infection, and a number of observations were made. It was found that CD4⁺ and CD8⁺ T cells can be separated into different differentiation subpopulations: naïve, early, late and fully differentiated cells based on their cell surface expression of CD27 and CD45RO. In addition, a unique subpopulation (intermediate cells) within the CD8⁺ compartment was also identified, and this subpopulation has also been previously reported by Burgers *et al.* (2009). These findings are comparable to what has been previously reported (De Rosa *et al.*, 2001, Appay *et al.*, 2002a, Emu *et al.*, 2005). The distinct memory T cell subpopulations have different proliferation, survival and homing capabilities (Hamann *et al.*, 1997, Sallusto *et al.*, 1999, Geginat *et al.*, 2001). For example, ED-memory (CD27⁺CD45RO⁺) T cells home to lymph nodes, whereas LD-memory (CD27⁻CD45RO⁺) cells are localized in the periphery (blood, and non-lymphoid tissues). With regard to cell phenotype and function, ED-memory cells are endowed with ability to proliferate, self-renew, but present little antiviral activity (Palmer *et al.*, 2004, Decrion *et al.*, 2007, Appay *et al.*, 2008). In contrast, LD- and/or FD-memory (CD27⁻CD45RO⁻) have reduced proliferative capacities, but mediate effector functions against pathogens and have powerful antiviral capabilities (Champagne *et al.*, 2001, Tomiyama *et al.*, 2002b, Romero *et al.*, 2007). FD-memory cells however, are also considered to be senescent and may die upon antigen re-stimulation (Brenchley *et al.*, 2003, Riou *et al.*, 2007).

Contrary to previous reports (Oswald-Richter *et al.*, 2007, Sakai *et al.*, 2010), when examining the differentiation status of total CD4⁺ T cells, surprisingly no differences were noted in the differentiation profile of HIV-infected individuals and HIV-uninfected controls. Thus, studies by Oswald-Richter *et al.* (2007) and Sakai *et al.* (2010), reported a skewing of total CD4⁺ subpopulations from naïve towards a more LD and FD status in HIV infected individuals. The explanation for these discrepancies are unclear, however it could be postulated that the differentiation status of total CD4⁺ T cells observed in HIV-infected individuals may be reflection of homeostatic responses/mechanisms that may be in place in order to maintain a certain number of CD4⁺ naïve and memory T cells. With regard to CD8⁺ T cells, HIV-infected individuals on the other hand presented lower proportions of naïve cells and higher proportions of ED- and/or FD-memory cells relative to HIV-uninfected individuals. These data are consistent with studies by Roederer *et al.* (1995), which showed decreased numbers of naïve T cells during HIV infection. The low frequencies of naïve CD8⁺ T cells have been attributed to thymic atrophy or involution caused by HIV (Kalayjian *et al.*, 2003). The elevated numbers of ED-, and/or FD-memory CD8⁺ T cells in early infected individuals, may be attributed to high and constant HIV antigen exposure, which could result in increased numbers of differentiated T cells (Papagno *et al.*, 2004). In addition, the loss of naïve cells may be attributed to continuous differentiation of CD8⁺ T cells out of the naïve phenotype driven by immune activation, bystander activation or antigen-specific activation. In line with previous studies (Harari *et al.*, 2002, Yue *et al.*, 2004, Jagannathan *et al.*, 2009), HIV-Gag-specific cells exhibited an early differentiated phenotype compared to CMV-specific cells that were mainly characterized by a late differentiated phenotype in both CD4⁺ and CD8⁺ compartments. The distinct differentiation profiles between HIV-Gag and CMV may be related to distinct antigen exposure and/or persistence (Kostense *et*

al., 2001, Tussey *et al.*, 2003, Harari *et al.*, 2004b, Bronke *et al.*, 2007). Additionally, the distinct differentiation profiles exhibited by different antigen-specific cells may also be associated with the phenotypic or functional characteristics that may be required to control the virus during infection. The failure of HIV-specific T cells to fully mature has been attributed to increased susceptibility to CD95/apoptosis (Mueller *et al.*, 2001). Furthermore the destruction of HIV-specific CD4⁺ T cell numbers have also been postulated to result in the lack differentiation in HIV infection (Rosenberg *et al.*, 1997, Champagne *et al.*, 2001). Further examination of antigen-specific cells, showed greater proportions of Gag- and/or CMV-specific memory CD4⁺ T cells than total CD4⁺ T cells within ED- and LD-memory subpopulations cells in HIV-infected and/or -uninfected individuals. In the CD8⁺ compartment, slightly higher proportions of Gag-specific memory ED cells compared to total memory ED were observed. Together, these findings suggest that antigen-specific memory cells present distinct differentiation profiles relative to total memory T cells

Consistent with previous findings (Northfield *et al.*, 2007, Mojumdar *et al.*, 2012) this chapter has shown a marked increase of CD57 expression within the fully differentiated CD4⁺ and CD8⁺ T cells than on late or early differentiated memory cells in both HIV-infected and -uninfected individuals. Furthermore, CD57 expression was most apparent within the intermediate and the fully differentiated CD8⁺ T cell subpopulations of early HIV-infected individuals compared to HIV-uninfected controls. These findings suggest that early HIV-1 replication could also contribute towards an increase in the expression of CD57 within the intermediate and fully differentiated T cell compartments. These data are in accordance with previous studies conducted by Mojumdar *et al.* (2011) showing elevated levels of CD57 within HIV-infected

individuals. Given that immunosenescent cells associate with an aged immune system and also display high rates of spontaneous apoptosis (Brenchley *et al.*, 2003, Palmer *et al.*, 2005) the elevated levels of the CD57⁺ cells in early infection may further indicate cellular dysfunction and subsequent HIV-1 pathogenesis.

The differentiation profile of naïve, ED- and FD-memory CD4⁺ T cells in HIV infected individuals was found to be stable over the first 15 months of HIV-1 infection. A similar analysis on CD8⁺ T cells, showed stable levels of naïve and LD-memory cells over time. There was however an accumulation of LD-memory CD4⁺ T cells and FD-memory CD8⁺ T cells over time, which could be related to constant antigen stimulation. Contrary to total CD4⁺ and CD8⁺ T cells, the differentiation profile of Gag-specific cells remained relatively stable during the course of early infection, suggesting that there may have been an establishment of steady-state differentiation of these cells prior to analysis. Consistent with a steady-state, significant and positive correlations were also apparent with total CD4⁺ and CD8⁺ T cells, suggesting that the differentiation profiles of total CD4⁺ and CD8⁺ T cells observed during the first 15 months of HIV infection may be a reflection of pre-existing and established events very early in infection. The differentiation profile of CMV-specific CD8⁺ T cells was found to increase within the first 15 months after HIV infection. These results are in line with observations (Fletcher *et al.*, 2005, Bronke *et al.*, 2007), which showed the skewing of CMV-specific T cells towards a late differentiated phenotype during the course of infection, and this skewing of the differentiation profile was linked with the repeated exposure to CMV antigen.

In conclusion, this chapter showed that HIV- and CMV-specific T cells display different memory differentiation profiles, whereby HIV-specific cells were mostly characterized by an early-differentiated phenotype. Furthermore, the differentiation profile of total memory cells was also found to be different compared to that of antigen-specific memory T cells. It was also evident that the memory T cell differentiation steady-state level appears to have been established during the early stages of HIV-1 infection. The next chapter will explore the levels of immune activation and how this relates to T cell memory differentiation.

CHAPTER 4: T CELL ACTIVATION

TABLE OF CONTENTS

4.1 Introduction.....	97
4.2 Results.....	98
4.2.1 Defining the activation profiles of memory T cells during at 3 months post-infection...	98
4.2.1.1 Activation profile of memory CD4 ⁺ T cells.....	98
4.2.1.2 Activation profile of memory CD8 ⁺ T cells.....	103
4.2.1.3 Comparison of the activation profile between memory CD4 ⁺ and CD8 ⁺ T cells ...	106
4.2.2 The relationship between CD4 ⁺ T cell activation and memory differentiation	109
4.2.2.1 Distribution of activation markers within memory CD4 ⁺ subpopulations	109
4.2.3 Susceptibility of Gag-specific and total activated memory CD4 ⁺ T cells to <i>in vivo</i> HIV-1 infection	117
4.2.4 The relationship between CD8 ⁺ T cell activation and memory differentiation	120
4.2.4.1 Distribution of activation markers within memory CD8 ⁺ subpopulations	120
4.2.3 Longitudinal changes in the activation profile of memory T cells during the course of HIV infection	125
4.2.3.1 CD4 ⁺ T cells.....	125
4.2.3.2 CD8 ⁺ T cells.....	128
4.2.4 Examination of the T cell memory activation steady-state.....	130
4.2.4.1 CD4 ⁺ T cells.....	130
4.2.4.2 CD8 ⁺ T cells.....	132
4.3 Discussion.....	134

4.1 Introduction

HIV-1 infection is characterized by generalized immune activation (Hazenbergh *et al.*, 2003), which is thought to be an import driver of CD4⁺ T cell decline and progressive immune deficiency (Sousa *et al.*, 2002, Choudhary *et al.*, 2007, Hunt *et al.*, 2008). Although stimulation of the immune system is likely to result in anti-viral immunity that could play a role in suppression of viral replication, constant stimulation of the immune system could lead to increased T cell turnover, loss of T cell regenerative capacity and subsequent immune exhaustion (Liu *et al.*, 1997, Hazenbergh *et al.*, 2000a, Appay and Sauce, 2008). Immunologic and virologic events following acute or early infection have been associated with the subsequent rate of disease progression (Pantaleo *et al.*, 1997, Mellors *et al.*, 2007, Streeck *et al.*, 2009). However, much of the current knowledge on persistent immune activation is based on events occurring during the chronic stages of HIV infection (Hazenbergh *et al.*, 2003, Hunt *et al.*, 2008, Catalfamo *et al.*, 2011). Moreover, there has been limited work measuring the simultaneous expression of activation and differentiation markers on both total and HIV-specific T cells (Papagno *et al.*, 2004, Barbour *et al.*, 2009). Examining and understanding the activation events during early HIV-1 infection may provide key information on how acute or early HIV-1 replication influences immune activation, and thus may also provide prognostic value or development of therapeutic strategies targeting hyperimmune activation. During acute or early/primary HIV infection, intense viral replication may result in increased stimulation of CD4⁺ and CD8⁺ T cells, and thus causing excessive T cell activation. It is therefore hypothesized **that early HIV-1 infection is characterized by elevated activation of both total and HIV-specific CD4⁺ and CD8⁺ memory T cells**. To examine this, early HIV-infected and -uninfected cohorts (Chapter 2, page 51-52) were used to (i) examine and compare the status of total memory T cell activation

between early HIV-infected and -uninfected individuals; (ii) compare the activation status of different antigen-specific memory T cells (Gag- and CMV-specific) in HIV-infected and -uninfected individuals, and (iii) investigate the longitudinal patterns of total and antigen-specific memory T cells over 15 months of HIV-1 infection. Whilst persistent immune activation is a feature of chronic HIV infection, the dynamics of immune activation from the early to the late stages to infection remains to be resolved. There has been limited work in examining the relationship between T cell activation and T cell memory differentiation in early HIV-1 infection and how this contributes toward disease outcome. Findings in chapter 3 showed an accumulation of fully differentiated CD8⁺ T cells during early HIV-1 infection. It was therefore further hypothesized that activation of T cells during early HIV infection drives the differentiation of T cells toward a more differentiated phenotype. The focus of this chapter will explore the relationship between T cell activation and memory differentiation during primary HIV-1 infection.

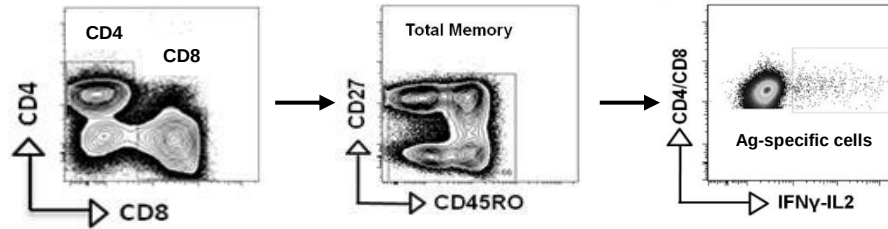
4.2 Results

4.2.1 Defining the activation profiles of memory T cells during at 3 months post-infection

4.2.1.1 Activation profile of memory CD4⁺ T cells

Using the memory and activation panel (Chapter 2, Section 2.4.2, page 56), Figure 4.1 shows representative dot plots, in which distinct populations of activated (CD38 and HLA-DR) and proliferating (Ki67) memory T cells could be discerned. HIV-Gag and CMV-specific memory T responses are given in Chapter 3, Table 3.1 page 69). To understand the impact of HIV-1 infection on the profile of activated T cells, the proportions of total memory activated cells were compared between early HIV-infected individuals and HIV-uninfected controls (Figure 4.2A).

A



B

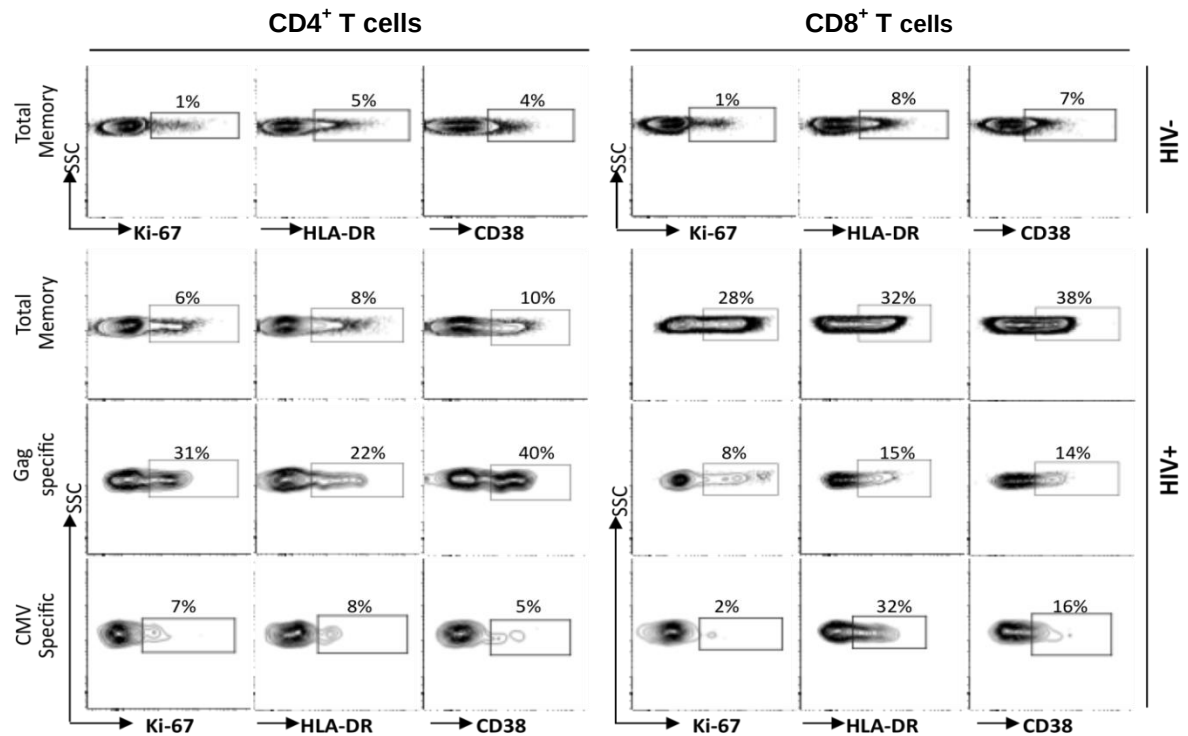


Figure 4.1: Gating scheme for activated total and antigen-specific memory CD4⁺ and CD8⁺ T cells in HIV-infected and -uninfected controls. Polychromatic flow cytometry was used to determine the activation profile in HIV-infected and HIV-uninfected controls based on the surface expression of CD38 and HLA-DR and intracytoplasmic expression of Ki67 in total and antigen-specific memory T cells. Initial gating was done by the removal of doublets, dead cells, CD14⁺ and CD19⁺ cells from the analysis. The total CD4⁺ and CD8⁺ T cell populations were based from the lymphocyte gate population (FSC-H and SSC-A), and (A) total memory T cells were then subsequently gated from the CD27 and CD45RO gate. Antigen-specific T cells were gated from either a CD4⁺/CD8⁺ versus an IFN γ -IL2 gate. (B) Representative contour plots showing expression levels of CD38, HLA-DR and Ki67 on total and antigen-specific CD4⁺ and CD8⁺ memory T cells. Naïve T cells (CD27⁺CD45RO⁻) were removed from the analysis.

During primary HIV-1 infection, the proportions of total memory CD38, HLA-DR and Ki67 CD4⁺ T cells were found to be significantly higher compared to HIV-uninfected controls (p=0.0006, p=0.007 and p<0.0001 respectively, Figure 4.2A). There were higher expression levels of CD38 and Ki67 on Gag-specific memory CD4⁺ T cells, compared to CMV-specific cells from HIV-infected individuals (p=0.0001 and p=0.0002, respectively, Figure 4.2B). In turn, CMV-specific CD4⁺ T cells from HIV-infected individuals were more activated compared to CMV-specific CD4⁺ T cells of HIV-uninfected controls (CD38: p=0.03; Ki67:p=0.01). There were no significant differences in the expression levels of HLA-DR between Gag and CMV-specific cells.

A

B

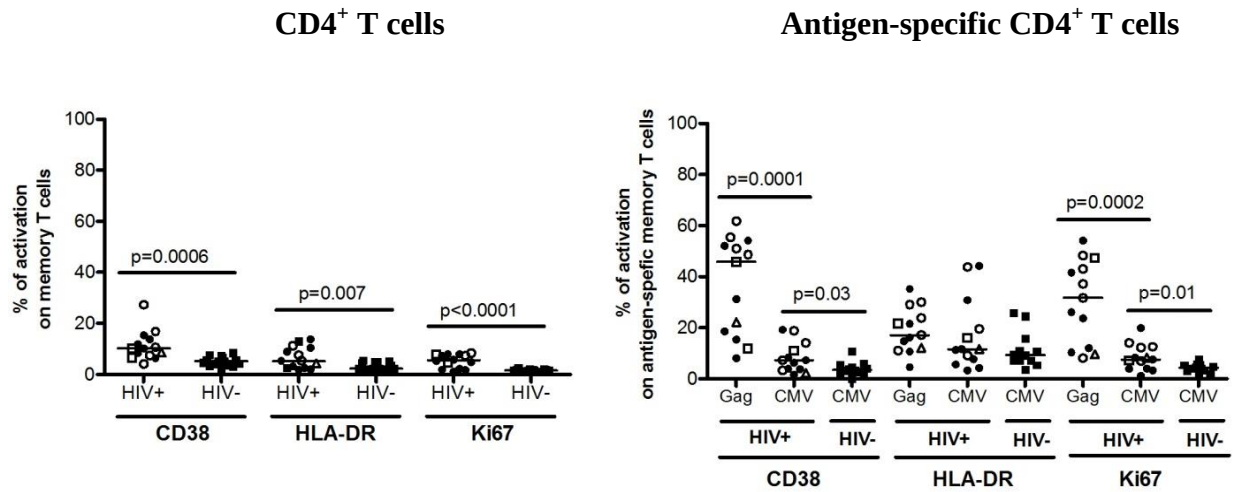


Figure 4.2: The activation profile of total and antigen-specific memory CD4⁺ T cells in HIV-infected and -uninfected controls. Comparison between the proportion of activation markers on (A) total memory, and (B) antigen-specific CD4⁺ T cells, from HIV-infected (n=14) and -uninfected controls (n=15, ■). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by either Mann-Whitney *U* non-parametric *t* test.

Comparison of activation levels between total memory and antigen-specific CD4⁺ T cells showed that Gag-specific cells were significantly more activated and proliferating than total memory CD4⁺ T cells in HIV-infected individuals (CD38: $p<0.001$; HLA-DR: $p<0.0001$ and Ki67: $p<0.0001$) (Figure 4.3A). The expression level of HLA-DR on CMV-specific memory cells was significantly higher compared to total memory CD4⁺ T cells in both HIV-infected ($p<0.05$) and -uninfected ($p=0.0003$) individuals (Figure 4.3A and B). The increased proportions

of antigen-specific T cells expressing CD38, HLA-DR and Ki67 compared to total memory T cells may suggest that virus-induced activation is a major contributor to the marked activation and expansion status of antigen-specific T cells. Furthermore, these data also show that Gag-specific CD4⁺ T cells during early HIV-1 infection are characterized by an activated phenotype (using CD38) and also show a higher proliferative potential than CMV-specific memory CD4⁺ T cells. These data reflect differing T cell dynamics between two distinct viral infections in the same individual.

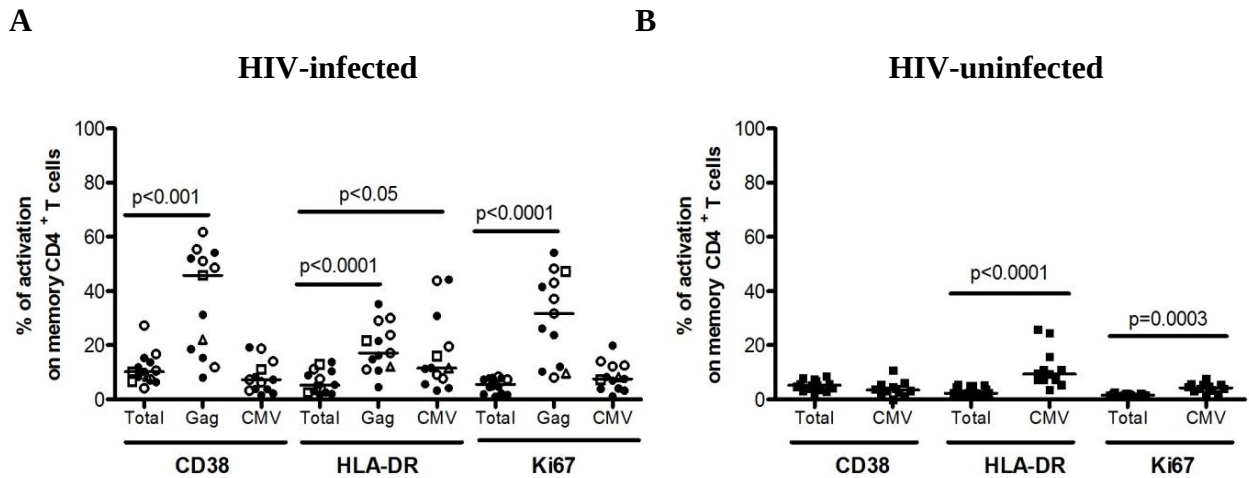


Figure 4.3: Comparison of the activation profiles of total with Gag- and CMV-specific memory CD4⁺ T cells in both HIV-infected and -uninfected controls. The comparison of the frequencies of activation between total memory and antigen-specific CD4⁺ T cells in (A) HIV-infected individuals (n=14), and (B) HIV-uninfected controls (n=15, ■). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5log₁₀ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within ±0.5log₁₀ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase >0.5log₁₀ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months Median values are shown as a horizontal bar. Statistical comparisons were performed by non-parametric One-way ANOVA.

4.2.1.2 Activation profile of memory CD8⁺ T cells

For the CD8⁺ T cell compartment, the activation profile of total memory cells in early HIV-infected individuals was also found to be significantly higher compared to HIV-uninfected controls (CD38: $p < 0.0001$; HLA-DR: $p = 0.0064$ and Ki67: $p < 0.0001$, Figure 4.4A). With regard to antigen-specific memory cells, there was higher CD38 expression on Gag-specific cells compared to CMV-specific cells ($p = 0.002$) within HIV-infected individuals (Figure 4.4B). No differences were observed with the expression on HLA-DR and Ki67 between Gag- and CMV-specific cells. However, comparative analysis between CMV-specific cells showed a higher expression of HLA-DR and Ki67 in HIV-infected individuals than in HIV-uninfected subjects ($p = 0.001$ and $p = 0.036$, respectively).

A

B

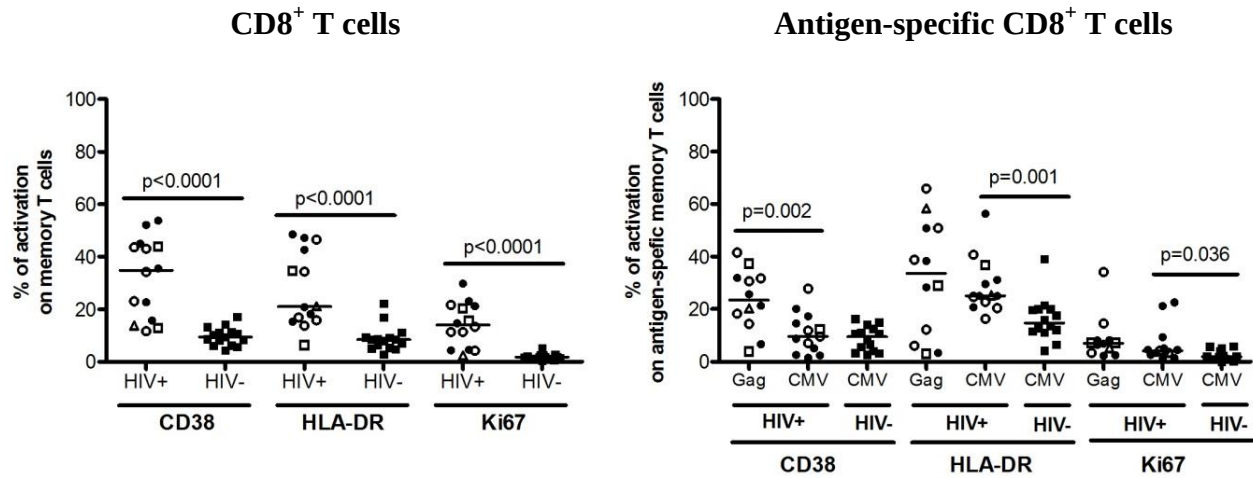


Figure 4.4: The activation profile of total and antigen-specific memory CD8⁺ T cells in HIV-infected and -uninfected controls. Comparison between the frequencies of activation markers on (A) total memory, and (B) antigen-specific memory CD8⁺ T cells, from HIV-infected (n=14) and -uninfected controls (n=15, ■). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by either Mann-Whitney *U* or non-parametric *t* test.

By comparing the activation levels of total memory to antigen-specific memory cells, significant differences were noted where there was a lower expression of CD38 and Ki67 on CMV-specific cells compared to total memory cells in HIV-infected individuals ($p < 0.001$ and $p < 0.001$, respectively, Figure 4.5A). Although there were no statistical significant differences between the expression levels of HLA-DR on antigen-specific cells, the median value of Gag-specific cells expressing HLA-DR was higher compared to total memory cells (median: 33.6% vs. 17.15%). In

HIV-uninfected individuals, the level of HLA-DR on CMV-specific cells was significantly higher than total memory cells ($p=0.0064$) whilst the level of activation (using CD38) and proliferation (using Ki67) was found to be comparable between total memory and CMV-specific memory cells (Figure 4.5B).

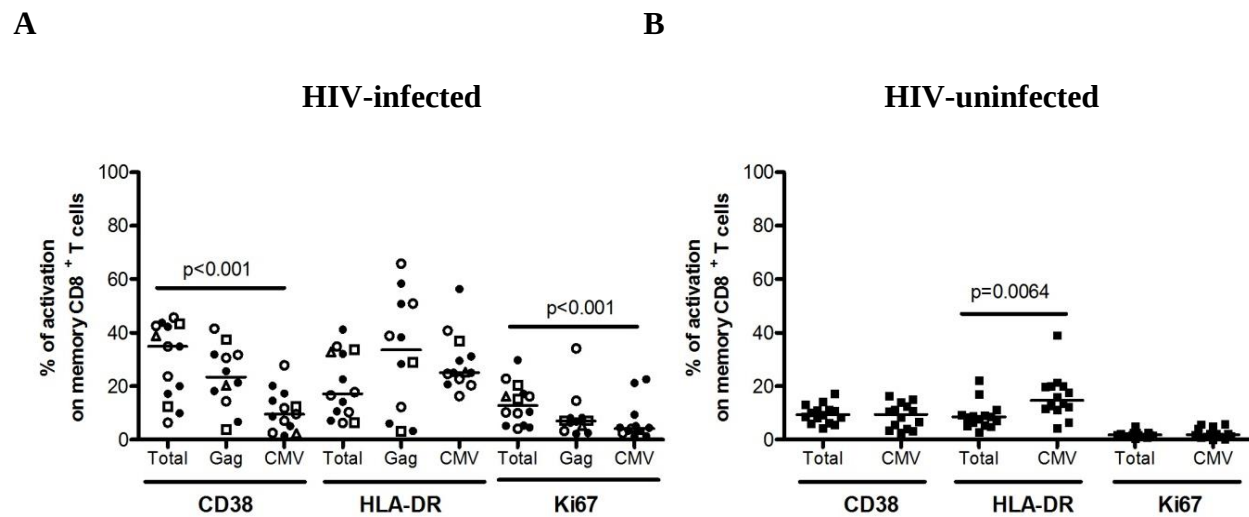


Figure 4.5: Comparison of the activation profiles of total with Gag- and CMV-specific memory CD8⁺ T cells in both HIV-infected and -uninfected controls. The comparison of the frequencies of activation between total memory and antigen-specific CD8⁺ T cells in (A) HIV-infected ($n=14$), and (B) HIV-uninfected controls ($n=15$, ■). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by non-parametric One-way ANOVA.

Overall, data from the CD8⁺ T cell compartment also confirms that there was a generalized increase of activated total memory T cells during early HIV-1 infection as opposed to HIV-uninfected controls. Additionally, CMV-specific cells within the HIV-infected individuals showed a highly activated and proliferative phenotype (HLA-DR and Ki67) compared to CMV-specific cells within the HIV-uninfected controls. The increased numbers of activated CMV-specific T cells in HIV-infected individuals may also be attributed to direct consequences of virus-specific activation, accompanied by other factors such as generalized bystander activation.

4.2.1.3 Comparison of the activation profile between memory CD4⁺ and CD8⁺ T cells

Previous studies have shown that upon antigen stimulation, expansion and/or responses appear to differ between CD4⁺ and CD8⁺ T cells (Foulds *et al.*, 2002), and thus understanding the activation profiles between CD4⁺ and CD8⁺ T cells during primary HIV-1 infection may be essential when developing T cell-based vaccines. To explore this, the proportions of activated cells were compared between memory CD4⁺ T cells and CD8⁺ T cells. Figures 4.6A and B show that total memory CD8⁺ T cells are significantly more activated than total memory CD4⁺ T cells in both HIV-infected (CD38: $p=0.0002$; HLA-DR: $p<0.0001$ and Ki67: $p=0.0072$) and HIV-uninfected controls (CD38: $p=0.0003$ and HLA-DR: $p<0.0001$). However, in HIV-uninfected individuals, Ki67 expression levels were found to be similar between CD4⁺ and CD8⁺ T cell subsets. The dissimilarity in the activation status of CD4⁺ and CD8⁺ cell compartments, may highlight distinct mechanisms of activation, which involve homeostatic and TCR-specific expansion (Catalfamo *et al.*, 2008) or differences in their intrinsic proliferative capacity (Foulds *et al.*, 2002).

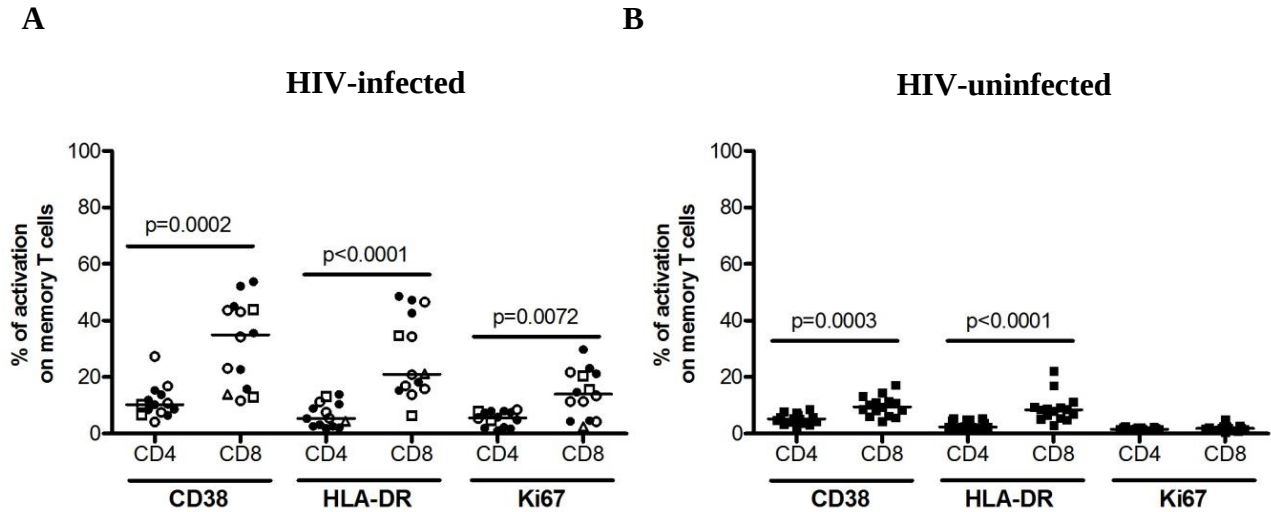


Figure 4.6: Comparison of the activation levels between memory CD4⁺ and CD8⁺ T cells in HIV-infected and HIV-uninfected controls. The comparison of the proportions of activated cells between total memory CD4⁺ and CD8⁺ T cells in (A) HIV-infected individuals (n=14) and (B) HIV-uninfected controls (n=15, ■). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5log₁₀ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within ±0.5log₁₀ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase >0.5log₁₀ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months Median values are shown as a horizontal bar. Statistical comparisons were performed by a Mann-Whitney *U* non-parametric *t* test.

Further analysis of antigen-specific cells showed high expression of HLA-DR on CMV-specific CD8⁺ T cells compared to CMV-specific CD4⁺ T cells within HIV-infected individuals (Figure 4.7A). The level of Ki67 expression was found to be significantly higher (p=0.0004) within Gag-specific CD4⁺ T cells relative to Gag-specific CD8⁺ T cells, suggesting that HIV-Gag specific CD4⁺ T cells have a higher turnover than HIV-Gag-specific CD8⁺ T cells during primary infection. Furthermore, there was a trend towards elevated proportions of Gag-specific

CD38⁺CD4⁺ T cells (median: 45.8%) than Gag-specific CD38⁺CD8⁺ T cells (median: 23.4%) (p=0.09). Collectively, the marked activation levels HIV-Gag specific CD4⁺ T cells could be attributed to preferential targeting of these cells by HIV (Douek *et al.*, 2002). Moreover the heightened activation status of Gag-specific memory CD4⁺ T cells could subsequently result in increased numbers of target cells that could ultimately favour HIV-1 replication during the early stages of infection.

With regard to HIV-uninfected controls, the proportion of activated cells expressing CD38 was significantly higher (p=0.01) on CMV-specific CD8⁺ T cells than CMV-specific CD4⁺ T cells (Figure 4.7B). The expression of HLA-DR was also found to be slightly higher on CMV-specific CD8⁺ T cells compared to CD4⁺ T cells (p=0.06). These data reflect distinct virus-specific T cell activation between CD4⁺ and CD8⁺ T cell subsets. Additionally, CMV-specific CD4⁺ T cells appeared to be expanding more (Ki67: p=0.03) than CMV-specific CD8⁺ T cells within the HIV-uninfected controls, and thus the elevated frequencies of proliferating CMV-specific CD4⁺ T cells may also be related to increased CMV-specific CD4⁺ T cell proliferative responses directed against the persistent CMV antigens during CMV-infection (Sester *et al.*, 2002). Collectively, these data show that: 1) regardless of HIV infection, there are differences in activation profile of total memory CD8⁺ and CD4⁺ T cells, whereby CD8⁺ populations seem to experience extensive expansion over CD4⁺ T cells; 2) the distinct activation profiles of antigen-specific T cells between CD4⁺ and CD8⁺ T cell compartments may be directly related to antigen-induced activation.

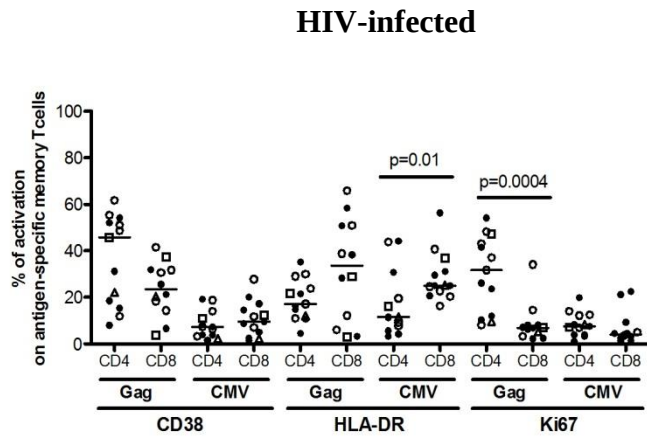
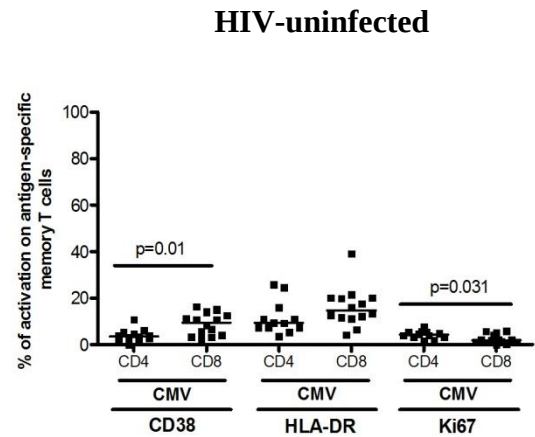
A**B**

Figure 4.7: Comparison of the activation levels between antigen-specific memory $CD4^+$ and $CD8^+$ T cells in HIV-infected and -uninfected controls. Comparing the proportions of activated cells between antigen-specific memory $CD4^+$ and $CD8^+$ T cells in (A) HIV-infected individuals ($n=14$), and (B) HIV-uninfected controls ($n=15$, ■). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months Median values are shown as a horizontal bar. Statistical comparisons were performed by a Mann-Whitney U non-parametric t test.

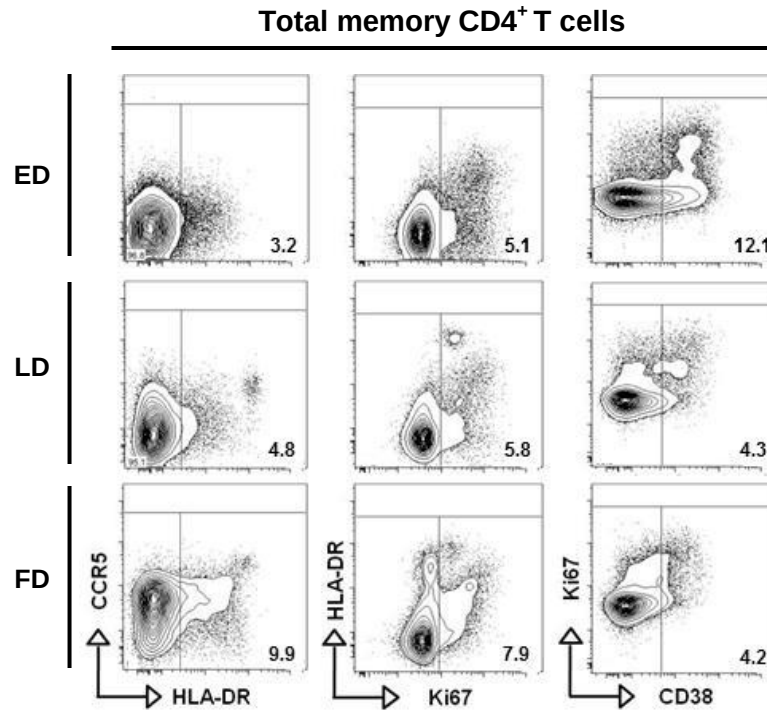
4.2.2 The relationship between $CD4^+$ T cell activation and memory differentiation

4.2.2.1 Distribution of activation markers within memory $CD4^+$ subpopulations

Immune activation is thought to play a major role in driving T cell memory differentiation (Papagno *et al.*, 2004, Burgers *et al.*, 2009). To examine whether this process was apparent during primary HIV-1 infection, Boolean gating was used to measure and identify the pattern of activated T cells within different memory T cell subpopulations. Boolean analysis identified eight T cell patterns by creating every possible combination of the three individual activation

markers (CD38, HLA-DR and Ki67). The profile of these eight activation T cell patterns were assessed and compared between the different stages of memory differentiation (ED, LD, FD and Int cells for CD8⁺ T cells) in both total and antigen-specific cells. Representative pie charts were used to illustrate the profile of activated cells within each memory T cell subpopulation. For this analysis, naive cells were gated out (as expression of activation markers are low to absent) and the focus was to assess the different T cell phenotypes on total memory cells (Figure 4.8). Figure 4.9 shows proportions of triple- (e.g. CD38⁺HLADR⁺Ki67⁺) in red, double- (e.g. (CD38⁺HLADR⁻Ki67⁺) in orange, single-positive (e.g. CD38⁺HLADR⁻Ki67⁻) in yellow and triple-negative combinations (e.g. CD38⁻HLADR⁻Ki67⁻) in green at the single cell level within the ED, LD and FD of total memory CD4⁺ T cells. The level of activated cells was found to be similar across each memory subpopulation in both HIV-infected individuals and HIV-uninfected controls. However, the comparison of activated cells between HIV-infected individuals and -uninfected controls, showed significantly larger proportions of activated CD4⁺ T cells (represented as pie distributions of 0, 1, 2 or 3 permutations) across the ED, LD and FD of HIV-infected individuals than HIV-uninfected controls (p=0.0001, p<0.0002 and p=0.0052, respectively). Commensurate with the higher activation status, there were significantly less triple negative cells (CD38⁻HLADR⁻Ki67⁻) in HIV-infected individuals when compared with uninfected controls). In summary, these data show that activated CD4⁺ T cells were; a) uniformly distributed across ED-, LD- and FD-memory CD4⁺ T cells, and b) more populous in HIV-infected individuals

A



B

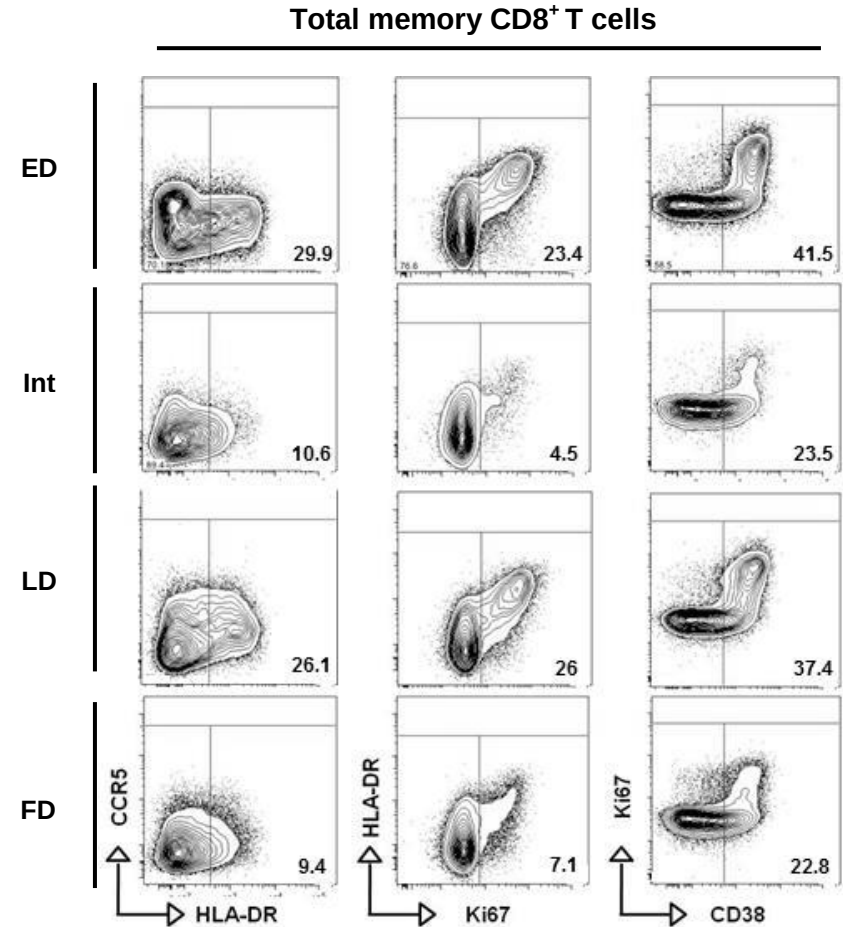


Figure 4.8: Gating strategy of activated cells within the ED-, Int-, LD- and FD-memory T cell subpopulations. After gating CD4⁺ and CD8⁺ populations, total memory cells were gated from a CD27 versus a CD45RO gate, followed by the gating of ED-, Int-, LD- and FD-memory subpopulations. Naïve cells (CD27⁺CD45RO⁻) were removed from the analysis. The level of T cell activation was then gated within the different memory subpopulations. Representative contour plots showing the level of activation within the memory (A) CD4⁺, and (B) CD8⁺ T cell subpopulations.

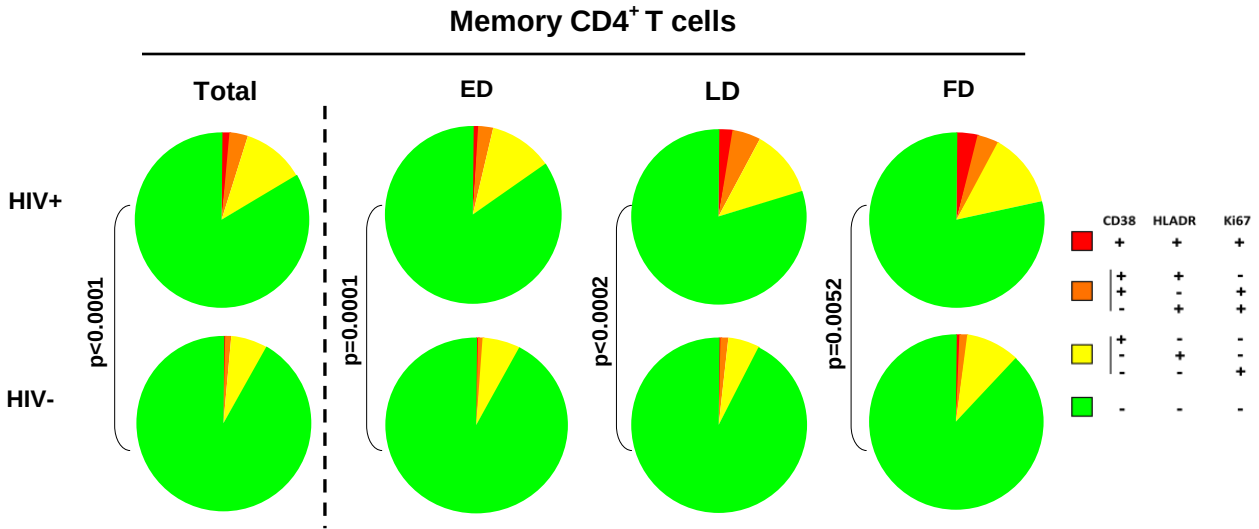


Figure 4.9: Activation profiles of the different memory CD4⁺ T cell subpopulations at 3 months post-infection.

Boolean gating analysis was used to assess the activation profile of total ED-, LD and FD-memory CD4⁺ T cell subsets in HIV-infected (n=14) and HIV-uninfected (n=15) controls. Activated T cells within each subset is represented as pie charts where red corresponds to the frequency of cells expressing all three CD38, HLADR and Ki67 markers; orange corresponds to the frequency of cells expressing two of the three markers (i.e.: CD38⁺HLADR⁺Ki67⁻; CD38⁺HLADR⁻Ki67⁺ and CD38⁺HLADR⁺Ki67⁺); yellow represents the frequency of cells expressing at last one of the activation markers (CD38⁺HLADR⁻Ki67⁻; CD38⁺HLADR⁺Ki67⁻ and CD38⁺HLADR⁻Ki67⁺) and green corresponds to the frequency of cells not expressing any of the markers (CD38⁻HLADR⁻Ki67⁻, triple negative). Statistical comparisons were performed in Spice (version 4.3) using permutation analysis of the pie distributions on proportions of single- double, triple-positive and triple negative cells. *P* values are shown for comparisons between HIV-infected and -uninfected individuals.

Similar observations were made for antigen specific memory populations, where no significant differences were observed with the proportions of activated T cells across each memory CD4⁺ subpopulations of either CMV- or Gag-specificities (Figure 4.10). However, Gag-Specific memory CD4⁺ T cells were characterized by significantly higher levels of activation relative to

CMV-specific cells within the LD cells in HIV-infected individuals ($p=0.0347$). There was also an increasing trend in the proportion of activated Gag-specific memory ED $CD4^+$ T cells compared to CMV-specific memory ED cells ($p=0.058$). No differences were observed in the activation profile of FD cells between Gag- and CMV-specific memory $CD4^+$ T cells. The proportions of activated Gag-specific memory $CD4^+$ T cells within the different differentiation stages of HIV-infected individuals were found to be significantly higher compared to CMV-specific cells of HIV-uninfected controls (ED; $p<0.0001$, LD; $p=0.0001$, and FD; $p=0.018$). The proportions of activated CMV-specific memory cells were also found to be significantly higher in HIV-infected individuals than in HIV-uninfected individuals.

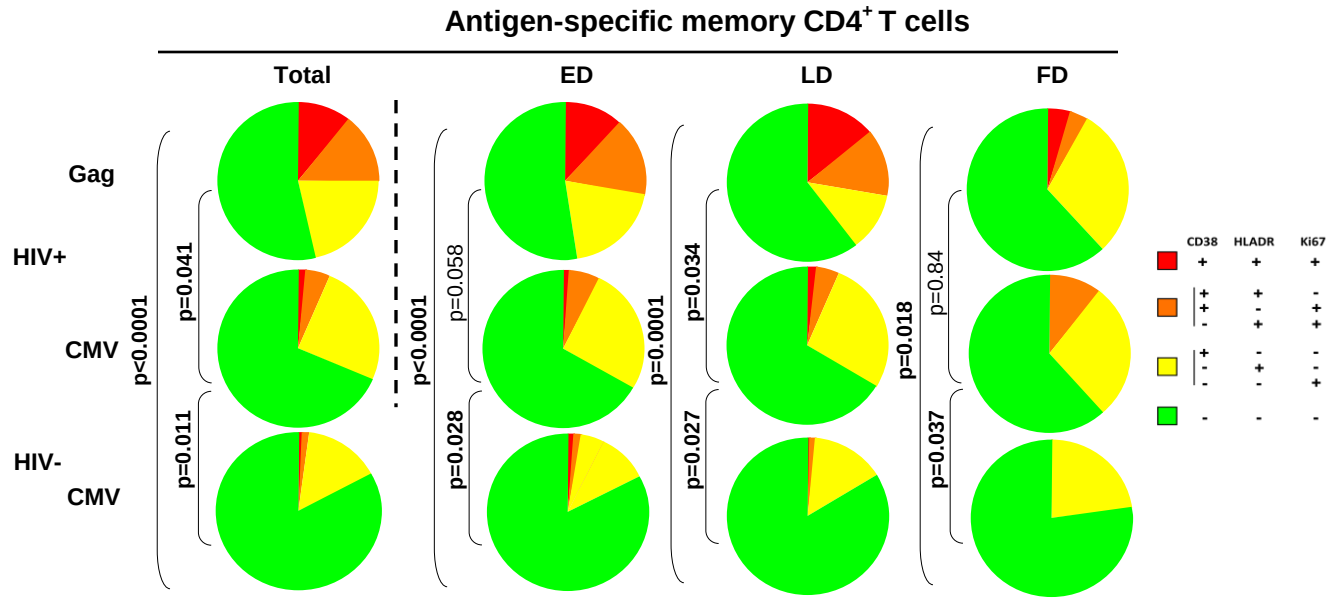
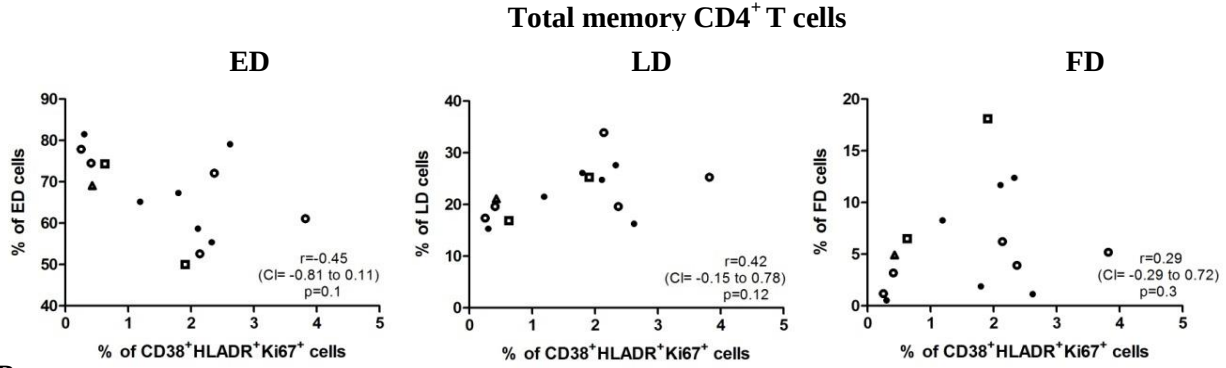


Figure 4.10: Activation profiles of the antigen-specific CD4⁺ T cells within different memory subpopulations at 3 months post-infection. Boolean gating analysis was used to assess the activation profile of antigen-specific ED-, LD and FD-memory CD4⁺ T cell subsets in HIV-infected (n=14) and HIV-uninfected (n=15) controls. The level of T cell activation within each subset is represented as pie charts where red corresponds to the frequency of cells expressing all three CD38, HLADR and Ki67 markers; orange corresponds to the frequency of cells expressing two of the three markers; yellow represents the frequency of cells expressing at least one of the activation markers and green corresponds to the frequency of cells not expressing any of the markers. Statistical comparisons were performed in Spice (version 4.3) using permutation analysis of the pie distributions, and *P* values are shown for comparisons between HIV-infected and -uninfected individuals.

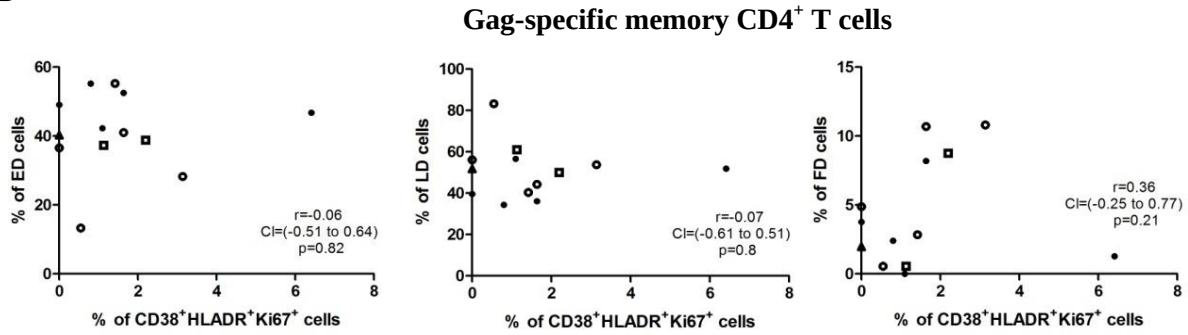
These data demonstrate equal distribution of activated total and Gag-specific cells across all the measured stages of memory subset differentiation, and this would suggest that activation signals may not be directly linked with CD4⁺ T cell memory differentiation. To further investigate this, the proportions of highly activated total memory CD4⁺ T cells (cells that express CD38, HLA-DR and Ki67 simultaneously) were correlated with the proportions of total memory T cell

subpopulations. Although a negative trend was observed between total memory ED cells and activated cells, there were no significant associations between the proportions of highly activated total memory CD4⁺ T cells with ED, LD or FD total memory subpopulations (Figure 4.11A). This held true for the correlations between Gag-specific (Figure 4.11B) and CMV-specific memory CD4⁺ T cells (Figure 4.11C) with ED, LD or FD antigen-specific T cells, suggesting that the activation status of both total and antigen-specific (Gag and CMV) CD4⁺ T cells are not necessarily linked to the memory status of CD4⁺ T cells.

A



B



C

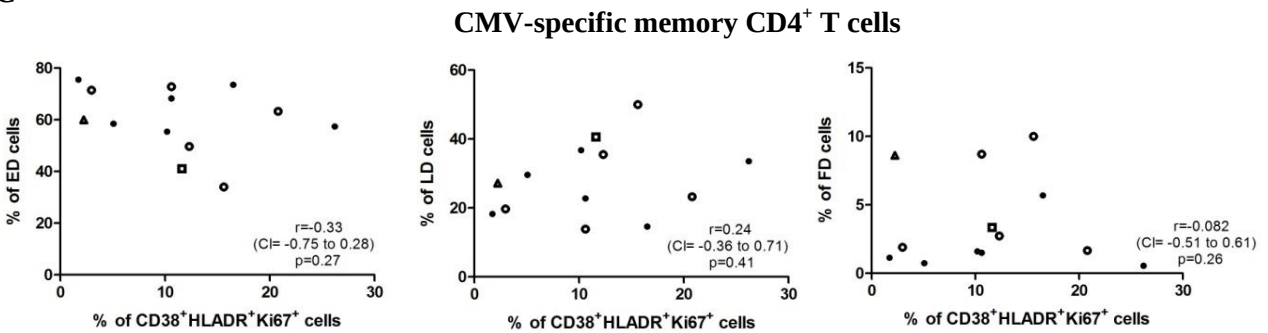


Figure 4.11: The relationship between the activation and the memory differentiation status of CD4⁺ T cells at 3 months-post infection. Correlations between proportions of highly activated cells (CD38⁺HLADR⁺Ki67⁺) and the proportions of ED, LD and FD cells in (A) total, (B) Gag- and (C) CMV-specific memory CD4⁺ T cells. Open circles (O) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months Median values are shown as a horizontal bar. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

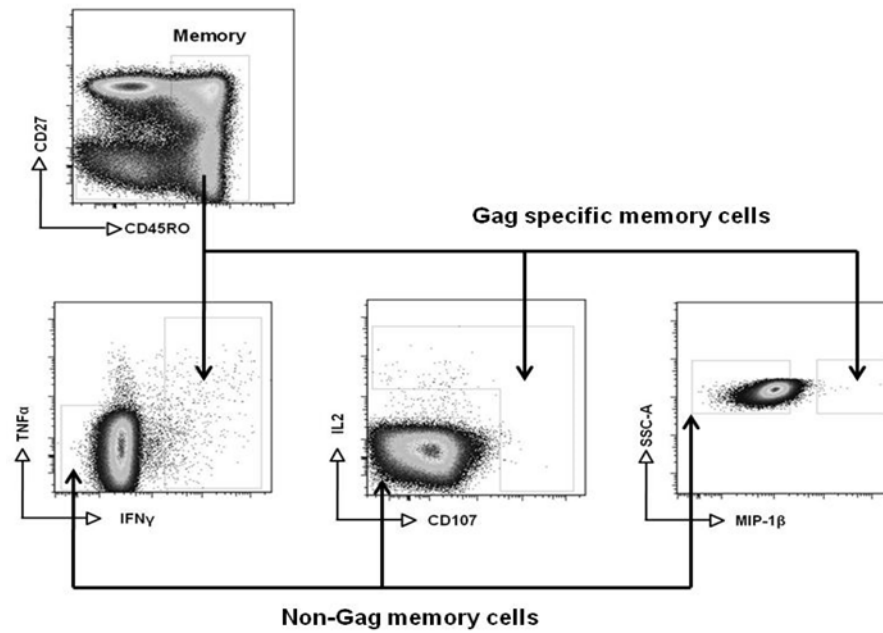
4.2.3 Susceptibility of Gag-specific and total activated memory CD4⁺ T cells to *in vivo* HIV-1 infection

A study conducted by Zhang *et al.* (1999) previously showed that activated CD4⁺ T cells harbour more SIV RNA compared to quiescent cells, which could then lead to the spread infection towards neighbouring cells (Zhang *et al.*, 2004). Moreover, it has also been shown that T cell activation signals are required in order for the virus to infect cells (resting or activated cells) (Oswald-Richter *et al.*, 2004). There is however limited data on the susceptibility of activated cells during primary HIV-1 infection and how this relates to HIV-1 replication. In an attempt to understand the underlying relationships between T cell activation and viraemia, this section sought to examine the *in vivo* susceptibility of activated memory CD4⁺ T cells to the HIV-1 virus, by comparing the levels of proviral *gag* DNA within activated and non-activated memory T cells.

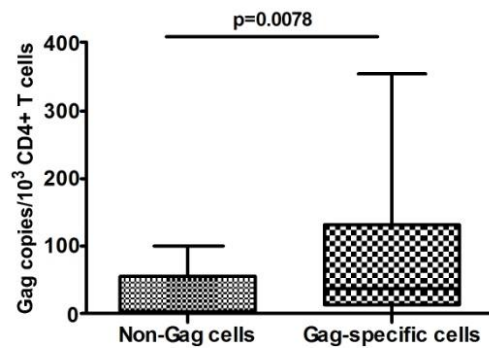
Firstly, HIV-specific CD4⁺ T cells were sorted by tightly gating on CD45RO⁺CD107⁺IFN- γ ⁺IL-2⁺MIP-1 β ⁺TNF α ⁺ cells outside the range of background staining, which was always less than 0.05% (Figure 4.12A). Although sorting of virus-specific is usually done by the use of tetramers and/or pentamers, sorting of virus-specific T cells based on cytokine secretion has also been shown in previous studies (Casazza *et al.*, 2009). How these distinct procedures affect the yield and/or quality of cell associated HIV-DNA is unclear and further studies may be warranted to clarify this. The level of proviral *gag*-DNA was then quantified using quantitative real time PCR (Chapter 2, Section 2.7.2.1, page 60), where proviral *gag*-DNA within Gag-specific memory cells was compared to non-Gag-specific memory cells (CD107⁻IFN γ ⁻IL2⁻MIP-1 β ⁻TNF α ⁻). Figure 4.12B shows significantly high levels of proviral *gag*-DNA within Gag-specific cells relative to

non-Gag-specific memory CD4⁺ T cells, and in line with what has been previously shown (Douek *et al.*, 2002). Secondly, the *in vivo* susceptibility of activated memory CD4⁺ T cell subpopulations were assessed by sorting on any of the three activation markers, CD38, HLA-DR or Ki67, and CD45RO⁺. The number of *gag* proviral DNA copies/cell were compared to non-activated memory CD4⁺ T cells (CD38⁻HLADR⁻Ki67⁻CD45RO⁺) assessed as described (Chapter 2, Section 2.7.2, page 59). Although found at very low frequencies of CD4⁺ T cells (0.15 *gag* copies/cell maximum), activated cells possessed significantly higher quantities of *gag* proviral copies when compared to with sorted non-activated cell fractions (p=0.04) (Figure 4.12C).). Collectively, these data directly show directly that; a) both HIV-specific and total activated memory CD4⁺ T cells are preferred targets for *in vivo* HIV-1 replication, and b) that the activation of memory CD4⁺ T cells support ongoing viral replication during primary HIV-1 infection.

A



B



C

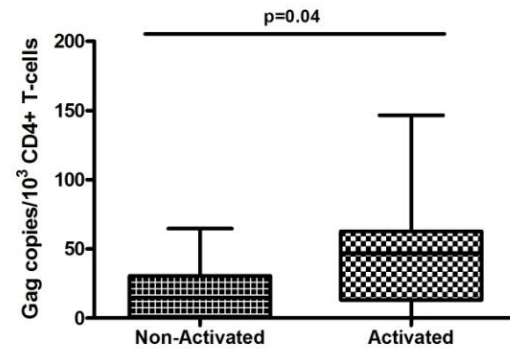


Figure 4.12: HIV-1 infection of Gag-specific and total memory activated CD4 $^{+}$ T cells at 3 months post-infection. (A) Representative dot plots showing the sorting strategy of memory CD4 $^{+}$ T cell subpopulations. (B) Comparison of the quantity of proviral *gag*-DNA copies between non-Gag memory and Gag-specific (based on the expression of CD107, IFN γ , IL2, MIP-1 β and TNF α in response to HIV-Gag peptides) memory CD4 $^{+}$ T cell subpopulations (n=8). (C) Comparing of the level of proviral *gag*-DNA within non-activated (CD38 $^{+}$ HLA-DR $^{+}$ Ki67 $^{-}$) and activated (cells expressing at least one activation marker) memory CD4 $^{+}$ T cells at 3 months post-infection (n=8). Statistical significance was performed by Wilcoxon signed-rank *t* test.

4.2.4 The relationship between CD8⁺ T cell activation and memory differentiation

4.2.4.1 Distribution of activation markers within memory CD8⁺ subpopulations

Measurement of activated cells within CD8⁺ T cell memory subpopulations showed increased proportions of activated cells within the ED and LD memory compartments relative to the Int and FD compartments (Figure 4.13). There were no significant differences observed in activation levels between ED and LD subpopulations or between Int and FD subpopulations. No significant differences were found in the level of T cell activation across different memory subpopulations in HIV-uninfected controls. The comparison of activation levels between different memory subpopulations in HIV-infected and -uninfected controls showed greater proportions activated ED and LD ($p=0.0001$ and $p=0.0001$) in HIV-infected individuals.

When examining the distribution of activated antigen-specific cells in memory subpopulations, the activated Gag-specific CD8⁺ T cells were found to be uniformly distributed across the memory CD8⁺ T cell subpopulations and no significant differences observed between ED, Int, LD and FD cells (Figure 4.14). There was however an increasing trend in the proportions of activated cells within the ED-memory cells relative to Int-, LD- and FD-memory cells. Similar findings were observed when comparing the profiles of activated CMV-specific cells, where there was an even spread of activation markers across memory subpopulations in HIV-infected and -uninfected individuals. However, when comparing different antigen specificities, proportions of activated Gag-specific ED cells in HIV infected individuals were found to be significantly higher than activated CMV-specific memory ED cells ($p=0.048$) in HIV-uninfected controls.

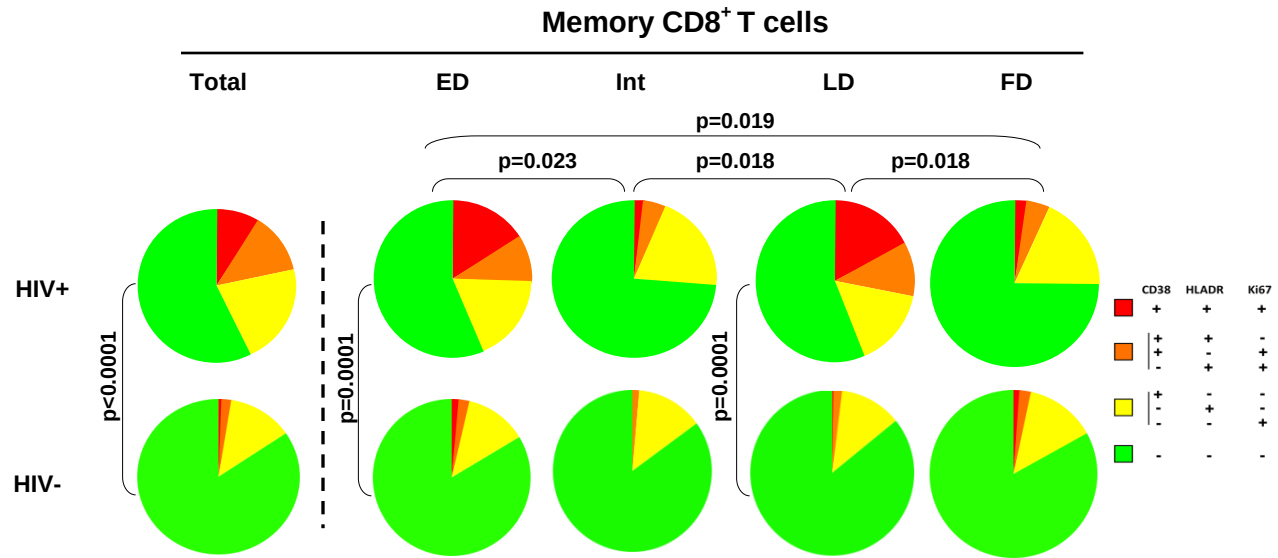


Figure 4.13: Activation profiles of the different memory CD8⁺ T cell subpopulations at 3 months post-infection. Boolean gating analysis was used to assess the activation profile of total ED-, LD and FD-memory CD8⁺ T cell subpopulations in HIV-infected (n=14) and HIV-uninfected (n=15) controls. The proportions of activated cells in each subpopulation is represented as pie charts where red corresponds to the frequency of cells expressing all three CD38, HLADR and Ki67 markers; orange corresponds to the frequency of cells expressing two of the three markers (i.e.: CD38⁺HLADR⁺Ki67⁺; CD38⁺HLADR⁺Ki67⁻ and CD38⁺HLADR⁻Ki67⁺); yellow represents the frequency of cells expressing at last one of the activation markers (CD38⁺HLADR⁻Ki67⁻; CD38⁻HLADR⁺Ki67⁻ and CD38⁻HLADR⁻Ki67⁺) and green corresponds to the frequency of cells not expressing any of the markers (CD38⁻HLADR⁻Ki67⁻, triple negative). Statistical comparisons were performed in Spice (version 4.3) using permutation analysis of the pie distributions on proportions of single- double, triple-positive and triple negative cells. *P* values are shown for comparisons between HIV-infected and -uninfected individuals.

Further examination of the relationships between activated CD8⁺ T cells with T cell differentiation showed a positive trend between total memory ED cells and highly activated cells ($r=0.45$, $p=0.09$, Figure 4.15A). Figure 4.15A also shows a positive correlation between the proportions of memory LD cells and an inverse correlation for FD cells with total memory CD38⁺HLADR⁺Ki67⁺ CD8⁺ T cells ($r=0.57$, $p=0.03$ and $r=-0.54$, $p=0.04$). No associations were

found between the proportions of intermediate cells with the level of total memory T cell activation. In summary, the positive associations between LD cells and highly activated cells could suggest that activation may be driving the differentiation of total CD8⁺ T cells towards a late differentiated memory status. On the other hand, an inverse relationship found between FD cells and activated cells, may be a reflection of cells that are in a state of exhaustion or a senescent state within the FD compartment (Barber *et al.*, 2006, Trautmann *et al.*, 2006). With regard to antigen-specific cells, no associations were observed between the levels of activated Gag-specific CD8⁺ T cells with the proportions of Gag-specific memory ED, Int, LD and FD cells (Figure 4.15B). The proportion of CMV-specific ED cells were found to correlate with CMV-specific CD38⁺HLADR⁺Ki67⁺ cells ($r=0.73$, $p=0.0065$), both CMV-specific Int and FD cells negatively correlated with activation ($r=0.66$, $p=0.019$ and $r=-0.68$, $p=0.021$) (Figure 4.15C). Collectively, these findings suggest that the activation of Gag-specific CD8⁺ T cells may not be influencing the differentiation profile of Gag-specific memory CD8⁺ T cells during primary HIV-1 infection. On the other hand, activation appears to play a role in the differentiation profile of CMV-specific memory CD8⁺ T cell responses.

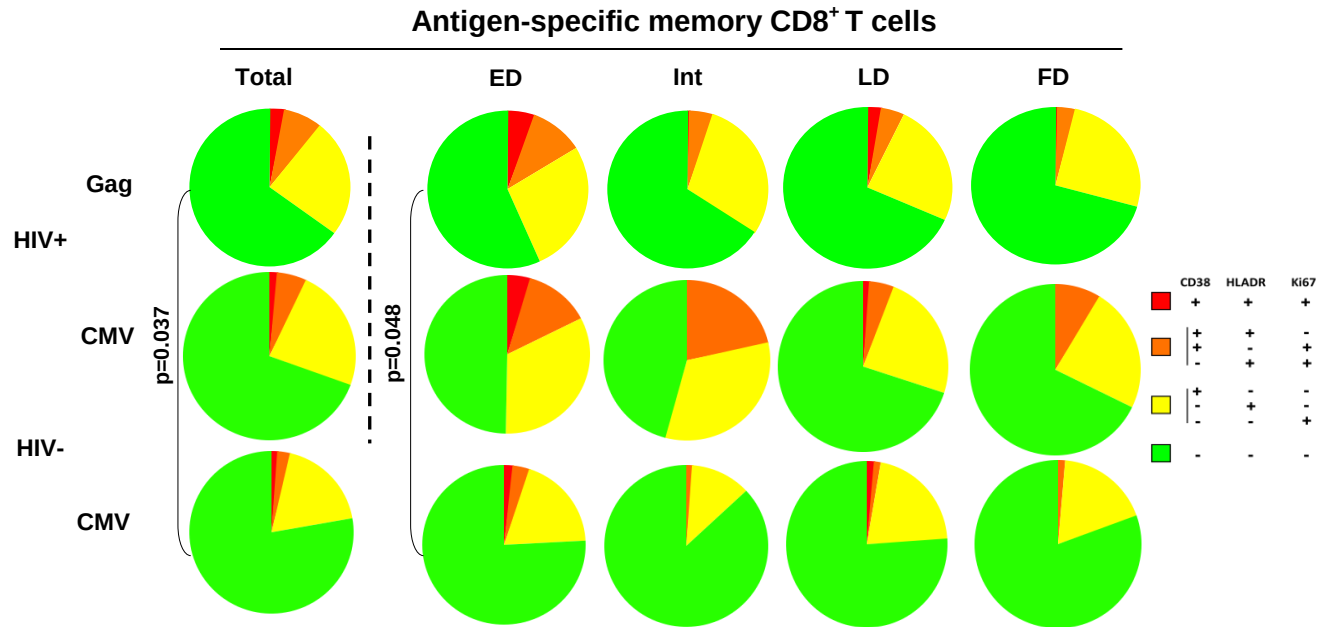
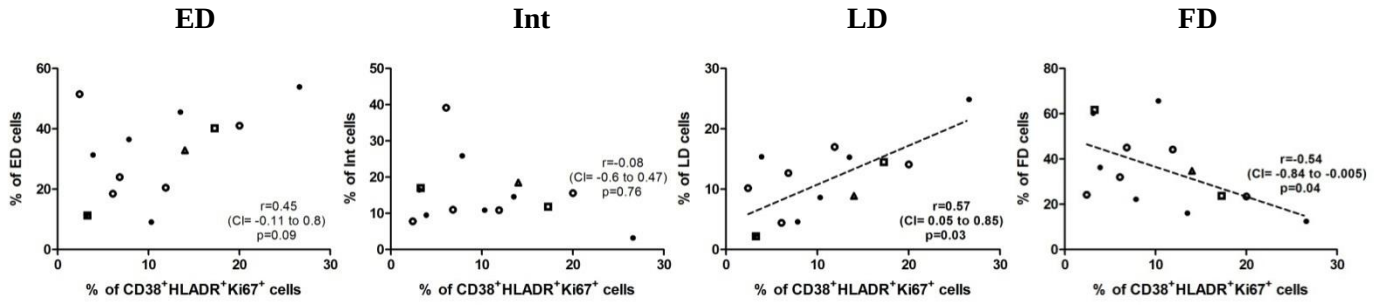


Figure 4.14: Activation profiles of the antigen-specific CD8⁺ T cells within different memory subpopulations at 3 months post-infection. Boolean gating analysis was used to assess the activation profile of antigen-specific ED-, LD and FD-memory CD8⁺ T cell subsets in HIV-infected (n=14) and HIV-uninfected (n=15) controls. The proportions of activated cells in each subset is represented as pie charts where red corresponds to the frequency of cells expressing all three CD38, HLADR and Ki67 markers; orange corresponds to the frequency of cells expressing two of the three markers (i.e.: CD38⁺HLADR⁺Ki67⁻; CD38⁺HLADR⁻Ki67⁺ and CD38⁻HLADR⁺Ki67⁺); yellow represents the frequency of cells expressing at least one of the activation markers (CD38⁺HLADR⁻Ki67⁻; CD38⁻HLADR⁺Ki67⁻ and CD38⁻HLADR⁻Ki67⁺) and green corresponds to the frequency of cells not expressing any of the markers (CD38⁻HLADR⁻Ki67⁻, triple negative). Statistical comparisons were performed in Spice (version 4.3) using permutation analysis of the pie distributions on proportions of single- double, triple-positive and triple negative cells. *P* values are shown for comparisons between HIV-infected and -uninfected individuals.

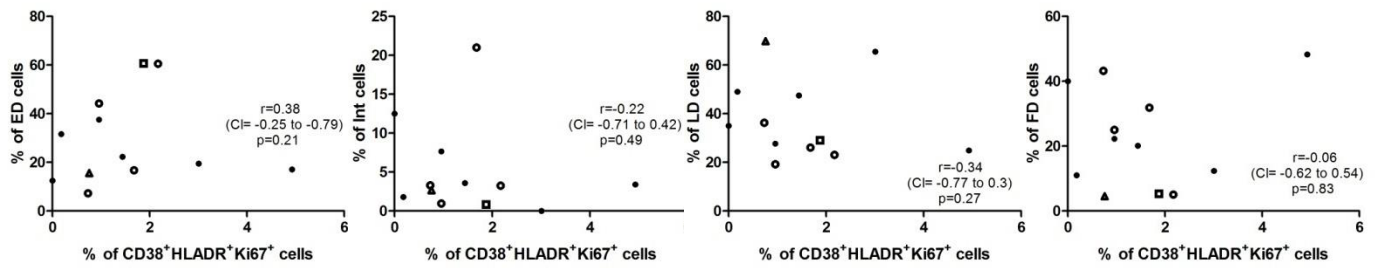
A

Total memory CD8⁺ T cells



B

Gag-specific CD8⁺ T cells



C

CMV-specific memory CD8⁺ T cells

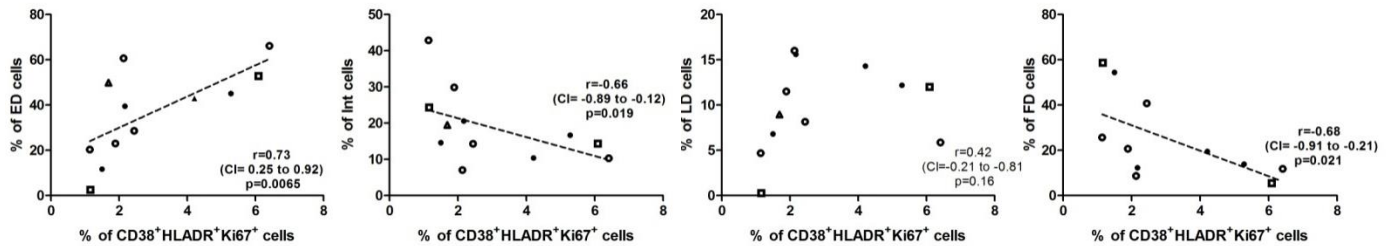


Figure 4.15: The relationship between the activation and the memory differentiation status of CD8⁺ T cells at 3 months-post infection.

Correlations between proportions of highly activated cells (CD38⁺HLADR⁺Ki67⁺) and the proportions of ED, LD and FD cells in (A) total, (B) Gag-specific, and (C) CMV-specific memory CD8⁺ T cells.

Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months Median values are shown as a horizontal bar.

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

4.2.3 Longitudinal changes in the activation profile of memory T cells during the course of HIV infection

4.2.3.1 CD4⁺ T cells

Persistent immune activation has been associated with the gradual loss of CD4⁺ T cell numbers and poor disease outcome in untreated HIV infection (Liu *et al.*, 1998, Grossman *et al.*, 2002). In an attempt to further understand the role of T cell activation in HIV pathogenesis, this chapter seeks to explore the dynamics of CD38, HLA-DR and Ki67 over the first 15 months of HIV-1 infection. Since the single expression of activation markers CD38 and HLA-DR may differ during the course of HIV infection (Kestens *et al.*, 1992), this chapter sought examine single expression of CD38 and HLA-DR rather than double positive cells (CD38⁺HLA-DR⁺) over time. The proportions of activated T cells were examined at 3, 4, 5, 6, 9, 12 and 15 months post-infection and analysed using a linear mixed-effects model across all time points. Slopes were used to determine significant changes of the T cell memory activation over time (Chapter 2, Model 1, page 62). Figure 4.16 shows that the profiles of activated total memory CD4⁺ T cells are widely dispersed, especially for cells expressing Ki67 over the period of investigation. However, there were no significant changes in the profile of total memory CD4⁺ T cells co-expressing HLA-DR and Ki67 over time. The proportions of Ki67, however, trended upwards over time, but was not significant (slope=0.0088, p=0.06). However, there was a significant increase in proportions of total memory CD4⁺ T cells expressing CD38 (slope=0.018, p<0.0001).

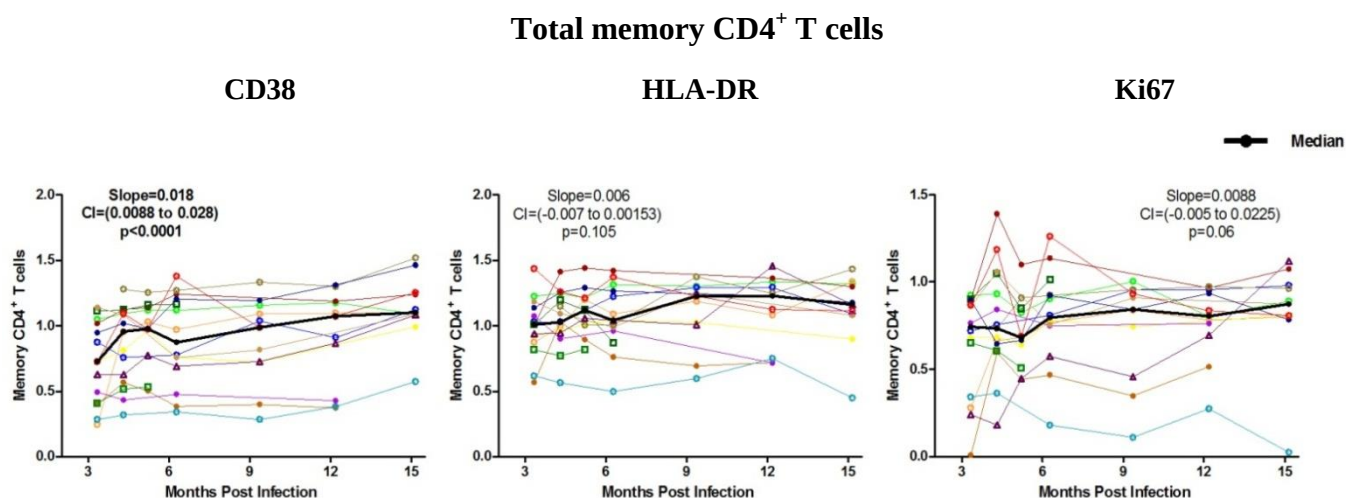


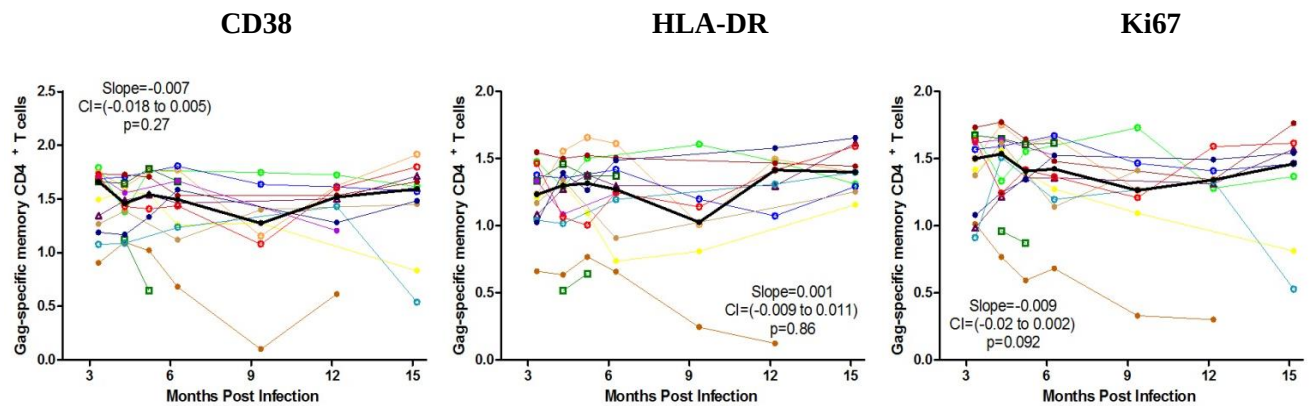
Figure 4.16: The longitudinal patterns of activated memory CD4⁺ T cells over the first 15 months of HIV infection. Longitudinal changes in the log expression of CD38, HLA-DR and Ki67 in HIV infected individuals (n=15) over time was determined using a linear mixed-effects model. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). The median change across time is represented by a bold sold line. Patient-specific colour-coded lines show proportions of activated cells at each time point, and coloured lines are consistent for patients in each represented graph.

Analysis of Gag-specific specific memory CD4⁺ T cells showed no significant changes in the proportion of cells expressing CD38, HLA-DR and Ki67 over time (Figure 4.17A). Persisting pathogens such as CMV may also participate in the activation and expansion of T cells during infection, and thus examining the dynamics of activated CMV-specific T cells during the course of HIV infection can be used to understand the impact of other infections on immune activation in HIV-1 infection. The activation of profile of CMV-specific memory CD4⁺ T cells showed a significant increase in the proportions of cells expressing CD38 and HLA-DR over time (slope=0.021, p=0.02 and slope=0.024, p<0.0001) (Figure 4.17B). The proportions of CMV-specific cells expressing Ki67, however, showed no significant changes over time. The increase

in the proportions of activated CMV-specific memory cells may be a reflection of generalized immune activation during HIV-1 infection or possibly reactivation of latent CMV.

A

Gag-specific memory CD4⁺ T cells



B

CMV-specific memory CD4⁺ T cells

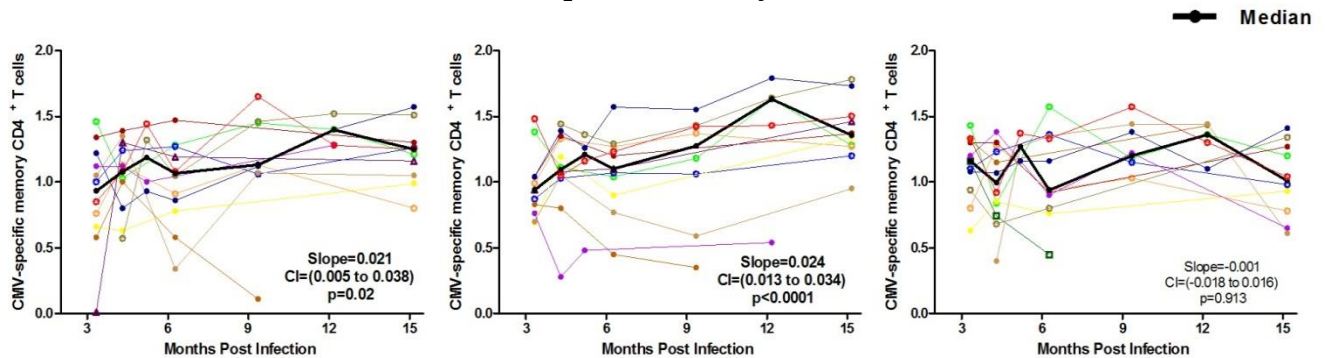


Figure 4.17: The longitudinal changes on the expression of CD38, HLA-DR and Ki67 on antigen-specific CD4⁺ T cells during the first 15 months of HIV infection. Longitudinal profile of (A) Gag- and (B) CMV-specific memory CD4⁺ T cells expressing log CD38, HLA-DR and Ki67 over time were determined with a linear multilevel model (n=15). The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). The median change across time is represented by a bold sold line. Patient-specific colour-coded lines show proportions of activated cells at each time point, and coloured lines are consistent for patients in each represented graph.

4.2.3.2 CD8⁺ T cells

Total memory CD8⁺ T cells were marked by a significant decrease in the proportion of activated cells expressing CD38 over time (slope=-0.012, p=0.001) (Figure 4.18). There was no change in the proportion of activated cells expressing HLA-DR over time. The proportions of activated total memory CD8⁺ T cells were also widely dispersed between time points. Of note, there was also a significant decrease in the proportion of proliferating (Ki67⁺) memory CD8⁺ T cells over time (slope=-0.017, p<0.001), which postulated from the CD57 staining (Chapter 3, Section 3.2.3, page 78) may reflect the replicative senescence of cells (Bestilny *et al.*, 2000, Papagno *et al.*, 2004). With regard to Gag-specific memory CD8⁺ T cells, there were no significant changes in the proportion of cells expressing CD38, HLA-DR, and Ki67 over time (Figure 4.19A). Activated CMV-specific memory CD8⁺ T cells also remained stable over time (Figure 4.19B).

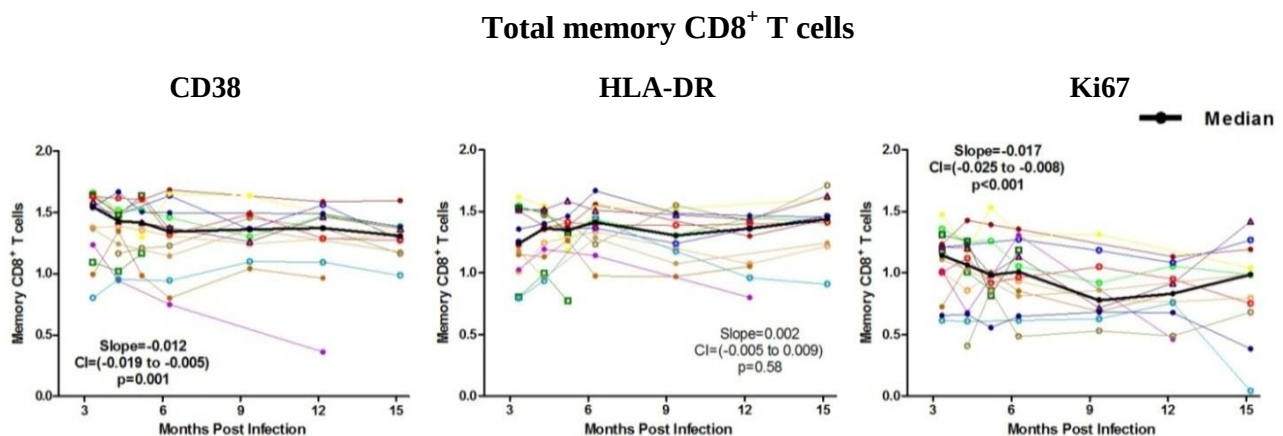
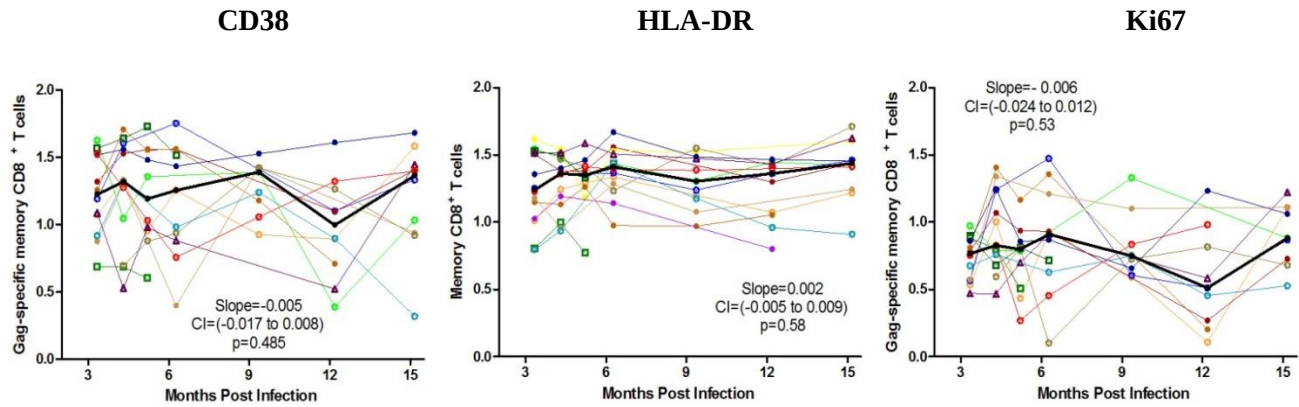


Figure 4.18: The longitudinal patterns of activated memory CD8⁺ T cells over the first 15 months of HIV infection. Longitudinal changes in the log expression levels of CD38, HLA-DR and Ki67 in HIV infected individuals (n=15) were determined with a linear multilevel model. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coded lines show proportions of activated cells at each time point, and coloured lines are consistent for patients in each represented graph.

A

Gag-specific memory CD8⁺ T cells



B

CMV-specific memory CD8⁺ T cells

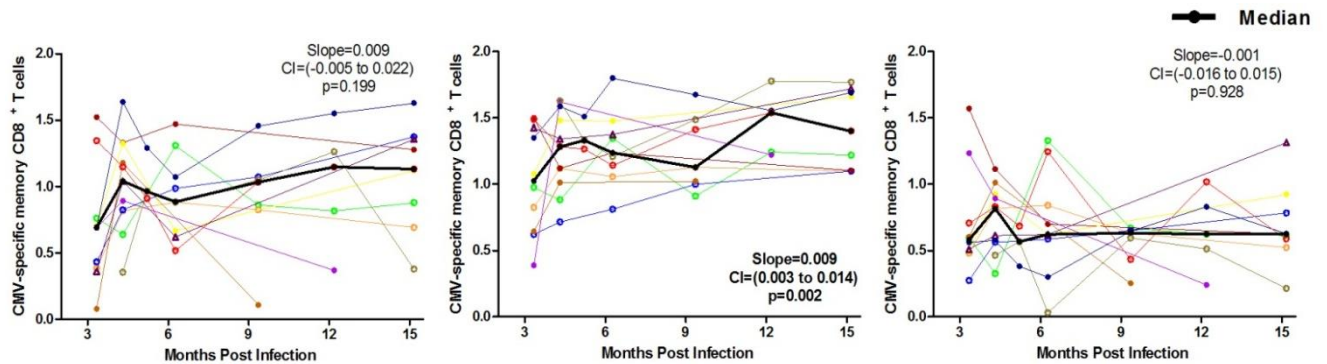


Figure 4.19: The longitudinal changes on the expression of CD38, HLA-DR and Ki67 on antigen-specific CD8⁺ T cells during the first 15 months of HIV infection. Longitudinal profile of (A) Gag- and (B) CMV-specific memory CD8⁺ T cells expressing log CD38, HLA-DR and Ki67 over time were determined with a linear multilevel model (n=15). The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coded lines show proportions of activated cells at each time point, and coloured lines are consistent for patients in each represented graph.

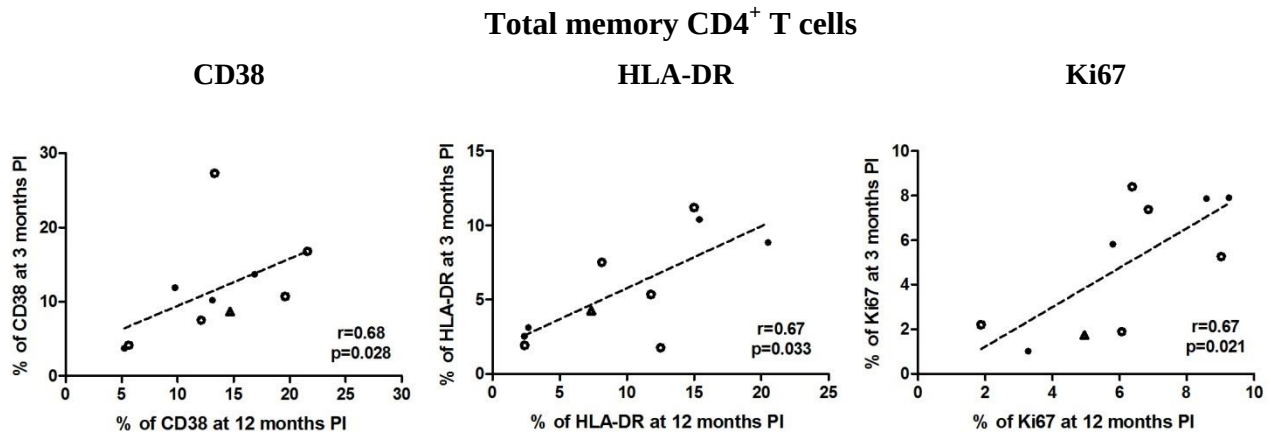
4.2.4 Examination of the T cell memory activation steady-state

4.2.4.1 CD4⁺ T cells

To investigate whether a steady-state of T cell activation was achieved, proportions of activated total memory CD4⁺ T cells at 3 months post-infection were correlated with those at 4, 5, 6, 9, 12 and 15 months post-infection. The correlations shown in this chapter are shown between 3 and 12 months post-infection, whilst a complete table showing all the associations between the proportions of activated cells between 3 months and all-time points (4, 5, 6, 12 and 15) are shown in Appendix L, Table L1, page 276. These multiple comparisons were also adjusted by Bonferroni correction. Figure 4.20A shows positive associations between 3 and 12 months for measurements of total memory CD4⁺ CD38⁺, HLA-DR⁺ and Ki67⁺ when using unadjusted correlations ($r=0.68$, $p=0.028$, $r=0.67$, $p=0.033$ and $r=0.67$, $p=0.021$, respectively). However, statistical significance was lost when adjusted using Bonferroni correction and the loss statistical significance may be related to low sample size.

Similar analysis of antigen-specific memory CD4⁺ T cells, showed a positive and significant association between Gag-specific cells expressing CD38 at 3 and 12 months measurements with unadjusted correlations ($r=0.7$, $p=0.01$, Figure 4.20B). Again the statistical significance did not hold when adjusted according Bonferroni method. No significant correlations were found between the proportion of Gag-specific memory CD4⁺ T cells expressing HLA-DR and Ki67 at 3 and 12 months post-infection (Appendix L, Table L2, page 276). Gag-specific memory CD4⁺ T cells expressing Ki67 at 3 months post-infection were found to correlate with those at 5 months PI ($r=0.81$, $p=0.0072$, respectively).

A



B

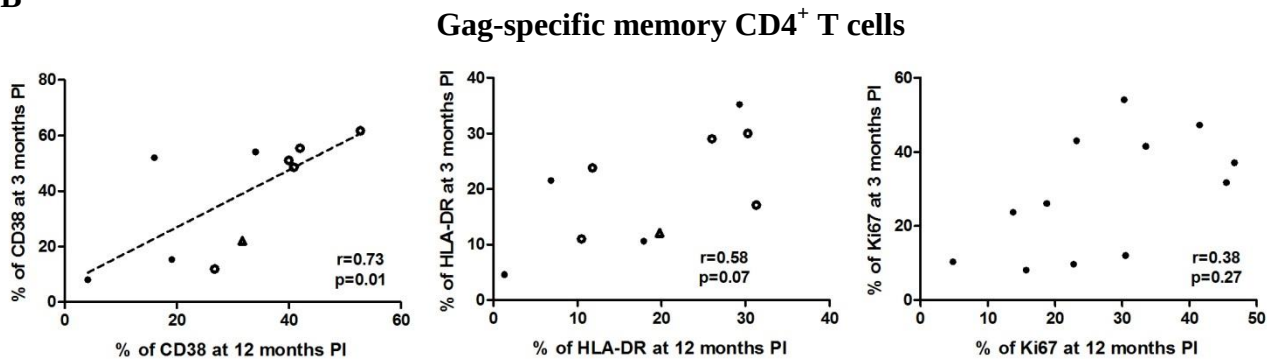
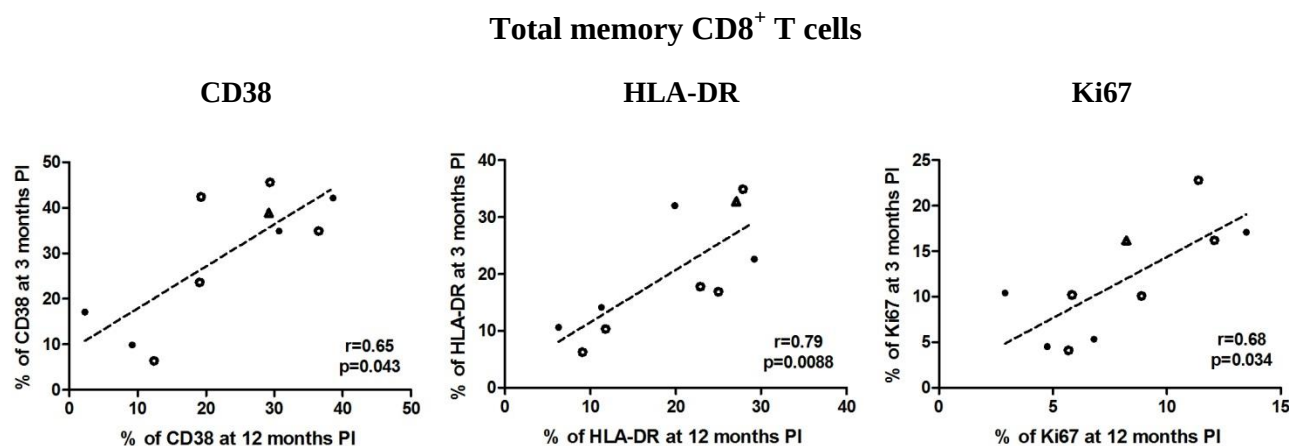


Figure 4.20: The relationship between the proportions of activated memory CD4⁺ T cells at 3 and 12 months post-infection. (A) Correlation between the proportions of total memory CD38, HLA-DR and Ki67 CD4⁺ T cells at 3 and 12 months post-infection. (B) Correlation between the proportions of antigen-specific memory CD38, HLA-DR and Ki67 CD4⁺ T cells at 3 and 12 months post-infection. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

4.2.4.2 CD8⁺ T cells

When associating the activation levels of memory CD8⁺ T cells at 3 and 12 months, positive and significant correlations were observed in the expression levels of CD38, HLA-DR and Ki67 ($r=0.65$, $p=0.043$; $r=0.79$, $p=0.0088$ and $r=0.68$, $p=0.034$, respectively, Figure 4.21A) when using unadjusted correlations. Although these correlations did not hold when adjusted according to Bonferroni correction, associations observed at 4 and 6 months for CD38, at 4 months for HLA-DR and at 6 months for Ki67 may suggest that activated total memory CD8⁺ T cells have also reached a set-point during early infection (Appendix L, Table L3, page 277). However, no significant associations were observed between the proportions of activated Gag-specific CD8⁺ T cells at 3 months and 12 months post-infection (Figure 4.21B). Other associations were observed between the activation levels of Gag-specific memory CD8⁺ T cells at 3 months with other time points (4, 5, 6, 9 and 15 months), but were lost after adjusting for multiple comparison (Appendix L, Table L4, page 277). Altogether, these data suggest that activated total memory cells were reflecting the steady-state of memory cells during primary HIV-1 infection. Moreover, this appeared to be independent of the course of viraemia in which, in some individuals, there was spontaneous control of viraemia despite possessing increased populations of activated memory CD4⁺ and CD8⁺ T cells (Figure 4.20 and 4.21, open symbols).

A



B

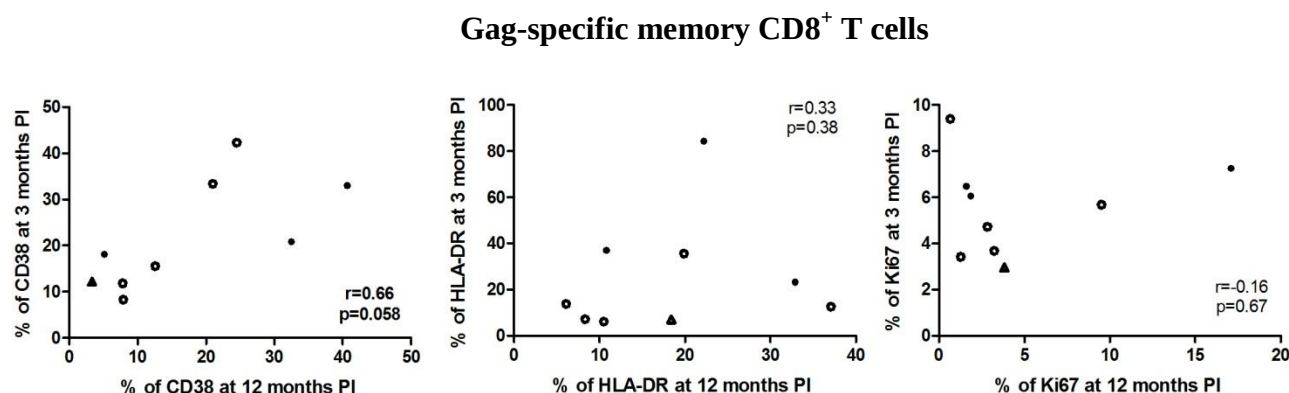


Figure 4.21: Correlations between memory CD8⁺ T cell activation profiles at 3 and 12 months post-infection.

(A) Correlation between the proportions of total memory CD38, HLA-DR and Ki67 CD8⁺ T cells at 3 and 12 months post-infection. (B) Correlation between the proportions of antigen-specific memory CD38, HLA-DR and Ki67 CD8⁺ T cells at 3 and 12 months post-infection. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

4.3 Discussion

Hyperimmune activation has been considered to be an important driver of immunopathogenesis and disease progression in chronic HIV infection (Liu *et al.*, 1997, Giorgi *et al.*, 1999). However, the degree to which acute or early HIV-1 infection impacts immune activation is poorly defined. Using the cell surface expression of CD38, HLA-DR and Ki67, this chapter characterized the status of activated and proliferating T cells in a cohort of early HIV-infected individuals. Data in this chapter showed several pieces of information.

There was extensive activation and expansion of both total memory CD4⁺ and CD8⁺ T cells in early HIV-infected individuals compared to HIV-uninfected controls. These findings support the hypothesis that high levels of viral replication, which occurs during the early stages of HIV infection (Kassutto and Rosenberg, 2004), could be a major determinant for the T cell activation during early HIV-1 infection. The findings that suppression of HIV replication with anti-retroviral therapy leads to reduction in T cell activation and proliferation (Hazenberg *et al.*, 2000b, Lempicki *et al.*, 2000, Barbour *et al.*, 2009) would support the link between viral replication and activation of T cells. Although increased activation is mainly attributed to HIV replication, other factors such as microbial products across the intestinal mucosa (Brenchley *et al.*, 2006b), co-infections and pathogens other than HIV (Eggena *et al.*, 2005b), or non-antigen specific bystander activation (Tough *et al.*, 1996, Boasso and Shearer, 2008) may also be responsible for the marked activation in primary HIV-1 infection.

HIV-specific memory CD4⁺ and CD8⁺ T cells showed significantly higher numbers of activated and proliferating cells when compared with CMV-specific T cells within the same individual.

Such differences may be related to distinct T cell responses towards differing types of viral antigens (HIV versus CMV) and are consistent with previous studies conducted by Doisne et al. (2004), which showed that HIV-specific CD8⁺ T cells are more activated compared to activated CD8⁺ T cells specific for other viruses (CMV, EBV and FLU). These findings are also corroborated by Barbour et al. (2009), who demonstrated increased Gag-specific T cell activation than CMV-specific activation. It is also shown in this chapter, that the proportions of activated and proliferating CMV-specific T cells were significantly higher in HIV-infected than in HIV-uninfected controls and possibly reflect bystander activation that could result in generalized activation of all T cells within the HIV-infected individual (Doisne *et al.*, 2004).

The proportions of activated and proliferating Gag-specific memory CD4⁺ T cells were significantly greater than the proportions of activated total memory CD4⁺ T cells, showing an expansion of Gag-specific cells during primary HIV-1 infection, which may be a direct consequence of repeated antigenic stimulation. This suggests that Gag-specific CD4⁺ T cells actively respond to HIV, but also underscores a paradox. It has been shown that HIV-specific CD4⁺ T cells are preferentially infected by HIV (Douek *et al.*, 2002), and thereby the rapid expansion of these activated cells represents a greater target pool. These findings are comparable with studies conducted by Scriba et al. (2005), who also demonstrated increased expression of CD38 and Ki67 within the HIV Gag p24-specific CD4⁺ T cell pool compared to the total memory CD4⁺ T cell compartment.

When comparing the activation status between memory CD4⁺ and CD8⁺ T cells, activation levels of total memory CD4⁺ T cells were found to be significantly lower relative to total memory

CD8⁺ T cells, and was evident in both HIV-infected and -uninfected individuals. The marked differences in the activation levels between memory CD4⁺ and CD8⁺ T cells may be related to a number of factors that include period of exposure to antigen (Iezzi *et al.*, 1998, Kaech and Ahmed, 2001) and/or the distinct inherent proliferative abilities between CD4⁺ and CD8⁺ T cells (Foulds *et al.*, 2002), or may also be related to preferential depletion and/or direct viral elimination of CD4⁺ T cell numbers during primary HIV-1 infection. Contrary to the total memory pool, Gag-specific memory CD4⁺ T cells were characterized by a much higher expression of CD38 and Ki67 compared to Gag-specific memory CD8⁺ T cells. These data are supported by recent findings, which demonstrated that higher cell-associated viral burden could result in increased expansion of activated HIV-specific CD4⁺ T cells (Hunt *et al.*, 2011a).

Although HIV-specific CD4⁺ T cells are preferential targets of HIV (Douek *et al.*, 2002), it is still not fully understood what other sources of infected cells exist, and how they contribute towards HIV-1 replication during the early stages of HIV-1 infection. Therefore, this chapter also set to investigate the susceptibility of activated total memory CD4⁺ T cells during primary HIV-1 infection. Extending previous reports (Demoustier *et al.*, 2002, Harari *et al.*, 2002, Douek *et al.*, 2002), Gag-specific memory CD4⁺ T cells were found to contain higher levels of proviral HIV-1 Gag-DNA compared to pool of total memory CD4⁺ T cells. Furthermore, activated memory CD4⁺ T cells harboured more cell-associated Gag-DNA than non-activated memory CD4⁺ T cells, and thus reflecting the susceptibility of activated memory CD4⁺ T cells to HIV-1 infection. Susceptibility of activated CD4⁺ T cells to HIV has also been previously demonstrated in SIV infections (Zhang *et al.*, 2004), and in a cohort of HIV controllers (Hunt *et al.*, 2011a). Given that T cell activation could also induce virus transcription from latently infected cells

(Grossman *et al.*, 1998), it could be suggested that memory CD4⁺ T cell activation promotes persistent viral replication by serving as a continuous reservoir for the virus during the early stages of HIV-1 infection. Moreover, the generation of activated CD4⁺ T cells due to HIV-1 could further create more target cells for efficient HIV-1 replication (Biancotto *et al.*, 2008), and thus resulting in a self-perpetuating cycle that allows the virus to thrive at the host's expense.

Excessive immune activation has been associated accelerated immune expansion and differentiation, which could ultimately lead to premature exhaustion of immune functions (Papagno *et al.*, 2004, Appay *et al.*, 2007). This chapter also aimed to explore the relationship between T cell memory activation and differentiation in primary HIV-1 infection. The activation level of both total and antigen-specific memory CD4⁺ T cells were found to be similar across different memory CD4⁺ T cell subpopulations (ED, LD and FD) and suggest that activation signals in early HIV-1 infection may not be the primary driver of memory CD4⁺ T cell differentiation. These findings were further supported by the lack of significant associations between activated memory CD4⁺ T cells with memory differentiation, but also appear to be contrary to previous reports showing preferential accumulation of CD38⁺HLA-DR⁺ cells within the total and Gag-specific late memory CD4⁺ T cell subpopulations (Barbour *et al.*, 2009). These apparent discrepancies are unclear, however there might be four possible scenarios that could explain the observations in this study: 1) there is preferential infection and depletion of either ED or LD memory subpopulations, and thereby giving an equal distribution of activation markers across the CD4⁺ subpopulations; 2) even in an activated state, ED CD4⁺ memory subpopulations are more resistant to HIV; and 3) activation and differentiation in with the CD4⁺ compartment are independent events. On the other hand, analysis of total memory CD8⁺ T cells showed

increased proportions of activated cells within the ED and LD compartments, together with positive and significant associations between activation and memory differentiation. These data suggest that total memory CD8⁺ T cell differentiation may be a consequence of immune activation. Thus, immune activation may be driving the early skewing of total memory CD8⁺ T cells towards a more differentiated state during early HIV-1 infection, and this notion is supported by the decreased proportions of naive cells and increased proportions of both ED and LD CD8⁺ subpopulations in early HIV-infected individuals relative to HIV-uninfected controls (observations in Chapter 3).

When examining the longitudinal profile of activated T cells, both total memory CD4⁺ and CD8⁺ T cells were found to display fluctuating patterns that may be related to the changes in HIV load during the course of infection. Oscillatory patterns of activated CD4⁺ and CD8⁺ T cells using spectral analysis have recently been demonstrated in HIV infection (Kitchen *et al.*, 2011), whereby subjects with elevated viraemia were characterized by frequent oscillations and increased amplitudes of T cell activation. These observations further demonstrated the strong relationships that exist between the dynamics of T cell activation and HIV replication over time. Furthermore, fluctuations observed in this chapter, were not only limited to total memory T cells, but were also found to occur on activated and proliferating antigen-specific memory T cells (both Gag- and CMV-specific). The fluctuation of activated T cells during HIV infection could also be attributed to other factors such as the diversity of the T cell receptor, whereby functionally different T cell clonotypes could be generated over the course of infection (Meyer-Olson *et al.*, 2006). Furthermore, infection with multiple viral variants (Sagar *et al.*, 2003), in addition to viral fitness (Liu *et al.*, 2007) may also contribute towards this phenomenon.

Further analysis of the dynamics of activation showed a significant increase in the proportions of total memory CD4⁺ T cells expressing HLA-DR over time and, in addition, a slight increase in the proportion of proliferating memory cells (Ki67⁺). With regard to total memory CD8⁺ T cells, the proportions of activated and expanding (as measured by the expression of CD38 and Ki67, respectively) appeared to decrease considerably over the first 15 months of infection. The increased numbers of activated total memory CD4⁺ T cells over time may be attributed to persistent antigen exposure, however other mechanisms such as the homeostatic responses to the loss CD4⁺ T cells during HIV infection (Catalfamo *et al.*, 2008) may in part play a role in the sustained numbers of activated memory CD4⁺ T cells. The decrease in proportions of activated memory CD8⁺ T cells may be a reflection of a clonal senescence of CD8⁺ T cells that might occur over the course of infection (Papagno *et al.*, 2004). Furthermore, the increased susceptibility of activated CD8 T cells to apoptosis (as measured by CD38⁺Fas/CD95⁺, (Chun *et al.*, 2004, Petrovas *et al.*, 2007), in addition to their increased ability to up-regulate markers associated with exhaustion (as measured by CD38⁺PD-1⁺) (Sauce *et al.*, 2007), may further explain the decline of CD8⁺ T cells over time.

Although total memory CD4⁺ or CD8⁺ T cells showed either an increase or a decrease in the proportions of activated cells over the first 15 months of infection, significant and positive correlations observed between the activation profiles at 3 months post-infection with those at later time points (i.e. 12 months post-infection). These findings demonstrate the establishment of a steady-state T cell activation during primary HIV-1 infection, and in agreement with previous observations suggesting that immunologic activation set-point is achieved during the early stages of HIV infection (Deeks *et al.*, 2004). Although it may be difficult to discern the causes of an

early activation set-point, factors such as the initial decline in HIV load during early infection (Borrow *et al.*, 1994, Koup *et al.*, 1994) may to some degree contribute towards T cell activation set-point. Analysis on antigen-specific T cells also showed relatively stable levels of activated Gag-specific memory CD4⁺ and CD8⁺ T cells over time. In contrast, the activation profile of activated CMV-specific memory CD4⁺ T cells (as measured by CD38 and HLA-DR) showed a significant increase over time. The increased and sustained numbers of activated CMV-specific CD4⁺ T cells over time may be interpreted as a consequence to the reactivation of latent forms of CMV (Dunn *et al.*, 2002, Hummel and Abecassis, 2002, Sester *et al.*, 2002), or as result of HIV-induced generalized immune activation (i.e. bystander activation).

In summary, data in this chapter adds important information regarding T cell activation during primary HIV-1 infection. Primary HIV-1 infection is characterized by the hyperactivation and expansion of total and Gag-specific CD4⁺ and CD8⁺ T cells, which possibly reflect viraemia. Moreover, the elevated activation status of CMV-specific T cells in HIV-infected individuals could also reflect generalized immune activation (i.e. bystander activation) in early HIV-1 infection. Results in this chapter also suggest that differentiation of memory CD4⁺ and CD8⁺ T cell subsets occur probably by different mechanisms and CD4⁺ activation and memory differentiation are possibly independent processes, but CD8⁺ activation may be linked with memory differentiation.

In conclusion, given that peak viraemia in primary HIV-1 infection may be related to elevated status of activated T cells, strategies such as the lowering of antigen load by means of anti-retroviral therapy in primary or acute HIV infection (Berrey *et al.*, 2001, Kaufmann *et al.*, 2004)

may serve as important interventions for reducing excessive T cell activation and turnover during the early phases of HIV-1 infection. The next chapter will explore the link between memory differentiation and polyfunctional capacity of CD4⁺ and CD8⁺ T cells.

CHAPTER 5: T CELL POLYFUNCTION

TABLE OF CONTENTS

5.1 Introduction.....	143
5.2 Results.....	144
5.2.1 Comparison between Gag- and CMV-specific memory responses in HIV-infected and -uninfected controls.....	144
5.2.2 Comparison between the polyfunctional profile of Gag- and CMV-specific memory CD4 ⁺ T cells in HIV-infected and -uninfected controls.....	151
5.2.3 Comparison between the polyfunctional profile of Gag- and CMV-specific memory CD8 ⁺ T cells in HIV-infected and -uninfected controls.....	155
5.2.4 The relationship between T cell polyfunction and memory differentiation	160
5.2.4.1 Polyfunctional profile of antigen-specific CD4 ⁺ T cell memory subpopulations...	160
5.2.5.2 Polyfunctional profile of antigen-specific CD8 ⁺ T cell memory subpopulations...	164
5.2.5 The longitudinal changes in the functional profile of HIV-specific memory T cells during HIV-1 infection	167
5.3 Discussion.....	175

5.1 Introduction

Cellular immunity against HIV is thought to play a key role in the control of HIV replication (Rowland-Jones *et al.*, 1997, McMichael and Rowland-Jones, 2001), whereby the production of soluble factors that are able to mediate effector functions and support the expansion of protective immune responses are considered to be important characteristics of a protective immune system (Slifka and Whitton, 2000). These effector functions may include the production of cytokines such as IFN γ or TNF α , which are capable of inhibiting viral replication (Wong *et al.*, 1988, Guidotti and Chisari, 1996, Lichterfeld *et al.*, 2004b). However, the induction of broad and robust effector functions with potent antiviral efficacy remains an elusive goal in HIV vaccine development (Virgin and Walker, 2010). To gain insight into what may constitute a protective immune response, delineating the specificity and the quality of immune responses during early HIV infection has been the focus of many studies. Thus, studies have suggested that the maintenance and preservation of HIV-specific T cells endowed with the ability to express multiple cytokines simultaneously may be important in controlling HIV disease progression (Betts *et al.*, 2006, Almeida *et al.*, 2007, Kannanganat *et al.*, 2007a). Despite T cell polyfunction being used as a marker for understating the quality of HIV-specific host responses, less is known whether the polyfunctional nature of HIV-specific memory T cells associates with memory differentiation.

Infection with other persistent viruses such as CMV is to some extent controlled during the course of infection, of which CMV-specific T cell responses have been shown to play a critical role in limiting CMV replication (Walter *et al.*, 1995, Einsele *et al.*, 2002, Sylwester *et al.*, 2005, Sacre *et al.*, 2005). The control of CMV infection by CMV-specific T cells could either be

related to differentiation phenotype (Harari *et al.*, 2004b) or to direct antiviral capabilities (Casazza *et al.*, 2006). Therefore understanding these factors could provide insight to the type of HIV-specific T cell responses that are required by an effective vaccine. As a result this chapter focuses on (i) examining and comparing the functional quality of HIV-1 Gag-specific memory CD4⁺ and CD8⁺ T cells with CMV-specific memory T cells; (ii) comparing the differentiation profile of polyfunctional HIV-specific T cells with CMV-specific T cells and (iii) examining the longitudinal profiles of the frequency and functional profile of HIV-specific memory CD4⁺ and CD8⁺ T cells over the first 15 months of HIV -1 infection.

5.2 Results

5.2.1 Comparison between Gag- and CMV-specific memory responses in HIV-infected and -uninfected controls

The first level of analysis sought to examine the frequency of Gag-specific memory T cell responses in early HIV-1 infection and compare with CMV-specific responses. Due to lack of material of early HIV-infected individuals, Gag-specific T cell responses in early HIV-infected individuals were compared to CMV-specific T cell responses within a cohort of chronically HIV-infected individuals and HIV-uninfected individuals. PBMC were stimulated with a pool of either Gag or CMV peptides, and antigen-specific responses evaluated based on expression CD107, IFN γ , IL-2, MIP-1 β and TNF α (Chapter 2, Section 2.4.1, page 55). Figure 5.1 and 5.2 show representative dot plots of the cytokine gating strategy employed to characterize the CD4⁺ and CD8⁺ T cell response patterns in HIV-1 infected individuals. The total frequency of HIV-1 Gag-specific T cell responses was calculated based on the contribution of all individual cytokine responses and compared to the total CMV-specific T cell responses.

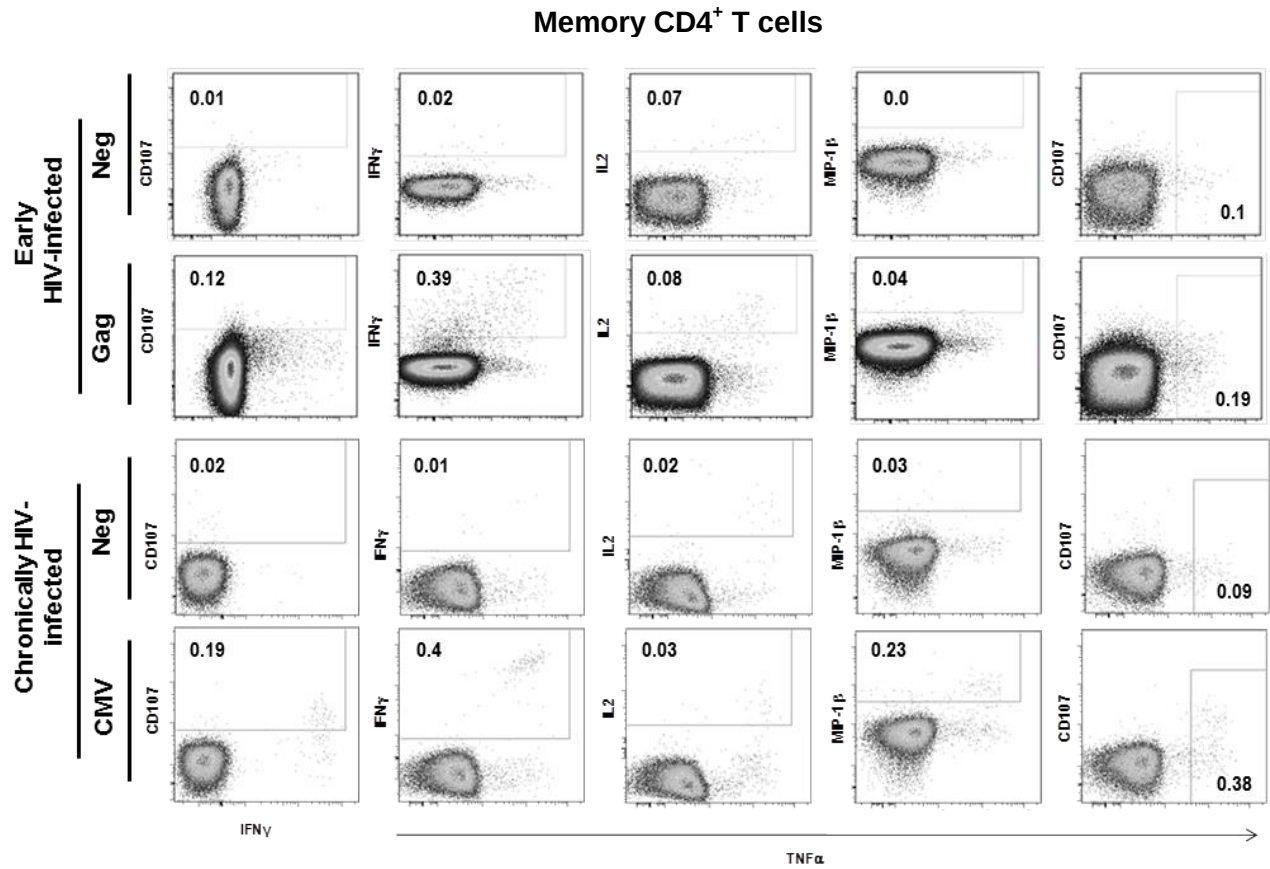


Figure 5.1: Representative dot plots showing the cytokine gating strategy for the determination of multi-functional memory CD4⁺ T cell responses. PBMC from HIV-infected individuals were stimulated for 6 hours with Gag and/or CMV peptide pools, stained and analysed using flow cytometry for the expression of CD107, IFN γ , IL-2, MIP-1 β and TNF α . Initial gating was performed on memory T cells based on the CD27 versus CD45RO plot. The cytokine gating scheme was used to determine both Gag- and CMV-specific memory CD4⁺ T cell responses in early HIV-infected and chronically HIV-infected individuals. The gates for each of the 5 functions were set based on the negative control (unstimulated cells) (Neg) for each individual.

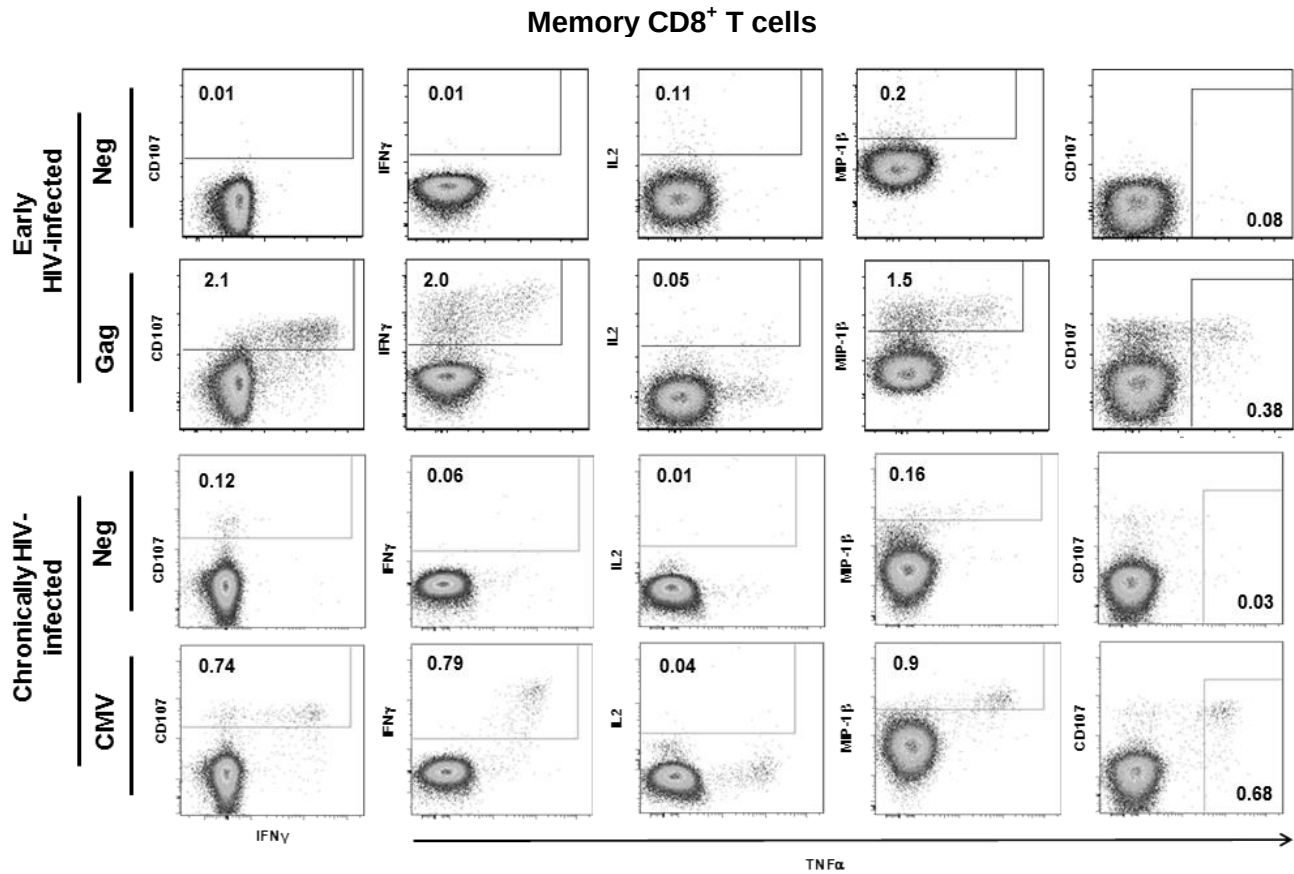
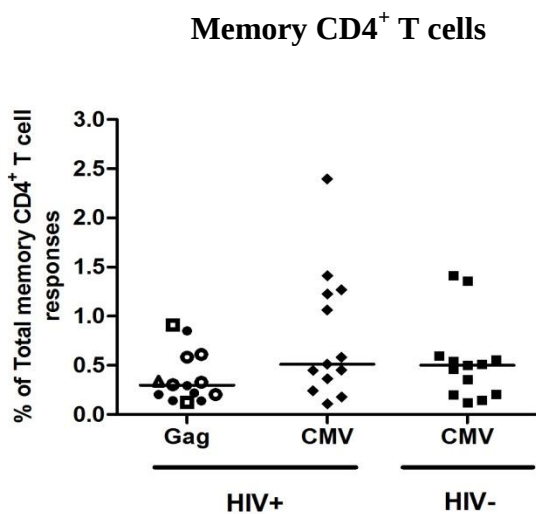


Figure 5.2: Representative dot plots showing the cytokine gating strategy for the determination of multi-functional memory CD8⁺ T cell responses. PBMC from HIV-infected individuals were stimulated for 6 hours with Gag and/or CMV peptide pools, stained and analysed using flow cytometry for the expression of CD107, IFN γ , IL-2, MIP-1 β and TNF α . Initial gating was performed on memory T cells based on the CD27 versus CD45RO plot. The cytokine gating scheme was used to determine both Gag- and CMV-specific memory CD8⁺ T cell responses in early HIV-infected and chronically HIV-infected individuals. The gates for each of the 5 functions were set based on the negative control (unstimulated cells) (Neg) for each individual.

The frequency of total Gag-specific memory CD4⁺ T cells in early HIV-1 infected individuals was found to be comparable with the frequency of CMV-specific memory CD4⁺ T cells of chronically HIV-1 infected individuals (Figure 5.3A). There was however a trend toward increased numbers of CMV-specific memory CD4⁺ T cells when compared with Gag-specific memory CD4⁺ T cells (median: 0.51% vs. 0.29%). No significant differences were observed between total Gag-specific CD4⁺ T cell responses of early HIV-1 infected individuals and total CMV-specific CD4⁺ T cell responses of HIV-uninfected controls. The total frequency of CMV-specific memory CD4⁺ T cell responses was similar between chronically HIV-infected individuals and HIV-uninfected controls. With regard to memory CD8⁺ T cells, no significant differences were observed when comparing total Gag-specific T cell responses in early HIV-1 infected individuals to CMV-specific responses in either HIV-infected or -uninfected individuals (Figure 5.3B). The frequency of total CMV-specific CD8⁺ T cell responses was also found to be similar between chronically HIV-infected and -uninfected controls.

A



B

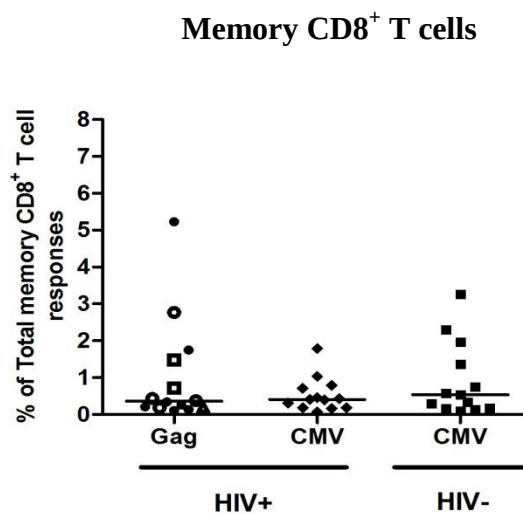


Figure 5.3: The total frequency of HIV-1 Gag- and CMV-specific T cell responses in early, chronically HIV-infected and -uninfected controls. Total T cell responses were calculated from the contribution of all five functions (CD107, IFN γ , IL2, MIP-1 β and TNF α). (A) The comparison between the total frequency of Gag-specific and CMV-specific memory CD4⁺ T cells responses in early (n=14), and chronically (n=13, $\blacklozenge$) HIV-infected and -uninfected controls (n=13, $\blacksquare$) (B) The comparison between the total frequency of Gag-specific and CMV-specific memory CD8⁺ T cells responses in early, chronically HIV-infected and -uninfected controls. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by non-parametric One-way ANOVA.

Further analysis sought to investigate the individual cytokine responses between HIV-Gag-specific and CMV-specific T cells. No significant differences were observed in the expression of CD107 and IFN γ between Gag- and CMV-specific memory CD4⁺ T cells of both HIV-infected

(early and chronic infection) and -uninfected controls (Figure 5.4A). The frequency of Gag-specific memory CD4⁺ T cells expressing IL-2, MIP-1 β and TNF α in early HIV-infected individuals were significantly lower compared with CMV-specific memory T cells in chronically HIV-infected individuals ($p < 0.05$, $p < 0.005$ and $p < 0.05$, respectively). The frequency of Gag-specific memory CD4⁺ T cells expressing MIP-1 β were also found be significantly lower in early HIV-infected individuals when compared with CMV-specific cells from HIV-uninfected controls. Although no significant differences were found in the frequency of T cells expressing IL-2 or TNF α between Gag-specific and CMV-specific memory CD4⁺ T cells, CMV-specific memory CD4⁺ T cells showed slightly higher frequencies of cells producing IL-2 (median: 0% vs. 0.06%) and TNF α (median: 0.08% vs. 0.34%) compared with Gag-specific CD4⁺ T cells. Analysis on memory CD8⁺ T cells also showed significantly low numbers of Gag-specific CD8⁺ T cells expressing IL-2 in early HIV-infected individuals compared with CMV-specific CD8⁺ T cells from chronically HIV-infected individuals ($p < 0.05$) (Figure 5.4B). There were no significant differences in the frequency of T cells expressing CD107, IFN γ , MIP-1 β and TNF α between Gag- and CMV-specific memory CD8⁺ T cells. However, there was a trend towards higher frequencies of CMV-specific CD8⁺ T cells expressing TNF α compared with Gag-specific T cells (median: 0.027% vs. 0.27%).

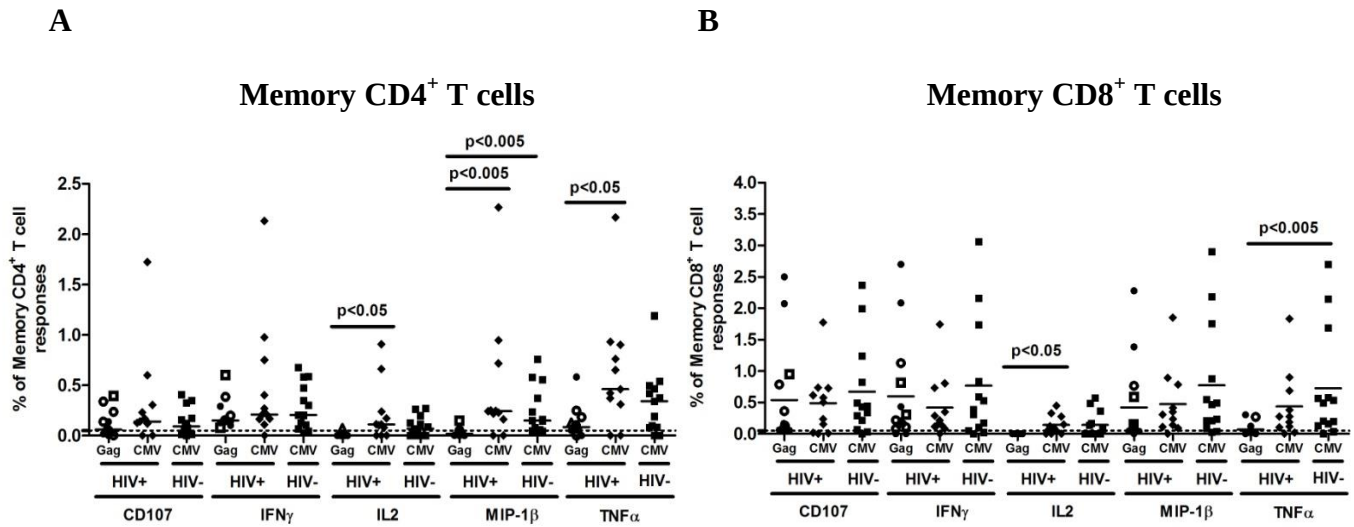


Figure 5.4: The frequency of HIV-1 Gag- and CMV-specific T cell responses in early, chronically HIV-infected and -uninfected controls. (A) Comparison between the frequency of Gag-specific and CMV-specific memory CD4⁺ T cells in early (n=14), chronically (n=13, $\blacklozenge$) HIV-infected and -uninfected controls (n=13, $\blacksquare$). (B) Comparison between the frequency of Gag-specific and CMV-specific memory CD8⁺ T cells in early, chronically HIV-1-infected and -uninfected controls. The dotted line represents the 0.05% cutoff used to define positive T cell responses. Open circles (O) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles ($\bullet$) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons were performed by non-parametric One-way ANOVA.

The frequency of Gag-specific CD8⁺ T cells expressing TNF α in early HIV-infected individuals was found to be significantly lower compared with CMV-specific CD8⁺ T cells from HIV-uninfected controls (p<0.005). There were no significant differences in the frequency of T cells that produced CD107, IFN γ or MIP-1 β between Gag-specific cells of early HIV-infected

individuals and CMV-specific cells of HIV-uninfected individuals. In summary, these data show that a greater fraction of CMV-specific T cells are characterized by the ability to mediate effector responses such as MIP-1 β and TNF α , and proliferative responses such as IL-2 compared to HIV-Gag-specific T cells, and thus may reflect the difference in the effectiveness of the two antiviral T cell functions. Furthermore, while the capacity to produce IL-2, MIP-1 β and TNF α appears to be limited in primary HIV-1 infection, the capacity to degranulate (CD107a) and produce IFN γ remained largely unchanged.

5.2.2 Comparison between the polyfunctional profile of Gag- and CMV-specific memory CD4⁺ T cells in HIV-infected and -uninfected controls

The preservation of polyfunctional HIV-specific T cells has been suggested to contribute to the control of HIV replication (Zimmerli *et al.*, 2005, Betts *et al.*, 2006, Almeida *et al.*, 2007, Duvall *et al.*, 2008). This chapter aimed to examine and compare the polyfunctional profile between Gag- and CMV-specific T cells. To determine the polyfunctional profile of antigen-specific memory CD4⁺ T cells, Boolean gating analysis (as described by Betts *et al.* (2006)) was employed to identify all possible 32 response patterns generated from the five individual cytokine parameters (CD107, IFN γ , IL-2, MIP-1 β and TNF α). The HIV-Gag-specific memory CD4⁺ T cell responses in early HIV-infected individuals were found to be significantly less polyfunctional than CMV-specific memory CD4⁺ T cells of both chronically HIV-infected and HIV-uninfected controls ($p < 0.0001$ and $p = 0.006$, respectively, Figure 5.5). In early HIV-infected individuals, monofunctional cells made up most of the global Gag-specific CD4⁺ T cell response (69%), followed by dual-functional (23%) and triple-functional cells (7%). Cells that expressed four or five functions made the least contribution to the total Gag-specific CD4⁺ T cell response (1%

combined). The monofunctional production of IFN γ (median: 27.4%) was the most common functional response observed in Gag-specific memory CD4 $^{+}$ T cells, followed by CD107 (median: 10.8%), the simultaneous production of IFN γ and TNF α (median: 6.5%), CD107 $^{+}$ IFN γ $^{+}$ cells (median: 4.5%) and IFN γ $^{+}$ MIP-1 β $^{+}$ TNF α $^{+}$ (median: 1.4%). The single expression of IFN γ , TNF α and CD107 also contributed mostly to the total Gag-specific memory CD4 $^{+}$ T cell response (24.5%, 22.7% and 12.6% respectively), while IFN γ $^{+}$ TNF α $^{+}$, CD107 $^{+}$ IFN γ $^{+}$ and IFN γ $^{+}$ MIP-1 β $^{+}$ TNF α $^{+}$ responses contributed 7%, 4%, and 1.7%, respectively.

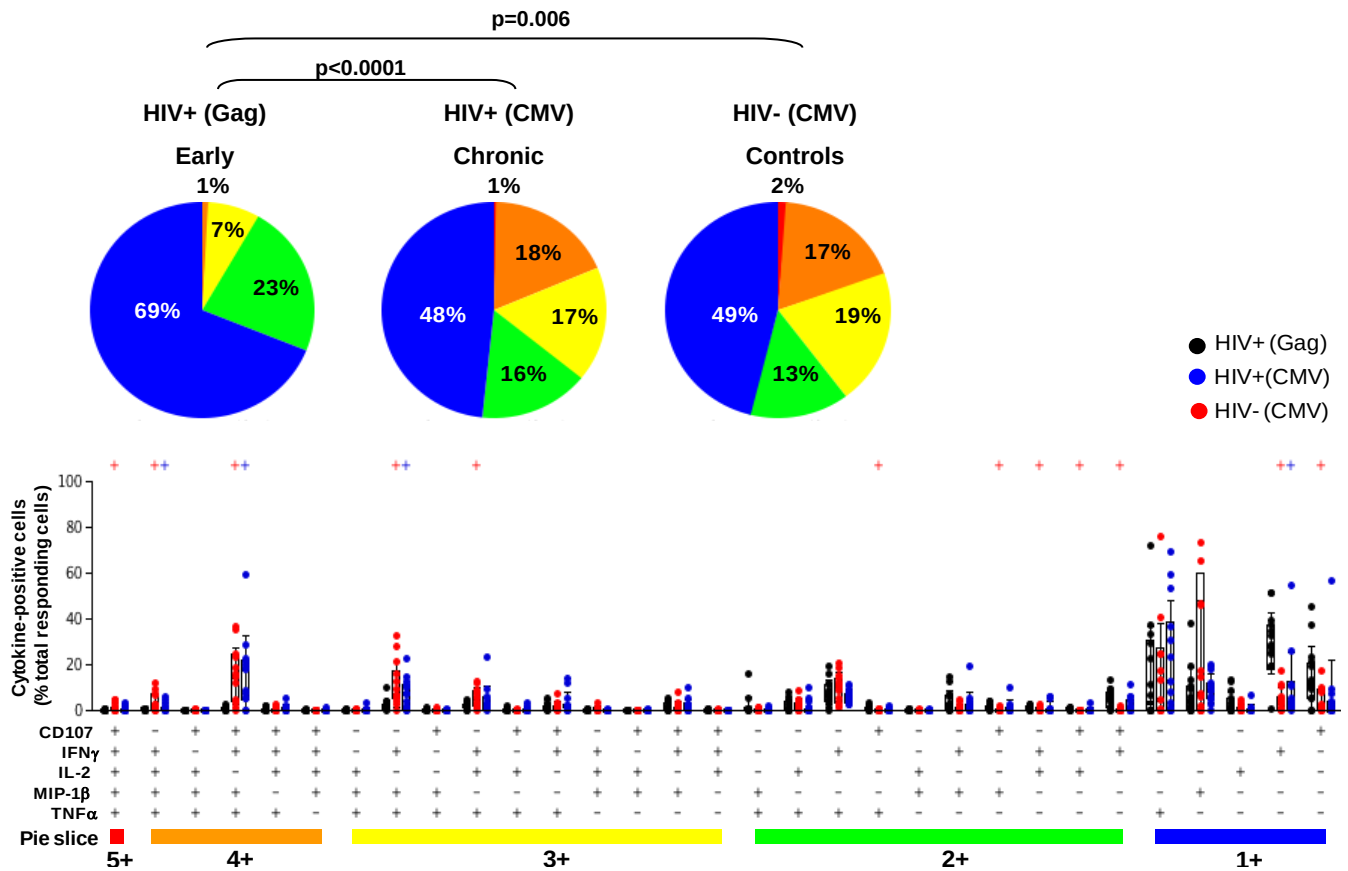


Figure 5.5: Comparison between the polyfunctionality of HIV-Gag-specific and CMV-specific memory CD4⁺ T cells in early, chronically HIV-infected and HIV-uninfected controls. The polyfunctional profile of antigen-specific T cells was compared between early (Gag-specific, n=14), chronically HIV-infected (CMV-specific, n=13), and HIV-uninfected controls (CMV-specific, n=13). Boolean gating was employed to assess the distribution of antigen-specific T cells between the 32 possible functional combinations as indicated on the x-axis. The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag/CMV response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of Gag- and CMV-specific T cells were performed by a student's test ⁺ ($p < 0.05$).

The CMV-specific memory CD4⁺ T cell responses in chronically HIV-infected individuals were also found to be dominated by monofunctional cells (48%), while dual- and triple-cytokine producing cells contributed 16% and 17%, respectively. Cells expressing more than four functions made up 18% of the global CMV-specific memory CD4⁺ T cell response. The median percentages of monofunctional CMV-specific CD4⁺ T cells producing IFN γ (1%) and CD107a (0.0), were found to be significantly lower than the median percentages of HIV-Gag-specific memory CD4⁺ T cells producing these cytokines (27.4% and 10.8%, respectively) (p=0.0033 and p=0.019, respectively). No significant differences were found between the median percentages of monofunctional CMV-specific CD4⁺ T cells producing TNF α (12.3%), MIP-1 β (8.5%), IL-2 (0.0) with those of Gag-specific memory CD4⁺ T cells (3.1%, 3.8% and 0.1%, respectively). The monofunctional TNF α response made up 24.8% of the total CMV-specific memory CD4⁺ T cell response, while MIP-1 β and IFN γ contributed 9.9% and 5.3%, respectively. The functional combination of IFN γ ⁺MIP-1 β ⁺TNF α ⁺ cells was also found to be a frequent response pattern among CMV-specific memory CD4⁺ T cells (median: 7%), whereby this combination made up 9.5% of the total CMV-specific CD4⁺ T response. The median percentage of IFN γ ⁺MIP-1 β ⁺TNF α ⁺ cells (7%) among CMV-specific cells was significantly higher compared to Gag-specific CD4⁺ T cells expressing this functional combination (median: 1.4%) (p=0.034). The simultaneous expression of CD107a, IFN γ , MIP-1 β and TNF α (median: 17.3%) was the most common functional combination within CMV-specific memory CD4⁺ T cells, and made up 22.9% of the total CMV-specific memory CD4⁺ T cell response. The median percentage of CMV-specific memory cells expressing CD107a⁺IFN γ ⁺MIP-1 β ⁺TNF α ⁺ (17.3%) was significantly higher compared to that of Gag-specific memory CD4⁺ T cells expressing this functional combination (median: 0.19%.) (p=0.001).

No significant differences were found between the functional profiles of CMV-specific CD4⁺ T cells between chronically HIV-infected and HIV-uninfected controls. Moreover CMV-specific CD4⁺ T cells displayed similar response patterns in both HIV-infected and HIV-uninfected controls. For instance, TNF α and MIP-1 β production were found to be the dominant cytokines within the single-cytokine expressing cells and followed IFN γ expression in both chronically HIV-infected and HIV-uninfected controls. The functional combination of CMV-specific CD4⁺ T cells producing IFN γ ⁺TNF α ⁺ found to be the highest among dual-cytokine producing cells followed by the combination of IL-2⁺TNF α ⁺ cells, while the expression of IFN γ ⁺MIP-1 β ⁺TNF α ⁺ cells was the most common among triple-cytokine producing cells, followed by IFN γ ⁺IL-2⁺TNF α ⁺ producing cells.

5.2.3 Comparison between the polyfunctional profile of Gag- and CMV-specific memory CD8⁺ T cells in HIV-infected and -uninfected controls

The next level of analysis sought to compare the functional profile of Gag- and CMV-specific memory T cells within the CD8⁺ T cell compartment. Similar to CD4⁺ T cells, HIV-Gag-specific memory CD8⁺ T cells detected during early HIV-infection were found to be significantly less polyfunctional than CMV-specific memory CD8⁺ T cells detected from chronically HIV-infected and HIV-uninfected controls ($p < 0.0001$ and $p < 0.0001$, respectively, Figure 5.6). Again, monofunctional cells contributed over two thirds of the CD8⁺ T cell response (71%), particularly those mobilizing CD107a (median: 29.7%), producing IFN γ (median: 22.1%) and MIP-1 β (median: 6.15%). Among monofunctional cells, the surface mobilization of CD107a contributed 32.9% to the total Gag-specific memory CD8⁺ T cell response, followed by IFN γ and MIP-1 β , which contributed 21.9% and 8.7%, respectively. The dual- and triple-functional cells made up

21% and 7% of the global Gag-specific CD8⁺ T cell response, while cells expressing more than four functions made the least contribution (1%). The simultaneous expression of CD107a and IFN γ was also a frequent response pattern among the Gag-specific memory CD8⁺ T cells (median: 6.1%), and this functional combination made up 8.3 % of the total Gag-specific memory CD8⁺ T cell response.

In contrast to HIV-specific CD8⁺ T cells, monofunctional cells made up 39% of the CMV-specific memory CD8⁺ T cell response in chronically HIV-infected individuals, while dual- and triple-functional cells contributed 16% and 16%, respectively. Cells expressing more than four functions made up 29% of the CMV-specific memory CD8⁺ T cell response. The median percentages of monofunctional CMV-specific CD8⁺ T cells expressing CD107a (2.3%) and IFN γ (3.6%) were significantly lower than the median percentages of HIV-Gag-specific CD8⁺ T cells expressing these cytokines (CD107: 27.2% and IFN γ : 22.1%) ($p=0.002$ and $p=0.0008$, respectively). The monofunctional MIP-1 β response (median: 14.3%) was however found to be significantly higher in CMV-specific memory CD8⁺ T cells relative to HIV-Gag-specific memory CD8⁺ T cells (median: 6.1%) ($p=0.0094$). The frequency of CD8⁺ T cells producing only MIP-1 β contributed 11.5% of the total CMV-specific T cell response, while those producing CD107a and IFN γ contributed 9.1% and 3.2%, respectively.

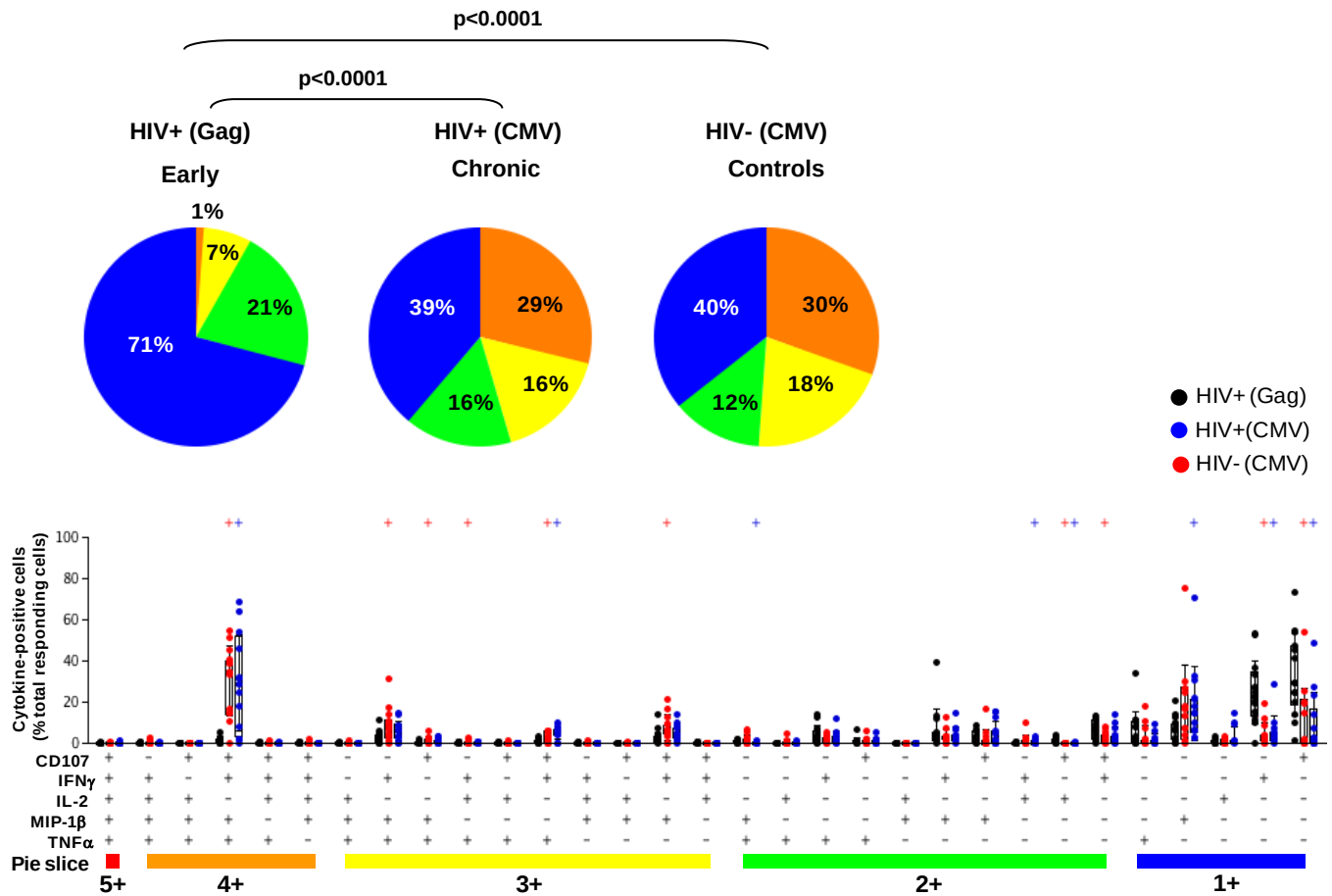
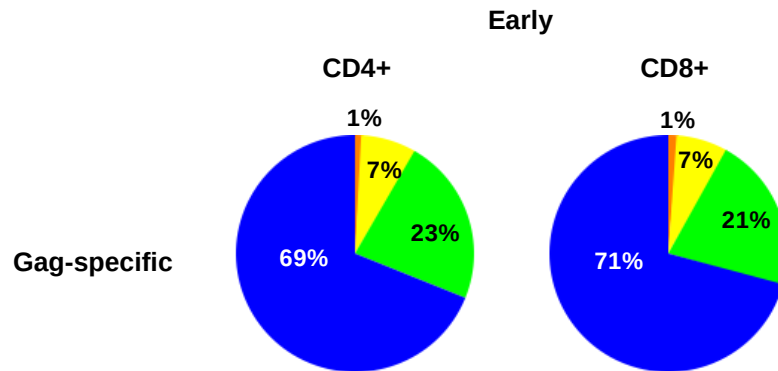


Figure 5.6: Comparison between the polyfunctionality of HIV-Gag-specific and CMV-specific memory CD8⁺ T cells in early, chronically HIV-infected and HIV-uninfected controls. The polyfunctional profile of antigen-specific T cells was compared between early (Gag-specific, n=14), chronically HIV-infected (CMV-specific, n=13), and HIV-uninfected controls (CMV-specific, n=13). Boolean gating was employed to assess the distribution of antigen-specific T cells showing all 32 possible functional combinations as indicated on the x-axis. The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag/CMV response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of Gag- and CMV-specific T cells were performed by a student's test ⁺ (p<0.05).

The functional combination of CD107⁺IFN γ ⁺MIP-1 β ⁺TNF α ⁺ cells was also the most frequent response pattern of CMV-specific memory CD8⁺ T cells (median: 28.6%), and found to contribute 41.3% of the total CMV-specific CD8⁺ T cell response. This functional combination was significantly more frequent for CMV-specific responses (median: 28.6%) than Gag-specific responses (median: 0.4%) (p=0.0008). The profile of CMV-specific memory CD8⁺ T cell responses between chronically HIV-infected individuals and HIV-uninfected controls displayed comparable levels of polyfunctionality and also presented similar hierarchy of functional response patterns. Taken together, these data show that CMV-specific CD4⁺ and CD8⁺ T cells possess distinct and enhanced functionality when compared with HIV-Gag-specific T cells.

This chapter has shown in sections 5.2.2 and 5.2.3 the polyfunctional profiles of total HIV-specific memory CD4⁺ and CD8⁺ T cells. Figure 5.7 shows again the functional differences between CD4⁺ and CD8⁺ T cells (Gag and CMV-specific) in early and chronically HIV-1 infected individuals. Gag-specific memory CD4⁺ T cells displayed similar frequencies of polyfunctional cells to Gag-specific memory CD8⁺ T cells (Figure 5.7A). In chronically HIV-infected individuals, CMV-specific memory CD4⁺ T cells were found to be less polyfunctional than CMV-specific CD8⁺ T cells (p=0.0059, Figure 5.7B). These observations held true in HIV-uninfected individuals, where CMV-specific memory CD4⁺ T cells were also found to be less polyfunctional than CMV-specific memory CD8⁺ T cells (p=0.037) (Appendix M, Figure M1, page 278). The observed differences between the functional patterns of CD4⁺ and CD8⁺ memory T cells may be related to the disparity in the efficiency of antigen presentation by the MHC class I and II pathways in the context of CMV or HIV-1 infection.

A



B

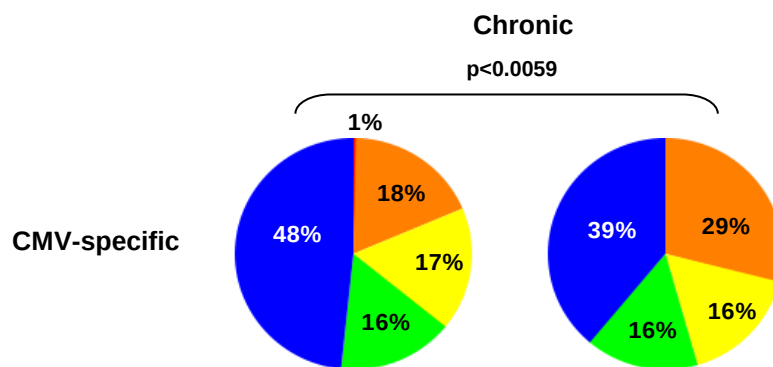


Figure 5.7: Comparison of the polyfunctional profile between antigen-specific memory $CD4^+$ and $CD8^+$ T cells in at 3 months post-infection. (A) Comparison of the polyfunctional profiles between HIV-Gag-specific memory $CD4^+$ and $CD8^+$ T cells in early HIV-infected individuals ($n=14$) (B) Comparison of the polyfunctional profiles between CMV-specific memory $CD4^+$ and $CD8^+$ T cells in chronically HIV-infected individuals ($n=13$). The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific $CD4^+$ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag-specific response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of Gag-specific T cells were performed by a student's test $^+$ ($p < 0.05$).

5.2.4 The relationship between T cell polyfunction and memory differentiation

5.2.4.1 Polyfunctional profile of antigen-specific CD4⁺ T cell memory subpopulations.

Increasing evidence suggests that the differences in the memory differentiation phenotype appear to associate with improved HIV control (Hess *et al.*, 2004, Addo *et al.*, 2007). While data in chapter 3 showed that most CMV-specific T cells are more mature than HIV-Gag-specific cells, it is still unclear whether the polyfunctional capacity of CMV- or Gag-specific cells associates with memory differentiation. To investigate this, the polyfunctional profiles of Gag and CMV-specific memory CD4⁺ T cells were examined based by using various permutations of CD107, IFN γ , IL-2, MIP-1 β and TNF α . Memory differentiation was assessed by CD27 and CD45RO (as described on Chapter 3, Section 3.2.2.1, page 67). The FD (fully differentiated)-memory CD4⁺ T cell subpopulations were omitted from this analysis as they presented low antigen-specific T cell numbers. Figure 5.8 shows that at 3 months post-infection, both ED (early differentiated) and LD (late differentiated)-memory Gag-specific CD4⁺ T cells possess similar functional profiles, and were mostly monofunctional in nature. Thus in both the ED and LD compartments, the presence of cells characterized by three and four functions was low and negligible.

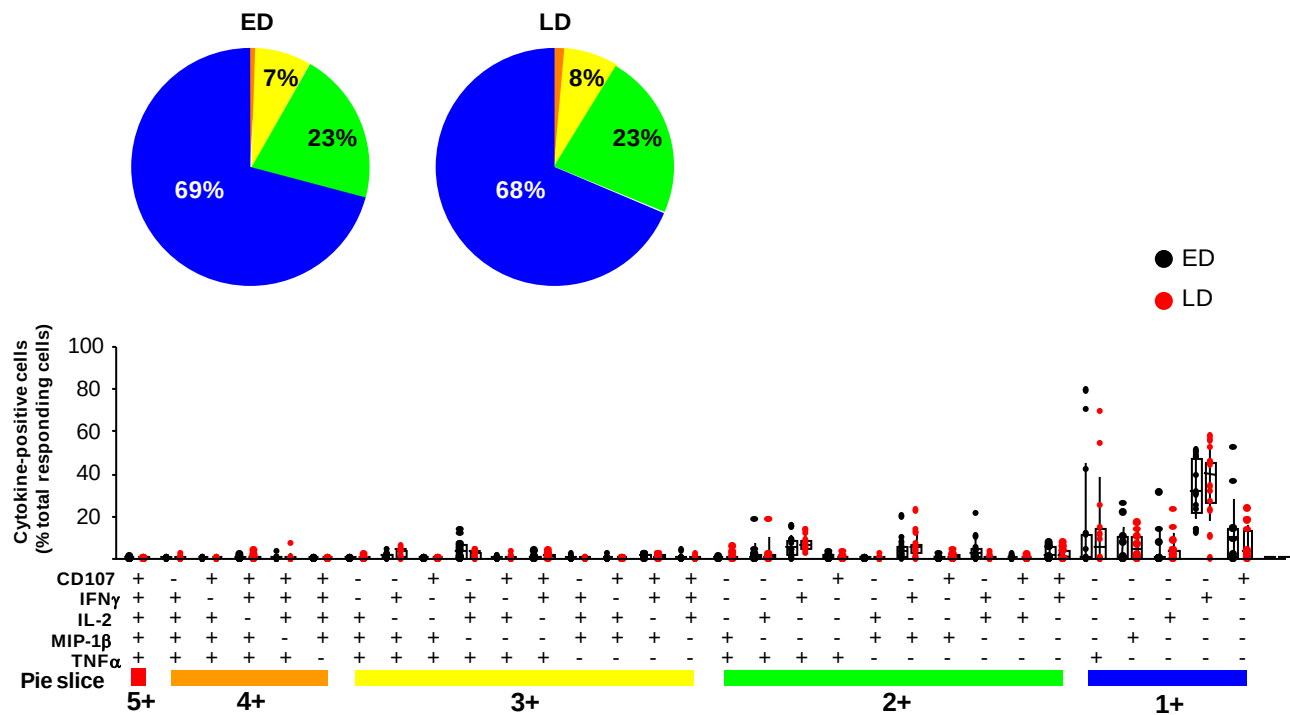


Figure 5.8: The polyfunctionality of Gag-specific ED- and LD-memory CD4⁺ T cells at 3 months post-infection. Using Boolean gating, the distribution of cells showing any combination of functional profiles was determined for Early (ED, red dots) and Late (LD, black dots) differentiated memory cells in early HIV-infected individuals (n=14). The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of Gag-specific T cells were performed by a student's test[†] (p<0.05).

Contrary to HIV-Gag-specific T cells, CMV-specific ED-memory CD4⁺ T cells were found to be less polyfunctional than CMV-specific LD-memory cells (p=0.0038) (Figure 5.9). Having shown CD107⁺IFN γ ⁺MIP-1 β ⁺TNF α ⁺ to be most common functional combination of CMV-specific memory CD4⁺ T cells, the proportion of CMV-specific CD4⁺ T cells expressing this combination of functions was significantly lower within ED-memory compartment relative to the LD-memory compartment (1.5% versus 21.7%) (p=0.003). A similar pattern was also observed for CMV-specific cells expressing IFN γ , MIP-1 β and TNF α simultaneously, where the median percentage of this functional combination was found to differ between ED cells (0.0%) and LD cells (12.2%, p=0.0009). With regard to monofunctional cells, TNF α was mostly associated with an ED phenotype, while CD107a, IFN γ , IL-2 and MIP-1 β were heterogeneous and spread across different memory subsets. It is also worth noting that a similar trend was observed in polyfunctionality of CMV-specific CD4⁺ T cells within HIV-uninfected controls (Appendix M, Figure M2, page 279), where greater proportions of polyfunctional cells were found among the LD-memory phenotype compared to the ED-memory phenotype. Further analysis showed increased levels of polyfunctionality within the LD-memory CMV-specific CD4⁺ T compared to the LD-memory HIV Gag-specific memory CD4⁺ T cells (p<0.0001). Jointly, these data show that CMV-specific memory CD4⁺ cells are both skewed towards a more differentiated phenotype and show increased functionality in more mature memory cells.

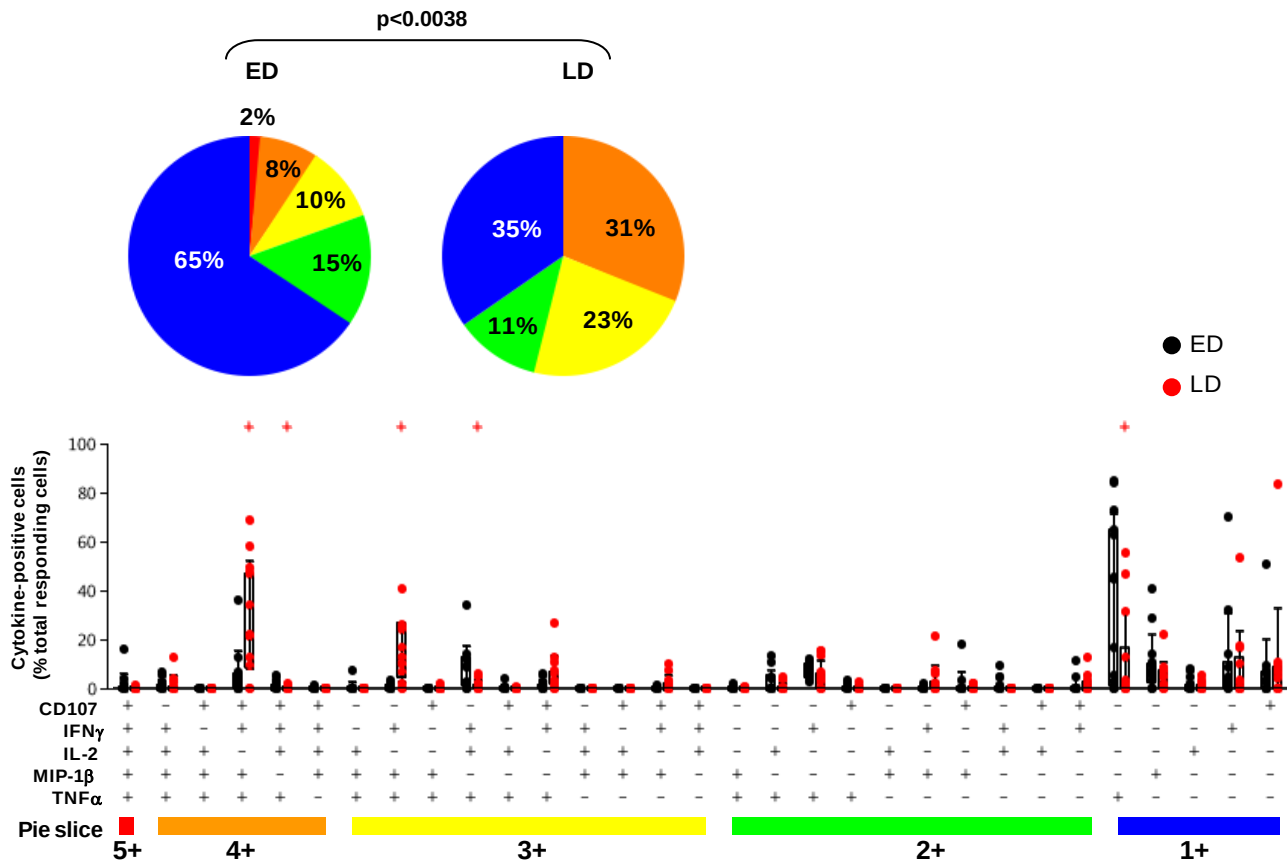


Figure 5.9: The polyfunctionality of CMV-specific ED- and LD-memory CD4⁺ T cells in chronically HIV-infected individuals. Using Boolean gating, the distribution of cells with any combination of functional profiles was determined for Early Differentiated (ED, red dots) and Late Differentiated (LD, black dots) cells in chronically HIV-infected individuals (n=13). The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of CMV-specific T cells were performed by a student's test ⁺ (p<0.05).

5.2.5.2 Polyfunctional profile of antigen-specific CD8⁺ T cell memory subpopulations

Using surface expression of CD27 and CD45RO, antigen-specific memory CD8⁺ T cells were divided into four distinct memory subpopulations (early differentiated, intermediate, late differentiated and fully differentiated) (Chapter 3, Section 3.2.2.3, page 74). The polyfunctional profile of Gag-specific memory CD8⁺ T cells was found to be no different for each memory subpopulation when analysed at the earliest time point. Memory subpopulations were in fact dominated by monofunctional cells (Figure 5.10) and the functional capacity of Gag-specific CD8⁺ T cells was similar among the different CD8⁺ memory subsets (Figure 5.10). No major differences were also observed in the frequency of polyfunctional CMV-specific memory CD8⁺ T cells between the different memory subpopulations of chronically HIV-infected individuals (Figure 5.11). This held true for the differentiation profile of CMV-specific memory CD8⁺ T cells within HIV-uninfected controls (Appendix M, Figure M3, page 280). Although CMV-specific cells displayed similar functional characteristics across the different memory subsets, non-significant differences were noted among the dual-functional CMV-specific CD8⁺ T cells, where greater proportions of cells expressing IFN γ and IL-2 were found within the ED-memory compartment (median: 4.7%) relative to the Int, LD and FD cells of which each compartment showed a median percentage of 0.0%. Again, when comparing the polyfunctionality between Gag- and CMV-specific cells on the different memory subsets, CMV-specific memory CD8⁺ T cells showed higher frequencies of polyfunctional cells within the ED, Int, LD and FD compartments than Gag-specific memory CD8⁺ T cells. Together, these data show that, unlike memory CD4⁺ T cells, there is no association between memory differentiation and the polyfunctionality of either Gag- or CMV-specific CD8⁺ T cells, although CMV-specific CD8⁺ T cells are more polyfunctional.

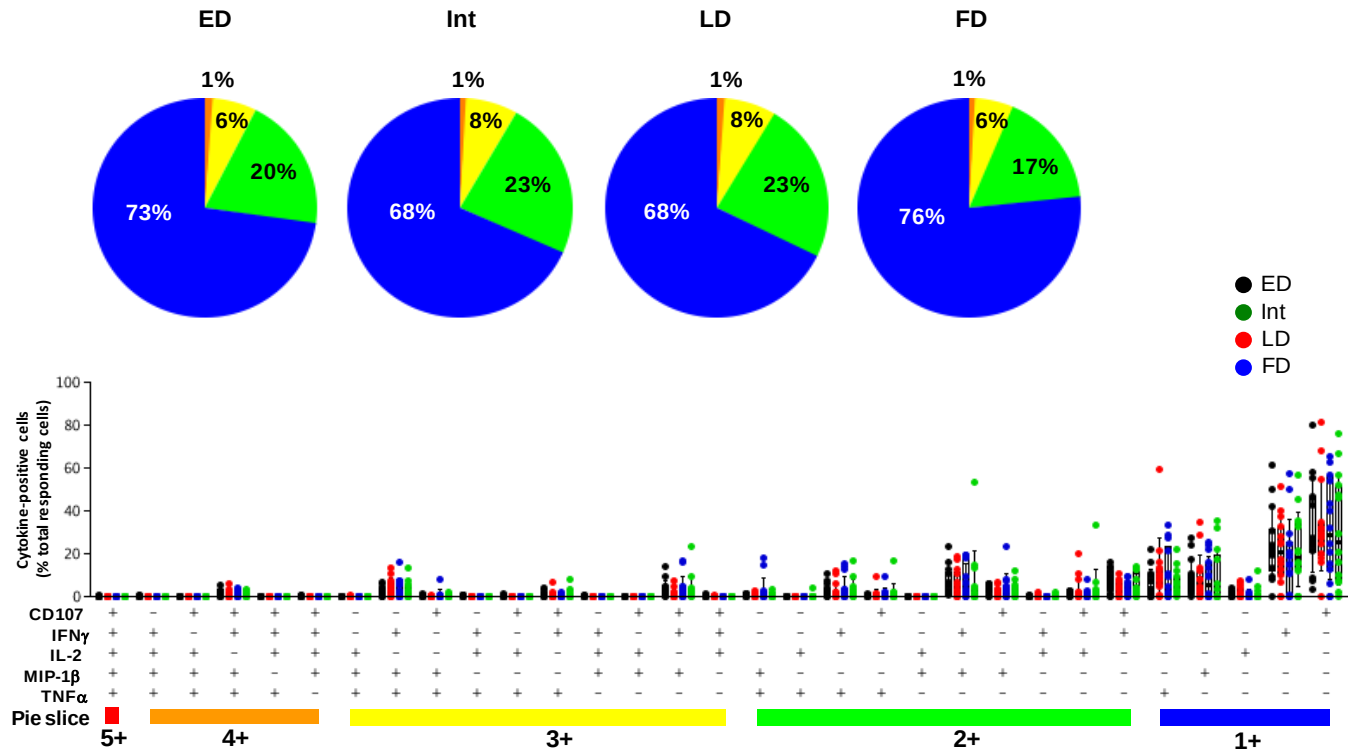


Figure 5.10: The polyfunctionality of Gag-specific ED-, Int-, LD- and FD-memory CD8⁺ T cells at 3 months post-infection. Using Boolean gating, the distribution of cells any combination of functional profiles was determined for ED (black dots), Int (green dots), LD (red dots) and FD (blue dots) in early HIV-infected individuals (n=14). The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of Gag-specific T cells were performed by a student's test ⁺ (p<0.05).

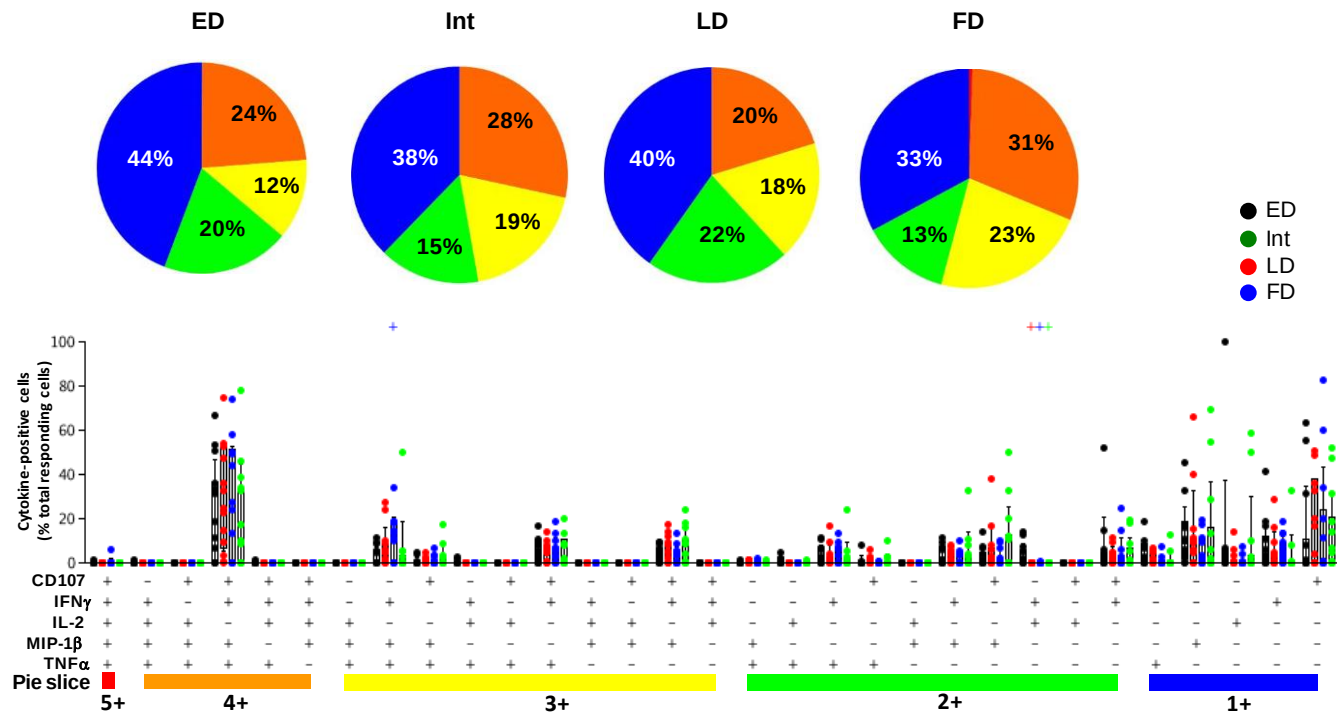


Figure 5.11: The polyfunctionality of CMV-specific ED-, Int-, LD- and FD-memory CD8⁺ T cells in chronically HIV-infected individuals. Using Boolean gating, the distribution of cells any combination of functional profiles was determined for ED (black dots), Int (green dots), LD (red dots) and FD (blue dots) in chronically HIV-infected individuals (n=13). The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total CMV response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of CMV-specific T cells were performed by a student's test ⁺ (p<0.05).

5.2.5 The longitudinal changes in the functional profile of HIV-specific memory T cells during HIV-1 infection

It has been previously shown that persistent exposure to viral antigens could result in the functional impairment of antigen-specific T cells (Wherry *et al.*, 2003). Thus, antigen-specific T cells may lose the ability to proliferate and produce multiple effector functions, which could ultimately lead to the incapability of clearing the virus. To explore this further, the evolution of multifunctional HIV-specific T cells following primary HIV-1 infection was assessed by measuring the ability of Gag-specific T cells to express CD107a, IFN γ , IL-2, MIP-1 β and TNF α over time. A linear mixed-effects model was used to assess changes over the first 15 months of HIV-1 infection (Chapter 2, Model 1, page 62). Figure 5.12A shows a significant increase in the frequency of total Gag-specific memory CD4⁺ T cells over time (slope=0.018, p=0.004). Likewise, the frequency of total Gag-specific memory CD8⁺ T cell responses significantly increased over the first 15 months of infection (slope=0.032, p=0.032) (Figure 5.12B). Although the total frequency of both Gag-specific memory CD4⁺ and CD8⁺ T cells significantly increased over time, the responses appeared to fluctuate during the course of infection.

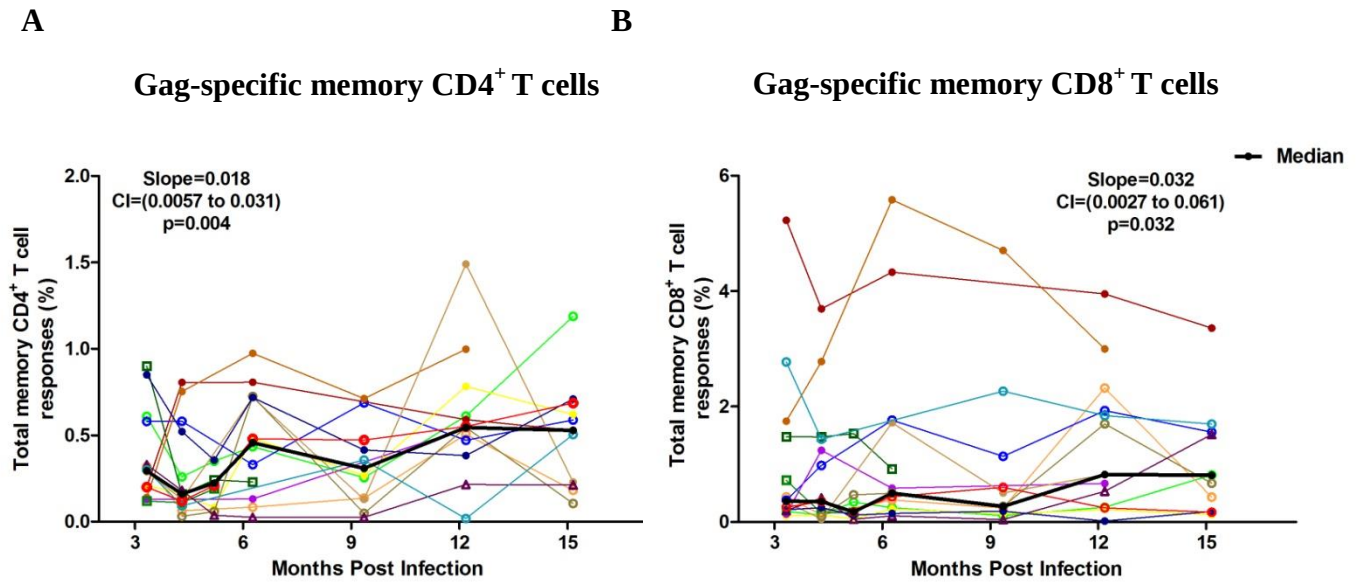


Figure 5.12: Longitudinal characterization of HIV-Gag-specific memory T cells over the first 15 months of infection. Longitudinal changes were determined with a linear mixed-effects model in early HIV-infected individuals (n=15), and slopes used to determine the changes in T cell responses over time with significant p-values ($p < 0.05$). The median change across time is represented by a bold solid line. Patient-specific colour-coded lines show the frequency of T cell responses at each time point, and coloured lines are consistent for patients in each represented graph.

The next level of analysis examined the polyfunctional profile of Gag-specific memory T cells over time. Although the frequency of Gag-specific memory CD4⁺ T cell responses was found to significantly increase over time, the polyfunctional profile remained unchanged between 3 and 15 months after infection (Figure 5.13). The Gag-specific CD4⁺ T cell response profiles were predominantly monofunctional throughout the first 15 months of infection. Mixed-effects linear model was also employed to examine the evolution of the functional profile of cells exhibiting 1+, 2+, 3+, 4+ and 5+ functions at each time point, and no significant changes were observed in the contribution of each functional group over time (Figure 5.13).

Having shown that the monofunctional production of IFN γ was the most frequent Gag-specific CD4⁺ T cell response pattern (median: 27.4%), and also contributed mostly to the global Gag-specific CD4⁺ T cell response (24.5%) at early infection, the contribution of IFN γ to the total Gag-specific CD4⁺ T cell response also remained unchanged over time (slope=-0.0064, p=0.42) (Figure 5.14A). Conversely, a significant increase was observed in the contribution of monofunctional TNF α to the global Gag-specific memory CD4⁺ T cell response (slope=0.04, p=0.008) (Figure 5.14B). Further analysis showed no significant changes in the contribution of CD107a, simultaneous production of IFN γ and TNF α and IFN γ ⁺MIP-1 β ⁺TNF α ⁺ to the total Gag-specific memory T cell response over time

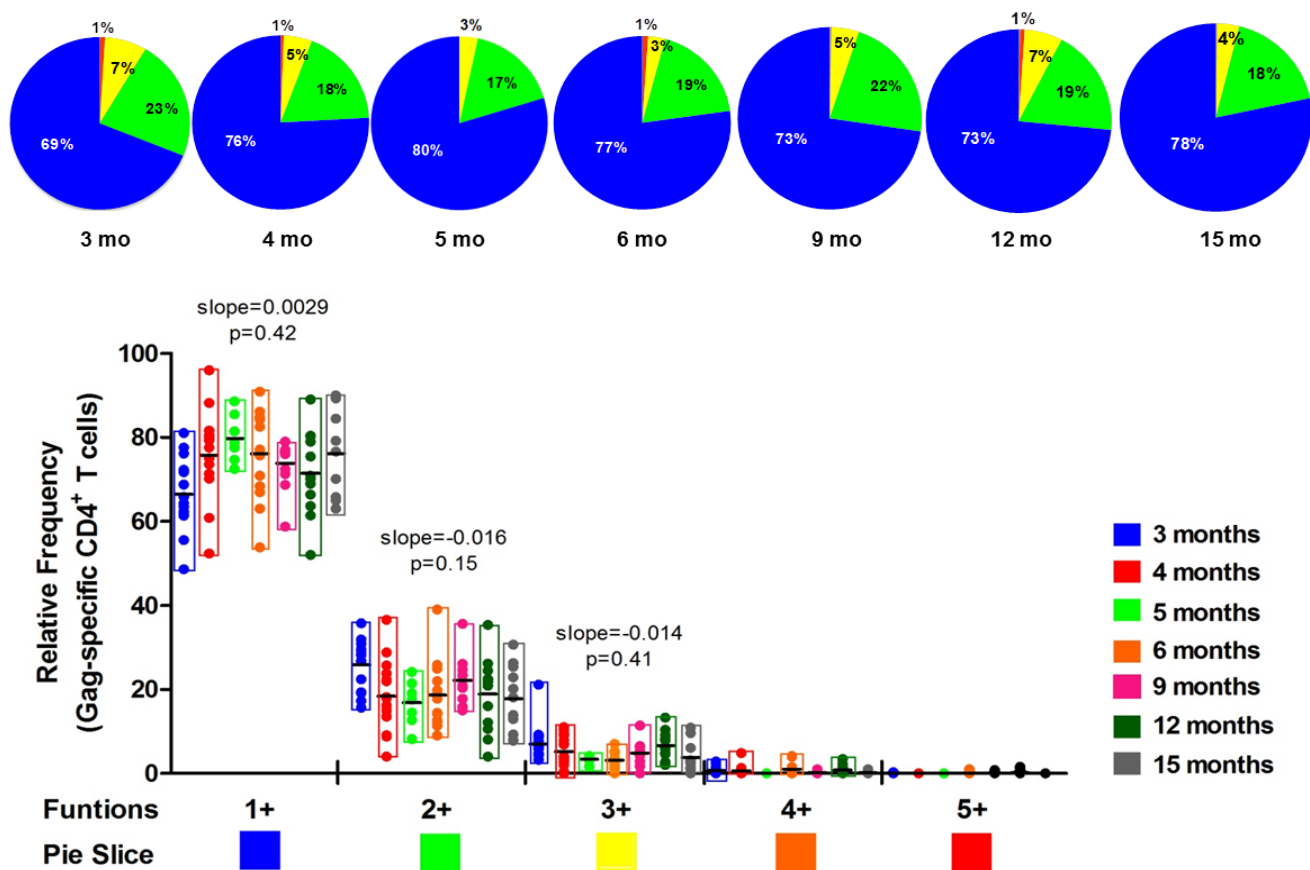


Figure 5.13: The functional profile of Gag-specific memory CD4⁺ T cells over the first 15 months of infection. Individual pie charts show the proportion of the contribution of 5+, 4+, 3+, 2+ and 1+ functional populations to the global Gag-specific memory CD4⁺ T cell responses over time (months; mo) (n=15). The responses are grouped and colour-coded according to the number functions (1+ = blue, 2+ = green, 3+ = yellow, 4+ = orange and 5+ = red). The colour-coded bar chart shows the median and ranges of the frequencies of each individual response at each time point. Longitudinal changes of each group of functions (1+, 2+, 3+, 4+ and 5+) was determined with a linear mixed-effects model, and slopes used to determine the changes in T cell responses over time. The slopes are shown with significant p-values (p<0.05).

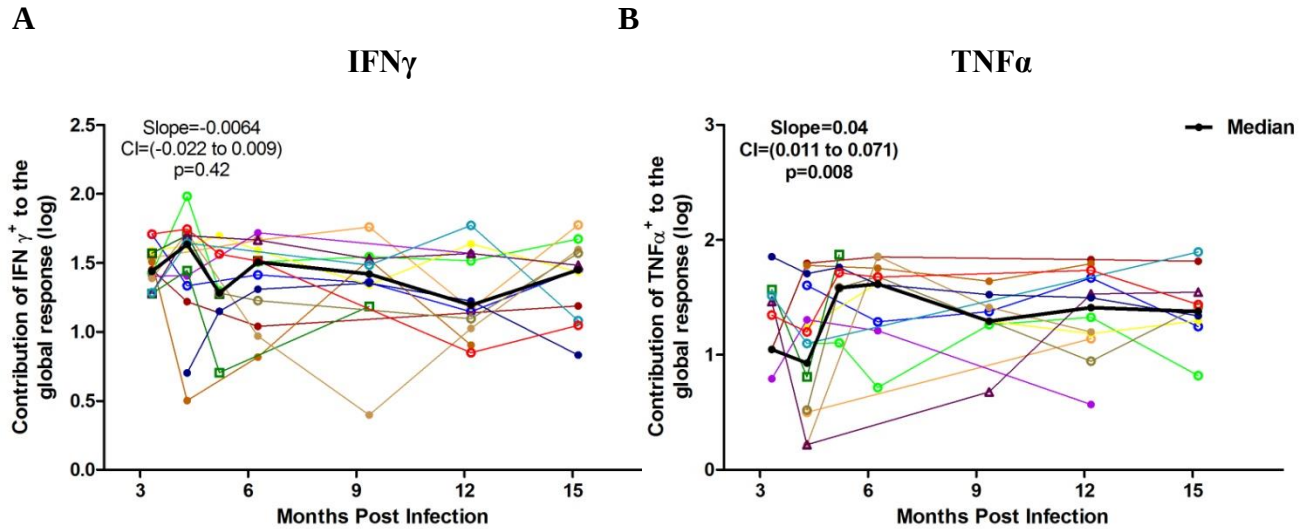


Figure 5.14: The functional contribution of monofunctional IFN γ and TNF α to the global of Gag-specific CD4⁺ T cell response over the first 15 months of HIV infection. Longitudinal changes were determined with a linear mixed-effects model in early HIV-infected individuals (n=15), and slopes used to determine the changes in T cell responses over time with significant p-values (p<0.05). The median change across time is represented by a bold sold line. Patient-specific colour-coded lines show the frequency of T cell responses at each time point, and coloured lines are consistent for patients in each represented graph.

Analysis of Gag-specific memory CD8⁺ T cells showed a significant increase in the frequency of polyfunctional cells at 12 months post-infection relative to 3 months post-infection (p=0.045) (Figure 5.15). There were however no significant changes in the level of polyfunctionality between 3 months and other time points (4, 5, 6, 9 and 15). Gag-specific memory CD8⁺ T cells were mostly characterized by a monofunctional response profile over the first 15 months of infection. The longitudinal analysis of the functional profile of cells exhibiting 1+, 2+, 3+, 4+ and 5+ functions, showed a significant decline in the frequency monofunctional cells over time (slope=-0.0071, p=0.004), whilst cells expressing 3 functions were found to be significantly higher at later time points (slope=0.021, p=0.017). There were no significant changes in cells

exhibiting 2 and 4 functions over time, however a trend toward an increase in the frequency of cells expressing 4 functions was noted (slope=0.11, $p=0.09$).

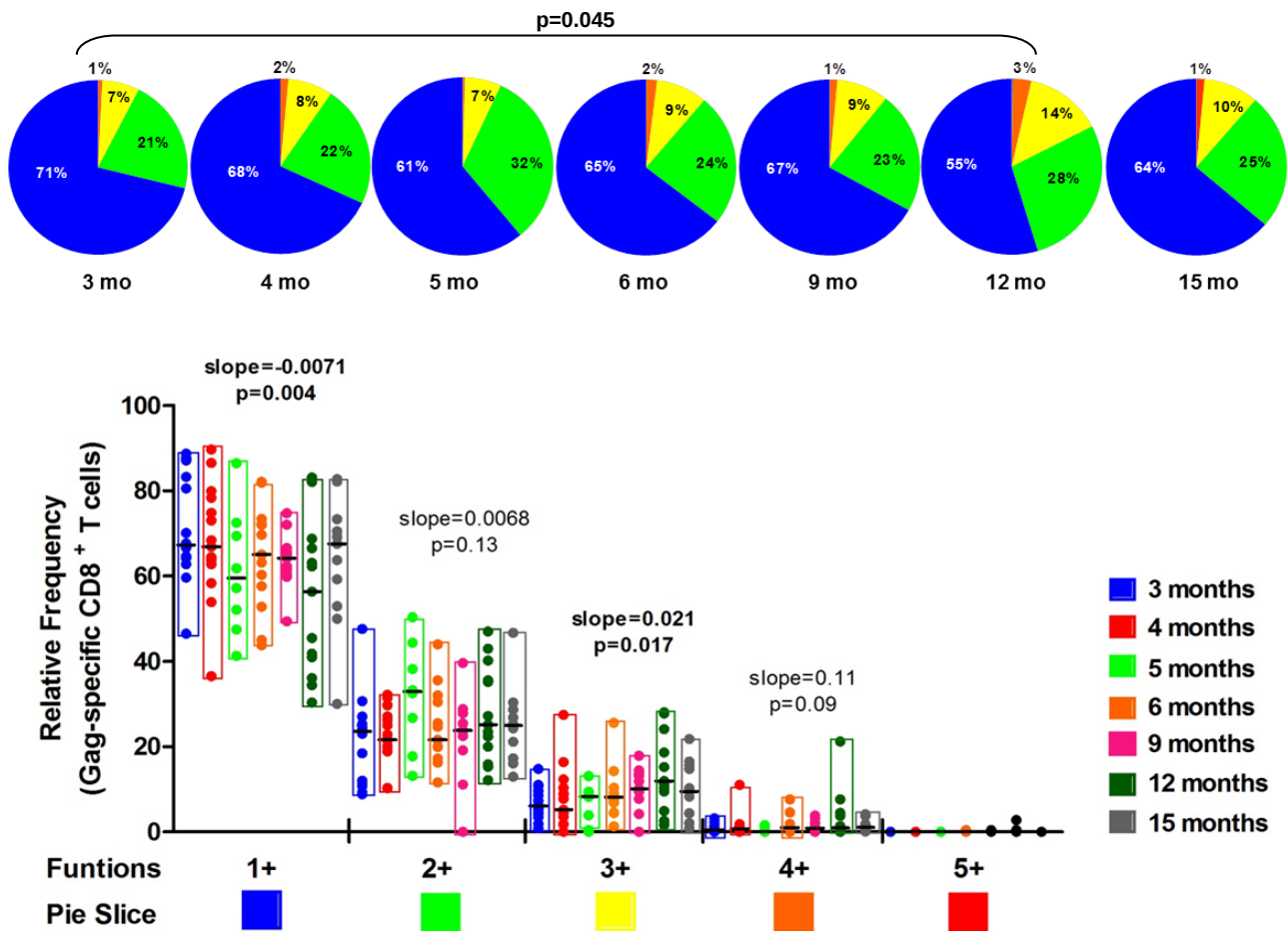


Figure 5.15: The functional profile of Gag-specific memory CD8⁺ T cells over the first 15 months of infection.

Individual pie charts show the proportion of the contribution of 5+, 4+, 3+, 2+ and 1+ functional populations to the global Gag-specific memory CD8⁺ T cell responses over time (months; mo). The responses are grouped and colour-coded according to the number functions (5+ = red, 4+ = orange, 3+ = yellow, 2+ = green and 1+ = blue). The colour-coded bar chart shows the median and ranges of the frequencies of each individual response at each time point. Longitudinal changes of each group of functions (5+, 4+, 3+, 2+ and 1+) was determined with a linear mixed-effects model, and slopes used to determine the changes in T cell responses over time. The slopes are shown with significant p-values ($p<0.05$).

The monofunctional production of CD107a and IFN γ were found to contribute most to the total Gag-specific memory CD8⁺ T cell response at 3 months post-infection (32.9% and 21.9%, respectively). The contribution of both CD107a and IFN γ to the total Gag-specific memory CD8⁺ T cell response remained stable over the first 15 months of infection (Figure 5.16A and B). In contrast, the contribution of Gag-specific CD8⁺ T cells expressing MIP-1 β alone increased significantly over time (slope=0.044, p=0.001) (Figure 5.16C). Among the dual-cytokine producing cells, IFN γ ⁺MIP-1 β ⁺ and CD107a⁺IFN γ ⁺ made up 8.4% and 8.3% of the total Gag-specific memory CD8⁺ T cell response at primary HIV infection. The contribution of IFN γ ⁺MIP-1 β ⁺ to the global Gag-specific memory CD8⁺ T cell response was also found to increase over time (slope=0.032, p=0.004) (Figure 5.16D), whilst the contribution of CD107a⁺IFN γ ⁺ cells remained unchanged. The functional combination CD107a⁺IFN γ ⁺MIP-1 β ⁺ also showed a marked contribution to the total Gag-specific CD8⁺ T cell response over time (slope=0.041, p<0.0001) (Figure 5.16E). With regard to cells expressing 4 functions, CD107a⁺IFN γ ⁺MIP-1 β ⁺TNF α ⁺ cells showed a slight contribution to the total Gag-specific CD8⁺ T cell response over the first 15 months of infection (slope=0.097, p=0.092) (Figure 5.16F). Collectively, these data demonstrate distinct and dynamic functional trajectories of Gag-specific memory CD4⁺ and CD8⁺ T cells over time. This could be attributed to either viral load dynamics or viral sequence diversification over the course of infection (Price *et al.*, 1999). Furthermore, the evolution of cytokine responses over time may also be related to the asynchronous cytokine production together with the various states of T cell differentiation (Han *et al.*, 2012).

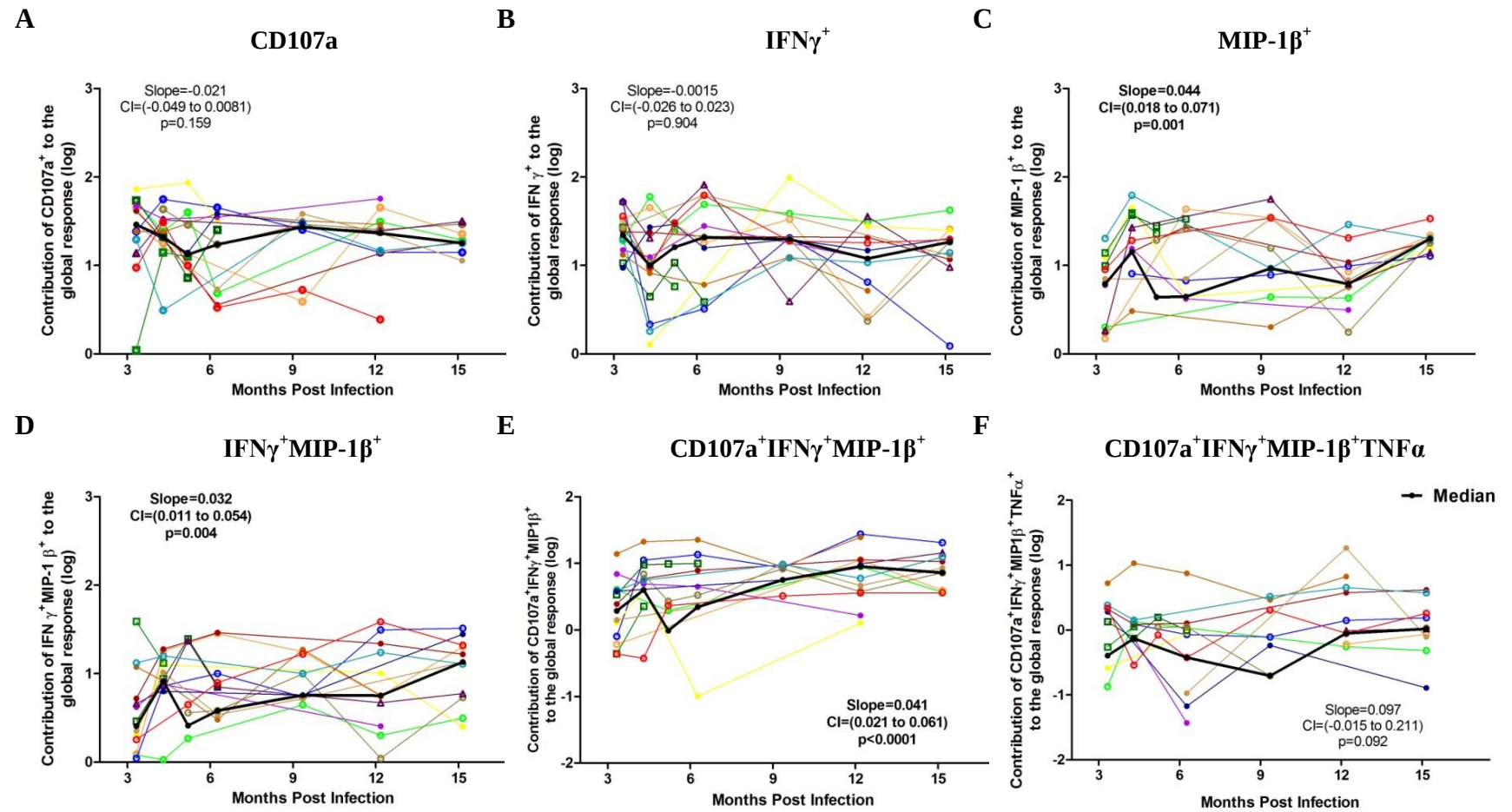


Figure 5.16: The contribution of functional combinations to the global Gag-specific memory CD8⁺ T cell response over the first 15 months of HIV infection. The functional contribution of (A) monofunctional CD107a, (B) IFN γ , (C) MIP-1 β , (D) IFN γ ⁺MIP-1 β ⁺, (E) CD107a⁺IFN γ ⁺MIP-1 β ⁺ and (F) CD107a⁺IFN γ ⁺MIP-1 β ⁺TNF α ⁺ cells to the total Gag-specific memory CD8⁺ T cell response in early HIV-infected individuals (n=15). The Longitudinal changes were determined with a linear mixed-effects model, and slopes used to determine the changes in T cell responses over time. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05). Patient-specific colour-coding is consistent in each represented graph.

5.3 Discussion

Multiple arms of the immune system may contribute to the control of acute or early HIV replication, however, virus-specific T cell responses are known to play an important role in the initial reduction of early viral replication (Borrow *et al.*, 1994, Koup *et al.*, 1994, Goonetilleke *et al.*, 2009). Despite significant numbers of virus-specific T cells, most untreated individuals eventually develop uncontrolled viraemia and progressive disease (Addo *et al.*, 2003). Increasing evidence suggests that T cell dysfunction is one of the key factors in the immunopathogenesis of HIV infection. Thus, impaired HIV-specific T cells may display poor proliferative capacity (Migueles *et al.*, 2002), produce limited array of cytokines (Betts *et al.*, 2006) and express low levels of cytotoxic molecules (Appay *et al.*, 2000). The aim of this chapter has been to examine the magnitude, polyfunctional profile and the dynamics of Gag- and CMV-specific T cells expressing CD107a, IFN γ , IL-2, MIP-1 β and TNF α .

The total magnitude of both Gag-specific memory CD4⁺ and CD8⁺ T cells was found to be comparable with that of CMV-specific memory CD4⁺ and CD8⁺ T cells, though a trend toward increased numbers of CMV-specific CD4⁺ T cells was observed relative to Gag-specific CD4⁺ T cells. These results appear to be in contrast to prior reports showing increased frequencies of IFN γ -producing CMV-specific CD4⁺ T cells (Papagno *et al.*, 2002, Harari *et al.*, 2002) and CD8⁺ T cells (Rehr *et al.*, 2008) than Gag-specific CD4⁺ and CD8⁺ T cells. The apparent discrepancies between these results are unclear, however differences in experimental design may contribute to the following differences: 1) Papagno *et al.* (2002) used a cohort of long-term HIV progressors as opposed to early HIV infected individuals used in this study; 2) Although a cohort of primary HIV infected individuals was used in studies conducted by Harari *et al.* (2002), CMV viral

lysates were used to trigger CMV-specific IFN γ ⁺CD4⁺ T cell responses. Stimulation with viral lysates has been shown to elicit differing immune responses compared to the use of viral proteins (Maecker *et al.*, 2001); and 3) Rehr *et al.* (2008) used a cohort of chronic viraemic subjects to assess HIV- and CMV-specific CD8⁺ T cell responses. When comparing the individual cytokine responses, the frequency of Gag-specific memory CD4⁺ T cells producing IL-2, MIP-1 β and TNF α were significantly lower than those of CMV-specific T cells. This held true for Gag-specific memory CD8⁺ T cells expressing IL-2 and TNF α . The production of IL-2 has been shown to be critical for T cell proliferation and also plays an important role in the establishment and maintenance of long-lived memory cells (Boaz *et al.*, 2002, Younes *et al.*, 2003, Wilson and Livingstone, 2008), while TNF α has been shown to induce apoptosis of cells infected with HIV (Kuwano *et al.*, 1993). Furthermore, increased production of β -chemokines such as MIP-1 β has been associated with a reduction of CCR5-tropic (R5) HIV CD4⁺ T cell infectivity *in vivo* (Casazza *et al.*, 2009). Thus, the binding of β -chemokines to the CCR5 ligands could protect against HIV infection by blocking of the CCR5 receptor binding site and downregulating the surface expression of CCR5 (Alkhatib *et al.*, 1996, Brandt *et al.*, 2002). Therefore, the low frequency of HIV Gag-specific CD4⁺ and CD8⁺ T cells expressing IL-2, MIP-1 β and TNF α may suggest functional impairment of HIV-specific T cells in mediating key functions that associate with the control of viral replication during early HIV-1 infection.

Consistent with previous reports (Casazza *et al.*, 2009), HIV Gag-specific memory CD4⁺ and CD8⁺ T cells displayed lower polyfunctional profiles than CMV-specific memory T cells. Additionally, Betts *et al.* (2006) have shown using a similar phenotype panel, that CMV-specific

CD8⁺ T cell responses maintain high polyfunctional profiles relative to HIV-specific T cells found in progressors. Although Gag- and CMV-specific memory cells exhibited distinct functional profiles, both were found to be predominantly monofunctional responses, followed by dual expression patterns and then responses with three and four functions. The diminished capacity of polyfunctional HIV-specific memory T cells in early infection may be explained by a number of factors: 1) high antigen exposure, which can induce a state of functional exhaustion (Day *et al.*, 2006, Geldmacher *et al.*, 2007b, Rehr *et al.*, 2008, Streeck *et al.*, 2008); 2) rapid viral sequence variation, that could result in less recognition of the variant epitopes (Turnbull *et al.*, 2009); 3) increased susceptibility of HIV-specific T cells to apoptosis compared to CMV-specific T cells (Mueller *et al.*, 2001); 4) high TCR avidity, which could consequently lead to susceptibility to activation-induced cell death, and increased viral escape mutations (Harari *et al.*, 2007). It should also be noted that some differences in the frequency and functional profile among T cells specific for HIV and CMV may be attributed to other factors such as the differences in virus biology, efficiency of antigen presentation, the impact of viral replication kinetics and reactivation, period of antigen encounter and recirculation pathways of virus-specific T cells (Papagno *et al.*, 2002, Appay *et al.*, 2002b, Ellefsen *et al.*, 2002, Jagannathan *et al.*, 2009).

Further analysis showed that monofunctional Gag-specific CD4⁺ and CD8⁺ T cells were characterized by elevated numbers of IFN γ and CD107 than CMV-specific T cells, suggesting that HIV-specific memory T cells in early infection still maintains the ability to produce IFN γ and degranulate (CD107a). Moreover these cytokine responses were found to be the major contributors toward the total Gag-specific memory CD4⁺ and CD8⁺ T cell response in early

infection. Although some T cell functions may be impaired in HIV infection, these data support and extend previous findings showing the preservation of degranulation capacity (CD107a) by HIV-specific T cells during HIV infection (Snyder-Cappione *et al.*, 2006, Rehr *et al.*, 2008, Nemes *et al.*, 2010). Conversely, the functional profile of CMV-specific CD4⁺ and CD8⁺ T cells were mainly comprised of cells expressing CD107, IFN γ , MIP-1 β and TNF α simultaneously, which also made up most of total CMV-specific memory T cell response. Thus, the ability to simultaneously mediate functions may reflect one of the underlying effector mechanisms against CMV infection. Moreover, the ability to degranulate (CD107a) and produce cytokines such as IFN γ and MIP-1 β has been suggested to contribute to the control of viral replication (Emilie *et al.*, 1992, Cocchi *et al.*, 1995, Casazza *et al.*, 2006, Freel *et al.*, 2010, Saunders *et al.*, 2011). Findings in this chapter also showed similar polyfunctionality and functional hierarchies of CMV-specific memory T cell responses in chronically HIV infected and HIV-uninfected individuals, suggesting that CMV-specific T cells present similar antigen sensitivity in HIV-infected and -uninfected individuals. This notion is supported by studies conducted by Almeida *et al.* (2009) showing that the functional hierarchy of T cell responses is largely determined by antigen sensitivity.

The differences in memory differentiation phenotype have been previously associated with improved control of viral replication (Burgers *et al.*, 2009). Different memory subpopulations have different functional capabilities and roles (Sallusto *et al.*, 1999, Sallusto *et al.*, 2004), and thus memory differentiation may impact on the functional abilities of antigen-specific T cells. However, less is known regarding the association between memory differentiation and the

polyfunctional ability of antigen-specific T cells during primary HIV-1 infection. Data in chapter 3 showed that HIV-Gag-specific T cells present an ED-memory phenotype, while CMV-specific T cells were characterized mainly by a LD-memory phenotype. Examination of the functional profile of antigen-specific T cells with regard to differentiation showed equal distribution of polyfunctional cells across the ED- and LD-memory Gag-specific CD4⁺ T cell subpopulations in early HIV-infected individuals. This held true for Gag-specific memory CD8⁺ T cells, which presented similar frequencies of polyfunctional cells within the ED, Int, LD and FD memory compartments. Moreover, all the Gag-specific memory T cell subpopulations presented a less polyfunctional profile than CMV-specific memory T cell subpopulations. Judging from these findings, the lack of functional differences between the different memory subpopulations may suggest that the polyfunctional nature of Gag-specific memory T cells diminishes during primary HIV-1 infection, regardless of memory differentiation phenotypes. On the other hand, the polyfunctionality of CMV-specific memory CD4⁺ T cells was enhanced with differentiation toward a LD-memory phenotype and this was observed in both chronically HIV-infected individuals and HIV-uninfected controls. These findings appear to be in agreement with previous reports, using a similar phenotype panel, demonstrating high numbers of polyfunctional CMV-specific CD4⁺ T cells within the late differentiated memory cells (Casazza *et al.*, 2009). Of note, the functional profile of CMV-specific memory CD8⁺ T cells was found to be equally expanded across the different memory subpopulations. Although data in Chapter 3, Section 3.2.2.3, page 76 showed a skewing of CMV-specific CD8⁺ T cell numbers toward a LD- and FD-memory phenotype, unlike CD4⁺ T cells, this did not translate into an improvement in polyfunctionality with differentiation, suggesting that the polyfunctionality of CMV-specific memory CD8⁺ T cells may be independent of a state of differentiation.

Memory CD4⁺ and CD8⁺ T cells are known to recognize different classes of antigens and execute distinct immune functions (Seder and Ahmed, 2003), and understanding these differences may provide insights for developing T cell-based vaccines aimed at generating effective and optimal HIV-specific CD4⁺ and CD8⁺ T cell responses. Therefore this chapter also sought to examine and compare the functional differences between antigen-specific memory CD4⁺ and CD8⁺ T cells. No difference in the polyfunctionality was observed between Gag-specific memory CD4⁺ and CD8⁺ cells. Conversely, CMV-specific memory CD4⁺ T cells were less polyfunctional than CMV-specific memory CD8⁺ T cells. The superior functionality of CMV-specific CD8⁺ T cells could mostly likely be attributed to higher intrinsic proliferative capacity of CD8⁺ T cells compared to CD4⁺ T cells (Foulds *et al.*, 2002). In addition, factors that also could contribute to the enhanced functionality of CMV-specific CD8⁺ T cells may be related to better efficiency of antigen presentation by MHC class I than MHC class II molecules (Seder and Ahmed, 2003). Given the link between increased proliferative capacity and improved ability to mediate anti-viral T cell functions (Day *et al.*, 2007, Julg *et al.*, 2010, Richmond *et al.*, 2011), in addition to direct associations between proliferation and polyfunction (Day *et al.*, 2011), the limited polyfunctionality of Gag-specific memory CD8⁺ T cells in the context of HIV infection, may further highlight their functional impairment during early stages of infection.

The quality of HIV-specific T cell responses appears to be important for controlling HIV replication (Almeida *et al.*, 2007, Duvall *et al.*, 2008). This chapter examined the quality and evolution of the functional profile of HIV Gag-specific memory T cells during the first 15 months of HIV infection. Longitudinal analysis showed that the magnitude of total Gag-specific memory CD4⁺ and CD8⁺ T cell responses significantly increases over time. Furthermore, the

pattern of responses was characterized by distinct hierarchy of evolving responses that appeared to expand and contract during the course of infection. The increase in the frequency of responses could be attributed to constant antigen exposure. These findings appear to be in line with previous findings showing that HIV-specific T cell responses during acute and/or early infection are narrowly directed against limited epitopic regions (Turnbull *et al.*, 2009, Radebe *et al.*, 2011), but broaden during the late stages of infection (Addo *et al.*, 2003, Karlsson *et al.*, 2007). Moreover, the pattern and frequency of epitope recognition have been shown to change considerably over time (Streeck *et al.*, 2009). The waxing and waning of T cell responses observed in this chapter, confirms and extends previous findings showing oscillations of T cell responses over time (Turnbull *et al.*, 2009, Mlotshwa *et al.*, 2010). The fluctuations of T cell responses could be attributed to a number of factors: 1) HIV antigen load fluctuations; 2) viral sequence diversification and escape mutations may account for the decline of immune functions (Price *et al.*, 1999, Turnbull *et al.*, 2009); 3) T cell exhaustion and susceptibility to apoptosis could also result in the loss of T cell functions (Andersson *et al.*, 2002, Lichterfeld *et al.*, 2004a, Day *et al.*, 2006, Turnbull *et al.*, 2009); while 4) divergence of the TcR (T cell receptor) repertoire could also lead to fluctuations in T cell responses (Meyer-Olson *et al.*, 2006). Moreover, recent findings suggest that changes in TCR repertoire could also result in improved T cell functionality during HIV infection (Janbazian *et al.*, 2012).

Longitudinal analysis on monofunctional cells, showed stable levels in the contribution of IFN γ -producing cells to the total Gag-specific memory CD4⁺ T cell response, whilst the contribution of TNF α increased significantly over time. The changes in the dominance of one cytokine function over another over time may be a related of the asynchronous evolution of cytokine

production under persistent antigen stimulation (Han *et al.*, 2012). Nonetheless, results in this chapter appear to support recent studies conducted by Riou *et al.* (2012), which showed an increase in the frequency of TNF α -producing cells within the IFN γ ⁺CD4⁺ T cell response, over time. Similarly, various functional combinations appeared to be stable, while others tend to contribute more to the global Gag-specific memory CD8⁺ T cell response over time. For example, the contribution of monofunctional MIP-1 β , dual-functional IFN γ ⁺MIP-1 β ⁺ and triple-functional CD107⁺IFN γ ⁺MIP-1 β ⁺ significantly increased over time, while the contribution of monofunctional CD107 and IFN γ remained unchanged. The data appears to be inconsistent with a recent study, which showed stable contributions of monofunctional MIP-1 β to the total Gag-specific CD8⁺ T cell response over time in individuals of different HIV disease progression (Peris-Pertusa *et al.*, 2010). Furthermore, in the latter study, the contribution of MIP-1 β ⁺TNF α ⁺ increased with time, and was primarily associated with elite HIV controllers. The inconsistency between these results is that, Peris-Pertusa *et al.* (2010) examined only two time-points (baseline and after 4 four years), and thus the certain functional profiles could have been missed between the extended time periods.

The polyfunctional response of Gag-specific memory CD4⁺ T cell was found to be stable during the 15 months of infection. Additionally, detailed analysis of each functional group showed no changes in the profile of monofunctional, dual-functional or triple-functional Gag-specific memory CD4⁺ T cells over time. Contrary to previous reports that showed a decline in the polyfunctionality of Gag-specific CD8⁺ T cells with time (Mendiratta *et al.*, 2011), Gag-specific memory CD8⁺ T cells were more polyfunctional during the late stages of infection (12 months PI). Moreover, the frequency of cells exhibiting 3 functions was also found to significantly

increase over time. These findings are in line with recent studies showing an increase in functionality of Gag-specific CD8⁺ T cell responses over time (Ferrari *et al.*, 2011). The increase in T cell polyfunctionality over time has been paralleled with emergence of escape mutations (Streeck *et al.*, 2008), while the recruitment of functionally superior clonotypes have also been linked with functional improvement of HIV-specific CD8⁺ T cells over time (Janbazian *et al.*, 2012).

In conclusion, although the polyfunctional profile of HIV- and CMV-specific memory T cells was compared between different cohorts of early HIV-infected, chronically HIV-infected and -uninfected individuals, this chapter demonstrates key features of the polyfunctional nature between HIV- and CMV-specific memory CD4⁺ and CD8⁺ T cells. CMV-specific memory T cells display a more mature and polyfunctional profile than HIV-specific memory T cells. Although it is still unclear what may constitute an effective or optimal T cell response against HIV-1 infection, the enhanced functionality exhibited by CMV-specific T cells (either during early or late CMV infection) may reflect key functional characteristics that may need to be elicited in HIV-specific T cells for an effective response against HIV infection. This chapter also shows distinct emergent polyfunctional profiles of Gag-specific memory CD4⁺ and CD8⁺ T cell responses over time, whether these responses are paralleled by viral load dynamics in this study remains to be elucidated. The next chapter explores potential mechanisms behind what drives T cell activation during early HIV-1 infection.

CHAPTER 6: MICROBIAL TRANSLOCATION AND INFLAMMATORY

CYTOKINES

TABLE OF CONTENTS

6.1 Introduction.....	185
6.2 Results.....	185
6.2.1 Plasma LBP, I-FABP, sCD14 and EndoCAb levels in HIV infection	185
6.2.2 Plasma cytokines concentrations and their association with microbial translocation ...	190
6.2.3 Associations between markers of microbial translocation and plasma cytokines	192
6.2.4 Associations between microbial translocation, plasma cytokines with HIV-1 viraemia	194
6.2.5 Associations between microbial translocation, plasma cytokines with T cell activation	198
6.2.6 Multivariate associations between microbial translocation, plasma cytokines, HIV-1 viraemia and T cell activation.....	203
6.2.7 The longitudinal changes of microbial translocation markers and plasma cytokines ...	206
6.3 Discussion.....	209

6.1 Introduction

This chapter aims to explore the contribution of microbial translocation and inflammation to the observed T cell activation status during early HIV-1 infection and to tease out whether this represents the major driving force behind early immune activation observed in Chapter 4 and how this impacts on viral replication. Given the strong associations between immune activation and poor HIV-1 disease outcome, understanding the interactions between microbial translocation-induced immune activation and inflammation during the early stages in HIV-1 infection may be essential in developing new strategies aimed at controlling HIV-1 pathogenesis.

It is hypothesized in this chapter **that the T cell activation during primary HIV-1 infection is associated with microbial translocation.** The transit of microbial products into the periphery would generate inflammatory conditions that lead to activation of cells. To test this, the specific aims of this chapter are to; (i) measure the levels and compare the markers associated with microbial translocation (LBP, I-FABP, sCD14 and EndoCAb); (ii) compared the level of plasma inflammatory cytokines (IL-1 β , IL-2, IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1) between HIV-infected and -uninfected individuals; (iii) investigate the relationships between activated memory T cells with the markers of microbial translocation and plasma cytokines and (iv) to assess the longitudinal profile of these solutes over time.

6.2 Results

6.2.1 Plasma LBP, I-FABP, sCD14 and EndoCAb levels in HIV infection

Intestinal permeability as measured by LBP, which is produced by hepatic cells in response to LPS (Albillos *et al.*, 2003), sCD14, an indicator of monocyte/macrophage activation upon LPS

stimulation (Lien *et al.*, 1998), and I-FABP, a marker of intestinal cell damage (Pelsers *et al.*, 2003, Relja *et al.*, 2010), together with IgM EndoCAb were examined in HIV-1 infected individuals at 3 months post-infection and compared to HIV-uninfected controls and chronically HIV-infected individuals. Plasma concentrations of LBP were found to be comparable between HIV-uninfected controls, early and chronically HIV-infected individuals (Figure 6.1A). Although, no significant differences were found between these groups, there was a trend toward greater levels of LBP in early HIV-infected individuals relative to HIV-uninfected controls and chronically HIV-infected individuals (median: 32269ng/ml vs. 18148ng/ml and 19777ng/ml, respectively). Plasma concentrations of I-FABP, were significantly increased in early HIV-infected individuals compared to HIV-uninfected controls and chronically HIV-infected individuals ($p<0.05$ and $p<0.0001$, respectively, Figure 6.1B). Plasma concentrations of I-FABP were found to be similar between HIV-uninfected controls and chronically HIV-infected individuals.

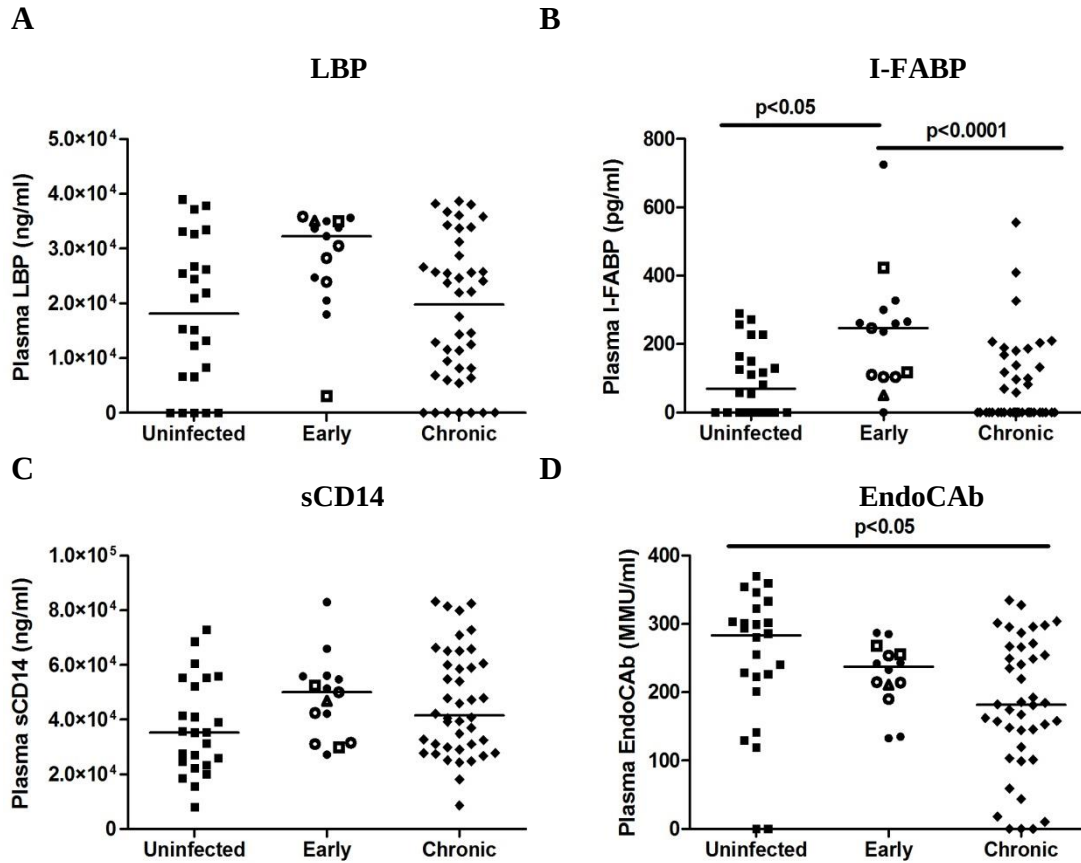


Figure 6.1: Plasma levels of markers associated with microbial translocation at early and chronic HIV-1 infection. Comparison of plasma concentrations of (A) LBP, (B) I-FABP, (C) sCD14 and (D) IgM EndoCAb in HIV-uninfected (n=24, ■) and early (n=15) and chronically (n=42, ◆) HIV-infected individuals. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons between different cohorts where performed by a Kruskal-Wallis ANOVA and multiple comparisons using Dunn's test.

Plasma levels of sCD14 were also found to be similar between HIV-uninfected controls, early and chronically HIV-infected individuals (Figure 6.1C). However, a trend toward greater levels of sCD14 in early HIV-infected individuals compared to HIV-uninfected controls was observed (median: 50013ng/ml vs. 35313ng/ml). No significant differences were found between the plasma concentrations of sCD14 in early and chronically HIV-infected individuals. Although the plasma concentrations of sCD14 did not differ significantly between HIV-uninfected controls and chronically HIV-infected individuals, the latter presented slightly higher plasma levels of sCD14 than control subjects (median: 41548ng/ml vs. 35313ng/ml). The IgM endotoxin core antibodies against LPS were found to be significantly lower in chronically HIV-infected individuals compared to HIV-uninfected controls ($p<0.05$, Figure 6.1D), while no significant differences were observed between HIV-uninfected controls and early HIV-infected individuals. Together, these data suggest that microbial translocation, assessed as by the plasma levels of LBP, I-FABP, sCD14 are to some degree elevated during the early stages of HIV-1 infection.

To further evaluate the occurrence of microbial translocation during chronic HIV infection, plasma concentrations of LBP, I-FABP, sCD14 and IgM endoCAb measured in a group of chronically HIV-infected individuals that had absolute CD4 counts of less than 200 cells/mm³ and compared to HIV-uninfected controls (Figure 6.2). Chronically HIV-infected individuals with CD4 counts below 200 cells/mm³ showed slightly greater levels of LBP than HIV-uninfected controls ($p<0.067$, Figure 6.2A), while sCD14 levels were found to be significantly higher in chronically HIV-infected individuals relative to HIV-uninfected controls ($p=0.032$, Figure 6.2C). In contrast, IgM endoCAb were found to be significantly higher in HIV-uninfected controls relative to chronically HIV-infected individuals ($p=0.017$, Figure 6.2D). No significant

differences were found in the plasma concentrations of I-FABP between chronically HIV-infected individuals with CD4 counts below 200 cell/mm³ and HIV-uninfected controls (Figure 6.2B). These findings may to some extent suggest a possible role of microbial translocation in HIV pathogenesis during the chronic stages of HIV infection.

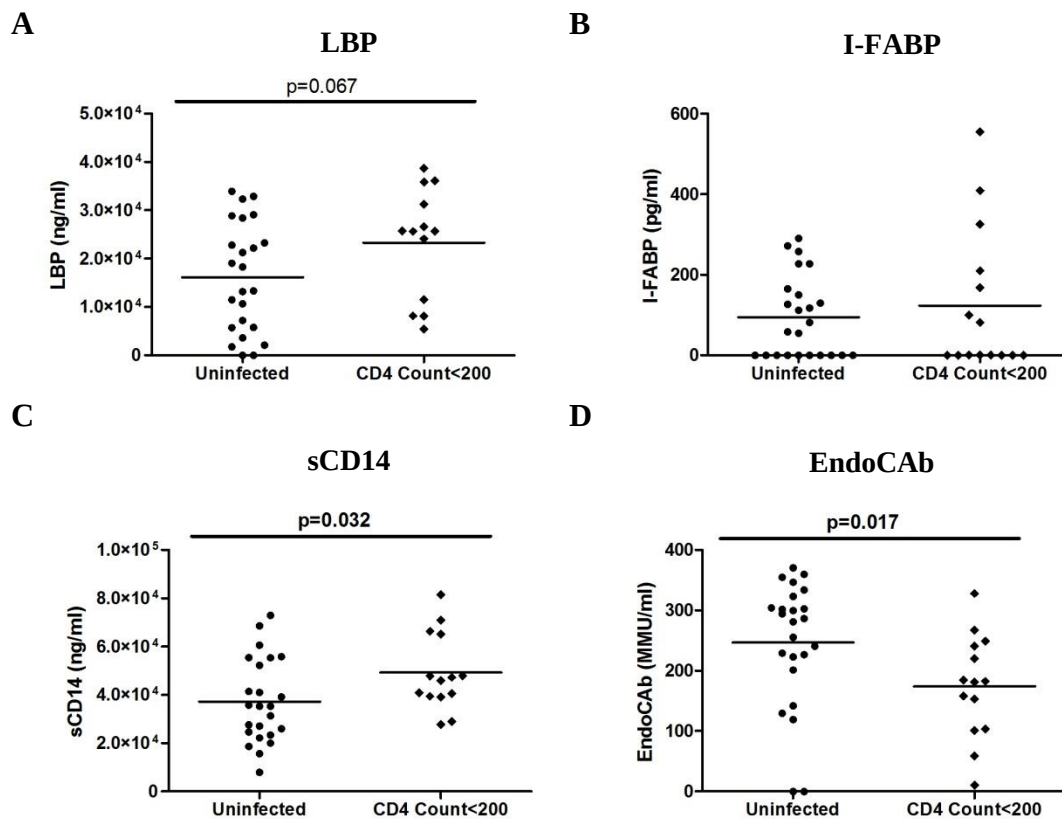


Figure 6.2: Plasma concentrations of markers of microbial translocation in chronically HIV-infected individuals with CD4 count less than 200 cells/mm³. Comparison between the plasma levels of (A) LBP, (B) I-FABP, (C) sCD14 and (D) IgM EndoCAB, in HIV-uninfected (n=24, ■) and chronically HIV-infected individuals with absolute CD4 count below 200 cells/mm³ (n=15, ♦). Median values are shown as a horizontal bar. Statistical comparisons between different cohorts were performed by a Mann-Whitney *U* non-parametric *t* test.

6.2.2 Plasma cytokines concentrations and their association with microbial translocation

HIV infection is also characterized by the up-regulation of inflammatory cytokines (Decrion *et al.*, 2005). Plasma cytokines and/or chemokine concentrations in early and chronic HIV-infected individuals were measured in parallel with the other factors and compared to HIV-uninfected controls. Figure 6.3 shows significantly elevated plasma levels of inflammatory IL-6 ($p<0.005$), hematopoietic IL-7 ($p<0.005$), anti-inflammatory IL-10 ($p<0.005$), and chemokine MCP-1 ($p<0.05$) in primary HIV infection when compared to HIV-uninfected controls. Plasma concentrations of IL-12p70, an isoform of IL-12 (Ethin *et al.*, 2003), were found to be significantly reduced in HIV-1 infected individuals compared to HIV uninfected controls ($p<0.05$). No significant differences were observed in plasma concentrations of TNF α and IL-1 β between HIV-infected individuals and HIV-uninfected controls. There was however a trend towards elevated levels of TNF α in HIV-infected individuals (median: 8.37pg/ml vs. 3.76pg/ml). Plasma cytokine and/or chemokine concentrations were comparable between chronically HIV-infected individuals and HIV-uninfected controls. The plasma concentrations of IL-7 and MCP-1 were found to be significantly lower in chronically HIV-infected individuals when compared to early HIV-infected individuals ($p<0.005$ and $p<0.05$, respectively).

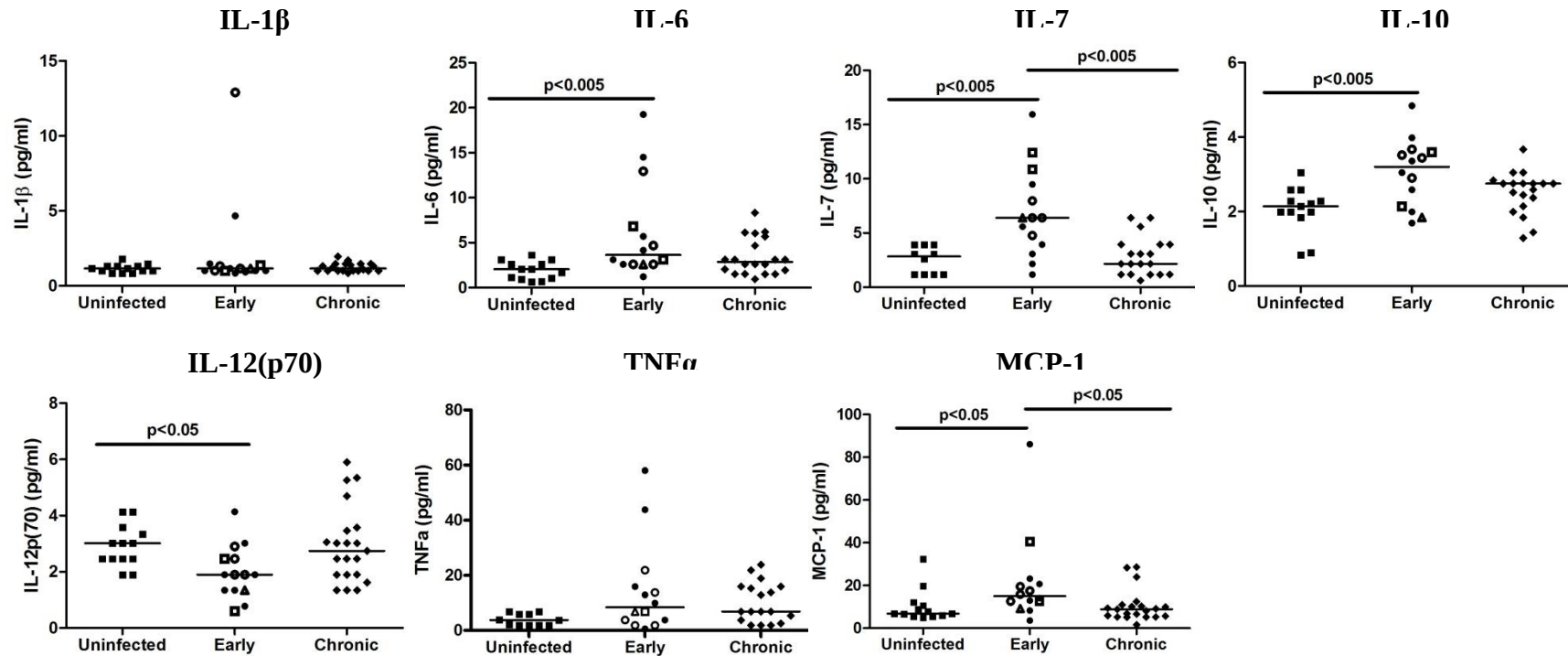
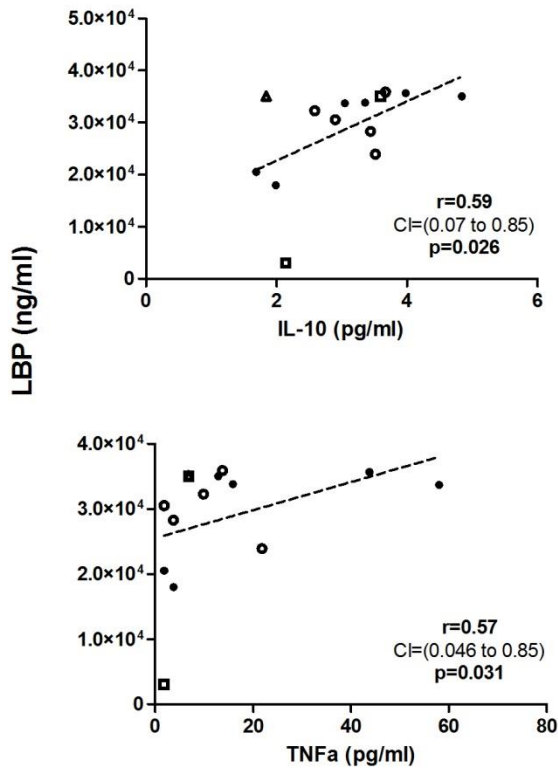


Figure 6.3: Comparison of plasma cytokines levels in HIV-uninfected, early and chronically HIV-infected individuals. Comparison of plasma levels of IL-1 β , IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1 between HIV-uninfected individuals (n=13, ■), early HIV-infected (n=15) and chronically HIV-infected individuals (n=21, ◆) infected individuals. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Median values are shown as a horizontal bar. Statistical comparisons between different cohorts were performed by a Kruskal-Wallis ANOVA and multiple comparisons using Dunn's test.

6.2.3 Associations between markers of microbial translocation and plasma cytokines

The involvement of sCD14-TLR4 complex with microbial products results in the activation of monocyte or macrophages and subsequent production of a wide range of inflammatory molecules (Miller *et al.*, 2005, Miyake, 2006, Lu *et al.*, 2008). To explore the possible associations between microbial translocation and inflammation during primary HIV-1 infection, plasma concentrations of LBP, I-FABP, sCD14 and IgM endoCAb were associated with plasma cytokine and/chemokine concentrations. Plasma levels of LBP correlated significantly with IL-10 and TNF α ($r=0.59$, $p=0.031$, and $r=0.57$, $p=0.031$, respectively, Figure 6.4A), while plasma levels of sCD14 positively associated with plasma levels of IL-6 and TNF α ($r=0.55$, $p=0.037$ and $r=0.73$, $p=0.0025$, respectively, Figure 6.4B). A trend toward a negative correlation was observed between the levels of sCD14 and IL-12p70 levels ($r=-0.46$, $p=0.09$) (Appendix N, Figure N1, page 281). No significant correlations were found between the plasma concentrations of IL-7, IL12-p70 or MCP-1 with any of the measured markers of microbial translocation. In contrast to HIV-infected individuals, no associations were found between the markers of microbial translocation and plasma cytokines in HIV-uninfected controls (Figure 6.5A and B). Although significant correlations found between markers of microbial translocation and plasma cytokines in HIV-infected individuals infer that the up-regulation of IL-10 or TNF α may be consequence of gut-associated microbial translocation and/or monocyte activation, the lack of correlations between markers of microbial translocation and these cytokines in HIV-uninfected controls suggest that these associations are not causative.

A



B

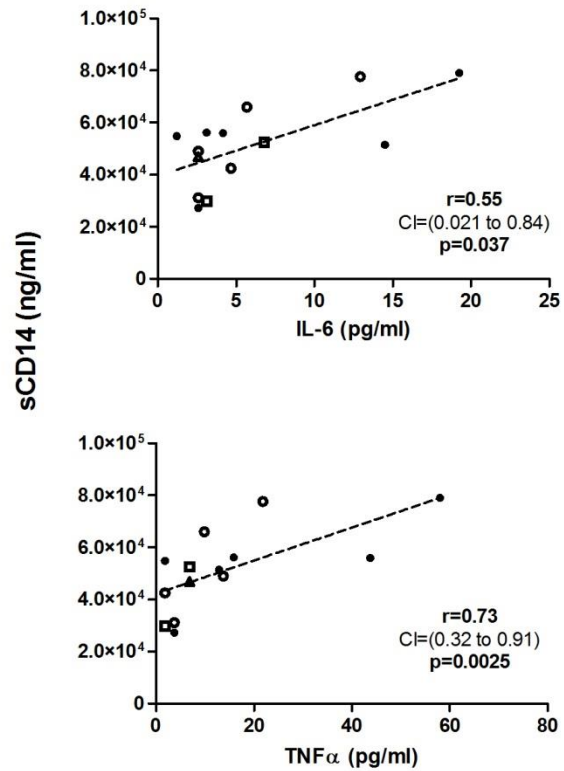
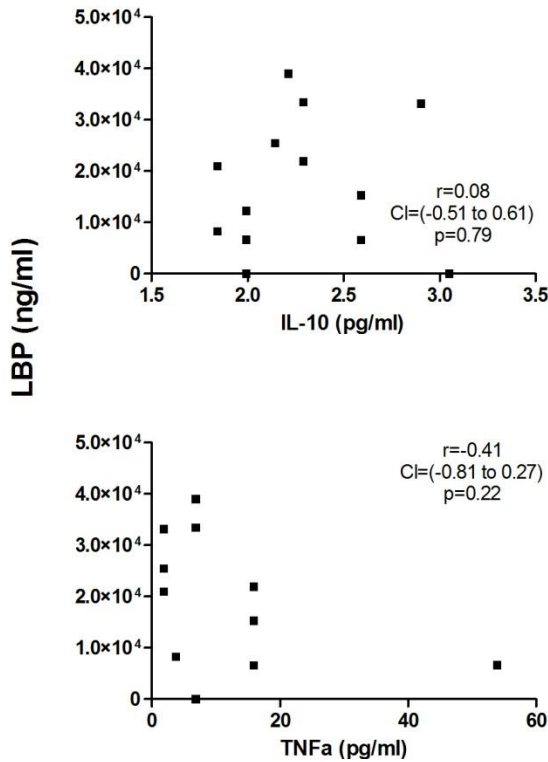


Figure 6.4: The association between markers of microbial translocation and cytokines at 3 months post-infection. Correlations between plasma concentrations of LBP and sCD14 with IL-10, IL-6 and TNFα at 3 months post-infection (n=14). Open circles (O) represent individuals who showed an HIV viral load decline of $>0.5 \log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5 \log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5 \log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

A



B

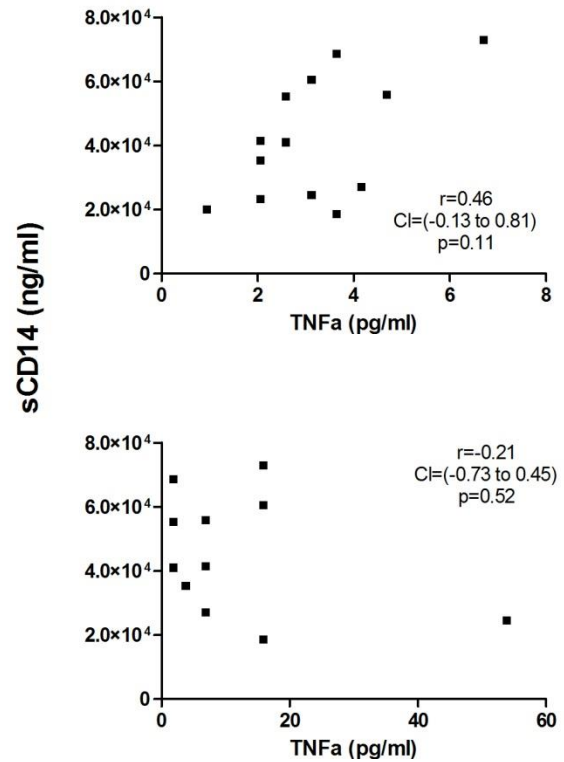


Figure 6.5: The relationship between plasma markers of microbial translocation and plasma cytokine concentrations in HIV-uninfected individuals. (A) Correlations between plasma concentrations of LBP with IL-10, TNF α and MCP-1. (B) Correlations between plasma concentrations of sCD14 with IL-6 and TNF α . Statistical associations were performed by a two tailed non-parametric Spearman rank correlation.

6.2.4 Associations between microbial translocation, plasma cytokines with HIV-1 viraemia

Microbial translocation has been previously associated with a number of immunodeficiency diseases (Chapter 1, page 44). One fundamental question is whether microbial translocation was associated with HIV-1 viral load at 3 months post-infection. Figure 6.6A shows a trend towards a positive correlation between plasma LBP levels and viral load ($r=0.49$, $p=0.069$), while plasma sCD14 concentrations significantly correlated viral load ($r=0.86$, $p<0.0001$, Figure 6.6C). An

inverse and significant association was found between plasma concentrations of IgM EndoCAb and HIV viraemia at 3 months post-infection ($r=-0.69$, $p=0.0056$, Figure 6.6D). No significant associations were found between I-FABP levels and viral load (Figure 6.6B).

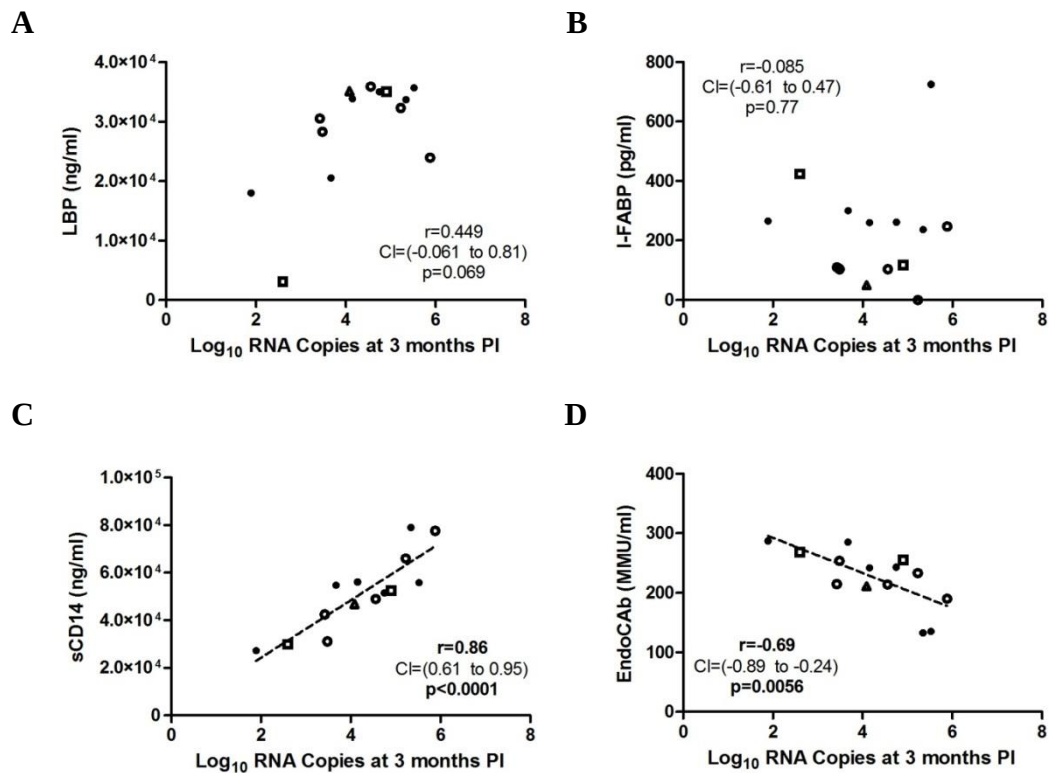


Figure 6.6: Correlations of plasma levels of markers associated microbial translocation and plasma cytokines with viral load at 3 months post-infection. Correlations between plasma concentrations of (A) LBP, (B) I-FABP, (C) sCD14 and (D) IgM EndoCAb with concurrent viral load at 3 months post-infection ($n=14$). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\text{log}_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\text{log}_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\text{log}_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

To assess the relationship between host immune responses to HIV-1 antigens during HIV-1 infection, plasma cytokine concentrations were correlated with the concurrent viral load at 3 months post-infection. Plasma IL-6, IL-10 and TNF α concentrations significantly correlated with viral load at 3 months post-infection ($r=0.65$, $p=0.011$; $r=0.57$, $p=0.032$; and $r=0.84$, $p=0.0002$, respectively) (Figure 6.7A, B and C), suggesting that HIV-1 viraemia may contribute towards the up-regulation of these cytokines during the early stages of infection. Plasma IL-12p70 levels negatively correlated with viral load ($r=-0.54$, $p=0.043$), while no significant associations were found between the plasma concentrations of IL-1 β , IL-7 and MCP-1 with viral load (Appendix N, Figure N2, page 281).

A mixed-effects linear regression model was used to examine the longitudinal relationship between plasma markers of microbial translocation and inflammatory molecules with viral load over the first 12 months of HIV-1 infection (Chapter 2, Model 2, page 63). Significant and positive correlations were found between plasma sCD14 levels and viral load over time (slope=0.084, $p=0.0024$, Table 6.1), suggesting that microbial translocation coincides with ongoing HIV-1 replication during the course of infection. Increased plasma levels of IL-6, IL-10 and TNF α also correlated with increased viraemia over time (Table 6.1) and no significant associations were observed between plasma concentrations of IL-1 β , IL-7, IL-12p70 and MCP-1 with viral load over time. Collectively, these data show that microbial translocation, as measured by sCD14 together with IL-6, IL-10 and TNF α , associate with continuous HIV-1 replication during the early stages of infection and throughout the first year of HIV-1 infection.

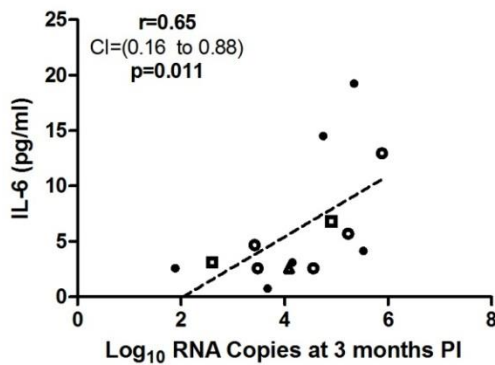
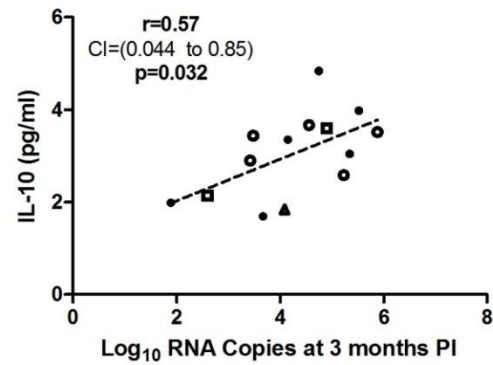
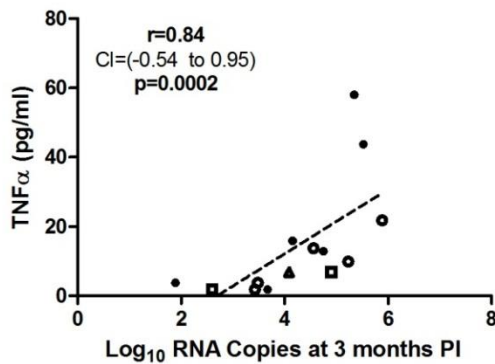
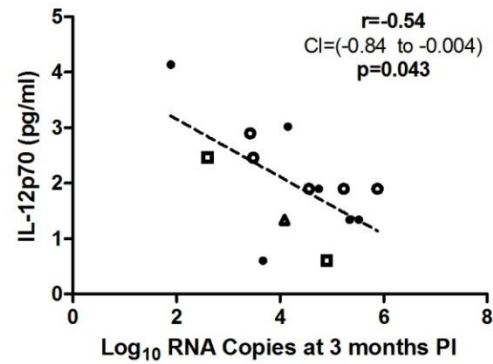
A**B****C****D**

Figure 6.7: The relationship between plasma cytokines with viral load at 3 months post-infection. (A) Correlations between plasma concentrations of IL-6, IL-10, TNF α and IL-12p70 with concurrent viral load at 3 months post-infection ($n=14$). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

Table 6.1: Mixed-effects linear associations between the plasma markers of microbial translocation, cytokines and viral load over the first 12 months of HIV infection

Markers of Microbial Translocation	Slope	<i>P</i>	95% CI
LBP	-0.0003	0.992	(-0.058 to 0.057)
I-FABP	0.0030	0.949	(-0.103 to 0.110)
sCD14	0.0840	0.002	(0.030 to 0.1370)
EndoCAb	-0.0290	0.013	(-0.051 to -0.006)
Plasma Cytokines			
IL-1 β	0.012	0.330	(-0.057 to 0.081)
IL-6	0.138	0.009	(0.032 to 0.227)
IL-7	0.029	0.545	(-0.064 to 0.121)
IL-10	0.048	0.036	(0.003 to 0.093)
IL-12(p70)	-0.014	0.665	(-0.075 to 0.048)
TNF α	0.254	0.007	(0.069 to 0.4390)
MCP-1	-0.029	0.710	(-0.074 to 0.05)

Longitudinal associations between plasma markers of microbial translocation, cytokines and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

6.2.5 Associations between microbial translocation, plasma cytokines with T cell activation

Having established that early HIV-1 infection is characterized by increased proportions of activated memory CD4⁺ and CD8⁺ T cells relative to HIV-uninfected controls (observations in Chapter 4), this section examined whether the process of microbial translocation influenced the level of immune activation. To understand the potential role that microbial translocation plays on T cell activation, correlations between activated proliferating memory T cells (CD38⁺HLADR⁺Ki67⁺), and plasma levels of LBP, I-FABP and sCD14 were examined. In HIV-

infected individuals, increased plasma sCD14 levels correlated with increased proportions of activated total memory CD4⁺ T cells ($r=0.71$, $p=0.0045$, Figure 6.8). There was a trend toward a positive correlation between plasma LBP levels and activated total memory CD4⁺ T cells ($r=0.52$, $p=0.053$). No significant associations were found between plasma I-FABP levels and activated memory CD4⁺ T cells. A positive correlation was also noted between LBP plasma concentrations and the proportion of activated total memory CD8⁺ T cells ($r=0.64$, $p=0.0129$), while no associations were found between the proportions activated memory CD8⁺ T cells with either I-FABP or sCD14 levels. Moreover, significant and positive associations between plasma markers of microbial translocation and activation were upheld when using double activation marker expression (CD38⁺HLADR⁺) (Appendix N, Figure N3A, page 282). Analysis on antigen-specific T cells showed no significant associations between the markers of microbial translocation with any of the activated Gag- and CMV-specific CD4⁺ or CD8⁺ memory T cells (Appendix N, Figure N4A and B, page 283).

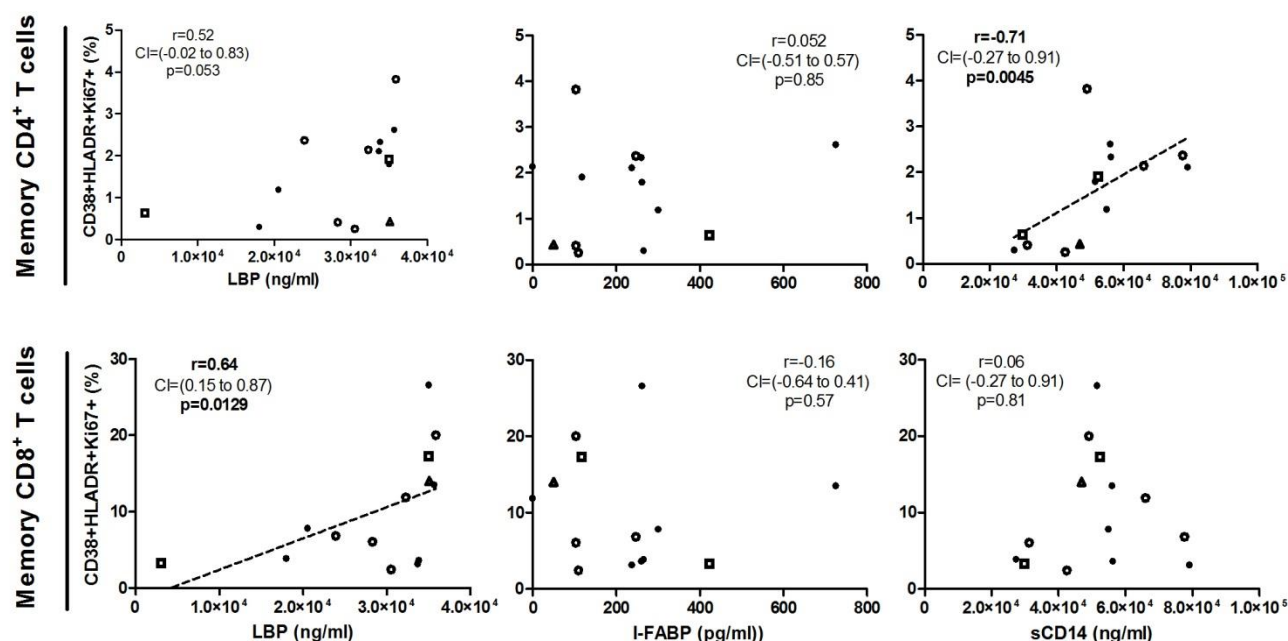


Figure 6.8: The relationship between plasma markers of microbial translocation with memory T cell activation at 3 month post-infection. Correlations between the plasma concentrations of LBP, I-FABP and sCD14 with the proportions of activated total memory CD4⁺ and CD8⁺ T cells. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

Given that T cell activation could also be regulated by cytokines independent of TCR-triggering (Unutmaz *et al.*, 1994, Geginat *et al.*, 2003), the relationship between plasma cytokine concentrations and T cell activation levels were also investigated. There were significant associations between plasma concentrations of IL-10 and TNF α with activated total memory

CD4⁺ T cells ($r=0.56$, $p=0.033$ and $r=0.79$, $p=0.0007$, respectively, Figure 6.9). No significant associations were found between plasma cytokines and activated total memory CD8⁺ T cells. Similarly, there was a positive and significant correlation between memory CD4⁺ T cells expressing CD38⁺HLADR⁺ with TNF α and a trend toward a positive correlation with IL-10, and no significant associations were noted between memory CD8⁺ T cells expressing CD38⁺HLADR⁺ with any of the measured plasma cytokines (Appendix N, Figure N3B, page 282). Of note, no associations were found between activated Gag-specific CD4⁺ and CD8⁺ T cells with any of the measured cytokines (Appendix N, Figure N5, page 284 and Figure N6, page 285). Significant and positive correlations were however noted between activated CMV-specific CD4⁺ T and CD8⁺ T cells with IL-10 ($r=0.53$, $p=0.049$ and $r=0.83$, $p=0.0007$, respectively) (Appendix N, Figure N7, page 286). In HIV-uninfected controls, no significant associations were found between plasma markers of microbial translocation and inflammatory molecules with activated total memory T cells (Figure 6.10) or CMV-specific memory T cell activation.

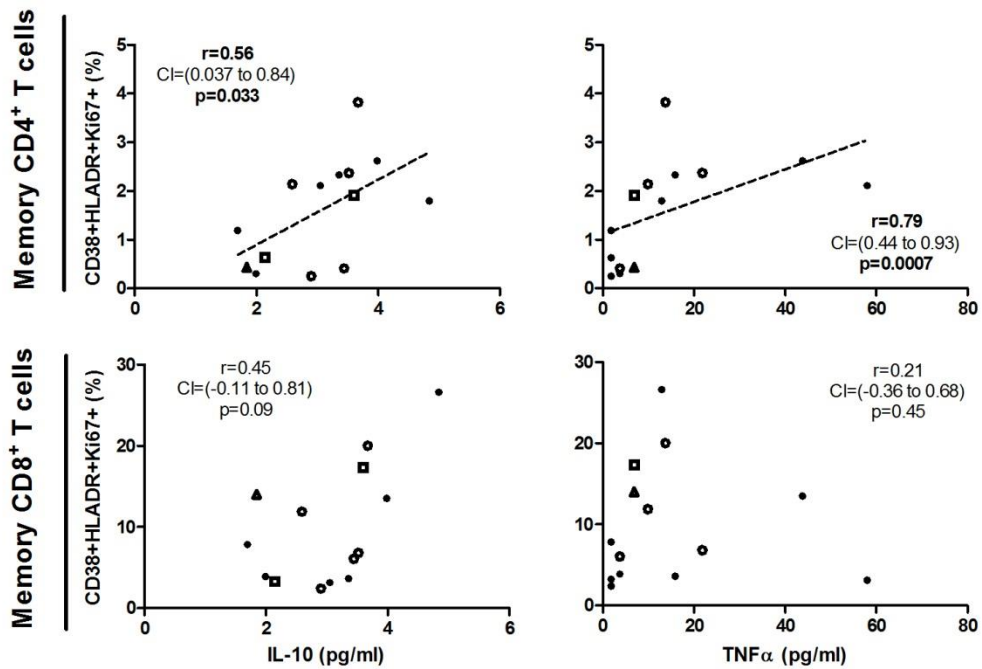


Figure 6.9: The relationship between plasma cytokines with memory T cell activation at 3 month post-infection. Correlations between the plasma concentrations of IL-10 and TNF α with the proportions of activated memory CD4⁺ and CD8⁺ T cells. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

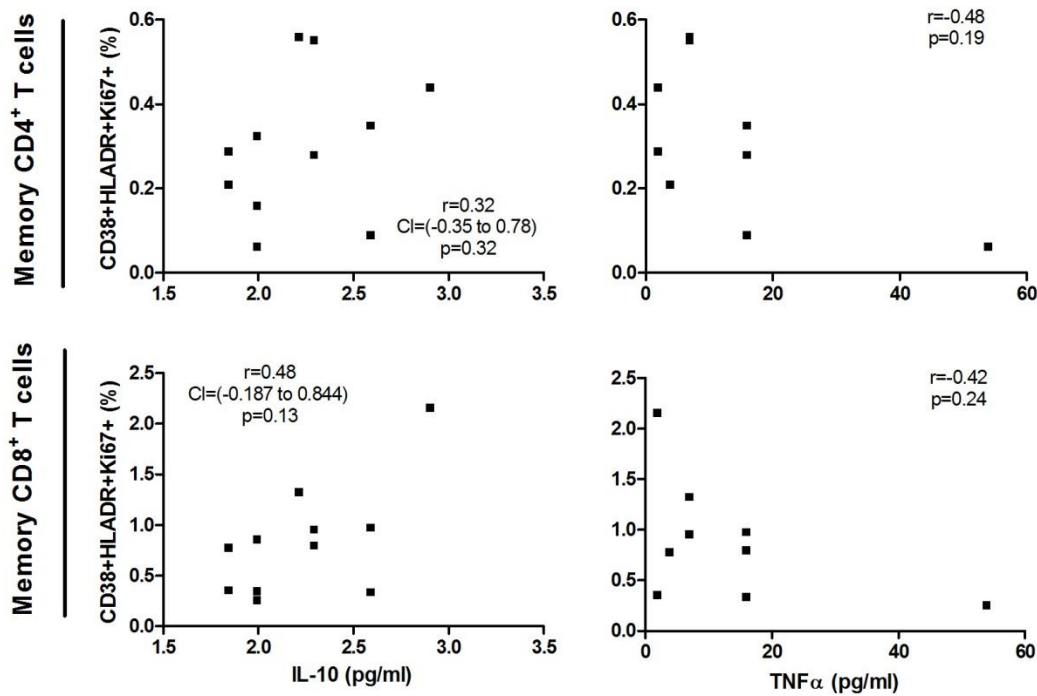


Figure 6.10: The relationship between total memory T cell activation with plasma cytokines in HIV-uninfected individuals. (A) Correlations between the plasma levels of, IL-10 and TNFα with the proportions of activated memory CD4⁺ and CD8⁺ T cells. Statistical associations were performed by a two tailed non-parametric Spearman rank correlation.

6.2.6 Multivariate associations between microbial translocation, plasma cytokines, HIV-1 viraemia and T cell activation.

Having shown direct correlations between plasma sCD14, LBP, IL-10 and TNFα with either activated memory CD4⁺ or CD8⁺ memory T cells using the univariate analysis, the relationships between markers of microbial translocation and cytokines were more accurately defined by adjusting for HIV-1 viral load. Of note, for the inclusion in the multivariate analyses, variables were chosen with an observed $p < 0.05$ in the univariate analysis (i.e. sCD14, LBP, IL-10 and

TNF α). Multivariate analysis showed no significant associations between sCD14 and LBP with T cell activation, while viral load significantly correlated with both activated total memory CD4⁺ and CD8⁺ T cells (p=0.036 and p=0.014, respectively, Table 6.2). Similarly, no associations were observed between plasma IL-10 and TNF α concentrations with activated memory CD4⁺ T cells when adjusting for viral load (Table 6.3). Collectively, these data indicate that *in vivo* replicating virus appears to be a major contributor to T cell activation, and that microbial translocation and specific cytokine responses, appear not to be primary activation signals to T cells during early HIV infection.

Table 6.2: Multivariate regression analysis associating plasma sCD14 and viral load with T cell activation at 3 months post-infection

Variable vs. CD4 Activation	Model(R ²)(P)	Standard Error	(P)	Coefficient (95% CI)
sCD14	0.53 (p=0.0001)	0.000023	0.85	-4.22e-06 (-0.00005 to 0.00004)
Viral load		0.307605	0.036	0.7325628 (0.05553 to 1.40959)
Variable vs. CD8 Activation	Model(R ²)(P)	Standard Error	(P)	Coefficient (95% CI)
LBP	0.43 (p=0.026)	0.000021	0.050	-0.0004731 (-0.00094 to -4.23e-07)
Viral load		2.852667	0.014	8.344614 (2.065936 to 14.62329)

Multivariate associations between plasma markers of microbial translocation and viral load with and T cell activation were performed according to linear regression models. Both models for CD4⁺ and CD8⁺ activation, fitted the data significantly (p=0.0001 and p=0.026, respectively), and the coefficient is shown with 95% Confidence Intervals (CI) and significant p-values (p<0.05).

Table 6.3: Multivariate regression analysis associating plasma IL-10 and TNF α with CD4⁺ T cell activation at 3 months post-infection

Variable vs. CD4 Activation	Model(R ²)(P)	Standard Error	(P)	Coefficient (95% CI)
IL-10	0.56 (p=0.0004)	0.27537	0.360	0.26073 (-0.35282 to 0.87430)
TNF α		0.00834	0.470	0.00617 (-0.01241 to 0.02476)
Viral load		0.14568	0.006	0.50520 (0.18058 to 0.82981)

Multivariate associations between plasma cytokines and viral load with and T cell activation were performed according to linear regression models. The model fitted the data significantly (p=0.0004) and the coefficient is shown with 95% Confidence Intervals (CI) and significant p-values (p<0.05).

6.2.7 The longitudinal changes of microbial translocation markers and plasma cytokines

In order to explore the longitudinal changes in the markers of microbial translocation during the first 12 months of HIV-1 infection, plasma LBP, I-FABP, sCD14 and IgM endoCAb levels from 15 participants were collected at 3 different time points; 3, 6 and 12 months after HIV-1 infection. These data was then analysed by a linear mixed-effects model across all time points (Chapter 2, Model 1, page 62). No significant changes were observed in the markers of microbial translocation over 12 months (Figure 6.11). These findings suggest that the levels of markers associated with microbial translocation may have reached a steady state during the first year of HIV-1 infection. The longitudinal changes on plasma cytokines were also evaluated using a linear mixed-effects model, whereby the slopes were assessed to determine the changes in the plasma levels of cytokines over time. Similarly, no significant changes were observed in the plasma levels of IL-1 β , IL-6, IL-7, IL-10, IL-12p70, TNF α and MCP-1 over the first 12 months of HIV infection (Figure 6.12).

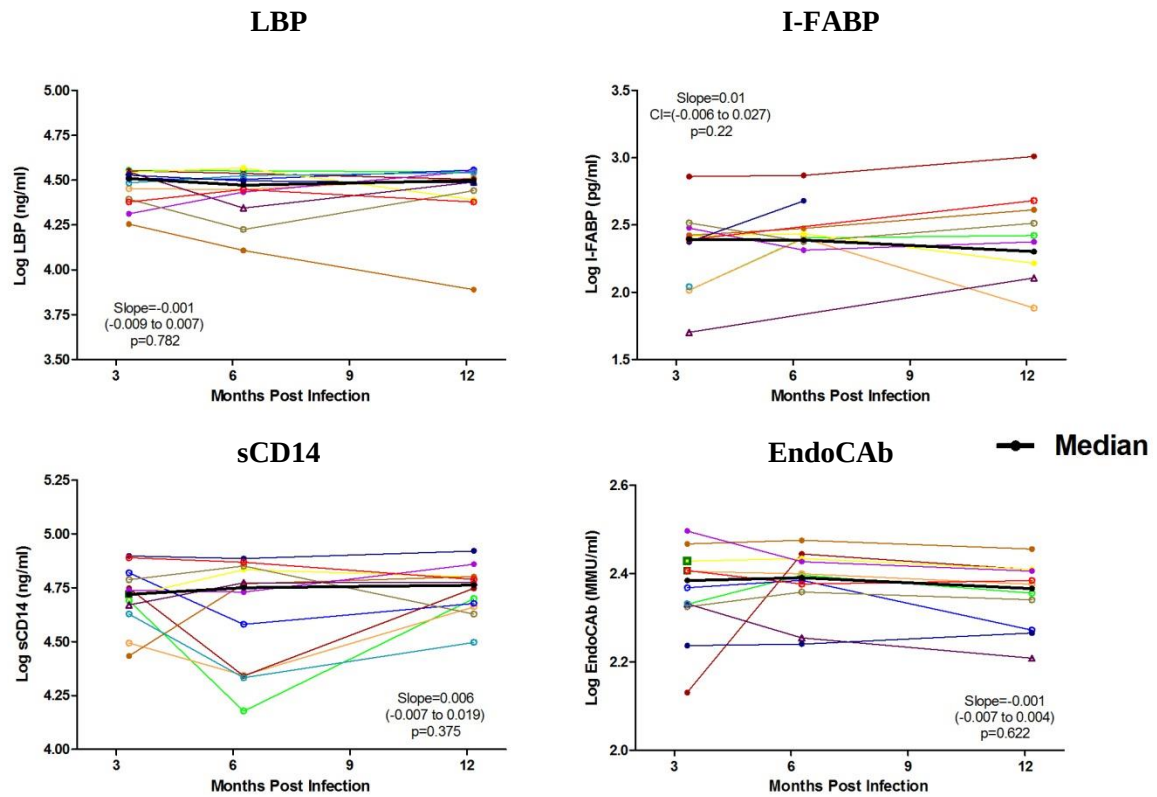


Figure 6.11: The longitudinal changes of the markers of microbial translocation during the first 12 months of HIV infection. Representative plots indicating the longitudinal changes in the plasma levels of LBP, I-FABP, sCD14 and IgM endoCAb in early HIV-infected individuals (n=15). Longitudinal changes in markers of microbial translocation were assessed by using a linear multilevel model. The median change across time is represented by a bold sold line. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$). Patient-specific colour-coding is consistent in each represented graph.

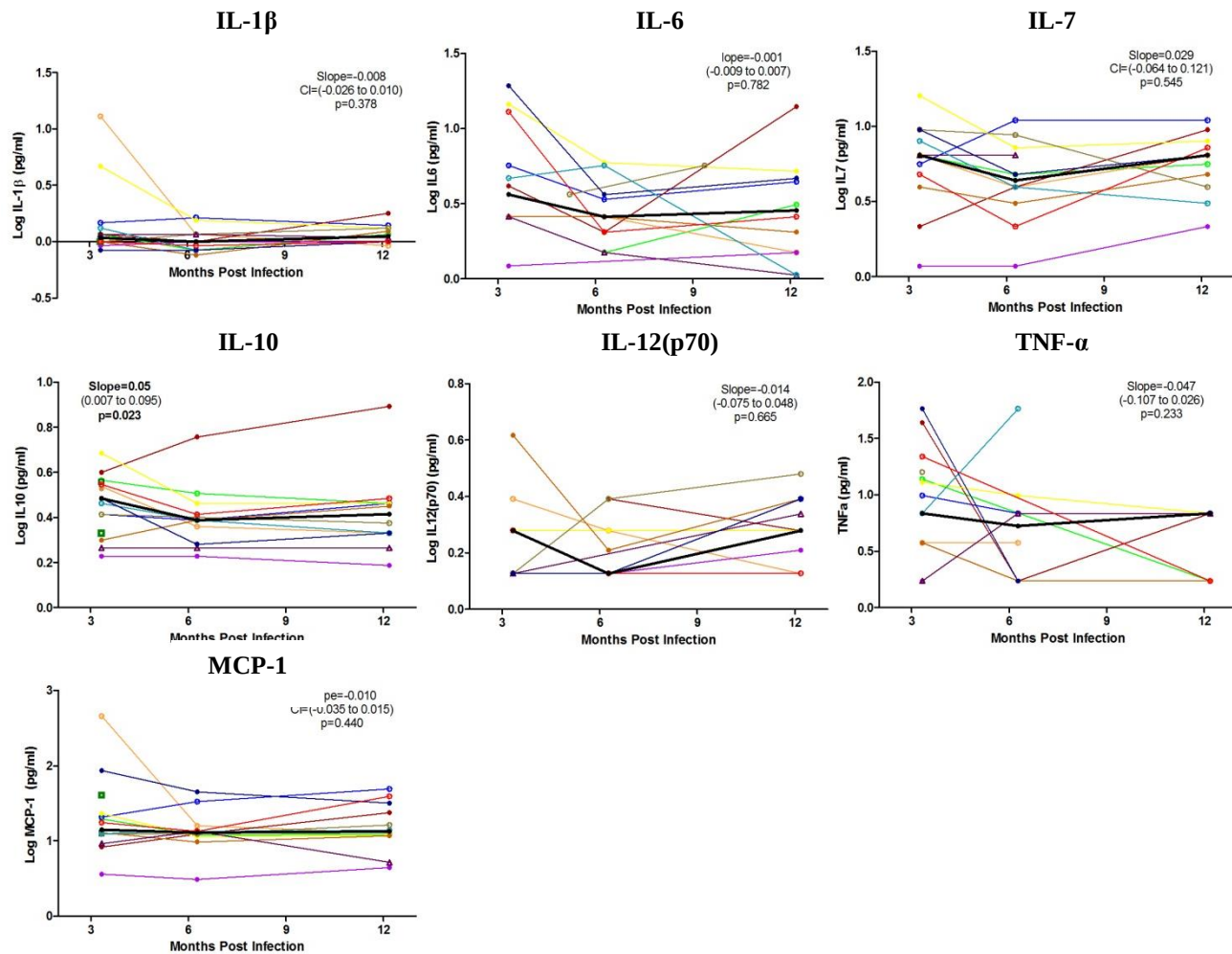


Figure 6.12: The longitudinal changes of the markers of plasma cytokines during HIV infection.

Representative plots indicating the longitudinal changes in the plasma concentrations of IL-1 β , IL6, IL-7, IL-10, IL-12p70, TNF α and MCP-1 in early HIV-infected individuals (n=15). Longitudinal changes in markers of microbial translocation were assessed by using a linear multilevel model. The median change across time is represented by a bold solid line. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$). Patient-specific colour-coding is consistent in each represented graph.

6.3 Discussion

Microbial translocation has been implicated as one of the underlying causes of persistent chronic immune activation (Brenchley *et al.*, 2006b), which in turn predicts HIV-1 disease progression (Hazenberg *et al.*, 2003, Deeks *et al.*, 2004, Appay and Sauce, 2008). Whether this occurs to the same degree in early or primary HIV-1 infection remains unclear. However, the disruption of the gastrointestinal barrier in early HIV-1 infection (Veazey *et al.*, 1998, Mehandru *et al.*, 2004, Brenchley *et al.*, 2004b) could result in the infiltration of bacterial products from the gut lumen into the blood stream, and potentially lead to systemic inflammation and enhanced immune activation. In addition, given that immunological events that take place during primary HIV infection likely determine subsequent disease progression, understanding the mechanisms that influence T cell activation during the early stages of HIV-1 infection may be important in understanding HIV-1 pathogenesis in the early phases of HIV-1 infection.

Monocyte activation in response to microbial products results in the release of sCD14 and the production of LBP (Wright *et al.*, 1990). The plasma concentrations of sCD14 and LBP were found to be slightly greater during primary HIV-infected relative to healthy HIV-uninfected controls, while I-FABP levels were significantly increased during primary HIV-1 infection. Since the presence of LPS promotes hepatic synthesis and production of LBP, and binding of LPS to CD14⁺ monocytes or macrophages via the MD2/TLR pathway induces the shedding of sCD14 (Caradonna *et al.*, 2000, Kitchens and Thompson, 2005), the slightly increased levels of LBP and sCD14 may reflect the *in vivo* bioactivity of LPS and/or alteration of the gut epithelial integrity in the early months after HIV-1 infection. These findings appear to be in line with microbial translocation being shown in the late acute stages of SIV infection (Estes *et al.*, 2010).

Furthermore, the elevated levels of I-FABP, which is an indicator for enterocyte damage (Lieberman *et al.*, 1997, Pelsers *et al.*, 2003), may also suggest an increase in the disruption and permeability of the intestinal epithelial barrier, where damage to the epithelial integrity could be attributed to viral replication and significant loss of CD4⁺ T cells of the gut mucosa (Mehandru *et al.*, 2004, Brenchley *et al.*, 2004b, Gori *et al.*, 2008). Direct effects of HIV-1 exposure on the intestinal barrier could also result in the decrease of tight junction protein expression, leading to increased permeability and dysfunction of the intestinal barrier (Nazli *et al.*, 2010). Endotoxin IgM antibodies against gram negative bacteria are normally present in healthy individuals (Cohen and Norins, 1966) and represents an early immune response to microbial seepage. However, levels of IgM endotoxin antibodies to LPS (EndCAb) are known to be significantly reduced in HIV infection (Ancuta *et al.*, 2008, Wallet *et al.*, 2010) possibly due to B-cell impairment that occurs during infection (Titanji *et al.*, 2006). In this study, there were no differences in the plasma concentrations of IgM EndoCAb between HIV-infected and -uninfected controls, suggesting that either the production of EndoCAb appears to be unaffected by early HIV-1 infection or that levels of IgM had subsided at the time of analysis.

Although microbial translocation appears to manifest itself later on during the chronic stages of HIV infection (Brenchley *et al.*, 2006b), no significant differences were found when comparing the plasma levels of LBP, I-FABP and sCD14 between HIV-uninfected controls and chronically HIV-infected individuals. The reasons for these discrepancies are unclear, but may to some degree be related to the grouping or characterization of chronic HIV-1 infected subjects between the studies. In the study conducted by Brenchley *et al.* (2006b), chronic HIV-infected individuals were grouped according to plasma viral loads above 400 copies/ml and CD4⁺ T cell counts above

200 cells/ μ l, whereas the characterization of chronic HIV-1 individuals in this chapter were not based upon this criteria. To understand the impact of microbial translocation during chronic HIV-1 infection, the level of microbial translocation was compared between chronically HIV-infected individuals (with CD4⁺ T cell counts below 200 cells/mm³) and HIV-uninfected controls. Based on this analysis, chronically HIV-infected individuals with absolute CD4⁺ T cell counts below (200 cells/mm³) were found have elevated plasma levels of LBP and sCD14 compared to HIV-uninfected controls. These data may thus reflect a relationship between the increase of microbial translocation and the severity of HIV-1 disease. Furthermore, these data are supported by recent findings from other studies also showing the association between microbial translocation and the loss CD4⁺ T cells in either HIV-1 or HIV-2 infections (Nowroozalizadeh *et al.*, 2010).

A number of studies have shown that acute or primary HIV infection is accompanied by a plethora of pro-inflammatory molecules (Norris *et al.*, 2006, Barqasho *et al.*, 2009, Stacey *et al.*, 2009) and consistent with these studies, findings in this chapter showed that there was up-regulation IL-6, IL-7, IL-10 and MCP-1 in HIV-1 infection compared to HIV-uninfected controls. These findings are also corroborated by Roberts *et al.* (2010), who demonstrated elevated plasma concentrations of IL-6, IL-7 and IL-10 in women with acute HIV-1 infection. Increased levels of IL-6 have been shown to promote reactivation of the virus in latently HIV infected cells (Hoshino *et al.*, 2010), while over-production of IL-10 could lead to CD4⁺ T cell dysfunction (Said *et al.*, 2010). Additionally, elevated levels of IL-7 have been associated with CD4⁺ T cell lymphopaenic conditions in HIV-1 infection, together with increased HIV-1 load (Napolitano *et al.*, 2001). The up-regulation of the inflammatory chemokine MCP-1 has also been previously implicated in cerebrospinal fluid of AIDS patients with CMV encephalitis

(Bernasconi *et al.*, 1996) or with AIDS dementia (Kelder *et al.*, 1998). Collectively, these data suggest that increased production of cytokines observed during primary HIV-1 infection most likely establishes immune impairment. Significantly low plasma concentrations of IL-12p70 relative to HIV-uninfected controls were noted, and these observations appear to be consistent with other studies (Norris *et al.*, 2006, Stacey *et al.*, 2009), while contradicting other findings (Byrnes *et al.*, 2008) demonstrating increased production of IL-12p70 in acute/early HIV infection. Since IL-12 plays a key role in the regulation of cell-mediated immunity (Watford *et al.*, 2003), and is mainly secreted by polymorphonuclear neutrophils, monocytes and macrophages, its down-regulation may to some degree reflect the functional impairment of these cells during primary HIV-1 infection. Furthermore, the limited IL-12 production could also be a consequence of HIV, which blocks the differentiation of mDC's and subsequent decrease of IL-12 expression (Granelli-Piperno *et al.*, 2004).

The stimulation of mononuclear cells by microbial products has been shown to promote increased secretion of inflammatory cytokines (Miller *et al.*, 2005, Lu *et al.*, 2008). Findings in this study showed that elevated levels of sCD14 and LBP associate with increased levels of IL-6, IL-10 and TNF α during primary HIV-1 infection. The lack of these associations among HIV-uninfected controls would suggest that these associations are unlikely to be causal and possibly more related to HIV replication. The positive significant associations between plasma levels of IL-6, IL-10 and TNF α with HIV-1 load supports the notion that viral antigens may be playing a direct role in triggering some of the inflammatory mediators. Our data also revealed that elevated plasma sCD14 levels, and to some extent LBP, were associated with elevated HIV-1 load and suggests a mechanism where microbial translocation could be driving viral replication. Thus, the

presence of microbial products could support HIV persistence by causing ongoing immune activation (Brenchley *et al.*, 2006b, Jiang *et al.*, 2009), which in turn favours viral replication (Zhang *et al.*, 2004, Hunt *et al.*, 2011a). On the other hand, given that HIV is largely responsible for gut structural damage and dysfunction in early infection (Mehandru *et al.*, 2004, Nazli *et al.*, 2010), these data could also suggest that elevated levels of LBP and sCD14 are attributed to high HIV-1 antigen load. Furthermore, since HIV antigens (i.e.gp120) could also result in the activation of monocyte and/or macrophages and subsequent release of sCD14 (Lien *et al.*, 1998), the associations between HIV antigen load and sCD14 may also explain the elevated levels of sCD14 during early HIV-1 infection.

Given controversies regarding the role of microbial translocation and inflammatory responses towards immune activation and disease progression in HIV-infected African populations (Redd *et al.*, 2009, Lester *et al.*, 2009b, Cassol *et al.*, 2010), this chapter evaluated the relationship between T cell activation with markers of microbial translocation and inflammation in early HIV-1 infection. Using unadjusted correlations, plasma levels of sCD14, IL-10 and TNF α correlated positively with activated memory CD4⁺ T cells, while LBP levels correlated with activated memory CD8⁺ T cells. These data appeared to support previous findings of microbial translocation-driven immune activation. However, in adjusted correlations only HIV-1 viral load correlated significantly with T cell activation, suggesting that activation during early HIV-1 infection may not be a direct result of microbial translocation, but rather an association with HIV-1 replication itself. When assessing the relationships between the activation status of Gag-specific CD4⁺ and CD8⁺ T cells with markers of microbial translocation and inflammation, no significant associations were found between activated Gag-specific T cells with markers of

microbial translocation and plasma cytokines evaluated in this chapter. The lack of significant correlations suggests that the activation of Gag-specific cells T cells could primarily be driven by HIV-antigen load during primary HIV-1 infection.

In summary, there have been uncertainties regarding the role of microbial translocation in HIV-infected African populations (Redd *et al.*, 2009, Lester *et al.*, 2009b). Although findings in this chapter are unable to provide causality between microbial translocation, inflammation, immune activation and HIV-1 viraemia, the data in this chapter suggest that HIV-1 antigen burden appears to be a central driver behind inflammation and T cell activation during early HIV-1 infection. This notion is supported by the absence of independent relationships between markers of microbial translocation or inflammatory molecules with T cell activation. The next chapter will focus on the implications for these findings and disease progression as measured by viral load and CD4⁺ counts.

CHAPTER 7: THE RELATIONSHIP BETWEEN T CELL RESPONSES
AND DISEASE PROGRESSION

TABLE OF CONTENTS

7.1 Introduction.....	216
7.2 Results.....	217
7.2.1 Associations between the CD4 ⁺ T cell differentiation and HIV-1 viral load.....	217
7.2.2 Associations between the CD8 ⁺ T cell differentiation and HIV-1 viral load.....	222
7.2.3 Associations between memory CD4 ⁺ T cell activation and HIV-1 viral load	226
7.2.4 Associations between the memory CD4 ⁺ T cell activation and CD4 count	229
7.2.5 Associations between the memory CD8 ⁺ T cell activation and HIV-1 viral load	233
7.2.6 Associations between the memory CD8 ⁺ T cell activation and CD4 count	236
7.2.7 Associations between T cell function and HIV-1 viral load.....	239
7.3 Discussion	246

7.1 Introduction

HIV-1 disease progression is greatly influenced by host immune factors, such as memory differentiation, activation and functional characteristics of T cells. Thus far, this thesis has described memory differentiation, activation and proliferative profile and frequencies of Gag-specific memory CD4⁺ and CD8⁺ T cell responses over time, in 15 untreated HIV-1 infected individuals. Primary HIV-1 infection was characterized by skewing of Gag-specific memory CD4⁺ and CD8⁺ T cells towards an early differentiated phenotype relative to CMV-specific memory T cells. Furthermore, this study also showed elevated levels of total and Gag-specific memory T cell activation and proliferation during early HIV-1 infection, which appeared to be stable over time. Moreover, the differentiation and activation profiles of total and Gag-specific memory T cells at 3 months were found to associate with those at 12 months post-infection, suggesting an establishment of a steady-state memory differentiation and activation during the early stages of HIV-1 infection. Observations in this study also showed disassociations between immune activation and differentiation in both total and Gag-specific memory CD4⁺ T cells, suggesting that these events may be independent from each other. Further analyses also showed that Gag-specific memory T cells were less polyfunctional than CMV-specific memory T cells, and as with the activation status of cells, the polyfunctionality of Gag-specific T cells was found to be equally distributed among memory T cell subpopulations of CD4⁺ and CD8⁺ T cells during early HIV-1 infection. Examination of the relationships between microbial translocation, inflammation and viraemia with activation status of memory T cells suggested that viral replication more likely plays a dominant role in driving hyperimmune activation and inflammation during primary HIV-1 infection, and may also to some extent drive microbial

translocation. Thus, this chapter now wishes to understand the relationships between all these parameters and HIV-1 disease progression.

Identifying host immune factors such as memory differentiation or activation profiles and functional capabilities of T cells that correlate with viral control during primary HIV-1 infection, may provide information into vaccine strategies or therapeutic interventions that could aim to control viraemia. This chapter investigates the relationship between (i) memory differentiation (ii) activation status and (iii) polyfunctionality of T cells with viraemia during the 15 months of HIV-1 infection.

7.2 Results

7.2.1 Associations between the CD4⁺ T cell differentiation and HIV-1 viral load

The first level of analysis sought to explore the associations between the memory differentiation phenotype of T cells and viraemia. The proportions of total and Gag-specific memory CD4⁺ T cell subpopulations were correlated with viral load at 3 months post HIV-1 infection. No significant associations were observed between the proportions of total naive, ED, LD and FD CD4⁺ T cells with viral load at 3 months post-infection (Figure 7.1). A negative trend was however observed between the proportions of total memory ED with viral load, while FD cells showed a positive trend with viral load at 3 months post-infection. There was significant and negative correlation between the frequencies of Gag-specific ED memory CD4⁺ T cells with viraemia at 3 months post-infection ($r=-0.87$, $p<0.0001$) and a positive correlation with LD-memory cells ($r=0.85$, $p=0.0002$, Figure 7.2). These data also show a uniform spread of total and Gag-specific CD4⁺ T cell subpopulations of individuals who were able to subsequently reduce

viral load in the first 12 months of infection, and those who did not (Figure 7.1 and 7.2, open and closed symbols). This suggests that the proportions of these subpopulations were not determining the trajectory of viraemia, and rather infer that viraemia could be driving the differentiation of Gag-specific memory, and to some extent that of total memory CD4⁺ T cells.

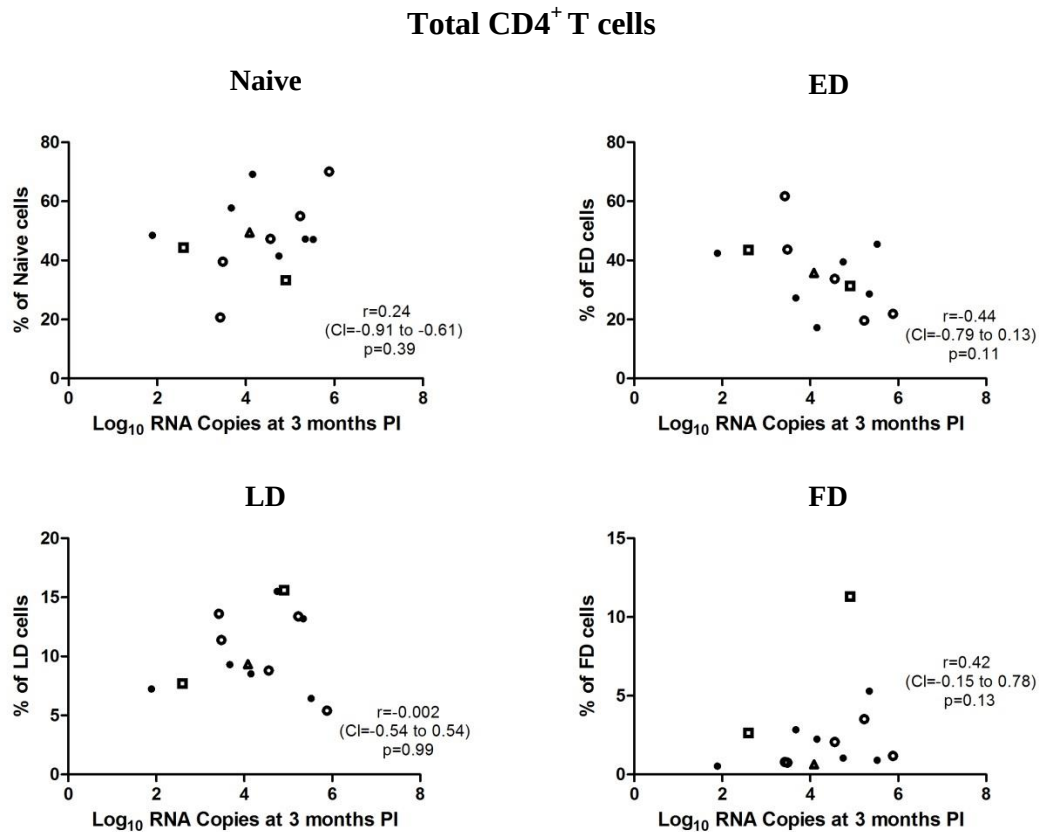


Figure 7.1: The relationship between the total CD4⁺ T cell subpopulations with viral load at 3 months post-infection. Correlations between the proportions total Naïve, ED-, Int-, LD- and FD-memory CD4⁺ T cell subpopulations with viral load in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

Gag-specific memory CD4⁺ T cells

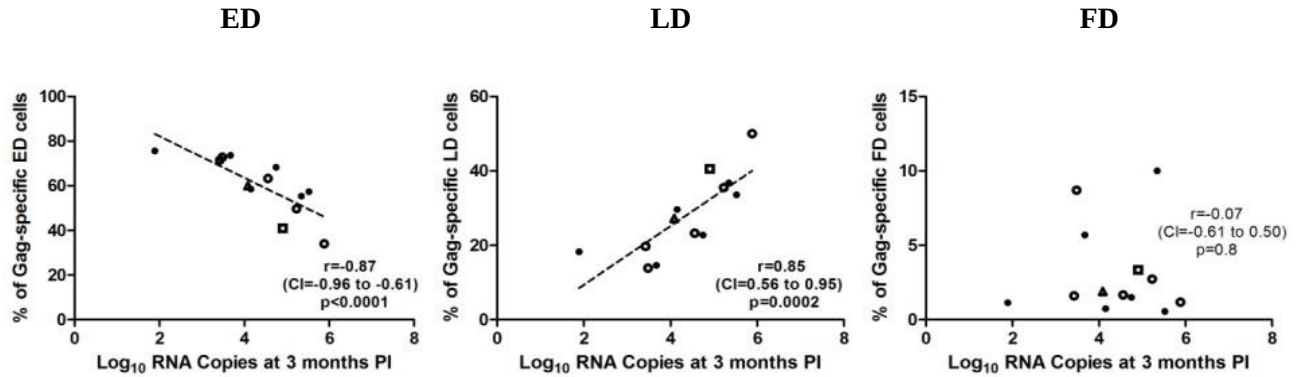


Figure 7.2: The relationship between the Gag-specific memory CD4⁺ T cell subpopulations with viral load at 3 months post-infection. Correlations between the proportion Gag-specific memory CD4⁺ T cell subpopulations with viral load in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

To further understand the relationships between memory T cell differentiation and HIV-1 viral load during the course of HIV infection, associations using mixed-effects linear regression were performed across all time points (3 to 15 months PI) with CD4⁺ T cell memory subpopulations as outcome variables (Chapter 2, Model 2, page 63). A significant and negative association was observed between the proportion of total ED CD4⁺ T cells and viral load over time (slope = -0.035, $p = 0.019$, Table 7.1). No associations were noted between the proportions of naive, LD or FD cells with viraemia over the first 15 months after HIV-1 infection. With regard to Gag-

specific cells, a significant and inverse association was observed between the proportion of ED-memory Gag-specific cells with viral load over time (slope=-0.05, p=0.004), while LD-memory Gag-specific cells positively associated with viraemia over time (slope=0.05, p=0.003). There were no significant associations between the proportion FD-memory Gag-specific cells with viraemia over time. In summary, these data support the suggestion that viraemia could be driving the differentiation of Gag-specific memory CD4⁺ T cells from an early differentiated phenotype towards a late phenotype during the course of infection.

Table 7.1: Mixed-effects linear regression associating the differentiation CD4⁺ T cell with viral load over time

Maturation markers	Slope	P	95% CI
Total			
Naïve cells	-0.019	0.187	(-0.048 to 0.009)
ED cells	-0.035	0.019	(-0.064 to -0.006)
LD cells	0.016	0.495	(-0.030 to 0.059)
FD cells	0.020	0.66	(-0.070 to 0.111)
Gag-specific memory			
ED cells	-0.050	0.004	(-0.083 to -0.016)
LD cells	0.057	0.003	(0.0190 to 0.095)
FD cells	-0.033	0.66	(-0.168 to 0.102)

Longitudinal associations between T cell differentiation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05).

7.2.2 Associations between the CD8⁺ T cell differentiation and HIV-1 viral load

The next level of analysis explored the correlations between the differentiation status of CD8⁺ T cells and viraemia, the proportions of naive, ED, Int, LD and FD CD8⁺ T cells were correlated with viral load at 3 months post-infection. Figure 7.3 shows a negative and significant association between the proportion of naive CD8⁺ T cells with viral load at 3 months post-infection ($r=-0.58$, $p=0.028$), and a positive association with LD CD8⁺ T cells ($r=0.61$, $p=0.018$). No significant correlations were observed between the proportions of Gag-specific ED, Int, LD and FD memory CD8⁺ T cells with viraemia at 3 months post-infection (Figure 7.4). These data also suggest that viral load could be playing a role in the differentiation of total CD8⁺ T cells during primary HIV-1 infection.

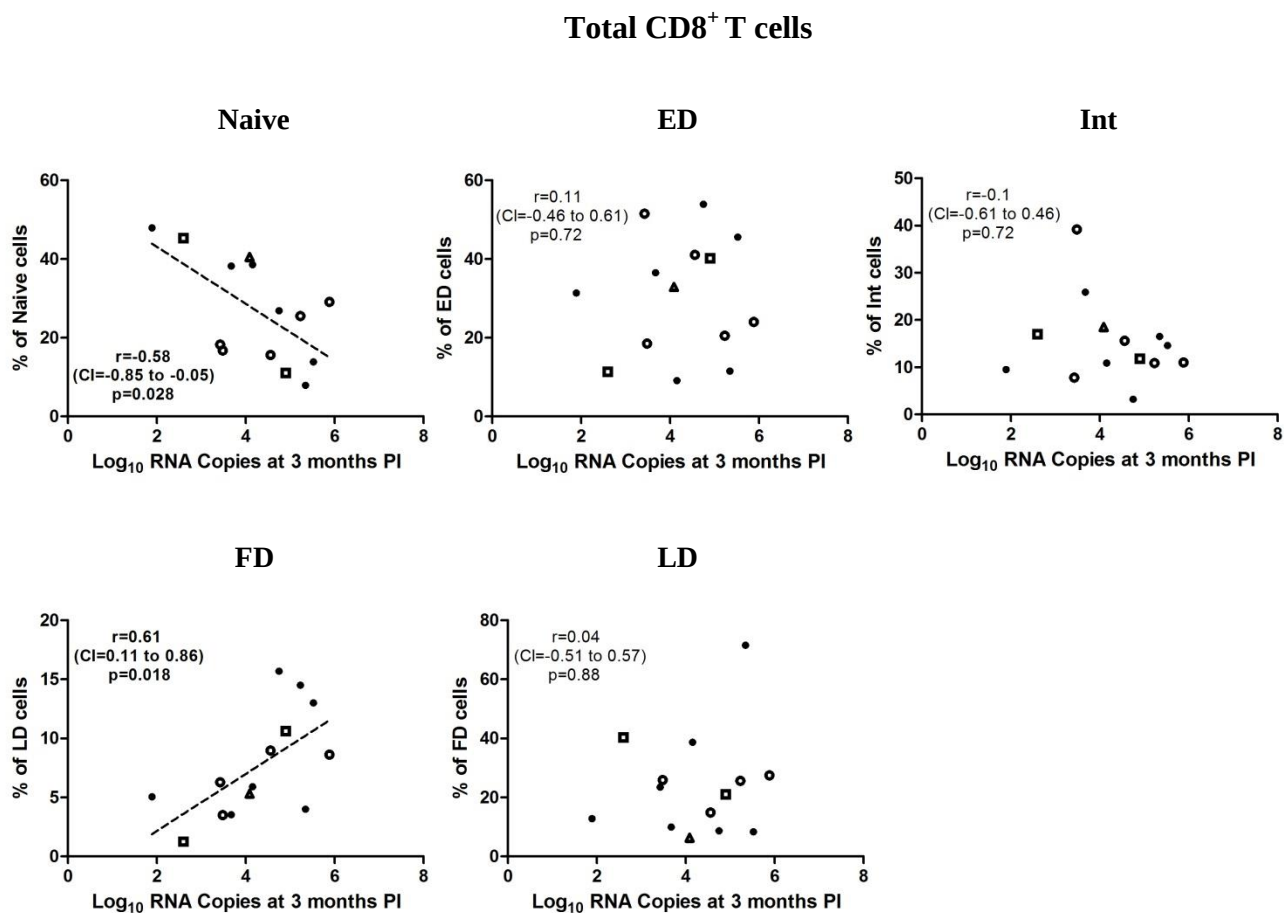


Figure 7.3: The relationship between the total CD8⁺ T cell subpopulations with viral load at 3 months post-infection. Correlations between the proportion of total Naïve, ED-, Int-, LD- and FD-memory CD8⁺ T cell subpopulations with viral load in early HIV-infected individuals (n=14). Open circles (O) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

Gag-specific memory CD8⁺ T cells

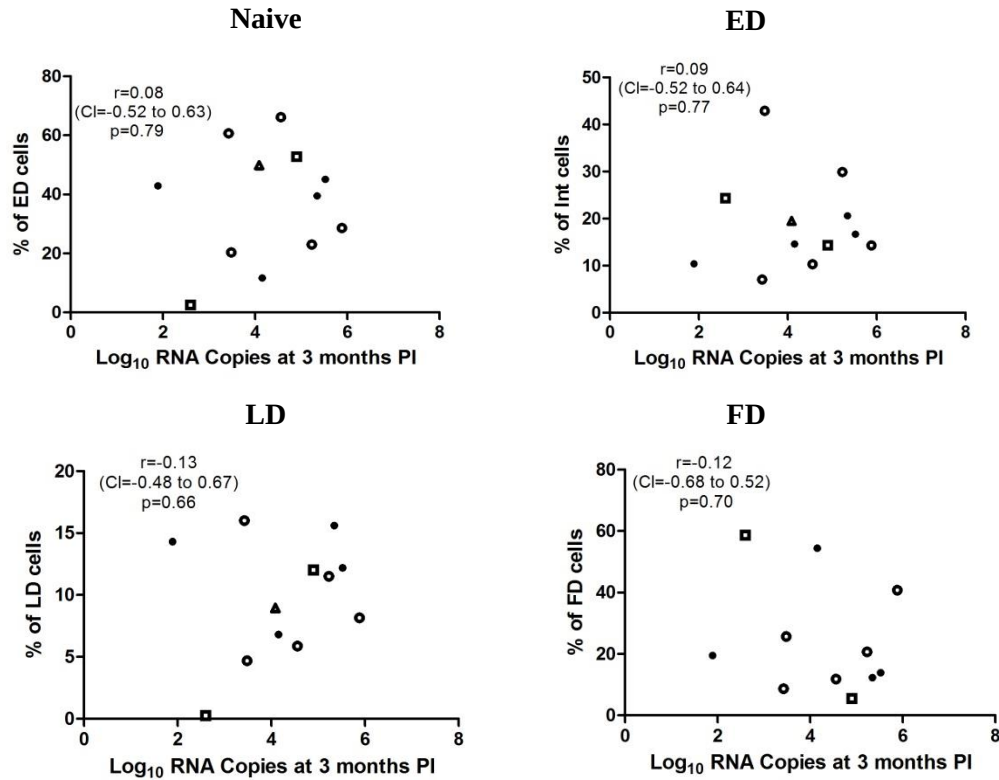


Figure 7.4: The relationship between the Gag-specific memory CD8⁺ T cell subpopulations with viral load at 3 months post-infection. Correlations between the proportion Gag-specific memory CD8⁺ T cell subpopulations with viral load in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\text{log}_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\text{log}_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\text{log}_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

The relationship between the differentiation of CD8⁺ T cells and viral load over time was also examined using mixed-effects regression analysis (Table 7.2). A negative and significant association continued to persist between the proportion of naive CD8⁺ T cells and viraemia from

3 to 15 months post-infection (slope=-0.052, p=0.023). Although the positive correlation between the proportion of LD-memory CD8⁺ T cells and viral load disappears over time, a positive and significant association was reached between the proportion of ED cells and viral load over time (slope=0.062, p=0.017). And thus still supports the suggestion that viral load could be playing a significant role in driving the differentiation of total CD8⁺ T cells. In contrast, no significant associations were observed between Gag-specific memory CD8⁺ T cell subpopulations with viraemia over time.

Table 7.2: Mixed-effects linear regression associating the differentiation CD8⁺ T cell with viral load over time

Maturation markers	Slope	P	95% CI
Total			
Naïve cells	-0.052	0.023	(-0.096 to -0.007)
ED cells	0.064	0.017	(0.011 to 0.116)
Int cells	0.027	0.252	(-0.022 to 0.076)
LD cells	0.035	0.288	(-0.025 to 0.096)
FD cells	-0.035	0.288	(-0.022 to 0.076)
Gag-specific memory			
ED cells	0.030	0.470	(-0.052 to 0.113)
Int cells	-0.005	0.925	(-0.104 to 0.095)
LD cells	0.004	0.941	(-0.108 to 0.117)
FD cells	-0.084	0.100	(-0.183 to 0.016)

Longitudinal associations between CD8⁺ T cell differentiation and viral load were determined using linear mixed-effects modeling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values (p<0.05).

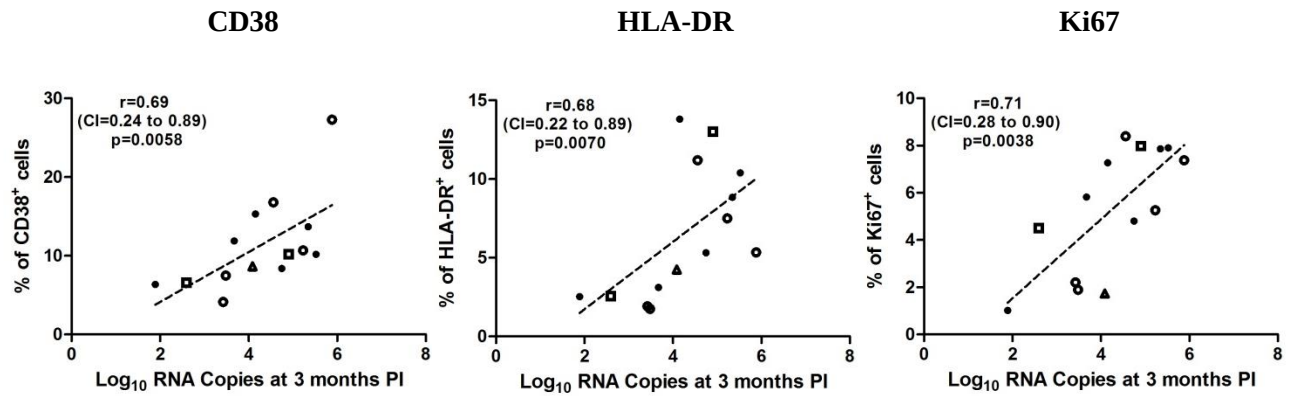
7.2.3 Associations between memory CD4⁺ T cell activation and HIV-1 viral load

Multivariate associations between markers of microbial translocation, cytokines and HIV viraemia with T cell activation showed that HIV viraemia appears to be a major driver of T cell activation at 3 months post-infection (observations in Chapter 6). To further these findings, this section aims to investigate the relationships between T cell activation and HIV viral load during primary HIV infection. Using univariate analysis, significant positive associations were found between the proportions of CD38, HLA-DR and Ki67 on memory CD4⁺ T cells with viral load at 3 months post-infection ($r=0.69$, $p=0.0058$; $r=0.68$, $p=0.007$ and $r=0.71$, $p=0.0038$, respectively, Figure 7.5A).

With regard to Gag-specific memory CD4⁺ T cells, significant and positive correlations were noted between the proportions of HLA-DR and Ki67 with viral load ($r=0.58$, $p=0.034$ and $r=0.58$, $p=0.036$, respectively, Figure 7.5B). However, this did not hold true for Gag-specific memory CD38⁺ CD4⁺ T cells. It is also worth noting that, when correlating non-activated CD4⁺ T cells (CD38⁻HLA-DR⁻Ki67⁻) with viral load, significant and inverse associations were observed for both total and Gag-specific memory cells ($r=-0.75$, $p=0.0018$ and $r=-0.66$, $p=0.0012$, respectively) (Appendix O, Figure O1, page 287). These data also show a uniform distribution of activated (both total and Gag-specific) cells regardless of individuals who subsequently reduced viraemia in first 12 months of infection, and those that did not (Figure 7.5 A and B, open and closed symbols). Overall, these data confirm that viraemia is a major driver of both total and Gag-specific CD4⁺ T cell activation at 3 months post-infection.

A

Total memory CD4⁺ T cells



B

Gag-specific memory CD4⁺ T cells

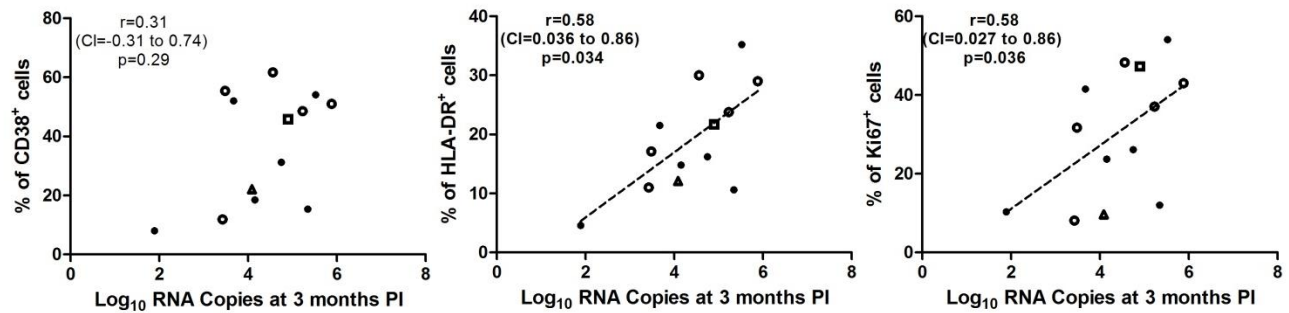


Figure 7.5: Correlations between the percentage of activated memory CD4⁺ T cells and viral load at 3 months post-infection. The associations between the proportion of activated (A) total memory, and (B) Gag-specific memory CD4⁺ T cells with viraemia at viral load in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5log₁₀ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within ±0.5log₁₀ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase >0.5log₁₀ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

To further understand the relationships between immune activation and viraemia, activated memory CD4⁺ T cells were subsequently analysed in relation to viral load over the first 15 months of HIV-1 infection. Given that the proportional expression of activation markers CD38 and HLA-DR on T cells may vary at different clinical stages of HIV disease, for example HLA-DR might be expressed in greater percentage in early infection relative to CD38, and may highlight varying prognostic significance of each marker towards HIV disease progression (Kestens et al., 1992). Thus, analysis in this Chapter, focused on assessing the relationships between single expression of CD38 and HLA-DR with the course of viraemia and absolute CD4⁺ T cell numbers. Moreover, analysis of the relationships between viraemia/CD4⁺ T cell count with the single expression of each activation marker rather than double positive cells (CD38⁺HLA-DR⁺) may provide more direct comparisons between the dynamics of activated cells (observed in Chapter 4, page 126), viraemia and CD4⁺ T cell counts over the first 15 months of infection.

Using the proportions of CD38, HLA-DR and Ki67 on memory CD4⁺ T cells as outcome variables, the correlations were performed by mixed-effects modeling (Chapter 2, Model 2, page 63). The relationship between the proportions of total memory CD38 and Ki67 persisted during the first 15 months of HIV-1 infection (slope=0.084, p=0.002 and slope=0.108, p<0.0001, respectively, Table 7.3). The relationship between total memory HLA-DR cells with viral load was however lost during the course of infection. No significant associations were observed between activated Gag-specific memory CD4⁺ T cells with viral load over time. However the expression of CD38 on Gag-specific memory CD4⁺ T cells showed a slight positive association with increased viraemia over time (slope=0.079, p=0.092). These data suggest that viral load

drives the activation (CD38) and the proliferation (Ki67) of memory CD4⁺ T cells over the first year of infection.

Table 7.3: Mixed-effects linear regression associating the proportions of activated memory CD4⁺ T cells with viral load over time

Activation markers	Slope	<i>P</i>	95% CI
Total memory			
CD38	0.084	0.0020	(0.031 to 0.137)
HLA-DR	0.011	0.7700	(-0.062 to 0.083)
Ki67	0.108	<0.0001	(0.048 to 0.169)
Gag-specific memory			
CD38	0.079	0.092	(-0.013 to 0.171)
HLA-DR	0.034	0.411	(-0.048 to 0.117)
Ki67	0.011	0.807	(-0.077 to 0.099)

Longitudinal associations between T cell activation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

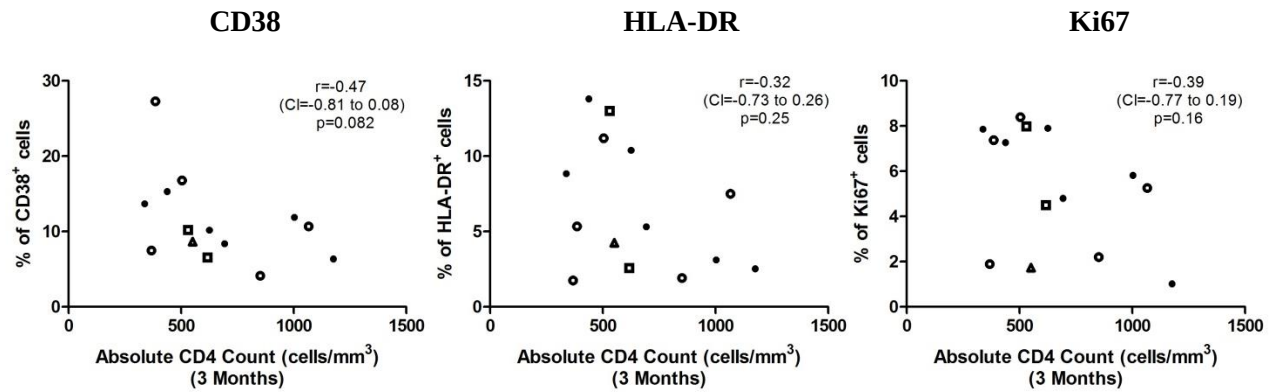
7.2.4 Associations between the memory CD4⁺ T cell activation and CD4 count

The depletion of absolute CD4⁺ T cell counts and subsequent disease progression has been attributed to the hyperactivation of T cells during HIV infection (Sousa *et al.*, 2002, Deeks *et al.*, 2004, Vajpayee *et al.*, 2009). This study sought to investigate relationships between activated memory CD4⁺ T cells with CD4⁺ T counts during early HIV-1 infection. No significant associations were observed between the proportions activated memory CD4⁺ T cells (either total or Gag-specific) with CD4⁺ T cell count at 3 months post-infection (Figure 7.6A and B). There was however negative trend in the relationship between the levels of total memory CD38⁺CD4⁺

T cells and CD4⁺ counts ($r=-0.47$, $p=0.082$). Univariate relationships between CD4⁺ T cell activation and CD4⁺ counts were further assessed over time by mixed-effects linear regression analysis (Chapter 2, Model 2, page 63). Using absolute CD4⁺ T cell counts as the outcome variable, significant and inverse correlations were found between the proportions of activated total memory CD4⁺ T cells with the absolute numbers of CD4⁺ T cells during the course of infection (CD38: slope=-0.384, $p<0.0001$; HLA-DR: slope=-0.271, $p<0.0001$ and Ki67: slope=-0.194, $p=0.0005$, Table 7.4). No significant associations were observed between the proportions of activated Gag-specific memory CD4⁺ T cells with CD4⁺ T cell counts over time. Negative associations observed between the activation of total memory CD4⁺ T cells and CD4⁺ T cell counts over time, may suggest that the hyperactivation of memory CD4⁺ T cells could be driving the depletion of absolute CD4⁺ T cells over the first 15 months of HIV-1 infection.

A

Total memory CD4⁺ T cells



B

Gag-specific memory CD4⁺ T cells

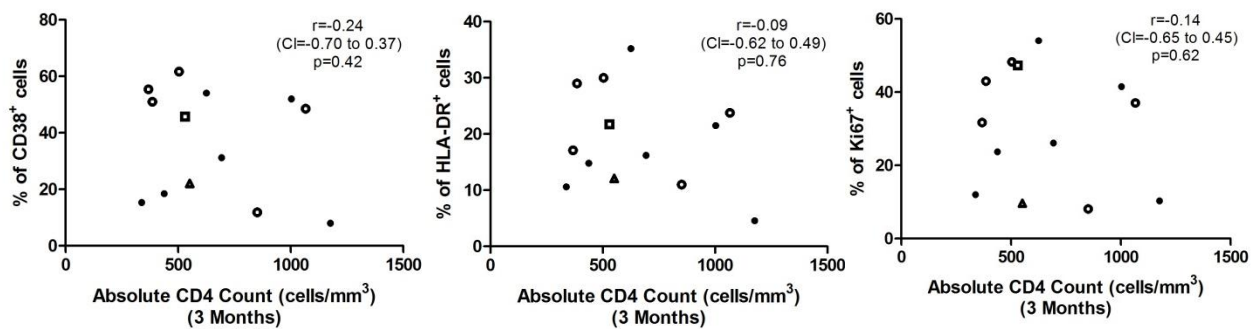


Figure 7.6: The relationship between the memory CD4⁺ T cell activation and CD4 T cell count at 3 months post-infection. Correlations between the proportions of CD38, HLA-DR and Ki67 on (A) total memory, and (B) Gag-specific memory CD4⁺ T cells with CD4⁺ T cell counts at 3 months post-infection. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

Table 7.4: Mixed-effects linear regression associating the proportions of activated memory CD4⁺ T cells with CD4⁺ T cell counts over time

Activation markers	Slope	<i>P</i>	95% CI
Total memory			
CD38	-0.384	<0.0001	(-0.541 to -0.227)
HLA-DR	-0.271	<0.0001	(-0.400 to -0.143)
Ki67	-0.194	0.0050	(-0.329 to -0.060)
Gag-specific memory			
CD38	-0.037	0.5240	(-0.151 to 0.077)
HLA-DR	0.027	0.6860	(-0.106 to 0.161)
Ki67	0.052	0.4180	(-0.073 to 0.177)

Longitudinal associations between T cell activation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

Univariate correlations between T cell activation and CD4⁺ T cell counts may be spurious, due to the indirect effects of the any associations that may exist between viral load and absolute CD4⁺ T cell counts. To resolve this, multivariate analysis using mixed-effects linear regression was employed to further assess the relationship between total memory CD4⁺ T cell activation, viral load and CD4⁺ T cell counts over the first 15 months of HIV-1 infection (Chapter 2, Model 3, page 64). Thus, by adjusting for both CD4⁺ T cell counts and viral load, this model corrected for any associations may exist between these two covariates (viral load and CD4⁺ counts). After adjusting for viral load, significant and inverse associations were maintained between the proportions of CD38, HLA-DR and Ki67 on total memory CD4⁺ T cells with absolute CD4⁺ counts, whilst significant and positive associations were observed between CD38 and Ki67 with

viral load when controlling for CD4⁺ count (Table 7.5). While viral load contributes towards the elevated activation status of total memory CD4⁺ T cells, the strong relationships between the decrease of CD4⁺ T cell numbers and activated total memory CD4⁺ T cells when adjusting for viraemia, further supports the suggestion that hyperactivation of total memory CD4⁺ T cells plays a major role in the depletion CD4⁺ T cells in HIV-1 infection.

Table 7.5: Multivariate analysis with mixed-effects linear regression associating activated memory CD4⁺ T cells with covariates viral load and CD4⁺ T cell counts over time

Activation markers	Slope	<i>P</i>	95% CI
CD38			
Log viral load (copies/ml)	0.077	0.0010	(0.0310 to 0.122)
Log CD4 count (cells/mm ³)	-0.508	<0.0001	(-0.721 to -0.294)
HLA-DR			
Log viral load (copies/ml)	-0.013	0.702	(-0.079 to 0.053)
Log CD4 count (cells/mm ³)	-0.571	<0.0001	(-0.867 to -0.276)
Ki67			
Log viral load (copies/ml)	0.092	0.0020	(0.033 to 0.152)
Log CD4 count (cells/mm ³)	-0.416	0.0040	(-0.699 to 0.134)

Longitudinal associations between T cell activation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

7.2.5 Associations between the memory CD8⁺ T cell activation and HIV-1 viral load

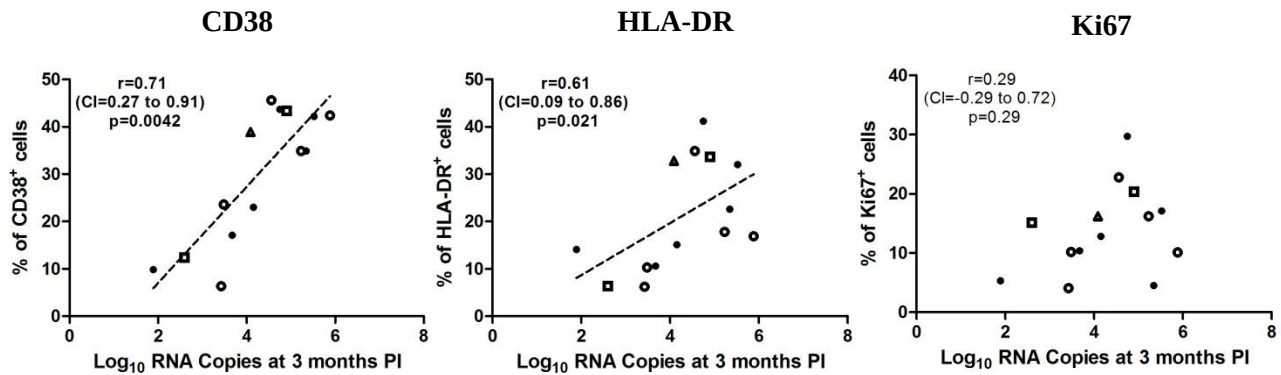
This chapter further examined the relationship between memory CD8⁺ T cell activation and viral load at 3 months post-infection. The expression of CD38 and HLA-DR on total memory CD8⁺ T

cells correlated positively with viral load at 3 months post-infection ($r=0.71$, $p=0.0042$ and $r=0.61$, $p=0.021$, respectively, Figure 7.7A). There were no significant associations between the proportions of Ki67 on total memory CD8⁺ T cells with viral load at 3 months post-infection. With regard to Gag-specific memory CD8⁺ T cells, significant and positive associations were only observed between the proportion of CD38⁺ T cells and viral load ($r=0.65$, $p=0.022$, Figure 7.7B). As expected significant and negative associations were found between non-activated total memory CD8⁺ T cells with viral load at 3 months post-infection ($r=-0.92$, $p=0.0002$), there was however a negative trend in the relationship between non-activated Gag-specific cells with viral load ($r=-0.51$, $p=0.084$) (Appendix O, Figure O1, page 287).

When assessing the relationship between activated memory CD8⁺ T cells with viral load over time (Chapter 2, Model 2, page 63), the correlations between proportions of CD38 and HLA-DR on total memory CD8⁺ T cells and viraemia persisted over the first 15 months of HIV infection (slope=0.103, $p<0.0001$ and slope=0.053, $p=0.045$, respectively, Table 7.6). The correlations between viraemia and total memory Ki67⁺CD8⁺ T cells, together with activated Gag-specific memory CD8⁺ T cells did not reach any statistical significance over time. Collectively these data show HIV antigen load is also a strong driver of total memory CD8⁺ T cell activation. The lack of associations between these antigens on cell surfaces of Gag-specific cells viral load may be attributed to additional mechanisms that could affect the activation of Gag-specific CD8⁺ T cells.

A

Total memory CD8⁺ T cells



B

Gag-specific memory CD8⁺ T cells

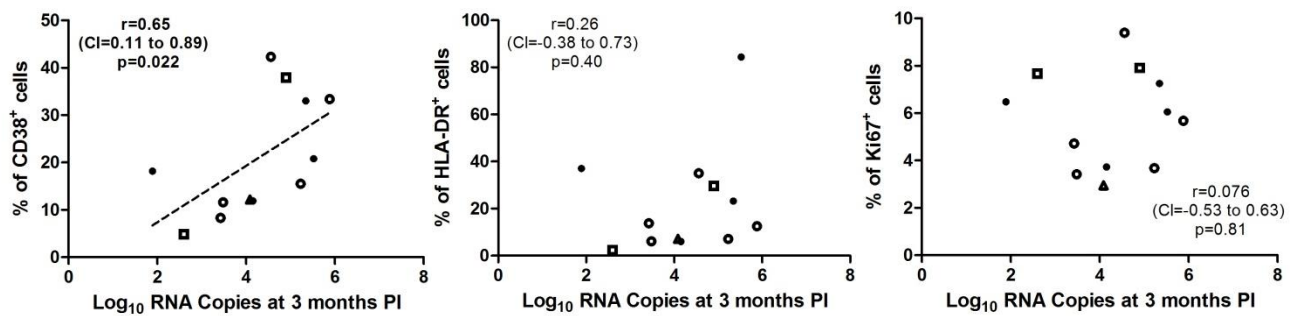


Figure 7.7: The relationship between the memory CD8⁺ T cell activation and viral load at 3 months post-infection. Correlations between the proportions of activated (A) total memory, and (B) Gag-specific memory CD8⁺ T cells with viral load in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\text{log}_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\text{log}_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\text{log}_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

Table 7.6: Mixed-effects linear regression associating the proportions of activated memory CD8⁺ T cells with viral load over time

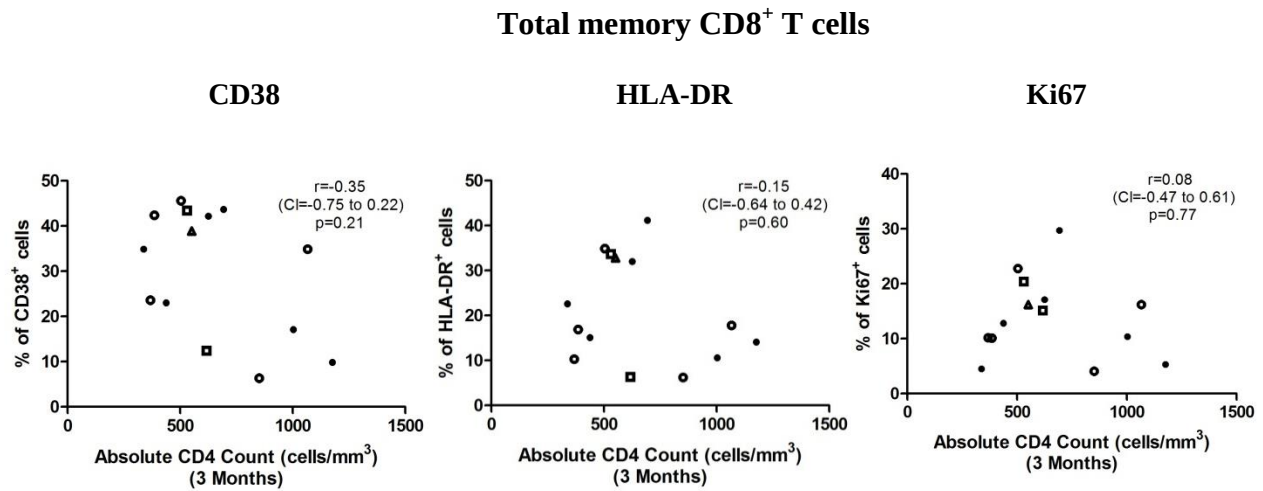
Activation markers	Slope	<i>P</i>	95% CI
Total memory			
CD38	0.103	<0.0001	(0.047 to 0.160)
HLA-DR	0.053	0.045	(0.001 to 0.106)
Ki67	0.061	0.104	(-0.012 to 0.134)
Gag-specific memory			
CD38	0.066	0.136	(-0.021 to 0.153)
HLA-DR	0.058	0.193	(-0.030 to 0.146)
Ki67	0.023	0.558	(-0.054 to 0.100)

Longitudinal associations between T cell activation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

7.2.6 Associations between the memory CD8⁺ T cell activation and CD4 count

In this chapter, inverse relationships were observed between activated memory CD4⁺ T cells and CD4⁺ T cell numbers during HIV infection. To assess whether similar relationships could be observed within the CD8⁺ T cell compartment, the proportion of memory CD8⁺ T cells expressing CD38, HLA-DR and Ki67 were correlated with the absolute CD4⁺ T cell counts at 3 months post-infection. No significant relationships were however observed between total and Gag-specific memory CD8⁺ T cells with CD4⁺ T cell numbers at 3 months post-infection (Figure 7.8A and B). The relationships between the activation of memory CD8⁺ T cells with CD4 T cells over time (Chapter 2, Model 2, page 63), showed negative trends between the proportions of CD38 and HLA-DR on total memory cells with CD4⁺ T cell counts (slope=-0.141, $p=0.086$ and slope=-0.16, $p=0.071$, respectively, Table 7.7).

A



B

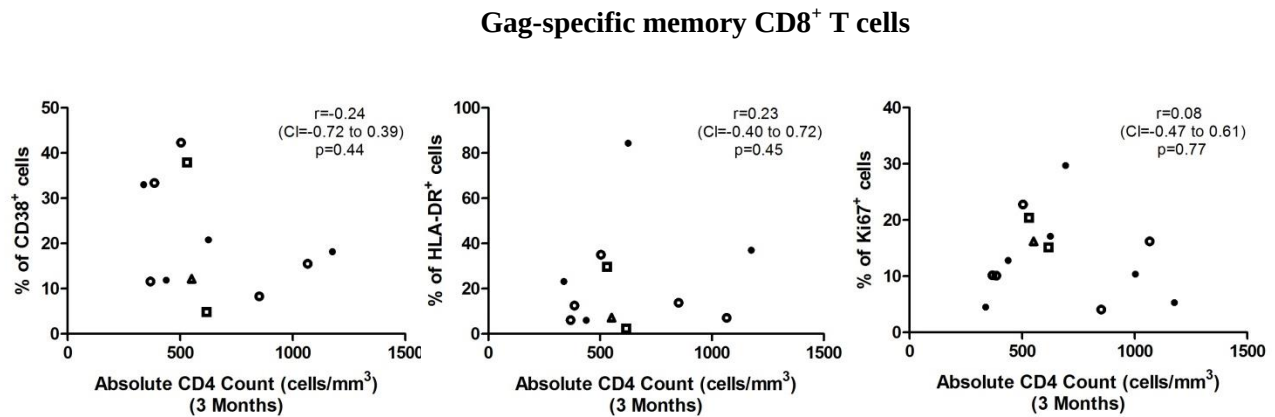


Figure 7.8: Correlations between memory T cell activation profiles with CD4⁺ T cell counts at 3 months post-infection. Associations between the proportions of CD38, HLA-DR and Ki67 on (A) total memory and (B) Gag-specific memory CD8⁺ T cells with CD4⁺ T cell counts in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

Table 7.7: Mixed-effects linear regression associating the proportions of activated memory CD8⁺ T cells with CD4⁺ T cell counts over time

Activation markers	Slope	<i>P</i>	95% CI
Total memory			
CD38	-0.141	0.086	(-0.301 to 0.020)
HLA-DR	-0.163	0.071	(-0.339 to 0.014)
Ki67	0.031	0.104	(-0.099 to 0.162)
Gag-specific memory			
CD38	-0.033	0.580	(-0.152 to 0.085)
HLA-DR	-0.028	0.643	(-0.147 to 0.091)
Ki67	-0.014	0.753	(-0.100 to 0.072)

Longitudinal associations between T cell activation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

Furthermore, when controlling for both viral load and CD4⁺ T cell count, the negative trends between the proportions of CD38 and HLA-DR with absolute CD4⁺ counts persisted over time (Table 7.8). The activation levels of CD38 and HLA-DR also continued to associate significantly with viral load over time, when controlling for CD4⁺ T cell counts. The proportion of Ki67 on memory CD8⁺ T cells showed a positive trend with viral load over time, and thus demonstrating that the proliferation of total memory CD8⁺ T cells over time could also be influenced by HIV antigen load. In summary, the data suggest the hyperactivation of total memory CD8⁺ T cells may to some degree contribute towards the decline of CD4⁺ T cells during the first 15 months of HIV-1 infection.

Table 7.8: Multivariate analysis with mixed-effects linear regression associating activated memory CD8⁺ T cells with covariates viral load and CD4⁺ T cell counts over time

Activation markers	Slope	<i>P</i>	95% CI
CD38			
Log viral load (copies/ml)	0.0098	0.001	(0.042 to 0.155)
Log CD4 count (cells/mm ³)	-0.2440	<i>0.060</i>	(-0.499 to 0.010)
HLA-DR			
Log viral load (copies/ml)	0.055	0.034	(0.0040 to 0.106)
Log CD4 count (cells/mm ³)	-0.231	<i>0.059</i>	(-0.471 to 0.008)
Ki67			
Log viral load (copies/ml)	0.065	<i>0.081</i>	(-0.008 to 0.138)
Log CD4 count (cells/mm ³)	0.168	0.329	(-0.169 to 0.505)

Longitudinal associations between T cell activation and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

7.2.7 Associations between T cell function and HIV-1 viral load

Studies conducted by Borrow et al. (1997) showed that the pressure exerted by cytotoxic CD8⁺ T lymphocytes results in rapid viral escape mutations during primary HIV infection, suggesting that immune responses during the early stages of infection may play an important role in HIV-1 replication. Therefore, this section sought to assess the relationships between the function of HIV-specific T cells with viraemia during early HIV-1 infection. Figure 7.9 shows no significant associations between the total magnitude either Gag-specific memory CD4⁺ or CD8⁺ T cell responses with viral load at 3 months post-infection suggesting that the total responding T cells infection had no impact on viraemia during early HIV-1 infection.

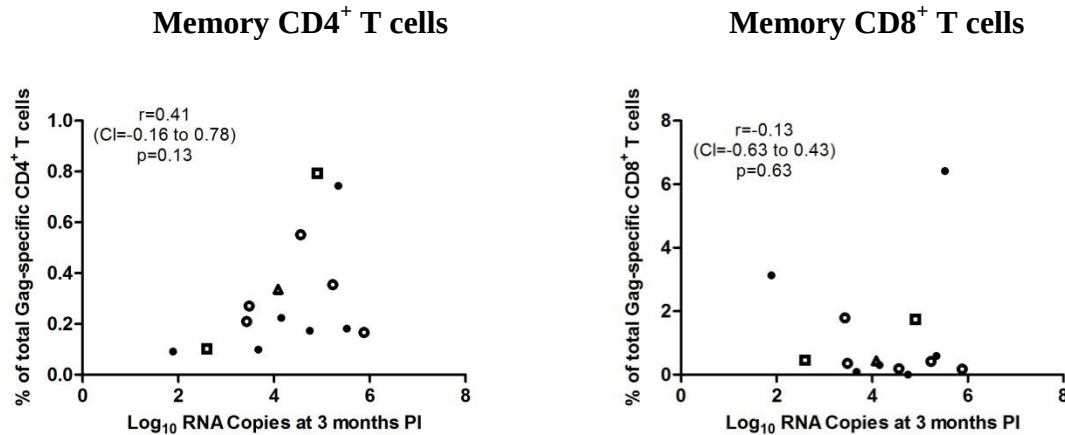
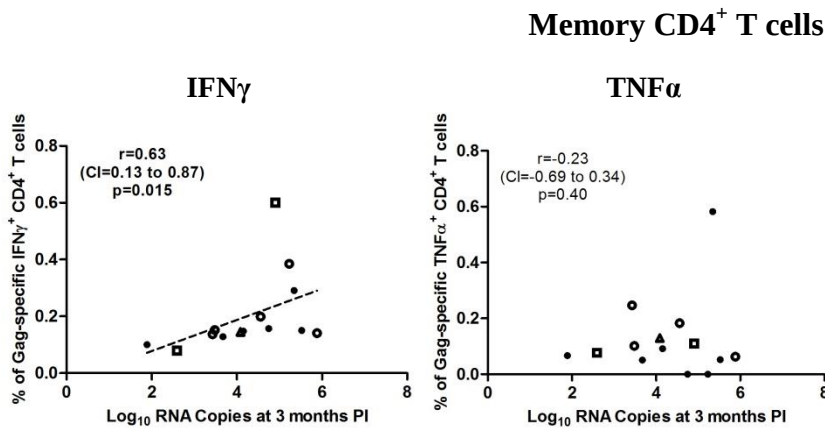


Figure 7.9: Correlations between total HIV-1 Gag-specific memory CD4⁺ and CD8⁺ T cells with viral load at 3 months post-infection. The total response Gag-specific responses were determined from the sum of responses across all the functional combinations (CD107, IFN γ , IL2, MIP-1 β and TNF α) in early HIV-infected individuals (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5log₁₀ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm$ 0.5log₁₀ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase >0.5log₁₀ RNA copies/ml and open square represent 2 individuals whose viral evolution could not been determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

The next level of analysis investigated the relations between the frequency of each HIV-1 Gag-specific T cell function (CD107, IFN γ , IL-2, MIP-1 β or TNF α) with viral load at primary infection. For CD4⁺ T cell analysis, the production of CD107, IL-2 and MIP-1 β were frequently below the positive response cut-off (<0.05) in most individuals. As a result, only the frequency of HIV-specific IFN γ ⁺ and TNF α ⁺ CD4⁺ T cells and HIV-specific CD107⁺, IFN γ ⁺ and MIP-1 β ⁺ CD8⁺ T cells were correlated with viral load. A positive and significant correlation was found between the frequency of Gag-specific IFN γ ⁺ CD4⁺ responses and viraemia (r=0.63, p=0.015),

but no associations were found between Gag-specific $\text{TNF}\alpha^+$ CD4^+ T cells with viral load (Figure 7.10A). Furthermore, no significant correlations were observed between the frequency of Gag-specific CD8^+ T cells that are able to degranulate (CD107^+), produce $\text{IFN}\gamma$, or MIP-1 β with viral load (Figure 7.10B).

A



B

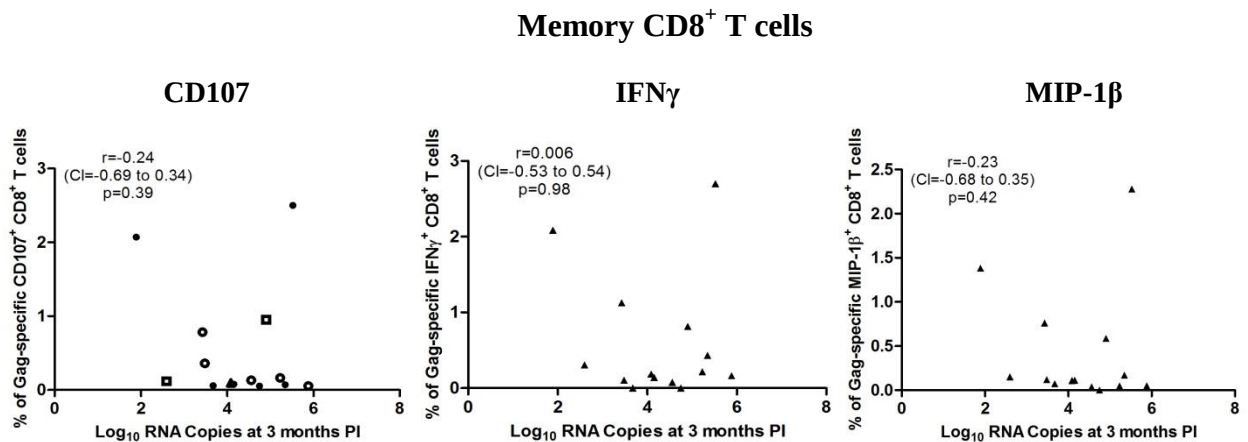


Figure 7.10: Relationships between HIV-1 Gag-specific T cells and viral load at 3 months post-infection. (A)

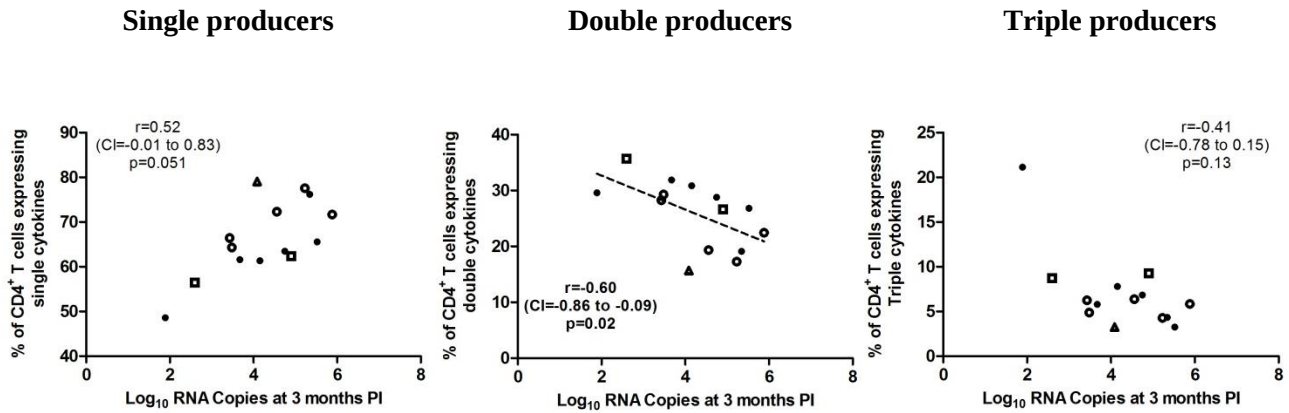
Correlations between the frequency of IFN γ - and TNF α -producing Gag-specific CD4⁺ T cells with viral load in early HIV-infected individuals (n=14). (B) Correlations between the Gag-specific CD107⁺, IFN γ ⁺ and MIP-1 β ⁺ CD8⁺ T cells and viral load at 3 months post-infection. Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

HIV-specific T cells that have the ability produce multiple cytokines or chemokines simultaneously, have been linked with a more effective response that could play a key role in the control of viraemia in HIV-1 infection (Betts *et al.*, 2006). To find out whether multifunctional responses play a part in the control of viraemia during primary HIV-1 infection, Boolean gating was employed to divide HIV-specific T cell responses into cells that able to co-express multiple cytokines, and then analysed in relation to concurrent viral load at 3 months post-infection. Data from Chapter 5, Section 5.2.2 page 151 and Section 5.2.3, page 155 showed that Gag-specific memory CD4⁺ and CD8⁺ T cells respectively, had low proportions of cells that are able to co-express 4 or 5 functions. As a result, associations were examined between the proportions of triple-, double-, and single-producing HIV-specific T cells and viral load. Figure 7.11A shows a positive trend in the relationship between the total proportions of single-cytokine producing cells and viral load ($r=0.52$, $p=0.051$), whereas a significant and inverse correlation was found between double-cytokine producers and viraemia ($r=-0.6$, $p=0.02$). Although no significant associations were found, a negative trend in the relationship between triple-cytokine producers and viral load was observed ($r=-0.41$, $p=0.13$).

Analysis of Gag-specific memory CD8⁺ T cells also showed a positive trend between the proportion single-cytokine producing cells and viral load ($r=0.49$, $p=0.069$, Figure 7.11B). A negative trend was also observed in the relationship between triple-cytokine producers and viraemia ($r=-0.45$, $p=0.1$), while no associations were observed when correlating double-cytokine producers with viraemia. Altogether, these data infer that HIV-1 Gag-specific cells that express single function to some extent associate with an increase in viral load, while those expressing two or more functions appear to associate with reduced viraemia.

A

Gag-specific memory CD4⁺ T cells



B

Gag-specific memory CD8⁺ T cells

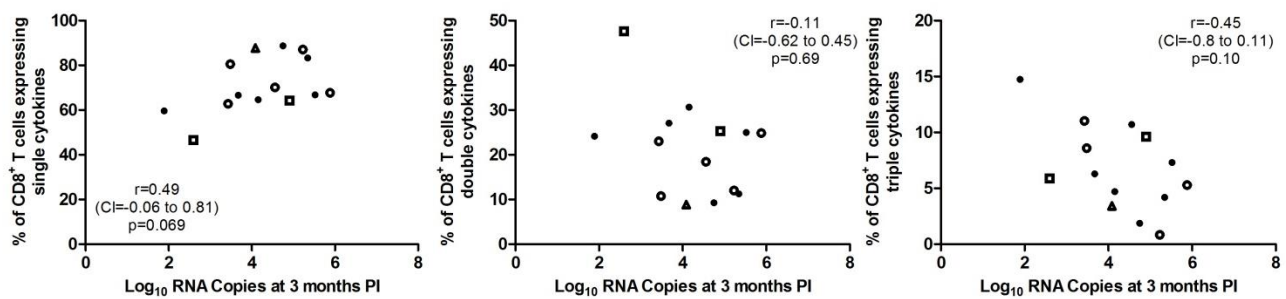


Figure 7.11: Relationships between the functional profile of Gag-specific memory T cells and viral load at 3 months post-infection. Correlations between the proportions of total single-, double-, and triple cytokine producers of (A) Gag-specific CD4⁺, and (B) CD8⁺ T cells with viral load (n=14). Open circles (○) represent individuals who showed an HIV viral load decline of $>0.5\log_{10}$ RNA copies/ml between 3 and 12 months post-infection; closed circles (●) represent individuals who showed a viral load change within $\pm 0.5\log_{10}$ RNA copies/ml between 3 and 12 months and the open triangle represents the one participant who showed a viral load increase $>0.5\log_{10}$ RNA copies/ml and open square represent 2 individuals whose viral evolution could not be determined due to missing viral load data at 12 months. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

To further investigate whether HIV-1 Gag-specific cellular immune responses over the first 15 months of infection were related to the course of viraemia, a linear mixed-effects model was used to correlate the proportions of the total magnitude and functional profile of T cells with viral load over time. No significant associations were found when examining associations between the total magnitude of Gag-specific memory CD4⁺ cells with viral load over time (Table 7.9). When correlating the functional profile of Gag-specific memory T cells with viraemia, positive and significant associations were found between the proportions of Gag-specific memory CD4⁺ T cells expressing single cytokines and viral load over time (slope=0.033, p=0.019), while cells expressing triple cytokines inversely correlated with viral load (slope=-0.19, p=0.019). No significant associations were observed between double-cytokine producing cells and viral load. There were no significant correlations found between the total magnitude and the polyfunctional profile of Gag-specific CD8⁺ T cell responses with viral load over time.

Table 7.9: Mixed-effects linear regression associating Gag-specific memory CD4⁺ and CD8⁺ T cell responses with viral load over time

Gag-specific memory T cells	Slope	<i>P</i>	95% CI
CD4⁺ responses			
Total responses	0.022	0.523	(-0.045 to 0.091)
Single cytokine producing cells	0.033	0.019	(0.005 to 0.061)
Double cytokine producing cells	-0.038	0.404	(-0.1299 to 0.052)
Triple cytokine producing cells	-0.966	0.019	(-0.177 to -0.161)
CD8⁺ responses			
Total responses	-0.004	0.967	(-0.266 to 0.258)
Single cytokine producing cells	0.005	0.681	(-0.021 to 0.031)
Double cytokine producing cells	0.001	0.945	(-0.038 to 0.041)
Triple cytokine producing cells	-0.063	0.200	(-0.161 to 0.033)

Longitudinal associations between Gag-specific memory T cell responses and viral load were determined using linear mixed-effects modelling fit in the log scale. The slopes are shown with the 95% Confidence Intervals (CI) with significant p-values ($p < 0.05$).

7.3 Discussion

Delineating the interplay between memory T cell differentiation, immune activation, T cell function and viraemia in acute or early HIV-1 infection may provide important insights toward understanding HIV-1 pathogenesis. Despite an increasing body of data on the phenotypic and T cell functions (Addo *et al.*, 2007, Barbour *et al.*, 2009), it is still not entirely clear which particular phenotypic enrichment or differentiation stage of memory T cells can provide necessary and sufficient protection from HIV-1 infection. Thus, one of the objectives this chapter aimed at defining the phenotype and functional capabilities of T cells that associate with viral control during the first 15 months of HIV-1 infection, and a number of observations were made.

Data in this chapter revealed that increased proportion of Gag-specific ED memory CD4⁺ T cells associates with low viral load while Gag-specific LD memory cells associates with increased viraemia during early HIV-1 infection, suggesting that the maintenance of less differentiated HIV-specific memory CD4⁺ T cells may favour better HIV-1 disease outcome. Similarly, other studies have suggested that the accumulation of less differentiated HIV-specific memory CD4⁺ T cell correlate with improved HIV-1 control (Emu *et al.*, 2005, Barbour *et al.*, 2009), whereby the protective ability of Gag-specific memory ED CD4⁺ T cells may be related to their ability to better proliferate and self-renew compared to LD or FD cells (Younes *et al.*, 2003, Palmer *et al.*, 2004). On the other hand, these associations also showed that some individuals that appeared to control viraemia over time, exhibited lower proportion of Gag-specific ED memory cells, suggesting that the apparent inverse correlations between less differentiated Gag-specific memory CD4⁺ T cells and lower viral load may more likely reflect HIV-1 antigen burden than a determining factor. Furthermore, longitudinal analysis showed significant associations between increased HIV-1 viraemia and the accumulation of LD Gag-specific memory CD4⁺ T cells, which are characterized by reduced capacity to proliferate and have alterations in their cytokine secretion profile (i.e. decreased IL-2 production) (Younes *et al.*, 2003).

Increase in viraemia was also found to associate with the reduction of the proportion of total naive CD8⁺ T cells, and an increase in the proportion LD total memory CD8⁺ T cells in early HIV-1 infection. Additionally, viraemia was also found associate with continuous generation of memory CD8⁺ T cells from a pool of naive CD8⁺ T cells over time. These data are concordant with previous studies showing that persistent HIV-1 replication results in the decrease of naive cells and accumulation of effector and memory CD8⁺ T cell subpopulations (Mojumdar *et al.*,

2011). Furthermore, emerging data also suggest that accumulation of late differentiated memory CD8⁺ T cells associate with higher HIV-1 viral set point (Burgers *et al.*, 2009), and thus reflecting a link between the maintenance of more mature T cells with poor HIV-1 disease outcome. Taken together that excessive differentiation could result in premature exhaustion and subsequent impairment of T cell functions (Appay and Rowland-Jones, 2002), these data could suggest that the skewing of memory T cells towards a more differentiated T cell phenotypes during HIV-1 infection, may to some degree reflect one of the pathogenic mechanisms through which HIV-1 depletes immune resources that may be required to offset HIV-1 disease progression.

It was found that primary HIV-1 infection was characterized by increased proportions of activated T cells that also appear to be susceptible to HIV-1 infection (observations in Chapter 4), however it remained to be resolved whether the heightened T cell activation was a consequence of HIV-1 replication or other factors such as microbial translocation or inflammation. As previously shown in Chapter 6, findings in this chapter further demonstrated significant associations between activated total memory CD4⁺ T cells with viral load at 3 months post-infection. Moreover, significant and positive correlations between activated Gag-specific memory CD4⁺ T cells and HIV-1 viral load suggest that generalized CD4⁺ T cell activation during primary HIV-1 infection was mainly driven by HIV-1 replication. Moreover, the relationship between the expression of CD38 and Ki67 and viral load over time, suggest that continuous HIV-1 replication maintains the hyperactivated status and continuous turnover of total memory CD4⁺ T cells throughout the first year of HIV-1 infection. These findings are

consistent with previous studies demonstrating direct associations between memory CD4⁺ T cell activation and elevated HIV viraemia (Deeks *et al.*, 2004, Hunt *et al.*, 2008).

As for memory CD8⁺ T cells, elevated proportions of activated total and Gag-specific memory CD8⁺ T cells, as measured by CD38, were also found to significantly correlate with increased levels of viraemia during primary HIV-1 infection. Furthermore, total memory CD8⁺ T cells that express CD38, strongly associated with viral load over the first 15 months of HIV-1 infection. These data supports an increasing body of data which suggest that the expression of CD38 on CD8⁺ T cells appears to be a strong indicator of HIV-1 disease progression, independent of CD4⁺ T cell counts and HIV viral load (Giorgi *et al.*, 1993, Bofill *et al.*, 1996, Liu *et al.*, 1997, Hazenberg *et al.*, 2003, Deeks *et al.*, 2004). Collectively, these data still shows that HIV-1 viral load, similarly to memory CD4⁺ T cells, modulates the activation of memory CD8⁺ T cells during the course of HIV-1 infection.

Although a major destruction of CD4⁺ T cells in HIV-1 infection has been attributed to either direct cytopathic effects of the virus (Gandhi *et al.*, 1998) or apoptosis (Groux *et al.*, 1992), associations found between the persistent hyperactivated status of total memory CD4⁺ and CD8⁺ T cells with low number of CD4⁺ T cells independent of HIV viraemia, suggest that generalized immune activation also accounts for the loss of absolute CD4⁺ T cell numbers during HIV-1 infection. These data appears to be in line with previous reports suggesting that T cell hyperactivation is more likely to cause rapid CD4⁺ T cell decline than HIV-1 replication (Sousa *et al.*, 2002, Grossman *et al.*, 2002, Choudhary *et al.*, 2007, Vajpayee *et al.*, 2009), either through decreased thymic T cell output (Douek *et al.*, 2001), low regenerative capacity (Hazenberg *et al.*,

2000a) or activation-induced bystander T cell apoptosis (Holm and Gabuzda, 2005). On the other hand, observations of strong and inverse correlations between CD4⁺ T cell counts with activated memory CD4⁺ T cells, and weak associations with activated memory CD8⁺ T cells, may also suggest the possibility that increased activation and proliferation of memory CD4⁺ T cells, in addition to viral load, may be a consequence of homeostatic mechanisms in response to CD4⁺ T cell decline. Consistent with this notion, recent studies have shown that homeostatic responses to CD4⁺ T cell depletion and HIV viral load contributes to the activation and proliferation of CD4⁺ T cells, whilst the activation of CD8⁺ T cells is primarily driven by the levels of HIV antigen load (Catalfamo *et al.*, 2008, Catalfamo *et al.*, 2011). Therefore it may be possible that the increased activation status of memory CD4⁺ T cells observed in this study, may be attributed to both viral replication and homeostatic forces in response to CD4⁺ T cell depletion.

Identifying immune responses that could mediate the control HIV-1 remain elusive (Pantaleo and Koup, 2004, Virgin and Walker, 2010), however there is evidence suggesting that T cell responses against HIV in acute or early stages of infection may be important for controlling HIV replication (Borrow *et al.*, 1994, Koup *et al.*, 1994). In addition, targeting of HIV-Gag has been previously shown to correlate with lower viral loads (Kiepiela *et al.*, 2007). Based on these reports, it was therefore hypothesized that HIV-Gag specific memory T cell responses during primary HIV-1 infection would associate with HIV-1 control. To examine this, Gag-specific T cell responses at 3 months post-infection were correlated with concomitant viral load. In line with previous findings (Betts *et al.*, 2001, Boaz *et al.*, 2002), this chapter found no significant associations between the total frequency of HIV-Gag-specific memory CD4⁺ T cells with viral load during primary HIV-1 infection. However, the frequency of Gag-specific IFN γ ⁺ CD4⁺ T

cells was positively correlated with viraemia, suggesting that increased numbers of Gag-specific IFN γ ⁺ CD4⁺ T cell during primary HIV-1 infection may be a reflection of intense and constant viral stimulation occurring during the early stages of HIV-1 infection. Neither total nor individual cytokine responses of Gag-specific memory CD8⁺ T cells were found to associate with viral load during primary HIV-1 infection. The lack of associations between Gag-specific memory CD8⁺ T cell responses and viraemia seems to suggest that the targeting of HIV-Gag may not be beneficial against HIV-replication during primary HIV-1 infection. These data are in agreement with data from Addo *et al.* (2003), but contradicts previous findings demonstrating direct inverse correlations between HIV-Gag CD8⁺ T cell responses and viraemia (Edwards *et al.*, 2002, Novitsky *et al.*, 2003, Kiepiela *et al.*, 2007, Mendiratta *et al.*, 2009). However, it may be possible that the lack of significant associations between Gag-specific CD8⁺ T cell responses and viraemia observed in this chapter may be related to the absence of protection-associated epitopes that have yet to emerge during the early stages of HIV-1 infection (Goulder *et al.*, 2001). Furthermore, the total frequency or the individual cytokine responses may also not completely reflect the true potential or the functional ability required to effectively control viraemia. Thus, converging evidence suggests that the measurement of polyfunctional T cells, may better explain the role of HIV-specific T cells against HIV replication (Betts *et al.*, 2006, Almeida *et al.*, 2007).

Data in this chapter appear to support and extend previous findings (Kannanganat *et al.*, 2007a) by showing inverse associations between the frequency of HIV-Gag specific memory CD4⁺ T cells that are able to co-express multiple cytokines, with viraemia. Moreover, Gag-specific CD4⁺ T cells with the ability to produce three cytokines inversely correlated with viraemia over the

first 15 months of infection. The resulting inverse associations between polyfunctional Gag-specific memory CD4⁺ T cell responses may on the other hand reflect the impact of high HIV-1 antigen burden on the functional profile of Gag-specific memory CD4⁺ T cells. Although not statistically significant, trends towards inverse relationships were also observed between polyfunctional Gag-specific memory CD8⁺ T cells and viral load at 3 months post-infection, however no associations were found with the polyfunctional profile of Gag-specific CD8⁺ T cell responses over time. These data appears to be in contrary to previous reports suggesting the importance of polyfunctional HIV-specific CD8⁺ T cells in the control of viral replication (Betts *et al.*, 2006, Almeida *et al.*, 2007, Julg *et al.*, 2010). The absence of associations between polyfunctional CD8⁺ T cells with viraemia may also be related to the progressive functional impairment of polyfunctional cells during the course of HIV-1 infection (Rehr *et al.*, 2008, Streeck *et al.*, 2008).

To summarize, this chapter shows that HIV-1 antigen burden appears to be a major driver of T cell differentiation of memory T cells towards a mature phenotype, which is associated with loss of HIV-1 control. It was also found that HIV-1 viraemia appears to contribute significantly to increased and persistent levels of immune activation, which could in turn provide more target cells that are susceptible to HIV-1 infection. Moreover, the heightened status of T cell activation and proliferation could also lead to rapid and continuous turnover of T cells that are destined to undergo increased rates of apoptosis, and subsequent decline of CD4⁺ T cell numbers and thereby contributing to HIV-1 pathogenesis. While host genetic factors play a crucial role in determining HIV-1 disease progression, this study is limited by the lack of individual HLA allele background, and thus could not address the relationship between HLA allele type and HIV-1

disease progression. Nonetheless, a broader interpretation of findings in this chapter is that the differentiation, activation and the functional profile of memory CD4⁺ and CD8⁺ T cell responses during HIV-1 infection are unlikely to play a role in determining the course of viraemia, but are most likely shaped by HIV itself.

CHAPTER 8: CONCLUDING REMARKS

8.1 Summarizing Discussion and Conclusions

An HIV-1 vaccine is of paramount importance to develop and implement as a public health option, especially in countries with a high HIV-1 prevalence. Understanding host immune responses to HIV-1 and biological events underlying HIV-1 pathogenesis could prove valuable in the development of an effective HIV-1 vaccine. While immune properties such as activation, differentiation status and functional responses of memory T cells have been shown to play a role during HIV-1 infection, less is known regarding the associations between these myriad of functional properties and how they influence HIV-1 disease outcome. In this context, this thesis takes a step forward from previous studies by delineating the interplay between cell activation, differentiation and functional profiles of total and HIV-specific memory CD4⁺ and CD8⁺ T cells with HIV-1 viraemia during primary HIV-1 infection. Furthermore, this work also focuses on understanding the mechanisms that relate to immune activation during primary HIV-1 infection.

When looking at the activation status of cells, primary HIV-1 infection was characterized by elevated numbers of activated and proliferating cells that correlated positively with viral load. Moreover, activation (CD38) and proliferation (Ki67) were found to be associated with viraemia over time, suggesting that continuous HIV-1 replication maintains an activated status and continuous turnover of total memory CD4⁺ and CD8⁺ T cells during the course of infection. Furthermore, upon sorting of activated total memory and HIV-Gag-specific memory CD4⁺ T cells, it was found that these cells were more susceptible to *in vivo* HIV-1 infection, further confirming that CD4⁺ activation events are most likely directly proportional to viral susceptibility. Further analysis showed inverse associations between increased immune

activation and CD4⁺ T cell counts, and thus it could be likely that the heightened immune activation during infection could reflect the *in vivo* pathogenicity of HIV-1 as defined by depletion of CD4⁺ T cell numbers, either through rapid turnover of memory cells that are destined to undergo increased rates of apoptosis and subsequent exhaustion or anergy of cells (Sousa *et al.*, 2002, Appay and Sauce, 2008).

Observations in this study also highlight an apparent paradox, if it is assumed that memory differentiation is linked with cell activation (Papagno *et al.*, 2004). In the CD4⁺ T cell compartment, activated cells were equally distributed across memory subpopulations and no significant associations were observed between cell memory activation and differentiation, suggesting that immune activation may not be directly driving memory differentiation of CD4⁺ T cells during early infection. On the other hand, the activation of memory CD8⁺ T cells was found to associate with the skewing of total memory T cell differentiation towards a more mature phenotype, and appears to support previous observations (Papagno *et al.*, 2004). These findings may also highlight the distinct mechanisms regulating CD4⁺ and CD8⁺ T cells. The differences between CD4⁺ and CD8⁺ subsets were further reflected by the distinct activation patterns observed in Chapters 4. Thus, memory CD4⁺ T cells were found to be less activated when compared with CD8⁺ T cells. These differences between CD4⁺ and CD8⁺ subsets may be related to factors such as the period of exposure to antigen and/or their inherent differentiation and proliferative capabilities (Seder and Ahmed, 2003). It was also evident from individuals examined in this study that the differentiation and activation profiles of both memory CD4⁺ and CD8⁺ T cells had reached a steady-state at some point during early HIV-1 infection. The findings were supported by associations between 3 months post-infection with those at subsequent time

points, 4 and 15 months post-infection, suggesting that these events were more a reflection of pre-existing and established events prior to analysis. The establishment of an early steady-state may be related to initial decline in HIV load during early infection (Deeks *et al.*, 2004), and thus it could further be postulated that the level of viraemia is likely determining the observed steady-state of memory differentiation and activation.

In order to tease out events that result in T cell activation during early HIV-1 infection, plasma markers of microbial translocation and inflammation were found to correlate with immune activation. The lack of these associations in HIV-uninfected controls suggests that microbial translocation and inflammation were unlikely causative. Multivariate analysis showed no significant associations between microbial translocation and inflammation or with T cell activation, whilst viral load did correlate with activated memory CD4⁺ and CD8⁺ T cells. Taken together, these findings argue in favour of *in vivo* HIV-1 replication being a major contributor to memory T cell activation during early HIV-1 infection, and that microbial translocation and the cytokines measured are proxy markers of inflammatory events. In addition, positive and significant associations between plasma markers of microbial translocation and cytokines with viraemia also suggest that microbial translocation and inflammation during early infection was more likely driven by HIV-1. The possible mechanisms may be related to increased viral replication resulting in preferential depletion of gut CD4⁺ T cell numbers and subsequent structural impairment and damage of the gut barrier thereby allowing the translocation of microbial products from the gut lumen into the periphery (Mehandru *et al.*, 2004, Mehandru *et al.*, 2007, Gori *et al.*, 2008, Nazli *et al.*, 2010).

Analysis on the polyfunctional profile of memory T cells during primary HIV-1 infection showed that Gag-specific memory CD4⁺ and CD8⁺ T cell responses were less polyfunctional compared to CMV-specific memory T cells. The low polyfunctional capacity may suggest functional impairment of HIV-specific memory CD4⁺ and CD8⁺ T cells responses during early HIV-1 infection, which may be related to functional exhaustion (Geldmacher *et al.*, 2007b, Rehr *et al.*, 2008, Streeck *et al.*, 2008) and/or rapid elimination of HIV-specific memory CD4⁺ (Douek *et al.*, 2002, Yue *et al.*, 2005) and CD8⁺ T cells (Mueller *et al.*, 2001). The resulting correlations between Gag-specific IFN γ -producing CD4⁺ T cells with viral load, may suggest that high antigenic burden in early infection may be driving functional profile of Gag-specific memory CD4⁺ T cells. Interestingly, no associations were observed between Gag-specific memory CD8⁺ T cell responses with viraemia, suggesting that the functional changes of Gag-specific memory CD8⁺ T cell responses may be multifactorial.

Examination of the differentiation profile of multicytokine-producing cells showed that the polyfunctionality of Gag-specific CD4⁺ and CD8⁺ T cells was equally expanded across memory subpopulations, whereas the polyfunctionality of CMV-specific memory CD4⁺ T cells increased with memory differentiation. Moreover, the polyfunctionality of CMV-specific T cells was also greater than that of Gag-specific memory T cells within each of the studied memory subpopulations, suggesting that early Gag-specific memory CD4⁺ and CD8⁺ T cells are less polyfunctional in nature regardless of the differentiation phenotype. Moreover, the polyfunctionality of Gag-specific memory CD4⁺ and CD8⁺ T cells remained unchanged over the 15 months of infection, though the frequency of CD8⁺ T cells expressing greater than 3 functions appeared to increase over time. Although the total frequencies of both Gag-specific memory

CD4⁺ and CD8⁺ T cells were found to significantly increase over time, these responses were unrelated to viraemia. In contrast, the kinetics of polyfunctional Gag-specific memory CD4⁺ T cells were found to associate with viral load, suggesting that HIV-1 replication may also be influencing the functional changes of HIV-specific memory T cells.

Although this study focuses on responses to HIV-Gag, it is unclear whether the observed functional profiles specific to Gag represent a general hallmark to HIV-specific T cell responses, and thus further studies may be required to examine the differentiation and functional profiles of HIV-specific CD4⁺ and CD8⁺ T cell responses toward other immunodominant HIV regions such as Env, Pol or Nef. Furthermore, the determination of correlates of immune protection may likely require more detailed understanding of host cellular immunity at single-epitope level and/or patterns of epitope recognition, TCR diversity, further examination of immune responses in individuals that are able to control viraemia (i.e. elite controllers and/or LTNP), host genetic factors such as HLA alleles and understanding mechanisms that relate to viral escape and diversification.

However, this thesis has shed light on the differentiation, activation and the polyfunctional status of memory CD4⁺ and CD8⁺ T cells during the early phases of HIV-1 infection. Moreover, this study further demonstrates the interplay between these events and how they associate with HIV-1 viraemia. In summary, the simplest interpretation from findings in this thesis is that the dynamics of total and Gag-specific memory T cell differentiation, activation, polyfunction along with microbial translocation and inflammation during primary HIV-1 infection did not appear to

influence the course of viraemia over the first year of infection. Indeed, it would appear that these events are rather determined by HIV-1 antigen load.

Findings in this thesis provide important insights on the impact of HIV viraemia on memory differentiation, immune activation, CD4⁺ T cell numbers, and function during primary HIV infection. The development of strategies aimed at either preventing HIV infection or reducing viraemia early after infection may be important in reducing viral set-point. Insights on how the SIV immune activation is controlled following SIV infection (Jacquelin *et al.*, 2009), may shed light in developing strategies aimed at attenuating the heightened immune activation during primary HIV infection. Thus, attenuated immune activation may be vital in limiting the size of viral reservoirs, preserving HIV-specific immunity (phenotype and function) and preserving normal CD4⁺ T cell homeostasis even in the face of actively replicating virus. Such interventions may include early diagnosis and ARV treatment of HIV during acute infection (O'Brien and Markowitz, 2012).

Control of viral replication in acute/early infection may also depend on developing HIV vaccines that are able to harness multiple antiviral arms of both innate and adaptive immunity. However, the design of such a vaccine requires fundamental insights regarding the nature, quantity, quality of immune responses that should be induced, and the ideal immunogens to include in a vaccine. This thesis also provides insights regarding the associations between the myriad functional properties (memory differentiation, immune activation, T cell function, microbial translocation and cytokine profiles) during primary infection, which should also be considered when developing novel approaches aimed at controlling viral replication during primary HIV infection.

APPENDICES

APPENDIX A: ETHICS CLEARANCE I

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

R14/49 de Bruyn

CLEARANCE CERTIFICATE

PROTOCOL NUMBER M050832

PROJECT

HIV-Specific CD4 T Cell Response among
Recent Seroconverters

INVESTIGATORS

Dr G de Bruyn

DEPARTMENT

Perinatal HIV Research Unit

DATE CONSIDERED

05.08.26

DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE

05.10.28

CHAIRPERSON



(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr C Gray

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B: ETHICS CLEARANCE II

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

R14/49 de Bruyn

CLEARANCE CERTIFICATE

PROTOCOL NUMBER M070249

PROJECT

Identification of signaling pathway networks
in T Cell Subpopulations and HIV CMV
antigen-specific CD8⁺ T Cells

INVESTIGATORS

Dr G de Bruyn

DEPARTMENT

Perinatal HIV Research Unit

DATE CONSIDERED

07.03.02

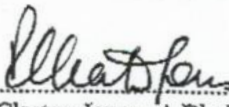
DECISION OF THE COMMITTEE*

APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.04.22

CHAIRPERSON


(Professors PE Cleaton-Jones, A Dhali, M Vorster,
C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX C: ETHICS CLEARANCE III



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Pholo W Maenetje

CLEARANCE CERTIFICATE

M120847

PROJECT

Activation and Memory Differentiation of Total
and HIV-Specific T Cells that Associate with
Viral Control during Subtype C HIV-1 Infection

INVESTIGATORS

Mr Pholo W Maenetje.

DEPARTMENT

School of Pathology/AIDS Research Unit

DATE CONSIDERED

31/08/2012

DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 31/08/2012

CHAIRPERSON 
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor Lynn Morris

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES..

APPENDIX D: PBMC AND PLASMA ISOLATION

15-30ml of blood was overlaid onto 15ml of Ficoll-Paque in 50ml Accuspin tube and centrifuged at 800g for 30 min at room temperature. Plasma was removed from the top gradient layer with a disposable sterile 10ml pipette and transferred into 2ml cryotubes at 1ml aliquots. Plasma containing cryotubes were immediately stored at -80°C until need. PBMC were collected from the buffy coat-mononuclear layer using a sterile pipette and transferred to a separate 50ml conical centrifuge tube. PBMC were then washed twice with approximately 45ml of HBSS (without calcium and magnesium) by centrifugation at 350g for 10min at room temperature. Cell pellet was re-suspended in 10ml of HBSS (cell suspension) and cell viability and cell count were determined using the Guava count or trypan blue dye method. After cell count and viability determination, the 10ml re-suspended cell pellet was centrifuged for 10 min at 350g at room temperature and supernatant discarded. Cell pellet was then re-suspended with the residual volume of HBSS (approximately 300µl) and 1 ml of ice-cold freezing media/solution (FBS plus 10% DMSO) was added slowly and mixed with cell suspension. Cell suspension (approximately 15×10^6 cells/ml) was transferred at 1ml aliquots into 2ml cryotubes and placed in Mr. Frosty (passive cell-freezing device) and stored at -80°C for 24h. After 24h, PBMC containing cryotubes were then transferred to liquid nitrogen, in vapour phase, for long term storage.

PBMC COUNTING AND VIABILITY DETERMINATION

PBMC counting and viability determination was performed using either the Neubauer hemocytometer and/or an automated cell counter Gauva PCA. When using the hemocytometer 10µl of the cell suspension was mixed with 10µl of 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) and allowed to incubate at room temperature for three to five minutes. 10µl of

trypan blue/cell mixture was then injected beneath the cover slip on the hemocytometer, and cells counted from the central large square of the hemocytometer using a binocular microscope.

Cell numbers were calculated as follows:

Total number cells = number of cells per square x 2 x 10 000 x total volume cell suspension (ml)

Viability was calculated as follows:

% viable cells = (number of viable cells counted/ number of total cells counted) x 100

Cell yield was calculated as follows:

Yield = total number of viable cells/processed whole blood volume (ml)

When using the Guava ViaCount method, in a microcentrifuge tube 20µl of cell suspension was mixed with 380µl of the Guava cell ViaCount stain and incubated for 5 min at room temperature. Cell acquisition was performed with the Guava PCA instrument (Guava Technologies Inc. 25801 Industrial Blvd. Hayward, CA 94545). Instrument calibration using Guava Check was performed according to manufacturer's recommendations. Cell count was analysed with Guava CytoSoft Software (Guava Technologies Inc. 25801 Industrial Blvd. Hayward, CA 94545). Number of total, viable and dead cells for each sample set was gated using a PM1 (Viability) vs. PM2 (Nucleated cells) dot plot.

APPENDIX E: LSRII 4-LASER SYSTEM

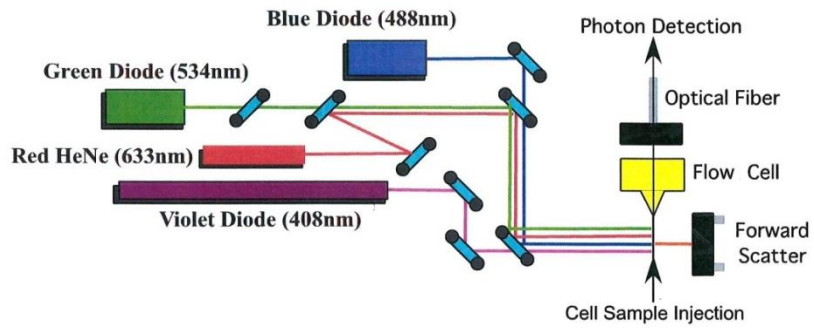
DETECTORS:

Table E1: Detector configuration and nomenclature.

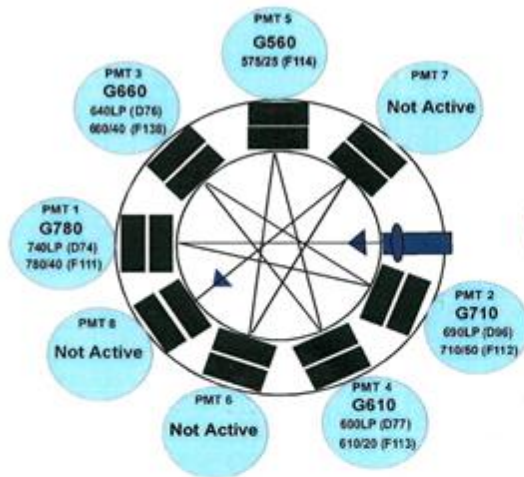
Panels		Fluorochromes			Laser (λ)	Dichroic Filter (LP)	Band Pass Filter
			Clones				
Functional	Activation	Stain	Function	Activation			
MIP-1β	IFNγ-IL2	PE	D21-1351	B27, & MQ1-17H12	532 nm	empty	575/25
CD27	CD27	PE-Cy5	1A4CD27	1A4CD27	532 nm	640 LP	660/40
CD4	CD4	PE-Cy5.5	S3.5	S3.5	532 nm	690 LP	710/50
TNFα	-	PE-Cy7	MAB11	-	532 nm	740 LP	780/40
CD45RO	CD45RO	Texas-Red PE	UCHL1	UCHL1	523 nm	600 LP	610/20
IL-2	CD38	APC	MQ1-17H12	HIT2	633 nm	empty	660/20
CD3	CD3	APC-Cy7	SK7	SK7	633 nm	740 LP	780/60
CD107	HLADR	Alexa-680	H4A3	G46-6	633 nm	685 LP	710/50
		Forward Scatter					
		Side Scatter			488 nm	488 LP	
IFNγ	Ki67	FITC	B27	B56	488 nm	505 LP	515/20
CD14	CD14	Pacific Blue	M5E2	M5E2	407 nm	empty	450/50
CD19	CD19	Pacific Blue	HIB19	HIB19	407 nm	empty	450/50
CD8	CD8	Q-dot 655	RPA-T8	RPA-T8	407 nm	630 LP	660/40
CD57	CD57	Q-dot 565	NK-1	NK-1	407 nm	557 LP	560/40

This LSRII instrument is equipped with 4 solid-state lasers (Green-532nm, Red-633nm, Blue-488nm and Violet-407nm).

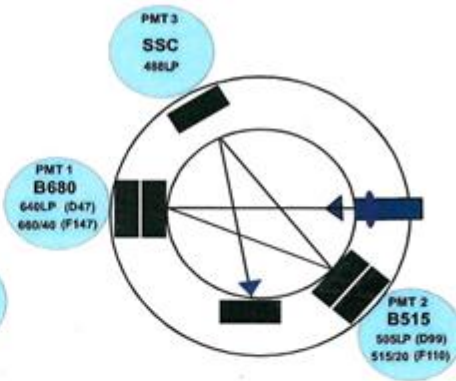
LASER PATH AND FILTER CONFIGURATION OF THE LSR II



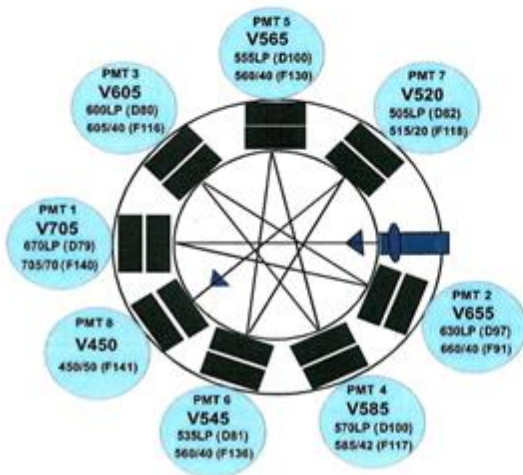
Green DPSS Laser 532nm Octagon



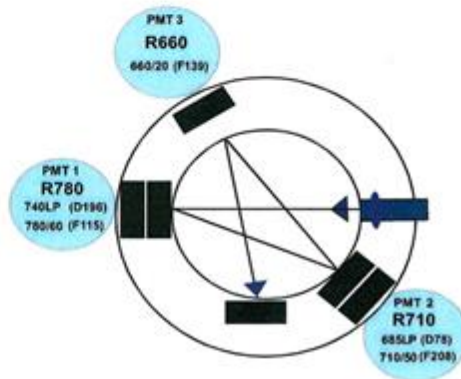
Blue DPSS Laser 488nm Octagon



Violet DPSS Laser 407nm Octagon



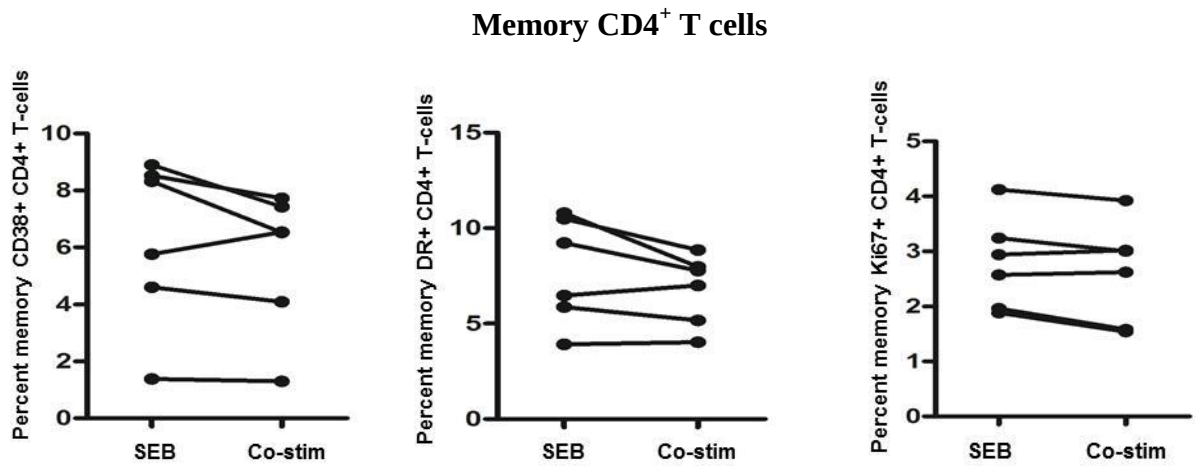
Red DPSS Laser 633nm Trigon



APPENDIX F: T CELL ACTIVATION PANEL OPTIMIZATION

In order to assess the effect of short-term antigen stimulation on activation marker expression levels, PBMC were stimulated with 1µg/ml SEB for 6 hours and stained with CD38, HLA-DR and Ki67. SEB unstimulated cells were used as controls (Figure F1A). SEB-specific memory CD4⁺ T cells were identified by the production of IFNγ and expression levels of CD38, HLA-DR and Ki67 and were compared with unstimulated and SEB-specific cells of the same phenotype. The expression levels of activation markers on SEB-specific cells and unstimulated memory cells were found to be equivalent after 6-hour stimulation. Furthermore, kinetic experiments were performed to examine the effect of BFA on the expression of activation makers on total memory CD4⁺ T cells (Figure F1B). PBMC were stimulated for 1, 3 and 6 hours with SEB ± BFA. Levels of CD38, HLA-DR and Ki-67 expression were analyzed on total memory CD4⁺ T cells and no significant differences in the expression of any of the markers ± BFA were observed between time points. Collectively, these data show that short-term stimulation had no effect on up-regulating the expression of CD38, HLA-DR and Ki67 and the the presence of brefeldin A during 6h stimulation did not limit the expression of activation markers.

A



B

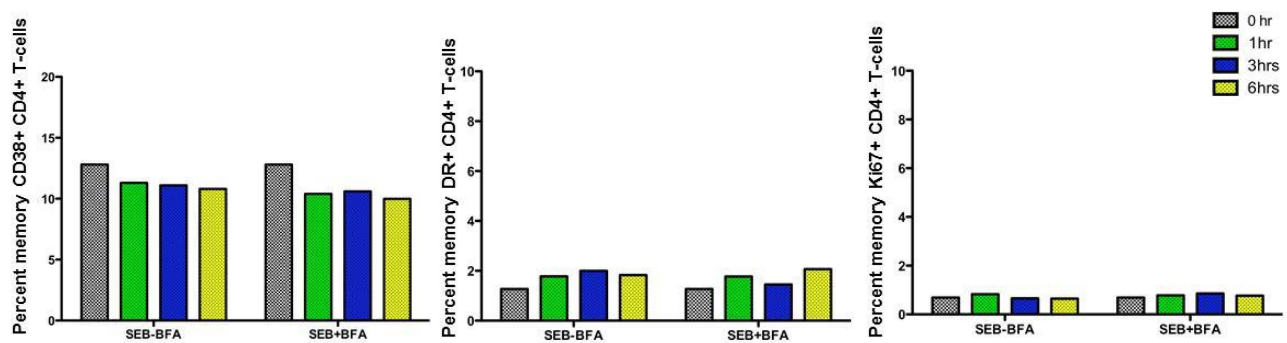


Figure F1: The expression of activation markers on CD4⁺ T cells after 6h stimulation. A) PBMC were stimulated with 1 μ g/ml SEB for 6h and the frequency of antigen-specific T cells was determined by the production of IFN γ -IL-2. The levels CD38, HLA-DR and Ki67 were compared between stimulated and un-stimulated cells. B) The kinetics of activation makers with and without the presence of brefeldin A. PBMC were stimulated for 1, 3, and 6 hours with 1 μ g/ml SEB in the presence or absence of brefeldin A and the expression of activation makers (CD38, HLA-DR and Ki67) on total memory CD4⁺ T cells were measured and analyzed using polychromatic flow cytometry.

APPENDIX G: CD4⁺ T CELL SORTING

SORTING STRATEGY OF GAG-SPECIFIC MEMORY CD4⁺ T CELLS

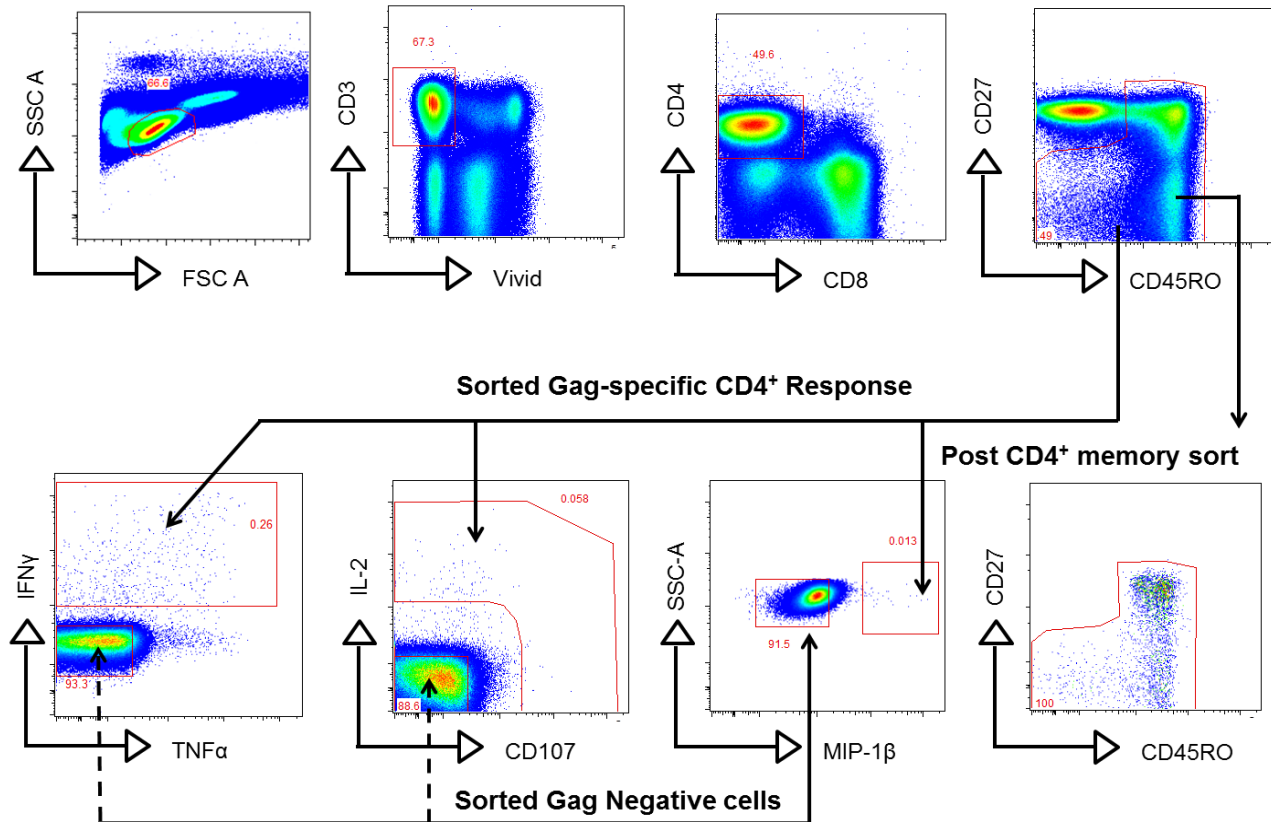


Figure G1: Gating strategy employed for sorting of Gag-specific memory CD4⁺ T cells. For the initial gating of each sample set, forward scatter area versus a forward scatter height plot was used for gating out cell aggregates. T cell lymphocytes were then gated from populations derived from a forward scatter area versus a side scatter area plot. Cells that were stained with Pacific blue: monocytes (CD14⁺), B cells (CD19⁺), and dead cells stained with violet amine dye, were removed from the analysis. The exclusion of these cells was done by gating on live CD3⁺ cells on a CD3 versus a pacific blue/violet amine plot. This was followed by gating on memory CD4⁺ T cells. Antigen-specific memory CD4⁺ T cells were sorted from combined gates cells expressing CD107, IFNγ, IL-2, MIP-1β and TNFα. The gates for sorting antigen-specific T cell populations were always placed outside the range of the background staining (unstimulated cells). Sorted total memory cells were also used to determine the purity of sorted populations (Post CD4⁺ memory sort). Sorted memory populations were constantly >98% pure.

SORTING STRATEGY OF ACTIVATED MEMORY CD4⁺ T CELLS

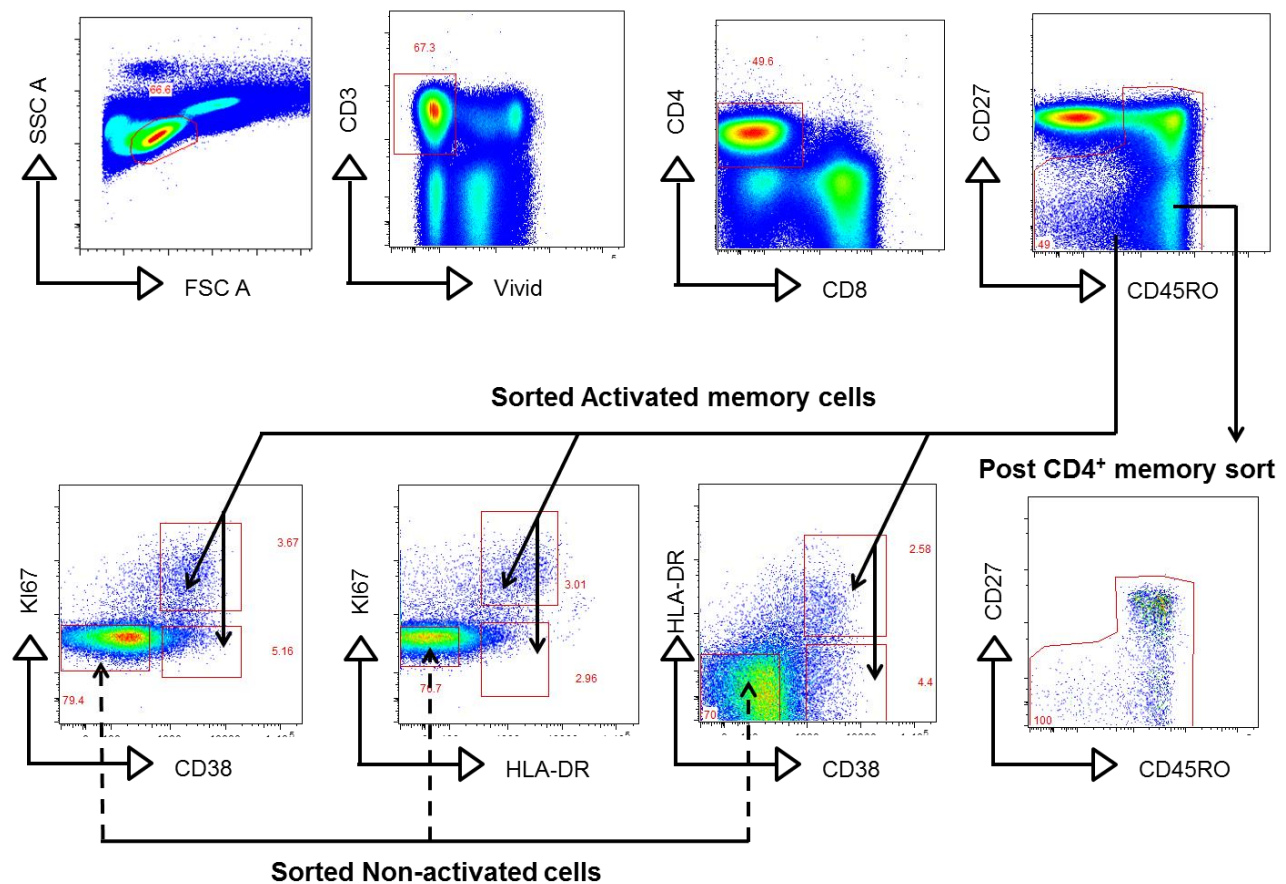
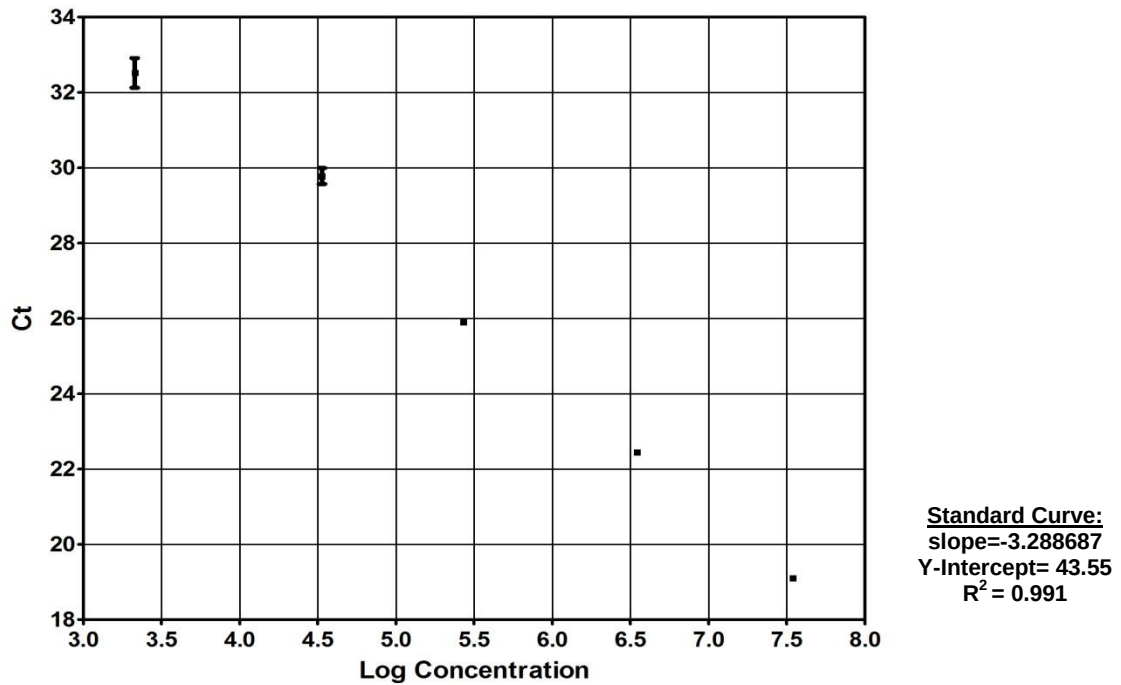


Figure G2: Gating strategy employed for sorting of activated total memory CD4⁺ T cells. Single cells were derived from a forward scatter area versus a side scatter area plot. Dead cells, monocytes (CD14⁺) and B-cells (CD19⁺) were excluded by staining with Pacific blue and live/dead violet dead stain. CD3⁺ and CD4⁺ were sequentially selected and memory CD4⁺ T cells were selected based on CD27 and CD45RO surface staining. Activated and/or proliferating memory CD4⁺ T cells were sorted based on cells expressing CD38, HLA-DR and Ki67. Non-activated cells (CD38⁻ HLA-DR⁻ Ki67⁻) were also sorted. Sorted total memory cells were also used to determine the purity of sorted populations (Post CD4⁺ memory sort). Sorted memory populations were constantly >98% pure.

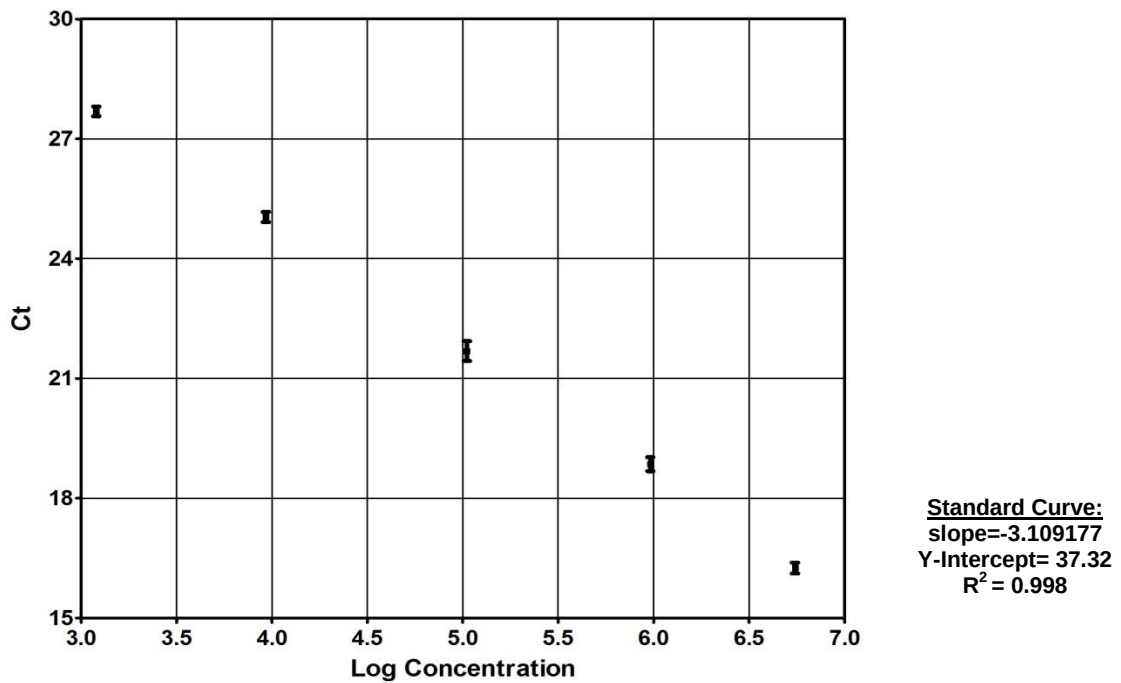
APPENDIX H: REAL TIME PCR STANDARD CURVES

Standard curves used for quantification of proviral DNA

Gag C Standards



Human Abumin Standards



APPENDIX I: QUANTITATIVE REAL TIME PCR TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	PHR001 (CD38-DR-Ki67-)		PHR001 (CD38+DR+Ki67+)		PHR002 (CD38-DR-Ki67-)		PHR002 (CD38+DR+Ki67+)		PHR003 (CD38-DR-Ki67-)		PHR003 (CD38+DR+Ki67+)	
B	PHR004 (CD38-DR-Ki67-)		PHR004 (CD38+DR+Ki67+)		PHR005 (CD38-DR-Ki67-)		PHR005 (CD38+DR+Ki67+)		PHR006 (CD38-DR-Ki67-)		PHR006 (CD38+DR+Ki67+)	
C	PHR014 (CD38-DR-Ki67-)		PHR014 (CD38+DR+Ki67+)		PHR015 (CD38-DR-Ki67-)		PHR015 (CD38+DR+Ki67+)					
D												
E	Gag C 10 ⁶		Gag C 10 ⁵		Gag C 10 ⁴		Gag C 10 ³		Gag C 10 ²		NTC	
F												
G												
H	Hum alb 10 ⁶		Hum alb 10 ⁵		Hum alb 10 ⁴		Hum alb 10 ³		Hum alb 10 ²		NTC	

No of wells: 60 (PCR contains 25ul/reaction)

Cycling Conditions:

Platinum Taq mix		
	(μl)	x60
10x Platinum Buffer	2.5	150
50mM MgCl ₂	1.75	105
10mM dNTP	0.5	30
5' primer (12μM)	1	60
3' primer (12μM)	1	60
Probe (5μM)	1	60
Platinum Taq	0.125	7.5
Rox Reference dye (100x)	0.05	3
Water	12.075	724.5
Template (Std/Sample)	5	

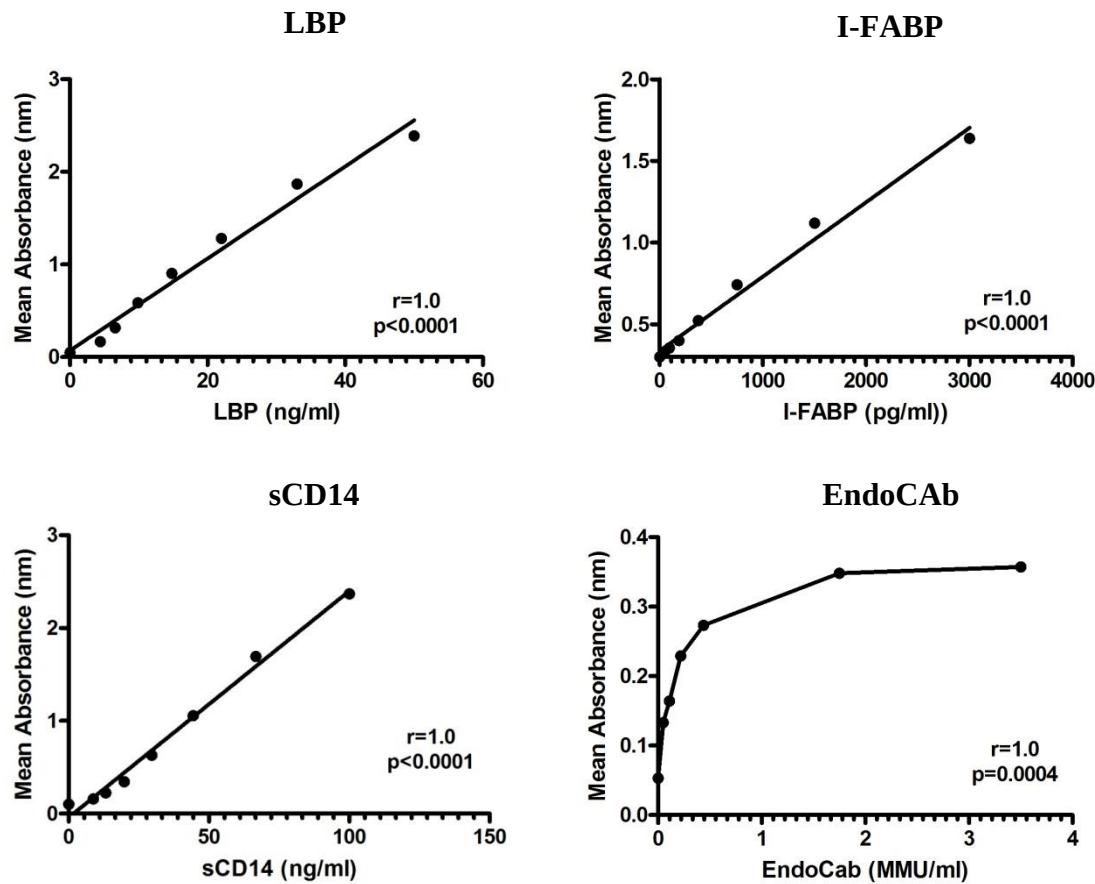
Temperature	Time	Repeat
94°C	2min	hold
94°C	15sec	
60°C	1min	40 cycles

*Add 5μl of template (or standard) to wells and make up to 25μl total with reaction mixture.

* NTC (No Template Control)

APPENDIX J: MICROBIAL TRANSLOCATION STANDARD CURVES

Standard curves for the determination of plasma LBP, I-FABP, sCD14 and EndoCAb



APPENDIX K: MEMORY SUBPOPULATIONS

Table K1: Correlations between the proportion of total memory CD4⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.

Subpopulation	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
Naïve	r	0.78	0.58	0.81	0.73	0.87	0.43
	p	0.0009	0.086	0.001	0.02	0.001	0.21
ED	r	0.75	0.60	0.41	0.46	0.92	0.67
	p	0.0017	0.073	0.16	0.17	0.0003	0.039
LD	r	0.41	0.78	0.73	0.51	0.57	0.73
	p	0.14	0.01	0.0041	0.13	0.088	0.02
FD	r	0.59	0.91	0.58	0.55	0.96	0.24
	p	0.032	0.0005	0.034	0.11	<0.0001	0.49

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

Table K2: Correlations between the proportion of Gag-specific memory CD4⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.

Subpopulation	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
ED	r	0.40	0.26	0.36	0.61	0.47	0.32
	p	0.17	0.49	0.24	0.11	0.23	0.36
LD	r	0.51	0.016	0.66	0.59	0.57	0.34
	p	0.07	0.96	0.018	0.11	0.08	0.32
FD	r	0.27	-0.51	0.097	-0.16	-0.1	-0.006
	p	0.36	0.15	-0.51	0.69	0.6	0.98

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

Table K3: Correlations between the proportion of total memory CD8⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.

Subpopulation	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
Naïve	r	0.87	0.79	0.84	0.64	0.79	0.66
	p	<0.0001	0.006	0.0003	0.042	0.001	0.037
ED	r	0.71	0.82	0.48	0.28	0.89	0.74
	p	0.0052	0.0052	0.09	0.42	0.0011	0.013
Int	r	0.81	0.87	0.85	0.85	0.57	0.52
	p	0.0003	0.0008	0.0002	0.0016	0.08	0.11
LD	r	0.91	0.72	0.84	0.66	0.80	0.86
	p	<0.0001	0.018	0.0003	0.037	0.0072	0.0012
FD	r	0.63	0.83	0.72	0.51	0.91	0.68
	p	0.013	0.0029	0.0052	0.12	0.0005	0.028

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

Table K4: Correlations between the proportion of Gag-specific memory CD8⁺ T cell subpopulations at 3 months post-infection with memory subpopulations at 4, 5, 6, 9, 12 and 15 months post-infection.

Subpopulation	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
ED	r	0.66	0.78	0.49	0.52	0.66	0.63
	p	0.058	0.012	0.14	0.18	0.058	0.067
Int	r	0.41	0.50	0.23	-0.19	0.75	-0.06
	p	0.17	0.17	0.52	0.65	0.025	0.86
LD	r	0.70	0.71	0.45	-0.16	0.28	-0.1
	p	0.01	0.01	0.18	0.69	0.36	0.79
FD	r	0.36	0.73	0.37	0.38	0.78	0.78
	p	0.24	0.024	0.28	0.35	0.017	0.012

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

APPENDIX L: MEMORY ACTIVATION

Table L1: Correlations between the proportions of activated total memory CD4⁺ T cells at 3 months post-infection with activated memory CD4⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection.

Activation Marker	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
CD38	r	0.63	0.85	0.57	0.78	0.63	0.2
	p	0.015	0.0029	0.039	0.011	0.028	0.58
HLA-DR	r	0.75	0.73	0.53	0.49	0.67	0.35
	p	0.0016	0.015	0.061	0.14	0.033	0.31
Ki67	r	0.78	0.85	0.78	0.79	0.68	0.09
	p	0.001	0.0016	0.0017	0.0061	0.028	0.8

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

Table L2: Correlations between the proportions of activated Gag-specific memory CD4⁺ T cells at 3 months post-infection with activated Gag-specific memory CD4⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection.

Activation Marker	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
CD38	r	0.54	0.75	0.32	0.60	0.73	0.68
	p	0.054	0.019	0.30	0.08	0.01	0.028
HLA-DR	r	0.39	0.56	0.43	0.70	0.58	-0.05
	p	0.18	0.11	0.15	0.035	0.07	0.8
Ki67	r	0.68	0.81	0.56	0.56	0.38	0.44
	p	0.0095	0.0072	0.056	0.11	0.27	0.19

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

Table L3: Correlations between the proportions of activated total memory CD8⁺ T cells at 3 months post-infection with activated memory CD8⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection.

Activation Marker	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs.9	3 vs. 12	3 vs. 15
CD38	r	0.79	0.62	0.71	0.62	0.65	0.62
	p	0.0007	0.061	0.0063	0.06	0.043	0.06
HLA-DR	r	0.91	0.22	0.57	0.79	0.79	0.81
	p	<0.0001	0.53	0.041	0.0088	0.0088	0.0072
Ki67	r	0.80	0.56	0.72	0.79	0.68	0.72
	p	0.0006	0.096	0.0054	0.0088	0.034	0.021

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

Table L4: Correlations between the proportions of activated Gag-specific memory CD8⁺ T cells at 3 months post-infection with activated Gag-specific memory CD8⁺ T cells at 4, 5, 6, 9, 12 and 15 months post-infection.

Activation Marker	Months Post-infection						
	Coefficient (p-values)	3 vs. 4	3 vs. 5	3 vs. 6	3 vs. 9	3 vs. 12	3 vs. 15
CD38	r	0.67	0.66	0.51	0.09	0.68	0.28
	p	0.015	0.049	0.13	0.82	0.058	0.46
HLA-DR	r	0.77	0.43	0.43	0.33	0.33	0.01
	p	0.003	0.24	0.21	0.41	0.38	0.96
Ki67	r	-0.05	0.31	-0.24	0.59	-0.16	-0.41
	p	0.86	0.41	0.48	0.11	0.67	0.26

Statistical associations were performed by a two tailed nonparametric Spearman rank correlation and adjusted with Bonferroni correction (Significant p-values 0.0083; **Bolded**).

APPENDIX M: MEMORY POLYFUNCTION

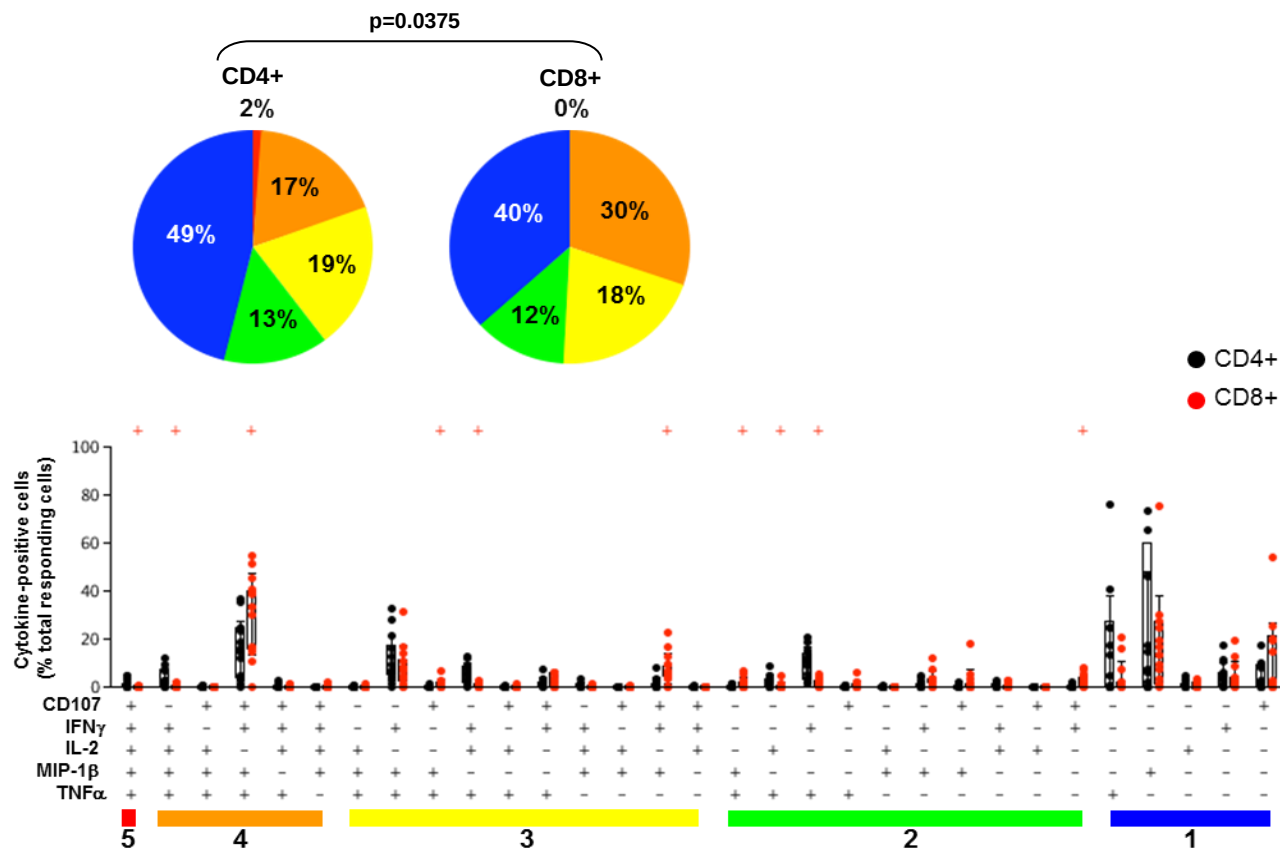


Figure M1: Comparison of the polyfunctional profile between CMV-specific memory CD4⁺ and CD8⁺ T cells in HIV-uninfected individuals. Using Boolean gating, the distribution of cells showing any combination of functional profiles was determined for CMV-specific memory CD4⁺ and CD8⁺ T cells in HIV-uninfected individuals. The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total CMV-specific response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of Gag-specific T cells were performed by a student's test (p<0.05).

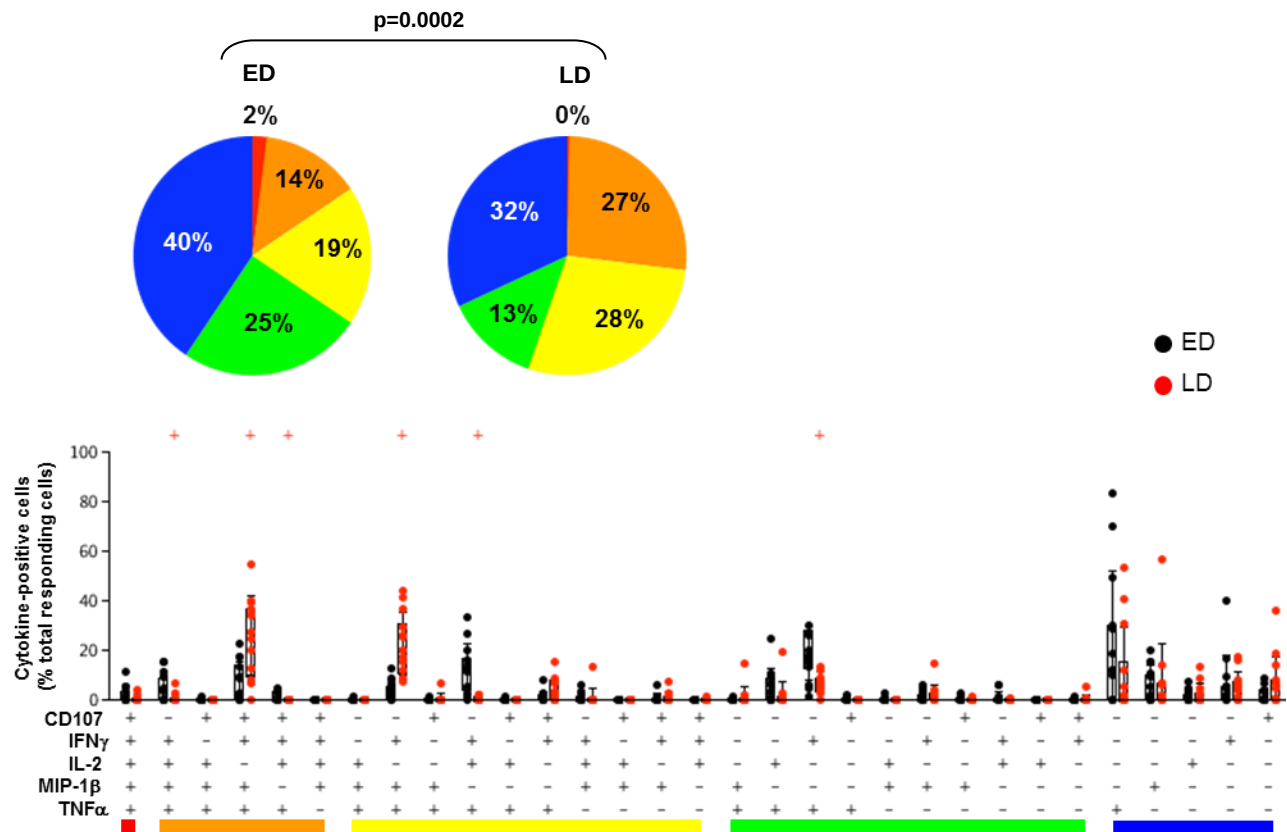


Figure M2: The polyfunctionality of CMV-specific ED- and LD-memory CD4⁺ T cells in HIV-uninfected individuals. Using Boolean gating, the distribution of cells with any combination of functional profiles was determined for Early Differentiated (ED, red dots) and Late Differentiated (LD, black dots) cells in HIV-uninfected individuals (n=13). The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total Gag response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of CMV-specific T cells were performed by a student's test ⁺ (p<0.05).

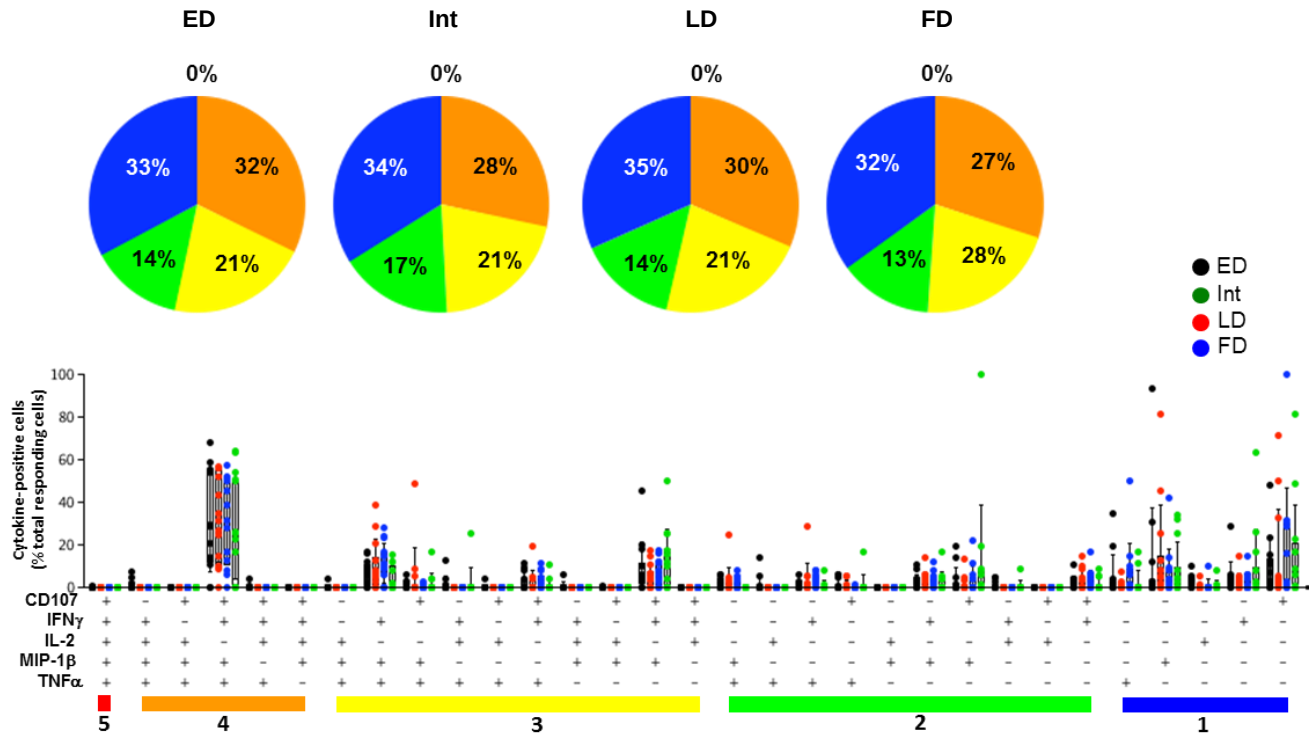


Figure M3: The polyfunctionality of CMV-specific ED-, Int-, LD- and FD-memory CD8⁺ T cells in HIV-uninfected individuals. Using Boolean gating, the distribution of cells any combination of functional profiles was determined for ED (black dots), Int (green dots), LD (red dots) and FD (blue dots) in HIV-uninfected individuals (n=13). The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The colour-coded bars show the median and interquartile ranges of the frequencies of each individual response pattern. The data are also summarized in colour-coded pie charts in which red (■) represents antigen-specific CD4⁺ T cells expressing 5 functions, orange (■): 4 functions, yellow (■): 3 functions, green (■): 2 functions and blue (■): one function. Each slice of the pie corresponds to the mean contribution of 5+, 4+, 3+, 2+ and 1+ functional population to the total CMV response. Statistical comparisons between pie charts were performed by a permutation test (Spice, version 4.3), while comparisons between the frequencies of different functional combinations of CMV-specific T cells were performed by a student's test ⁺ (p<0.05).

APPENDIX N: MICROBIAL TRANSLOCATION AND INFLAMMATORY MOLECULES

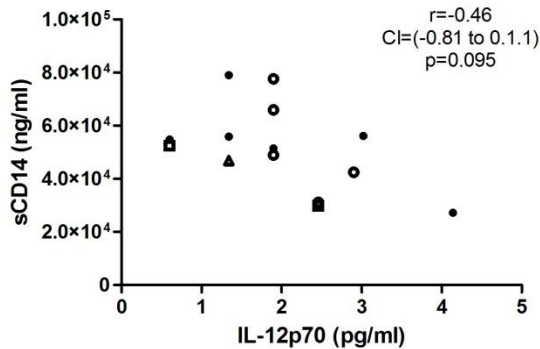


Figure N1: The association between sCD14 and IL-12(p70) at 3 months post-infection. Correlations between plasma concentrations of sCD14 and IL-12(p70) in early HIV-1 infected individuals ($n=14$). Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

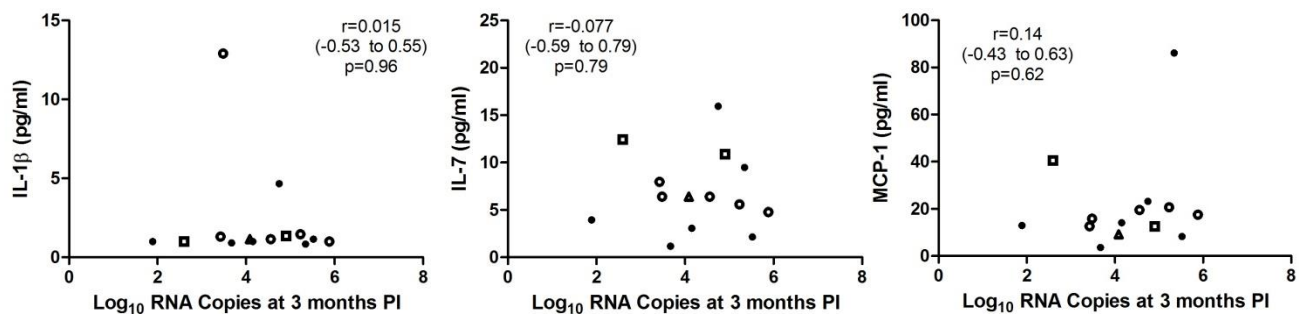
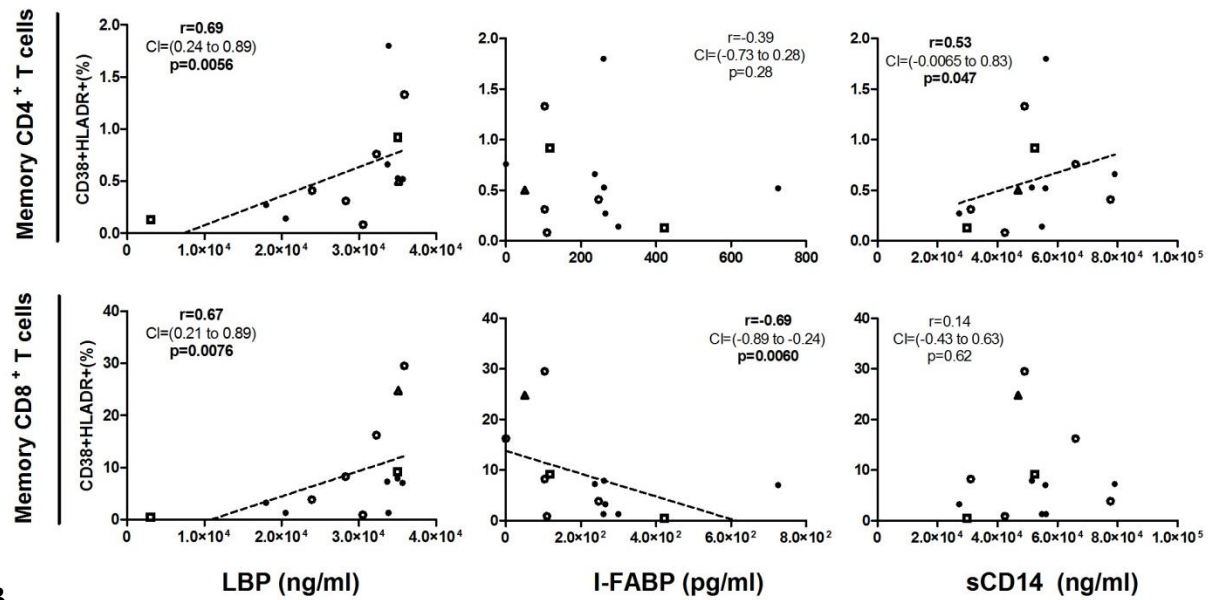


Figure N2: The relationship between plasma cytokines with viral load at 3 months post-infection. Correlations between plasma concentrations of IL-1 β , IL-7 and MCP-1 with concurrent viral load in early HIV-1 infected individuals ($n=14$). Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

A



B

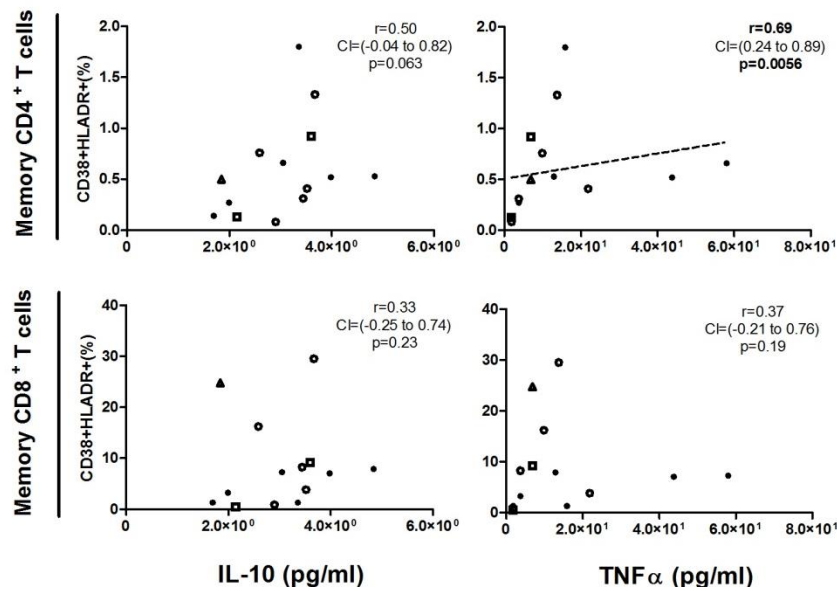
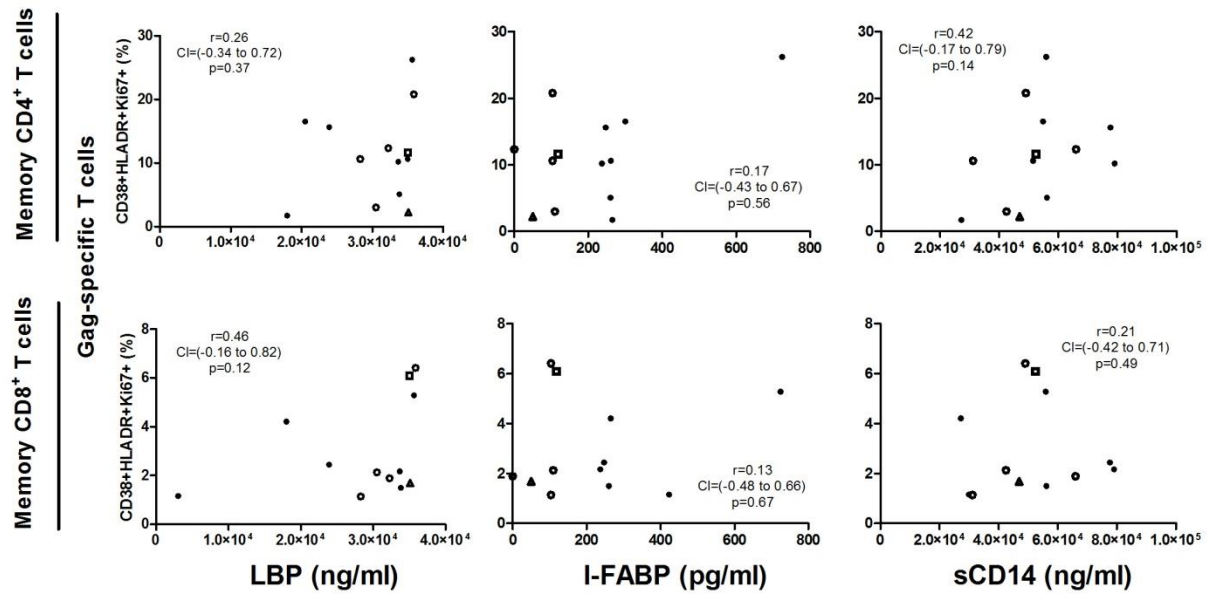


Figure N3: The relationship between plasma markers of microbial translocation and cytokines with total memory T cell activation in HIV-infected individuals. (A) Correlations between the plasma levels of sCD14, LBP and I-FABP with the proportions of activated total memory CD4⁺ and CD8⁺ T cells (CD38⁺HLADR⁺). (B) Correlations between the plasma levels of IL6, IL-10 and TNFα with the proportions of activated total memory CD4⁺ and CD8⁺ T cells (CD38⁺HLADR⁺). Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

A



B

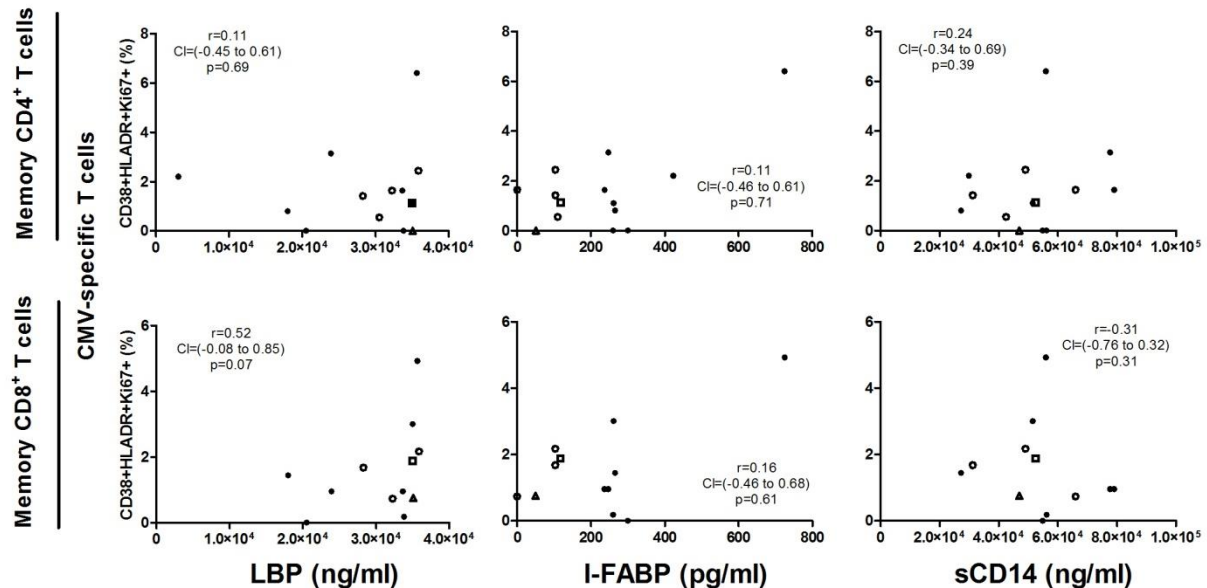


Figure N4: The relationship between plasma markers of microbial translocation with antigen-specific memory T cell activation at 3 months post-infection. (A) Correlations between plasma LBP, I-FABP and sCD14 levels with proportions of activated Gag-specific memory CD4⁺ and CD8⁺ T cells in early HIV-1 infected individuals. (B) Correlations between plasma LBP, I-FABP and sCD14 levels with proportions of activated CMV-specific memory CD4⁺ and CD8⁺ T cells in early HIV-1 infected individuals. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

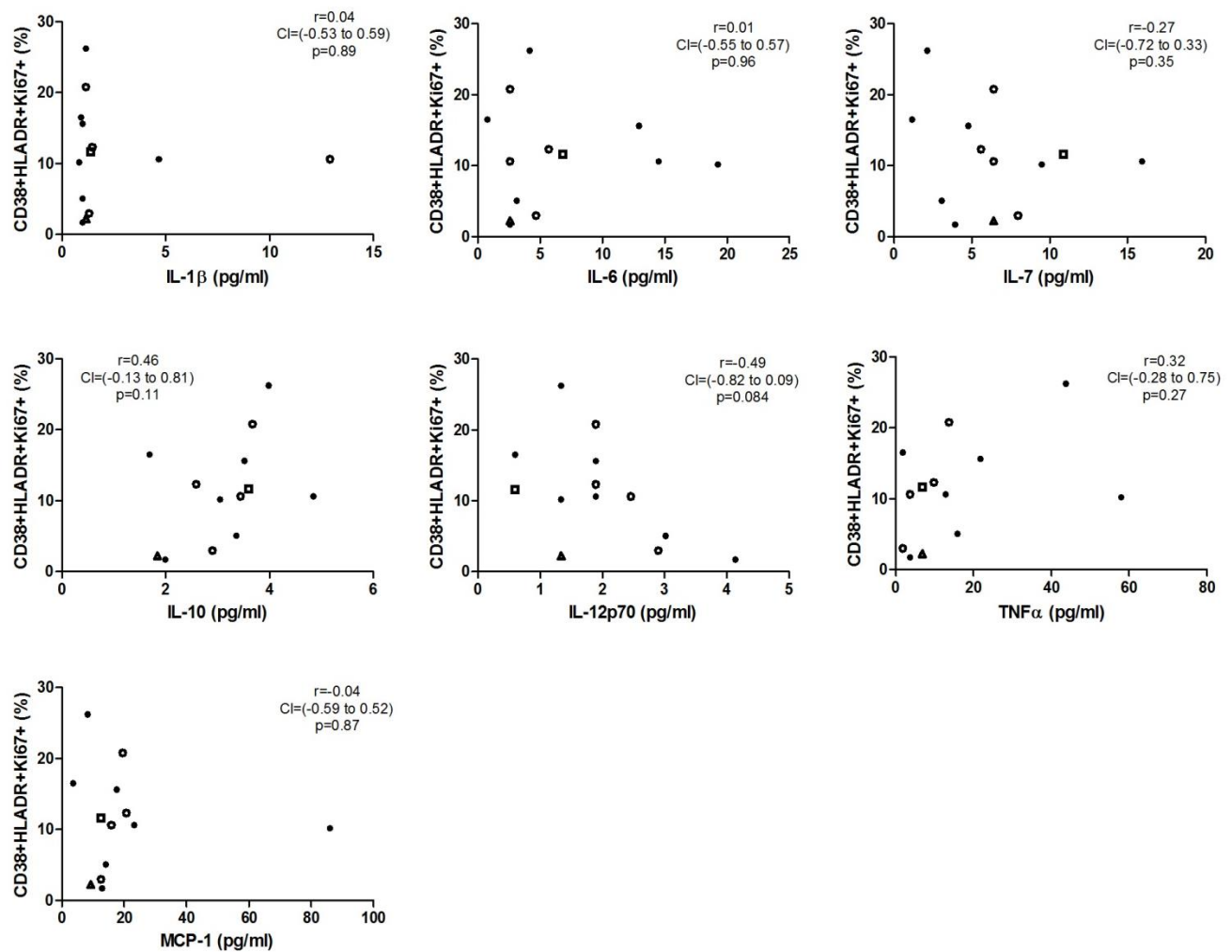


Figure N5: The relationship between plasma cytokines with activated Gag-specific memory CD4⁺ T cells activation at 3 months post-infection. (A) Correlations between plasma levels of IL-1β, IL-6, IL-7, IL-10, IL-12(p70), TNFα and MCP-1 with proportions of activated Gag-specific memory CD4⁺ T cells in early HIV-1 infected individuals (n=14). Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

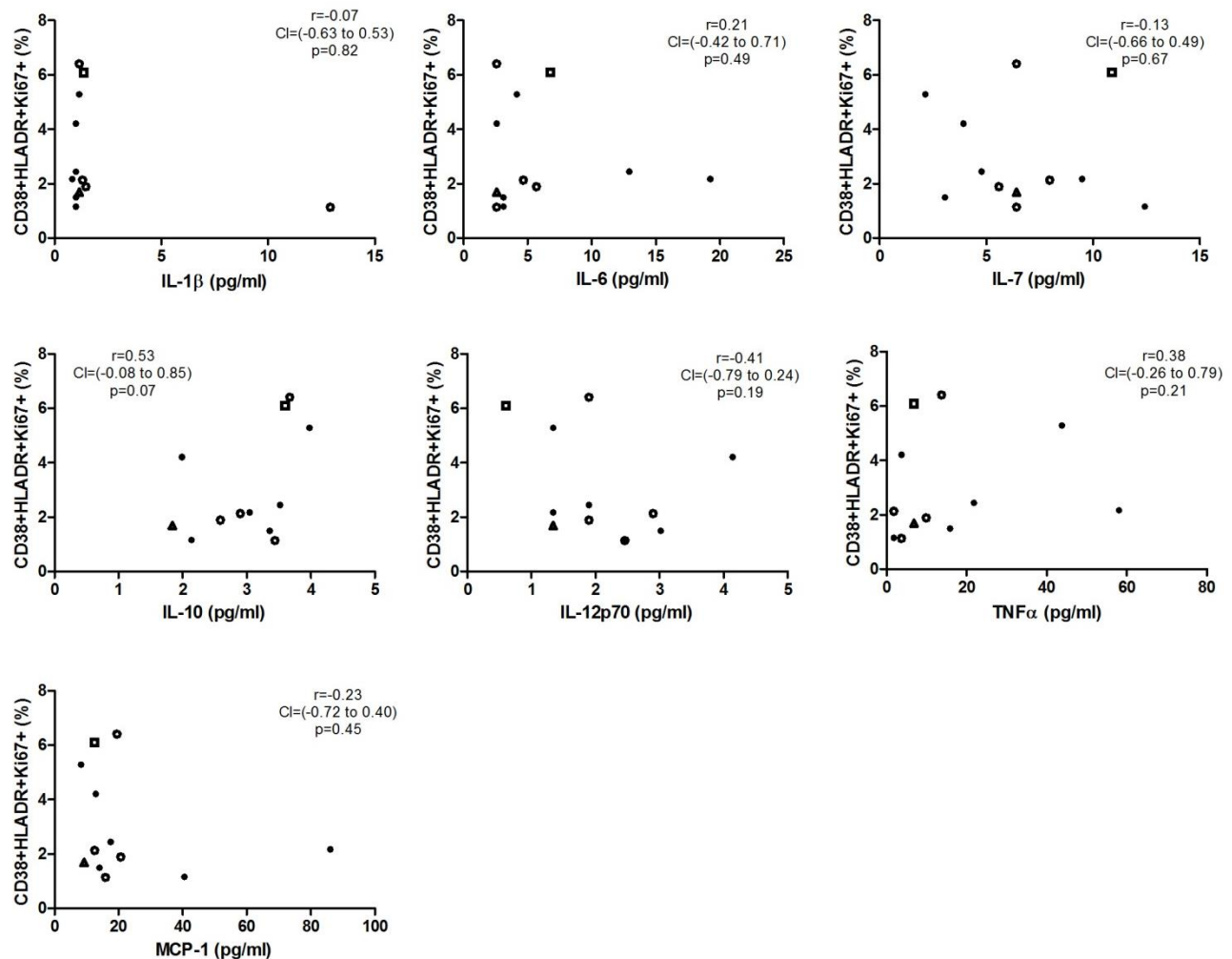
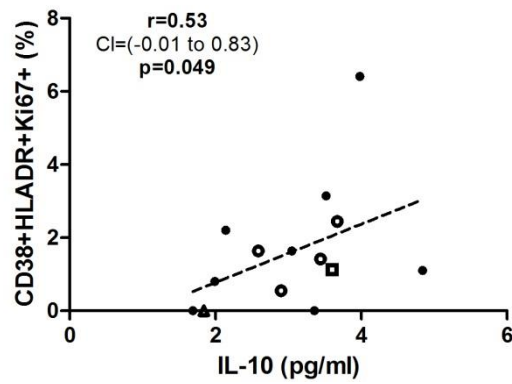


Figure N6: The relationship between plasma cytokines with activated Gag-specific memory CD8⁺ T cells activation at 3 months post-infection. (A) Correlations between plasma levels of IL-1β, IL-6, IL-7, IL-10, IL-12(p70), TNFα and MCP-1 with proportions of activated Gag-specific memory CD8⁺ T cells in early HIV-1 infected individuals (n=14). Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

CMV-specific memory CD4⁺ T cells



CMV-specific memory CD8⁺ T cells

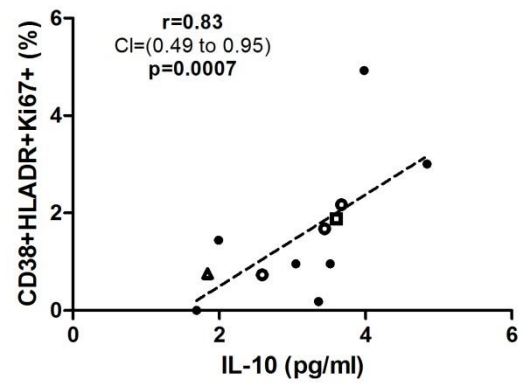
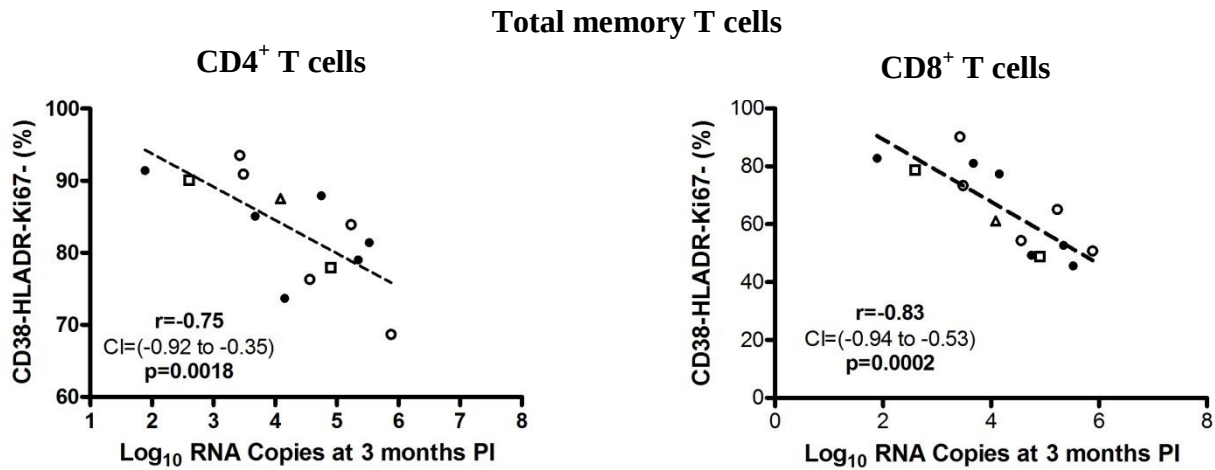


Figure N7: The relationship between memory T cell activation with IL-10 in HIV-infected individuals.

Correlations between the plasma levels of IL-10 with the proportions of activated CMV-specific memory CD4⁺ and CD8⁺ T cells (n=14). Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation.

APPENDIX O: NON-ACTIVATED MEMORY CELLS

A



B

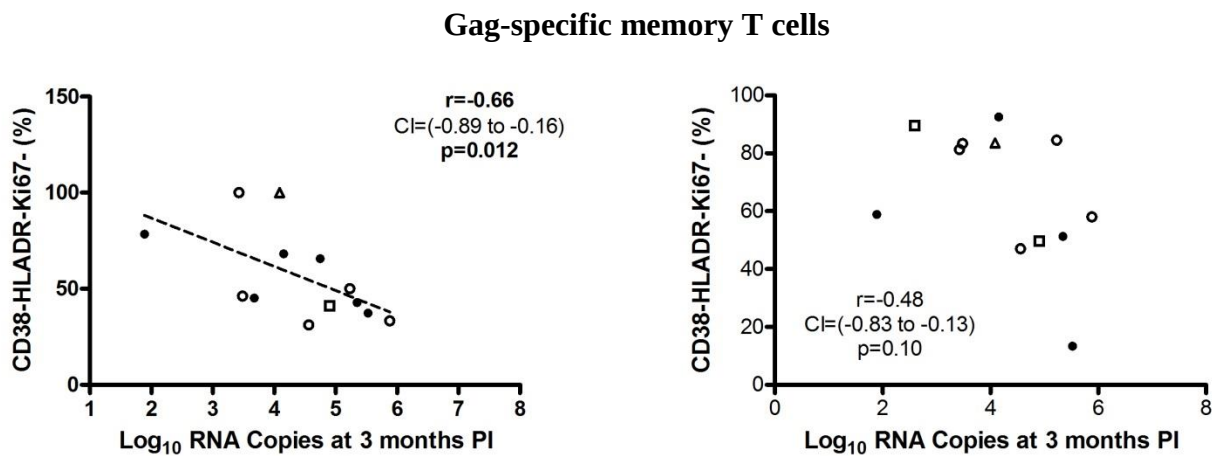


Figure O1: The relationship between non-activated total and Gag-specific memory T cells with viraemia at 3 months post-infection. (A) Correlation between non-activated total memory CD4⁺ and CD8⁺ cells with viraemia in early HIV infected individuals. (B) Correlation between non-activated Gag-specific memory CD4⁺ and CD8⁺ cells with viraemia in early HIV infected individuals. Statistical associations were performed by a two tailed nonparametric Spearman rank correlation.

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