Patterns and features of HIV-1-specific CD8+ T-cell responses during acute HIV-1 infection and their association with viral control

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Doris Duke Medical Research Institute
Nelson R Mandela School of Medicine

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Patterns and features of HIV-1-specific CD8+ T-cell responses during acute HIV-1 infection and their association with viral control

By

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Submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy (Immunology) in the School of Laboratory Medicine and Medical Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal

2015

DECLARATION

I, Mopo Radebe, declare that this is my original work except as acknowledged in the thesis and has not been submitted in any other form to another university. Most of the experiments were performed at the HIV Pathogenesis Programme (HPP) Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal. Some of the experiments were carried out at the Walker Laboratory at the The Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Boston, MA, USA. The work was supervised by Prof Thumbi Ndung'u (University of KwaZulu-Natal).

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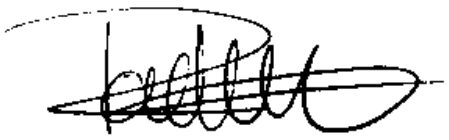
Signed:  Date: 25 February 2015

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PUBLICATIONS

First Author Publications:

1. Radebe M, Nair K, Chonco F, Bishop K, Wright JK, van der Stok M, Bassett IV, Mncube Z, Altfeld M, Walker BD and Ndung'u T. Limited immunogenicity of HIV CD8+ T-cell epitopes in acute clade C virus infection. Journal of Infectious Diseases 2011.

2. Radebe M, Kamini G, Mokgoro M, Ndlhovu Z, van der Stok M, Mkhize L, Walker BD and Ndung'u T. Broad and persistent Gag-specific CD8+ T-cell responses are associated with viral control but rarely drive viral escape during primary HIV-1 infection. *Manuscript submitted.*

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1. Wright JK, Novitsky V, Brockman MA, Brumme ZL, Brumme CJ, Carlson JM, Heckerman D, Wang B, Losina E, **Leshwedi M,** van der Stok M, Maphumulo L, Mkhwanazi N, Chonco F, Goulder PJ, Essex M, Walker BD, Ndung'u T.

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1. Sande's HIV/ AIDS Medicine, 2nd Edition Medical Management of AIDS 2012. Edited by Paul Volberding, Warner Greene, Joep M. A. Lange, MD, Joel E. Gallant, MD, MPH and Nelson Sewankambo (Chapter 4). Release Date: 25 May 2012.

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1. Radebe M, Zaza Ndlhovu, Mmamekwa Mokgoro, Mary van der Stok, Lungile Maphumulo, Walker BD and Ndung'u T. Longitudinal Analysis of the Human Immunodeficiency Virus-1 Specific T-Cell Response during primary HIV-1 infection and association with viral control. AIDS Vaccine 2012, October 7-10, Poster number: P01.02. Barcelona, Spain.

2. Radebe M, Zaza Ndlhovu, Mmamekwa Mokgoro, Mary van der Stok, Lungile Maphumulo, Walker BD and Ndung'u T. Longitudinal Assessment of HIV-1-Specific T-Cell Responses Generated During Acute Subtype C HIV-1 Infection and Associations with Viral Set Point. AIDS Vaccine 2012, September 9-12, Poster number: 247795. Boston, Massachusetts.

3. Radebe M, Nair K, Chonco F, Bishop K, Wright J, Stok M, Mncube Z, Altfeld M, Walker B, Ndung'u T. Limited Immunogenicity of HIV CD81 T-Cell Epitopes in Acute Clade C Virus Infection. AIDS Vaccine 2011, September 12-15, Oral Presentation. Bangkok, Thailand.

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5. Radebe M, Nair K, Chonco F, Bishop K, Altfeld M, Walker B, Ndung'u T. Immunodominance of Human Immunodeficiency Virus-Specific CD8+ T-cell Responses during acute HIV-1 Clade C Infection. Poster Presentation: HIV Acute Infection Meeting, September 22 – 23, 2009, Boston, MA.

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"Alas! What was there of mine in that work? Say not that it was I, but God in me. I labored – yet not I, but by the grace of God which was in me. Away with all merit, either in me or any other man whatsoever."

-Phillp de Mornay (1549-1623)

ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribonucleic acid
ELISPOT	Enzyme-linked immunosorbent spot
IFN-γ	Interferon gamma
IL-2	Interleukin 2
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12p70	Interleukin 12p70
Gag	Group-specific antigen
GM-CSF	granulocyte macrophage-colony stimulating
HIV	Human immunodeficiency virus
HAART	Highly active anti-retroviral treatment
HLA	Humal leukocyte antigen
FMO	Flourescence minus one
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1-alpha
MIP-1β	Macrophage inflammatory protein-1-beta
NEF	Negative regulatory factor
PBMC	Peripheral mononuclear cells
PD-1	Programmed Death 1

Rev	Regulator of gene expression
RNA	Ribonucleic acid
TCR	T-cell receptor
Tat	Trans-activator of transcription
TNF-α	Tumor necrosis factor alpha
SIV	Simian immunodeficiency virus
Vif	Viral infectivity factor
Vpu	Viral protein U
Vpr	Viral protein R
WHO	World Health Organization

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ABSTRACT

Evidence suggests that CD8+ T-cells play a major role in the control of HIV-1 viremia and apply significant immune pressure on HIV-1 replication. However, the presence of virus-specific CD8+ T-cells in individuals with varying levels of viral control suggests that CD8+ T-cells may differ in their antiviral function or efficacy. The mechanisms underlying differences in the control of viremia, particularly the reasons why particular individuals experience more effective acute viremia resolution, which is a good correlate of the subsequent rate of disease progression, are still not well understood. In order to uncover some of the features of CD8+ T-cell subsets responsible for the control of HIV replication, particularly during the critical early infection phase, we investigated the patterns and features of HIV-1-specific CD8+ T-cell responses during acute and primary HIV-1 infection and their association with viral control. We also sought to determine the impact of acute phase immune activation on the acute HIV-1-specific CD8+ T-cell response and on disease progression. We hypothesized that protein-specific and epitope-specific immunodominance patterns during the first 12 weeks of HIV-1 infection are associated with subsequent disease progression.

Our data show the presence of HIV-1 specific CD8+ T-cells with limited breadth during acute HIV-1 infection and also demonstrate that the magnitude and breadth of interferon gamma (IFN-γ) ELISPOT assay responses measured within 12 weeks post-infection are unrelated to the course of disease in the first year of infection. During the first weeks of infection Nef protein was most frequently recognized by T-

cells and was the target for the earliest response. Although initially subdominant, there was a broadening of the Gag-specific T-cell immune response such that these responses became immunodominant by one year post infection. The broadening and preservation of early Gag–specific T-cell responses during the follow up period was associated with better control of viremia and lower viral load set point. Although many of the acute/early HIV-1-specific IFN-γ enzyme linked immunospot assay (ELISPOT) CD8+ T-cell responses targeting Gag and Pol persisted, the majority of acute and early T-cell responses targeting Env, Nef and other regulatory proteins waxed and waned over time and could not be detected at the last time point evaluated. Some of the early T-cell responses which where no longer detectable when using overnight ELISPOT assay were detectable when PBMCs were stimulated with corresponding peptides and cultured for 10 days before measuring IFN-γ secretion via the ELISPOT assay. The presence of these cultured ELISPOT central memory type T-cell responses targeting epitopes in Pol, Env, Nef, Regulatory and Accessory proteins were not significantly associated with viral set point. However, cultured ELISPOT Gag-specific responses correlated with low plasma viremia, thus further providing evidence for the favourable role of Gag-specific T-cell responses in the control of viral replication. We also show that three cytokines IL-10, IP-10 and IL-12 were associated with changes in viral load set point and/or CD4+ T-cell dynamics during the first year of HIV-1 infection. Interestingly, the activation of the PD-1 inhibitory pathway in acute HIV-1 infection was associated with a slower disease progression.

The data indicate that induction and preservation of Gag-specific effector and central memory type CD8+ T-cell responses is required for the maintenance of low viral load levels in primary infection. Our study suggests that an early and broadly directed Gag-specific CD8+ T-cell response in acute infection may augment early control, and provide a rational goal for both prophylactic and therapeutic vaccination. The role of these plasma and T-cell markers of immune activation and dysfunction as early independent predictors of rapid disease progression was detected despite the relatively small number of subjects evaluated, supporting the predictive strength of these markers. In addition to or in combination with canonical parameters, early biomarkers, which are indicative of early immune dysfunction and are also predictive of subsequent HIV disease prognosis, may inform approaches for evaluating the ability of therapeutic HIV vaccines to control HIV infection and may be indicative of pathways that may be manipulated to achieve better viral control in natural infection or following vaccination.

ETHICS

The research reported study is registered with the Biomedical Research Ethics Committee of the University of KwaZulu-Natal as a subsidiary study to the parent study entitled Characterisation of the evolution of adaptive immune responses in acute HIV clade C virus infection *(BREC reference number: E036/06)*.

The Postgraduate Education Committee at the Nelson R Mandela School of Medicine, Faculty of Health Sciences, granted approval towards studying for a PhD degree.

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Outline of Thesis

Chapter 1 provides an overview of the HIV epidemic, acute and primary HIV-1 infection and the role of the innate and adaptive immune responses in the control of HIV-1 infection.

Chapter 2 gives a detailed overview of CD8+ T-cell responses, the magnitude and breadth of these responses and their targets and immunodominance patterns in 20 HIV-1 subtype C infected, antiretroviral naive subjects from 4 weeks up to 18 weeks post infection.

Chapter 3 provides a description of evolving breadth of CD8+ T-cell responses during the first year HIV-1 of infection and the association of these changes with viral load. The role of persistent immune responses and central memory-type CD8+ T-cells in viral control is also highlighted.

Chapter 4 provides evidence on the role of T-cell and plasma immune markers of activation and dysfunction in predicting disease progression.

Chapter 5 gives an overview of the study findings in relation the current body of knowledge on the immune response to HIV. Also highlighted is what we know and what we should know, in order to facilitate the development of an efficacious HIV vaccine.

Chapter 1 : The Immune Response to HIV-1

1. HIV and AIDS Pandemic:

1.1. The Global Perspective

In the more than three decades since the first clinical case of AIDS was identified, over 70 million people have been infected with the human immunodeficiency virus type 1 (HIV-1) and 35 million people have died of HIV-1-related illnesses (UNAIDS, 2013). At the end of 2012, it was estimated that there were 35.3 million (32.2–38.8 million) people living with HIV. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 20 adults (4.9%) living with HIV and accounting for 69% of the people living with HIV globally (Figure 1.1) and in this region women account for at least 58% of infections (UNAIDS, 2012, UNAIDS, 2013).

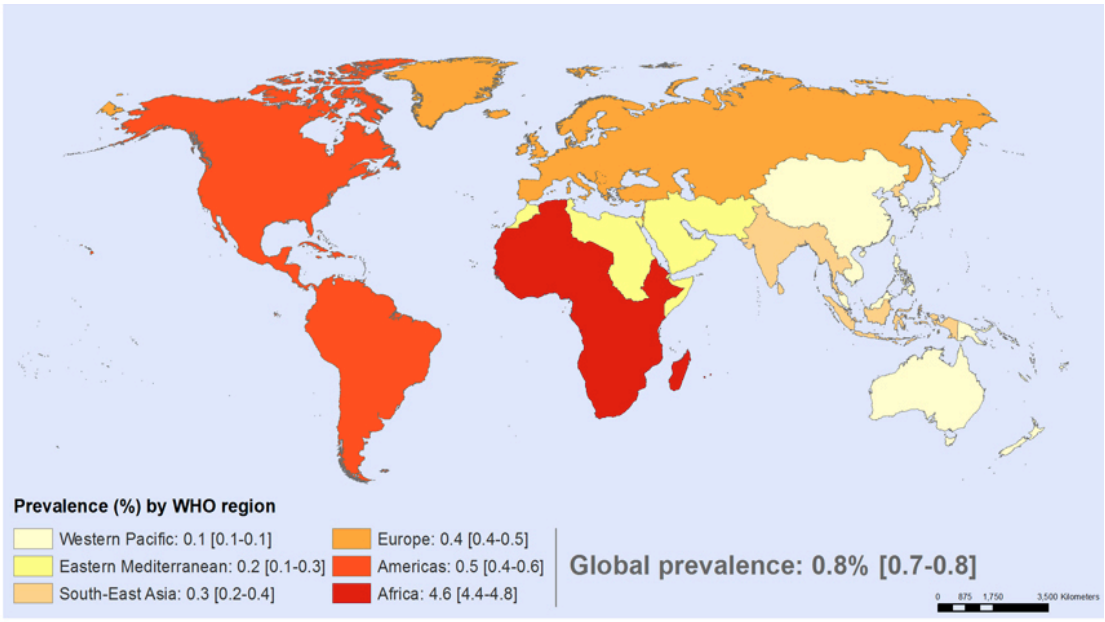


Figure 1.1. Global view of the estimated number of adults living with HIV/AIDS (according to region) in 2012 (WHO, 2013).

1.2. HIV and AIDS: South Africa

Within sub-Saharan Africa, South Africa with 5.26 million individuals infected with HIV is the country with the world's largest population of people living with HIV (UNAIDS, 2013). The estimated overall HIV prevalence rate is approximately 10%, with 16% of adults aged 15–49 years and 17% of South African women in their reproductive ages HIV infected (Stats SA., 2013). However, within the country, differences in HIV prevalence vary widely by region and risk group.

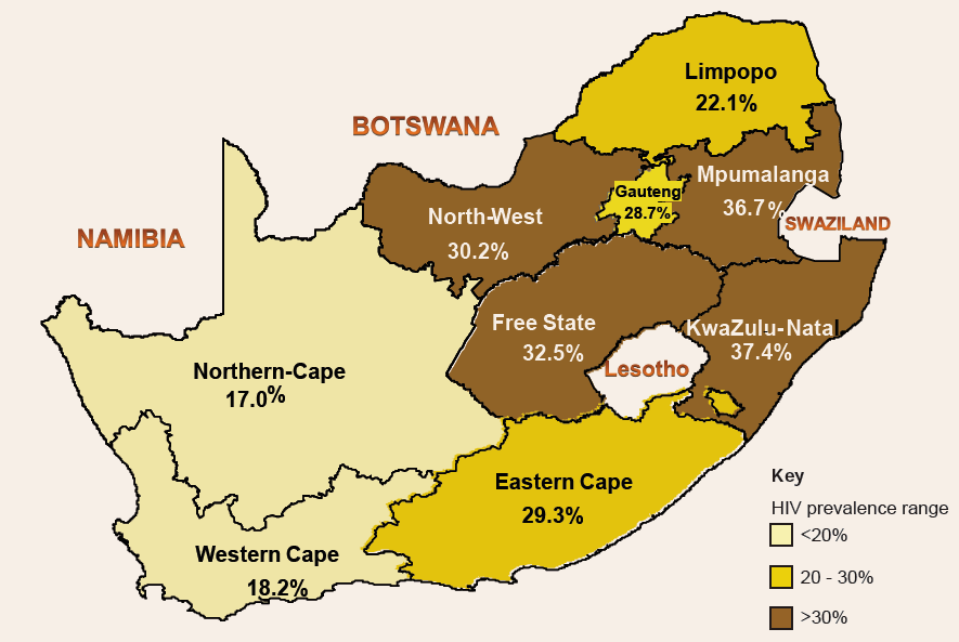


Figure 1.2. HIV prevalence distribution by province in South Africa in 2011 (Stats SA., 2013).

According to the 2011 National Antenatal Sentinel HIV & Syphilis Prevalence Survey, an estimated 29.5% of the antenatal population is HIV positive with the province of KwaZulu-Natal experiencing the most severe HIV epidemic with the highest HIV

prevalence of 37.4% in the antenatal population (South African Department of Health., 2011). Mpumalanga follows it closely with overall prevalence rates of 36.7%, and the Free State at 32.5% (Figure 1.2). The Northern Cape and Western Cape provinces have the lowest HIV prevalence rates of below 20% (Stats SA., 2013).

2. Therapeutic strategies for HIV

Although declines in new HIV infections have been reported since 2011, the burden of disease remains incredibly significant. So far the best results at curbing the epidemic have been the development of antiretroviral drugs (ARVs) that inhibit crucial HIV-1 functions. Whilst ARV therapy has significantly reduced the toll of AIDS related deaths, a significant gap still exists between those on therapy and those who need it. In South Africa, only 2 million of the 5.26 million individuals infected with HIV are on therapy (UNAIDS, 2013). Furthermore, the unique ability of HIV-1 to mutate and adapt (Korber et al., 2001, Wain-Hobson, 1995, Ndung'u and Weiss, 2012) also requires the use of multiple drug treatments that are often limited in their use mainly because of their side effects and cost (Maenza and Flexner, 1998).

Arresting the spread of the HIV/AIDS epidemic will require a multi-pronged approach, which consists of efficient HIV-1 prevention strategies, improved ARV therapy access for individuals infected with HIV-1, and the development of an efficient vaccine to protect people from HIV-1 infection and/or attenuate disease progression in those that are already infected. In order to facilitate the design of successful vaccination regimens that elicit specific immune responses, an in-depth

immunological knowledge as well as an enhanced understanding of host-virus interaction is crucial (Virgin and Walker, 2010).

Table 1.1. HIV vaccine products tested in clinical efficacy trials to date (U.S. Department of Health and Human Services, 2013).

1981	HIV was identified as the cause of AIDS.
1987	The first HIV vaccine clinical trial opened at the National Institutes of Health (NIH) Clinical Center. The gp160 subunit vaccine showed no serious adverse effects.
1992	NIAID launched the first Phase II HIV vaccine clinical trial.
1998	First large scale vaccine trial began: VaxGen initiates Phase III trial of AIDSVAX in North America and The Netherlands.
1999	NIAID begins first African preventive HIV vaccine trial in Uganda. First large scale vaccine trial in a developing country began: VaxGen initiates Phase III trial of AIDSVAX in Thailand.
2003	U.S. and Royal Thai governments jointly initiated RV144, a Phase III trial to evaluate a novel HIV vaccine strategy commonly referred to as "prime-boost."
2004	VaxGen candidate failed in Phase III trials.
2007	NIAID halted the Phase II Step and Phambili studies due to safety concerns.
2009	Phase II HVTN 505 study initiated to evaluate a "prime-boost" vaccine regimen developed by the VRC.
2009	Results of Phase III Thai Trial (RV144) show vaccine combination is first to demonstrate modest preventive effect in humans.
2010	Two potent antibodies that prevent most strains of HIV identified by the VRC (VRC01 and VRC02). Establishment of Pox-Protein Public-Private Partnership (P5)
2012	Additional analyses of samples from RV144 provide insight about what type of immune response may be needed for an effective vaccine.
2013	HVTN 505 immunizations stopped due to lack of efficacy.

2.1. The STEP and Phambili trials

Soon after HIV-1 was isolated and confirmed as the cause of AIDS, it was widely expected that an effective vaccine would be developed in a few years. Over thirty years later, we are still struggling to develop an effective vaccine. As shown in Table 1.1, decades of effort have been spent, and continue to be made, toward developing an efficacious HIV vaccine (U.S. Department of Health and Human Services, 2013). Two Phase IIb trials of a vaccine candidate created by the pharmaceutical company Merck were halted in September 2007. The STEP and Phambili clinical trials evaluated Merck's trivalent Ad5-HIV-1 vaccine in high-risk MSM and heterosexual men and women in the Americas and Australia (STEP) and heterosexual men and women in South Africa (Phambili). Despite inducing T-cell responses of similar magnitude and breadth to those observed in earlier trials, the vaccine failed to prevent infection or impact on early viraemia and was prematurely terminated in 2007 on grounds of futility (Buchbinder et al., 2008, McElrath et al., 2008). The measures of immunogenicity in phase I/II trials of the STEP trial were shown to be ambiguous since vaccinees who acquired HIV-1 infection showed a similar magnitude of CD8+ T-cell responses to vaccination in comparisons to those who were not vaccinated. Furthermore, the breadth of responses in vaccinees was particularly narrow with CD8+ T-cell responses targeting a median of one epitope per protein and with a bias towards less conserved epitopes (McElrath et al., 2008, Li et al., 2011).

2.2. The Thai Phase III HIV vaccine clinical trial

The Thai HIV vaccine efficacy trial (2003-2009), known as RV144, tested the "prime-boost" combination of two vaccines: ALVAC-HIV recombinant canarypox vaccine (the prime) and AIDSVAX gp120 B/E vaccine (the boost). The vaccine combination was based on HIV strains that commonly circulate in Thailand. In 2009, the RV144 trial conducted in Thailand revealed an estimated efficacy of 31% with the ALVAC vCP1521-AIDSVAX B/E vaccine regimen (Rerks-Ngarm et al., 2009). Exploring the immune correlates of risk for HIV-1 infection in this phase IIb trial led to the identification of two immune correlates of risk, IgG antibodies to HIV-1 Env V1-V2 and high-titre anti-Env IgA antibodies (Haynes et al., 2012). Initially, IgG antibodies to HIV-1 Env V1-V2 were shown to correlate inversely with infection risk, suggesting that antibodies binding to the V1V2 Env region were involved somewhat in preventing infection (Liao et al., 2013, Haynes et al., 2012). High titer anti-Env IgA antibodies were shown to be directly correlated with infection risk, moreover, in the presence of low-titer anti-Env IgA antibodies, antibody-dependenT-cell-mediated cytotoxicity (ADCC) levels inversely correlated with infection risk suggesting that anti-Env IgA antibodies could lessen the protective effects of IgG antibodies (Haynes et al., 2012, Tomaras et al., 2013).

Although attempts to produce a vaccine that prevents HIV infection have not been wholly successful to date, these HIV vaccine clinical trials have provided many valuable lessons towards the generation of promising HIV vaccine regimens. The clues gathered from the failed clinical trials and the partially successful RV144 trial, in addition to the promising ongoing preclinical vaccine data, offer a unique

opportunity to build on efforts towards an effective HIV vaccine (Spearman, 2003, Day and Kublin, 2013).

3. Origin and Structure of HIV

3.1. HIV-1 types

HIV belongs to a large family of ribonucleic acid (RNA) viruses and is a member of the genetically related Lentivirus genus of the Retroviridae family. Infection with lentiviruses is characterized mainly by immunosuppression with a long period of clinical latency following infection before manifestations of illness become apparent (Fauci, 1993). It has long been suspected that HIV-1 is of chimpanzee origin derived from the simian immunodeficiency virus of the *Pan troglodytes troglodytes* subspecies of chimpanzee (SIVcpz) (Gao et al., 1999). In 2006, extracts of SIVcpz were found in faecal samples collected from wild chimpanzees, thus confirming that they were indeed a reservoir of SIVcpz (Keele et al., 2006). It is now well established that SIVs from chimpanzees *(Pan troglodytes troglodytes)* and gorillas *(Gorilla gorilla gorilla)* in West central Africa are the progenitors of HIV-1 (Keele et al., 2006, Van Heuverswyn et al., 2006, Sharp and Hahn, 2011).

The HIV isolates are currently grouped into two types, HIV type 1 (HIV-1), which is the main causative agent of AIDS globally, and HIV type 2 (HIV-2), which is restricted to some regions of Western and Central Africa (Clavel et al., 1986, Sharp and Hahn, 2011, Whittle et al., 1994). HIV-1 comprises 3 distinct virus groups, termed M (the Main group), O (the Outlier group) and N (the Non-M, Non-O group). Group M was

the first to be discovered and is the pandemic form of HIV-1 that has infected millions of people and is found in nearly every country on the globe (Sharp and Hahn, 2011). It consists of at least nine genetically distinct subtypes (or clades), denoted subtype A, B, C, D, F, G, H, J and K (Perrin et al., 2003, Robertson et al., 2000). Subtype B is the dominant form in Europe, the Americas, Japan, and Australia, whilst subtype C is dominant in southern Africa, eastern Africa, India, and parts of China (Taylor and Hammer, 2008). Group O is less prevalent than group M and represents less than 1% of global HIV-1 infections restricted mainly to Cameroon, Gabon, and neighbouring countries (Mauclere et al., 1997, De Leys et al., 1990). Group N infections have been documented in 13 individuals, all from Cameroon (Simon et al., 1998, Vallari et al., 2010, Sharp and Hahn, 2011) and two cases of group P have been reported also in individuals from Cameroon (Plantier et al., 2009).

3.2. Structure of HIV-1

The genome of HIV, similar to retroviruses in general, contains three major genes *gag*, *pol*, and *env* (Figure 1.3). The *gag* gene (group-specific-antigen) encodes the core structural proteins (p24, p7, p6) and matrix (p17) and the *env* gene encodes the viral envelope glycoproteins gp120 and gp41, which are used for cell surface receptor recognition. The *pol* gene encodes for enzymes crucial for viral replication, namely reverse transcriptase which converts viral RNA into DNA, the integrase which incorporates the viral DNA into host chromosomal DNA (the provirus) and the

protease which is responsible for cleaving large Gag and Pol protein precursors into their components (Sierra et al., 2005, Sundquist and Krausslich, 2012).

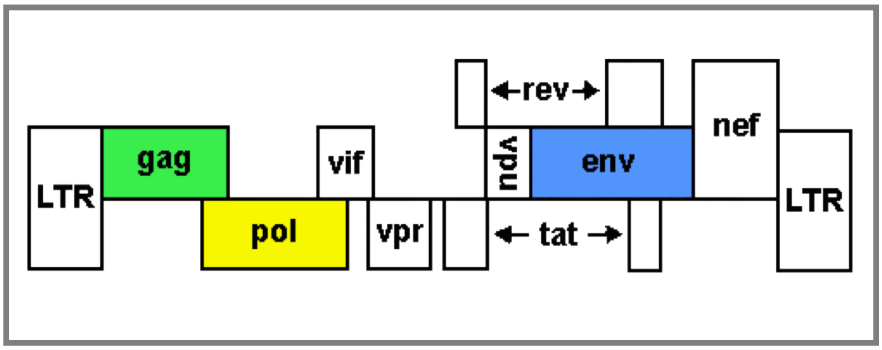


Figure 1.3. The viral genome for HIV-1 consists of nine open reading frames: *gag, pol and env*. *Gag* encodes the proteins that form the viral capsid. *Env* encodes the envelope surface proteins. *Pol* encodes the three catalytic enzymes: Reverse Transcriptase (RT), Integrase (IN), and the Protease (PR). The remaining six open reading frames encode accessory proteins: Vif, Vpr, Nef, Tat, Rev, and Vpu.

A defining feature of retroviruses is their ability to assemble into particles that are able to leave producer cells and spread infection to susceptible cells. Virion morphogenesis can be divided into three stages, 1) assembly: the virion is created and vital components are packaged; 2) budding: wherein the virion crosses the plasma membrane and acquires its lipid envelope; and 3) maturation: the virion changes structure and becomes infectious (Figure 1.4). The Gag polyprotein and its proteolytic maturation products, which function as the major structural proteins of the virus, coordinate these stages (Sundquist and Krausslich, 2012).

Six virally regulatory/accessory genes carried by HIV-1 in its 9,2 kB RNA include *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* and these contribute to the genetic complexity of the virus. *Tat* and *rev* stimulate the transcription of proviral HIV-1 DNA into RNA, enhance the

transportation of HIV RNA from the nucleus to the cytoplasm and are important for translation (Fanales-Belasio et al., 2010, Emerman and Malim, 1998).

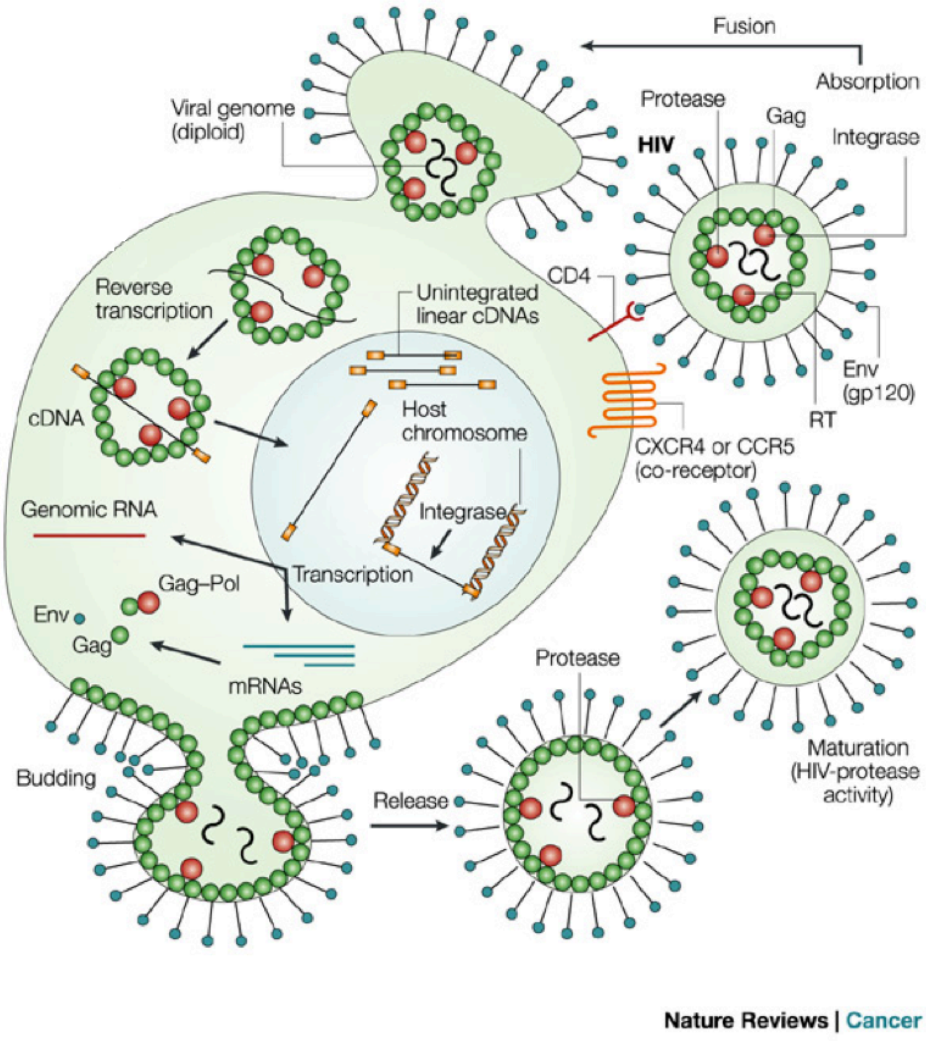


Figure 1.4. Representation of HIV's lifecycle: The HIV lifecycle begins with the interaction of a virus particle with a receptor on the cell's surface, resulting in the fusion of the viral and cellular membranes and transfer of the viral contents into the cell. The viral RNA genome is reverse transcribed and produces a DNA copy which is imported into the nucleus. Within the nucleus, the viral DNA is integrated into the hosT-cell genome and is transcribed from within the host genome, then exported from the nucleus. The translation of the viral proteins by hosT-cell machinery begins and the major structural protein, Gag, is transported to the plasma membrane where it directs assembly of the viral coat and incorporates other viral proteins and the viral genome. The virus buds through the cell membrane, and Gag is cleaved by the viral protease leading to a structural change of the virion into its mature infectious form, which is capable of fusing with a new susceptible cell (figure adapted from Monini et al. (2004), with permission from the Nature Publishing Group).

Nef has been shown to have a number of functions such as CD4 and HLA class I downregulation (Schwartz et al., 1996, Greenberg et al., 1998), activation of p21-activated protein kinase (Pak2) (Foster and Garcia, 2007, Foster and Garcia, 2008) and enhancement of virion infectivity (Miller et al., 1994). The downregulation of CD4 and MHC class I molecules from the surface of HIV-1-infected cells represents an important escape mechanism for the virus in evading recognition and attack mediated by cytotoxic CD8+ T-cells and to also avoid recognition by CD4+ T-cells (Foster and Garcia, 2007, Foster and Garcia, 2008). Downregulation of CD4+ T-cells is thought to be primarily for enhancing virion release, virus infectivity and this may also be a mechanism to avoid antibody mediated cytotoxicity (Veillette et al., 2014). The virion infectivity factor (Vif) suppresses the antiretroviral cellular enzyme APOBEC3G and other cytidine deaminases in the cytoplasm (Marin et al., 2003) and is essential for efficient viral replication in primary cells (Desrosiers et al., 1998). The virion-associated protein (Vpr) has the ability to delay or arrest infected cells in the G2 phase of the cell cycle (Fletcher et al., 1996), facilitates infection of macrophages (Eckstein et al., 2001) and activates HIV transcription (Felzien et al., 1998). By counteracting host restriction factors (particularly Tetherin) (Neil et al., 2008), Vpu enhances efficient release of virions from infected cells (Varthakavi et al., 2003) and downregulates the CD4 molecules during the late stages of HIV-1 infection (Levesque et al., 2003).

3.3. Transmission of HIV-1

The most common mode of HIV-1 infection is sexual transmission via the genital mucosa or rectal mucosa (Cohen et al., 1997, Royce et al., 1997). Upon sexual transmission, HIV-1 infects Langerhans cells and dendritic cells that reside in the submucosal tissues (Spira et al., 1996) as these cells express CD4 and CCR5 molecules on their surfaces, which serve to facilitate viral entry (Elsaesser et al., 2009). The lamina propria of the human vagina also contains many CD4+ T-cells, which express relatively high levels of CCR5 compared with T-cells found in peripheral blood (Hladik and Hope, 2009, Elsaesser et al., 2009, Spira et al., 1996). Entry of HIV-1 into host T-cells depends largely upon the interaction of the viral glycoprotein envelope gp120 subunit with cell surface CD4 (Dalgleish et al., 1984). Upon CD4 binding, subsequent conformational changes of gp120 trigger interactions of the Env with HIV-1 co-receptors CCR5 or CXCR4 (Moore, 1997). As a result, this binding to co-receptors exposes the Env gp41 transmembrane subunit and promotes fusion of viral and cellular membranes (Furuta et al., 1998).

Findings from studies of macaques infected intravaginally with SIV show that virus replication is initially confined to the mucosal infection site where small foci of infection are established locally, some of which expand and disseminate virus to the draining lymph node and blood (Haase, 2010). Single-genome amplification and sequencing of the first detectable virus has shown that approximately 80% of mucosally transmitted HIV-1 subtype B and C infections originate from single viruses (Keele et al., 2008) and these primary founder viruses have been shown to infect

CD4+ T-cells with greater efficiency than they can infect monocytes and macrophages (Salazar-Gonzalez et al., 2009). Productive infection has also been shown to arise from a single infecting virus in rhesus macaques inoculated intra-rectally with a complex SIV quasispecies thus further supporting the use of SIV infection of rhesus macaques as a model for HIV-1 transmission and vaccine studies (Keele et al., 2009, McMichael et al., 2010).

4. The acute phase of HIV-1 infection

4.1. Viral dynamics

Acute HIV-1 infection is the phase of HIV disease immediately after infection during which the initial burst of viremia in newly infected patients occurs whilst anti-HIV antibodies are undetectable. After transmission of the virus, there is an interval, known as the eclipse phase, which is the time before viral RNA becomes detectable in the plasma (Keele et al., 2008, Lee et al., 2009). The length of the eclipse phase before virus dissemination takes place may range from 5–6 days to several weeks, with exposure to lower doses of the virus being associated with longer eclipse periods (Keele et al., 2008, Lee et al., 2009, Liu et al., 2010, Stone et al., 2010).

Following infection and the replication of the virus in the mucosa, virus and/or virus infected cells are transported to draining lymph nodes, where they encounter activated CD4+CCR5+ T-cells and thus facilitating further viral amplification in these cells (Haase, 2010). Subsequently, the virus spreads via the blood to the other lymphoid tissues reaching peak plasma levels, usually more than a million RNA

copies per ml of blood at 21–28 days after HIV-1 infection in humans (McMichael et al., 2010).

4.2. The acute retroviral syndrome

The clinical effects of HIV-1 infection are diverse, ranging from an asymptomatic seroconversion to a severe illness that may require hospitalization of the patient. Acute HIV infection often remains undiagnosed partly because the symptoms are protean and are also typical of other viral infections like influenza or mononucleosis (Tindall et al., 1988, Niu et al., 1993). The onset of illness is usually between 2 and 6 weeks after viral transmission and most patients experience fever, disseminated lymphadenopathy, often associated with headache, myalgias, anorexia, rash, and/or diarrhoea (Busch, 1994). A more severe clinical syndrome during acute HIV infection has been associated with rapid HIV disease progression (Pedersen et al., 1989).

4.3. Staging of acute HIV-1 infection

Analysis of samples obtained from individuals during early HIV-1 infection has revealed that the first few weeks after infection can be divided into clinical stages that are defined by a sequential gain in positive HIV-1 clinical diagnostic assays (viral RNA measured by PCR, p24 and p31 viral antigens and HIV-1-specific antibodies measured by enzyme-linked immunosorbent assay (ELISA) and HIV-1-specific antibodies detected by Western blot) (Fiebig et al., 2003). Based on this sequential gain in positivity of these HIV-1 clinical diagnostic assays, patients can be categorized into Fiebig stages I–VI (Table 1.2).

Table 1.2. Laboratory stages of primary HIV infection based on the emergence of viral markers in seroconverting plasma donors (adapted from Fiebig et al., 2003).

Stage	Marker					Duration in days	
	RNA	P24 Antigen	Not sensitive 2nd generation EIA	Sensitive 3rd generation EIA	Western blot	Individual	Cumulative
I	+	–	–	–	–	5 (3.1, 8.1)	5 (3.1, 8.1)
II	+	+	–	–	–	5.3 (3.7, 7.7)	10.3 (7.1, 13.5)
III	+	+	–	+	–	3.2 (2.1, 4.8)	13.5 (10.0, 17.0)
IV	+	+/–	–	+	I	5.6 (3.8, 8.1)	19.1 (15.3, 22.9)
V	+	+/–	+/–	+	+ without p31 band	69.5 (39.7, 121.7)	88.6 (47.4, 129.8)
VI	+	+/–	+	+	+	Open-ended	Open-ended

Infected individuals progress from acute infection through to the early chronic stage of infection at the end of Fiebig stage V, approximately 100 days following infection, (Fiebig et al., 2003, McMichael et al., 2010) (Figure 1.5). At this time the plasma viral load begins to plateau and decreases to a steady level referred to as the viral set point. The mechanisms underlying the establishment of the viral set point are poorly understood, but may involve the interplay between host immune responses and viral replication. The viral load set point is an established predictor of HIV-1 disease progression and mortality where a high viral set point is a marker of rapid disease progression; the higher the set point, the more rapid the immunologic progression to disease (Mellors et al., 1997)

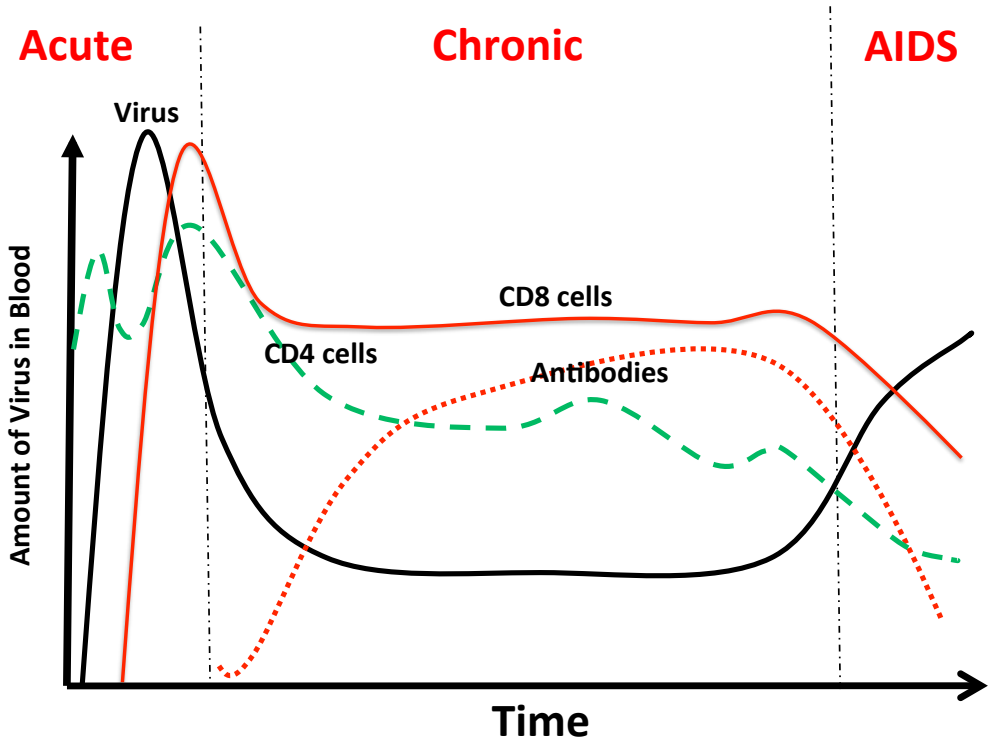


Figure 1.5. Schematic representation of the course of HIV-1 infection showing changes in viral load, antibodies, CD4 and CD8 T-cell counts in peripheral blood in untreated individuals.

Although several mechanisms such as the innate immune system, host factors, age, virulence of the infecting strain and other immunological factors are known to influence the rate of HIV disease progression, the adaptive immune response is thought to be the most critical component of the immune system in the control of HIV infection (Fauci, 1993, Pantaleo et al., 1993, Borrow et al., 1997, Borrow et al., 1994).

5. The innate immune response to HIV-1

5.1. Dendritic cells

The immune response to HIV-1 requires the successful activation of innate immunity, which stimulates the development of the subsequent adaptive immune response. The innate response triggered during acute HIV-1 infection is thought to be one of the key aspects of the immune system, which determines establishment of infection and the rate of subsequent disease progression (McMichael et al., 2010, Borrow and Bhardwaj, 2008, Borrow et al., 2010).

Dendritic cells (DCs) known as the sentinels of the immune system, are strategically located in skin, mucosal tissues (including oral and vaginal mucosal surfaces) and lymphoid tissues, and are proposed to be among the first cells that encounter HIV-1 during sexual transmission (Haase, 2005, Coleman et al., 2013). Through expression of a diverse array of receptors and signalling molecules, these professional antigen presenting cells play a critical role in the early host response to infection by acting as potent antigen-presenting cells and by producing cytokines that mediate direct effector and immunoregulatory functions which can stimulate adaptive immune responses and also mediate rapid antimicrobial effector functions (Banchereau et al., 2000, Borrow and Bhardwaj, 2008). Dendritic cells are highly efficient at stimulating HIV-specific T-cell responses as well as priming polyfunctional HIV-specific T-cell responses (Zarling et al., 1999, Colleton et al., 2009, Lubong Sabado et al., 2009, McMichael et al., 2010). However, during acute HIV-1 infection there is a rapid decline in the number of circulating DCs, mainly plasmacytoid DCs and this may be

the result of activation-induced cell death or the migration of activated DCs into lymph nodes (Malleret et al., 2008, Lore et al., 2002). In chronic HIV-1 infection, the depletion of pDCs has been associated with HIV disease progression and development of opportunistic infections (Soumelis et al., 2001).

5.2. Natural killer cells

Other key players in the innate immune system are natural killer (NK) cells, which contribute to innate defence against viral infections as they respond rapidly during the early events of HIV-1 infection. The classical NK cells are non-T-cell lymphocytes, lacking expression of T-cell receptors (Biron et al., 1999) as well as a small subset of T-lymphocytes expressing surface markers characteristic of both T-cells and NK (NKT) cells are now appreciated to form an important link between the innate and adaptive immune responses (Alter et al., 2007, Borrow and Bhardwaj, 2008). An increased percentage of NK cells has been observed in the blood during acute HIV-1 infection and longitudinal data also show that NK cell populations are expanded around the time of peak plasma viremia prior to seroconversion and the appearance of HIV-specific CD8+ T-cell responses (Alter et al., 2005, Alter et al., 2007). Because of their activation during acute HIV-1 infection and their heightened activity in HIV-exposed, seronegative individuals, it has been suggested that these cells may contribute directly or indirectly to control of primary viraemia and may also play a role in preventing the establishment of infection (Alter et al., 2005, Alter et al., 2007, Borrow and Bhardwaj, 2008, Titanji et al., 2008, Scott-Algara et al., 2003). NK cells may also inhibit HIV-1 replication and spread via lysis of virally infected cells or

production of antiviral cytokines (e.g. IFN-γ) and chemokines (Borrow and Bhardwaj, 2008). Although the significance of innate responses as determinants of the outcome of HIV-1 infection has been demonstrated, more work is still needed to understand how innate immune responses can be harnessed to combat HIV-1 (Borrow and Bhardwaj, 2008, McMichael et al., 2010).

5.3. Host restriction factors

As part of the innate immune response to HIV-1, cell-intrinsic factors also contribute in suppressing viral replication. These are known as restriction factors and/or intrinsic resistance factors, and they provide an early line of defence against infection as a part of, or even occurring before innate antiviral responses (Monajemi et al., 2012). The most widely recognized restriction factors, which restrict HIV-1 replication in target T-cells and provide the host with a pre-mobilized defence against retroviral infection are APOBEC3G (Sheehy et al., 2002), TRIM5α (Stremlau et al., 2004) and tetherin (Neil et al., 2008).

APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G) is a human enzyme encoded by the APOBEC3G gene belonging to the APOBEC super-family of proteins and has the ability to interfere with HIV replication (Monajemi et al., 2012). This family of proteins has been suggested to play an important role in innate anti-viral immunity by converting deoxycytidine (dC) to deoxyuridine (dU) in single stranded DNA (ssDNA) or RNA of human and viral genomes, most notable HIV, thereby affecting a variety of inhibiting proper viral replication (Conticello, 2008,

Monajemi et al., 2012). This protein is mainly expressed in CD4+ T-cells, macrophages, as wells as dendritic cells, however, expression may be induced by interferons in other tissues (Tanaka et al., 2006, Pion et al., 2006, Monajemi et al., 2012). APOBEC3G can interrupt dissemination of HIV by binding viral RNA, interfering with reverse transcription (cDNA), physically blocking reverse transcriptase and obstructing integration into the hosT-cell genome (Mbisa et al., 2007, Anderson and Hope, 2008). After the reverse transcription stage, incorporation of dU into the neo-synthesized minus strand DNA of the HIV genome has been shown to interfere with synthesis of the complementary plus strand (Klarmann et al., 2003, Monajemi et al., 2012). However, lentiviruses such as HIV have evolved several strategies, primarily in the form of auxiliary proteins such as Vif, which interacts with APOBEC3G and triggers its degradation via the ubiquitin-dependent proteosomal pathway (Yu et al., 2003, Goncalves and Santa-Marta, 2004, Sheehy et al., 2003).

TRIM5-alpha: Mammalian cells express several factors that inhibit lentiviral infection and one of these factors, tripartite motif-containing motif 5 alpha (TRIM5α), mediates species-specific and early block of retrovirus infection (Reymond et al., 2001). In Old World monkeys, rhesus TRIM5α may restrict HIV-1 infection by binding to the viral capsid, leading to its premature disassembly or by blocking virus production (Stremlau et al., 2004, Uchil et al., 2008, Zhang et al., 2008). Although human TRIM5 has also been shown to possess some anti-HIV activity, its effect however, has been shown to be significantly less potent than that of rhesus TRIM5α (Stremlau et al., 2004, Zhang et al., 2008, Kaumanns et al., 2006). Studies have

suggested that genetic variants of human TRIM5 may influence relative susceptibility to HIV-1 infection, antiretroviral function and the rate of disease progression (Javanbakht et al., 2006, Speelmon et al., 2006). Recently Richardson et al. (2014) investigated the role of protein stability in conferring TRIM5α-mediated HIV-1 restriction and they demonstrated that human TRIM5α protein was less stable than rhesus TRIM5α, and this difference correlated with higher self-ubiquitination activity. Using a stabilized form of human TRIM5α in which the steady-state expression level was more similar to rhesus TRIM5α, the authors were able to show comparable HIV-1 restriction activity in HIV-1 challenge assays. Interestingly, primary human CD4+ T-cells expressing a stabilized human TRIM5α were protected from HIV-1 mediated destruction *in vivo* (Richardson et al., 2014).

Tetherin: Human cells also possess an antiviral activity that inhibits the release of a broad-spectrum of enveloped viruses, including retroviruses and this antiviral activity can be constitutively expressed or induced by interferon-alpha (Neil et al., 2008, Pardieu et al., 2010, Xu et al., 2011). This antiviral activity consists of protein-based tether, termed 'tetherin' because it directly tethers virions to infected cells through the partition of its membrane anchors into both virion and cell membranes thus causing the retention of fully formed virions on infected cell surfaces (Perez-Caballero et al., 2009). However, HIV-1 can overcome the restriction imposed by tetherin through the activity of viral protein U (Vpu), which mediates surface downregulation and degradation of tetherin (Neil et al., 2008, Van Damme et al., 2008, Schindler et al., 2010, Douglas et al., 2009).

6. The adaptive immune response to HIV-1

6.1. The antibody response

The last few years have seen a rapid acceleration in our understanding of human antibodies that neutralize HIV-1 and much of this has been enabled by technologies which allow for the identification of effective neutralizers from HIV-1-infected donors capable of neutralizing a broad spectrum of HIV-1 isolates (Doria-Rose et al., 2014, Scheid et al., 2011, Walker et al., 2009b, Wu et al., 2010). Infection by HIV-1 elicits a strong antibody response to both the surface unit (gp120) and the transmembrane unit (gp41) envelope glycoprotein as well as to conformationally dependent epitopes formed by the trimeric Env spike (Pantophlet and Burton, 2006, Mascola and Montefiori, 2010). However, these Env-directed responses consist predominantly of non-neutralizing and neutralizing antibodies are usually detected several months later (Wei et al., 2003, Gray et al., 2007, Pantophlet and Burton, 2006, Shen et al., 2009, Stamatatos et al., 2009). Although the detection of cytotoxic T-cell activity coincides with the initial decrease in plasma viral RNA levels, neutralizing antibodies against autologous virus are detected in the majority of HIV-infected individuals from approximately 12 weeks or later after HIV-1 infection (Wei et al., 2003, Gray et al., 2007). Screening of thousands of HIV-1 infected individuals in different cohorts has revealed that some degree of neutralization of heterologous viruses eventually arises in 20-25% of patients and antibodies appear after 1-4 years of infection, however, evidence of their efficacy in the control of viremia is still lacking (Gray et al., 2007, Shen et al., 2009, Stamatatos et al., 2009). Interestingly, approximately 1% of infected individuals, referred to as elite neutralizers, develop an

exceptionally broad and potent neutralizing antibody response (Simek et al., 2009, Bello et al., 2009, Lambotte et al., 2009). In HIV-1 clade B infection, the range of epitopes bound by the earliest autologous-virus neutralizing antibodies is narrow and epitopes are mostly restricted to certain virus isolates (Wei et al., 2003, Richman et al., 2003). Similarly, in HIV-1 clade C, the initial autologous-virus neutralizing antibodies are induced with similar kinetics and are typically specific only for the initially transmitted Env variant (Moore et al., 2008). Moreover, antibodies specific for the more conserved regions of HIV-1 Env are rarely generated during the course of HIV-1 infection; when they do occur, they develop only after 20–30 months of infection (Calarese et al., 2003, Kwong et al., 2002, Shen et al., 2009).

During acute HIV-1 and SIV infection the virus replicates profusely in mucosal B cell inductive microenvironments such as Peyer's patches, high levels of HIV and SIV can induce lysis of uninfected bystander follicular B cells, apoptosis of B cells and loss of nearly 50% of germinal centers within the first 80 days of infection (Alam et al., 2008, Fox et al., 1991, Pantaleo et al., 1993, McMichael et al., 2010). The early loss of germinal centers may result in defects in the ability to rapidly generate high-affinity HIV-1 antibodies and result in the delayed induction of autologous-virus-neutralizing antibodies (McMichael et al., 2010, Pantaleo et al., 1991, Muro-Cacho et al., 1995). The finding that the generation of potentially protective antibodies is delayed until after initial control of viraemia and the narrowness of the breath of these neutralizing antibodies implies that key vaccine strategies which will enable effective control of early viral expansion have to focus on priming an early and broad antibody response targeting multiple neutralizing epitopes and also induce a rapid

secondary response (McMichael et al., 2010). However, vaccine protocols with a number of envelope presentations, including live and inactivated virus and recombinant envelope proteins lack the ability to elicit strong neutralizing responses to primary isolates of HIV-1 or SIV capable of protection against challenge with primary viruses (Mascola and Montefiori, 2010, Pantophlet and Burton, 2006, Burton, 1997).

6.2. The CD8+ T-cell response

There is compelling evidence that acute-phase HIV-1-specific CD8+ T-cell responses play an important role in determining the subsequent clinical course of disease. CD8+ T-cell depletion studies have demonstrated that in non-human primates infected with pathogenic SIV, the absence of CD8+ T-cells during acute or chronic infection resulted in a significant increase in virus replication and acceleration in disease progression (Schmitz et al., 1999, Jin et al., 1999). Numerous studies have also demonstrated that vaccine-induced CD8+ T-cells can protect against the development of AIDS when macaques were challenged with an SIV-HIV chimera (Amara et al., 2001, Barouch et al., 2001, Barouch et al., 2000, Barouch et al., 2012, Liu et al., 2009a, Barouch et al., 2013, Hansen et al., 2013).

In both HIV-1 and SIV infection the first wave of virus-specific CD8+ T-cell responses occurs as viremia approaches its peak, and the CD8+ T-cell response peaks 1–2 weeks later, coinciding with the decline in viraemia (Koup et al., 1994, Borrow et al., 1994, Goonetilleke et al., 2009, Pantaleo et al., 1994, Huber and Trkola, 2007,

Radebe et al., 2011, Liu et al., 2013). The range of epitopes recognized by the CD8+ T-cell response during acute HIV-1 infection is narrow, even at the time of maximal decline in peak viremia when only a very few epitopes are targeted (Altfeld et al., 2001, Turnbull et al., 2009, Radebe et al., 2011, Goonetilleke et al., 2009). The earliest CD8+ T-cell responses are often specific for Env and Nef with responses against Gag p24 and Pol proteins arising during later waves of T-cell responses (Turnbull et al., 2009, Goonetilleke et al., 2009, Radebe et al., 2011). Despite the narrowness of the acute phase CD8+ T-cell response, both human and animal studies have demonstrated that the emergence of a CD8+ T-cell response is important in the control of virus replication in early infection, before the appearance of anti-HIV binding and/or neutralising antibodies (Hansen et al., 2009, Bimber et al., 2009, Wilson et al., 2009, Streeck et al., 2009b, Borrow et al., 1994, Koup et al., 1994, Goonetilleke et al., 2009, Schmitz et al., 1999, Burton et al., 2004, Walker et al., 2009a, Hubner et al., 2009, Buchbinder et al., 2008, McElrath et al., 2008, Virgin and Walker, 2010).

6.3. Role of HLA class I in mediating CD8+ T-cell responses

CD8+ T-cells detect HIV-1 peptides through T-cell receptor (TCR)-mediated recognition of peptides which are selected and transported to the cell surface by HLA class I proteins inducing T-cell activation and clonal expansion, which is followed by lysis of infected target T-cells (Koup et al., 1989, Kaslow et al., 1996, Neefjes et al., 2011). Although many thousands of different peptides can be potentially generated for CD8+ T-cell recognition, the intricate system of intracellular peptide processing,

transport and binding to available HLA-I allomorphs, and TCR repertoire matching, constrain these candidates to a relatively small number of peptide epitopes that serve as targets for the effector CD8+ T-cells (Yewdell, 2006, Irvine and Bennink, 2006). The control of HIV replication has also been linked with the ability to mount a diverse class I restricted response, where HIV-infected individuals who are heterozygous at the HLA class I loci have been shown to have a slower disease progression (Carrington et al., 1999).

6.4. CD8+ T-cell immunodominance

Depending on the relative contribution of epitope-specific CD8+ T-cell responses to the overall magnitude of HIV-1-specific CD8+ T-cell responses in a given individual, these CD8+ T-cells responses can be classified as immunodominant, co-dominant and subdominant (Streeck and Nixon, 2010, Loffredo et al., 2008, Tenzer et al., 2009, Goulder et al., 1997b). Specific immunodominant patterns of CD8+ T-cell responses have been identified during acute HIV-1 infection that are linked to a low ensuing set point suggesting that the hierarchy of HIV-1-specific CD8+ T-cell responses in acute infection may be crucial for the effectiveness of the immune response (Liu et al., 2013, Streeck et al., 2009b). Indeed, individuals able to mount certain immunodominant responses in acute HIV-1 infection have been shown to on average have a lower viral set point than those who do not target those epitopes in acute HIV-1 infection (Streeck et al., 2009b, Allen et al., 2000, Liu et al., 2013). These data show that the generation of immunodominant CD8+ T-cell responses in the acute phase of infection may impact viral load set point and subsequent disease

progression. Owing to their early presentation, high immunogenicity and apparent involvement in the initial control of viremia, these early-recognized CD8+ T-cell epitopes represent key targets in vaccine development (Yu et al., 2002).

6.5. CD8+ T-cells exert selective pressure

An indicator of CD8+ T-cell immune pressure on the virus is the emergence of viral escape within targeted epitopes (Fischer et al., 2010, Goonetilleke et al., 2009, Brumme et al., 2008, Liu et al., 2013, Ferrari et al., 2011). CD8+ T-cells are able to exert significant selective pressure on HIV-1 and SIV during acute and chronic infection and thus lower viral replication (Cao et al., 2003b, Borrow et al., 1997, Allen et al., 2000, Moore et al., 2002, Allen et al., 2005, Kawashima et al., 2009, Pereyra et al., 2008). In fact, after the initial CD8+ T-cell response wave, significant changes in the sequence of the virus have been observed with rapid selection of mutations occurring at discrete sites in the virus genome (Salazar-Gonzalez et al., 2009, Bernardin et al., 2005, Goonetilleke et al., 2009, Liu et al., 2013). More rapid escape has been associated with the most immunodominant CD8+ T-cell response within an individual (Liu et al., 2013, Turk et al., 2013), and escape from such responses may be linked to establishment of viral set point (Henn et al., 2012).

Detailed analysis of four patients by Goonetilleke et al., 2009 showed that during acute infection HIV-1–specific T-cells rapidly select escape mutations concurrent with falling virus load and that CD8+ T-cell–mediated killing significantly contributed to the killing of productively infected cells in acute HIV-1 infection thus contributing

to the initial decline of plasma virus in acute infection. By single genome amplification they were able to detect viral evolution at sites of CD8+ T-cell pressure during peak viremia, thus clearly demonstrating that there is effective pressure being applied as it leads to a significant turnover in the replicating virus population. A mathematical model of these data postulates that 15%– 35% of infected cells can be killed by CD8+ T-cells of a single specificity per day in vivo during acute infection (Ganusov et al., 2011, Liu et al., 2013, Goonetilleke et al., 2009).

6.6. The fitness of the infecting virus

Current data convincingly demonstrate the role of CD8+ T-cells in combating HIV-1 infection and have been a driving force in strategies aimed at eliciting these responses with HIV-1 vaccines (Burton et al., 2004, Walker et al., 2009a, Hubner et al., 2009, Buchbinder et al., 2008, McElrath et al., 2008, Virgin and Walker, 2010, Hansen et al., 2011, Korber et al., 2009, Barouch et al., 2010, Stephenson et al., 2012). A major obstacle however, is CTL-mediated selection leading to viral escape, which occurs frequently in both acute/early and chronic HIV-1 infection (Boutwell et al., 2010, Wang et al., 2009, Brumme et al., 2009, Ganusov et al., 2011). Although the evasion of intrinsic immune responses through escape mutations may represent a survival benefit to the virus, the same mutations can carry associated fitness costs as evidenced by the reversion of transmitted escape mutations during acute and early HIV-1 infection (Leslie et al., 2004, Goonetilleke et al., 2009, Li et al., 2007). Many of the critical escape mutations which carry an associated fitness cost have been identified in the relatively conserved Gag protein (Wright et al., 2010, Boutwell

et al., 2009, Brockman et al., 2007, Schneidewind et al., 2008), whilst CTL escape mutations in Env protein have been shown to have a neutral effect on virus fitness, suggesting a balance between immunologic escape and replicative fitness costs (Troyer et al., 2009). A number of studies have reported that in chronic HIV-1 infection, there is a correlation between in vitro HIV-1 replication capacity and plasma viremia or disease progression (Blaak et al., 1998, Brumme et al., 2007, Wright et al., 2010). Studies of HIV-1 elite controllers who are able to maintain viremia to limits below detection, have revealed the presence of replication-competent viruses in these individuals, however, these viruses were shown to be less fit (Blankson et al., 2007, Lassen et al., 2009, Miura et al., 2009). There is also data demonstrating that acute/early stage viruses from individuals with a slower rate of disease progression also display reduced replication capacity during the early stages of infection, suggesting that viral dynamics during acute infection may have a major impact on HIV disease progression and outcome (Miura et al., 2010, Prince et al., 2012, Brockman et al., 2010, Wright et al., 2011).

6.7. CD4+ T-cells in HIV-1 infection

The hallmark of HIV-1 infection is the depletion of CD4+ T-cells in peripheral blood, lymphoid organs and mucosal sites (Haase, 2005). Since CD4+ T-cells play a vital role in immune defences against almost all pathogens, HIV-1-infected individuals are subject to a variety of opportunistic infections (Okoye et al., 2007, Andrews and Koup, 1996, Rosenberg et al., 1997). Activated CD4+ T helper cells are the main target of HIV-1 and are killed by infection and HIV-1 induced apoptosis (Groux et al.,

1992, Musey et al., 1999). Activated CD4+ T-helper cells mediate a Th1 response by producing IL-2 and IFN-γ, cytokines essential for maintaining an effective CD8+ T-cell response (Altfeld et al., 2001, Kalams et al., 1999, Rosenberg et al., 1997, Ramduth et al., 2005). However, as a result of continuous viral replication, the CD4+ T-cell population lose the ability to produce IL-2 or to proliferate and may result in lack of viral control (Iyasere et al., 2003, Younes et al., 2003, Palmer et al., 2005).

6.8. Immune activation and HIV pathogenesis

Data from animal models and human studies show that acute HIV-1 infection is characterized by a rapid and massive depletion of CCR5+CD4+ memory T-cells and these cells account for most of the CD4+ T-cells found in the mucosal surface of the intestinal tract (Veazey et al., 2000, Brenchley et al., 2004a). The depletion of CD4+ T-cells in mucosal lymphoid tissues results in the absence of key regulatory and effector functions that CD4+ T-cells perform in controlling immune response, thus the extent of the initial damage at the mucosal surface may subsequently determine the course of infection (Brenchley et al., 2006a, Hel et al., 2006, Gupta, 1993, Pedersen et al., 1990). Loss of mucosal CD4+ T-cells may also disrupt various immune components that constitute the mucosal barrier in the gut thus causing microbial translocation into circulation and the systemic immune system resulting in enhanced T-cell activation (Catalfamo et al., 2008, Equils et al., 2001, Faure et al., 2000, Brenchley et al., 2006b, Nowroozalizadeh et al., 2010, Deeks et al., 2004). The increased levels of activation result in increased CD4+ and CD8+ T-cell turnover and death of these activated cells (Alter et al., 2004, Brenchley et al., 2006b, Douek et al.,

2003, Hellerstein et al., 2003). In addition, T-cell immune activation during acute HIV-1 infection is characterized by an increased expression of pro-inflammatory cytokines (Godfried et al., 1993, Fahey et al., 1990, Miedema et al., 2013, Stacey et al., 2009, Roberts et al., 2010) which enhance HIV-1 replication and CD4+ T-cell loss by directly promoting proviral transcription, by recruiting and activating CD4+ T-cell targets for HIV-1 infection, and by inducing apoptosis of bystander T-cells (Osborn et al., 1989, Swingler et al., 1999, Katsikis et al., 2011).

7. Rationale for the study:

Evidence suggests that CD8+ T-cells play a major role in the control of HIV-1 viremia and apply significant immune pressure on HIV-1 replication. However, significant numbers of HIV-specific CD8+ T-cells responses are seen in both chronically infected rapid progressors and controllers. The presence of virus-specific CD8+ T-cells in individuals with varying levels of viral control suggests that CD8+ T-cells may differ in their antiviral function or efficacy. The mechanisms underlying differences in the control of viremia, particularly the reasons why particular individuals experience more effective primary viremia resolution are still not well understood. It is important to identify the specificity and patterns of HIV-1-specific CD8+ T-cell responses and how they mediate viral control in order to accurately define the immune correlates for protection. In order to uncover some of the features of CD8+ T-cell subsets responsible for the control of HIV replication, we investigated the patterns and features of HIV-1-specific CD8+ T-cell responses during acute and primary HIV-1 infection and their association with viral control. We also sought to determine the impact of acute phase immune activation on the acute HIV-1-specific CD8+ T-cell response and on disease progression. We hypothesized that protein-specific and epitope-specific immunodominance patterns during the first 12 weeks of HIV-1 infection are associated with disease progression.

8. Objectives of the Study:

1. To characterize HIV-1-specific CD8+ T-cell responses and their immunodominance patterns during primary HIV-1 infection.

2. To assess the impact of early HIV-1-specific CD8+ T-cell responses on viral control and viral evolution

3. To assess the impact of memory CD8+ T-cell responses during primary HIV-1 clade C infection on long-term disease progression.

4. To characterize fluctuations of early cytokine expression and levels of T-cell immune activation in acute HIV-1 infection and the impact of these factors on the acute CD8+ T-cell response and disease progression.

Chapter 2 : Limited immunogenicity of HIV CD8+ T-cell epitopes in acute clade C virus infection

1. Abstract

Background: HIV-1-specific CD8+ responses contribute to the decline in acute peak viremia following infection. However, data on the relative immunogenicity of CD8+ T-cell epitopes during and after acute viremia are lacking.

Methods: We characterized CD8+ T-cell responses in 20 HIV-1C acutely infected, antiretroviral-naive subjects using the IFN-γ ELISPOT assay. Eleven of these had not fully seroconverted at analysis. Viruses from plasma were sequenced within defined CTL epitopes for selected subjects.

Results: At approximately 28 days after estimated initial infection, CD8+ T-cell responses were directed against an average of 3 of the 410 peptides tested (range 0-6); two individuals had no detectable responses at this time. At 18 weeks, the average number of peptides targeted had increased to 5 (range 0-11). Of 56 optimal Gag CTL epitopes sequenced, 31 were wildtype in the infecting viruses, but only 11 of 31 elicited measurable CD8+ T-cell responses.

Conclusion: These data demonstrate that the majority of CD8+ responses that subsequently arise are not elicited during acute HIV infection despite the presence of the cognate epitope in the infecting strain. There is a need to define factors that influence lack of induction of effective immune responses and the parameters that dictate immunodominance in acute infection.

2. Introduction

HIV-1-specific CD8+ T-lymphocytes have been a key focus for HIV vaccine development efforts, in part because approaches based on the development of a neutralizing antibody based vaccine have thus far failed to induce broadly cross reactive neutralizing antibodies (Walker et al., 2009a, Hubner et al., 2009, Buchbinder et al., 2008, McElrath et al., 2008, Virgin and Walker, 2010) and in part because induction of T-cell responses in animal models of AIDS virus infection impact viral set point and disease progression (Hansen et al., 2009, Bimber et al., 2009, Wilson et al., 2009).

Numerous studies in clade B infection have shown that early CD8+ T-cell responses are narrowly directed, and specific immunodominant T-cell responses detected during acute HIV-1 infection are associated with the subsequent viral set point (Yu et al., 2002, Lichterfeld et al., 2005, Altfeld et al., 2006, Streeck et al., 2009b). However, these studies have had a number of limitations. In most studies to date, although subjects have been identified prior to seroconversion, the initial assays of T-cell function have been performed following seroconversion. Studies that link CD8+ T-cell responses to seroconversion status and contemporaneous viral load are lacking, and overall there is a paucity of data on HIV-1-specific CD8+ T-cell responses for the ethnicities most ravaged by the HIV-1 pandemic and for the clades responsible in these regions.

Moreover, most studies on immune responses in acute infection have relied on identification of subjects based on symptoms of acute infection staged as days following onset of symptoms (Koup et al., 1994, Streeck et al., 2009b, Turnbull et al., 2009), whereas the evolution of antibody responses used in the Fiebig staging of acute infection were obtained from a cohort of asymptomatic blood donors (Fiebig et al., 2003) who would not have qualified for donation if they had had typical acute infection symptoms such as fever and malaise.

To address these limitations and to define the characteristics of HIV-1-specific immunity in acute HIV-1C infection, we screened persons at voluntary counseling and testing (VCT) centers testing negative by standard antibody assays for evidence of HIV RNA (Bassett et al., 2011). Subjects were enrolled after initial screening, allowing us to document the decline in viral load concurrent with the measurement of immune responses. Using the ELISPOT assay, we evaluated the breadth and magnitude of HIV-specific CD8+ T-cell responses over the first few weeks of infection using synthetic overlapping peptides and defined HLA class I-restricted epitopes. Our data indicate that limited T-cell breadth is associated with the initial decline in viremia in acute HIV-1C infection, and that the majority of epitopes known to be targeted in the context of expressed class I molecules are not immunogenic in the earliest stages of infection.

3. Materials and methods

3.1. Study Participants

The HIV Pathogenesis Programme (HPP) Acute Infection Study is an ongoing effort to characterize acute HIV-1 subtype C infection in Durban, KwaZulu-Natal. Recruitment of subjects with acute and early HIV infection was achieved through the recruitment of subjects at 3 different sites: St. Mary's Hospital, McCord Hospital and Prince Mshiyeni. Individuals testing negative or discordant by dual commercial rapid HIV-1 tests (Bioline, Standard Diagnostics, Inc., Kyonggi-do, Korea); and Sensa (Hitech Healthcare LTC, China) were recruited for HIV-1 RNA testing. Acute HIV-1 infection was defined by a positive HIV-1 RNA test, negative HIV-1 enzyme immunoassay test (SD HIV1/2 ELISA 3.0 (Standard Diagnostics, Inc., Kyonggi, Korea)) and a negative or indeterminate Western blot (Genetic Systems, Bio-Rad, Redmond, USA). Centers for Disease Control (CDC) criteria were used for the interpretation of Western blot results such that a positive sample had at least two of the major bands (gp160, gp120, gp41 and p24), with either gp160 or gp120 present as well as gp41 or p24 (Centers for Disease, 1985). If the result did not meet these requirements but one or more bands were present; or where the band intensity was less than the weak positive control this result was classified as indeterminate. Negative results exhibited no reactive bands. We estimated the infection date as occurring 14 days prior to the first positive HIV RNA and negative HIV antibody test as previously described (van Loggerenberg et al., 2008).

A total of 42 subjects were identified, of whom 20 were enrolled. The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal. All study participants provided written informed consent for participation in the study.

3.2. Isolation of PBMCs

Blood was collected in EDTA tubes and processed within 6 hrs of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll-Histopaque (Sigma, St Louis, Mo) density gradient centrifugation and used fresh in ELISPOT assays.

3.3. Viral load quantification, and $CD4^+$ T-cell enumeration

HIV-1 RNA in plasma was measured using the Roche Amplicor version 1.5 or Cobas Taqman HIV-1 Test according to the manufacturer's instructions. CD4+ T-cells were enumerated using Tru-Count technology and analyzed on a four-color flow cytometer (Becton Dickinson) according to the manufacturer's instructions.

3.4. HLA typing

DNA for HLA typing was extracted using Puregene DNA isolation kit for blood (Gentra Systems, Minneapolis, Minn.) according to the manufacturer's instructions. HLA class I typing was done by DNA PCR using sequence specific primers as previously described (Kiepiela et al., 2004). Briefly, genomic DNA

samples were initially typed to an oligo-allelic level using Dynal RELITM reverse Sequence Specific Oligonucleotide (SSO) kits for the HLA-A, -B and -C loci (Dynal Biotech). Refining the genotype to the allele level was performed using the Dynal Biotech sequence-specific priming kits in conjunction with the previous SSO type.

3.5. Synthetic HIV-1 peptides

A panel of 410 peptides (18-mers overlapping by 10 amino acid residues), spanning the entire HIV-1 clade C consensus sequence together with optimal peptides with known HLA class I restriction patterns, were synthesized on an automated peptide synthesizer (MBS 396; Advanced ChemTech) and used in the ELISPOT assay (Chessman et al., 2008).

3.6. IFN-gamma ELISPOT Assay

Assessment of HIV-1-specific CD8+ responses was performed on peripheral blood mononuclear cell (PBMC) samples collected at 2-4, 4-6, 6-8, 8-12 and 12-18 weeks (14-28, 29-56, 57-84, 85-126 days) following initial viral load screening. PBMCs (50,000-100,000 cells/well) were plated in 96-well polyvinylidene plates (MAIP S45; Millipore), precoated with anti-human IFN-γ monoclonal antibody 1-D1k (0.5 µg/ml; Mabtech). Peptides were added at 2 µg/ml in a matrix (Thobakgale et al., 2007). Phytohemagglutinin (1 µg/ml) stimulation was included as a positive control, and medium alone as a negative control. Plates were incubated overnight at 37°C and 5% CO_2, then washed with phosphate-buffered saline before addition of the biotinylated anti-IFN- MAb, 7-B6-1 biotin

(Mabtech), at 0.5 µg/ml and incubation for 90 minutes. Following washing, streptavidin-conjugated alkaline phosphatase (Mabtech) was added for 45 minutes. IFN-γ-producing cells were noted by direct visualization following development with alkaline phosphatase colour reagents (Bio-Rad). A response was defined as positive when there were at least 100 spot forming cells (SFCs)/million PBMC and the total number of spots was 3 standard deviations above the negative control value. In addition to overlapping peptides, peptides corresponding to optimal HIV-1 CTL epitopes described for the individual's HLA class I type were tested at a peptide concentration of 2 µg/ml.

To verify peptides identified as positive in the peptide pool matrix, PBMCs from the same time point were stimulated with individual peptides that were common to two peptide pools in the initial ELISPOT screen. The criteria used to define positive responses in the peptide pool matrix were also used for the verification assay. In 98% of the matrix positive responses, at least one single peptide positive reaction was confirmed. The sensitivity of the matrix approach for the detection of peptide-specific T-cell responses was found to be 82% for Nef and Gag. The sensitivity was calculated by dividing the number of responses detected in the peptide matrix by the number of responses detected using individual peptides (Addo et al., 2003).

All responses at the first sampling time point were evaluated using fresh PBMC, which provide greater sensitivity in ELISPOT assays in comparison to viably frozen cells (Mooij et al., 2009). Fresh cells were also used in all but 10% of assays

performed at later time points. PBMC were frozen (90% calf serum and 10% DMSO) at -160°C until use. Cell viability was determined using a Guava automated counter (Guava Technologies, Hayward, CA) and the ELISPOT assay was performed only when PBMC viability was greater than 80%.

3.7. *Gag* amplification and sequencing

Viral RNA was isolated from 140μl of plasma samples using the QIAamp Viral RNA Extraction Mini Kit (Qiagen, Hilden, Germany). Viral RNA was then reverse transcribed using ThermoScript™ RT-PCR System kit (Invitrogen, Carlsbad, CA, USA) and the gene-specific primer, GagD reverse as previously described (Chopera et al., 2008). To amplify the HIV-1 gag region, a nested PCR was performed with two sets of primers, GagD forward (5′-TCT CTA GCA GTG GCG CCC G-3′) and GagD reverse for the first round and GagA forward (5′-CTC TCG ACG CAG GAC TCG GCT T-3′) and GagC reverse (5′-TCT TCT AAT ACT GTA TCA TCT GC-3′) for the second round as previoulsy described (Chopera et al., 2008). The resulting PCR product was then purified using purification columns from Illustra™ GFX™ PCR DNA Gel Band Purification Kit, (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and cloned into a PCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated from individual randomly picked white bacterial colonies (GeneJet Plasmid Mini Prep Kit; Fermentas, Vilnius, Lithuania) and were screened for the presence of the insert with the EcoRI restriction enzyme (New England BioLabs, Ipswich, MA, USA). Sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction

kit version 3.4 (Applied Biosystems, Foster City, CA, USA). A modified NAP algorithm (Huang et al., 1996) was used to align sequences to HXB2 (Genbank Acc. No. K03455) and insertions with respect to HXB2 were stripped before further analysis.

3.8. Statistical analysis

The Mann-Whitney nonparametric analysis was used to test for significant differences between HLA groups. For assessments of the relationship between immune responses and viral load, Spearman rank correlations were used. Statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California USA).

4. Results

4.1. Viral load and CD4 counts in HIV-1 clade C acute infection

HIV-specific CD8+ T-cell responses have been documented in primary HIV infection; however, very little is known about the breadth and magnitude of these responses in the earliest phase of infection, prior to seroconversion. Moreover, most studies have evaluated responses well after the documented decline in viremia, such that very little data are available during documented periods of viral load decline. To begin to address these issues, we recruited persons with acute clade C HIV infection who had not yet seroconverted. Twenty such persons were identified, all of whom initially tested negative or discordant by two rapid HIV tests currently in use at Voluntary Counseling and Testing (VCT) sites in KwaZulu-Natal, and were then screened for HIV RNA in plasma. In each case, $RNA^{+}Ab^{-}$ status was confirmed by positive quantitative HIV-1 reverse transcription–polymerase chain reaction (PCR), negative ELISA test and a negative Western blot, and all were subsequently documented to seroconvert to HIV. The representative serological and viral profile of subjects recruited into the study is given in Table 2.1.

The characteristics of each of the recruited subjects are listed in Table 2.2. Median age at enrolment was 29 years (range 21-66) and 60% of the participants were female. Subjects were heterogeneous with respect to viral load obtained at the time of diagnosis, ranging from 28,720 to 33,200,000 RNA copies/ml plasma (median 5,572,000 RNA copies/ml). The majority of individuals in the cohort exhibited a dramatic elevation in plasma viral load before seroconversion

indicative of acute HIV and this was followed by a steady decline in viremia over the first year of infection, as well as a decline in absolute CD4+ T-cell counts (Figure 2.1).

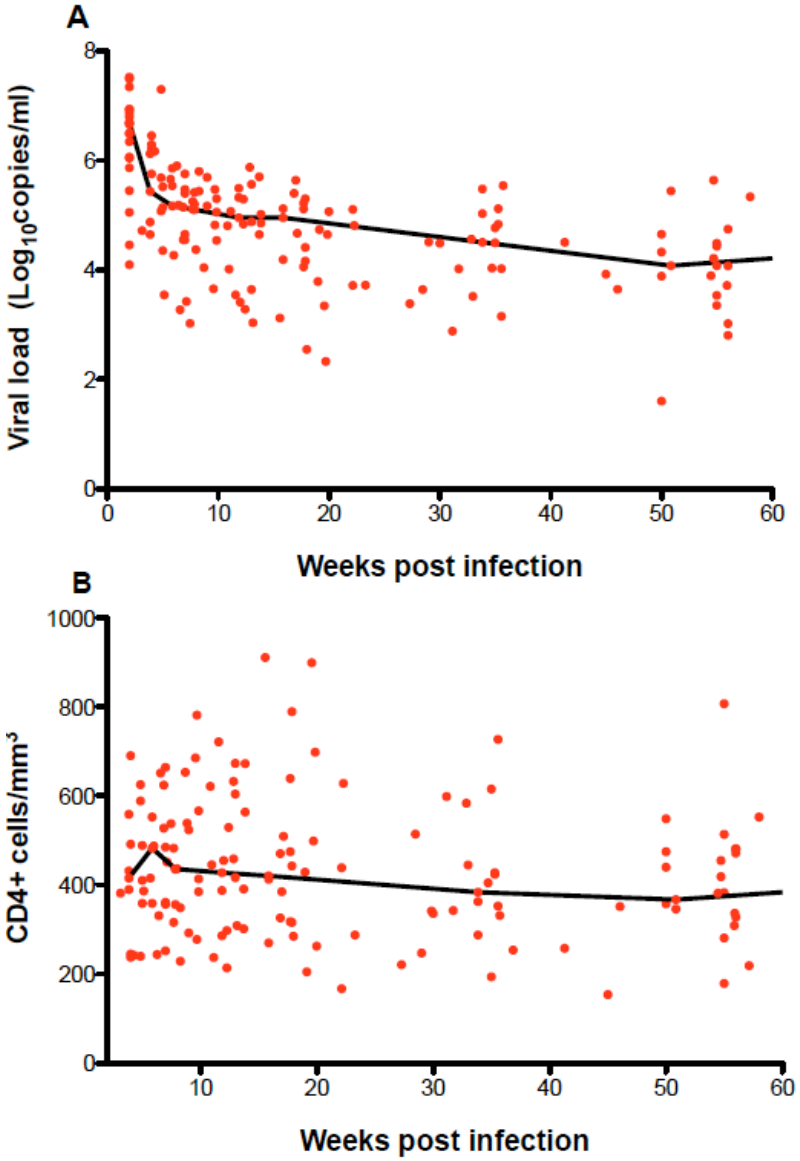


Figure 2.1. Plasma HIV-1 RNA titres and CD4+ T-cell counts in primary infection. Scatter plot of (A) plasma HIV-1 RNA levels and median plasma HIV-1 RNA levels in the cohort (solid black line) from the time of seroconversion up to one year post infection; (B) individual absolute CD4+ T-cell counts and median CD4+ count (solid black line) in the cohort during acute HIV-1 infection up to one year post infection.

Table 2.1. Typical evolution of key viral and serological markers during the first weeks following infection with HIV-1.

Variable	AS01-0703					
Days post infecion	14	26	40	54	110	138
Rapid screening or HIV-1	Negative					
EIA	Negative	Positive				
CD4+ T cell count (per mm^3)		625	782	674	640	616
gp160	-	+/-	+	+	+	
gp120	-	+/-	+	+	+	
p65	-	-	-	-	+	
p55	-	-	+	+	+	
p51	-	-	-	-	+	
gp41	-	-	-	-	+	
p40	-	-	+	+	+	
p31	-	-	-	+/-	+/-	
p24	-	-	+	+	+	
p18	-	-	+/-	+/-	+	
Interpretation of Western blot results	Negative	Indeterminate	Positive	Positive	Positive	
HIV-1 RNA copies/ml	4,560,000	119,000	140,000	66,200	365,000	166,000

The initial screening was limited to obtaining a plasma sample, thus no T-cell assays were performed at this time. The viral load at enrolment, when the first ELISPOT assays were performed (on average 14 days following the initial RNA assay) was 371,000 RNA/ml (range 1,090-2,840,000) and the average CD4+ cell counts obtained at this time were 389 cells/mm^3, again with a wide range from 151-691 cells/mm^3. Of note, four of the 20 subjects expressed the HLA-B*58:02 allele which is associated with rapid disease progression and poor prognosis (Ngumbela et al., 2008). Surprisingly, these four subjects had a lower median plasma viral load and a higher CD4+ count when compared to the rest of the cohort not expressing this allele, with a median viral load approximately 10 fold less than the cohort as a whole (506,000 RNA copies/ml, range 112,500-4,560,000 vs 7,425,000 RNA copies/ml range 28,700-33,200,000 and median

CD4+ cell count 519 cells/mm^3, range 238-691 vs. 385 cells/mm^3, range 151-652 cells/mm^3, respectively), however these differences did not reach statistical significance (p=0.199 and p=0.163, respectively). The two subjects with known protective alleles HLA-B*57:03 and HLA-B*58:01 had initial viral loads of 1.1M and 7.3M respectively, indicating even with these few numbers that pre-seroconversion viral loads in persons with protective HLA class I alleles can be in the range of the median for clade C virus infection.

Together these results indicate that a strategy based on nucleic acid testing on persons who test negative at VCT sites can identify acute HIV-1 virus infection during times of peak viremia following acute HIV infection, and these subjects can be successfully recruited for follow up studies. These data also indicate that acute HIV clade C virus infection is somewhat heterogeneous with respect to pre-seroconversion viral load, early CD4+ cell counts and expressed HLA class I alleles.

Table 2.2: Demographic and clinical characteristics of enrolled subjects.

Participant number	Sex	Age at Enrolment	HLA Type	*CD4+ T cell count/µl	HIV-1 RNA copies/ml	†Western Blot
AS1-703	M	54	A*2601/3004, B*4403/5802, C*0210/0602	626	4,560,000	-
AS1-876	F	29	A*2301/4301, B*1503/4403, C*0210/1800	309	1,160,000	-
AS1-919	M	58	A*2902/7400, B*3501/4201, C*0401/1700	589	6,280,000	-
AS2-016	F	28	A*2601/3002, B*1518/4201, C*1700/1800	559	31,000,000	-
AS2-110	M	23	A*0214/2902, B*4403/4403, C*0401/0701	304	634,000	-
AS2-174	M	33	A*2301/3002, B*0801/1402, C*0304/0802	359	7,390,000	-
AS2-207	M	39	A*0205/3001, B*5801/5801, C*0302/0701	246	7,460,000	-
AS2-358	F	34	A*2402/3001, B*4202/5301, C*0404/1700	242	8,560,000	-
AS2-483	M	27	A*3001/3402, B*1503/4201, C*0210/1700	390	33,200,000	-
AS2-802	F	42	A*3002/4301, B*0801/5802, C*0602/0701	411	732,265	-
AS2-945	M	26	A*2301/7400, B*1510/5703, C*0701/1601	652	1,109,915	-
AS2-973	M	66	A*3001/4301, B*4201/4201, C*1700/1700	151	10,000,000	-
AS2-1037	F	43	A*03/3402, B*1503/1510, C*0210/0304	418	12,133,226	-
AS3-017	F	34	A*2301/2601, B*3910/8100, C*0401/1501	245	22,000,000	-
AS3-268	F	21	A*2902/8001, B*1503/1801, C*0202/0210	416	8,640,000	-
AS3-369	F	28	A*6602/6802, B*1510/4403, C*0304/0701	432	3,180,000	-
AS3-458	F	25	A*2902/3402, B*4403/5802, C*0401/0602	691	280,000	-
AS3-513	F	25	A*2601/6601, B*1510/5802, C*0304/0602	238	112,448	-
AS3-740	F	26	A*0205/3402, B*1510/4403, C*0401/05	387	28,716	-
AS3-759	F	21	A*0101/7400, B*3501/8100, C*0401/1800	382	4,864,320	-
Median		28.5		389	5,572,160	

*CD4+ cell counts were not performed at screening; the values given are for 2-4 w post screening

† CDC criteria followed for the interpretation of Western Blot results

4.2. HIV-1 -specific CD8+ T-cell responses prior to and following seroconversion

Although studies of acute HIV infection have assessed CD8+ T-cell responses in persons identified prior to seroconversion, there are some important caveats: most of initial assays were performed post-seroconversion, and/or with cryopreserved samples (Altfeld et al., 2001, Alter et al., 2002, Koup et al., 1994). Moreover, few have assessed responses to all expressed viral proteins (Altfeld et al., 2001, Streeck et al., 2009b, Alter et al., 2002, Blankson, 2009). To address these gaps in knowledge, we performed a detailed analysis of CD8+ T-cell responses to all expressed HIV proteins using fresh PBMCs obtained prior to antibody seroconversion. Since only plasma was obtained during initial

screening, these assays were limited to the 11 subjects for whom subsequent samples were available prior to seroconversion (Table 2.3). All 11 subjects were Fiebig stage IV or earlier.

Table 2.3. Viral load characteristics and antibody pattern against HIV-1 western blot in 11 individuals who had not fully seroconverted when HIV-1-specific CD8+ T-cell responses to peptides spanning the entire HIV proteome were evaluated at ~28 days post the estimated time of infection.

Participant number	Screening VL (RNA copies/ml)	Enrollment VL (days after screening)	WB bands at enrollment	Fiebig stage
AS1-703	4 560 000	119,000 (14)	p24, p40, p55/51	IV
AS1-919	6 280 000	484,000 (15)	p24, p40	IV
AS2-174	7 390 000	330,000 (21)	p18, p24	IV
AS2-802	732 260	138,300 (15)	p18, p24, p40	IV
AS2-945	1 100 000	1,872 (32)	p24, p40, p55/51	IV
AS2-973	10 000 000	66,530 (15)	p24	IV
AS2-1037	12 100 000	708,000 (23)	p24, p40, p55/51	IV
AS3-017	22 000 000	2,840,000 (14)	0	II/III
AS3-458	280 000	1,650,000 (14)	p24, p55/51	IV
AS3-513	112 448	1,967,000 (14)	p24	IV
AS3-740	28 716	3,493 (22)	p24, p40, p55/51	IV

HIV-1-specific CD8+ T-cell responses were detected in 9 of the 11 subjects but were narrowly directed in each case (Table 2.4). The overlapping peptides targeted as well as their sequences are listed in Table 2.5. Two subjects with no detectable responses had each experienced an approximate 50-100 fold drop in viremia at the time of testing; both made detectable responses at later time points. Although the two most frequently recognized proteins at the earliest time point were Nef (peptides #69 to #84) and Gag (peptides #4 to #65), targeted by 50% and 36% of the subjects respectively, there was great heterogeneity among subjects with respect to the specificity of the initial responses. Moreover, among those with detectable responses, there were dramatic differences in magnitude, with total detectable epitope-specific responses ranging from as high as 4,000 SFC/million PBMC detected to as low as 100 SFC/million PBMC.

HIV-1-specific responses were subsequently tested longitudinally in these individuals up to 18 weeks (Table 2.4). At this time, responses were detectable in 10 of the 11 subjects, albeit at different magnitudes, and both subjects who initially tested negative (AS2-0945 and AS2-0973) now had detectable responses. Of the nine with initially positive responses, at least one of these responses was still detected in eight subjects at the later time point. There were only two subjects in whom the initial breadth of responses declined at the follow-up time point. Of the nine subjects in whom an initial immunodominant response was detected, this response was still present at follow-up weeks later in seven. However, there was no consistent pattern in terms of specificity, magnitude or evolution of responses in these individuals. These results indicate that the initial

responses largely persist, and that those with no initial responses were able to mount immune responses subsequently.

Table 2.4. Dynamics of HIV-CD8+ T-cell responses to peptides spanning the entire HIV proteome

Participant number	Peptides targeted at enrollment	Dominant response (SFC/million)	Total magnitude of responses (SFC/million)	Peptides targeted at 12-18 weeks	Total magnitude of responses (SFC/million)
AS1-703	Nef-81, Nef-83	Nef-83 (490)	850	#81	500
AS1-919	Nef-83, Nef-202, Nef-203	Nef-83 (280)	740	None	0
AS2-174	gp41-366	gp41-366 (700)	700	#25, #35, #36, #41, #65, #69, #231, #366	11240
AS2-802	Nef-82, Nef-83, gp120-289, gp120-294	gp120-294 (4000)	5710	#11, #35, #36, #44, #64, #65, #82, #83, #294	2740
AS2-945	None	None	0	#20, #22, #33, #84, #181	6390
AS2-973	None	None	0	#275, #294, #407	780
AS2-1037	Gag-41, Rev-100	Rev-100 (580)	720	#40, #41, #46, #76, #100, #101, #116, #411	5610
AS3-017	Gag-16, Gag-40, Nef-76, Tat-115	Gag-16 (1020)	2700	#40	1320
AS3-458	gp120-316	gp120-316 (420)	420	#42, #157, #194, #225, #252, #316	1990
AS3-513	Gag-41	Gag-41 (200)	200	#4, #40, #41	920
AS3-740	Gag-41, Nef-78, Nef-81, Nef-83	Nef-78 (700)	2280	#41, #78, #81, #83	1120

and viral load kinetics in individuals not fully seroconverted at ~28 days post the estimated time of infection.

4.3. Evolution of CD8+ T-cell responses following acute HIV infection

To further characterize the evolution of CD8+ T-cell responses following acute HIV-1 infection, we performed a longitudinal analysis in all 20 subjects. The magnitude of responses at approximately 4 weeks after the estimated date of infection ranged from 150-8,770 SFC/million PBMC. There was a 15-fold decline in viral loads from the initial screening value, CD8+ T-cell responses were directed against an average of 3 peptides (range 0–6) and the most targeted protein was Nef followed by Pol and Gag (Figure 2.2). At 18 weeks, the total magnitude of responses ranged from 500-11,240 SFC/mil PBMC and the average number of peptides targeted was 5 (range 0-11). The overlapping peptides targeted as well as their sequences are listed in Table 2.5.

At 6-8 weeks post the estimated date of infection, our analyses for all 20 subjects revealed that there was a significant positive correlation between the magnitude of Nef CD8+ T-cell responses and plasma viremia (P=0.003). Plotting the magnitude of virus-specific T-cell responses targeting other HIV-1 proteins against plasma viral load over time revealed that the kinetics of expansion and contraction of T-cell responses in the subjects did not correlate with their viral load dynamics. In subjects sampled prior to the documented peak in plasma viremia (AS3-458 and AS3-513), the magnitude of T-cell responses were found to be lower during peak viremia with an increase thereafter. Likewise, in all other subjects, the higher enrolment plasma viremia was not accompanied by a higher T-cell response; instead, the breadth and magnitude of responses increased following the decline in viremia.

Table 2.5. Overlapping peptides targeted in the cohort between 4-18 weeks post infection.

[a]Peptide number	Protein	OLP sequence
#3	Gag	EKIRLRPGGKKHYMLKHL
#4	Gag	GKKHYMLKHLVWASREL
#5	Gag	KHLVWASRELERFAL
#7	Gag	ERFALNPGLLETSEGCK
#11	Gag	TGTEELRSLYNTVATLY
#12	Gag	SLYNTVATLYCVHAGIEV
#13	Gag	LYCVHAGIEVRDTKEAL
#16	Gag	NKSQQKTQQKAAADKGKV
#18	Gag	GKVSQNYPIVQNLQGQMV
#20	Gag	QMVHQAISPRTLNAWVKV
#21	Gag	PRTLNAWVKVIEEKAF
#22	Gag	WVKVIEEKAFSPEVIPMF
#25	Gag	GATPQDLNTMLNTVGGH
#33	Gag	SDIAGTTSTLQEQIAWM
#34	Gag	STLQEQIAWMTSNPPVPV
#35	Gag	WMTSNPPVPVGDIYKRWI
#36	Gag	PVGDIYKRWIILGLNKIV
#40	Gag	GPKEPFRDYVDRFFKTLR
#41	Gag	YVDRFFKTLRAEQATQDV
#42	Gag	LRAEQATQDVKNWMTDTL
#44	Gag	MTDTLLVQNANPDCKTIL
#46	Gag	TILRALGPGASLEEMMTA
#54	Gag	CFNCGKEGHIARNCRAPR
#64	Gag	RFEETTPAPKQEPKDREPL
#65	Gag	PKQEPKDREPLTSLK
#66	Gag	KDREPLTSLKSLFGSDPLSQ
#69	Nef	WPAIRERMRRTEPAAEGV
#75	Nef	WLRAQEEEEEVGFPVRPQV
#76	Nef	EVGFPVRPQVPLRPMTFK
#77	Nef	QVPLRPMTFKGAFDLSFF
#78	Nef	FKGAFDLSFFLKEKGGL
#80	Nef	GLEGLIYSKKRQEILDLW
#81	Nef	KKRQEILDLWVYHTQGYF
#82	Nef	LWVYHTQGYFPDWQNY
#83	Nef	QGYFPDWQNYTPGPGVRY
#84	Nef	NYTPGPGVRYPLTFGWCF
#85	Nef	RYPLTFGWCFKLVPV
#95	Rev	DEALLQAVRIIKILY
#96	Rev	QAVRIIKILYQSNPY
#100	Rev	RWRARQRQIHSISERIL
#101	Rev	QIHSISERILSTCLGRPA
#102	Rev	ILSTCLGRPAEPVPLQL
#103	Rev	RPAEPVPLQLPPIERL
#111	Tat	MEPVDPNLEPWNHPGSQPK
#115	Tat	YHCLVCFQTKGLGISYGR
#116	Tat	TKGLGISYGRKKRRQRRS
#117	Tat	GRKKRRQRRSAPPSSEDH
#125	Vpu	LIVALIIAIVVWTIAYIEY
#151	Pro	GAERQGTLNFPQITLW
#157	Pro	DDTVLEEINLPGKWKPKM
#181	RT	LDVGDAYFSVPLDEDFRK
#194	RT	KIEELREHLLKWGFTTPDK
#202	RT	SKLNWASQIYPGIKVRQL
#203	RT	IYPGIKVRQLCKLLRGAK
#209	RT	YYDPSKDLIAEIQKQGHDQW
#216	RT	QKIAMESIVIWGKTPKFR
#218	RT	FRLPIQKETWETWWTDYW
#224	RT	GAETFYVDGAANRETKI
#225	RT	DGAANRETKIGKAGYV
#226	RT	ETKIGKAGYVTDRGRQKI
#228	RT	VSLTETTNQKTELQAIQL
#229	RT	QKTELQAIQLALQDSGSEV
#231	RT	EVNIVTDSQYALGII
#234	RT	AQPDKSESELVNQIIEQL
#240	RT	SSGIRKVLFLDGIDKA
#252	Int	GYIEAEVIPAETGQETAY
#258	Int	VKAACWWAGIQQEFGIPY
#262	Int	GIQQEFGIPYNPQSQGVV
#265	Int	AVFIHNFKRKGGIGGYSA
#275	Int	KVVPRRKAKIIKDYGKQM
#276	Int	KIIKDYGKQMAGADCVA
#279	Vpr	GPQREPYNEWTLELLEEL
#281	Vpr	ELKQEAVRHFPRPWLHGL
#287	Vpr	HFRIGCQHSRIGILRQRR
#289	gp120	MRVMGIQRNCQQWWRW
#294	gp120	TVYYGVPVWKEAKTTLF
#316	gp120	KVSFDPIPIHYCAPAGYA
#325	gp120	IRSENLTNNAKTIIVHL
#347	gp120	AGNITCKSNITGLLLTR
#348	gp120	SNITGLLLTRDGDTTTNT
#366	gp41	RVLAIERYLKDQQLLGIW
#405	Vif	VKHHMYVSRRANGWFYRH
#408	Vif	HPKVSSEVHIPLGEARLV
#409	Vif	HIPLGEARLVIKTYWGL
#411	Vif	LQTGERDWHLGHGVSIEW
#413	Vif	VSIEWRLRRYSTQVDPGL
#414	Vif	RYSTQVDPGLADQLIHMH

[a]**Peptides are numbered sequentially, as follows:** Gag peptides 1 to 66; Nef peptides 67-93; Rev peptides 94-107; Tat peptides 111-122; Vpu peptides 123-131; Protease peptides 145-164; RT peptides 165-240; Intergrase peptides 241-277; Vpr peptides 278-288; gp120 peptides 289-355; gp41 peptides 356-401; Vif peptides 402-425.

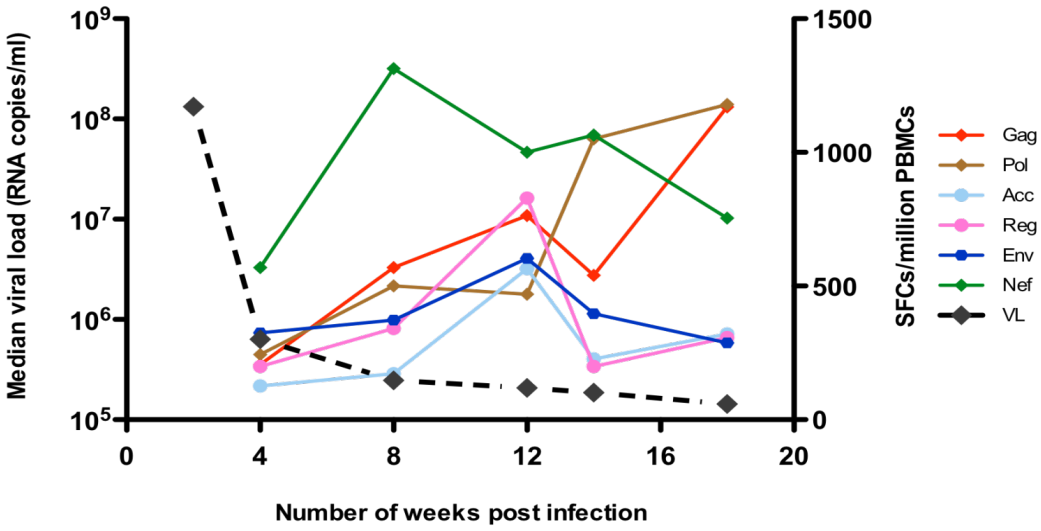


Figure 2.2. Average magnitude of HIV-1 specific CD8+ T-cell responses in early infection for all participants. The magnitude of HIV-1-specific CD8+ T-cell responses to overlapping HIV peptides spanning each of the designated proteins are shown on the *y* axis as SFC/million PBMCs and the *x* axis denotes when the assays were done (number of days post the estimated time of infection). Solid lines represent IFN-γ release in response to HIV-1 CD8+ T-cell peptides, and the dashed line represents viral load dynamics.

4.4. Limited breadth of HIV-1-specific T-cell epitopes in primary infection

The above analyses determined HIV-1–specific CD8+ T-cell responses using overlapping peptides, thus did not define the actual targeted epitopes within those peptides. To delineate the precise regions being targeted, a subset of peptides corresponding to previously described epitopes for each person's respective HLA class I allotypes were used (Table 2.6) (Brander and Goulder, 2000). The analyses were restricted to HLA class I alleles that were expressed in at least three participants, and for which at least three optimal CD8+ T-cell epitopes had been described and could be tested against the subjects' PBMC. The frequencies of these qualifying HLA class I alleles expressed in the study

cohort, as well as the number of epitopes tested for each HLA allele and the percentage of individuals with detectable CD8+ T-cell responses to the tested epitopic peptides restricted by the designated allele, are listed in Table 2.7. The majority of HIV-1 clade C epitopes that have been described in chronic HIV infection were not targeted in these early stages of infection, indicating that there are marked differences in the inductive phase of the immune response that must depend on the epitope rather than the restricting HLA allele.

Table 2.6. Optimal CD8+ T-cell peptides corresponding to defined epitopes for the HLA class-I alles represented in the cohort.

Class 1 family	Class 1 allele	Peptide Identification[a]	Peptide Sequence
HLA-A	HLA-A23	A23-HW9 (p17)	HYMLKHLVW
		A23-RW8 (Nef)	RYPLTFGW
		A23-IL9 (RT)	IYQEPFKNL
		A23-VW8 (gp120)	VYYGVPVW
		A23-LY10 (gp120)	LFCASDAKAY
		A23-RL9 (gp41)	RYLKDQQLL
		A23-KY9 (gp120)	KYLGSLVQY
		A23-NL10 (gp120)	NYTNTIYRLL
		A11-IN8 (RT)	IYQEPFKN
	HLA-A26	A26-EL9 (p24)	EVIPMFTAL
		A26-ER11 (RT)	ETFYVDGAANR
		A26-EY13 (Int)	EVIPAETGQETAY
	HLA-A29	A29-YY8 (Nef)	YFPDWQNY
		A29-RY11 (p17)	RSLYNTVATLY
		A29-QY9 (RT)	QLIKKERVY
		A29-SY9 (gp160)	SFDPIPIHY
		A29-SY10 (gp120)	SFNCRGEFFY
		A29-RW10 (p15)	RQANFLGKIW
		A29-FY9 (gp120)	FNCRGEFFY
		A 29 LY9 (p17)	LYNTVATLY
		A11-IN8 (RT)	IYQEPFKN
	HLA-A30	A3002-RLY10 (p17)	RLRPGGKKHY
		A3002-RY11 (p17)	RSLYNTVATLY
		A3002-AY11 (RT)	AQNPEIVIYQY
		A3002-KLY9 (RT)	KLNWASQIY
		A3002-KIY9 (Int)	KIQNFRVYY
		A3002-RY9 (gp41)	RIGPGQTFY
		A30-KQY9 (gp43)	KYLGSLVQY
		A30-KCY9 (gp41)	KYCWNLLQY
		A3002-SW11 (p17)	SILRGGKDKW
		A3002-RL9 (p17)	RFALNPGLL
		A3002-KV10 (p24)	KNWMTDTLLV
		A30-YY8 (Nef)	YFPDWQNY
		A3002-AF8 (p17)	ASRELERF
		A3002-RY10 (RT)	RAQNPEIVIY
	HLA-A43	A43-TK10 (gp120)	TVYYGVPVWK
		A43-GF13 (gp120)	GVPVWKEAKTTLF
		A43 PF11 (Env))	PVWKEAKTTLF
		A43 VL11 (Env)	VWKEAKTTL
	HLA-A68	A6801-RK9 (RT)	RTAHTNDVK
		A6801-FK8 (Vif)	FADSAIRK
		A6801-DA8 (p24)	DTINEEAA
		A6801 NK10 (Pol)	NTPVFAIKKK
		A6801 PK8 (Pol)	PAGLKKKK
		A6801 YR8 (Pol)	YVDGAANR
		A6801 ER10 (Vif)	EVHIPLGEAR
		A6802-DV9 (RT)	DVKQLTEAV
		A6802-IV9 (RT)	ITLWQRPLV
		A6802-DL9 (RT)	DTVLEEINL
		A6802-EV10 (Vpr)	ETYGDTWTGV
		A6802-IL9 (gp41)	IAARAVELL
		A6802-EL9 (Int)	ETAYFILKL
		A6802-VV8 (p17)	VSQNYPIV
		A6802-SA9 (p24	STLQEQIA
		A6802-GA10 (RT)	GAETFYVDGA
		A6802-PA9 (Int)	PAETGQETA
		A6802-EA10 (Int)	ETAYFILKLA
		A6802-EQA10 (RT)	ETWWTDYWQA
		A6802-EA9 (RT)	ETFYVDGAA

Class 1 family	Class 1 allele	Peptide identification[a]	Peptide sequence
HLA-B	B7/42/81	B7/42/81-SV9 (p24)	SPRTLNAWV
		B7/42/81-TL9 (p24)	TPQDLNTML
		B7/42/81-HA9 (p24)	HPVHAGPIA
		B7/42/81-GL9 (p24)	GPSHKARVL
		B7/42/81-FL9 (Nef)	FPVRPQVPL
		B7/42/81-RM9 (Nef)	RPQVPLRPM
		B7/42/81-RGF9 (Nef)	RPMTFKGAF
		B7/42/81-TL10 (Nef)	TPGPGVRYPL
		B7/42/81-SM9 (RT)	SPAIFQSSM
		B7/42/81-FL9 (Vpr)	FPRPWLHGL
		B7/42/81-RI10 (gp120)	RPNNNTRKSI
		B7/42/81-IA9 (gp41)	IPRRQGFEA
		B7/42/81-HI10 (Vif)	HKRVSSEVHI
		B7/42/81-LI9 (Int)	LPPIVAKEI
		B7/42/81-TL11 (Pro)	TPVNIIGRNML
		B7/42/81-YL9 (RT)	YPGIKVKQL
		B7/42/81-RL10 (Rev)	RPAEPVPLQL
		B7/42/81-RLY10 (p17)	RLRPGGKKHY
		B81/42/81-SL10 (RT)	SPIETVPVKL
		B7/42/81-VM9 (RT)	VPVKLKPGM
		B7/42/81-VL9 (RT)	VASCDKCQL
		B7/42/81-RQL11 (RT)	RPPLVKLWYQL
		B7/42/81-TM10 (Pro)	TPVNIIGRNM
		B7/42/81-KL9 (Vif)	KPKKIKPPL
		B7/42/81-TL11 (Pro)	TPGIRYQYNVL
		B7/42/81-GL9 (RT)	GPKVKQWPL
	HLA-B8	B8-GL8 (p17)	GGKKHYML
		B8-EV9 (p17)	ELRSLYNTV
		B8-DI8 (p24)	DIYKRWII
		B8-DL9 (p24)	DCKTILRAL
		B8-WM8 (Nef)	WPAIRERM
		B8-FL8 (Nef)	FLKEKGGL
		B8-GL9 (RT)	GPKVKQWPL
		B8-YL8 (gp41)	YLKDQQLL
		B8-EL8 (p15)	EPKDREPL
		B8-VI8 (RT)	VPRRKAKI
		B8-NL11 (p24)	NPDCKTILRAL
		B8-MK15 (p24)	MTDTLLVQNANPD
		B8-LL13 (p24)	LVQNANPDCKTIL
	HLA-B15	B1503-FY10 (Tat)	FQTKGLGISY
		B1503-FY10 (Int)	FKRKGGIGGY
		B1503-VF9 (p24)	VKVIEEKAF
		B1503-IY9 (RT)	IQQEFGIPY
		B1503-RY9 (RT)	RKAKIIKDY
		B1503-GL9 (RT)	GKKAIGTVL
		B1510-RL9 (p17)	RFALNPGLL
		B1510-VL10 (p24)	VHQAISPRTL
		B1510-GL9 (p24)	GHQAAMQML
		B1510-WI9 (Vif)	WHLGHGVSI
		B1510-KL10 (p17)	KHLVWASREL
		B1510-GI9 (p17)	GKVSQNYPI
		B1510-IL9 (Rev)	IHSISERIL
		B1510-TIL9 (RT)	THLEGKIIL
		B1510-VL9 (RT)	VASCDKCQL
		B1510-RI11 (Vif)	RHPKVSSEVHI
		B1510-GL11 (Rev)	GRPAEPVPLQL
		B1510-KL9 (Pol)	KHQKEPPFL
		B1510-KL8 (p17)	KHYMLKHL

Table 2.6 continued….

Class 1 family	Class 1 allele	Peptide identification[a]	Peptide sequence
HLA-B	HLA-B44	B44-SL9 (p24)	SEGATPQDL
		B44-RL11 (p24)	RDYVDRFFKTL
		B44-AW11 (p24)	AEQATQDVKNW
		B44-GV11 (Nef)	GEVGFPVRPQV
		B44-MY9 (gp120)	MGNLWVTVY
		B44-AA9 (RT)	AETFYVDGA
		B44-II10 (p24)	IEEKAFSPEVI
		B44-SY11 (RT)	SEVNIVTDSQY
		B44-IW11 (RT)	IEELREHLLKW
		B44-QW11 (RT)	QEEHEKYHSNW
		B44-GL10 (p24)	GDIYKRWIIL
		B44-GL9 (p24)	GDIYKRWII
		B44 IV10 (p24)	IEEKAFSPEV
	HLA-B57	B57-WF9 (p17)	WASRELERF
		B57-ISW9 (p24)	ISPRTLNAW
		B57-KF11 (p24)	KAFSPEVIPMF
		B57-DW10 (p24)	DTINEEAAEW
		B57-TW10 (p24)	TSTLQEQIAW
		B57-QW9 (p24)	QATQDVKNW
		B57-HW9 (Nef)	HTQGYFPDW
		B57-QY10 (Rev)	QAVRIIKILY
		B57-IW9 (RT)	IQLPEKDSW
		B57-IAW9 (RT)	IAMESIVIW
		B57-SW10 (Int)	SAAVKAACWW
		B57-KF9 (Int)	KTAVQMAVF
		B57-AW9 (Vpr)	AVRHFPRPW
		B57-KW11 (Env)	KAYETEVHNVW
		B57-VF9 (Vif)	VSRRANGWF
		B57-KAF9 (Nef)	KAAFDLSFF
		B57-AF10 (RT)	QATWIPEWEF
		B57-FF9 (RT)	FSVPLDEDF
		B57-EY9 (RT)	ETKIGKAGY
		B57-KI13 (RT)	KAGYVTDRGRQKI
		B5802-QL11 (gp120)	QTRVLAIERYL
		B57-LW9 (Vif)	LGHGVSIEW
		B57-TWS10 (gp41)	TTAVPWNSSW
		B57-LL9 (RT)	LGIPHPAGL
		B57-FW11 (RT)	FAIKKKDSTKW
		B57 KR10 (Pol)	KAGYVTDRGR
		B57 VI9 (Pol)	VTDRGRQKI
HLA-Cw	HLA-Cw4	Cw4-QW9(p24)	QATQDVKNW
		Cw4-SF9 (gp120)	SFNCRGEFF
		Cw4-YY8(Nef)	YFPDWQNY
		Cw4-WM13 (gp41)	WSNKSQEEIWDNM
		Cw4-EW11 (gp41)	EIWDNMTWMQW
		Cw4-KY12 (RT)	KKSVTVLDVDAY
		Cw4-IW10 (Env)	IWDNMTWMQW
		Cw4-TF10 (RT)	TVLDVDAYF
		Cw4-IY11 (gp120)	IPIHYCAPAGY
	HLA-Cw6	Cw6-LR10 (RT)	LKTGKYAKMR
		Cw6-KK11 (RT)	KMRTAHTNDVK
		Cw6-II13 (gp120)	IRSENLTNNAKTI
		CW6-LL12 (gp120)	LTNNAKTIIVHL
		Cw06-MV9 (RT)	MRTAHTNDV
		Cw06-AL8 (gp120)	AKTIIVHL
		CW0602-KR9 (Pol)	KTGKYAKMR
	HLA-Cw17	Cw1701-HL10 (Vpu)	HLRLLDVNDL
		Cw1701-TL11 (RT)	TPGIRYQYNVL
		Cw1701-PL9 (Rev)	PAEPVPLQL
		Cw1701-KL9 (Vif)	KPKKIKPPL
		Cw1701-RL10 (Rev)	RPAEPVPLQL

[a]Peptide identification provides the restricting HLA Class I allele, the first and last amino acid of the respective epitope, the length of the epitope, and the HIV-1 protein containing the epitope.

Table 2.7. HLA frequencies in study cohort and the percentage of subjects with responses against peptides associated with individual HLA expression.

HLA Class I Allele	Frequency (%)	Epitopes Targeted / Epitopes Tested at:		Subjects (%) with Responses at:	
		6-8 w	12-18 w	6-8 w	12-18 w
A23	21	3/9	5/9	40	60
A26	17	1/3	1/3	25	50
A29	25	4/9	5/9	60	67
A30	38	5/14	8/14	75	63
B1503	25	4/6	6/6	100	100
B1510	21	6/11	7/11	100	100
B42	29	8/26	13/26	100	100
B44	33	4/13	4/13	100	67
B58	21	3/27	5/27	20	67
Cw4	25	4/9	2/9	67	50
Cw6	21	1/7	2/7	20	40
Cw17	25	1/5	1/5	17	25
Median Values	**25**	**4/9 (44%)**	**5/9 (56%)**	**63**	**65**

To further define these relationships, we assessed the persistence of early epitope-specific immune responses for the most frequently targeted early epitopes (Figure 2.3). For five of the seven epitopes tested, there was a decrease over time in the targeting of these epitopes in persons expressing the restricting HLA allele, but the immunodominance patterns of CD8+ T-cells remained unchanged in the individuals with detectable responses. There was no shift in the immunodominance hierarchy in HLA-B*1503 restricted responses measured at 6-8 and 8-12 weeks against the HLA-B*1503-restricted epitopes RT(Int)-FY10 and p24-IY9, and the epitopes were found to be immunodominant and subdominant, respectively.

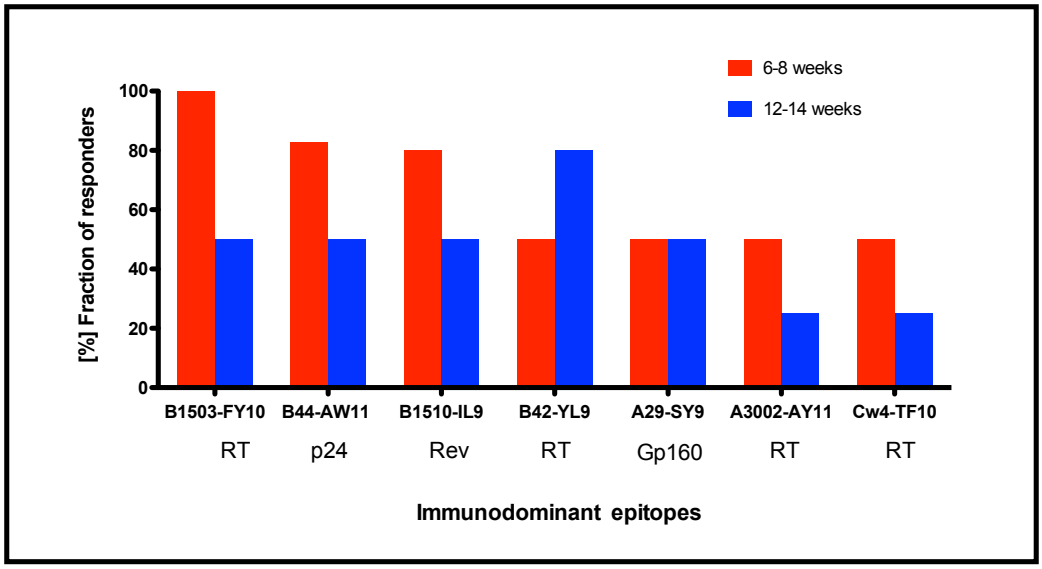


Figure 2.3. Immunodominance patterns of HIV-1-specific CD8+ T-cell responses restricted by individual HLA class I alleles at 4-6 weeks and 12-18 weeks post the estimated time of infection. The percentage of participants expressing the respective allele that had detectable peptide-specific CD8+ T-cell responses is shown on the *y* axis. HLA-restricted HIV-specific CD8+ T-cell epitopes are aligned on the *x* axis and their location on the major HIV-1 proteins is also indicated. Data are shown only for HLA class I alleles that were expressed in at least three individuals, and for which at least three HIV-1-specific optimal CD8+ T-cell epitopes had been defined.

Similarly, CD8+ T-cell responses against the HLA-B*44-restricted epitope p24-AW11 and the HLA-B*1510 restricted epitope Rev-IL9 remained immunodominant during the earliest and latest tested time points in individuals expressing these respective alleles. However, there was a shift in the hierarchy of responses in individuals expressing the HLA-A*30 where the initially immunodominant RT-AY11 epitope later became subdominant and CD8+ T-cell responses against the initially subdominant Int-KIY9 epitope became immunodominant (Figure 2.3). Overall, these data demonstrate the immunodominance patterns of specific epitopes during acute infection are persistent for the majority of HLA class I alleles which could be assessed, but that

many class I alleles are infrequently utilized in early infection, and many potentially immunogenic epitopes are not targeted.

4.5. Effect of sequence variation on recognition by specific CTL

Others (Turnbull et al., 2009) and we (Altfeld et al., 2003) have shown that up to 30% of early responses may be missed when using peptides representing a reference strain of virus. We therefore sequenced plasma virus to determine if lack of CD8+ recognition related to lack of expression of the cognate epitope. We focused these efforts on the *gag* region, as targeting of Gag has been associated with lower viral loads (Kiepiela et al., 2007). Plasma virus sequencing revealed that of the 56 epitope sequences evaluated, 31 were wild-type virus sequences; however, these did not elicit detectable CD8+ T-cell responses in 20 of 31 (65%) of cases (Table 2.8). In the two subjects in whom we failed to detect any CD8+ T-cell responses prior to seroconversion (Table 2.4; AS2-945 and AS2-973), both were infected with viruses that contained epitopes present in the reference set of peptides used in the assays. These data indicate that despite expression of the restricting HLA class I allele and the presence of cognate viral sequence, CD8+ responses to the virus were often not mounted, suggesting that the immunogenicity of these epitopes is suboptimal, and that factors other than IFN-gamma expressing CD8+ T-cells may be involved in the initial decline in viremia.

Table 2.8. Sequence analysis of autologous virus Gag epitopes at 6-8 weeks post infection.

Participant Number	HLA Type	HLA Restricted Epitope	CD8+ T cell Response
		EVIPMFTAL	
AS2-0016	A26	.....	-
AS3-0017		.....	+
AS1-0703		.I.....	-
AS3-0513		.....	-
		RSLYNTVATLY	
AS3-0268	A29	K..F.....	+
AS3-0458		.....I....	-
AS1-0919		K..F.....	-
AS1-0323		K..F.....	-
AS2-0184		K..F.....C	-
AS2-0110		K.....V..	-
		RLRPGGKKHY	
AS2-0802	A30	.....Q.	-
AS2-0050		.....	+
AS2-0973		.....	-
AS2-0483		.....Q.	-
AS2-0358		.....R.	-
AS2-0016		.....C.	+
AS1-0703		.....	-
AS2-0174		K.....Q.	-
		RSLYNTVATLY	
AS2-0802	A30	.....	+
AS2-0050		.....	-
AS2-0973		K.....I....	-
AS2-0483		K.....	-
AS2-0358		...F.....	-
AS2-0016		...F.....	-
AS1-0703		...F.....	-
AS2-0174		.....	-
		VKVIEEKAF	
AS2-1037	B1503	.....	-
AS1-0876		.....	-
AS3-0268		.....	+
AS2-0483		.....G.	-
AS2-0341		.....G.	-
AS2-0050		.....	+

Table 2.8. ***continued.***

Participant Number	HLA Type	HLA Restricted Epitope	CD8+ T cell Response
		VHQAISPRTL	
AS2-0945	B1510	....	-
AS3-0369		....	-
AS2-1037		....	+
AS3-0513		....	-
		GHQAAMQML	
AS2-0945	B1510	....	-
AS3-0369		....	-
AS2-1037		....	-
AS3-0513		.......I.	+
		EQATQDVKNW	
AS1-0703	B44	....	+
AS3-0458		....	+
AS2-0184		....	+
AS3-0369		....S.....	+
AS1-0876		...G..E....	-
		SEGATPQDL	
AS1-0703	B44	....	-
AS3-0458		....	-
AS2-0184		......T..	-
AS3-0369		....	-
AS1-0876		....	+
		RDYVDRFFKTL	
AS2-0110	B44	....	-
AS1-0703		........R..	-
AS3-0458		........R..	-
AS2-0184		....	+
AS3-0369		....	-
AS1-0876		....	-

5. Discussion

Despite the dominance of HIV-1 subtype C worldwide, the evolution of virologic and immunologic parameters in acute HIV-1C infection have not been extensively characterized. By RNA screening of persons testing negative by standard antibody assays, we were able to recruit 20 subjects before seroconversion, and characterize viral kinetics and evolving HIV-specific CD8+ T-cell responses during acute infection. Despite high viremia, responses were narrowly directed, and the majority of epitopes targeted in chronic infection (Kiepiela et al., 2007) did not induce detectable responses during the rapid decline of viremia. Although the ELISPOT assay can underestimate the true magnitude of T-cell responses (Sun et al., 2003, Turnbull et al., 2009, Goonetilleke et al., 2009) and autologous virus sequences can differ from the reference strains used, HIV-1-specific CD8+ responses ultimately arose in these persons, indicating that many immunogenic epitopes are not targeted in the earliest stages of infection, at a time when viral load is rapidly declining.

Longitudinal assays for responses during and following acute infection allowed us to address not only the specificity of responses but also their persistence. In the entire cohort, responses to Nef-derived peptides were dominant in the earliest stages of infection, consistent with other data (Blankson, 2009, Celum et al., 2010, Harari et al., 2009). However, only approximately half of the individuals tested targeted Nef in the early stages, although 82% targeted this protein at some time during the average 5 months of follow up (data not shown), again

suggesting impaired induction of responses in acute infection. A trend was observed where high T-cell responses against the Nef proteins correlated positively with high viral loads, as has been reported in other studies (Lichterfeld et al., 2004b, Blankson, 2009, Novitsky et al., 2003, Masemola et al., 2004a, Masemola et al., 2004b), suggesting that these responses are driven by level of antigenemia, rather than they being causal in lowering viral load, again suggesting impaired functional CD8+ T-cell responses in the earliest stages of acute infection. However, there was no correlation between the magnitude and breadth of CD8+ T-cell responses for other viral proteins and the concurrent plasma viral load, consistent with other reports (Addo et al., 2003, Betts et al., 2001, Cao et al., 2003a).

By assessing responses using peptides representing optimal epitopes, we were able to assess the hierarchy in the development of epitope-specific CD8+ T-cell responses restricted by specific HLA alleles, their immunodominance patterns, and the timing of induction of these responses. Within a month of the estimated time of infection, CD8+ T-cell responses were detected against 44% of peptide epitopes matched for each subjects' HLA and against 56% of the epitopes presented by their expressed alleles at 3 months. These data confirmed that the detectable responses in early infection are largely maintained, although immunodominance often shifts. Further investigation is required to determine whether the changes in the magnitude of responses and immunodominance are the result of sequence evolution within targeted epitopes or their flanking

regions (Altfeld et al., 2006), immunoregulation (Day et al., 2006, Trautmann et al., 2006, Petrovas et al., 2006, Kaufmann et al., 2007), or other mechanisms.

6. Conclusion

We demonstrate that HIV-1-specific CD8+ T-cell responses can be detected before complete seroconversion, but many of the epitopes that elicit responses in chronic infection are not immunogenic during acute infection. We also confirm previous studies showing that the initial HIV-1 specific immune responses are narrowly directed, and extend these by showing the paucity of responses even when dramatic drops in viremia have occurred. Moreover, although response breadth and magnitude expands with duration of infection, a significant proportion of individuals are still unable to make responses despite the presence of cognate peptides restricted by the corresponding HLA allele. Further studies are needed to address why recognition of HIV-1 peptides appears to be selective during acute infection as the lack of recognition may contribute to the failure to control viremia to a low set point. Moreover the paucity of responses during the dramatic reduction in viremia suggests that tissue specific responses as well as measures of CD8+ T-cell function other than IFN-gamma should be examined to better define the role of HIV-specific CD8+ T-cells in the initial decline in viremia. Further studies are needed to understand the determinants and role of HIV-1-specific CD8+ T-cell responses in high burden settings where an effective HIV-1 vaccine is urgently needed.

Chapter 3 : Progressive broadening of HIV-1 Gag-specific CD8+ T-cell responses is associated with viral control despite limited immunogenicity and infrequent immune escape in early HIV-1 infection

1. Abstract

Background: We characterized protein-specific CD8+ T-cell immunodominance patterns during the first year of HIV-1 infection, and their impact on viral evolution and immune control.

Methods: We analyzed CD8+ T-cell responses to the full HIV-1 proteome during the first year of infection in eighteen antiretroviral-naïve individuals with acute HIV-1 subtype C infection, all identified prior to seroconversion. E*x vivo* and cultured IFN-γ ELISPOT assays were performed and viruses from plasma were sequenced within defined CTL Gag epitopes.

Results: Nef-specific CD8+ T-cell responses were dominant during the first 4 weeks post infection and made up 40% of total responses at this time, yet by 1 year responses against this region had declined and Gag responses made up 47% of all T-cell responses measured. An inverse correlation between the breadth of Gag-specific responses and viral load set point was evident at 26 weeks ($p=0.0081$; $r= -0.60$) and beyond. An inverse correlation between the number of persistent responses targeting Gag and viral set point was also identified ($p=0.01$; $r=-0.58$). Gag-specific responses detectable by cultured ELISPOT correlated negatively with viral load set point ($p=0.0013$; $r=-0.91$). Sequence evolution in targeted and non-targeted Gag epitopes in this cohort was infrequent.

Conclusions: These data underscore the importance of HIV-specific CD8+ T-cell responses, particularly to the Gag protein, in the maintenance of low viral load levels during primary infection and show that these responses are initially poorly elicited by natural infection. These data have implications for vaccine design strategies.

2. Introduction:

There is compelling evidence that acute-phase HIV-specific CD8+ T-cell responses play an important role in determining the subsequent clinical course of disease. Experimental data and mathematical models show that the appearance of HIV-1-specific CD8+ T-cells coincides with and contributes to the decline in peak viremia in acute infection (Borrow et al., 1994, Koup et al., 1994, Goonetilleke et al., 2009, Pantaleo et al., 1994, Liu et al., 2013), and CD8+ T-cell responses have been detected in some cases when antibodies are not yet detectable by ELISA and Western blot assays (Huber and Trkola, 2007, Radebe et al., 2011, Liu et al., 2013, Goonetilleke et al., 2009). An indicator of CD8+ T-cell immune pressure on the virus is the emergence of viral escape within CTL epitopes, which has been demonstrated to occur as early as 10 days after the first detection of a CD8+ T-cell response during acute HIV-1 infections (Fischer et al., 2010, Goonetilleke et al., 2009), but the kinetics of escape is quite variable (Brumme et al., 2008, Liu et al., 2013). Together these data indicate that early immune responses are critical for immune containment of HIV infection.

Despite strong data that HIV-1-specific CD8+ T-cells play a critical role in viral control, there is also evidence of marked heterogeneity in the antiviral effectiveness of these cells. First, is the observation that almost all HIV-1-infected individuals have detectable CD8+ T-cell responses, irrespective of viral control or disease progression status (Betts et al., 2001, Addo et al., 2003), suggesting that some CD8+ T-cells are inefficient at suppressing virus replication (Gray et al., 2009, Chen et al., 2009, Ferrari et al., 2011, Perreau et al., 2013).

Indeed, even in late stage infection the majority of detectable HIV-specific CD8+ T-cell responses are able to target the circulating virus (Draenert et al., 2004, Koibuchi et al., 2005). Secondly, it has been demonstrated that CD8+ T-cells targeting different viral proteins differ in their antiviral effectiveness (Chen et al., 2009), consistent with clinical data that Gag but not Env- or Nef-specific responses are associated with lower viremia (Saez-Cirion et al., 2009, Julg et al., 2010, Chen et al., 2009, Perez et al., 2013, Kiepiela et al., 2007, Turk et al., 2013). Third, there is variability in the hierarchy of responses following infection and the ability of HLA-restricted CD8+ T-cell responses to drive viral immune escape is not consistent and remains incompletely characterized (Brumme et al., 2008, Streeck et al., 2009b, Liu et al., 2013).

Studies conducted during acute and early HIV-1 infection offer an important opportunity to characterize and understand better the quality and phenotype of CD8+ T-cells that mediate control of viral replication and disease progression. Numerous studies have examined these responses in early stages of infection (Turk et al., 2013, Mlotshwa et al., 2010, Gray et al., 2009, Streeck et al., 2009b, Henn et al., 2012, Freel et al., 2011, Borrow et al., 1994, Koup et al., 1994, McMichael et al., 2010, Fischer et al., 2010, Lichterfeld et al., 2004b) but less is known about the impact and fate of CD8+ T-cell immune responses in cohorts limited to persons with clearly defined dates of acute infection in whom studies have been initiated within a month of exposure to the virus (Goonetilleke et al., 2009, Ferrari et al., 2011, Turk et al., 2013, Mlotshwa et al., 2010, Radebe et al., 2011, Jones et al., 2004, Gray et al., 2009). A number of studies have reported on

the longitudinal analysis of CD8+ T-cell responses in relation to time from initial acute or primary infection and viral load, in order to determine the kinetics and fate of these responses and their impact on the virus over time (Ferrari et al., 2011, Mlotshwa et al., 2010, Streeck et al., 2009b, Perez et al., 2013, Liu et al., 2013, Goonetilleke et al., 2009, Allen et al., 2005, Brumme et al., 2008, Turk et al., 2013). However, very few studies have assessed CD8+ T-cell immune responses to the entire viral proteome during acute infection and at one year in antiretroviral therapy naïve persons with clearly defined dates of infection, in high prevalence and incidence settings (Mlotshwa et al., 2010, Liu et al., 2013). This is an important consideration in view of evidence of viral adaptation to endemic population HLA and loss of CTL epitopes over the course of the epidemic (Kawashima et al., 2009, Schellens et al., 2011) and the emerging evidence of high transmission of escape variants (Goepfert et al., 2008, Wright et al., 2011).

Here, using a cohort of acutely infected, antiretroviral therapy naive individuals with a reliable estimated time of infection in a high HIV-1 prevalence and incidence setting, we have conducted a longitudinal analysis of immune responses in persons with acute HIV-1 subtype C infection identified prior to full seroconversion in order to define the specificity of the earliest CD8+ T-cell responses, the fate of these responses over time, the evolving immunodominance patterns and impact on viral control within the first year of infection. Our data provide evidence of marked differences in immunogenicity of individual viral proteins for induction of HIV-specific CD8+ T-cell responses during

acute HIV infection, and lack of a clear relationship of early CD8+ T-cell responses to viral load. However, over the one year period of follow up, the number of targeted Gag CD8+ T-cell epitopes increased resulting in a significant negative association between these responses and viral load set point. CD8+ T-cell immune responses generated during the acute phase of infection persisted or waned irrespective of cognate epitope sequence, with persistence of initial responses correlating with lower viral set point. Moreover, some Gag-specific immune responses that were no longer detected by standard IFN-gamma ELISPOT assay persisted as memory responses and appeared to contribute to lower viremia. This information sheds light on the characteristics and antiviral efficacy of evolving CD8+ T-cell immune responses responsible for long-term control of HIV-1 infection in a high incidence setting and has important implications for both prophylactic and therapeutic vaccine design for this population.

3. Materials and methods

3.1. Study participants

The cohort consisted of 18 antiretroviral treatment-naïve individuals with acute HIV-1 subtype C infection enrolled as part of the HIV Pathogenesis Programme (HPP) Acute Infection Study in Durban, KwaZulu-Natal, South Africa (Radebe et al., 2011). Briefly, study participants were recruited from HIV Counseling and Testing (HCT) centers in the greater Durban area based on negative HIV rapid testing, and those testing positive by plasma HIV RNA were offered enrollment into the study. Blood samples were obtained from study participants at 2-4, 4-6, 6–8, 8–12, 12–18, 24-26, and 50-52 weeks post-infection. The date of infection was estimated to be 14 days prior to screening, as previously described (Radebe et al., 2011). The median age at recruitment was 28 years (interquartile range [IQR] 26 to 40 years), and 60% of the participants were female. The median plasma viral load at enrollment was 5,572,160 RNA copies/ml, (IQR, 921,090 to 9,320,000 RNA copies/ml) and the median CD4 count was 389 cells/mm^3 (IQR, 275 to 496 cells/mm^3). The average viral load between 3 and 12 months post infection was the measure of viral load set point used in this study and could be calculated for all study subjects. The Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal and the institutional review board of Massachusetts General Hospital approved this study, and all the participants provided written informed consent for participation in this study.

3.2. Synthetic HIV-1 subtype C peptides

A panel of 410 consensus subtype C peptides (18-mers overlapping by 10 amino acid residues), spanning the entire HIV-1 proteome corresponding to the gene products Gag, Pol, Nef, Env, Vif, Vpr, Vpu, Tat, and Rev were synthesized on an automated peptide synthesizer (MBS 396; Advanced ChemTech, Louisville, Kentucky) and used in the ELISPOT assay as previously described (Thobakgale et al., 2007). HLA class I-restricted epitopes predicted from the published epitopes on the HIV immunology database were also tested based on the HLA type (Llano et al., 2013) of the individual.

3.3. Viral load determination, CD4 T-cell counts, and HLA typing

Plasma HIV-1 RNA levels were quantified using the Roche Amplicor version 1.5 or Cobas Taqman HIV-1 Test according to the manufacturer's instructions (Roche Diagnostics, Branchburg, NJ). Absolute blood CD4 T-cell counts were enumerated using Tru-Count technology and analysed on a FACSCalibur flow cytometer (Becton Dickinson) and were expressed as cells/mm^3. High-resolution HLA typing was performed as previously described (Radebe et al., 2011).

3.4. Interferon-γ ELISPOT Assay

IFN-γ enzyme-linked immunosorbent spot assays (ELISPOT) were performed as described previously (Streeck et al., 2009a, Radebe et al., 2011, Thobakgale et al., 2007). Briefly, peripheral blood mononuclear cell (PBMC) samples were stimulated with HIV-1 peptide pools (2 μg/ml) and incubated overnight at 37°C

with 5% CO_2. Phytohemagglutinin (PHA) was used as a positive control (duplicate wells) at a final concentration of 4 μg/ml and negative controls consisted of cells without stimuli (quadruplicate wells). Confirmation of positive responses at the single-peptide level within peptide pools was undertaken in a second ELISPOT assay. A response was defined as positive when there were at least 100 spot-forming cells (SFCs)/million PBMCs and the total number of spots was 3 standard deviations above the negative control value (Novitsky et al., 2001, Ngumbela et al., 2008). To be conservative in estimating the breadth of a response, responses to 2 overlapping peptides were classified as one response; to 3 overlapping peptides were classified as 2 responses. In addition to overlapping peptides, HLA class I-restricted epitopes predicted from the published epitopes on the HIV immunology database (Llano et al., 2013), based on the expressed HLA alleles of the participants were also tested at a peptide concentration of 2 μg/mL.

3.5. Cultured IFN-γ ELISPOT Assay

The cultured IFN-γ enzyme-linked immunosorbent spot assays were performed as described previously (Ndhlovu et al., 2012, Calarota et al., 2008). Briefly, 5-10 × 10^6 peptide-stimulated were cultured at 37°C and 5% CO_2 for 12 days in 5-10 ml of RPMI medium containing 10% heat-inactivated fetal calf serum (R10 medium) and supplemented with 50 units/ml of recombinant human interleukin 2 (IL-2)(R10/50 medium). Unstimulated cells cultured with IL-2 (R10/50 medium) alone were used as controls. A peptide concentration of 100 ng/ml (the final concentration of each peptide within the pool) was selected for these

experiments. Fresh R10/50 medium was added to the cultures at day 3, day 7, and day 10 as needed. On day 12, cells were harvested, washed three times with fresh R10 medium and rested at 37°C and 5% CO_2 overnight in fresh R10 medium. Cells were then plated for a standard ELISPOT assay as described for the *ex vivo* ELISPOT.

3.6. Virus sequencing

Viral RNA was isolated from 140μl of plasma samples using the QIAamp Viral RNA Extraction Mini Kit (Qiagen, Hilden, Germany). Viral RNA was then reverse transcribed using ThermoScript™ RT-PCR System kit (Invitrogen, Carlsbad, CA, USA) and the gene-specific primer, GagD reverse as previously described (Chopera et al., 2008). To amplify the HIV-1 Gag region, a nested PCR was performed with two sets of primers, Gag forward (5′-TCT CTA GCA GTG GCG CCC G-3′) and GagD reverse for the first round and GagA forward (5′-CTC TCG ACG CAG GAC TCG GCT T-3′) and GagC reverse (5′-TCT TCT AAT ACT GTA TCA TCT GC-3′) for the second round as previoulsy described (Chopera et al., 2008). Resulting PCR products were purified using purification columns from Illustra™ GFX™ PCR DNA Gel Band Purification Kit, (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and cloned into a PCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was isolated from individual randomly picked white bacterial colonies (GeneJet Plasmid Mini Prep Kit; Fermentas, Vilnius, Lithuania) and were screened for the presence of the insert with the EcoRI restriction enzyme (New England BioLabs, Ipswich, Massachusetts, USA). Sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.4

(Applied Biosystems, Foster City, CA, USA). Sequences were assembled and edited using the Sequencher Program v5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

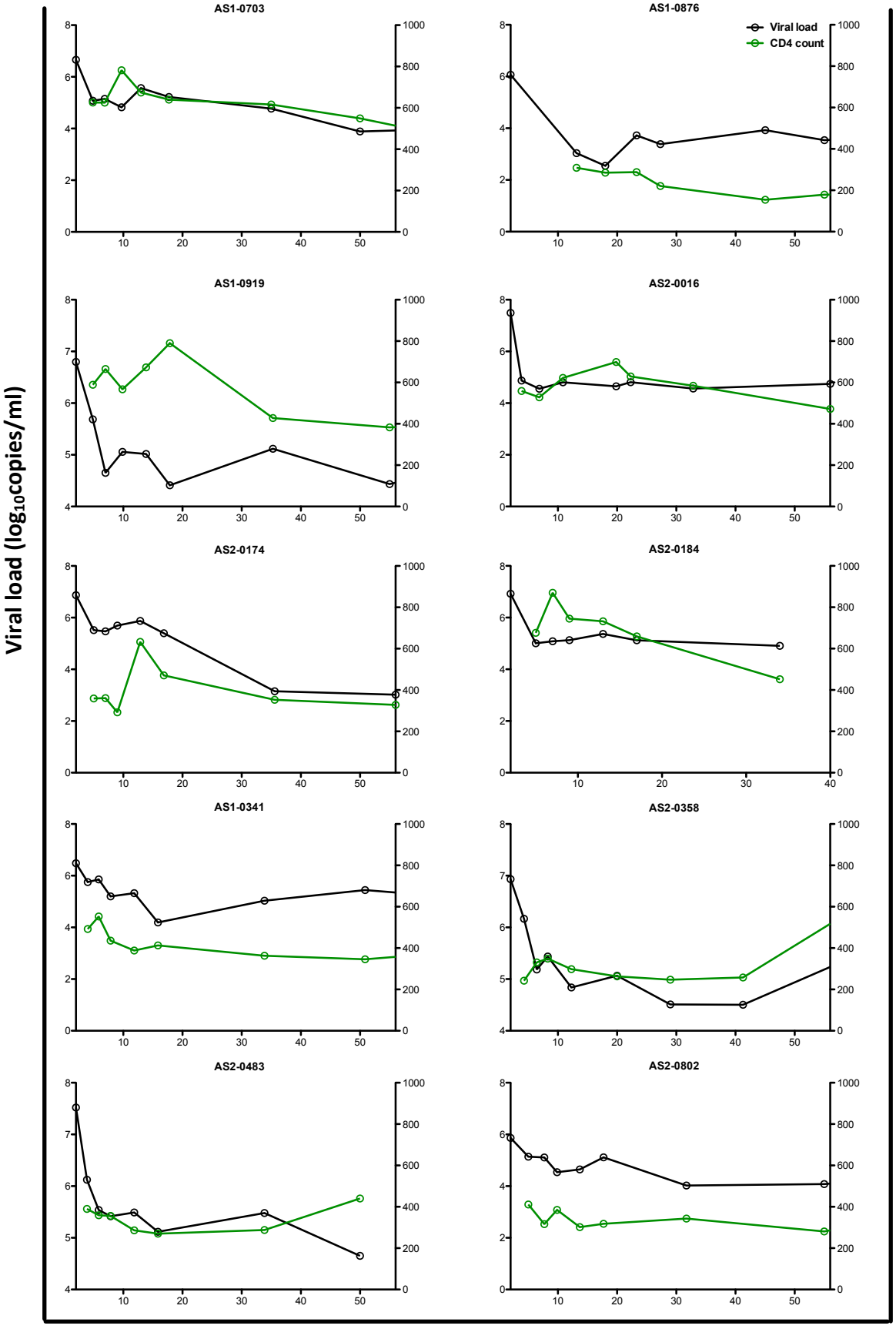
3.7. Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism software version 5.0 for Windows. For assessments of the relationship between immune responses and viral load, statistical analysis of significance was based on two-tailed *t* tests using Spearman rank correlations. A value of <0.05 was considered statistically significant.

4. Results

4.1. Association of CD8+ T-cell responses with viral load

In earlier studies, we examined the breadth and specificity of CD8+ T-cell responses to HIV during the first 18 weeks of infection in twenty subjects with acute HIV infection (Radebe et al., 2011). Here we extended these studies by examining changes in the frequency of recognition, magnitude and breadth of HIV-1-specific CD8+ T-cell responses during the entire first year of infection. Viral load measurements and CD4+ T-cell counts from the 18 individuals from whom blood samples were available at sequential time points over the first year of infection are shown in Figure 3.1. We screened 18 subjects using the IFN-γ ELISPOT assay with synthetic peptides spanning HIV-1 Gag, Pol, Env, Nef, Tat, Vpr, Rev, Vif, and Vpu. PBMCs were stimulated with pools of overlapping 15- to 20-mer peptides, in addition, each subject's PBMCs were evaluated according to their class I HLA type for selective recognition of 8- to 11-mers corresponding to previously defined class I MHC-restricted HIV-1-specific CTL epitopes (Llano et al., 2013). A summary of the defined class I MHC-restricted HIV-1 epitopes targeted per subject is given in Table 3.1 and depicted in Figures 3.2 and 3.3.


AS1-0703
AS1-0876
Viral load
CD4 count
AS1-0919
AS2-0016
AS2-0174
AS2-0184
AS1-0341
AS2-0358
AS2-0483
AS2-0802
Viral load (log10copies/ml)
CD4+ cells/mm3
Weeks post infection

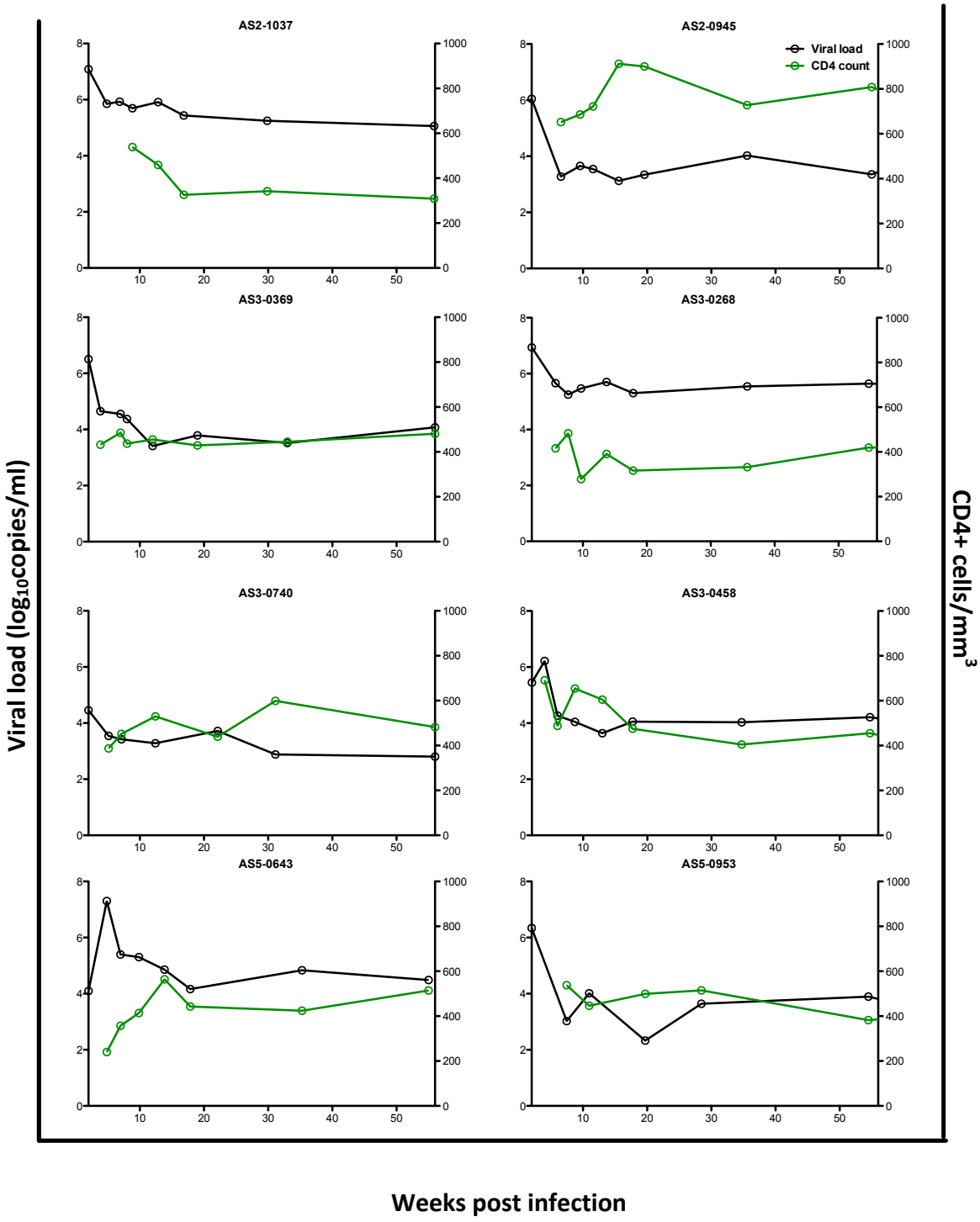


Figure 3.1. Alignment of viral load data from 18 subjects during acute HIV infection and over the first year of infection. For each patient, the viral load (log_{10}copies/ml plasma) and CD4+ T-cell counts determined at sequential time points throughout the course of acute and early infection are plotted against time, measured weeks post infection.

Table 3.1. HIV-1-specific $CD8^+$-T-cell responses: breadth, HLA class I usage, and total frequency during the first year of HIV-1C infection.

Participant ID	HLA Class I Type	No. of MHC class I defined epitopes targeted in:									Total no. of epitopes per subject	No. of restricting class I alleles	Viral load set point (Log_{10}copies/ml)
		Nef	Gag	Pol	Vif	Vpr	Vpu	Env	Rev	Tat			
AS1-0703	A*2601/3004, B*4403/5802, C*0210/0602	1	5	3				1		1	11	3	4,89
AS1-0876	A*2301/4301, B*1503/4400, C*2100/1008	1	3	5							9	4	3,61
AS1-0919	A*2900/7400, B*3500/4201, C*0400/1700	3	4	6	1			1		1	16	5	4,44
AS2-0016	A*2600/3000, B*1518/4201, C*1700/1800	3	5	4		1					13	4	4,70
AS2-0174	A*2301/3002, B*0801/1402, C*0304/0802	1	10	4				2	1		18	6	3,20
AS2-0184	A*2300/2900, B*0800/4400 C*0700/0700	4	4		1			3			12	3	4,60
AS2-0341	A*3400/6800, B*1503/5802, C*0200/0600	1		3	1					1	6	2	4,93
AS2-0358	A*2400/3000, B*4202/5300, C*0400/1700	6	7	8				2	1		24	6	5,18
AS2-0483	A*3000/3400, B*1503/4201, C*0200/1700	2	7	7		1				1	18	3	4,79
AS2-0802	A*3000/4301, B*0800/5802, C*0600/0700	1	6	3				3			13	4	5,20
AS2-0945	A*2300/7400, B*1510/5703, C*0700/1600	2	6	5				3	1		17	4	4,40
AS2-1037	A*0300/3400, B*1503/1510, C*0200/0300	2	5	1	2			1	1	1	13	4	3,61
AS3-0268	A*2902/8001, B*1503/1801, C*0202/0210	2	3	2	1			2		1	11	3	5,57
AS3-0369	A*6602/6800, B*1510/4400, C*0300/0700	1	7	3					1		12	6	3,78
AS3-0458	A*2900/3400, B*4400/5802, C*0400/0600	4	7	5							16	4	3,97
AS3-0740	A*0200/3400, B*1510/4400, C*0400/0500	1	6	1	1				3		12	4	3,38
AS5-0643	A*0201/3001, B*1503/4201, C*0210/1700	1	2	2		1		2			8	5	4,67
AS5-0953	A*2402/7400, B*0702/5703, C*0701/0702	1	5	3	1	1		3			14	4	3,62

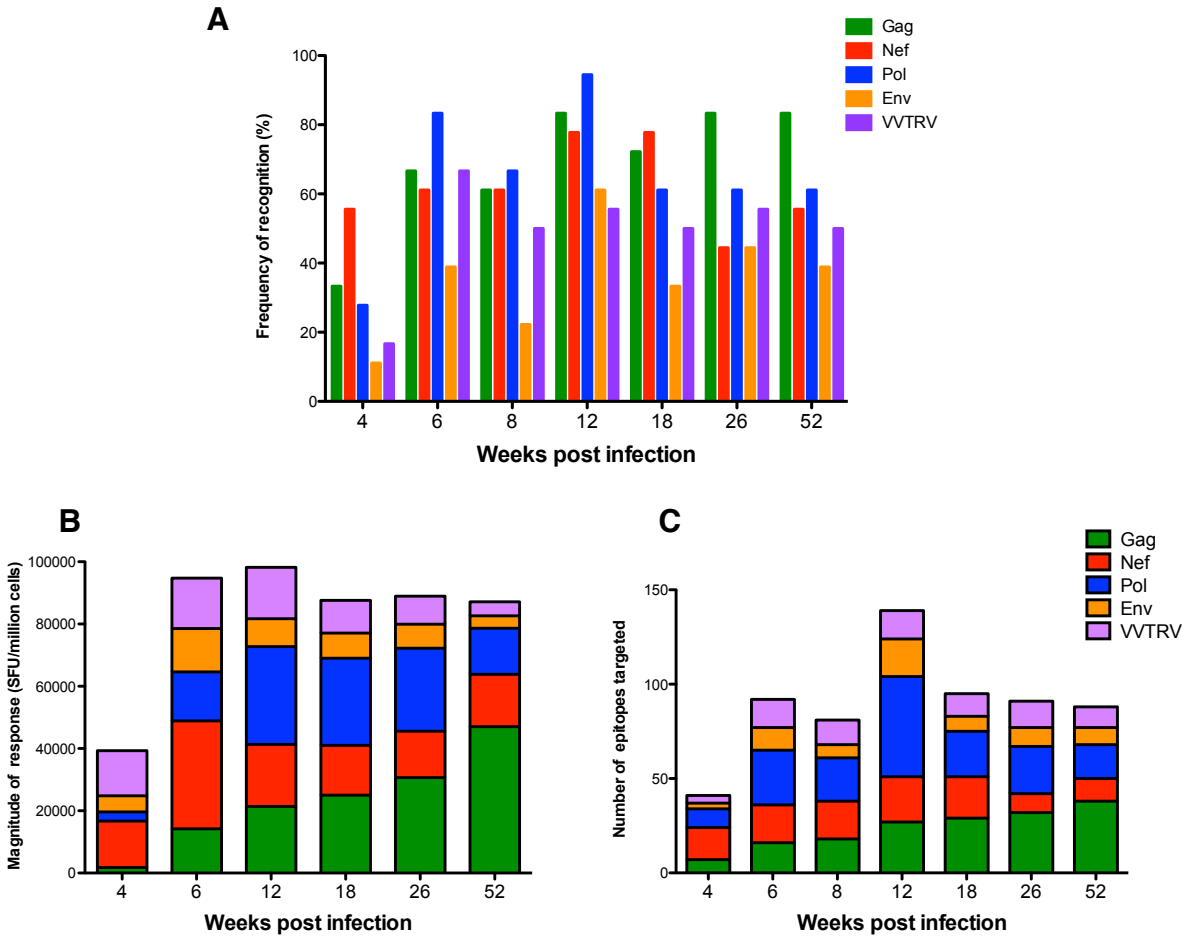


Figure 3.2. Kinetics of HIV-1-specific CD8+ T-cell responses during the 1 year follow-up period. (A) Frequency of T-cell recognition across the entire HIV-1 subtype C proteome. The bars represent response frequencies to Nef, Pol, Gag, Env, Regulatory (Rev, Tat) and Accessory (Vpr, Vpu, and Vif) proteins from 4 to 52 weeks post infection; (B) Bar graphs depicting the relative contributions of Nef, Gag, Pol, Env, and Regulatory (Rev, Tat) and Accessory (Vpr, Vpu, and Vif) proteins the total magnitude and (C) breadth of HIV-1-specific T-cell responses at the specified time points.

At 4 weeks post infection, the earliest time point of measurement of HIV-specific CD8+ T-cell responses, 55% of the subjects made Nef-specific T-cell responses, followed by Gag (33%) and Pol (27%), and VVTRV (Vif, Vpr, Vpu, Rev and Tat) (16%) and Env (10%) (Figure 4.2A). Responses against Nef increased in frequency

and at 18 and 26 weeks, 77% of the subjects had detectable Nef responses, subsequently, these responses underwent a marked decline and at 52 weeks only 50% of the subjects had detectable anti-Nef responses. In contrast, epitopes in the Gag region were targeted by only 33% of the subjects at 4 weeks and this number increased to 94% at 52 weeks. The number of individuals with detectable responses against Pol peaked at 12 weeks when 88% of subjects had detectable anti-Pol responses. The sequences of the defined class I MHC-restricted HIV-1-specific CD8+ epitopes that were targeted, the restricting MHC class I allele, and position of these epitopes in the HIV-1 genome as well as OLPs that were targeted but have not been previously defined are listed in Tables 3.2 and 3.3.

Evolution of immune responses was also apparent in terms of the breadth of responses to individual viral proteins. For this analysis we examined the sum total responses detected in the 18 study subjects. A total of 41 T-cell responses against epitopes in Gag, Pol, Nef, Env, Vif, Vpr, Vpu, Tat, and Rev were measured at 4 weeks. As expected, Nef responses were dominant in breadth and made 42% of the total detectable HIV-specific CD8+ T-cell response at this time, whilst Pol and Gag-specific responses contributed 24% and 17%, respectively (Figure 3.3). In those in whom HIV-1-specific T-cell responses were detected, the median magnitude of responses was quite variable over time, and the median values for Nef-specific responses were generally higher in magnitude than those directed against other regions.

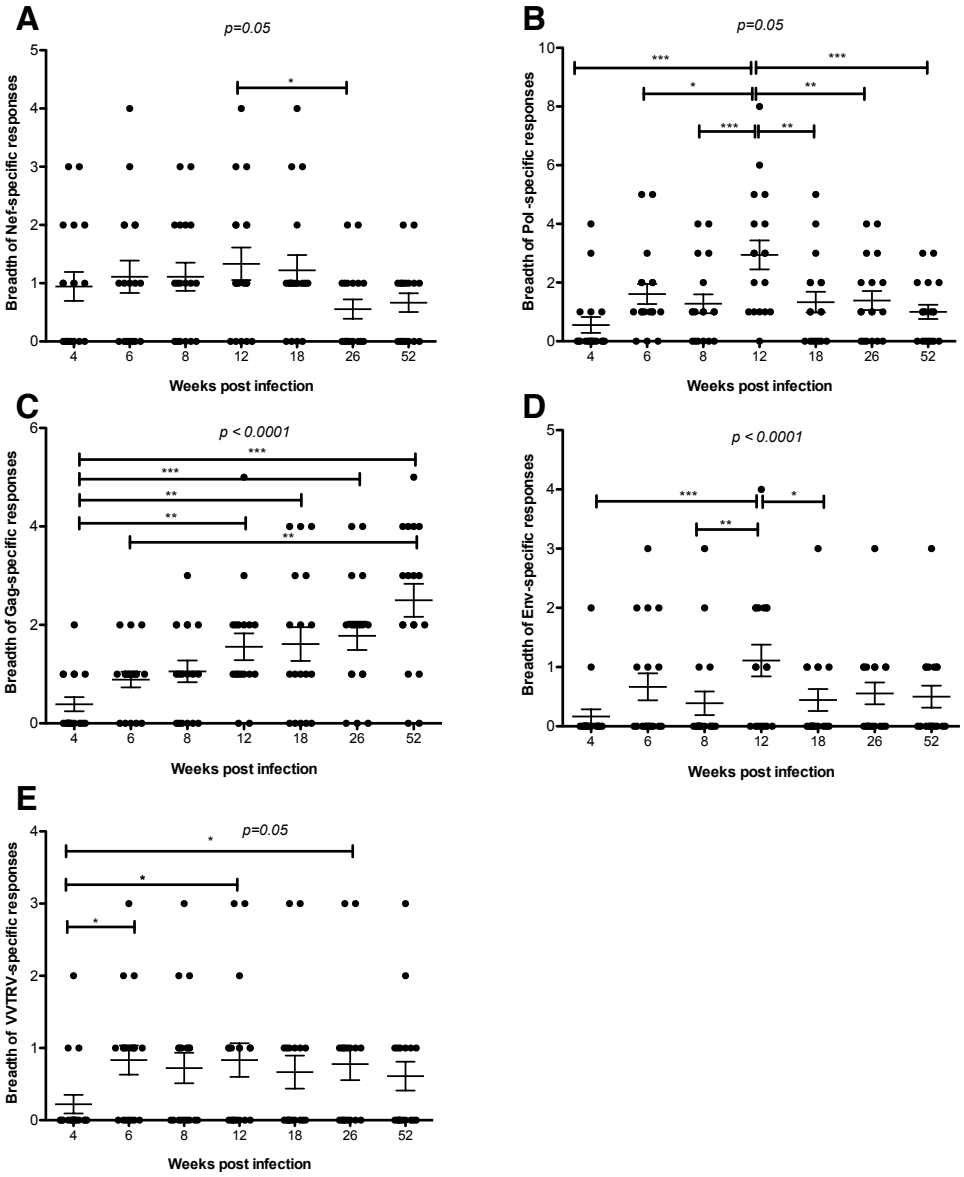


Figure 3.3. Evolution of IFN-γ ELISPOT CD8+ T-cell responses targeting epitopes in (A) Nef, (B) Pol, (C) Gag, (D) Env and (E) Regulatory (Rev, Tat) and Accessory (Vpr, Vpu, and Vif) proteins HIV-during the 1 year follow-up period. measured from 4-52 weeks post infection in a cohort of 18 subjects. Each symbol represents a single response against an epitope in the specified protein. *P* values are indicated (Repeated measures ANOVA test followed by Bonferroni adjustment).

We next examined the relationship between evolving breadth of responses and viral load. Responses against Pol, Nef and Env peaked at 12 weeks and subsequently declined; yet with increased duration of infection there was a steady rise in the overall number of Gag epitopes targeted (Figure 3.2 and 3.3C). The highest breadth of Gag-specific responses was recorded at 52 weeks post-infection, the last time point to be analyzed in this study (Figure 3.3C). We next aimed to identify whether the magnitude or breadth of CD8+ T-cell responses against epitopes in all HIV-1 regions had any impact on early disease progression in the first 12 months post infection. We observed no correlation between magnitude and breadth of all of responses to Pol, Env, Rev, Tat, Vpu, Vpr and Vif and viral load set point. There was also no significant association between the breadth of Nef T-cell responses and viral set point at all measured time points, yet a trend towards significance was observed at 8 weeks (p=0.098; r=0,4)(Figure 3.4A). Although there was no correlation observed between the breadth of Gag-specific responses measured up to 18 weeks and viral set point (Figure 3.4B and data not shown), subsequent measures showed a significant negative association from 26 weeks (p=0.0081; r=-0.61) and at 52 weeks (p=0.027; r= -0.52) (Figure 3.4B-D). When we fitted a linear regression model to viral load set point, accounting for the repeated measurements and adjusting for weeks post infection, there remained a significant association between breadth and viral load set point in this analysis. For every 1 unit increase in breadth, viral load decreases by 0.15 log copies/ml (SE=0.067; p=0.0225).

Table 3.2. HIV-1-specific CD8$^+$ T-cell responses in acute and early infection, the epitopes targeted and restricting MHC class I alleles.

HLA	Protein	Epitope code	Clade-C Sequence	Corresponding OLP
A2	p17	SL9	SLYNTVATL	#11, #12
A2	RT	AM9	ALTAICEEM	#170
A2	RT	VL9	VIYQYMDDL	#190
A2	RT	IV9	ILKEPVHGV	#207
A2	gp41	SV10	SLLDTIAIAV	#396
A2	Nef	PL10	PLTFGWCFKL	#85
A2	Nef	VL10	VLQWKFDSQL	#91
A2	Vpr	AL9	ALIRILQQL	#285
A2	Nef	GL9	GAFDLSFFL	#78
A2	p24	IA9	ILRALGPGA	#45
A2	p24	GA9	GVGGPSHKA	#48
A2	Rev	QL9	QAVRIIKIL	#96
A2	P15	QW9	QANFLGKIW	#58
A2	RT	QV8	QIYPGIKV	#202
A2	Pol	QL9	QVRDQAEHL	#262
A2	RT	LI10	LVGPTPVNII	#162
A0205	Pol	YI9	YTAFTIPSI	#183
A3	p17	KK9	KIRLRPGGK	#2, #3
A3	p17	RH9	RLRPGGKKH	#3
A3	p17	RLY10	RLRPGGKKHY	#3
A3	RT	AM9	ALTAICEEM	#170
A3	RT	GK9	GIPHPAGLK	#178
A3	RT	KK11	KMRTAHTNDVK	#213
A3	Vif	KK11	KIKPPLPSVRK	#422
A3	gp120	TK10	TVYYGVPVWK	#293, #294
A3	gp41	RR11	RLRDLILIAAR	#391
A3	RT	KLK9	KLAGRWPVK	#254
A11	RT	IN8	IYQEPFKN	#212
A23/24	p17	HW9	HYMLKHLVW	#4
A23/24	Nef	RW8	RYPLTFGW	#84
A23/24	RT	IL9	IYQEPFKNL	#211, #212
A23/24	gp120	VW8	VYYGVPVW	#293, #294
A23/24	gp120	LY10	LFCASDAKAY	#295
A23/24	gp41	RL9	RYLKDQQLL	#366
A23/24	gp120	NL10	NYTNTIYRLL	#373
A26	p24	EL9	EVIPMFTAL	#23
A29	Nef	YY8	YFPDWQNY	#82, #83
A29	p17	RY11	RSLYNTVATLY	#11, #12
A29	gp160	SY9	SFDPIPIHY	#316
A29	gp120	SY10	SFNCRGEFFY	#338
A29	gp120	FY9	FNCRGEFFY	#338
A29	p17	LY9	LYNTVATLY	#11
A30	Nef	YY8	YFPDWQNY	#82, #83
A3002	p17	RLY10	RLRPGGKKHY	#3
A3002	p17	RY11	RSLYNTVATLY	#11, #12
A3002	RT	AY11	AQNPEIVIYQY	#190
A3002	RT	KLY9	KLNWASQIY	#201, #202
A3002	Int	KIY9	KIQNFRVYY	#269, #270
A3002	gp41	RY9	RIGPGQTFY	#330
A3002	p17	SW11	SILRGGKDKW	#1
A3002	p17	RL9	RFALNPGLL	#6, #7
A3002	p24	KV10	KNWMTDTLLV	#43
A3002	p17	AF8	ASRELERF	#6
A3002	RT	RY10	RAQNPEIVIY	#190
A66	RT	ER11	ETFYVDGAANR	#224
A6801	p24	DA8	DTINEEAA	#28
A6801	VIF	ER10	EVHIPLGEAR	#408
A6802	Int	EL9	ETAYFILKL	#253
A6802	Int	EA10	ETAYFILKLA	#253
A6802	RT	EA9	ETFYVDGAA	#223, #224
B7/42/81	p24	SV9	SPRTLNAWV	#20
B7/42/81	p24	TL9	TPQDLNTML	#25
B7/42/81	p24	HA9	HPVHAGPIA	#29, #30
B7/42/81	p24	GL9	GPSHKARVL	#48
B7/42/81	Nef	FL9	FPVRPQVPL	#76
B7/42/81	Nef	RM9	RPQVPLRPM	#76
B7/42/81	Nef	RGF9	RPMTFKGAF	#77
B7/42/81	Nef	TL10	TPGPGVRYPL	#84
B7/42/81	RT	SM9	SPAIFQSSM	#187
B7/42/81	vpr	FL9	FPRPWLHGL	#281, #282
B7/42/81	gp120	RI10	RPNNNTRKSI	#328
B7/42/81	Int	LI9	LPPIVAKEI	#244
B7/42/81	Pro	TL11	TPVNIIGRNML	#163
B7/42/81	RT	YL9	YPGIKVKQL	#202, #203
B7/42/81	Rev	RL10	RPAEPVPLQL	#102
B7/42/81	p17	RLY10	RLRPGGKKHY	#3
B7/42/81	RT	SL10	SPIETVPVKL	#166
B7/42/81	RT	RQL11	RPPLVKLWYQL	#221
B7/42/81	Vif	KL9	KPKKIKPPL	#422
B7/42/81	RT	TL11	TPGIRYQYNVL	#184
B7/42/81	RT	GL9	GPKVKQWPL	#168
B8	p17	EV9	ELRSLYNTV	#11
B8	p24	DI8	DIYKRWII	#36
B8	Nef	WM8	WPAIRERM	#68
B8	RT	GL9	GPKVKQWPL	#168
B8	gp41	YL8	YLKDQQLL	#366
B8	P15	EL8	EPKDREPL	#64
B8	RT	VI8	VPRRKAKI	#274, #275
B14	p24	DA9	DRFFKTLRA	#41
B14	p15	CC9	CRAPRKKGC	#55
B14	gp41	EL9	ERYLKDQQL	#366
B1503	Tat	FY10	FQTKGLGISY	#115
B1503	RT	FY10	FKRKGGIGGY	#265
B1503	p24	VF9	VKVIEEKAF	#21
B1503	RT	IY9	IQQEFGIPY	#258, #259
B1503	RT	RY9	RKAKIIKDY	#275
B1503	RT	GL9	GKKAIGTVL	#162
B1510	p17	RL9	RFALNPGLL	#6, #7
B1510	p24	VL10	VHQAISPRTL	#20
B1510	p24	GL9	GHQAAMQML	#26, #27
B1510	Vif	WI9	WHLGHGVSI	#411
B1510	p17	GI9	GKVSQNYPI	#18
B1510	Rev	IL9	IHSISERIL	#100
B1510	RT	TIL9	THLEGKIIL	#249
B1510	Rev	GL11	GRPAEPVPLQL	#102
B1510	p17	KL8	KHYMLKHL	#4
B18	Nef	YF9	YPLTFGWCF	#84, #85
B18	Vif	LY10	LADQLIHMHY	#415
B18	RT	NY10	NETPGIRYQY	#184
B18	Int	VL9	VASCDKCQL	#246
B18	RT	NY9	NPEIVIYQY	#190
B18	RT	SY11	SEVNIVTDSQY	#230
B18	Rev	DI10	DEALLQAVRI	#95
B18	Vif	IY8	IEWRLRRY	#413
B18	Nef	QY9	QEILDLWVY	#81
B18	VPR	NL10	NEWTELLEEL	#279
B18	VPR	GW10	GPQREPYNEW	#279
B35	Nef	VF8	VPLRPMTF	#76, #77
B35	RT	VY10	VPLDEDFRKY	#182
B35	RT	NQY9	NPEIVIYQY	#190

Table 3.2. *continued*

HLA	Protein	Epitope code	Clade-C Sequence	Corresponding OLP	HLA	Protein	Epitope code	Clade-C Sequence	Corresponding OLP
B35	gp120	DL9	DPNPQEMVL	#299	B57	RT	AF10	QATWIPEWEF	#220
B35	Tat	EW10	EPVDPNLEPW	#111	B57	RT	FF9	FSVPLDEDF	#181
B35	Nef	YF9	YPLTFGWCF	#84, #85	B57	RT	EY9	ETKIGKAGY	#226
B39	RT	IL9	IVTDSQYAL	#231	B57	RT	KI13	KAGYVTDRGRQKI	#226
B44	Vif	SL9	SEGATPQDL	#24	B57	Pol	VI9	VTDRGRQKI	#226
B44	p24	RL11	RDYVDRFFKTL	#40	Cw0202	RT	IY10	ILKEPVHGVY	#207
B44	p24	AW11	AEQATQDVKNW	#42	Cw0202	Pol	EY11	EILKEPVHGVY	#207
B44	Nef	GV11	GEVGFPVRPQV	#75	Cw3	p24	YL9	YVDRFFKTL	#40
B45	RT	AA9	AETFYVDGA	#223, #224	Cw4	p24	QW9	QATQDVKNW	#42
B45	p24	II10	IEEKAFSPEVI	#22	Cw4	Nef	YY8	YFPDWQNY	#82, #83
B44	RT	SY11	SEVNIVTDSQY	#230	Cw4	gp41	EW11	EIWDNMTWMQV	#371
B44	RT	IW11	IEELREHLLKW	#194	Cw4	RT	TF10	TVLDVDAYF	#180
B44	Int	QW11	QEEHEKYHSNW	#242	Cw6	gp120	II13	IRSENLTNNAKTI	#325
B53	p17	WF9	WASRELERF	#5	CW6	gp120	LL12	LTNNAKTIIVHL	#325
B53	p24	QW9	QATQDVKNW	#42	Cw06	gp120	AL8	AKTIIVHL	#325
B53	Nef	YF9	YPLTFGWCF	#84, #85	CW0602	Pol	KR9	KTGKYAKMR	#213
B53	RT	EW10	EPVDPNLEPW	#111	Cw7	Nef	KY11	KRQEILDLWVY	#81
B53	RT	QW11	QPIQLPEKDSW	#199	Cw8	p24	TL9	TPQDLNTML	#25
B57	p17	WF9	WASRELERF	#5	Cw8	Rev	PL9	PAEPVPLQL	#102, #103
B57	p24	ISW9	ISPRTLNAW	#20	Cw8	RT	IL9	IVTDSQYAL	#231
B57	p24	KF11	KAFSPEVIPMF	#22	Cw12	RT	IL9	IVTDSQYAL	#231
B57	p24	TW10	TSTLQEQIAW	#33, #34	Cw1701	Rev	RL10	RPAEPVPLQL	#102
B57	p24	QW9	QATQDVKNW	#42	Cw18	p24	FFF9	FRDYVDRFF	#40
B57	Nef	HW9	HTQGYFPDW	#82	Cw18	p24	VI9	VRMYSPVSI	#37
B57	RT	IAW9	IAMESIVIW	#216	Cw1801	RT	VL8	VRDQAEHL	#262
B57	Vif	VF9	VSRRANGWF	#405					

Table 3.3. T-cell responses to OLPs that are not known to contain defined CD8+ T-cell epitopes.

PID	OLP #	Region	*Sequence*	No. of time-points where response was detected	No. of SFC/10^6PBMC
AS2-1037	#9	p17	EGCKQIMKQLQPALQTGT	1	260
AS3-0017	#16	p24	NKSQQKTQQKAAADKGKV	4	1020; 420; 580
AS5-0643	#52	p15	QRSNFKGPKRIVKCF	1	200
AS1-0703	#117	Tat	GRKKRRQRRSAPPSSEDH	2	4000; 520
AS3-0458	#125	Vpu	LIVALIIAIVVWTIAYIEY	1	240
AS2-0110	#229	RT	VSLTETTNQKTELQAIQL	2	360
AS2-0050	#229	RT	QKTELQAIQLALQDSGSEV	5	4000; 1500
AS3-0458	#252	INT	GYIEAEVIPAETGQETAY	1	180
AS3-0268	#287	Vpr	HFRIGCQHSRIGILRQRR	1	280

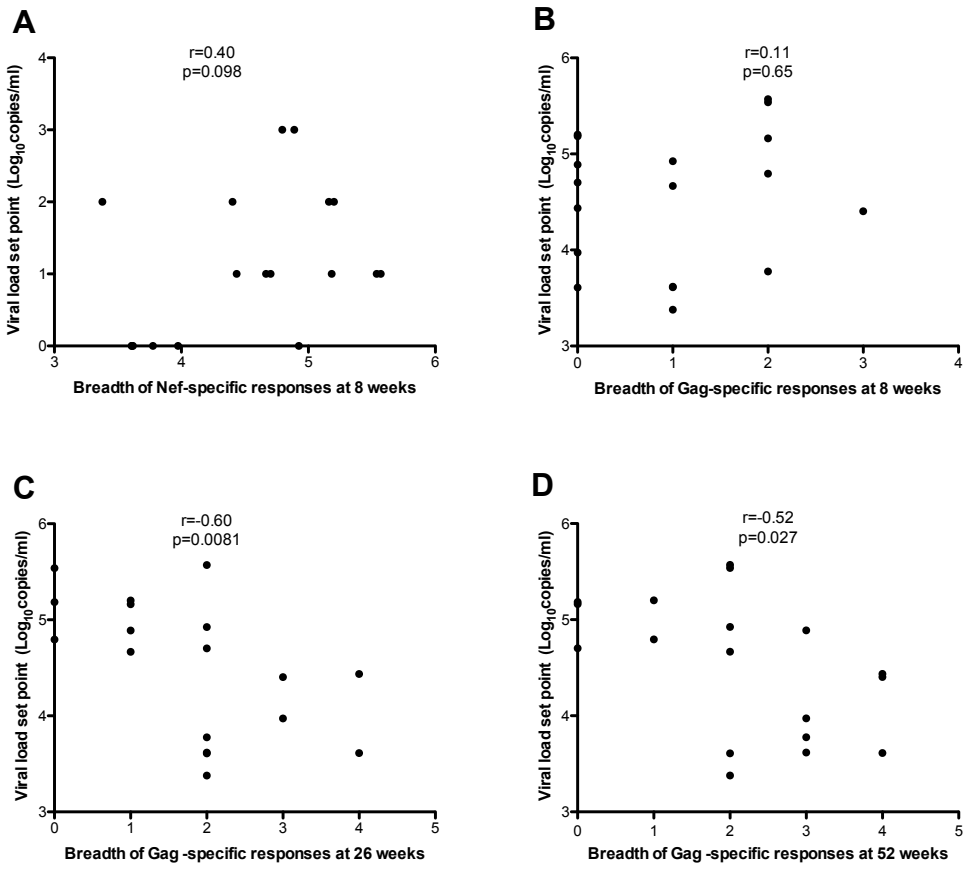


Figure 3.4. Association of the breadth of HIV-specific CD8+ T-cell responses and viral load set point. (A) The breadth of Nef-specific T-cell responses; (B, C and D) The breadth of Gag-specific T-cell responses. Each symbol represents the total number of epitopes targeted per participant. Spearman correlations between the breadth of responses and viral load set point at 8, 26, and 56 weeks post infection.

4.2. Fate of the early T-cell IFN-γ ELISPOT responses

As illustrated in Figure 3.3 over the first year of infection, CD8+ T-cell responses are highly variable following acute infection, with an ultimate overall decline of anti-Nef and Pol responses and a gradual increase of Gag-specific T-cell responses. It is noteworthy that even at an epitope-specific level within each viral protein, immune responses fluctuated with some persisting throughout, some no longer detectable at later time points while others were undetectable

at some time points and later re-emerged (Radebe et al., 2011). Few studies have examined the relationship between early loss and gain in CD8+ T-cell responses and viral load (Turnbull et al., 2009, Mlotshwa et al., 2010, Goonetilleke et al., 2009) and this led us to investigate the fate of these early T-cell responses and their impact on viral load set point over the one year follow up period. We focused our analysis on two distinct recognition profiles, persistent and lost responses. Persistent responses were defined as responses that were detected as early as 6 weeks and persisted up to 52 weeks. Lost responses were defined as responses which appeared at any time during the follow up period but had decreased by more than 80% and/or had fallen below the threshold of 100 SFC/10^6 PBMC when the last evaluation was performed at 52 weeks post infection.

Analysis of persistent HIV-1-specific CD8+ T-cell responses revealed that although 92 responses were measured at 6 weeks, only 45% (41) of these responses were still detectable at 52 weeks. Of the 16 Gag-specific responses measured at 6 weeks, at least 12 (75%) persisted and were still detectable at 52 weeks. Table 3.4 shows the epitopes that were persistently targeted by all the subjects in the cohort and the HLA class I alleles restricting these responses. All eighteen subjects had persisting T-cell responses against either optimal epitopes (36 in total) and/or OLPs (5 in total), ranging from 1 to 5 responses per subject.

Table 3.4. Confirmed persistent HLA class I-restricted CD8+ T-cell epitopes and OLPs targeted between 4-52 weeks post infection.

Participant ID	Epitopes and OLPs targeted: between 6-52 weeks post infection		Locations of the targeted epitopes:									Viral load set point (Log_{10}copies/ml)
	Defined HLA class I restricted epitopes	OLPs	Gag	Pol	Nef	Vif	Vpr	Vpu	Env	Rev	Tat	
AS1-0703	none detected		1	1								4,89
AS1-0876	B1503-VF9(p24)		1									3,61
AS1-0919	A29-YY8(Nef)	#202(RT)		1	1							4,44
AS2-0016	A3002-KIY9(Int), B42-FL9(Vpr), B42-YL9(RT), B42-RM9(Nef)	#275(Int)		3	1		1					4,70
AS2-1037	B1510-IL9(Rev)									1		5,54
AS2-0174	A3002-AY11(RT), A3002-KIY9 (Int), Cw3-YL9(p24)		1	2								3,20
AS2-0184	A23-RW8(Nef)				1							5,16
AS2-0341	None detected	#81(Nef), #408(Vif)			1	1						5,18
AS2-0358	A23-HW9(p17), A23-RW8(Nef), B42-RL10(Rev), B53-EW10(RT)		1	1	1					1		4,79
AS2-0483	B42-TL9(p24)		1									5,20
AS2-0802	A3002-RY11(p17)		1									4,40
AS2-0945	B57-TW10(p24), B1510-IL9(Rev)		1							1		3,61
AS3-0268	A29-SY9(gp160)								1			5,57
AS3-0369	A66-ER11(RT), Cw3-YL9(p24)		1	1								3,78
AS3-0458	A29-SY9(gp160), B44-AW11(p24), B57-FF9(RT), B57-KI13(RT), Cw4-EW11(gp120)		1	2					2			3,97
AS3-0740	B44-AW11(p24), B44-IW11(RT)	#78(Nef)	1	1	1							3,38
AS5-0643	A2-SL9(p17), A2-AL9(Vpr), B42-YL9(RT), Cw0202-EY11(RT)		1	2			1					4,67
AS5-0953	A3-RLY10(p17)		1									3,62

Responses to Pol made up 34% of all the HIV-1-specific CD8+ T-cell responses that were classified as persistent, followed by Gag (29%), and Nef (15%). When T-cell responses were analysed irrespective of viral protein or region targeted, there was no significant negative association between the breadth of these responses and viral load set point (P=0.37; r=-0.22) (Figure 3.5A). Even though Pol-specific responses made up 35% of all persistent responses, there was no correlation between the breadth of these responses and viral load set point (P=0.77; r=-0.073) (Figure 3.5B). In contrast, a significant association was observed between persistent responses targeting Gag and viral set point (P=0.010; r=-0.59) (Figure 3.5C). These data suggest that persistent Gag-specific CD8+ T-cell responses may be advantageous in providing a sustained reduction in viremia.

Whilst some of the early HIV-1-specific IFN-γ ELISPOT CD8+ T-cell responses were shown to persist, 55% of these T-cell responses waned and were below the threshold of detection at 52 weeks. In order to determine the fate of some of these T-cell responses which were no longer detectable in circulation, we used the cultured IFN-γ ELISPOT assay which assesses the proliferation of low frequency T-cells which may be missed by the *ex vivo* ELISPOT assay (Calarota et al., 2008, Pala et al., 2013, Ndhlovu et al., 2012). Due to sample availability limitations, not all subjects could be evaluated for immune responses using the cultured IFN-γ ELISPOT assay. In the 8 subjects evaluated, a total of 96 (median=11) *ex vivo* IFN-γ ELISPOT responses were measured between the 4 and 26 weeks post infection (Table 3.5). With the duration of infection, these

responses fluctuated over time and only 30% of these T-cell responses were still detectable by the *ex vivo* ELISPOT assay at 52 weeks. At least 40% of T-cell responses (27 of the 67 individual epitope-specific T-cell responses), which had disappeared and were presumed lost, could be detected with the cultured ELISPOT assay. These T-cell responses were directed against epitopes in all HIV-1 regions (magnitude of response ranging from 200–4,000 SFC/10^6 PBMC), and 30% of these responses targeted epitopes in Gag. There was no significant correlation between the presence of cultured ELISPOT responses targeting all HIV-1 regions and viral set point (*P*=0.24; r=0.47 and data not shown). However, a significant negative correlation between cultured ELISPOT Gag-specific responses and viral set point (*P*=0.017; r=-0.85) (Figure 3.5D) was observed. Our data show that the cultured ELISPOT could amplify and enhance the detection of at least 40% of low frequency T-cell responses which had become undetectable when measured by the ex vivo ELISPOT. It is also plausible that these effector T-cell responses that were below the limit of detection by ex vivo ELISPOT had converted to a memory phenotype, which is not detectable by standard ELISPOT (Godkin et al., 2002, Reece et al., 2004, Todryk et al., 2008, Ndhlovu et al., 2012).

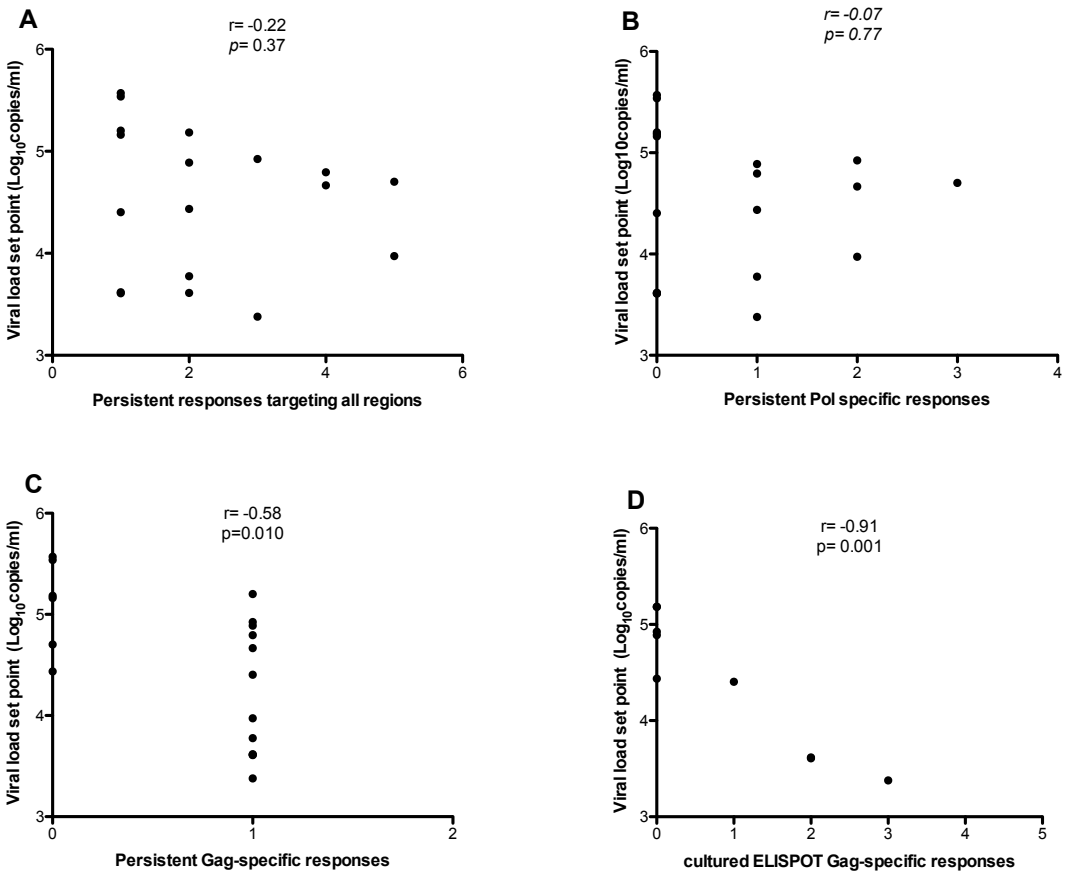


Figure 3.5. The impact of persistent T-cell responses measured between 4 weeks and 52 weeks post infection on viral load set point. (A) A lack of association between persistent responses targeting epitopes within all proteins and viral load set point; (B) A lack of association between persistent responses targeting epitopes within Pol and viral load set point; (C) A significant negative association between the breadth of Gag-specific persistent T-cell responses and viral load set point; (D) A significant negative association between the breadth of Gag-specific cultured ELISPOT T-cell responses and viral load set point.

Table 3.5. HIV-1-specific T-cell responses determined by the ex vivo ELISPOT assay during the entire follow up period and responses measured using cultured ELISPOT assay at 52 weeks.

Participant ID	Pre-culture		Post-culture	
	Responses detected from 4 to 26 weeks	Responses detected at 52 weeks	All responses detected at 52 weeks	Gag specific responses at 52 weeks
AS1-0703	10	3	4	0
AS1-0876	9	4	3	2
AS1-0919	16	4	9	0
AS2-0341	6	0	4	0
AS2-0358	20	5	0	0
AS2-0945	13	7	2	1
AS3-0740	12	3	3	3
AS5-0953	10	3	2	2
TOTAL	96	29	27	8

4.3. Gag immune responses and viral evolution

To investigate the relationship between the dynamics of Gag-specific T-cell responses and sequence evolution within targeted Gag epitopes, we sequenced HIV-1 Gag from plasma samples obtained at 2-6 weeks and 52 weeks post infection. We evaluated a cumulative total of 210 epitopes restricted by the HLA class I alleles expressed by subjects in the cohort. Of the epitope sequences we assessed at 2-6 weeks, in spite of expression of the restricting HLA class I allele and the presence of the cognate viral sequence, T-cell responses were only mounted against 14% (11 of 78) of the wild type epitopes known to be presented in the context of HLA alleles expressed by the subject. Interestingly, 7% (9 of 135) of epitopes with variant sequences either in the putative epitope or in the flanking region also induced a detectable T-cell response. Analysis at 52 weeks showed that a significantly higher number of both wild type (24 of 75, 32%) (P=0.006) and variant epitopes (21 of 135, 15%) (P=0.01) respectively induced a

detectable CD8+ T-cell immune response at this time. Although a total of 11 Gag-specific T-cell responses against wild type epitopes were measured at 2-6 weeks, at one year post infection mutations had occurred in only two of these epitopes, the HLA-B*57 restricted TW10 and B*81-TL9; yet these epitopes were still able to induce a detectable response at 52 weeks (Figure 3.6A). In contrast, initial CD8+ T-cell responses against HLA-B*8 restricted EV9 and HLA-B*15:10-VL10 were lost after 6 weeks and these wild type epitope sequences failed to induce a detectable response even up to 52 weeks post infection. Responses targeting 6 of the epitopes with variant sequences at 2-6 weeks declined over time yet this loss of response did not coincide with further sequence changes within the epitopes or their flanking regions (Figure 3.6B). Further analysis showed that there was no significant difference in viral set point between participants who made early T-cell responses to wild type epitopes and retained these responses even when variant sequences arose (median= 4 log copies/ml) and participants who made early T-cell responses against variant epitope sequences and retained these responses (median= 5 log copies RNA copies/ml) ($p=0.416$). However, the former had a significantly lower viral load set point values (median=4 log copies/ml) in comparison to participants who made responses to epitopes with variant sequences but subsequently lost these responses (median=5.2 log copies/ml) ($p=0.003$).

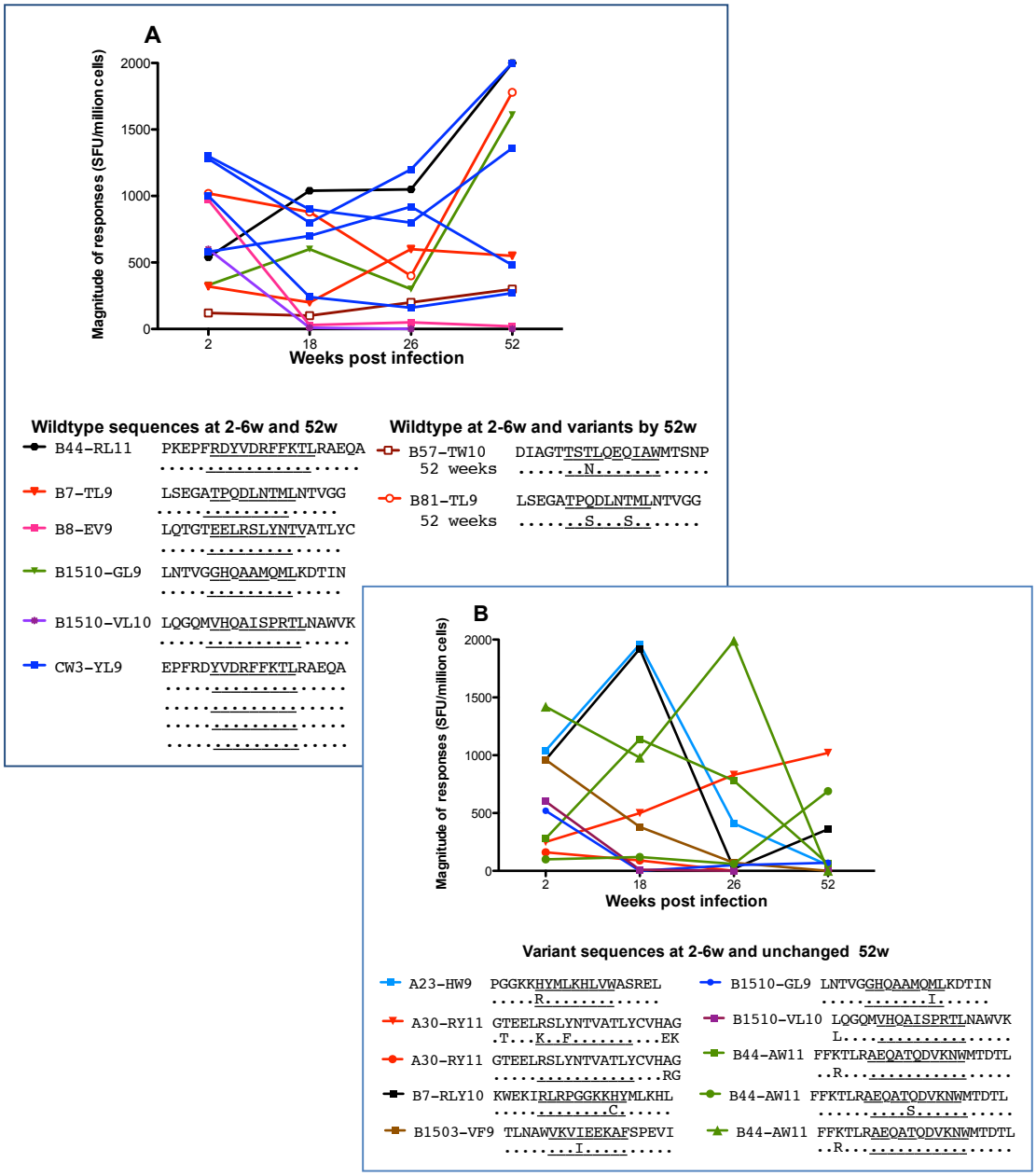


Figure 3.6. Recognition patterns associated with wild type and variant Gag sequences at 2-4 weeks and 52 weeks post infection. (A) T-cell recognition in relation to wild type Gag sequences shown as the magnitudes of IFN-γ ELISPOT responses at 2-4 weeks and 52 weeks post infection. (B) T-cell recognition in relation to sequence variations in Gag at 2-4 weeks and 52 weeks post infection. Each line represents the specific epitope targeted. The sequences of the targeted epitopes at 2-4 weeks and 52 weeks post infection are shown underneath the graphs. The underlined areas indicate the putative targeted epitope.

5. Discussion:

Data accumulated over the past years have established that early immune events determine the rate of disease progression and thus a detailed understanding of the patterns, fate, functions and phenotypes of early CD8+ T-cell subsets which best correlate with control of viremia is needed to inform vaccine and therapeutic strategies that aim to contain the AIDS pandemic (McDermott and Koup, 2012, Freel et al., 2011, McMichael et al., 2010). In the current study we have analysed the evolution and fate of HIV-specific T-cell responses during the first year of infection, investigated their association with viral load set point and their ability to induce viral escape or persist in the presence of escape. We report that a diverse pattern of T-cell recognition across the HIV-1 proteome was evident during primary infection. Despite significant heterogeneity among participants in the timing of appearance and dominance of virus specific T-cell responses, Nef and responses were dominant during earliest phase of HIV-1 infection, however, the breadth of Gag-specific responses increased over time and by one year Gag responses were dominant and contributed over 40% to the total magnitude of response. We cannot rule out that the true magnitude and breadth of T-cell responses may have been underestimated through the use of consensus virus sequences (Goonetilleke et al., 2009, Altfeld et al., 2003).

A significant finding in the current study is the inverse association between the breadth of Gag responses measured during primary infection (26 weeks and later) but not at the acute phase of infection (within the first 12 weeks post infection). Our findings provide a clear demonstration of the broadening of Gag–

specific T-cell responses over the course of one year following HIV-1 infection, with this increased immunogenicity associated with better control of viremia and lower viral load set point. Our findings are in line with reports from other studies, which have shown that the presence of a high proportion of Gag-specific CD8+ T-cell responses correlates with delayed disease progression (Turk et al., 2013, Perez et al., 2013, Zuniga et al., 2006, Geldmacher et al., 2007, Kiepiela et al., 2007, Masemola et al., 2004a, Streeck et al., 2009b, Mudd et al., 2012). Possible mechanisms by which Gag-specific responses in reducing viral replication include Gag fitness constrains to escape immune pressure (Wright et al., 2010, Miura et al., 2010, Henn et al., 2012) and the efficacious antiviral activity of Gag-specific CD8+ T-cells (Sacha et al., 2007, Payne et al., 2010, Julg et al., 2010, Turk et al., 2013). However, because responses were measured in the peripheral blood we cannot rule out that Gag responses were already present in mucosal and lymphoid tissues in higher levels in comparison to the responses in the blood as has been observed (Altfeld et al., 2002).

We also show that individuals with controlled viremia had significantly higher levels of Gag-specific T-cell responses, which persisted from the first 6 weeks up to 52 weeks post infection. These findings confirm that fluctuations in levels of T-cell responses and viral load are related even in early HIV-1 infection. The paucity of Gag-specific responses during the acute stages of HIV-1 infection when dramatic declines in viremia are recorded suggest that Gag-specific T-cell responses may be more important in maintaining the viral load at the set point than for controlling early viremia (Goonetilleke et al., 2009, Turnbull et al., 2009,

Wang et al., 2009). Importantly, and relevant for vaccine design, our results imply that relatively limited Gag immunogenicity during acute HIV-1 infection may lead to suboptimal viral control. Thus, a vaccine designed to induce greater breadth of CD8+ T-cell responses recognizing Gag epitopes, may tip the balance towards control during the earliest phases of infection and result in viremic or elite controller phenotype more efficiently than would be expected in natural infection.

The fluctuation of the recognition patterns of HIV-specific CD8+ T-cell responses during primary infection led us to investigate the impact of these changes on disease progression. We used the peptide-based cultured IFN-γ ELISPOT assay, (Godkin et al., 2002, Keating et al., 2005, Calarota et al., 2008) to determine the fate the HIV-1 specific T-cell responses which had decreased and had fallen below the threshold of 100 SFC/10^6 PBMC at one year. Previous studies indicate that some antigen-specific CD8+ T-cell responses, undetectable by the *ex vivo* 18h ELISPOT assay, may be maintained as low frequency central memory precursors (Calarota et al., 2008, Ndhlovu et al., 2012), suggesting that these are the responses detected using the cultured ELISPOT assay in our study. However, because we did not phenotypically characterize the T-cells detected in the cultured ELISPOT assay, we cannot rule out the possibility that these may represent low frequency HIV-1–specific CD8+ T-cells functionally and phenotypically indistinguishable from those detected by the regular ELISPOT assay (Lichterfeld et al., 2004a).

Although we observed a significant negative correlation between the presence of cultured ELISPOT Gag-specific T-cell responses and viral set point,

the small sample size limits our ability to draw conclusions from this data. Our findings however, are in line with data which show that preservation of memory T-cells is essential for better outcome and survival in HIV-1 (Burgers et al., 2009, Champagne et al., 2001, Northfield et al., 2007, Addo et al., 2007, Calarota et al., 2008) as the memory subset of Gag-specific CD8+ T-cells have viral inhibition properties in HIV-1 infected individuals (Ndhlovu et al., 2012).

We sequenced HIV-1 Gag from plasma samples obtained during acute HIV-1 infection and at one year post infection. In agreement with our earlier findings, we report that despite the expression of the restricting HLA class I alleles and the presence of the wild type epitopes, T-cell responses were only mounted against a few of the wild type epitopes and analysis at 1 year revealed that a significantly higher but still relatively low number of both wild type and variant epitopes were targeted. Because of limited immunogenicity observed during acute HIV-1 infection we are unable to draw definitive conclusions about the overall impact of acute T-cell responses on viral evolution. However, our data show that the initial lack of induction of Gag-specific T-cell responses is not primarily the result of sequence mismatch within the targeted epitopes. Given that our analysis of CD8+ T-cell-mediated viral escape was restricted to the Gag protein, it is possible that CD8+ T-cells induced escape in other proteins (Goonetilleke et al., 2009, Liu et al., 2013). A variant was considered full escape only when it was no longer recognized by CD8+ T-cells, thus not all variants constituted escape. Although mutations had occurred in some of the initially targeted wild type epitopes by one year, these epitopes still induced a detectable T-cell response, suggesting

that whilst selection pressure mediated by early T-cell responses led to viral escape, HIV-1-specific T-cell responses persisted, resulting in the relative control of viral replication (Haas et al., 1996, Turnbull et al., 2006). Since 66% of T-cell responses targeting epitopes with variant sequences at the earliest time point subsequently declined, this may suggest that there was immune selection pressure already being applied to these epitopes during AHI and the virus successfully escaped recognition, as indicated by the declining T-cell responses and higher viral loads. Interestingly, the participants with persisting detectable T-cell responses against invariant epitopes, had significantly lower viral load set point values when compared to participants with responses to epitopes containing variant sequences during acute HIV-1 infection but subsequently lost these responses. These data suggest that persisting Gag-specific T-cell responses, which may be the result of continual invariant viral epitope presentation or the generation of *de novo* responses to arising variant epitopes, bear the greatest burden on control of early HIV-1 replication and disease progression (Wang et al., 2009, Mlotshwa et al., 2010, Perez et al., 2013). Thus although the quality (in terms of breadth and capacity to respond upon stimulation) of HIV-1-specific CD8+ T-cell responses during acute infection is generally poor, it is not entirely ineffective. Thus, indicating the need to better understand what distinguishes ineffective from ineffective CD8+ T-cells and raising the intriguing possibility that there is room for improvement of vaccine-induced CD8+ T-cells to achieve better immune control than that achieved by natural infection induced CD8+ T-cells.

Conclusions

The data indicate that induction and preservation of Gag-specific effector and central memory type CD8+ T-cell responses is required for the maintenance of low viral load levels in primary infection. Acute and primary infection phase CD8+ T-cells in this study mostly failed to induce immune escape over the first year of infection, highlighting the need to better understand the characteristics of effective HIV-specific CD8+ T-cells. Our study suggests that an early and broadly directed Gag-specific CD8+ T-cell response in acute infection may augment early control, and provide a rational goal for both prophylactic and therapeutic vaccination.

Chapter 4 : The prognostic value of plasma and T-cell markers of immune activation in predicting viral load set point and CD4+ T-cell loss in HIV infection

1. Abstract

Background: Few studies have investigated the coupled effect of acute phase T-cell activation and cytokine expression on disease progression. We sought to determine the prognostic value of combinations of these immunologic parameters in predicting viral load set point and CD4+ T-cell loss during acute HIV-1 infection.

Methods: The study included 33 subjects that were followed longitudinally from acute HIV-1 infection, up to 1-year post infection. Plasma concentrations of 11 cytokines and T-cell markers of immune activation were assessed. Associations between acute phase markers of immune activation and T-cell responses and markers of disease progression (viral load and CD+ T-cell counts) were assessed.

Results: Analyses were performed using general linear models. Acute CD8+ T-cell responses were inversely associated with MIP-1β plasma concentrations (p=0.001). Expression of IL-12p70 was associated with lower viral load set point (P=0.026), whereas IP-10 and IL-10 were associated with higher viral load set point (P=0.024 and P=0.015, respectively). PD-1 expression on CD8+ T-cells during acute infection was associated with preservation of CD4+ T-cells (p=0.029), whereas IL-10 and TNF-α were associated with more rapid CD4+ loss (p=0.016 and p=0.0057, respectively).

Conclusion: Our study identifies immune signatures that are stronger predictors of long-term HIV disease progression than CD8+ T-cell responses measured during acute HIV-1 infection. In combination with canonical parameters, these early biomarkers may inform approaches for evaluating the ability of therapeutic HIV-1 vaccines to control HIV infection.

2. Introduction

Human immunodeficiency virus (HIV-1) infection is characterized by CD4+ T-cell depletion, CD8+ T-cell expansion and chronic immune activation resulting in immune dysfunction demonstrated by increased T-cell proliferation and high T-cells death rates (Hellerstein et al., 2003, Grossman and Paul, 2000). This HIV-1-induced expansion and increased turnover of T-cells ultimately results in the exhaustion of the regenerative capacity of the immune system (Alter et al., 2004, Brenchley et al., 2006b, Douek et al., 2003, Hellerstein et al., 2003, Deeks et al., 2004). Chronic immune activation is therefore a hallmark of pathogenic HIV infection and the level of immune activation has been shown to be the best predictor of progression to AIDS and death independent of HIV viral load (Fahey et al., 1990, Liu et al., 1998, Hazenberg et al., 2003, Giorgi et al., 1999, Zangerle et al., 1998, Deeks et al., 2004). The mechanisms of HIV-associated immune activation are not completely understood, however, studies have suggested that HIV-1 infection induces chronic immune activation through activation of the innate and the adaptive immune system, via single-stranded (ss) RNA or through intracellular viral DNA which activate plasmacytoid dendritic cells (pDCs) via endosomal Toll-like receptors 7 and 8 (Grossman and Paul, 2000, Hazenberg et al., 2000). Translocation of bacterial products from the gut is thought to also contribute to the systemic immune activation associated with HIV infection through the stimulation of innate immune cells via the Toll-like receptors 2, 4 and 5 pathways (Brenchley et al., 2004b, Li et al., 2005, Brenchley et al., 2006b). The activation of innate and adaptive immune systems enhances expression of cell surface molecules such as programmed death-1 (PD-1), CD95, CD38, HLA-DR,

CD69 and CD57; and high expression of these markers is often predictive of an adverse prognosis (Giorgi et al., 1993, Hazenberg et al., 2003, Deeks et al., 2004, Lederman et al., 2000, Kaufmann et al., 2007, Day et al., 2006, Brenchley et al., 2003, Kestens et al., 1992, Krowka et al., 1996, Westendorp et al., 1995). Cytokines have been shown to play a key role in numerous infectious diseases, by shaping the immune response mounted against pathogens and also contributing to disease pathogenesis (Murdoch and Finn, 2000, Dinarello, 1992, Kedzierska and Crowe, 2001). HIV-associated immune activation contributes to the induction of a potent inflammatory response marked by elevated levels of pro-inflammatory cytokines, and this inflammatory response induces a potent and sustained immunoregulatory response marked by production of anti-inflammatory cytokines (Stacey et al., 2009, Roberts et al., 2010, Nilsson et al., 2007, Boasso and Shearer, 2008). Some reports have established that plasma cytokine levels measured during acute HIV infection may predict subsequent disease progression and a marked increase in pro-inflammatory cytokine levels can be used as a surrogate marker for HIV-disease progression (Godfried et al., 1993, Fahey et al., 1990, Miedema et al., 2013, Stacey et al., 2009, Roberts et al., 2010, Ausiello et al., 1996).

Our earlier findings revealed that acute HIV-1 subtype C CD8+ T-cell responses were very narrow in breadth and that early Nef responses were associated with higher viral loads. In light of these findings, we aimed to determine whether an association exists between acute plasma and T-cell immune activation levels and the magnitude and breadth of CD8+ T-cell responses during acute HIV-1

infection. In addition, we aimed to identify acute phase plasma and T-cell immune activation signatures associated with rapid disease progression. Although studies have reported that a number of plasma and T-cell markers of immune activation predict disease progression, no single study has assessed the combined predictive value of these parameters during acute HIV-1 infection. The predictive value of immune activation biomarkers has largely been investigated during chronic HIV infection, but the benefits of being able to predict, and perhaps modify, disease course during early HIV infection would be beneficial. In addition to shedding light on the narrowness and general ineffectiveness of the early immune response, determining which biomarkers are dysregulated during acute and primary HIV-1 infection may provide direction in understanding mechanisms of pathogenesis.

Using a cohort of acutely infected, antiretroviral therapy-naive individuals, we sought to establish the impact of cytokine expression patterns on the quality of the CD8+ T-cell responses during acute HIV-1 infection and determine the prognostic value of coupled estimates of T-cell activation and plasma cytokine levels in predicting viral load set point and CD4+ T-cell decline during acute HIV-1 infection.

3. Materials and methods

3.1. Study cohort

The study population for this analysis consisted of 33 participants from the previously described cohort (Chapter 2 and 3). The median age at baseline was 27 years (IQR, 25–34 years) and seventeen of the subjects (58%) were female. A median of seven CD4+ T-cell counts per individual was performed over the first year of infection. The rate of CD4+ T-cell decline (median of -4 cells/mm^3 per month; IQR, -8 to 0.3 cells/mm^3 per month) was calculated by linear regression over a median treatment-free follow up period of 234 days (IQR, 58 to 362 days). Viral load set point was calculated as the average viral load from 3 to 12 months post-infection (median of 4.5 log10 copies/ml; IQR, 4 to 5 log10 copies/ml). Sequential plasma samples collected at 4 weeks, 16 weeks and 52 weeks post infection were assayed for cytokine levels. T-cell expression markers of immune activation and dysfunction were measured on PBMC samples collected at 4 weeks post infection. Of the 33 participants in the study, IFN-γ ELISPOT CD8+ T-cell responses to the entire proteome were measured in 25 subjects as explained in chapters 2 and 3, plasma samples for cytokine analysis were available for 21 subjects, and only 19 PBMC samples were available for the evaluation of cell expression markers of immune activation and dysfunction (Figure 4.1).

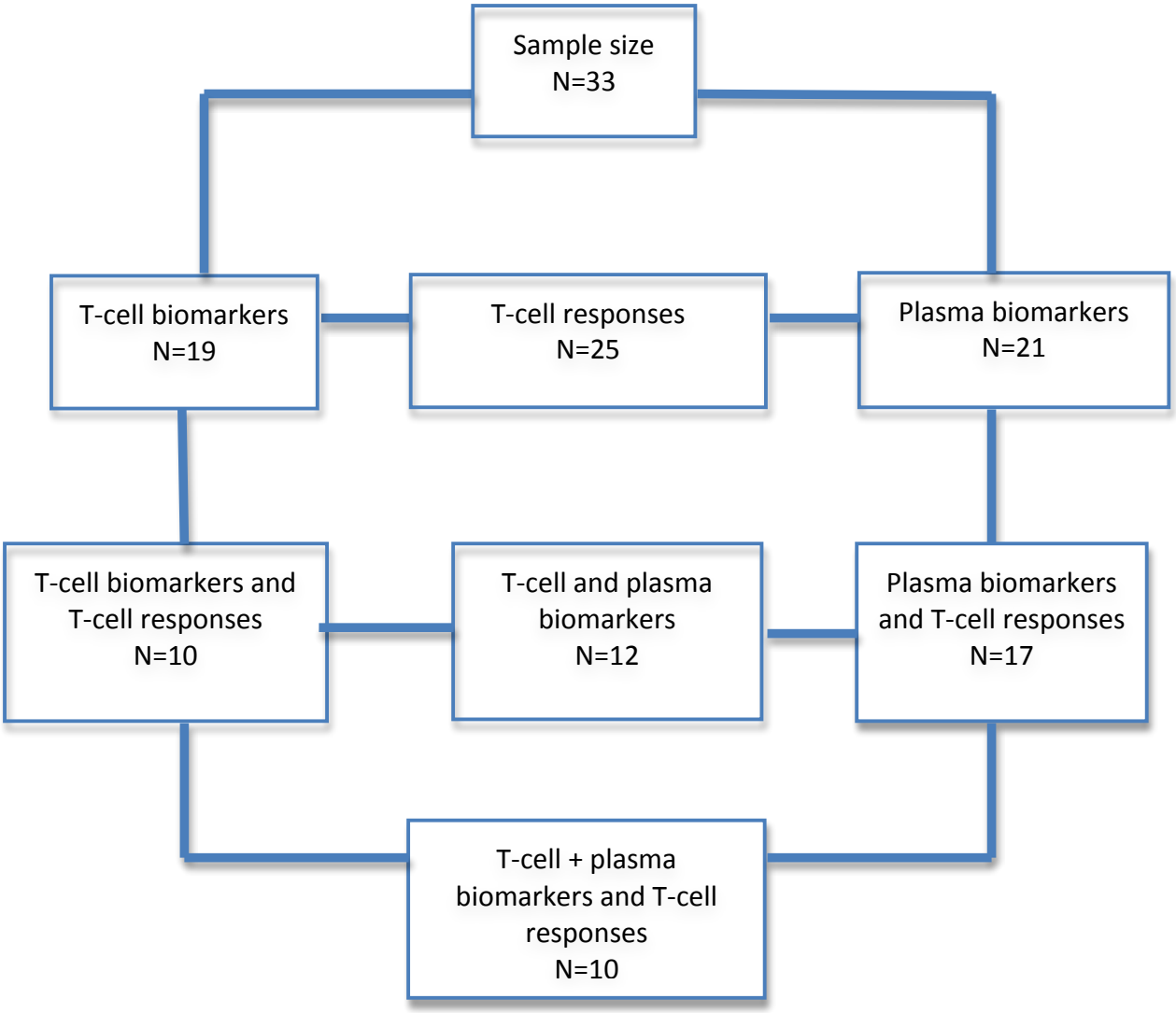


Figure 4.1. Summary of the distribution of the data sets used for analyses and general linear models.

3.2. Viral load determination, CD4+ T-cell counts and HLA typing

Plasma viral loads were measured using either the Roche Amplicor Monitor Assay detection limit of 400 HIV-1 RNA copies/ml plasma,) or the Roche Ultra-sensitive assay detection limit of 50 RNA copies/ml plasma), according to the manufacturer's instructions. CD4 counts were determined from fresh whole blood using Tru-Count technology and analyzed on a four-color flow cytometer (Becton Dickinson) according to the manufacturer's instructions. High-resolution HLA class I typing was performed as previously described (Radebe et al., 2011).

3.3. Measurement of plasma cytokines

Eleven cytokines were measured in plasma from the cohort using the MILLIPLEX™ MAP high sensitivity human cytokine/chemokine kits (Millipore, Melbourne, Australia) according to the manufacturer's instructions for plasma samples. The following cytokines were assessed (minimal detection limits are shown in brackets in pg/ml): interleukin (IL)-1beta (0.06), IL-2 (0.16), IL-8 (0.11), IL-10 (0.15), IL-12p70 (0.11), granulocyte macrophage-colony stimulating factor (GM-CSF) (0.46), interferon (IFN)-gamma (0.10), IFN-gamma-induced protein (IP-10) (1.20), macrophage inflammatory protein (MIP)-1 alpha (4.50), MIP-1 beta (3.50) and tumour necrosis factor (TNF)-alpha (0.40). Samples were studied undiluted in triplicates and the average value computed for each individual analyte. Data were collected and read on a Bio-Plex 200 reader (Bio-Rad Laboratories). Concentration values below the lower limits of detection were

reported as the midpoint between the lowest concentration for each cytokine measured and zero (Roberts et al., 2010, Bebell et al., 2008).

3.4. Assessment of T-cell activation by multicolor flow cytometry

Ex vivo measurement of T-cells for the expression of markers of activation (CD38, HLA-DR, CD69) (Kestens et al., 1992, Krowka et al., 1996), exhaustion (PD-1) (Day et al., 2006), senescence (CD57) (Brenchley et al., 2003) and apoptosis (CD95) (Westendorp et al., 1995) were performed. Briefly, thawed cryopreserved PBMCs were resuspended to 1-2 x 10^6 cells/ml in R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS), 100 U/ml penicillin, 1.7mM sodium glutamate, 5.5ml HEPES buffer) and rested overnight at 37^0C; 5% CO_2. Cells were then adjusted to 1x10^6 cells/ml, washed with PBS (2% PBS/FCS), stained for intracellular amine groups to differentiate between live/dead using the violet viability dye (Invitrogen) and incubated for 30 min at 4^0C. Following incubation, cells were washed and stained with the following surface antibodies: CD3-phycoerithrincarbocyanin (PE-Cy5.5), CD8 phycoerithrincarbocyanin (PE-Cy7) and CD4 quantum dot (Qdot 605), in addition to: CD38 phytoerythrin (PE), CD57 allophycocyanin (APC), CD69 allophycocyanin carbocyanin, CD95 fluorescein isothiocyanate (FITC), HLA-DR- peridin chlorophyll protein (PerCP) and PD-1 pacific blue (Pac Blue) all from BD PharMingen (BD BioSciences, San Jose, CA, USA). Cells were again washed and fixed in 1% paraformaldehyde (Fix Perm A, Caltag) for 20 min in the dark at room temperature. Following incubation, cells were washed twice with PBS and resuspended in 200µl of PBS.

Samples were acquired on the LSR II flow cytometer, using FACSDiva software (BD Biosciences), and analyzed with FlowJo software (Tree Star, San Carlo, CA). Fluorescence voltages were determined using matched unstained cells. Fluorescence-minus-one (FMO) was used for gating strategy (Figure 4.2) (Roederer, 2001). For each sample a minimum of 300,000 events we acquired.

3.5. Statistical analysis

Univariate analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). Spearman rank tests were used to test for correlations. P values less than 0.05 were considered significant. General linear models were performed using Stata (Stata Corp., College Station, Texas, USA). A general linear model was used to determine the cytokines and T-cell markers that best correlated with magnitude or breadth of acute CD8+ T-cell responses, viral load set points and the rate of CD4+ T-cell decline. Log transformed viral loads and cytokine concentrations were used. Using a univariate regression as a starting point, variables that were significantly associated with (1) breadth and magnitude of CD8+ T-cell responses or (2) viral load set point or (3) CD4+ T-cell loss, while controlling for each of the markers already included, were added to the model in a stepwise manner.

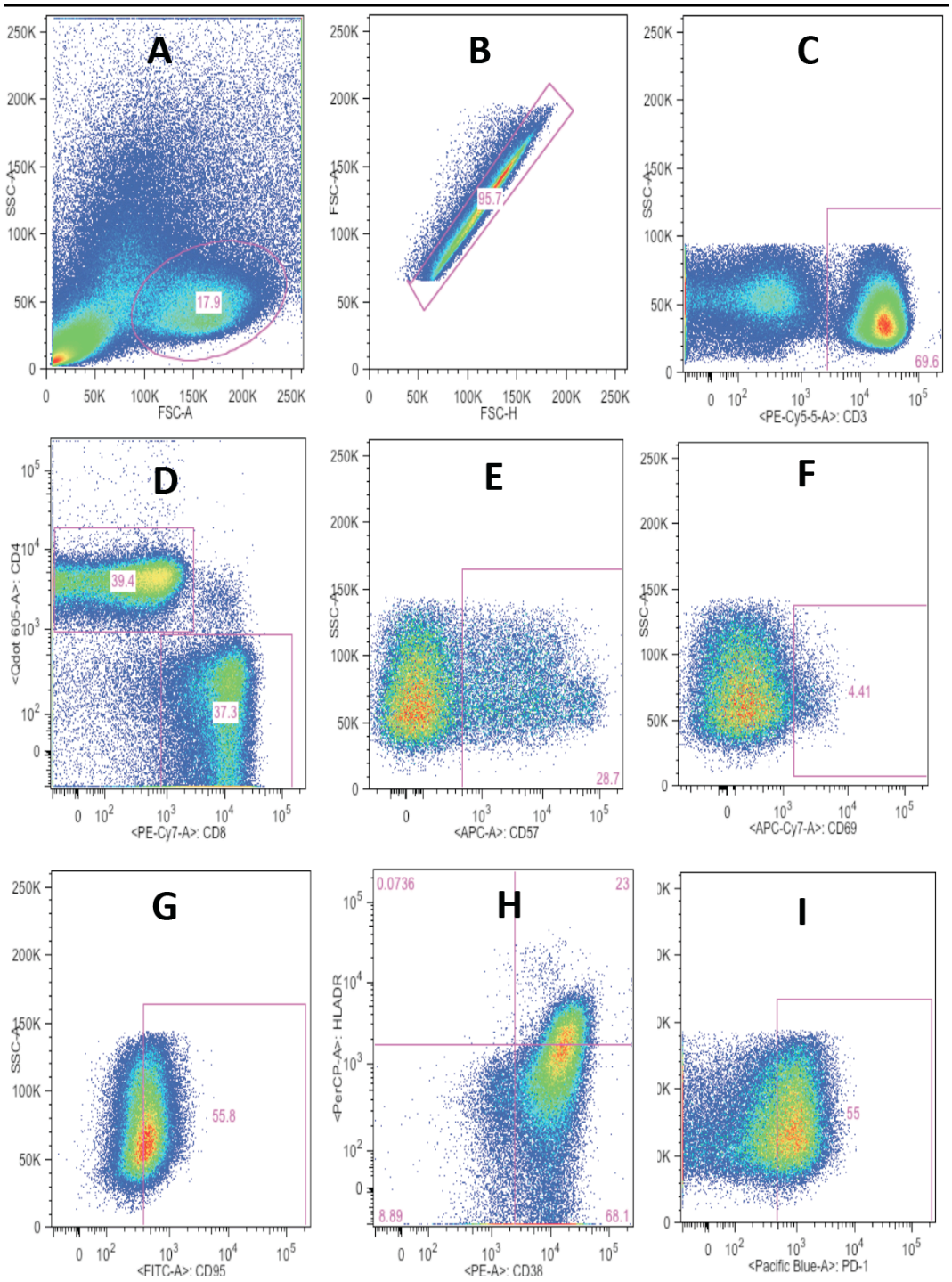


Figure 4.2. Gating strategy for identification of CD4+ and CD8+ T-cell subsets. Representative flow cytometry plots from the PBMCs of one subject within the cohort to demonstrate the gating strategy. Gating Strategy on (A) lymphocyte population, (B) singlet, (C) CD3+ cells (D) CD4+ and CD8+ cells and (E) CD8+ T-cells expressing CD57, (F) CD8+ T-cells expressing CD69 (G) CD8+ T-cells expressing CD95, (H) CD8+ T-cells expressing HLA-DR and CD38 (I) CD8+ T-cells expressing PD-1.

4. Results

4.1. Exponential viral load expansion in plasma donors with acute HIV infection

Sequential samples collected during acute HIV infection present a rare opportunity to analyze kinetic changes in cytokine levels during the earliest stages of infection. Plasma panels from 33 participants enrolled into the study during acute HIV-1 infection were studied. At the time when the first samples were taken, subjects were Fiebig stages III or IV (Fiebig et al., 2003) and the median plasma viral load was elevated at 6 log_{10}copies/ml (IQR 4.7 to 6.1 log_{10} copies/ml), thus indicative of acute HIV infection. The level of viremia decreased over time, to a median of 4 log_{10} copies/ml (IQR 3.5 to 4.6 log_{10} copies/ml) at 1 year post infection. CD4+ T-cell kinetics over time were less pronounced, with a decrease from 420 T-cells/mm^3 (IQR 383 to 586 cells/mm^3) to 419 cells/mm^3 (IQR 316-466 cells/mm^3) at one year. The relationship between viremia and CD4+ T-cell counts at baseline was examined using data from all 33 subjects. There was a negative correlation between plasma HIV RNA levels and CD4+ T-cell counts at study baseline (Figure 4.3A) (Spearman *rho =–0.40, P =0.04*, n = 33). We performed a linear regression analysis to estimate the independent associations between CD4+ T-cell counts and contemporaneous viral load during acute HIV-1 infection. A linear regression analysis showed that on average, there was a 0.2-log10 drop in viral load per 100 cells/mm^3 increase in CD4+ T-cell count. There was no correlation observed between the CD4+ T-cell count measured at baseline and viral load set point, however a marginal trend toward significance

was observed for higher CD4+ T-cell counts and lower viral load set point (P=0.08, r=-0.32 (Figure 4.3B).

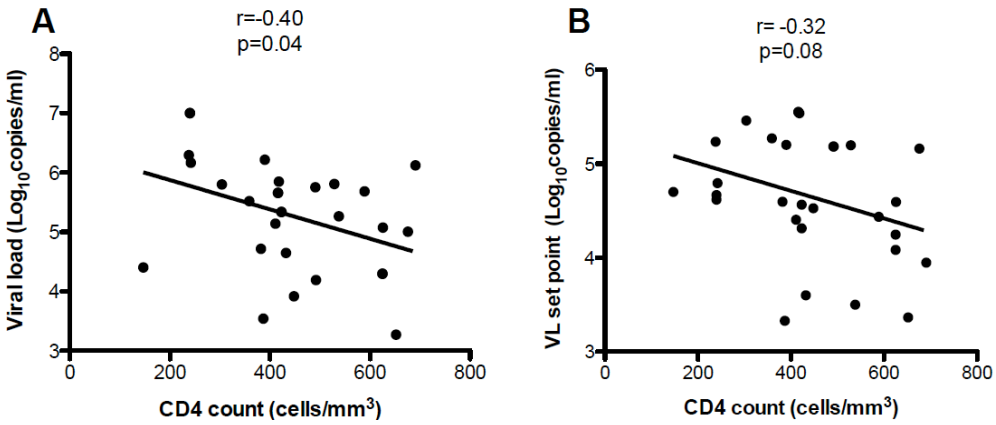


Figure 4.3. The relationship between CD4+ T-cell counts and (A) concurrent plasma HIV RNA levels and (B) viral load set point (both log_{10} transformed) during acute and early HIV-1 infection. A smooth line was generated by linear regression with quadratic equations.

4.2. Plasma levels of cytokines and chemokines in primary HIV-1 infection.

At 4 weeks post infection, a total of 11 plasma cytokines and chemokines were quantified in the plasma of 21 subjects. Figures 4.4 shows examples of the changes occurring in all analytes in each of the 21 subjects. Baseline expression levels were measured at 4 weeks post infection, and analyte levels measured at 16 and 52 weeks were deemed elevated if there was a >2 fold increase from baseline. Between 4 and 16 weeks post infection we observed an increase (>2) in the levels of IL-12p70, GM-CSF and IL-1 beta, the increase in these factors was sustained up to 52 weeks post infection (Figure 4.4 and 4.5). There was also a notable increase in the expression of the anti-inflammatory cytokine IL-10

between 16 weeks and 52 weeks post infection. However, although modest changes in the levels of a handful of cytokines and chemokines were observed, no significant changes in the kinetics of many of the cytokines and chemokines known to be produced very early on in acute and primary HIV-1 infection were detected (Stacey et al., 2009, Gay et al., 2011). The modest changes in the levels of cytokines may be explained by the fact that the time points analyzed corresponded to Fiebig stages III and IV instead of the earliest (eclipse and Fiebig stages I or II) stages of HIV-1 infection.

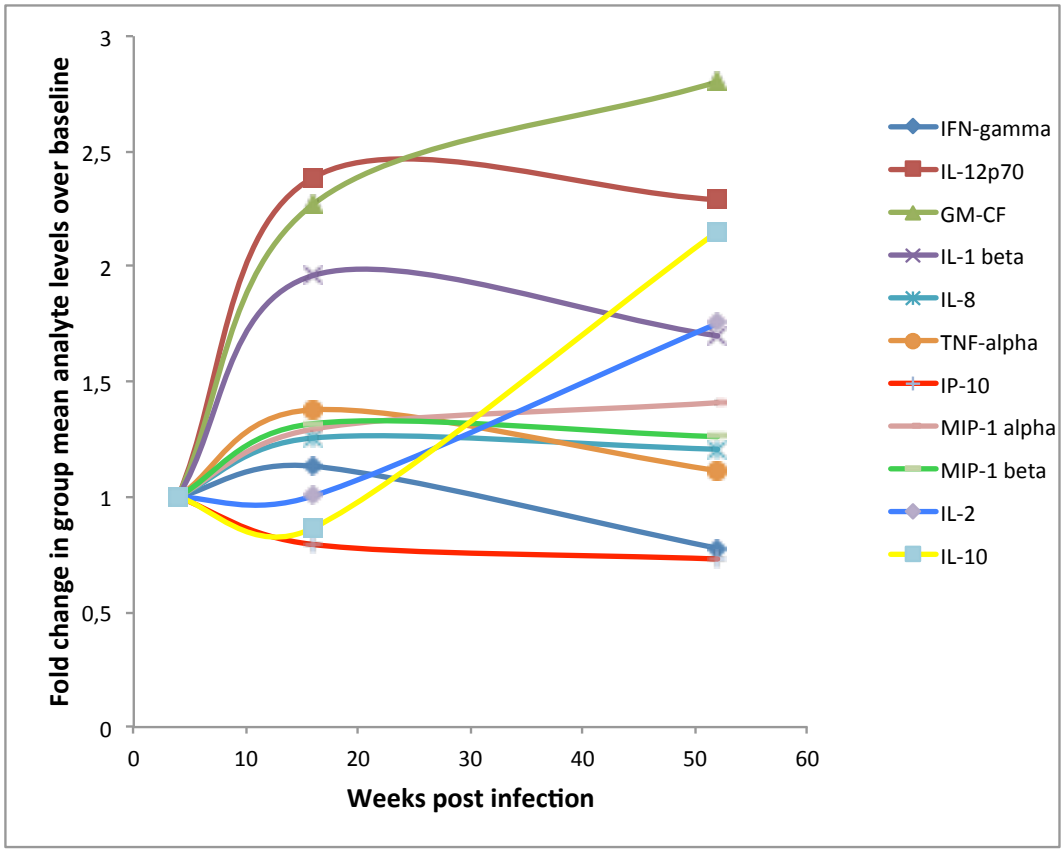
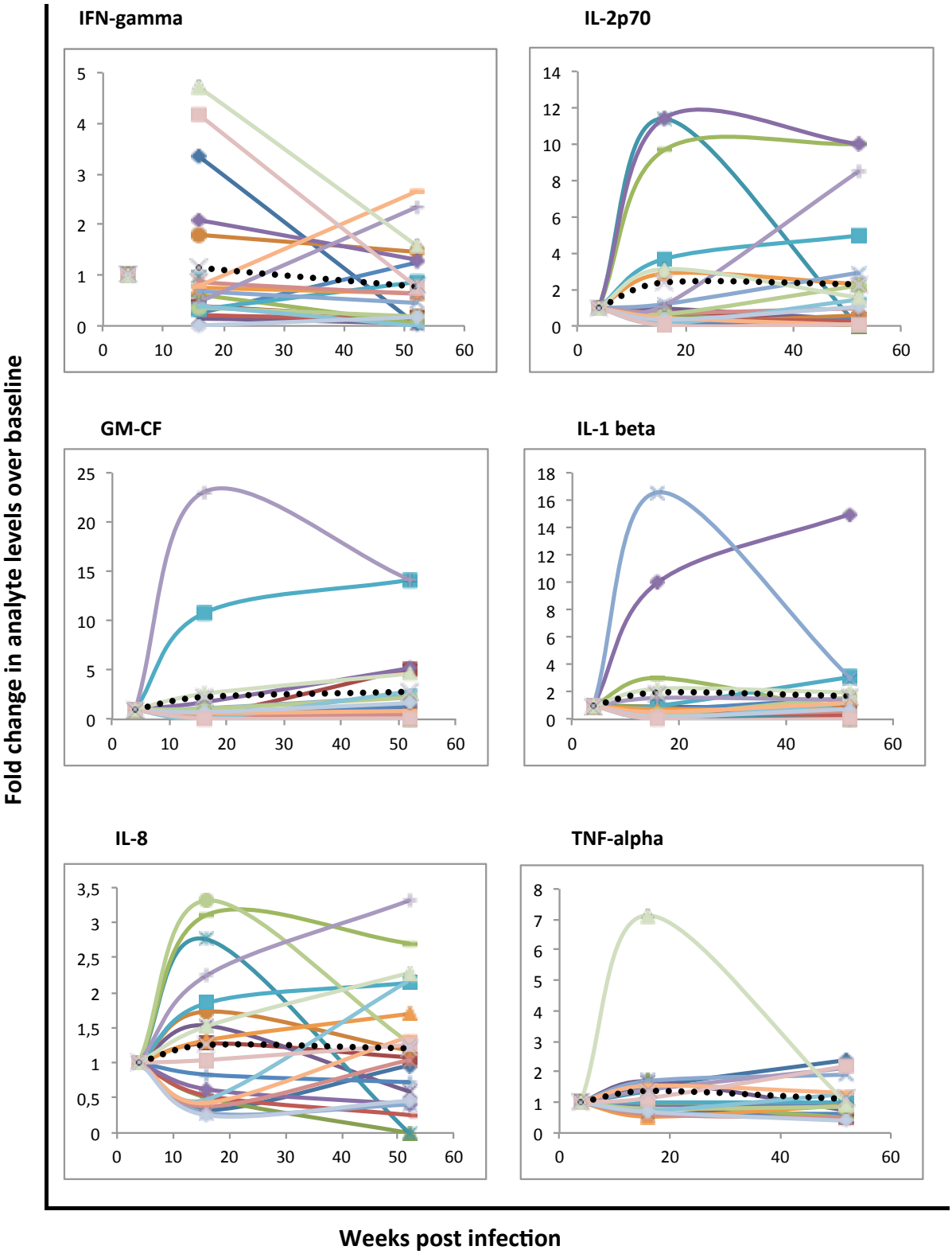


Figure 4.4. The mean fold changes of analytes from baseline (4 weeks) up to 52 weeks post infection. The graph is a composite of the results for all 11 analytes.


IFN-gamma
IL-2p70
GM-CF
IL-1 beta
IL-8
TNF-alpha
Fold change in analyte levels over baseline
Weeks post infection

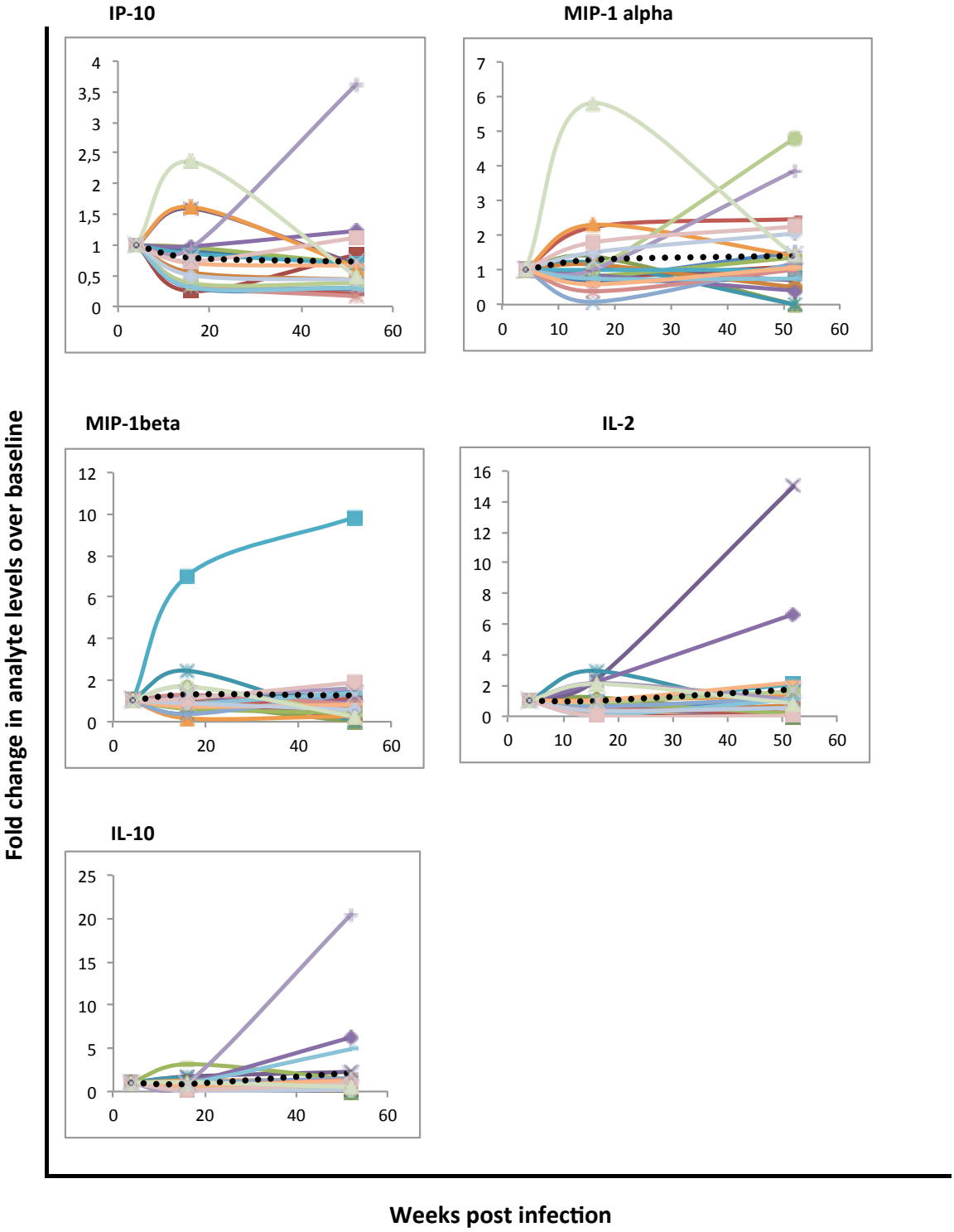


Figure 4.5. Changes of plasma analyte levels relative to baseline during primary HIV-1 infection. Plasma levels of 11 cytokines (IL-1β, TNF-α, IL-8, IFN-γ and IL-10, MIP-1α, MIP-1β, IL-12p70) collected at 3 sequential time points from 21 HIV-infected subjects are shown (colored lines), the mean values for each analyte are also given (black dotted lines).

4.3. Cytokine expression is associated with CD8+ T-cell magnitude and breadth

Univariate regression analysis was used to determine the relationship between plasma and T-cell markers of immune activation and the breadth of CD8+ T-cell responses during acute HIV-1 infection. Interestingly, MIP-1β, a cytokine that is produced by HIV-specific CD8+ T-cells was the only marker significantly associated with the breadth CD8+ T-cell responses in the unadjusted analysis, and this was an inverse relationship such that with every one log increase in MIP-1β, breadth decreased by 1.36 units (p=0.0240) (Table 4.1). IL-10 showed a trend of positive association with breadth (p=0.0720). No other variables were associated with breadth. When both MIP-1β and IL-10 were added to the general linear model, MIP-1β remained statistically significant (p=0.0335).

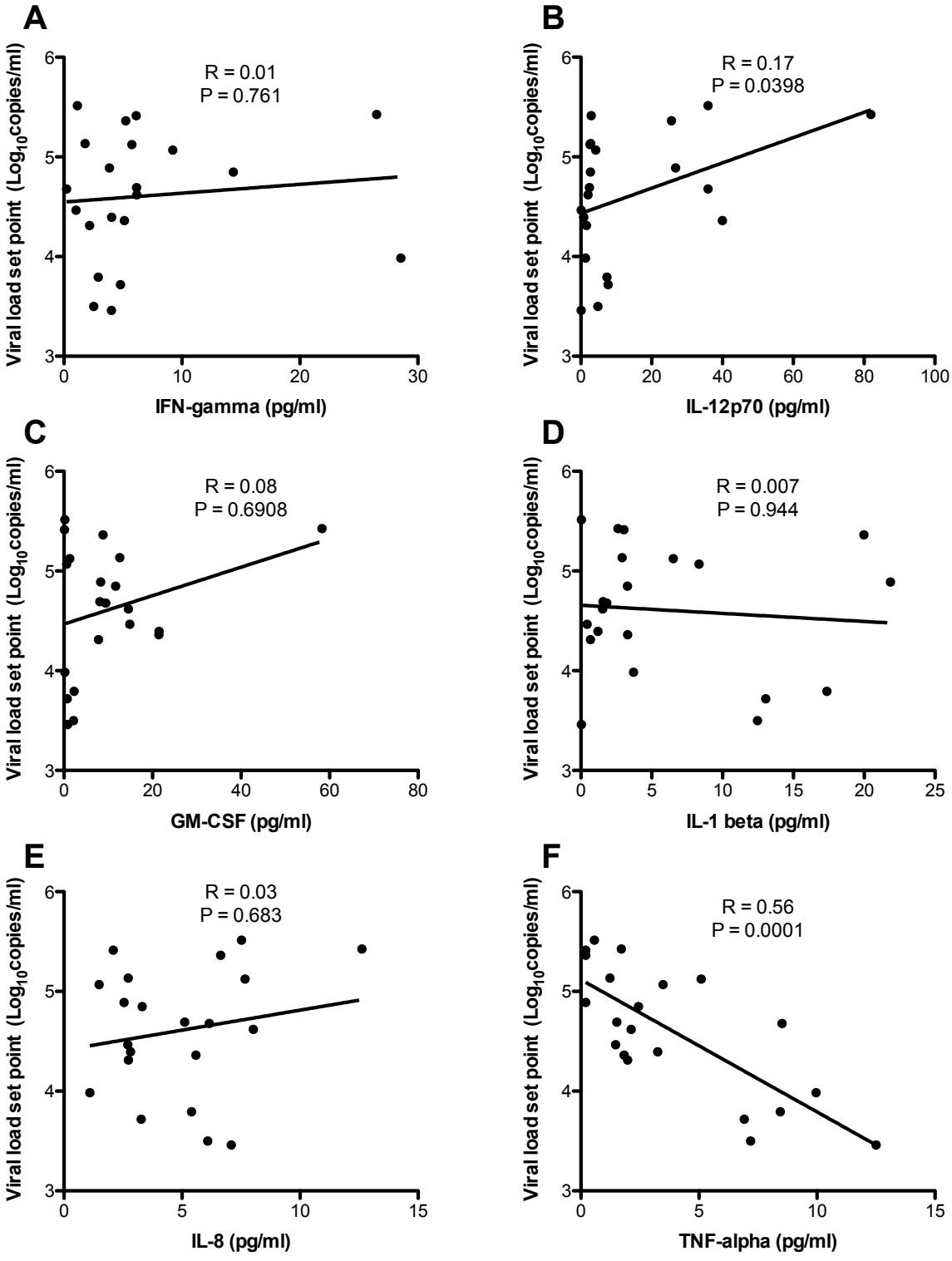
A univariate regression analysis was used to determine the impact of acute phase plasma and T-cell markers of immune activation cytokines on the magnitude of CD8+ T-cell responses. A square root transformation was applied to the magnitude in order to ensure normality. As observed previously, only MIP-1β was significantly associated with the magnitude of the earliest CD8+ T-cell response in the unadjusted analysis; with every one log increase in MIP-1β, magnitude decreases by 32.96 square root units – or 1,086 SFU/10^6 PBMCs. When we fitted an adjusted model with MIP-1β and IL-10, MIP-1β remained statistically significant (p=0.0015) (Table 4.1).

Table 4.1. General linear model analysis showing associations between plasma T-cell markers of immune activation and the acute phase CD8+ T-cell response.

Independent Variable	Variable	Unadjusted analysis		Adjusted analysis	
		Effect estimate (SE)	p-value	Effect estimate (SE)	p-value
Breadth	MIP-1β	-1.36 (0.54)	0.0240	-1.20 (0.51)	0.0335
	IL-10	1.08 (0.56)	0.0720	0.87 (0.50)	0.1011
Magnitude	MIP-1β	-32.96 (8.30)	0.0011	-31.01 (8.04)	0.0015
	IL-10	16.03 (10.42)	0.1434	10.63 (7.83)	0.1948

4.4. Early cytokine expression patterns predict viral load set point

A general linear model was used to determine the relationship between plasma cytokine concentrations at 4 weeks post infection and viral load set point. Using univariate regression as a starting point, cytokines that were significantly associated with viral load set point were added to the model in a stepwise manner. Only a handful of the 11 variables were significantly associated with changes in viral load set point (Figure 4.6 B, F, G and K). Higher plasma levels of IL-10, IL-12p70 and IP-10 were significantly associated with an increase in viral load set point (p=0.0003, p=0.0398 and p=0.0529, respectively) whilst high TNF-α expression was associated with a significant decrease in viral load set point (p=0.0001) (Table 4.2). When we adjusted for other cytokines, TNF-α came up as negatively associated with viral set point (p=0.0136), while the effect of IL-10 remained but was less dramatic (p=0.0193).


A
R = 0.01
P = 0.761
Viral load set point (Log10copies/ml)
IFN-gamma (pg/ml)
B
R = 0.17
P = 0.0398
Viral load set point (Log10copies/ml)
IL-12p70 (pg/ml)
C
R = 0.08
P = 0.6908
Viral load set point (Log10copies/ml)
GM-CSF (pg/ml)
D
R = 0.007
P = 0.944
Viral load set point (Log10copies/ml)
IL-1 beta (pg/ml)
E
R = 0.03
P = 0.683
Viral load set point (Log10copies/ml)
IL-8 (pg/ml)
F
R = 0.56
P = 0.0001
Viral load set point (Log10copies/ml)
TNF-alpha (pg/ml)

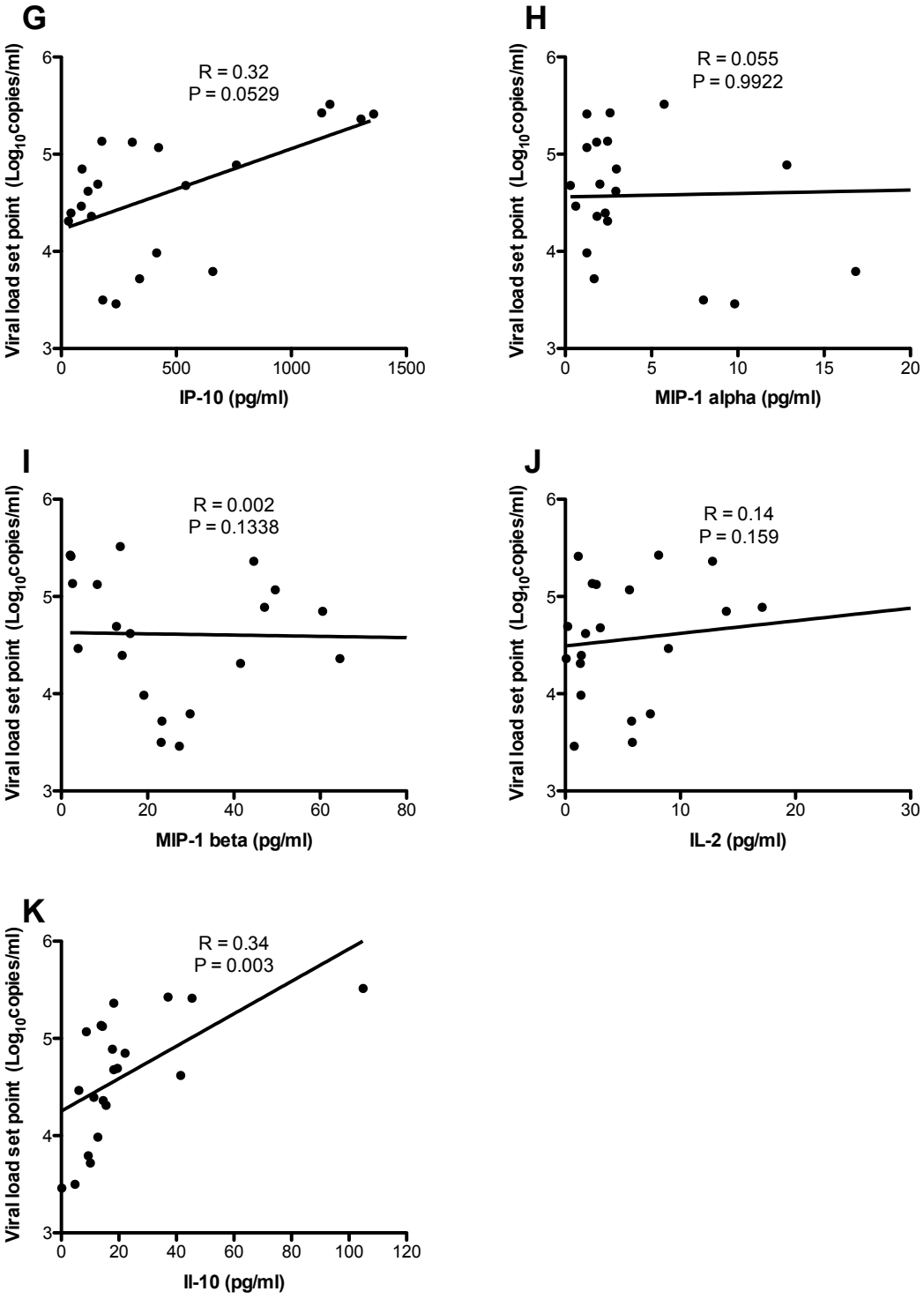
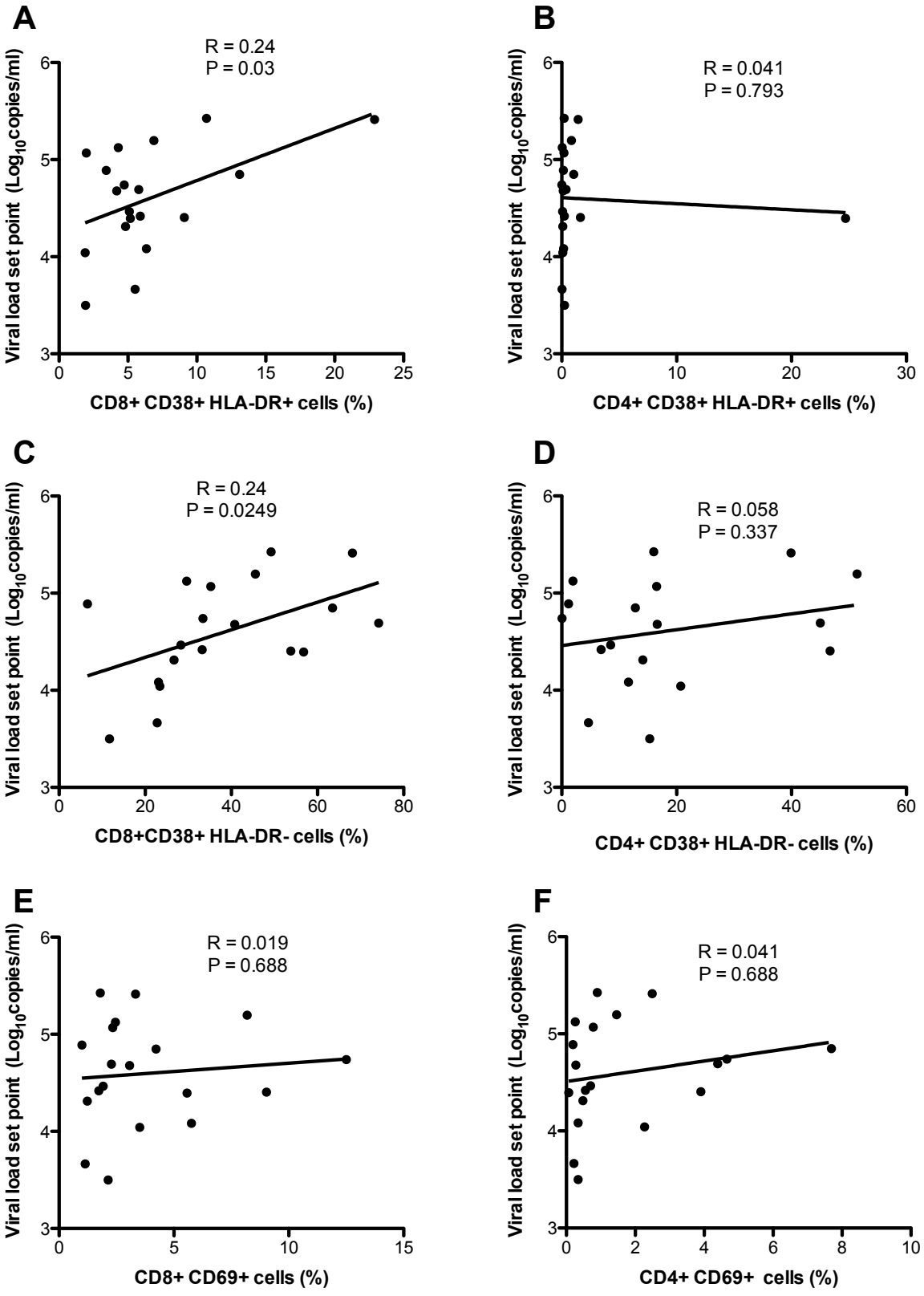


Figure 4.5. The relationship between viral load set point and cytokines measured at 4 weeks post infection in 21 individuals. A smooth line was generated by linear regression with quadratic equations.

4.5. T-cell markers of immune activation and viral load set point

In order to assess the impact of T-cell markers of activation measured at 4 weeks post infection on viral load, we fitted a general linear model to viral set point using data from 19 subjects. Using a univariate regression as a starting point, T-cell markers that were significantly associated with viral load set point were added to the model in a stepwise manner. We observed no associations between the expression of the various markers on CD4+ T-cells and viral load set point. However, there was a positive correlation between viral load set point and CD8+ T-cell activation levels as measured by CD38+HLA-DR- expression ($p=0.0240$) (Figure 4.7 C) (Table 4.2). Elevated CD38 expression may be used as a marker of CD8+ T-cell activation and appears to be a better correlate of HIV-1 disease progression than when combined with HLA-DR expression (Liu et al., 1997). There was also a positive correlation between viral load set point and CD8+ T-cell expression of CD95+ ($p=0.0267$), PD1+ ($p=0.0312$) (Figure 4.7G and I). However, when we adjusted the model for other T-cell markers, none of the T-cell markers came up as significantly associated with viral set point (Table 4.2).


A
R = 0.24
P = 0.03
Viral load set point (Log10copies/ml)
CD8+ CD38+ HLA-DR+ cells (%)
B
R = 0.041
P = 0.793
Viral load set point (Log10copies/ml)
CD4+ CD38+ HLA-DR+ cells (%)
C
R = 0.24
P = 0.0249
Viral load set point (Log10copies/ml)
CD8+CD38+ HLA-DR- cells (%)
D
R = 0.058
P = 0.337
Viral load set point (Log10copies/ml)
CD4+ CD38+ HLA-DR- cells (%)
E
R = 0.019
P = 0.688
Viral load set point (Log10copies/ml)
CD8+ CD69+ cells (%)
F
R = 0.041
P = 0.688
Viral load set point (Log10copies/ml)
CD4+ CD69+ cells (%)

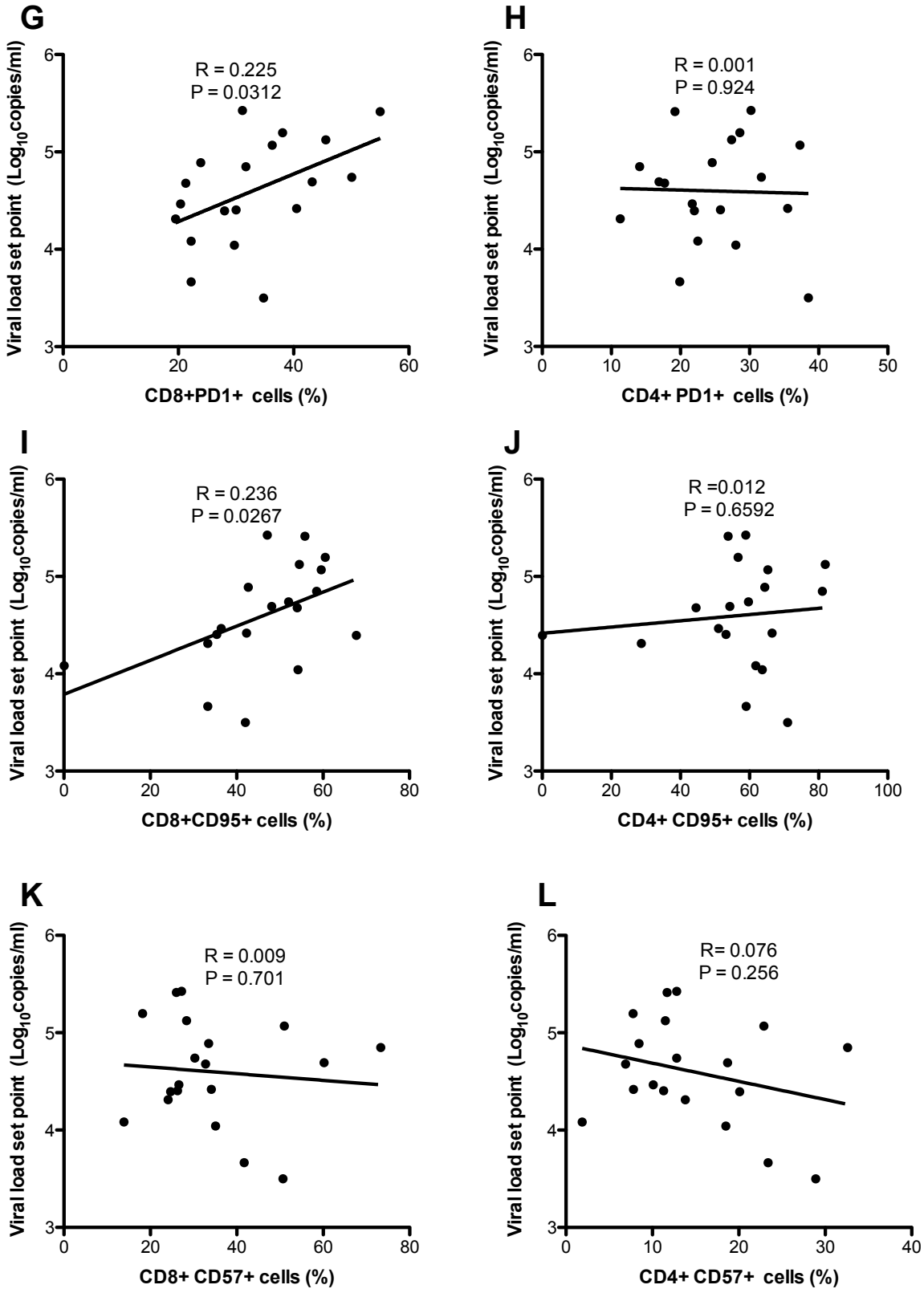


Figure 4.6. The relationship between CD8+ and CD4+ T-cell activation (A-F), exhaustion (G-H), apoptosis (I-J) and senescence (K-L) and viral load set point (log_{10}transformed) in 19 individuals during acute HIV infection. A smooth line was generated by linear regression with quadratic equations.

Table 4.2. General linear model analysis for the evaluation of associations between acute phase plasma and T-cell markers of immune activation and viral load set point.

Variable	Unadjusted analysis		Adjusted analysis Plasma + T-cell markers (n=12)	
	Effect estimate (SE)	p-value	Effect estimate (SE)	p-value
PD-1	0.02 (0.01)	0,0312	-0.005 (0.007)	0,5515
CD95	0.02 (0.007)	0,0267	0.02 (0.007)	0,1029
CD38	0.01 (0.006)	0,024	-0.006 (0.005)	0,3414
IL12p70	0.33 (0.15)	0,0398	-0.62 (0.15)	0,0259
TNF-α	-0.83 (0.18)	0,0001	0.15 (0.16)	0,4199
IP-10	0.55 (0.26)	0,0529	0.80 (0.19)	0,0242
IL-10	0.82 (0.19)	0,0003	2.65 (0.54)	0,0158

Table 4.3. General linear model analysis for the evaluation of associations between acute phase plasma and T-cell markers of immune activation and CD4+ T-cell loss.

Variable	Unadjusted analysis		Adjusted analysis Plasma + T-cell markers (n=12)	
	Effect estimate (SE)	p-value	Effect estimate (SE)	p-value
PD-1	0.14 (0.10)	0,1827	0.23 (0.06)	0,029
CD95	0.02 (0.07)	0,7693	-0.05 (0.06)	0,4208
IL12p70	2.19 (3.22)	0,5041	2.69 (1.27)	0,1236
TNF-α	-2.35 (4.85)	0,6337	-9.70 (1.36)	0,0057
IP-10	4.04 (5.55)	0,4761	-4.07 (1.60)	0,0839
IL-10	3.28 (4.95)	0,5159	-21.79 (4.50)	0,0168

4.6. Plasma cytokine concentrations alone predict viral load set point

To assess the coupled impact of cellular expression markers and cytokines measured during acute HIV-1 infection on viral load, a general linear model was fitted to viral set point and the model was adjusted for both variables. Only 12 individuals could be included in this analysis (i.e., had both cytokine and T-cell surface expression data). Four cytokines (IL-12p70, TNF-α, IL-10 and IP-10) and cellular expression markers (CD38, PD-1 and CD95) were incorporated into this model, each of which was significantly associated with viral load set point in the unadjusted models. Higher levels of IL-12p70 were significantly associated with a lower viral load set point – with every one log increase in IL-12p70, viral set point decreased by 0.62 log copies/ml (p=0.0259) (Table 4.2). High expression levels of IP-10 and IL-10 were significantly associated with an increase in viral set point (p=0.0242 and p=0.0158, respectively). After adjusting the model, none of the cellular expression markers came up as significantly associated with viral set point, however the small sample size may have been a limitation in this analysis.

4.7. Variables predicting the rate of CD4+ T-cell decline

A general linear model was used to determine the effect of plasma and T-cell markers of immune activation measured during acute HIV-1 infection and on the rate CD4+ T-cell decline (Table 4.3). When the model was adjusted for plasma and T-cell markers of immune activation associated with viral load set point in the univariate analysis, PD-1 expression on CD8+ T-cells was significantly associated with CD4+ T-cell preservation (p=0.029). Plasma concentrations of IL-

10 and TNF-α were significantly associated with a decline in CD4+ T-cell counts (p=0.0057 and p=0.0168, respectively), a notable but not significant trend for IP-10 expression and lower CD4+ T-cell counts was also observed (p=0.083). We observed no associations between the expression of the various markers on CD4+ T-cells and the rate of CD4+ decline.

5. Discussion:

Immune activation during HIV infection has been shown to be a major contributor to HIV disease progression and is the product of inflammatory responses to HIV-1 antigens, microbial translocation, HIV-encoded Toll-like receptor ligands, and the homeostatic response to CD4+T-cell depletion (Deeks et al., 2004, Brenchley et al., 2006b, Meier et al., 2007). Although the contribution of immune activation in HIV disease pathogenesis has been extensively evaluated in chronic HIV infection, its role in primary infection remains poorly defined. Moreover, the impact of cytokine and chemokine expression patterns on the quality of the CD8+ T-cell responses during acute HIV-1 infection is not well understood. One of the main findings of this study is that the breadth and magnitude of the acute CD8+ T-cell response was inversely correlated with plasma MIP-1β. It is noteworthy to mention that the acute CD8+ T-cell response was dominated by Nef-specific responses and although we cannot ascertain the type of cells which where responsible for the secretion of this chemokine, these findings are in line with a recent report from Turk et al. (2013) showing that Nef-specific cells inversely correlated with plasma MIP-1β. These data are of importance, since MIP-1β is produced by CD8+ T-cells upon stimulation and the percentage of cells expressing MIP-1β alone or in combination with other functions have been previously associated with virus control and inhibition (Ferrari et al., 2011, Betts et al., 2006, Freel et al., 2010, Saunders et al., 2011). Previous studies have also demonstrated that MIP-1β-producing CD8+ T-cells exert considerable immune pressure over viral replication

(Ferrari et al., 2011, Almeida et al., 2009). It is intriguing that there exists an inverse association between this important soluble antiviral mediator and the acute CD8+ T-cell responses. Given that MIP-1β has been identified as a key determinant of protective immunity, our current findings are in line with earlier reports (Chapters 2 and 3), which indicate that early Nef CD8+ T-cell may be ineffective and are related to higher viral loads. These data provide some rationale for the association between early Nef responses and viremia. It could also be that the MIP-1β measured in plasma is not necessarily expressed by HIV-specific CD8+ T-cells but rather a product of bystander activated cells. Thus, MIP-1β in plasma may not reflect the potency of the HIV-specific immune response but just generalized immune activation. Further investigations will be needed to determine the nature of the relationship between acute Nef-specific responses and MIP-1β-production, the lack of expression of this key chemokine may be related to the generation of an ineffective antiviral immune response leading to faster disease progression.

We show that plasma pro-inflammatory TNF-α levels were directly associated with rapid CD4+ T-cell loss and higher IP-10 levels were significantly associated with higher HIV viral loads and rapid CD4+ T-cell loss. Recently, IP-10 was shown to be the only cytokine among the 26 cytokines tested that was always positively correlated with disease progression during Fiebig stages III-V in HIV-1 infection (Liovat et al., 2012, Jiao et al., 2012). The strong predictive capacity of IP-10 was confirmed in an independent group of 88 HIV-1 infected individuals where plasma IP-10 levels were an earlier marker with strong predictive value for

disease progression (Liovat et al., 2012). Widespread acute-phase CD4+ T-cell destruction is a hallmark of pathogenic immunodeficiency virus infections in humans and primates, and cytokines induced in acute HIV infection may contribute to the high levels of CD4+ T-cell loss that occurs at this time (Klein et al., 1996, Gasper-Smith et al., 2008). Although the apoptosis of HIV-infected CD4+ T-cells may restrict viral spread, cytokines may also drive the destruction of bystander uninfected CD4+ T-cells (Gasper-Smith et al., 2008). The upregulation of TNF-α in acute/early HIV-1 infection is thought to drive apoptosis through binding to cellular TNF receptors (Klein et al., 1996, Gasper-Smith et al., 2008). This is supported by the observation that peak plasma levels of apoptotic microparticles, TNFR2, and Fas ligand were observed at the time of maximal TNF-α when viremia was also at its peak (Gasper-Smith et al., 2008).

Previous studies have demonstrated that HIV-infected patients have significantly higher circulating IL-10 levels than healthy controls, with the highest levels in patients with a high viral load and a more rapid disease progression (Stylianou et al., 1999, Brockman et al., 2009, Srikanth et al., 2000, Roberts et al., 2010). Similarly, we report that high plasma immunoregulatory IL-10 levels were significantly associated with higher HIV viral load and rapid CD4+ T-cell loss. Data from HIV-1 and SIVmac infections have demonstrated that IL-10 is induced after the first burst of pro-inflammatory cytokines, possibly to dampen the acute "cytokine storm" (Kornfeld et al., 2005, Katsikis et al., 2011, Stacey et al., 2009). Production of this anti-inflammatory cytokine might participate in disease progression by inhibiting HIV-1-specific T-cell responses during acute infection

and thus contribute to viral persistence (Couper et al., 2008, Estes et al., 2006, Brooks et al., 2008, Brooks et al., 2006). However, the role of IL-10 in HIV-1 infection is complex as other studies have also shown that polymorphisms associated with decreased IL-10 production are associated with an increased likelihood of HIV-1 acquisition and an accelerated rate of $CD4^+$ T cell decline, particularly in late-stage disease, suggesting that high IL-10 expression may reduce susceptibility to HIV-1 infection and protect against disease progression (Erikstrup et al., 2007, Naicker et al., 2009, Shin et al., 2000). Based on our findings, we hypothesize that high CD4+ T-cell loss rates and viral loads observed in individuals with higher levels of IL-10 may be the result of a stunted adaptive immune response or immunosuppression that allows viruses to persist unchecked by the immune system.

We found that higher concentrations of IL-12p70 associated with lower viral set point. Our findings corroborate prior work describing the role of IL-12p70 in delaying HIV-1 disease progression (Roberts et al., 2010). In the macaque model of SIV infection, IL-12p70 treatment during acute infection was associated with decreased viral loads, preservation of HIV-specific CD8+ T-cells and survival (Ansari et al., 2002) and data from *in vitro* studies also suggest that IL-12 can restore HIV-specific cell-mediated immunity in HIV-infected individuals (Clerici et al., 1993).

Expression of the inhibitory marker PD-1 is known to play an important role in resolving immune activation and T-cell proliferation (Sharpe et al., 2007, Keir et al., 2007, Estes et al., 2008, Freeman et al., 2000, Day et al., 2006). We show in

this study that early elevation of PD-1 expression correlated directly with preservation of CD4+ T-cells. Our results are in line with a report from an SIV study, which demonstrated that the early upregulation of PD-1 is associated with the resolution of immune activation and slower disease progression (Estes et al., 2008). The study suggested that decreased immune activation mediated by early PD-1 expression in sooty Mangabeys may represent an important cause for the lower viral set point levels compared with rhesus macaques (Estes et al., 2008). In this study, we hypothesize that early activation of PD-1 expression may lower immune activation during acute infection and therefore attenuate the immunopathogenic effects driven by persistent immune activation such as CD4+ T-cell loss. The delayed induction of PD-1 possibly fails to diminish T-cell activation after the acute window of infection, resulting in sustained activation and dysfunction seen in chronic HIV-1 infection (Estes et al., 2008, Zhang et al., 2007, Day et al., 2006, Trautmann et al., 2006). Our findings suggest that early control of immune activation by host immunoregulatory mechanisms may be a determinant of disease outcomes. Although progression of HIV-1 infection in terms of CD4+ T-cell decline, has been correlated to PD-1 T-cell subsets in chronic infection (Liovat et al., 2012, Palmer et al., 2013), to our knowledge, this is the first time this association has been described in primary HIV-1 infection.

6. Conclusion

In summary, the impact of acute phase immune activation and plasma cytokine levels were characterized and an association of these factors with the magnitude and breadth of CD8+ T-cell responses was studied. We showed that acute phase expression of MIB-1β was inversely associated with the magnitude and breadth of acute CD8+ T-cell responses. We also demonstrate the potential to use plasma cytokine concentrations and estimates of T-cell dysfunction during acute HIV-1 infection to predict subsequent disease progression. Early IL-10, IP-10 and IL-12p70 levels were more predictive of viral load set point than either CD38 and PD-1 expression or CD4+ T-cell counts and IL-10, IP-10 and PD-1 expression levels also correlated with CD4+T-cell loss. The role of these markers as an early independent predictor of rapid disease progression was detected here despite the relatively small number of subjects evaluated, supporting their predictive strength. These data suggest that high levels of antigenaemia in early HIV-1 infection are associated with immune regulatory signals mediated through and/or by these biomarkers and thus limit or impair the ability of HIV-specific cellular immune responses to effectively control viraemia to a low set point. In addition to or in combination with canonical parameters, novel early biomarkers, which are indicative of early immune dysfunction and are also predictive of subsequent HIV disease prognosis, may inform approaches for evaluating the ability of therapeutic HIV vaccines to control HIV infection.

Chapter 5 : General Discussion and Future Directions

1. Discussion and Future Directions

Three decades since research of the development of a vaccine to prevent or control HIV-1 infection began and despite strong optimism from some, definitive evidence that a robustly protective vaccine can be made is still lacking (Schiffner et al., 2013). HIV is currently the most studied and most understood virus that has affected humankind and we have seen many advances in the field of HIV-1 immunology and virology. Recent advances in the understanding of the kinetics and quality of early immune responses to HIV-1 have emphasized the importance of events in early infection as determinants of the subsequent disease course (McMichael et al., 2010). The acute phase, which includes the eclipse phase before viremia is detected and the viremic phase before viral load set-point is reached, is crucial for target T-cell availability, seeding of latent reservoirs, and the initiation and expansion of antiviral immune responses by the host (Katsikis et al., 2011). The appreciation of the role of acute-phase immune events in HIV pathogenesis highlights the need for vaccine-associated immune responses to intervene during the earliest stages of infection in order to modulate these critical events.

The key role of CD8+ T-cells in the initial control of HIV-1 replication is a concept that has stood the test of time and multiple lines of evidence support the importance of virus-specific CD8+ T-cells in control of HIV replication. The induction of HIV-specific CD8+ T-cell responses in primary infection is temporally associated with containment of the acute viremia and the depletion of CD8+ T-cells from simian SIV-infected macaques impairs control of viremia. In addition,

strong HIV-specific CD8+ T-cell responses are sustained in elite controllers and the emergence of viral variants able to escape recognition by epitope-specific CD8+ T-cell responses in both HIV-1 and SIV illustrates the selective pressure exerted by CD8+ T-cells on viral replication *in vivo* (Walker and McMichael, 2012, McMichael et al., 2010). However, the correlates of immune protection against HIV-1 infection remain incompletely understood mainly because induction of HIV-1-specific CD8+ T-cell responses in natural infection does not result in protective immunity or complete control but results in a lifelong infection, which in the absence of antiretroviral therapy, may lead to AIDS and death (Garber and Feinberg, 2006). The development of an efficacious vaccine will be facilitated by knowing the type of immune responses that will be protective against infection and/or disease progression (Plotkin, 2013). Indeed, knowledge on the precise functional profile of CD8+ T-cells that might lead to permanent control of HIV-1, or prevention of disseminated infection is still lacking.

We aimed to characterize CD8+ T-cell responses measured during acute and primary HIV-1 infection and assess their role in the control of HIV-1 infection in subtype C infected, antiretroviral naive subjects from 4 weeks up to 52 weeks post infection. The impact of acute phase cytokine expression and immune activation on disease progression was also assessed. Our findings are summarized below and depicted in Figure 5.1, a number of recommendations for future research are also given.

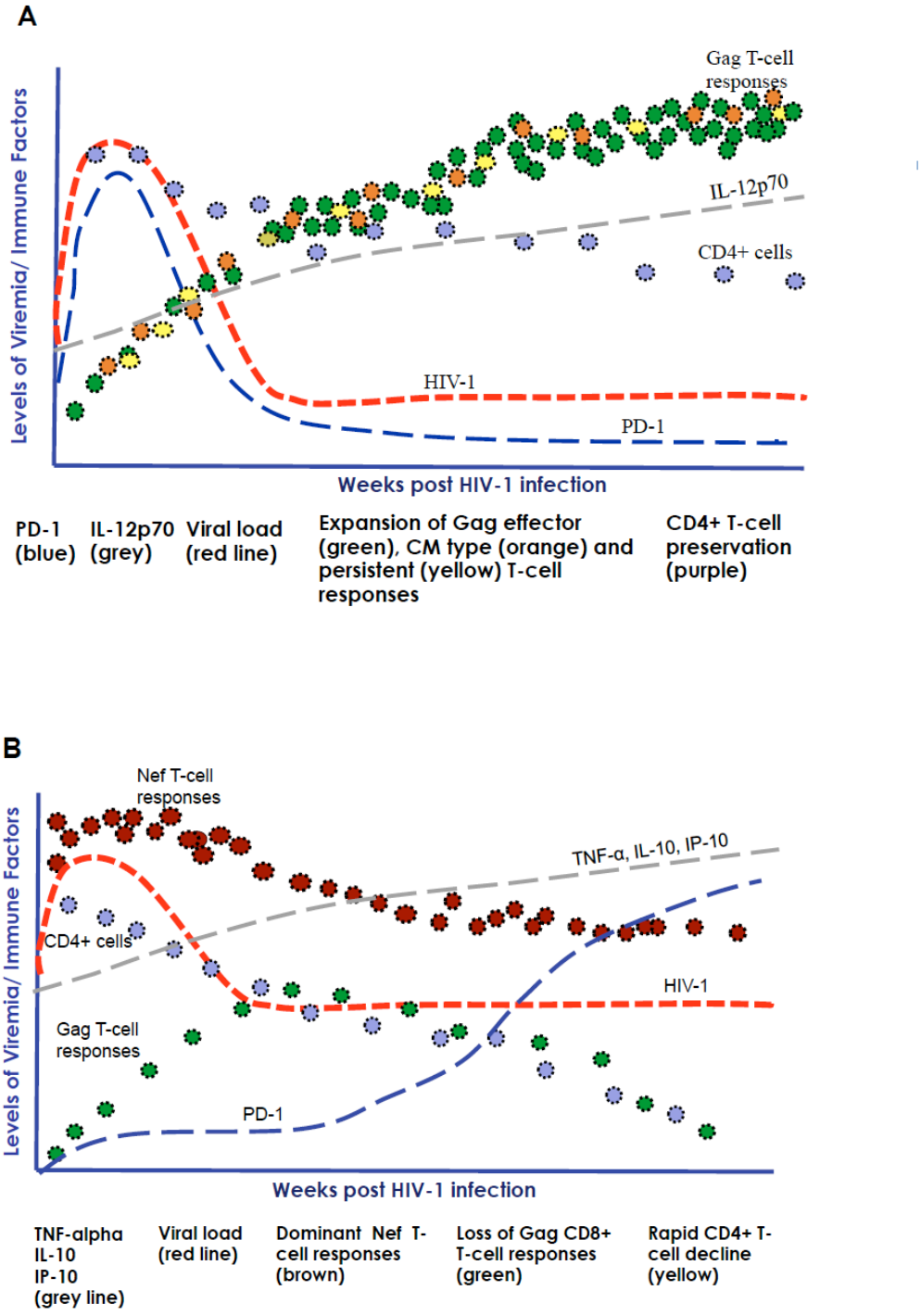


Figure 5.1 Schematic diagram summarising the main findings of the current study. (A) The proposed model of control of viral replication or slow disease progression is associated with the acute phase expression of IL-12p70 and high early expression of PD-1 on CD8+ T cells that subsequently resolves; the preservation of CD4+ T-cells and induction of broad and persistent Gag-specific effector and central memory CD8+ T-cell responses; (B) Lack of control of viral replication or rapid disease progression is associated with the acute phase expression of IL-10, IP-10, TNF-α and the rapid loss of CD4+ T-cells; the majority of the epitopes targeted in primary infection are within Nef, Gag-specific CD8+ T-cell responses are narrow and do not persist, and PD-1 expression kinetics on CD8+ T cells are slow and fail to resolve following acute viremia.

2. The acute CD8+ T-cell response and HIV-1 dynamics

Earlier studies have demonstrated the presence of HIV-1 specific CD8+ T-cells with limited breadth during acute HIV-1 infection and our study confirms this. Our data corroborate findings from a clade C cohort demonstrating that the magnitude and breadth of IFN-γ ELISPOT assay responses measured within 3 months post-infection are unrelated to the course of disease in the first year of infection and have low predictive power for the viral set point (Gray et al., 2009). Also consistent with previous reports, during the first weeks of infection Nef protein was most frequently recognized by CD8+ T-cells and was the target for the earliest response (Frahm et al., 2004, Lichterfeld et al., 2004b). The initial HIV-1-specific CD8+ T-cell responses which are narrowly directed against a few epitopes have been shown to suppress HIV-1 as viral load is declining from its peak (Goonetilleke et al., 2009). In contrast, acute Nef-specific responses were not associated with control of viremia from its acute phase peak suggesting that CD8+ T-cells targeting Nef during acute HIV-1 infection are ineffective. In fact, we observed that an increase in the magnitude and breadth of Nef-specific CD8+ T-cell responses was associated with higher viral loads during acute and primary infection. We also found no correlation between the presence of acute phase T-cell responses targeting all other regions of the HIV-1 proteome and the concurrent decline in viremia. In addition, CD8+ T-cell responses measured within the first 18 weeks of HIV-1 infection and their immunodominance patterns did not have a significant demonstrable impact on viral load set point.

3. Acute Nef-specific CD8+ T-cell responses: the good and the bad

Our findings that Nef-specific CD8+ T-cell responses were dominant but did not suppress viral replication during and after acute infection are similar to findings from other studies (Turk et al., 2013, De La Cruz et al., 2014, Novitsky et al., 2003), but are also in contrast with recent data from Riou et al. (2014) and Adland et al. (2013) which show a correlation between Nef-specific CD8+ T-cells and a lower levels of viral replication (Riou et al., 2014, Adland et al., 2013). Similarly, SIV studies have also yielded conflicting results as shown by a recent study which revealed that vaccine-induced Nef-specific T-cells failed to control viremia and easily escaped during acute infection (Iwamoto et al., 2014), whilst results from a recent SIV-macaque vaccine study suggest that Nef-specific CD8+ T-cell responses have an important contribution in the control of viremia (Takahashi et al., 2013). Nef has been shown to exhibit a variety of *in vitro* functions that may modulate pathogenesis, including HLA class I down-regulation (Schwartz et al., 1996), CD4+ T-cell down-regulation (Garcia and Miller, 1991), enhancement of virion infectivity (Munch et al., 2007) and stimulation of viral replication in PBMC (Miller et al., 1994). Although the Nef protein is variable overall, its central region is as conserved as that of Gag (Fischer et al., 2006) and the conflicting reports on the role of Nef in HIV-1 infection may be attributed to the different targets of CD8+ T-cell responses within the Nef region, where CD8+ T-cell responses implicated in reducing early viremia are directed against more conserved functional domains that promote viral persistence *in vivo*, such those for MHC class I downregulation (Adland et al., 2013, Mothe et al., 2011, De La Cruz et al., 2014, Mwimanzi et al., 2013).

There is enough data from acute HIV-1 and nonhuman primate vaccine studies that clearly suggest that Nef-specific CD8+ T-cell responses have the potential to significantly control viral replication. Although limited by the number of subjects in our cohort, our data suggest that even though epitopes in Nef are highly immunogenic as well as immunodominant during acute HIV-1 infection, using the whole Nef protein as a vaccine immunogen may likely lead to targeting of T-cell responses that are rapidly lost or escape with little effect on the course of disease progression. Thus further studies should aim to characterize CD8+ T-cell epitopes within conserved regions of Nef, likely representing important functional domains. Epitopes within these conserved regions may eventually be used as immunogens in future vaccines.

4. Viral load kinetics mirror evolving Gag CD8+ T-cell responses

Consistent with previous studies, there was a broadening of the T-cell immune response within the follow up period. Similar to reports from elite controller SIV and HIV-1 studies, the broadening and preservation of Gag–specific T-cell responses during the follow up period was associated with better control of viremia and lower viral load set point (Turk et al., 2013, Perez et al., 2013, Zuniga et al., 2006, Geldmacher et al., 2007, Kiepiela et al., 2007, Masemola et al., 2004a, Streeck et al., 2009b, Schieffer et al., 2014, Rolland et al., 2008, Honeyborne et al., 2007, Borghans et al., 2007). Interestingly, insights have recently emerged from the STEP trial showing that vaccine recipients with T-cell responses targeting three or more Gag epitopes pre-infection showed

significantly lower plasma viremia compared to subjects without Gag responses following infection, thus further confirming the role of Gag responses in controlling viremia (Janes et al., 2013).

During the 52 week follow up period we also observed that although many of the acute/early HIV-1-specific IFN-γ ELISPOT CD8+ T-cell responses targeting Gag and Pol persisted, the majority of acute and early T-cell responses targeting Env, Nef and other regulatory proteins waxed and waned over time and could not be detected at the last time point evaluated. Indeed, although the earliest T-cell responses against epitopes in Gag and Pol were subdominant in relation to Nef (fewer than the epitopes targeted in Nef), many of these responses were maintained during the first year of infection. Our findings are in line with reports from studies showing that targeting of dominant epitopes within highly variable regions of the HIV-1 proteome often result in rapid escape and limited fitness cost to the virus, although we could not directly confirm this in this study because Nef was not sequenced. However, in T-cells targeting more conserved and fitness constrained HIV-1 regions escape is slow and although these conserved epitopes are targeted in acute infection, responses are often subdominant (Liu et al., 2009b, Goonetilleke et al., 2009, Mlotshwa et al., 2010, Martinez-Picado et al., 2006, Schneidewind et al., 2007, Liu et al., 2013). Although many of the persistent responses targeted epitopes in Pol, these responses did not have a significant impact on viral load set point. However, the preservation of an acute CD8+ T-cell response against even a single epitope in Gag had a demonstrable impact on viral load set point.

Interestingly, some of the early T-cell responses which where no longer detectable when using overnight ELISPOT assay were detectable when PBMCs were stimulated with corresponding peptides and cultured for 10 days before measuring IFN-γ secretion via the ELISPOT assay. The presence of these cultured ELISPOT central memory type T-cell responses targeting epitopes in Pol, Env, Nef, Regulatory and Accessory proteins were not significantly associated with viral set point. However, cultured ELISPOT Gag-specific responses correlated with low plasma viremia, thus further providing evidence on the favourable role of Gag-specific T-cell responses in the control of viral replication. This is in line with earlier studies conducted in human and non-human models demonstrating that central memory cells are critical determinants of cytotoxicity in HIV and other infectious diseases (Migueles et al., 2008, Calarota et al., 2008, Ndhlovu et al., 2012).

When we sequenced Gag in order to assess the impact of T-cell responses on the viral landscape, our results revealed that the majority of epitopes that stimulated T-cell responses remained invariant over the first year after infection, consistent with data from previous studies (Liu et al., 2013, Goonetilleke et al., 2009, Mlotshwa et al., 2010). Although the contribution of T-cells specific for epitopes that did not mutate over the first year of infection is hard to assess, the significant negative association between the breadth of responses against these invariant epitopes and viral load set point suggests that these responses are important. Moreover, these data are consistent with the observation that invariant epitopes occurring in conserved regions of HIV-1 (mainly Gag) are rare

during the first year of infection since escape mutations might have too great a fitness cost for the virus.

In order to decrease the incidence of HIV acquisition after sexual exposure and/or to contain virus replication at an initial site of infection, an effective AIDS vaccine will most likely need to elicit high levels of fully functional T-cell precursors that migrate to relevant mucosal tissues and are also competent to respond to HIV infection and expand into effector cells more rapidly than occurs in natural HIV infection (Garber and Feinberg, 2006, Blankson et al., 2002, Reynolds et al., 2005). Our data suggest that in order to combat viral replication more rapidly than occurs in natural infection, HIV vaccines would need to induce greater breadth of CD8+ T-cell responses which can recognize numerous Gag epitopes resulting in lower levels of viremia and a slower disease progression. In a recent study, monkeys were vaccinated with replicating RhCMV vectors before challenge with SIV and the breadth of responses to Gag epitopes were strongly associated with the striking control of SIV observed in 50% of the challenged monkeys, an effect attributed to the robust CD8+ T-cell responses that was induced by the vaccine since SIV specific antibody responses were weak with no neutralization activity (Hansen et al., 2013). It is noteworthy to point out that this RhCMV vaccine did not include Env as an immunogen and thus, the protection could not have been mediated by anti-Env antibodies. However, one of the hurdles limiting the success of CD8+ T-cell responses is the tendency for HIV to evade these responses through sequence evolution (Price et al., 1997, Goulder et al., 1997a, Reece et al., 2013, Mudd et al., 2012). Studies have illustrated that vaccinations can alter immune targets patterns (Vogel et al., 2002, Rodriguez et

al., 2002, Hansen et al., 2013), thus vaccine approaches that can re-direct CD8+ T-cell responses to focus on particular conserved epitopes with high fitness costs following escape should be considered. Although effector T-cell responses may be necessary to circumvent the rapid spread and the establishment of viral reservoirs during acute infection, our data also suggest that generation and maintenance of a substantial Gag-specific central memory pool is still desirable for viral control. The pivotal role of central memory responses in the control of viral replication have been validated in an SIV vaccine study which demonstrated that vaccine-induced virus-specific CD8+ central memory T-cell responses, not the CD8+ effector T-cell response inversely correlated with the level of SIVmac251 replication, (Vaccari et al., 2005). Thus in conjunction with the induction of Gag-specific effector T-cell responses, vaccination regimens should also be focused on programming and maintaining Gag-specific central memory CD8+ T-cells able to expand adequately upon virus encounter.

Robust Gag-specific CD8+ T-cell responses have been shown to control viral replication and reduce or abort disease in non-human primate models, however, it is still unclear whether CD8+ T-cells can actually eliminate an established infection completely in the HIV-1 model (Liu et al., 2009a, Buchbinder et al., 2008, Hansen et al., 2009, Hansen et al., 2011, Hansen et al., 2013). Nonetheless, a non-sterilizing vaccine may be beneficial as reductions in set point viral loads will provide benefit to an infected individual by slowing down the rate of disease progression while simultaneously reducing the probability of

transmitting the infection to others, and thus provide the additional benefit of reducing infection at the population level (Mellors et al., 1995, Gray et al., 2001).

5. Impact of acute immune activation on HIV-1 disease progression

We show that three cytokines IL-10, IP-10 and IL-12 were associated with changes in viral load set point and/or CD4+ T-cell dynamics during the first year of HIV-1 infection. Both human and non-human primate studies suggest that cytokine profiles during acute infection impact viral set point and disease progression (Ansari et al., 2002, Mueller et al., 2008, Campillo-Gimenez et al., 2010, Roberts et al., 2010, Stacey et al., 2009). The notion that disease progression can be influenced by cytokines during acute HIV-1 infection is intriguing and raises the question of whether cytokines expressed during acute HIV infection can be targeted through vaccination or immunotherapeutics to alter viral set point or to preserve CD4+ T-cells. The rationale for HIV-1-specific therapeutic vaccines stems from the observation that the severe immune deficiency caused by HIV-1 infection is related to the levels of immune dysfunction or immune activation seen in HIV-1 infection (Autran et al., 2004). These immunotherapeutic vaccines thus aim to augment a host's immune response to HIV-1 so that viral proliferation and immune damage is slowed or blocked (Ha et al., 2008, MacGregor et al., 1998, Autran et al., 2004). There is also the anticipation that any immune response to a therapeutic vaccine strong enough to control established infection would also be effective in preventing initial infection in HIV-naive subjects (Autran et al., 2004). It has been proposed

that one of the reasons that current therapeutic vaccination strategies fail to sustain or resurrect T-cell responses is because they do not improve the immunosuppressive environment which may be the result of increased levels of anti-inflammatory cytokines such as IL-10 (Brooks et al., 2008).

Cytokines provide one of the most targeted factors used to investigate alterations of viral kinetics, the recruitment of viral targets, and the development of anti-viral immunity (Katsikis et al., 2011). Blocking key suppressive factors such as IL-10 could allow immune-mediated control of persistent viral infection and thus render ineffective vaccines more efficient at improving T-cell immunity (Brooks et al., 2008). Indeed, interventional studies to block IL-10/IL-10Rα have been proposed as a potential adjuvant to vaccination regimens or as a strategy to enhance underlying immunity in chronic viral infections, such as HIV (Blackburn and Wherry, 2007, Brooks et al., 2008). Similarly, IL-12 administration in rhesus macaques acutely infected with SIV lowered viral set point by 100-fold and also slowed disease progression (Ansari et al., 2002) and *in vitro* and *in vivo* responses to IL-12 are usually maintained during late SIV infection (Villinger et al., 2000, Watanabe et al., 1998). Based on the current findings, we hypothesize that the use of cytokine adjuvants for therapeutic vaccines during acute HIV infection may boost HIV-specific immune responses or imprint an immune response, which would alter viral load set point in the host whilst preserving CD4+ T-cells.

HIV-1 infection is associated with chronic immune activation ultimately resulting in the functional exhaustion of T-cells and this defect in responding T-cells is linked to the inability of the host to eliminate the persisting pathogen (Wherry et al., 2003). Exhausted virus-specific CD8+ T-cells up-regulate PD-1 expression during chronic HIV-1 infection and this expression correlates with viral load in blood plasma of HIV-infected patients. However, we show that the activation of this PD-1 inhibitory pathway in acute HIV-1 infection is associated with a slower disease progression and this may due to the inhibitory impact of PD-1 on the detrimental effects of immune activation (Estes et al., 2008). Ha et al. (2008) have suggested the use of therapeutic vaccination strategies, in combination with blockade of the PD-1 pathway as treatment for chronic HIV-1 infection. Although we are limited by the small sample size, our data suggest that using this immunotherapeutic two-pronged approach during acute HIV-1 infection would require activation of the PD-1 inhibitory pathway in order to dampen early immune activation. This may possibly lead to the containment of the spread of virus thus limiting its pathogenic effect on CD4+ T-cells resulting in enhanced viral clearance and a less rapid disease progression.

6. Future directions

Despite the hurdles that HIV presents to vaccine development, the historic success of vaccines for other pathogens argue that HIV vaccine research must be continued and accelerated (Plotkin, 2009). T-cell vaccine approaches to HIV have dominated much of the vaccine research agenda over the last decade mainly because there is an abundance of data from HIV-infected individuals indicating that CD8+ T-cells are key in viral control and also because of the inability to stimulate broad neutralizing antibodies through vaccination. However, evidence from many other vaccine-preventable infectious diseases demonstrates that although antibody titers correlate with protection from infection, CD8+ T-cell-mediated immune responses are essential for protection against disease suggesting that a dual approach is still warranted in HIV-1 vaccine development (Plotkin, 2013, Plotkin, 2010, Schiffner et al., 2013).

Despite the extensive characterization of CD8+ T-cell responses induced during acute and chronic HIV infection, there still remains a general lack of consensus regarding the association of these responses with effective control of virus replication and in fact a clear "correlate of immunity" based on any existing CD8+ T-cell measurements is still lacking (Ogg et al., 1998, Addo et al., 2003, Betts et al., 2001, Goonetilleke et al., 2009, Gray et al., 2009). Although the reasons for this have been deemed as multifaceted, others attribute this to limitations of the most commonly employed ex-vivo cytokine-production assays mainly the ELISPOT (used in the current study) (Wilson et al., 2000, Gray et al., 2009).

Currently, interferon-γ ELISPOT assays rank among the most validated assays for HIV-1 vaccine trials because of ease of reproducibility, high sensitivity and quantitative precision in mapping the targeting and magnitude of CD8+ T-cell responses particularly in samples with low-frequency responses (Dubey et al., 2007, Fu et al., 2007, Valentine and Watkins, 2008, Saade et al., 2012). The major limitation of ELISPOT is that it does not provide information on the phenotype of the responding T-cell or its antiviral function and properties (Yang, 2003, Valentine et al., 2008, Ogg et al., 1998, Addo et al., 2003, Betts et al., 2001, Goonetilleke et al., 2009, Gray et al., 2009). Future studies should pay greater attention on qualitative features of CD8+ T-cell responses and the qualitative features most closely associated with immunologic control of HIV, such as the ability of HIV-specific CD8+ T-cells to increase their contents of proteins known to mediate cytotoxicity appears to be a critical means by which HIV-specific cytotoxic capacity is regulated (Wherry et al., 2005, Migueles et al., 2002, Migueles et al., 2008).

One of the findings in this study is that the immune response is narrowly directed during acute HIV infection. An effective vaccine would need to stimulate a broad CD8+ T-cell response to multiple epitopes that are highly conserved and to common variants of the founder virus epitope sequence as this would limit escape options (McMichael et al., 2010). Thus future studies should also aim to establish mechanisms responsible for the narrow CD8+ T-cell immune response during acute HIV-1 infection. The relevance of immune activation pathways and acute phase cytokines in directing or influencing T-cell signalling pathways and responsiveness should also be investigated. Answers to these questions may

enable better vaccine design and immune measurement assays, which could significantly advance the field.

The initial analysis of the STEP trial showed that neither protection against infection nor control of initial viraemia despite the presence of vaccine-induced T-cell responses (Buchbinder et al., 2008, McElrath et al., 2008). However, more recent reports show that individuals with 3 or more Gag-specific responses have lower viral loads (Janes et al., 2013). These data provide much-needed momentum for the development of new approaches and for rigorous re-evaluation of current approaches. Based on our findings and reports from others, what appears to be the logical direction that vaccine research should follow is the application of modalities designed to stimulate effector memory and central memory CD8+ T-cells, which differ in quality, quantity, phenotype, breadth, from the vaccines tested previously, for which multiple approaches are available. In order to identify the precise immunological correlates of protection an immunological portfolio comprised of a multi-faceted evaluation, rather than relying on the measurement of IFN-γ secretion should be generated for the evaluation of vaccine efficacy.

Future studies should also focus on elucidating the complex interactions between HIV-1 and cytokines expressed in the host during acute HIV-1 infection in order to more accurately predict the effect of the cytokine interventions as vaccine adjuvants. Moreover, the cell types and tissues expressing these cytokines and antigens responsible for triggering these cytokines will have to be identified. In-depth understanding of immune activation and exhaustion

pathways during acute HIV-1 infection is also much needed as this may help uncover additional pathways critical for control of viral replication. Finally, understanding kinetics of cytokines such as IL-10 or the underlying mechanisms of PD-1 expression and regulation, and how these biomarkers affect the quality and quantity of T-cell responses, may not only provide fundamental insight into the poorly understood mechanisms that control viral replication but may also inform the development of novel immunotherapeutics or vaccine adjuvants against HIV.

Chapter 6 : References

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