Role of Polyfunctional and Proliferative CD8+ T cell Responses in HIV-1 Infection

by

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Abstract

The limited success of HIV vaccine candidates to date highlights our need to better characterize protective cell-mediated immunity. Understanding correlates CD8+ T cell protection against HIV infection and progressive disease is essential for informing effective vaccine development, design and evaluation. CD8+ T cell responses with a robust polyfunctional and proliferative component are strongly linked to better disease outcomes. However, the specificity of polyfunctional and proliferative CD8+ T cell responses has not been thoroughly investigated. Additionally, the specificity of memory subsets and their connection to polyfunctionality and proliferation responses has not been adequately assessed. We address these gaps in knowledge and provide a better understanding of the fine specificity of HIV-specific CD8+ T cell responses. We hypothesize that the epitopes recognized by central memory (T_{CM}) and effector memory (T_{EM}) CD8+ T cells, defined by functional attributes, differ in chronic HIV-1 infection. Additionally, we hypothesize that polyfunctional and proliferative responses will better correlate with protection in HIV disease progression.

The qualities of CD8+ T cell responses were evaluated using polyfunctional flow cytometry measuring both functional and phenotypic attributes of both T_{EM} and T_{CM} subsets in HIV infected individuals. We evaluated the quality and evolution of CD8+ T cell responses in HIV infected individuals shortly after seroconversion through to the chronic phase of infection, finding that early polyfunctional responses may result in better HIV disease outcomes. Additionally, we show that epitope-specificity differs between short-term cytokine/chemokine secretion and long-term proliferative assays. Importantly, we show that, at a cohort level, particular epitopes preferentially elicit specific qualities of CD8+ T cell responses in preference to others.

This research improves our understanding of HIV pathogenesis and indicates that we can identify specific epitopes that can elicit protective responses and that early polyfunctional responses may slow HIV disease progression. Understanding the polyfunctional and proliferative capacities of HIV-specific effector and memory cells at various stages of HIV infection is of critical importance to the design of vaccines intended to elicit protective cell-mediated responses.

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Dedicated to the memory of my Grandpa,
Mr. Sydney McMurray

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1. Chapter 1. Introduction

1.1. The HIV Pandemic

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), continues to be one of the world's worst public health crises. HIV/AIDS is the sixth leading cause of death worldwide and the third leading cause of death in low-income countries (1). Globally, there are approximately 34.0 million people infected with HIV (2). Since the virus was first clinically observed, the United Nations on HIV/AIDS (UNAIDS) reports that there have been approximately 30 million deaths due to HIV infection (3). Although cases of new infections have declined in recent years, there were still an estimated 2.5 million new infections in 2011, more than 40% of which occurred in those aged 15-24 years. Seventy-one percent (1.8 million) of new infections occurred in sub-Saharan Africa, with the vast majority of these new infections occurring in young people (aged 15-24 years) (4). Sub-Saharan Africa remains the most severely affected region in the world (2). Additionally, young women are disproportionately affected by HIV/AIDS, representing 22% of all new infections globally and 31% of all HIV-infected individuals in sub-Saharan Africa (4). Sadly, millions of orphans have been created as their parents have died of AIDS (5). Although treatment options are available for those infected, prevention of new infections is imperative if we are to curb this devastating pandemic.

HIV prevention research has focused on the development of HIV vaccines and microbicides. Vaccines and microbicides would serve as an important option for women who are often unable to access current prevention options, such as condoms, due to gender inequality issues. Unfortunately, despite some progress in the field of biomedical

interventions, to date these options remain elusive. Understanding the mechanisms by which some individuals are able to control virus replication in the absence of therapy has become a crucial means to inform vaccine and microbicide design. The aim of this thesis was to characterize immunological mechanisms that may control viral replication and slow HIV disease progression using a well-described longitudinal cohort of HIV infected individuals based in Kenya.

1.1.1. *Kenya*

Although Kenya has made great strides in curbing their epidemic, HIV remains one of the greatest public health challenges. As of December 2011, there were 1.6 million people in Kenya living with HIV (6). The adult HIV prevalence in 2010 was 6.2%, about 40% lower than at the epidemic's peak in the early '90s (prevalence 10-14%) (6). New infections among adults have also decreased in recent years; currently there are a third fewer new infections than in 1993, which was the country's epidemic peak.

Heterosexual transmission within a union or primary partnership is thought to be the principal driver of the Kenyan epidemic, accounting for 44% of new infections (6). As with the global pandemic, Kenyan women are disproportionally affected by HIV, representing 58% of prevalent infections. Tragically, this has resulted in a substantial number of transmissions to newborns. In 2011, there were an estimated 12,894 Kenyan children newly infected with HIV (6).

Kenya has made progress in controlling their epidemic through behavioural and social interventions. Condom use has doubled since the early '90s and on average Kenyans are less likely to have multiple sex partners (6). Additionally, Kenya has made a strong push to increase male circumcision which reduces the risk of transmission during heterosexual intercourse by 60% (6, 7). Despite this progress, many social factors continue to perpetuate the epidemic including: gender inequity, sexual violence, poverty, and anti-HIV stigma. Particular segments and regions of the population continue to be at greater risk of infection than others. Marginalized segments of the population including men who have sex with men (MSM) and people who inject drugs (IDU) have an HIV prevalence that exceeds 18% while 29.3% of all female sex workers (FSW) in Kenya are living with HIV (6).

1.1.2. *History of the HIV/AIDS Pandemic*

AIDS first came to the world's attention in 1981, when a number of cases of opportunistic infections including *pneumocystis pneumonia* (PCP) and opertunistic cancer, Kaposi's Sarcoma were identified in otherwise healthy young MSM in New York City and Los Angeles (8-10). Both of these illnesses typically only affect immunocompromised individuals and are extremely rare in individuals under 65 years of age (8, 9). Epidemiologic risk factors for the newly termed Acquired Immunodeficiency Syndrome (AIDS) included being a bisexual or gay male, a Haitian immigrant, an IDU, or a haemophiliac (11). Patients with AIDS had a distinct reduction of cellular immunity and a depletion of T Lymphocytes which suggested that T cells or a subset of T cells were the preferential target for the putative infectious agent. In 1983, two separate research groups in France and the United States of America identified the causative agent

of AIDS as the virus originally named lymphadenopathy-associated virus (LAV) and The Human T-lymphotropic virus Type III (HTLV-III) respectively (both now known as HIV-1) (12, 13). Quickly following the discovery of HIV-1, the T cell surface marker CD4 was identified as the major receptor for viral entry into host cells (14). The availability of laboratory reagents and techniques to identify HIV led to rapid discovery of widespread HIV infection in both men and women across the globe (9, 15-18).

1.2. HIV Transmission, Prevention and Treatment

1.2.1. *HIV Transmission*

The transmission of HIV occurs through contact with infected body fluids, including mucosal secretions and blood. The primary paths of transmission are via sexual activity (heterosexual and homosexual, both penile-vagina and penile-anal), vertical transmission (from mother-to-child in utero, during delivery, or through breast-milk) and parenteral transmission (IDU or through contaminated blood products). Rarely occurring routes of transmission include oral sex, dental surgery, and occupational exposure such as needle stick injuries (19).

HIV transmission methods vary greatly in different population groups, however, heterosexual transmission accounts for the majority of new HIV infections globally (20). Although the risk of acquiring HIV through heterosexual sex is low, 0.04 % per female-to-male unprotected sex act and 0.08% from male-to-female excluding commercial sex exposure, sexual transmission accounts for 75-90% of HIV infections worldwide (20-23). Heterosexual transmission of HIV through vaginal intercourse is the dominant transmission pathway in sub-Saharan Africa (20).

The relative risk of HIV transmission varies with numerous confounding factors including: viral load (24-26), concurrence of sexually transmitted infections (STI) (27-30), antiretroviral therapy (ART) (31, 32), male circumcision (33-36), condom use (37-40), and use of hormonal contraception (41). The risk of transmitting the virus to a sexual partner is greatest during acute HIV infection when viral load is at its highest and most people are unaware they are infected. Fifty to sixty percent of HIV transmission occurs during the first 6 months of infection (42-45).

Concurrent STIs including, but not limited to: *Chlamydia trachtomatis*, *Neisseria gonorrhea,* and herpes simplex virus increase the risk of HIV acquisition (46-48). STIs increase the pool of T cells and macrophages in the genital tract thus increasing the number of HIV-1 susceptible cells at the site of HIV exposure (49). Additionally, STIs cause disruption of the epithelial barrier and enabling viral entry (50). Furthermore, STIs increase abnormal vaginal microflora leading to bacterial vaginosis which correlates with HIV acquisition in women (51).

1.2.2. *HIV Prevention: Behavioural and Biomedical interventions*

The UNAID's mission to reduce the level of new transmissions by 50% by 2015 will require the continued use of proven behavioural and biomedical interventions (2). Traditionally, behavioural strategies include encouraging a delay of sexual debut or abstinence, condom use, and a reduction in concurrent sexual partnerships are the most widely used approach to avert sexual transmission. In the late '80s to mid '90s while the rest of sub-Saharan Africa was being ravaged by HIV, Uganda was surprisingly able to

keep the virus contained. This unexpected feat has been largely credited to the early implementation of national prevention program known as the ABCs (Abstinence, Be faithful, use Condoms) (40, 52, 53). Globally, the decline of new HIV infections has been associated with behavioural and social changes; however, the best methods to motivate behaviour change remain unclear. Although the implementation of age-appropriate sexual education can increase HIV knowledge and lead to more responsible sexual behaviours, there remains a significant gap in even basic knowledge about HIV and its transmission (2). UNAIDS reports that in countries with generalized epidemics less than 50% of young people have comprehensive and correct knowledge about HIV. Notably, young women are lacking in knowledge concerning the effectiveness of condoms in preventing HIV transmission (2). Currently behavioural prevention services reach less than 10% of persons at risk (2). These data highlight the importance of persistent focus on social and behavioural interventions as it continues to be one of our most effective HIV prevention strategies. However, these efforts should be strategically focused to target groups where it will make the greatest impact.

Regardless of the effectiveness of social interventions, behavioural issues will remain central to the spread of infection and the need for other prevention strategies is critical. Therefore, biomedical approaches to reduce the risk of infection have gained favour in recent years as an alternative or in combination with behavioural strategies. However, to date, reduction of infections with biomedical techniques has had limited success. Treatment of STIs at a population level has produced mixed results in terms of reduction in HIV incidence (46, 47, 54, 55). A recent observational study demonstrated that a

decrease in HIV incidence coincided very closely with decreased rates of *N. gonorrhea* infection (47). However, clinical trials to assess the feasibility of treating HSV-2 to decrease HIV incidence have not shown efficacy (48). Conversely, three successful efficacy trials have focused on medical male circumcision (MMC), which has long been epidemiologically associated with decreased HIV susceptibility. The protective effect of MMC has now been confirmed as approximately 60% in these studies (7, 33, 56, 57). Unfortunately, the benefits of MMC are mainly one-sided and only reduce the risk of female-to-male transmission in heterosexual partners. Currently there is little evidence as to whether MMC benefits females at the community level. Additionally, there is limited data on the benefits MMC for MSM particularly those in the receptive anal role (58). These data suggest a need for more effective HIV preventative strategies especially biomedical interventions that would benefit both sexual partners and the greater population.

Currently the most effective tool for HIV prevention is the condom. Condoms are estimated to be as effective at preventing HIV transmission as they are at preventing pregnancy which is 90.7-98.6% effective (37, 38). For comparison, the average Influenza vaccine only has an efficacy of 60% (59). The effectiveness of condoms at preventing HIV infection surpasses that of most licensed vaccines for any other infectious disease. However, HIV continues to be one of the globe's most widespread infections since the effectiveness of condoms is almost entirely dependent on behavioural modifications as individuals must continually make the conscious decision to use them. Additionally, even when used, if condoms are not used properly there is risk of condom

breakage and slippage, which abrogates protection. Condom failure due to breakage and slippage rates have varied from 0.5% to 6.7% for breakage and 0.1% to 16.6% for slippage (38). This further emphasizes the need for foolproof HIV prevention strategies that eliminate the need for behavioral modifications.

1.2.3. *HIV Prevention and Treatment: Antiretroviral Therapy*

The advent of combination highly active antiretroviral therapy (HAART, also known as drug *cocktail* and triple-therapy) has changed what was once a fatal infection to a potentially manageable chronic infection with significant advances in life expectancy among those able to access treatment. Standard ART consists of the combination of at least three antiretroviral (ARV) drugs to maximally suppress the HIV virus and stop the progression of HIV disease. Large reductions have been seen in rates of death and suffering when potent ARV regiments are used particularly in early stages of the disease. Currently there are 36 approved drugs from seven drug classes targeting various steps in the virus' life cycle including: Nucleoside Reverse Transcription Inhibitors, Non-nucleoside Reverse Transcription Inhibitors, Protease Inhibitors, Fusion Inhibitors, Entry Inhibitors, Integrase Inhibitors, and Multi-class Combinations (60). According to the WHO guidelines, when a patient's CD4 count drops below 500 cells/ml, they should be placed on ART with priority given to those with CD4 counts below 350 cells/ml. Although in developing countries where resources are limited, this is not always achieved (61, 62).

When treatment is initiated in a timely manner and with proper drug adherence it results in the control of viral replication in the majority of cases (23). Unfortunately, the mass of the world's new HIV infections occur in the poorest areas which have inadequate access to health care or in high risk-taking, stigmatized populations. Often these people are also the hardest to access and have challenges complying with daily ART. WHO and UNAIDS estimate that at least 15 million people were in need of ART in 2011. As of the end of 2011, only 8 million people had access to ART in low- and middle-income countries (2). Many low-and middle-income countries rely heavily on international aid for treatment, care, and support. International funding accounted for more than 50% of the HIV treatment spending in 59 countries and for more than 75% in 43 of the 102 low- and middle-income countries (2). Consistent, well-managed, and well-funded access to HIV treatment and care is critical in ensuring proper ART adherence and controlling the pandemic.

Adherence is a major determinant in the success of ART (63). Interruption of therapy can abrogate any positive effects of treatment and can lead to the emergence of drug-resistant viruses. Unfortunately, one of the main challenges with ART is drug toxicity which can make adherence difficult. Minimizing side effects is considered to be one of the keys to high adherence. In developing counties, supplies of ART, delivery to those in need, and maintenance of the health care infrastructure required to monitor individuals continued access to ART are additional challenges (64). These individuals will need to continue ART for the duration of their lifetimes.

This lifelong dependency on ART can be subverted in newborns by strategic implementation of HIV treatment during pregnancy. Prevention of mother-to-child transmission of HIV (PMTCT) can be drastically improved with appropriate ART use. The current WHO guidelines recommends pregnant women be put on ART early in pregnancy and continue through breastfeeding and the postpartum risk period (65). Treatment of both mother and child during delivery reduces the likelihood of vertical transmission (66). Combination ART treatment of infected mothers has decreased the chance of mother-to-child transmission from 15-45% to <2% (67, 68). Unfortunately, such prophylaxes are not available to all women, especially those in developing countries. Additionally breast milk replacement formula is often unavailable, expensive, and unsuitable in some rural areas (69, 70).

Expanded access to ART can also reduce HIV transmission at the population level. HIV transmission is critically dependent on the level of HIV viral load within blood and genital secretions. HIV viral load can be reduced to undetectable levels with the appropriate use of ART. Additionally pre-exposure prophylaxis, given to HIV negative high-risk individuals has the potential to reduce transmission.

1.2.4. *HIV Prevention: HIV Microbicide, PrEP and Vaccine development:*

For women, heterosexual transmission occurs when HIV crosses the vaginal epithelium and infects underlying target cells (71). Vaginal microbicides are a potentially powerful tool for HIV prevention as they give women, who are more vulnerable to infection due to biology and gender inequalities, the ability to protect themselves without their partner's

input (72-74). Until recently, clinical trials of microbicides in humans have had limited success as demonstrated by a spermicide ingredient nonoxynol-9, that showed *in vitro* anti-HIV activity, but resulted in increased ulcers and HIV incidence in clinical trials (75)(76-79). The failure of this and many other microbicides was thought to be due to inclusion of surfactants, which compromise the integrity of the genital tract mucosa (72). Surfactants or detergents are often used for vaginal douching. Vaginal douching is associated with an increased risk HIV-1 infection in female sex workers, giving insight into the failure of these microbicide formulations (80).

Recent studies have been more encouraging; a microbicide candidate gel containing 1% tenofovir (nucleotide reverse transcriptase inhibitor) was recently tested in a South African population (CAPRISA 004 phase IIb trial). Trial results were positive and provided support for the development of similar microbicides. The tenofovir gel reduced HIV infections by 39% across the whole data set and importantly reduced infection in women with superior gel adherence (>80%) by 54%.

Pre-exposure prophylaxis (PrEP) - introducing HIV drugs as a preventative strategy, whether in a topical microbicide formulation similar to the CAPRISA 004 trial or the drug administered orally - is thought to be one of the more promising strategies for HIV prevention. However, to date, of the seven PrEP trials with available HIV endpoints, only four have demonstrated effectiveness. These trials include the microbicide CAPRISA 004 and oral tablets iPrEx (Truavada), TDF2 (Truvada) and Partners for PrEP (Tenofovir and Truvada), ranging in efficacy from 39% to 80% in reducing HIV acquisition. The sizeable range in efficacy and the failure of other trials (TFV phase II,

FEM-PrEP and MTN-003 (VOICE)) is believed to be largely the result of differences in adherence. Drug levels correlated with protection in the CAPRISA and iPrEx trials, strongly suggesting that adherence determines the level of protection. Despite the potential benefits of PrEP as a strategy for HIV protection again, these data emphasize the need to identify a prevention strategy that eliminated the reliance on behavioral changes.

After 30 years of intense research and development, an HIV vaccine has remained elusive. There have been four HIV vaccine products tested in clinical phase IIb or III efficacy trials to date (81). Two of these were protein vaccines derived from the HIV-1 envelope AIDSVAX B/E (VAX003) and AIDSVAX B/B (VAX004) (VaxGen, San Francisco, CA, USA). These were tested in phase III efficacy trials and found ineffective. The vaccines were designed to induce Env-specific neutralizing antibodies to either clade B or E viruses, but unfortunately the titres of neutralizing antibodies induced were low. Neither vaccine prevented HIV infection nor delayed disease progression in those who were infected (81). Two phase IIb trials, the STEP (HVTN 502) and the Phambili (HVTN 503) studies (sponsored by Merck and the US National Institutes of Health (NIH)), were designed to induce strong HIV-specific T cell responses. The vaccine candidate in both trails was the MRKAd5 Gag/Pol/Nef vaccine, a recombinant Adenovirus 5 (Ad5) vector expressing HIV-1 clade B Gag, Pol and Nef antigens. The STEP study was unexpectedly terminated at the first interim analysis due to futility and the Phambili study quickly followed suit. Despite the fact that MRKAd5 Gag/Pol/Nef HIV-1 vaccine elicited the expected immune responses, it neither prevented infection with HIV nor lowered the viral load setpoint of those who became infected.

Additionally, further analyses revealed that vaccinated uncircumcised Ad5 seropositive males had a 4-fold increase in the risk of HIV infection. Thus, it is clear that the discovery of an HIV vaccine remains tenuous and raises many doubts as to whether these efficacy trials were justified considering the burden on the trail populations, the large associated costs, and the tremendous gaps that remain in knowledge in HIV pathogeneses and immune responses. These failed trials highlight the importance of continued investigation of basic HIV immunobiology, including potential correlates of protection and slowed disease progression.

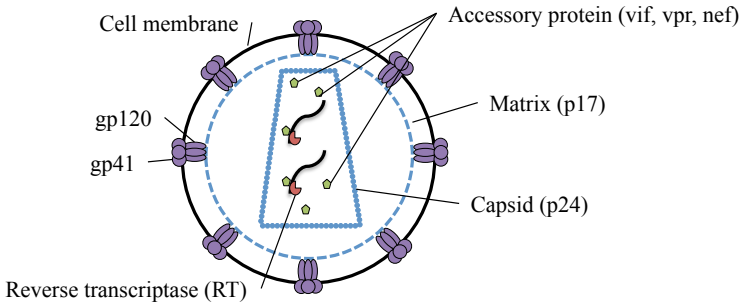
To date, the only trial to show any efficacy in preventing HIV infection is the RV144 vaccine study or "Thai Trail". This vaccine candidate has two components ALVAC-HIV (Sanofi-Pasteur) and AIDSVAX B/E (VaxGen). ALVAC-HIV was a recombinant Canarypox vector expressing HIV-1 clade B Gag, and Pro, and clade AE recombinant gp120 and gp41, while AIDSVAX B/E (VaxGen) was a recombinant gp120 subunit vaccine. The vaccine had moderate efficacy ranging from 26.2% in the per-protocol, 26.4% in the intent-to-treat, and 31.2% in the modified intent-to-treat (82). This vaccine strategy was meant to induce both CD8+ T cell responses and neutralizing antibodies, however, both of these responses failed to correlate with protection. However, binding antibodies to Env were induced and correlated with protection. The vaccine also induced proliferative CD4+ T cell responses. Currently there are substantial efforts underway to describe the correlates of immunity of this vaccination regimen. This is essential if these results are to contribute to future rational vaccine design and development. The results from this trial stress the need for a better understanding of HIV pathogenesis and immunity.

1.3. HIV Virology

1.3.1. *HIV Classification and Structure*

HIV belongs to the viral family *Retroviridae* and the genus lentivirus (lente-, latin for slow), due to their long latency periods. HIV is an enveloped retrovirus, which reverse transcribes its single-stranded positive sense RNA genome (9.2kb) into DNA that is integrated into the host genome. The genomic make up of HIV consists of three structural proteins (Gag: p24, p17 and p6), three enzymes (protease (Pro), reverse transcriptase (RT), and integrase (IN)), six accessory proteins (Vif, Vpr, Vpu, Rev, Tat, and Nef), and two glycosylated envelope proteins (gp120 and gp41) (Figure 1.1).

A



B

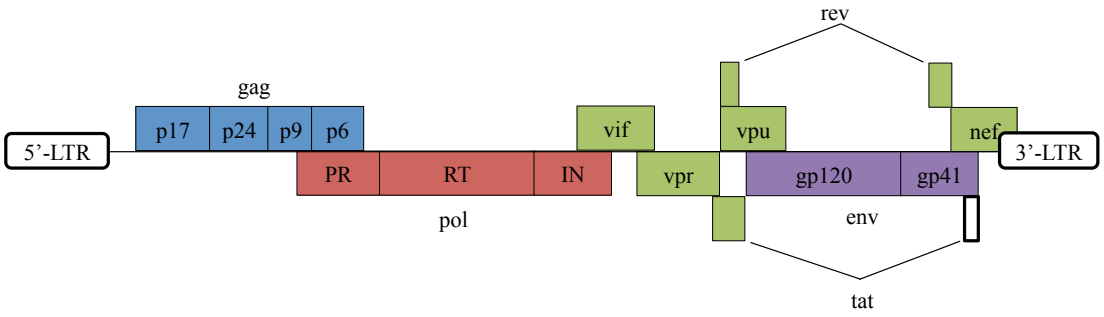


Figure 1.1. HIV-1 viral protein and genetic arrangement A) HIV virion structure. B) HIV genome. (83-85)

1.3.2. *HIV Life Cycle*

The initial phase of HIV infection occurs when the virion enters target cells expressing the host protein cluster of differentiation 4 (CD4), found mainly on T cells and macrophage (14, 86-89). Viral entry is mediated by the binding of HIV envelope gp120 and gp41 proteins to CD4. Following conformational changes gp120 binds to the co-receptor C-C chemokine receptor 5 (CCR5) on the T cell or macrophage. Similarly gp120 can also bind C-X-C chemokine receptor type 4 (CXCR4) on T cells. The co-receptor used for entry is dependent on the virus and is used to define the virus' tropism (90-95). CCR5-tropic viruses are prominent in early infection, while CXCR4-tropic viruses are largely found in chronic infection (96, 97). Fusion of the virus to the host cell is facilitated by conformational changes between HIV protein gp41 and the host cell membrane (98, 99), which is followed by viral entry and un-coating. Following un-coating, viral RT transcribes a single stranded complementary DNA (ssDNA). Subsequently, the ssDNA is copied to produce the double stranded DNA (dsDNA) proviral genome. Provial DNA is transported into the cell nucleus and the viral IN enzyme integrates proviral DNA into the host genome. Integrated viral DNA serves as a lifelong HIV reservoir which can be activated at anytime.

Upon activation integrated viral DNA is transcribed into mRNA and viral replication begins. This mRNA is then translated into proteins, followed by assembly in the cytoplasm, and finally budding of new virions. Viral replication is more efficient in activated host cells (100, 101). When host cells are activated the long terminal repeats (LTR) of the integrated genome acts as a promoter and enhancer region for viral gene transcription (100).

HIV mRNAs are translated into polyproteins that are consequently cleaved into individual viral proteins by cellular and viral proteases. The full length mRNA translate into Gag/Pol polyprotein synthesis, which are later cleaved by viral protease, while smaller spliced mRNA are translated into Env and accessory proteins (83). The Env protein is sent to the endoplasmic reticulum (ER) and the golgi apparatus for glycosylation. Lastly the virion is transported to the hosts plasma membrane, assembled, and then budded out of the host cell as progeny virions (100).

1.3.3. *Origin and diversity of the HIV/AIDS Pandemic*

The identification of HIV as the causative agent of AIDS has allowed for rapid progress in understanding the natural history of HIV infection. Phylogenetic analysis of simian immunodeficiency virus (SIV) and HIV isolates suggests that HIV originated in Western Africa from four independent viral zoonotic transmission events from primates to humans (102-104). Genetic sequencing and phylogenetic analyses reveal that HIV-1 groups M (Major) and N (Non major and Non outlier) originated from chimpanzee (105, 106), HIV-1 group O (Outlier) originated from gorillas (107), and lastly sooty mangabey to human transmission resulted in HIV-2 (108). Evolutional estimates suggest that HIV was first introduced to humans between 1902 and 1921 (109, 110). This estimate is based on the oldest HIV-1 isolates available, from Kinshasa, Democratic Republic of the Congo (DRC) circa 1960. These isolates demonstrated that diverse HIV strains were already present in the 1960s. It is thought that urbanization and increased international travel have helped to facilitate the spread of HIV-1 from its origin in West Africa (111).

The majority of the global pandemic is caused by HIV-1 clade M which is believed to have been transmitted to humans in southern Cameroon in the early 20th century (110, 112). Group M has been divided into subtypes (clades) denoted with letters, and sub-subtypes denoted with numerals. HIV clades display distinct geographic distributions (Table 1) and include A1, A2, A3, A4, B, C, D, F1, F2, G, H, J and K (112, 113). The dominant clades in Kenya are clade A and D. Super-infection of multiple HIV clades in a single target cell can result in recombination. Circulating recombinant forms (CRF) can occur if these recombinants are established in the population (Table 1.1) (113).

Table 1.1. Global distribution of Prominent HIV-1 Clades and CRF[a]

Main Clades	Main geographic locations
A	Central Africa, East Africa (Kenya, Rwanda Burundi), Eastern Europe (Russia)
A1	Eastern Europe (Russia), East Africa (Kenya)
A2	Kenya
AE (CRF)	Angola, Congo, DRC, Gabon, South East Asia
AG (CRF)	Central and West Africa
A1D (CRF)	Kenya, Tanzania, Uganda, Europe
AB (CRF)	Eastern Europe, Central Africa
B	North America (USA/Canada), South America, Europe, Australia, East Asia (China, Thailand, Japan)
BF (CRF)	Eastern South America
BC (CRF)	China
C	China, India, Eastern and Southern African countries except Kenya
D	East Africa (Uganda, Sudan)
F	Brazil
F1	South America
G	North, Central and West Africa, Central Europe
H	North, Central and West Africa (DRC, Cameroon)
J	
K	

[a](113)

1.3.4. *HIV Genetic Diversity and Evolution*

HIV continues to evolve over the course of the infection and pandemic resulting in substantial viral genetic diversity. Estimates suggest that a billion new virions are produced within a single infected host daily. Rapid viral replication combined with the highly error-prone HIV RT leads to this extensive diversity. An important implication of this diversity is that HIV is capable of rapid evolution to escape ART and immunological responses. Additionally, substantial diversity is generated through genetic recombination between clades resulting in HIV genomes that are a mixture of more than one clade (114).

The genetic diversity between and within clades is extensive. For example, the divergence of amino acids in HIV-1 Gag is >20% between the clades, while HIV-1 Env is >30% (115). Additionally, intra-clade amino acid diversity can be more than 20%. Only one or very few virions establish primary infection however, over the course of infection mutations begin to accumulate forming quasispecies (116). The amino acid diversity in quasispices can be as great as 10% (117, 118). The rapid evolution and extensive HIV diversity present a difficult challenge to both immune control of HIV and vaccine design.

1.4. HIV Pathogenesis

1.4.1. *HIV Disease Progression*

Following transmission, HIV patients experience an acute phase of disease that typically lasts up to three months. During this time, an individual may experience acute seroconversion syndrome which can include signs and symptoms of a flu-like illness and rash. Viral load (copies of HIV per ml of blood) peaks within a few weeks post infection and is followed by a sharp drop in virus levels reaching a steady state condition, termed viral set point. This viral set point varies widely between individuals (>1000 fold) and coincides with robust HIV-specific CD8+ T cell responses (101, 119-124). The viral set point is a strong predictor of disease progression (120), with low viral set point correlating with slower progression to AIDS (120, 125).

The primary stage of HIV infection is characterized by a massive decline in CD4+ T cell levels in the periphery but rebound as viral replication is contained. Additionally, HIV significantly depletes CD4+ T cells from the gut-associated lymphoid tissue (GALT) resulting in dysfunction of the gut immune system (126-128). The initial dysfunction of the gut immune system does not recover consequently leading to systemic CD4+ T cell depletion stemming overall immunodeficiency as the disease progresses (129, 130).

Primary infection is followed by the chronic phase of infection which includes a high level of HIV replication (131). The duration of this stage varies widely between individuals, often depending on viral set point. The life expectancy of an HIV infected patient without antiretroviral therapy (ART) can range from 2 to over 50 years. A

combination of both viral and host factors contribute to the variation in infection rates. WHO and CDC guidelines define AIDS as being present when CD4 counts drop below 200 cell/μl and/or the development of characteristic opportunistic infections (101, 119). Low CD4 counts suggest the host's immune system is losing control of the virus and the patient ultimately expires. Figure 1.2 illustrates a characteristic ART naïve individual's HIV disease progression.

Once HIV infection becomes established, there is no evidence to date that infection can be cleared. There are two cases where HIV infected individuals have been "cured" of their infections. First was a leukemia patient in Berlin who received a hematopoietic stem cell transplant (132, 133) and second was a baby in Mississippi who was born with HIV and given an ART cocktail for 18 months (134). The Berlin patient had undetectable HIV viral load following treatment, while the Mississippi child had low levels of viral DNA in her cells (132-134). There is still much debate in the field whether these individuals were truly cured of their infections, particularly in the latter case. Even treated subjects with undetectable viral load continue to have a latent viral reservoirs that last for the lifespan of the individual (135). As a result of these viral reservoirs, HIV is a chronic infection that is never cleared.

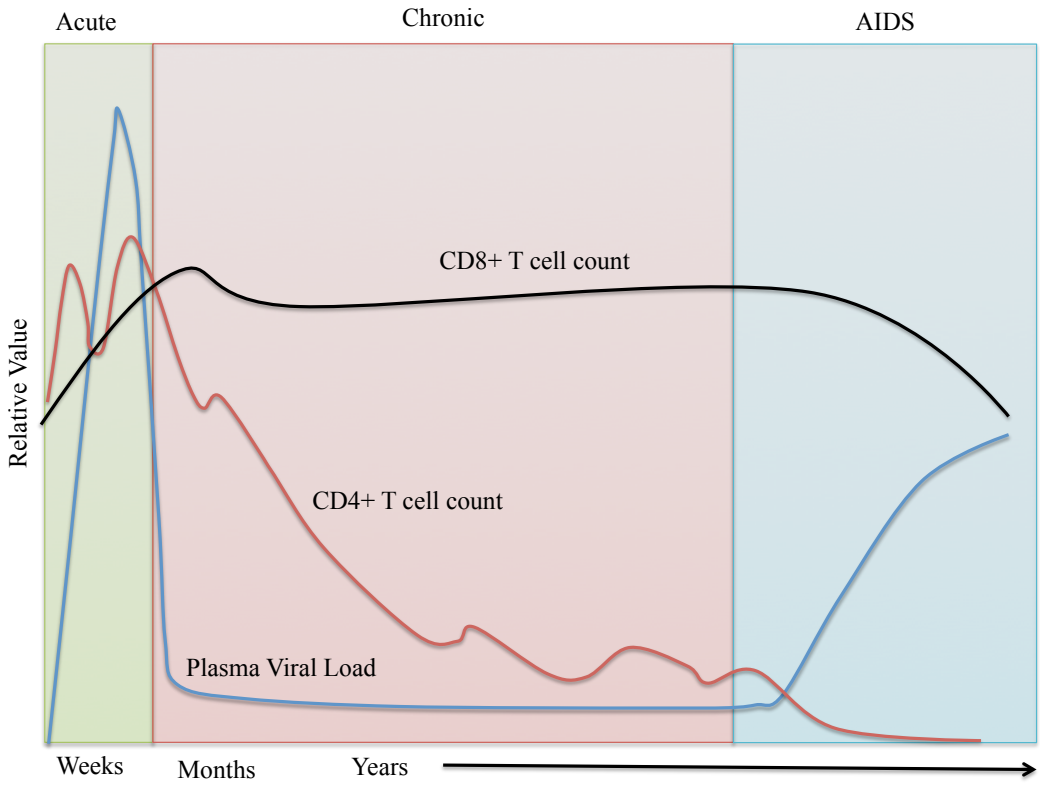


Figure 1.2. Schematic representation of clinical and immunological events associated with typical untreated HIV disease progression. (Reviewed in (136, 137)

1.4.2. *Models of Natural Protection*

There are several well-known models of individuals who remain HIV negative (HIV-exposed seronegative (HESN)) despite numerous exposures to HIV. HESNs include infants of HIV-infected mothers exposed *in utero*, health care workers exposed parenterally, HIV negative partners of HIV positive individuals (heterosexual or homosexual), IDU, and commercial sex workers (CSW) (138). Unfortunately, HESNs are rare and the majority of individuals are susceptible to HIV infection.

Most untreated HIV-infected individuals exhibit high levels of viral replication and progressive decline in CD4+ T cells. However there are some individuals who experience slower progression to AIDS and are able to maintain high CD4 counts or contain viral replication for extended periods of time in the absence of ART. These individuals are termed long-term non-progressors (LTNP, maintain high CD4 counts >500 cells per μl of blood for >6 years), elite controllers (ECs, spontaneously achieve undetectable viral loads for > 1 year), or viremic controllers (VCs, spontaneously achieve low viral loads for > 1 year) (139, 140). In contrast to HIV-1 infection, where LTNPs are rare, LTNP are normal in HIV-2 infection (141). Slow progressing individuals and HIV-2 infections provide a valuable model for the study of HIV-specific immunity that may be capable of controlling HIV in already infected subjects.

1.5. Basic Immunology

The immune system protects the host from a myriad of pathogens. The human immune system is divided into the innate and adaptive arms. The innate immune system provides rapid protection against invading pathogens by non-specific recognition and containment. In contrast, the adaptive immune system is slower to respond due to the more antigen-specific nature of the response. However, the adaptive immune response has a memory component which allows swift and strong responses upon repeated exposure to the same antigen (142, 143).

1.5.1. *Innate Immune System*

The primary characteristic of the innate immune system is its rapid response time, within hours of exposure, but does not generate or maintain memory. Components of the innate immune system include mucosal barriers, basophils, dendritic cells (DC), eosinophils, macrophages, mast cells, monocytes, natural killer cells (NK), natural killer T cells (NKT), neutrophils, and γδ T cells (142, 143).

Innate responses are directed by germ line-encoded pathogen recognition receptor (PRR) genes and have limited diversity (143). Innate cells PRR bind common molecular patterns on pathogens known as pattern-associated molecular patterns (PAMP). Examples of PAMP include lipopolysaccharide (LPS), peptidoglycan, flagellin, and double-stranded RNA (dsRNA). PAMPS are found on a variety of organisms including bacteria, virus, fungi, and protozoa. Binding of PAMPs to their corresponding PRR initiates innate cell activation and maturation. Activated innate cells signal the presence of an infection and influence the function and migration of both innate and adaptive cells (142, 144).

Antigen presenting cells (APCs) provide an important link between the innate and adaptive immune systems. Macrophage, monocytes, DC, and B cells are key players in carrying out antigen surveillance and presentation. Macrophages survey the infection site and phagocytise the infectious agents which are then processed and presented to T cells (142, 144). DCs actively sample their environment through endocytosis. Once activated, DCs mature and travel to lymph nodes (LN) where they activate T cells. LNs are the sites were the preponderance of the adaptive immune system is activated. The LN is strategically arranged to improve T and B cell activation and are organized into zones that contain B and T lymphocytes (B and T zone respectively).

In addition to the cellular component, soluble factors also contribute to innate immunity. In particular, cytokine and chemokine secretions directly influence which cells are recruited to the LN and to the site of inflammation. In response to cytokine and chemokine gradients, both DC and T cells migrate toward the T cell zone leading to the activation of the adaptive immune response (143, 145). In addition to acting as messengers, some cytokines have a direct role in pathogen defense; for example, the interferons (IFN) and tumor necrosis factor (TNF).

1.5.2. *Adaptive Immune System*

The adaptive immune system differs from the innate system in two major ways: memory and specificity. The adaptive immune system consists of T and B lymphocytes. Adaptive responses are highly specific and are a product of somatic rearrangement of the variable (V), diversity (D), and joined (J) segments of the T cell and B cell receptor

genes. These segments recombine in unique patterns so that each cell expresses a very specific receptor, generating vast diversity. Due to immunological memory, upon exposure to the same pathogen, the adaptive immune system is capable of quick and vigorous antigen-specific responses.

1.5.3. *B cells and humoral immunity*

B cells and the antibodies (Ab) they produce are the bases of the humoral arm of the adaptive immune system. Ab can be divided into several classes and subclasses (immunoglobulin (Ig)G1-IgG4, IgA1, IgA2, IgM, IgD, and IgE) which direct antibody function and localization. When B cells are activated, via their membrane-anchored antibody called the B cell receptor (BCR), they begin to proliferate, undergoing affinity maturation and class switching. Affinity maturation is a process where the genes of the BCR undergo small point mutations which allow the selection of Ab with optimal binding affinity for the cognate antigen. Maturation of B cells and class switching is followed by differentiation into Ab-secreting plasma cells and memory B cells (142, 146). Plasma B cells secrete antigen-specific Ab that act immediately on foreign antigen and are short lived. Conversely, antigen-specific memory B cells persist for weeks or months and are quickly activated upon re-exposure to the pathogen.

1.5.4. *T cells:*

T cells originate from haematopoietic stem cells in the bone marrow and then mature in the thymus into either CD4+ or CD8+ T cells. T cell development, known as central tolerance, is the result of a positive and negative selection process. Positive selection

leads to survival of T cells, whose T cell receptor (TCR) are capable of optimal binding of major histocompatibility complex (MHC) molecules, known as human leukocyte antigen (HLA) in humans. T cells that bind too strongly to self-antigen are killed via apoptosis (negative selection) which provides protection from autoreactive T cells (142). These processes create a diverse TCR repertoire of approximately 10^7-10^9 different specificities. This diversity provides T cells with the potential to respond to almost any antigen, regardless of its origin. Fully developed antigen naïve T cells enter circulation then travel to LNs. An individual's total T cell count found in circulation is only 2%, the balance inhabit tissue and the lymphatic system. In their naïve state T cells can only survive for a few weeks if their TCR does not encounter its cognate antigen (143).

Following TCR recognition of a peptide-HLA complexes, transcriptional activation occurs via a signal transduction cascade leading to initiation of genes involved in T cell proliferation, differentiation, and cytokine production (146, 147). Activation of a T cell requires binding of the TCR to its cognate antigen presented in combination with an HLA molecule and a co-stimulatory signal. Co-stimulation requires the binding of cell-surface molecules on the T cell (e.g. CD28) to those on the APC (e.g. CD80 and CD86, which are up-regulated during infection) in combination with cytokine signals (such as interleukins (IL) -15 and IL-2). The type of T cell response that proceeds is determined by the co-stimulation signals. The absence of this second signal leads to the T cell undergoing apoptosis or to be permanently inactivated (anergy) in a process known as peripheral tolerance (142, 147).

Antigen-specific T cells become activated then undergo clonal expansion increasing the number of T cells specific for a given antigen exponentially (148). With continued proliferation, T cells differentiate and begin to display effector phenotypes intended to kill, disable, and clear the foreign antigen and infected cells. Typically the pathogen is cleared and the immune response enters a contraction phase where >95% of pathogen-specific T cells die by apoptosis. This contraction phase occurs regardless of pathogen clearance, for example in the case of chronic infections such as HIV. The persisting T cells are memory T cells which are longstanding and upon secondary challenge with the same pathogen are capable of quick expansion and differentiation to effector phenotypes (Figure 1.3). The memory T cell compartment for some pathogens can last the lifetime of the individual although this compartment fades with age (149, 150).

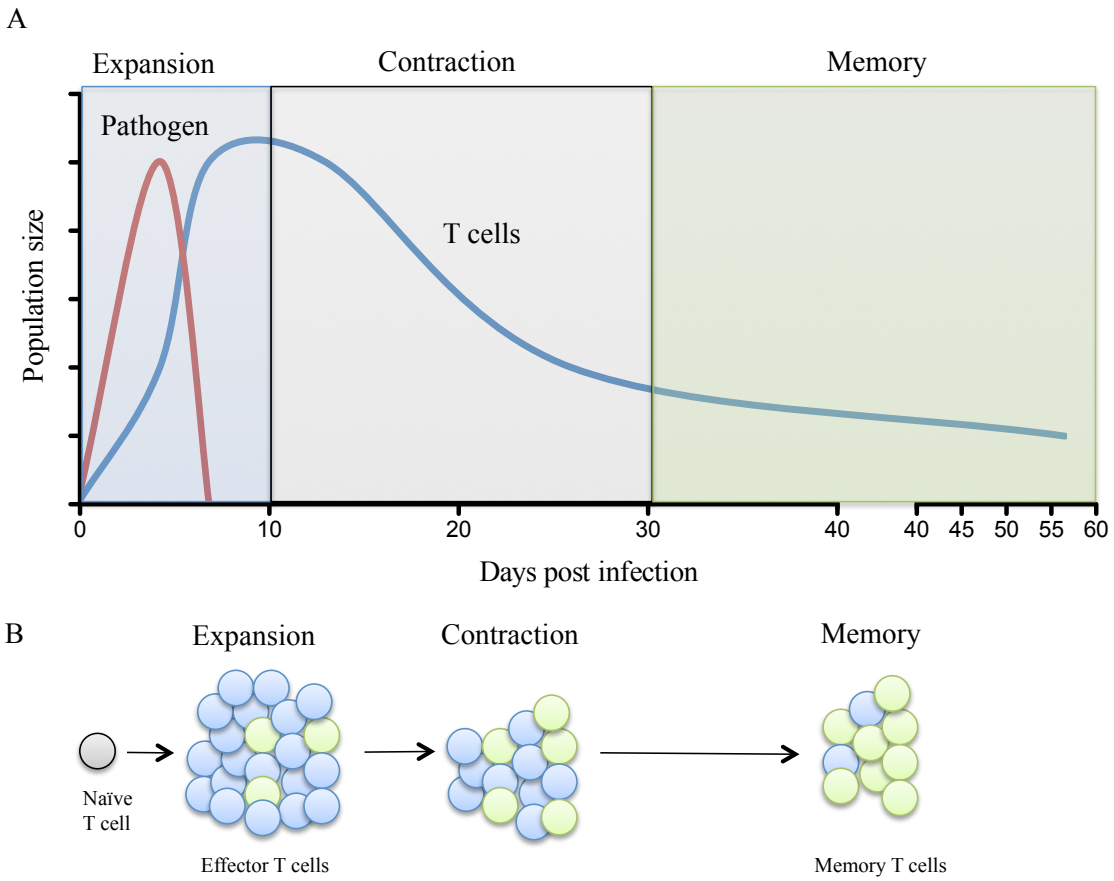


Figure 1.3. Schematic of T cell kinetics following antigen stimulation. A) Following activation, T cells rapidly expand, followed by clonal contraction and finally long lived memory. B) Over this time course T cells differentiate from naïve, to activate effectors than lastly memory cells. (151-155)

1.5.5. *CD4+ T cell immunology*

CD4+ T cells, also called T helper (Th) cells, recognize peptide bound to HLA-class II molecules on APCs (143) and are the major regulators of the adaptive immune system. Activated CD4+ T cells provide activation signals to both CD8+ T cells and B cells through secretion of cytokines that are crucial in determining the nature of the immune response. Traditionally, there were believed to be two key families of Th cells - Th1 and Th2. Th1 and Th2 cells have opposing patterns of cytokine secretion creating a dichotomous Th1/Th2 paradigm (156). Intracellular pathogens generally induce Th1 responses which are characterized by the production of IL-2, IFNγ, inflammatory cytokines, and cell-mediated immunity. Conversely, extracellular pathogens generate Th2 responses characterized by humoral immunity and the production of Ab, IL-4, IL-5, IL-10, and IL-13 (156).

The initial depiction of Th immunity has expanded in recent years with several other subsets of T_H cells having been recognized (157). Newly identified T cell subsets include regulatory T cells (Tregs), Th17 cells, follicular helper T cells (T_{FH}), Th9, and Th22 (158). Of these Tregs, Th17 and T_{FH} are the best studied. Tregs play a critical role in preventing self-directed immune responses (autoimmunity) and in controlling inflammation (159). Conversely, Th17 play a role in maintaining the integrity of the mucosal barrier and are essential for regulating effective anti-bacterial and fungal defenses (160-162). Finally, T_{FH} are pivotal for the regulation of plasma B cell activation and Ab production (163). These T_{FH} reside in the follicular region of secondary lymphoid tissues and are specialized for the regulation of effector and memory B cell responses (163-165).

These data demonstrate the functional flexibility of CD4+ T cells in guiding distinct immune responses (158). T cell flexibility allows thymocyte precursor cells to be continually generated, allowing the immune system to constantly deal with new circumstances and circulate within the body filling a multitude of distinct immunological niches. The plasticity and unstable phenotypes of T cells has important biological implications for designing therapeutic regimens to combat infections and control autoimmunity.

1.5.6. *CD8+ T cell immunology*

CD8+ T cells also called killer T cells and cytotoxic T lymphocytes (CTL) recognize peptide bound to HLA-class I molecules and kill target cells infected by viruses or intracellular bacteria. Upon activation CD8+ T cells clonally expand and differentiate into effector and memory phenotypes.

Activated effector CD8+ T cells migrate to the site of infection and secrete cytokines and chemokines that regulate the immune response: including IFNγ, TNFα, macrophage inflammatory protein-1β (MIP-1β), IL-2, and other soluble factors. Effector CD8+ T cells also carry out a cytolytic function that directly kills infected cells. This may occur through the release of cytotoxic granules containing effectors, such as perforin and granzyme B, into target cells or through induction of apoptosis via the Fas-FasL pathway (146). The perforin-granzyme method of apoptosis begins when perforin forms pores in the target cell's membrane; granzymes then enter the cell and activate caspases, leading to target cell death. Specifically removing infected cells restricts the spread of intracellular organisms to other uninfected cells.

CD8+ T cells typically recognize peptides (epitopes) 8-11 amino acids in length which are generated following proteolytic cleavage of foreign (and self) proteins in the cytoplasm of the APC. These proteins are acquired exogenously through antigen capture and cross-presentation by professional APCs or acquired endogenously through intracellular infection of the APC (145). HLA class I molecules are found on all nucleated cells and display epitopes to a cognate TCR on interacting CD8+ T cells. Before epitopes are presented on the cell surface for recognition, the HLA class I molecules bind the processed epitopes in the ER. Cytosolic peptides enter into the ER via the transporter associated with antigen processing protein (TAP). Proteins such as endoplasmic reticulum-associated aminopeptidase with antigen processing (ERAAP) aid the binding of the peptide to HLA inside the ER (Figure 1.4a) (166). Different HLAs have precise binding motifs and specific amino acids within the epitope play unique roles in the HLA-epitope-TCR interaction. The second and last amino acids are the anchor residues responsible for binding the epitope inside the pocket of the HLA class I molecule (Figure 1.4b). Different HLA alleles select different amino acids as anchors which play a critical role in dictating which epitopes are presented to T cells. Conversely, the middle residues within an epitope play an important role in contact with the TCR. Notably, the overall shape of the epitope can influence how the epitope will be presented and recognized. Amino acid substitutions at any point in the epitope can change the shape of the epitope and therefore impact whether it will and how efficiently it will interact with both its HLA and TCR.

An epitopes ability to be recognized by a cognate TCR greatly impacts the overall nature of the CD8+ T cell response. Immunodominance is defined by the observation that particular epitope-specific CD8+ T cell responses tend to correspond to the larger proportion of the total CD8+ T cell response than others (167). Some HLA alleles have specific immunodominance patterns although there is still a large diversity in recognition patterns within a given population. Immunodominace is affected by several factors including the ability of an epitope to be processed in the proteasome, binding by TAP and ERAAP, capacity to bind the HLA, and capacity to successfully stimulate a TCR from the collection of naïve T cells. The amino acid sequences flanking an epitope can also play a key role in the processing of the peptide. Flanking sequences can make the difference between an immunodominant and a sub-dominant epitope. This has been demonstrated by the substitution of sub-dominant epitopes into the flanking sequences of immunodominant epitopes, which resulted in increased responsiveness of the sub-dominant epitopes (168).

Immunodominance can also differ depending on the time point of pathogen infection with certain epitopes being commonly recognized in chronic infection, yet rarely recognized in acute infection (169). The strength with which the antigen binds, known as avidity, is a crucial determinate in steering immunodominance and TCR clonality in prolonged infections (170, 171). The implication of immunodominance on disease outcome is that responses to certain less dominant epitopes may be more beneficial and thus individuals whose responses are centred on immunodominant epitopes may be at a disadvantage. In some cases recognising one or a few sub-dominant epitope can be more successful than targeting dominant epitopes.

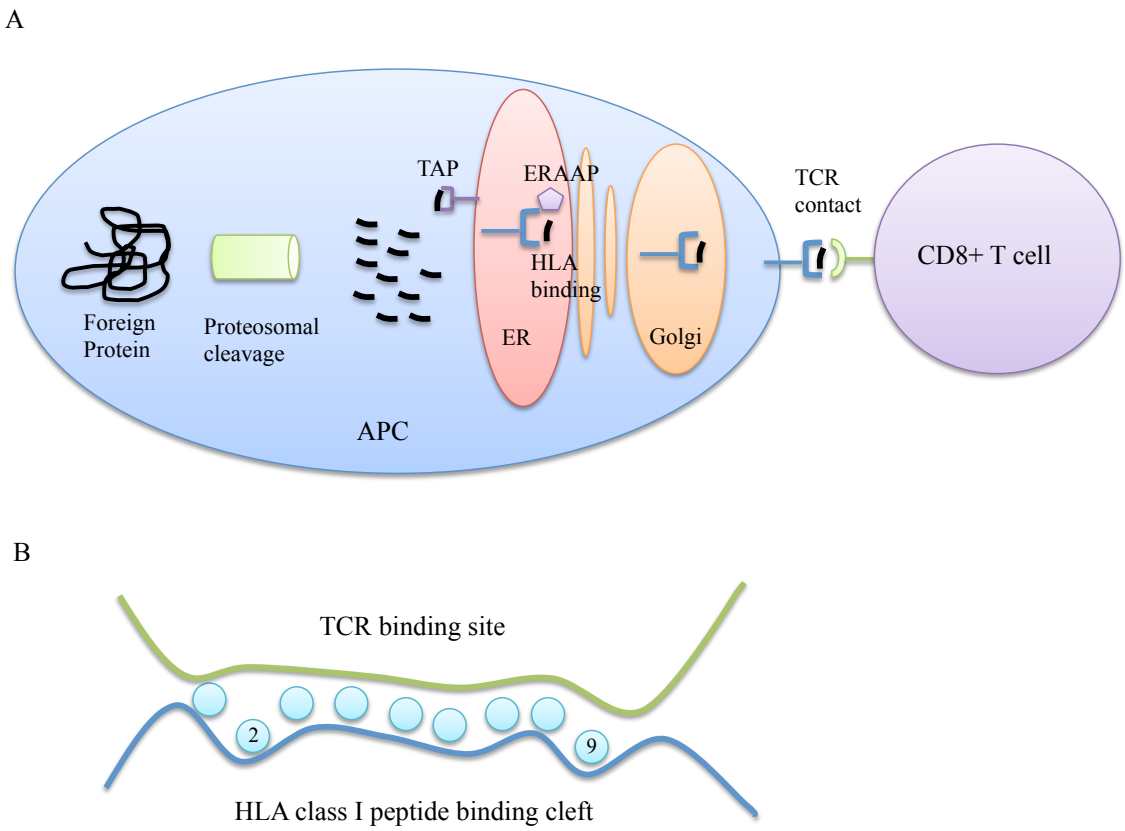


Figure 1.4. Epitope processing and TCR recognition. A) Epitope processing: foreign protein is cleaved in the cytoplasm and attached to specific HLA molecules in the ER. B) TCR:HLA complex: the ability of an epitope to be bound by a TCR following HLA presentation, is highly dependent on the amino acid sequence, particularly those in position 2 and 9.

1.5.7. *T cell memory paradigms*

Following the generation of effector cells and clonal expansion the immune response enters the contraction phase where the bulk of antigen-specific CD8+ T cells die by apoptosis. However, a small percentage (~5-10%) of the remaining antigen-specific T cells survive as memory T cells (155). These CD8+ T cells are long-lived and capable of more rapid expansion and differentiation to effector phenotype upon secondary challenge with the same pathogen (172). The concept of vaccination is based on these properties of immunological memory. Memory CD8+ T cell populations are, however, heterogeneous in their quality.

The physiological relevance of this heterogeneity and separating immunological memory into effector (T_{EM}) and central (T_{CM}) memory populations is significant (173). T_{EM} cells home preferentially to tissues and are capable of rapid effector activity but have limited proliferative capacity. Therefore upon re-infection, T_{EM} are critical for the quick memory response. Conversely, T_{CM} cells have slow effector activity, but have the capacity for quick expansion and differentiation to effector cells following persistent immune stimulation and can target pathogens on a larger scale. The combination of T_{EM} and T_{CM} ensure both a rapid and sustained secondary immune response (174).

Several models have been used to define memory populations, including one that splits memory CD8+ T cells into T_{CM} and T_{EM} cells, based on cell surface markers. The traditional approach measures expression of the LN homing marker CCR7 and the T cell differentiation marker CD45RA to define cells as T_{CM}, T_{EM}, effector, and naïve (175, 176). Based on these surface markers, T_{CM} are defined as CCR7+ CD45RA-, T_{EM} as

CCR7- CD45RA-, naïve cells as CCR7+ CD45RA+, and effectors as CCR7- CD45RA+ (175). CD62L, which is also a LN homing marker, can often be used in place of CCR7 to identify T_{CM}. An alternative model divides T cells on the basis of their differentiation past and antigen experience with cells termed naïve, early, intermediate, and late using CD28 and CD27 markers. The expression of CD27+CD28+high, CD27+CD28+low, CD27+CD28-, and CD27-CD28-, define naïve, early, intermediate, and late respectively (177). Using this model, late cells have superior effector potential while early cells have superior memory potential and have divided fewer times.

To further delineate memory CD8+ T cells, a combination of the two approaches can be used. The addition of CD27 to the CCR7 and CD45RA approach allows further delineation on the basis of differentiation status as CD27 is lost upon antigen stimulation (178). Traditionally, CD8+ T cells expressing CCR7+CD27+CD45RA+ are considered to be functionally naïve, while CCR7-CD27-CD45RA+/- are functionally active which represent terminally differentiated (T_{TD}) and T_{EM} cell subsets, respectively (178). The phenotype CCR7+CD27+CD45RA- is thought to define a T_{CM} subset. A transitional memory phenotype (CCR7-CD27+CD45RA-) can also be defined using this model (178). Unfortunately, accumulating evidence suggests that these memory markers may not necessarily correspond with the functional capacities they are suggested to represent (152, 179, 180). Therefore, further studies need to be conducted to evaluate whether surface markers routinely used to define memory T cells actually predict *ex vivo* function, particularly the wide range of polyfunctional CD8+ T cell responses that are now routinely measured.

Despite the complexities of properly identifying memory subsets, the selection of memory T cell pools following the contraction phase is not entirely random as all effector cells do not inherit memory potential (154). It appears that some CD8+ T cells are intrinsically better than others at persisting and populating the memory pool. The exact mechanism by which memory CD8+ T cells are created is controversial and varies between hosts and infection. Currently, the best-characterized models of memory T cell formation and maintenance have been described in mice for both acute and chronic infection. Several potential differentiation models that explain how heterogeneous pools of effector and memory CD8+ T cell arise during infection have been put forward. These include: "*Separate-precursor model*", "*Decreasing-potential model*", "*Signal-strength model*" and "*Asymmetric cell fate model*" (Figure 1.5).

The *separate-precursor model* suggests that a single naïve CD8+ T cell can give rise to either terminal effector or memory T cells. Differentiation states of the precursors following activation and development are based on information received during thymic development. However, there is little evidence for this model as it has only been shown using adoptive transfer of single naïve CD8+ T cells, which demonstrate multipotency giving rise to both effector and memory T cells, including both T_{CM} and T_{EM} (155, 181, 182). The caveat of these studies is that they used TCR-transgenic CD8+ T cells limited to only a single TCR clonotype and it is not known whether or not these same results would occur under more natural, highly diverse TCR colonotype conditions (155).

The *decreasing-potential model* suggests that repetitive stimulation of T cells with antigens and pro-inflammatory cytokines drives proliferation and differentiation into different memory populations. This model implies a linear progression from Naïve to T_{CM} to transitional memory (T_{TM}) to T_{EM} and finally T_{TD}. Studies supporting this model demonstrated that abbreviating antigen and pro-inflammatory exposure promoted memory T cell formation, particularly T_{CM} (155).

The *signal-strength model* suggests the formation of heterogeneous cell populations are dependent on the overall strength of the antigen signal, co-stimulation, and pro-inflammatory signals encountered during T cell activation. Strong signals can drive differentiation into T_{EM}, but too strong leads to T_{TD}. Conversely, weaker signals promote T_{CM} or T_{TM}. Unlike decreasing potential model, this model is not linear and different cell fates are specified early following antigen stimulation (155).

The *asymmetric cell fate model* suggests that T cell fates arise from a single precursor T cell through asymmetric cell division which occurs at activation and clonal expansion. Evidence for this model suggests that cell fate is dependent on the proximity of the daughter cell to the APC. Closer proximity to the TCR:HLA interaction provides greater effector potential while further proximity results in greater memory potential (155, 183).

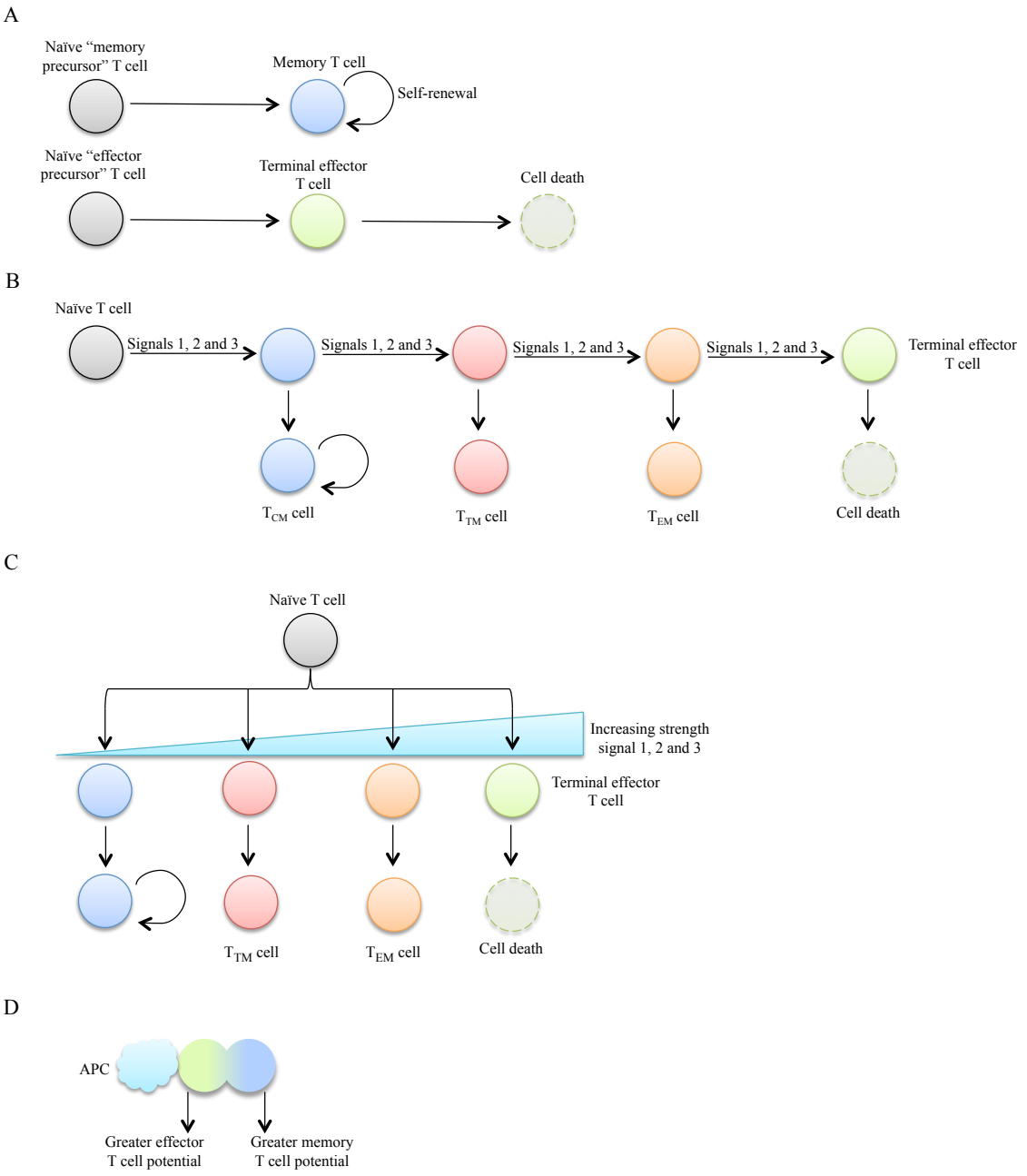


Figure 1.5. Differentiation models of heterogeneous effector and memory CD8+ T cell pools. A) Separate-precursor model: suggests naïve T cells are preprogramed in the thymus to develop into either T_{EM} or T_{CM}, little evidence exist for this model. B) Decreasing-potential model: suggests that repetitive stimulation of T cells with antigens and pro-inflammatory cytokines drives proliferation and differentiation into different memory populations. C) Signal-strength model: suggests the formation of heterogeneous cell population is dependent on the overall strength of the antigen signal, co-stimulation, and pro-inflammatory signal encountered during T cell activation D) Asymmetric cell fate model: suggests that T cell fates arise from a single precursor T cell through asymmetric cell division, which occurs at activation and clonal expansion.

Regardless of the model, several inflammatory cytokines and transcriptional regulators have been found to influence CD8+ T cell differentiation. The maintenance and homeostasis of memory T cells is mediated by signals from the cytokine environment. In particular, IFNγ, IL-2, IL-7, IL-12, and IL-15 are required for maintenance of CD4+ and CD8+ memory T cells (184-187). These cytokines support homeostatic proliferation and cell survival by regulating the relative levels of pro-survival and pro-apoptotic factors. IL-7Rα plays an important role in naïve T cell homeostasis and, during an immune response, effector cells expressing IL-7Rα are those that eventually form the pool of memory T cells (188). IL-15R plays an important role in the homeostatic long-term maintenance of T cell memory. Additionally, inflammatory cytokines, particularly IL-12, induce the expression of Bcl-2 and Bcl-3 which can enhance activated CD8+ T cell expansion (155, 180).

Furthermore, T-box transcription family members, T-bet and eomesodermin (EOMES), cooperate to sustain memory CD8+ T cell homeostasis enabling IL-15-mediated signalling. The ratio of T-bet and EOMES directs the differentiation of effector and memory CD8+ T cell. The ratio of T-bet to EOMES is highest at the effector stage and lowest at the memory stage. IFNγ is critical for T-bet induction and IL-2 is critical for EOMES while IL-12 augments T-bet expression and diminishes EOMES expression (155).

In addition to EOMES/T-bet, there are three more sets of antagonistic transcription factors that mediate effector and memory cell fate, BLIMP1 and BCL-6, ID2 and ID3, and STAT3, STAT4 and STAT5. All of these transcription factors interact and work together by unknown means to generate heterogeneous pools of T cells. Data on

transcription factors suggest that the memory differentiation models described above are not mutually exclusive and that there is much plasticity in the memory T cell lineage. As the discovery of signalling pathways and transcription factors involved in effector and memory CD8+ T cell development continues, it is essential to identify how these factors cooperate, or impede, each other's function. Discovering the mechanisms by which memory CD8+ T cells develop and differentiate along with their phenotype and function is important to understand if we are to appreciate their protective role in infectious diseases. This information will also improve vaccine design and treatment of infections.

1.5.8. *T cell receptor usage of CD8+ T cell responses*

The structural characteristics of the TCR repertoire are another important feature of CD8+ T cell immunity. Adaptive T cell responses have evolved to generate a large number of T cells capable of recognizing a large array of antigens. The TCR repertoire is extremely diverse as a consequence of gene segment (V, D, and J) recombination. TCRs are heterodimeric proteins consisting of an α and β subunit. The V (variable) segments provide the greatest amount of diversity and contain three complementarity-determining regions (CDRs) that form loops. These CDR3 regions are the TCR components which interact directly with the HLA-epitope complex.

Despite its potential diversity, several studies have shown that the TCR repertoire emerging in response to antigenic stimulation is restricted (189). The memory population consists of only a small number of clones which expand during a particular antigen-specific response. Although the mechanism for this restriction is unknown, it appears

that both thymic selection and immunodominant epitopes play a role in limiting the TCR repertoire. Some HLA-restricted epitope-specific responses display clear biases in TCR usage. These include favouring particular Vβ segments (Type 1 bias), particular CDR3 motifs (Type 2 bias), and less commonly, a particular clonal sequence (Type 3 bias)(190). Type 1 TCR bias is characterized by the persistent selection of a single TCR Vβ region but maintains large diversity in the CDR3 regions. Type 2 TCR bias is characterized by the frequent selection of conserved amino acids or 'motifs' in the CDR3 sequence of antigen-specific TCR Vα or Vβ chains. Lastly and the least common, type 3 TCR bias is characterized by the reproducible selection of the same clonal TCR sequences, sometimes termed public clonotype. Public clonotypes are defined as V-D-J amino acid sequences that are dominant and present in the majority of individuals while conversely, private clonotypes (Type 1 and 2 bias) are defined as those being rarely observed in the majority of individuals.

TCR bias and variation in the use of certain antigen-specific TCRs between individuals provides an additional level of complexity to the T cell response (190). Understanding the way TCR repertoires are selected can contribute to protective immunity and has implications for vaccine design and immunotherapy. For example, there are certain infections known for selecting an extremely low number of TCR clonotypes, including Epstein-Barr virus (EBV) and Influenza. These viruses are known for selecting public TCR clonotypes. Since EBV is a chronic infection and individuals encounter acute influenza repeatedly though their lives, it is hypothesised that successive and repetitive stimulation is responsible for an individual's TCR repertoire narrowing. The idea of

repertoire narrowing is, however, controversial, as the TCR repertoire during chronic Cytomegalovirus (CMV) infection remains quite heterogeneous. Elucidating the diversity of the TCR repertoires following infection particularly chronic infection has been highly sought after and can have important implications for the effectiveness of antigen-specific CD8+ T cell responses to chronic infection.

The recognition properties and antiviral efficacy of TCR clonotypes can also vary considerably depending on the quantity of presented antigen. Functional avidity, defined as the measure of the efficiency of antigen recognition by CD8+ T cells, plays an important role in cell functionality. Functional avidity is recognized to be of critical importance in determining pathogen clearance (191). High avidity CD8+ T cells have been shown to initiate the lysis of target cells more rapidly at any given antigen density and to be more effective at mediating viral clearance than low avidity cells (192, 193). Similarly, mouse models suggest a correlation between T cell functionality and avidity so that the quality of the CD8+ T cell response, as determined by cytokine profile, is enhanced for highly avid T cells (194). Thus, the efficiency with which CD8+ T cells are stimulated and exert their anti-viral effects to eradicate infected cells is dependent on avidity. Some studies suggest that "public" clonotypes exhibit high levels of TCR avidity and antigen sensitivity which impart functional advantages and enable effective suppression of viral infections (195). By understanding the molecular mechanisms involved in improving antigen-specific T cell responses, we may be able to develop better vaccines as well as better therapies for resolving acute and chronic viral infections.

1.6. HIV Immunology

Although, many individuals can contain their HIV infection for several years, inevitably, most individuals' immune systems lose control and become immunodeficient, leading to AIDS and ultimately succumbing to opportunistic infections. The course of an average HIV infection demonstrates how intensely the virus affects the immune system. However, the control of viral replication and disease progression has been associated with a number of innate and adaptive immune responses.

1.6.1. *Innate response to HIV infection*

Reduction in viral load during acute HIV infection often occurs before the induction of adaptive immune responses, strongly suggesting that the innate immune system plays an early essential role in HIV control (196, 197). NK cells are the first immune modulators in acute HIV infection and expand rapidly following infection during the window of acute seroconversion. NK cells survey the body for altered HLA class I expression on infected cells using killer immunoglobulin-like receptor (KIRs) and play a central role in controlling early HIV replication through killing infected cells (mainly CD4+ T cells) via production of IFNα, perforin and granzymes, and interaction with DCs (196). Unfortunately, NKs are not specific and substantial bystander death of uninfected CD4+ T cells can occur that may contribute to HIV associated CD4+ T cell depletion. However, NK cells are thought to place considerable pressure on HIV replication exemplified by the effects of particular KIR genotypes on rates of disease progression and the multiple strategies HIV has developed to escape NK cell recognition. The HIV protein Nef down regulates expression of HLA class I on the surface of infected cells

thus averting recognition by HIV-specific CD8+ T cells. Theoretically, the downregulation of HLA class I should alert NK cells to the infection however Nef has overcome this problem by downregulating HLA class I molecules differentially. Nef strongly down regulates HLA-A and partially down regulates HLA-B the dominant TCR ligands, but spares HLA-C the dominant ligand for inhibitory NK cell receptors (196). Thus the virus has evolved a balanced strategy to escape both T cells and NK cells.

Macrophages are also a key innate cell subset in HIV infection. Although macrophages act as important APCs, which activate the adaptive immune response, they also express CD4 and CCR5 and are susceptible to HIV infection. Macrophages are a major reservoir for latent HIV during chronic infection (198). Similarly, DCs play an important role in transmission of HIV at mucosal surfaces and the subsequent systemic propagation of infection. The inflammatory responses following DC activation in early infection contribute to the immunopathology observed throughout progressive disease and to generalized CD4+ T cell loss through induction of pro-apoptotic pathways (199). HIV infection results in dysregulation of DCs and DCs counts decline during acute infection due to activation-induced cell death (AICD) (200, 201). However, DCs are essential for establishing the optimal cytokine environment and signaling to develop an effective adaptive response. During HIV infection, DCs take up, process, and present HIV antigens via MHC I and II molecules for priming and boosting of HIV-specific CD8+ and CD4+ T cell responses. Researchers found that HIV peptide-loaded, mature DCs stimulated CD8+ and CD4+ T cells proliferate better than with peptide alone (202). These data suggests DCs can be harnessed to steer the immune system towards a more effective adaptive immune response.

1.6.2. *HIV-specific T cells*

HIV-specific CD4+ T cell responses can be observed during acute HIV-infection and associate with lowering set point viremia but contract rapidly (203). HIV infects CD4+ T cells attenuating many aspects of HIV-specific immunity. However, individuals with robust HIV-specific CD4+ T cell responses characterized by IFNγ, strong proliferation, and IL-2 are associated with better disease outcomes (204-209). These data suggest HIV-specific CD4+ T cell responses are playing a significant role in containing HIV replication. However, this role remains unclear and it is unknown why some individuals are less susceptible to the massive depletion of CD4+ T cells characteristic of a normal progressive infection.

HIV-specific CD8+ T cell responses are among the first antigen-specific responses observed during the first few weeks of HIV-infection and are thought to play a key role in lowering viremia and establishing viral set point. Following peak viremia, HIV-specific CD8+ T cells decline due to the emergence of viral escape mutations and lack of antigen stimulation (210). The earliest CD8+ T cell responses develop against Env and Nef viral targets followed by Gag responses later in infection (211, 212). Numerous studies have compellingly displayed the necessity for HIV-specific CD8+ T cells in controlling viral replication (204, 213-220).

1.6.3. *Escape from CD8+ T cell responses and Cross-reactivity:*

Inevitably, most HIV infected individuals lose immune control of the virus and one of the major reasons for this is viral escape from CD8+ T cells. HIV replicates rapidly, with high mutation rates, and escape mutations that can affect disease progression whether they occur in early or chronic infection (221-224). The main forms of escape mutations include epitope mutations that abolish effective epitope processing and alter HLA binding and/or TCR recognition (225-227). CD8+ T cell escape mutations can be detected as early as one month following infection, highlighting the pressure CD8+ T cells place on the virus. These early mutations are a combination of selection of new mutations and reversion of incoming virus to original sequence (228, 229). Population studies have found associations between specific HLA alleles and HIV polymorphism, signifying an accumulation of escape mutations in HIV sequences (230, 231). Although these escape mutations can have major consequences for the host, there is also substantial fitness cost to the virus. Evidence for the viral fitness cost is apparent by escape reversion after transmission to a subject lacking the restricting HLA allele (232, 233). Protective HLA alleles, such B*57 and B*27, place considerable pressure on HIV fitness as the immune escape mutations driven by these alleles impart considerable impairment to the virus, including replicative capacity (234).

CD8+ T cell escape mutations that do not impose fitness cost add considerable variation to the viral genome and contribute to HIV's high genetic diversity. This diversity, including variation between clades and escape mutations, is a continuous hurdle in HIV immunological and vaccine research. How well HIV-specific CD8+ T cell responses

cross-react with different clades and epitope variants are important issues to address for any candidate vaccine aimed at inducing cell-mediated immunity. An important determinant of an effective vaccine is whether it will provide coverage relevant to the virus to which a vaccinee is exposed.

Most HIV-specific CD8+ T cells have some level of cross-reactivity; however, this differs between individuals, the targeted protein and epitope. Restricting HLA alleles and presenting TCR also play an important role in CD8+ T cell cross-reactivity (235-241). Until recently the cross-reactivity of CD8+ T cell has largely been evaluated using IFNγ Elispot and chromium release which are not associated with reduced HIV replication (242, 243). The role cross-reactive HIV-specific CD8+ T cells play in 'protective' responses, including polyfunctionality and proliferative capacity, remains to be fully explored. However, studies have revealed that HIV controllers are more likely to have cross-reactive CD8+ T cells expressing IFNγ, TNFα, IL-2, CD107a, perforin, CD127 and/or BCL-2 with higher TCR clonotype promiscuity (244-246). These data suggest that within an individual, cross-reactive CD8+ T cells provide more efficient control of HIV and associate with better disease outcome (247, 248). Additionally, the data suggest that the capacity to elicit cross-protective CD8+ T cells able to recognize HIV diversity and viral escape is likely important for the development of effective CD8+ T cell based vaccine.

1.6.4. *Evidence for protective HIV-specific CD8+ T cells in HIV infection:*

Most current HIV vaccine candidates aim to induce HIV-specific CD8+ T cell responses capable of containing viral replication and slowing disease progression (249, 250). This vaccine concept is based on several lines of evidence suggesting that CD8+ T cell responses can control viral replication (137, 222, 251-253)**.** HIV-specific CD8+ T cell responses have been correlated with the initial control of HIV replication that establishes viral set-point (121, 122). Additionally, early data suggested an inverse correlation between Env-specific CD8+ T cell responses in the first year of infection and viral load (254). Furthermore, an inverse correlation is seen in the numbers of HIV-specific CD8+ T cells, as measured by HLA class I tetramers and viral load (255). Together these data highlight the importance in eliciting the CD8+ T cell compartment of the immune system in order to more effectively control HIV replication.

Evidence for the protective role of CD8+ T cells was provided by non-human primate experiments in which SIV-infected rhesus macaques showed dramatic increase in viremia when CD8+ T cells were depleted *in vivo* using monoclonal antibodies (251, 253). Conversely, when these same experiments were repeated in sooty mangabeys, the non-pathogenic SIV model, depletion of CD8+ T cells had limited impact on viral loads (256). Therefore, CD8+ T cells do play a role in viral control in rhesus macaques, but do not affect viral control in sooty mangabeys. The inconsistencies between these two models highlight the differences in host-pathogen interaction (257) and emphasise the need to identify correlates of protection in human models.

Subgroups of HIV-infected subjects, termed LTNP, EC, and VC (section 1.4.2), experience slower progression to AIDS and provide a valuable model for the study of cell mediated immune responses that may be capable of controlling HIV. Previous work has demonstrated that these individuals maintain higher levels of HIV-specific CD8+ T cell proliferation compared to progressing controls (258-260). LTNP were found to have more polyfunctional HIV-specific CD8+ T cells, as defined by the concurrent expression of IFNγ, IL-2, TNFα, MIP-1β, and CD107a (193, 216). Polyfunctional CD8+ T cell responses have been measured in humans vaccinated with the highly efficacious smallpox vaccine suggesting that these responses are protective (261). Polyfunctional CD4+ T cell responses have been found to be protective in settings where immunity is primarily cell-mediated, for example following tuberculosis vaccination and in murine models of *Leishmania major* (262, 263). Detection of polyfunctional CD8+ T cells in HESNs potentially demonstrate that these responses play a role in protecting against HIV infection (264).

Better disease outcomes have also been associated with distinct HLA class I alleles and HLA class I heterozygosity (265, 266). Responses that restrict HLA-B, which has the greatest degree of polymorphisms, are most frequent and are associated with a higher degree of polyfunctionality and slower disease progression (267, 268). A number of distinct HLA class I alleles have been correlated with slow progression to AIDS; however, the most widely studied are HLA-B*57 and B*27 (269). Individuals that carry the HLA-B*57 allele have a 2-fold lower chance of progressing to AIDS after 10 years of infection (270). CD8+ T cells that restrict HLA-B*57 are better able to cross-recognize

HIV epitope variants than those that restrict non-protective HLA alleles (247, 248). In addition, compared to other alleles, HLA-B*57 restricted epitopes are dominant in early infection which is critical in lowering early viral replication and establishing a lower viral set point and thus slowing disease progression (271). CD8+ T cells that restrict HLA-B*57 and B*27 are more likely to retain proliferative capacity and polyfunctionality in chronic infection (272, 273). A genome-wide association study in a multiethnic cohort of HIV-1 controllers and progressors revealed that three specific amino acids (position 67, 70, and 97) in the HLA-B peptide-binding grove showed the strongest association with HIV control. Moreover, position 97 was more significantly associated with HIV control than any single HLA allele, including B*5701. These results imply that the nature of the HLA-viral peptide interaction is a major factor in HIV control and suggest disease outcome could be mediated, at least in part, by these HLA-B amino acid positions. Together these data suggest that the MHC class I peptide-binding groove and the conformational presentation restricted epitopes play a key role in host control and evoking protective responses.

Similarly, the specific HIV protein and epitopes targeted by HIV-specific CD8+ T cells play an important role in slowing disease progression (274). For example, the breadth of CD8+ T cell responses to the HIV Gag protein are inversely correlated with viral load, while the breadth of CD8+ T cells responses to Env protein are positively correlated (275). CD8+ T cell responses to Gag, particularly the matrix protein p24, have also been associated with greater polyfunctionality and proliferative capacity and slower disease progression (269). These data suggest that HIV CD8+ T cell responses targeting Gag

confer clinical advantages (276-280). In addition to being expressed early in the viral replication cycle (as soon as 4 hours post infection) (278), Gag is more structurally conserved than other regions of the genome and thus less prone to immune escape and mutations which present substantial cost to viral fitness (269).

Properties of the interaction between the TCR, a specific HIV peptide, and certain HLA alleles may also define HIV disease outcome. Studies show that HIV infection alters the normal distribution of TCRs resulting in the reduction of TCR repertoire diversity and lack of optimal TCRs specific for HIV epitopes and opportunistic infections (281-283). Furthermore, reduced clonal TCR diversity leaves the host vulnerable to viral escape via single amino acid mutations within the TCRs target epitopes (284). However, data also shows that individuals with better disease outcomes have selected TCR clonotypes with superior epitope binding, cross-reactivity, and decreased viral escape (247, 285). Evidence for these is supported by studies on distinct HLA alleles that present the same epitopes, including HLA-B*5701/B*5703 (286) and HLA-B*8101/B*4201/B*0702 (287). These HLA pairs are associated with distinctive TCR repertoires. CD8+ T cell responses from HIV controllers with protective HLA alleles are more effective at inhibiting viral replication and more cross-reactive than those with non-protective alleles (287). Moreover, these effective TCR clonotypes were associated with upregulated perforin and granzyme B expression which provides a mechanistic explanation for the divergent disease outcomes in people with protective HLA alleles (287). Recently, a study of HIV controllors and progressors with the same ‘protective’ HLA allele, B*2705 or B*5701, stimulated with the same epitopes, further examined the role of TCR

selection in disease progression. This study demonstrated that despite having the exact same HLA allele, individuals with slower disease progression selected TCR clonotypes with better capacity to stimulate protective T cell attributes while progressors selected less or ineffective clonotypes (288).

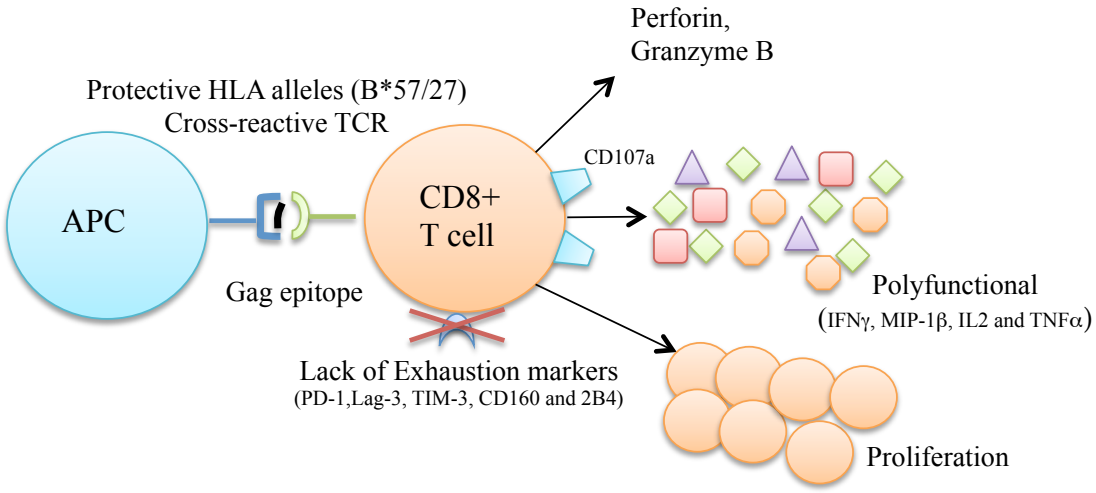


Figure 1.6. Attributes of CD8+ T cell associated with HIV control

1.6.5. *Evidence for insufficient CD8+ T cell activity in HIV infection:*

CD8+ T cell responses are functionally heterogeneous and there are data to suggest that specific aspects of CD8+ T cell function confer different capacities to protect against AIDS. CD8+ T cells remain detectable in nearly all HIV+ subjects, regardless of disease progression, and there is little difference in the quantity of HIV-specific CD8+ T cells measured by intracellular IFNγ staining, between progressors and non-progressors (178, 223, 224, 289). Several large-scale epitope mapping studies have assessed HIV-specific immune responses to the entire viral proteome and found no relationship with HIV-specific IFNγ T cell responses and viral load in chronic infection (279, 280, 286, 290). Furthermore, no correlation was found between HIV-specific CD8+ T cells secreting IFNγ and viral load in subjects progressing to AIDS who also have high avidity CD8+ T cell responses to autologous virus can be measured in these subjects (291, 292). HIV-specific CD8+ T cells are often exhausted or functionally inferior in chronic, progressive HIV infection, in some cases lacking perforin expression, cytokine secretion, and proliferative capacity (259, 260). These data suggest that not all CD8+ T cell responses are equally effective.

Virus-specific CD8+ T cells in chronic infections such as HIV experience substantial functional exhaustion. It has been suggested that this occurs in a hierarchical manner as the infection persists; where IL-2, cytolysis and proliferation responses are the first to be lost, followed by TNFα; and finally by the loss of IFNγ and anergy/deletion (252, 293-295). Class I tetramers studies have shown that HIV-specific CD8+ T cells in chronic HIV infection were perforin deficient while CMV-specific CD8+ T cells maintained

efficient perforin expression (296, 297). This suggests HIV-specific CD8+ T cells were functionally impaired while CMV-specific CD8+ T cells remain effective. Additionally, in some studies HIV-specific CD8+ T cells were found to be anergic and failed to produce IFNγ (298-300), further suggesting that HIV dramatically weakens HIV-specific CD8+ T cell functionality. HIV-specific CD8+ T cell proliferation is quickly lost following acute infection and CD8+ T cell proliferative capacity is absent in most HIV-specific responses (260, 301). This is contrary to responses to other persistent viruses such as CMV and EBV which maintain CD8+ T cell proliferative capacity and perforin expression (302, 303). The immune sytem is able to maintain much better control over CMV and EBV infections compared to HIV infection. Taken together, these data indicated that HIV-specific CD8+ T cell functionality is severely damaged as the infections persists and demonstrate substantial functional exhaustion.

The identification of functionally exhausted CD8+ T cells is an ongoing area of research. Signaling through Programmed Death-1 receptor (PD-1), a maker of T cell exhaustion (or inhibition marker), has been shown to play an important role in CD8+ T cell exhaustion in murine and rhesus macaque models of chronic viral infection, LCMV, and SIV, respectively (304). Studies also demonstrated that human HIV-specific CD8+ T cells have higher expression of PD-1 and that this expression was correlated with impaired CD8+ T cell immunity, higher viral loads, and lower CD4 counts (305). Comparison of HIV controllers and normal progressors found that normal progressors had higher levels of PD-1 expression (306). PD-1 is critical for inducing apoptosis of virus-specific CD8+ T cells and blocking PD-1 from interacting with its ligand restored CD8+ T cell function

(307). Similarly, other inhibitory or exhaustion markers have been associated with CD8+ T cell dysfunctional and progressive HIV infection including TIM-3, LAG-3, CD160, and 2B4 (308, 309). The functional relevance of these exhaustion markers in regulating T cell responses in HIV infection remains to be investigated. It is not yet established if the elevated levels of these exhaustion markers are part of the immune suppression seen in HIV infection. Recent data revealed a highly complex expression pattern of exhaustion markers on HIV-specific CD8+ T cells and suggested that specific combinations of these receptors, rather than individual markers, might provide a better definition of exhausted HIV-specific CD8+ T-cell populations and provide information that relates to the immune control of viremia and disease status (308).

Although it is clear CD8+ T cells play a central role in viral control in acute infection, this control is evidentially lost in the majority of HIV infected individuals. This loss of CD8+ T cell control is likely the result of viral escape (above section 1.6.4), exhaustion, and functional impairment. Understanding the nature of CD8+ T cells in progressive infection and those that may slow disease progression is imperative for the development of effective HIV treatments or vaccine.

1.7. Thesis Rationale

This thesis focuses on defining the role of CD8+ T cell responses in HIV infection. Not all individuals infected with HIV progress to AIDS. LTNP appear to be able to control HIV infection and provide a valuable opportunity to study viral-host interactions. While various host and viral factors can explain this apparent success of the immune system

against HIV, understanding inducible CD8+ T cell responses may offer the best hope for providing protective immunity to HIV infection and therefore, are the focus of several vaccine candidates aiming to induce a strong cell-mediated immunity component.

CD8+ T cells play an important role in controlling HIV disease progression. HIV-specific CD8+ T cell responses are temporally correlated with control of setpoint viremia and there is a strong correlation between the expression of certain HLA class I alleles, lack of escape, and non-progression. Despite extensive efforts, the definition of a CD8+ T cell-mediated immune correlate of protection in HIV infection has proven elusive. To better understand the pathogenesis of HIV, it is crucial to elucidate the role of CD8+ T cells in the control of viral replication and disease progression.

The majority of epitope mapping studies and study of HIV-specific CD8+ T cell responses have been characterized primarily using IFNγ Elispot assays, which do not always correlate with control of viral replication or disease progression. IFNγ is primarily thought to be an effector memory (T_{EM}) attribute. Mapping studies have largely ignored central memory (T_{CM}) proliferation responses which are thought to be more important in controlling HIV infection (216, 258, 259). Attributes typical of T_{CM} cells, including proliferation and IL-2 production, appear advantageous in HIV infection (258, 259, 302). Additionally, CD8+ T cell responses with a robust polyfunctional component are strongly linked to better disease outcome (214, 216). However, the specificity of polyfunctional and proliferative CD8+ T cell responses has not been thoroughly investigated. Moreover, the relationship between T_{CM} and polyfunctionality

remains to be determined, particularly as polyfunctional CD8+ T cells are the cells that express the highest levels of IFNγ, typical of effector and T_{EM} cells. The specificity of these memory subsets and connection to polyfunctionality has not been assessed. Previous data from our lab examining CD8+ T cell responses to HIV Env using IFNγ Elispot (T_{EM}) and 6 day CFSE proliferation (T_{CM}) assays revealed substantial differences in the responses to specific epitopes with each assay (310). Suggesting the epitope specificity of these two memory compartments may differ.

This thesis will address these gaps in knowledge and provide a better understanding of the fine specificity of HIV-specific T_{EM} and T_{CM} responses. The quality of CD8+ T cell responses will be evaluated using polyfunctional flow cytometry measuring attributes of both T_{EM} and T_{CM} function in LTNP and HIV+ patients with normal disease progression. As well, the quality and evolution of CD8+ T cell responses over time will be evaluated by examining HIV+ patients who are in the early phase of infection (newly seroconverted) through to the chronic phase. This research will improve our understanding of HIV pathogenesis. The polyfunctional assessment of HIV-specific T_{EM} and T_{CM} responses at various stages of HIV infection is of critical importance to the design of effective vaccines intended to elicit protective cell-mediated immunity or therapeutic vaccine development.

1.7.1. *Global Hypotheses*

We hypothesize that the epitopes recognized by T_{CM} and T_{EM} CD8+ T cells, defined by functional attributes, differ in chronic HIV-1 infection.

Additionally, we hypothesize that polyfunctional and proliferative responses will better correlate with protection in HIV disease progression.

These hypotheses will be tested in the following chapters:

Chapter 3: Memory phenotypes of HIV-specific CD8+ T cell responses are independent of functional activity as defined by cytokine output and proliferative capacity

Chapter 4: Epitope mapping of HIV-specific CD8+ T cell responses reveals distinct specificities defined by function

Chapter 5: Characterization of novel clade A HIV epitopes: measuring polyfunctional responses and proliferative capacity in CD8+ T cells

Chapter 6: Evolution of polyfunctional and proliferative CD8+ T cell responses from early to chronic HIV-1 infection

2. Chapter 2. Materials and Methods

2.1. General Reagents

The methodology used in this thesis was primarily flow cytometry based. We optimized intracellular cytokine staining protocols and used advanced methodology to evaluate and analyse polyfunctional and proliferative CD8+ T cell responses.

2.1.1. *Solutions*

Phosphate-buffered saline (PBS): 48.5g PBS powder: 137.93mM NaCl, 2.67mM KCl, 8.1mM Na_2HPO_4, 1.47mM KH_2PO_4 (Gibco, Invitrogen), dissolved in 1L of ddH_20.

Fluorescence-activated cell sorting wash (*FACS wash):* PBS with 2% fetal bovine serum (FBS) (Gibco, Invitrogen).

10% Paraformaldehyde (PFA): 48ml ddH_20, 2ml 5M NaCl, 5g PFA (Electron Microscopy Sciences). Solution was heated for 1 minute, and then 20ul of 10N NaOH was added to drive PFA into solution. PFA was made fresh weekly.

R10 Cell culture media (R10): Roswell Park Memorial Institute (RPMI) 1640 (HyClone, Thermo Scientific) supplemented with 10% FBS, heat inactivated at 56°C for 1 hour) (Gibco, Invitrogen) and 1% Penicillin/Streptomycin (Gibco, Invitrogen).

Freezing Media (FM): 10% dimethyl sulfoxide (DMSO, tissue culture grade, Sigma) and 90% FBS.

2.2. General Methods

2.2.1. *Study population*

Study participants were all HIV infected and enrolled in a well described longitudinal female sex worker (FSW) cohort based in the Pumwani District of Nairobi, Kenya (Majengo, ML cohort) (311). Written informed consent was obtained from all study participants and ethics review boards from the Universities of Manitoba and Nairobi approved these studies. The ML cohort, which has been recruiting FSW since 1985, includes >4,000 participants, with approximately 600 participants actively resurveyed biannually. HIV prevalence in the cohort in 2006 was approximately 47% (47). Active participants were involved in biannual resurvey visits at our clinic, based the Pumwani district, each woman completes a behavioural interview, was screened for sexually transmitted infections, and provided 21ml of heparinised blood for immunological assays and 7ml of blood in EDTA for CD4 counts and viral loads. HLA typing was performed using a high resolution, sequence-based method (312). The women have full access to free condoms, counselling, and basic medical treatment. As of 2004, this treatment includes antiretroviral therapy (ART) for eligible HIV-positive subjects following UNAIDS guidelines (2).

Participants who were ART naïve with CD4+ counts above 400 cells/μl for over 6 years were classified as long-term non-progressors (LTNP). Participants who did not meet the criteria for LTNP were considered to be treatment naïve normal progressors (NP). These cut offs were established based on time to AIDS which has been demonstrated to be 3.5 years from seroconvertion until AIDS in this cohort (313). Progression to AIDS in North American cohorts is commonly 8-11 years (313).

2.2.2. *Samples*

2.2.2.1. Blood Sample collection and processing

Heparinized whole blood samples were centrifuged at 595xg for 7 minutes using Beckman Coulter Allegra X15R to separate plasma, which was stored at -70°C. Following separation of blood plasma, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll density gradient centrifugation. Whole blood diluted with PBS was layered onto ficoll (Sigma-Aldrich), and then centrifuged at 455xg for 25 minutes with the break off. The PBMC layer was collected and diluted with sterile FACS wash. Cells were mixed then centrifuged at 595xg for 10 minutes. The supernatant was poured off and the cell pellet was re-suspended in R10 media and centrifuged at 595xg for 10 minutes. Supernatant was poured off and the cells were re-suspended in R10 media and counted using trypan blue (Sigma-Aldrich) exclusion method. Samples were used immediately or suspended in FM (10-20x10^6 cells/ml) and aliquoted into cryovials (SARSTEDT) then preserved at -70^{o}C overnight then liquid nitrogen. Frozen PBMCs were thawed in a 37°C water bath, washed twice in R10 media by centrifugation at 595xg for 10 minutes, and rested for 6 hours at 2 x 10^6 cells/ml at 37°C prior to use. Following rest, frozen cells were treated in an identical fashion to fresh PBMCs. Cell counts and viability were then determined by trypan blue staining.

2.2.2.2. HIV Testing and Confirmation

HIV testing was performed on plasma samples, from all participants, using Recombigen (Trinity Biotech) enzyme-linked immunosorbent assay (ELISA). All samples testing positive in the first assay were confirmed by Detect HIV1/2 immunoassay (Adaltis). Only those samples giving positive results in both assays are considered HIV-1 positive.

2.2.2.3. CD4+ T cell enumeration

CD4+ T cell counts were assessed for all HIV-infected participants in the cohort. Whole blood collected in EDTA tubes was assessed using the Tritest CD3/CD4/CD8 flow cytometry assay (BD Biosciences). To calculate CD4+ T cell count, lymphocyte counts were multiplied by the percentage of CD4+ T cells.

2.2.2.4. Plasma viral load determination

Plasma viral loads were determined for samples when possible. HIV RNA in Ethylenediaminetetraacetic acid (EDTA) plasma was extracted and quantified using the automated Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (Roche Diagnostics). The lower limit of detection of this assay is 40 HIV RNA copies/ml with a dynamic range of: 40-10^7 HIV RNA copies/ml.

2.2.2.5. HLA Typing

Genomic DNA was isolated from PBMCs using a QIAamp DNA Mini Kit (QIAGEN). HLA genes were amplified using PCR and purified using Amicon Microco-PCR Centrifugal filter device (MILLIPORE). The purified PCR products were sequenced with gene-specific primers using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems). The sequence was analyzed with ABI3100 Genetic Analyser (Applied Biosystems). Sequence outputs were genotyped with CodonExpress software developed based on a taxonomy-based sequence analysis (TBSA)(314).

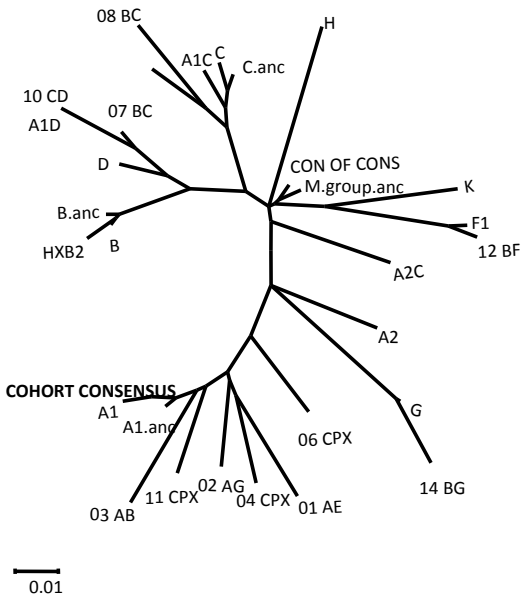
2.2.3. *Stimulations*

2.2.3.1. HIV gag Consensus sequence

To determine the appropriate HIV clade for antigen stimulation, Proviral DNA was isolated from HIV-1-positive women from the Majengo, ML cohort. Nested PCR amplification was used to amplify Gag genes. PCR amplification was confirmed using 1% agarose gel electrophoresis. PCR products were purified using the Multiscreen$_{HTS}$ PCR plate (Millipore Corp.). BigDye Terminator v3.1 was used to sequence Gag genes with specific primers. The sequencing products were purified by ethanol-sodium acetate precipitation. Purified sequencing products were analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were assembled and edited with Sequencher 4.5 (Genecodes Corp.). Samples with heterogeneous quasispecies sequence were gel purified and cloned using TOPO TA cloning kit (Invitrogen). Multiple clones were sequenced as described above.

Quasispecies sequences of the p24 protein were phylogenetically analysis with MEGA v3.1. (Molecular Evolutionary Genetics Analysis). A consensus sequence for the cohort was constructed and aligned using Clustal W, along with reference p24 sequences obtained from the Los Alamos HIV database. Phylogenetic trees were constructed using neighbor-joining algorithms with bootstrap testing of 1,000 replicates. Clade A1 is the predominant circulating clade in this study population(12, 35, 36) and the Clade A cohort consensus varied only slightly from reference clade A1 ancestral (Figure 2.1).

A



B

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PIVQNAQGQMVHQSLSPRTLNAWVKVIEEKAFSPEVIPMFSALSEGATPQDLNMMLNIVGGHQAAMQMLKDTINEEAA
............A.............................................................

EWDRLHPVHAGPIPPGQMREPRGSDIAGTTSTLQEQIGWMTGNPPIPVGDIYKRWIILGLNKIVRMYSPVSILDIRQG
.....................................................................K..

PKEPFRDYVDRFFKTLRAEQATQEVKNWMTETLLVQNANPDCKSILRALGPGATLEEMMTACQGVGGPGHKARVL
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Figure 2.1. Clade A1 is the predominant clade in the cohort. A) Phylogenetic tree constructed using neighbor-joining algorithms with bootstrap testing of 1,000 replicates, indicates the cohort consensus is closest to clade A1 ancestral. B) Comparison of clade A1 ancestral (top) with cohort consensus indicates variation in only 2 amino acids.

2.2.3.2. Antigen used for stimulation

All HIV peptides used were immunological grade >75% HPLC (Sigma-Genosys and United Biochemical Research), each confirmed by mass spectrometry for quality control. An overlapping peptide pool (9 mers overlapping by 8 amino acids) derived from the HIV-1 p24 clade A1 ancestral sequence was used. All peptides were resuspended at 4mg/ml in 100% DMSO and subsequently diluted to each relevant 10X concentration in R10. The final concentration of DMSO in assays never exceeded 1%. Details of specific peptides and peptide pools can be found in chapter specific methods.

Cytomegalovirus, Epstein-Barr virus and Influenza virus peptides (CEF, 32 peptides/pool, 2μg/ml/peptide; HPLC purity >90%, AnaSpec) and *Staphylococcus aureus* enterotoxin B (SEB, 0.1 μg/ml; Sigma-Aldrich) were used as positive controls in all immunological assays. The pool consisted of 32 optimal 9mer epitopes from the above-mentioned pathogens, and covered the majority of HLA alleles in a given population.

2.2.3.3. Increasing detection of functional CD8+ T Cell responses

To define the optimal conditions to measure multiple immunological functions by flow cytometry, fresh and rested PBMC (section 2.2.2.1) were stimulated with CEF peptides, SEB and duplicate negative controls, for 4, 6, 8, 12, 14, 16 and 18 hours and expression levels of CD107a, IFNγ, IL-2, MIP-1β and TNFα was determined (Figure 2.2 and 2.3). These data show that for most parameters the duration of stimulation did not impact expression levels, however for CD107a and IFNγ longer incubation (12-14 hours) appeared to be optimal. Based on these results we designed the CD8+ T cell experiments, conducted in this thesis, at a stimulation time of 14 hours in order to optimize the detection of all cytokines and chemokines.

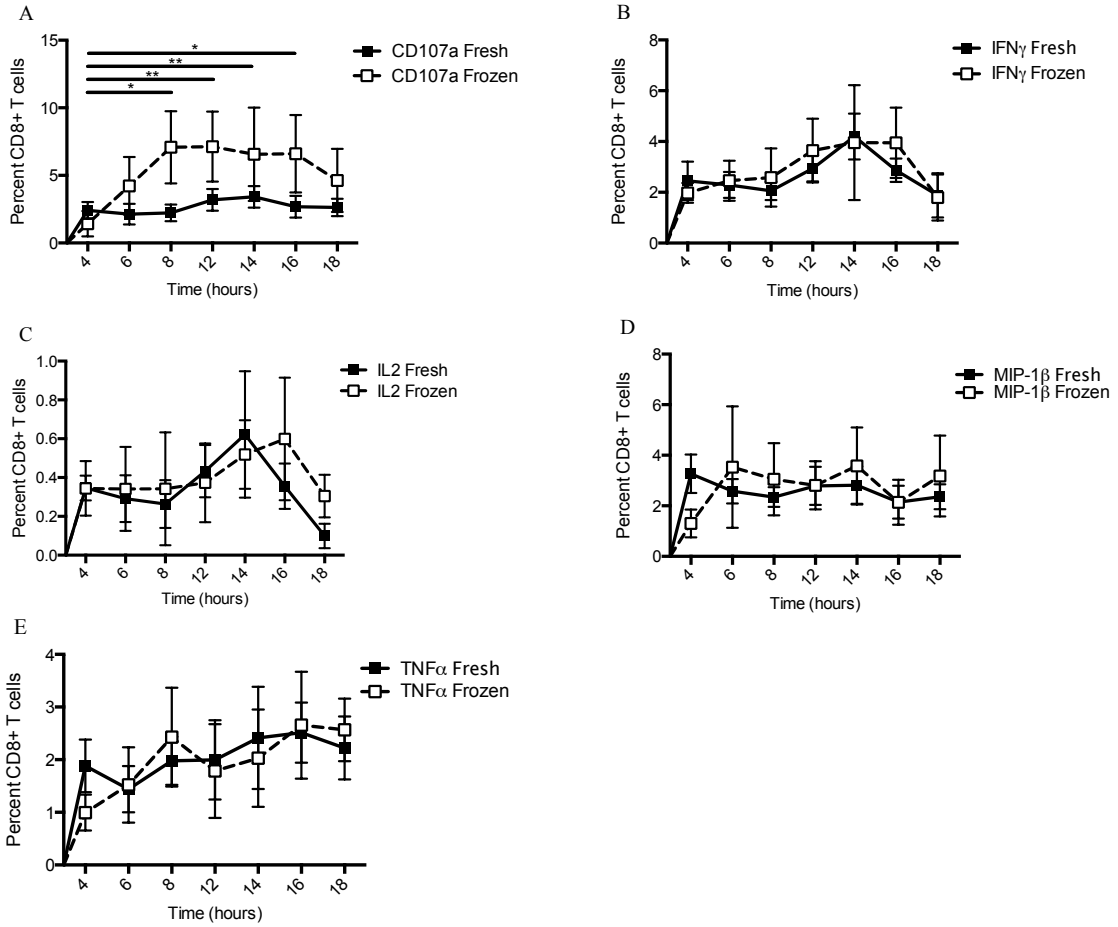

Figure 2.2. Expression levels of multiple CD8+ T cell functions overtime after stimulation with SEB. A) CD107a, B) IFNγ, C) IL2, D) MIP-1β and E) TNFα Solid and open squares represent fresh and frozen samples respectively. Error bars indicated SEM between individuals (n=5). * indicates significant difference CD107a Two-way RM ANOVA (p=0.0008) post test Holm-Sidak's multiple comparisons pair wise tests (4 vs 8 p=0.0163, 4 vs 12 p=0.0023, 4 vs 14 p=0.0046 and 4 vs 16 p=0.0163). IFNγ Two-way RM ANOVA (p=0.0227)

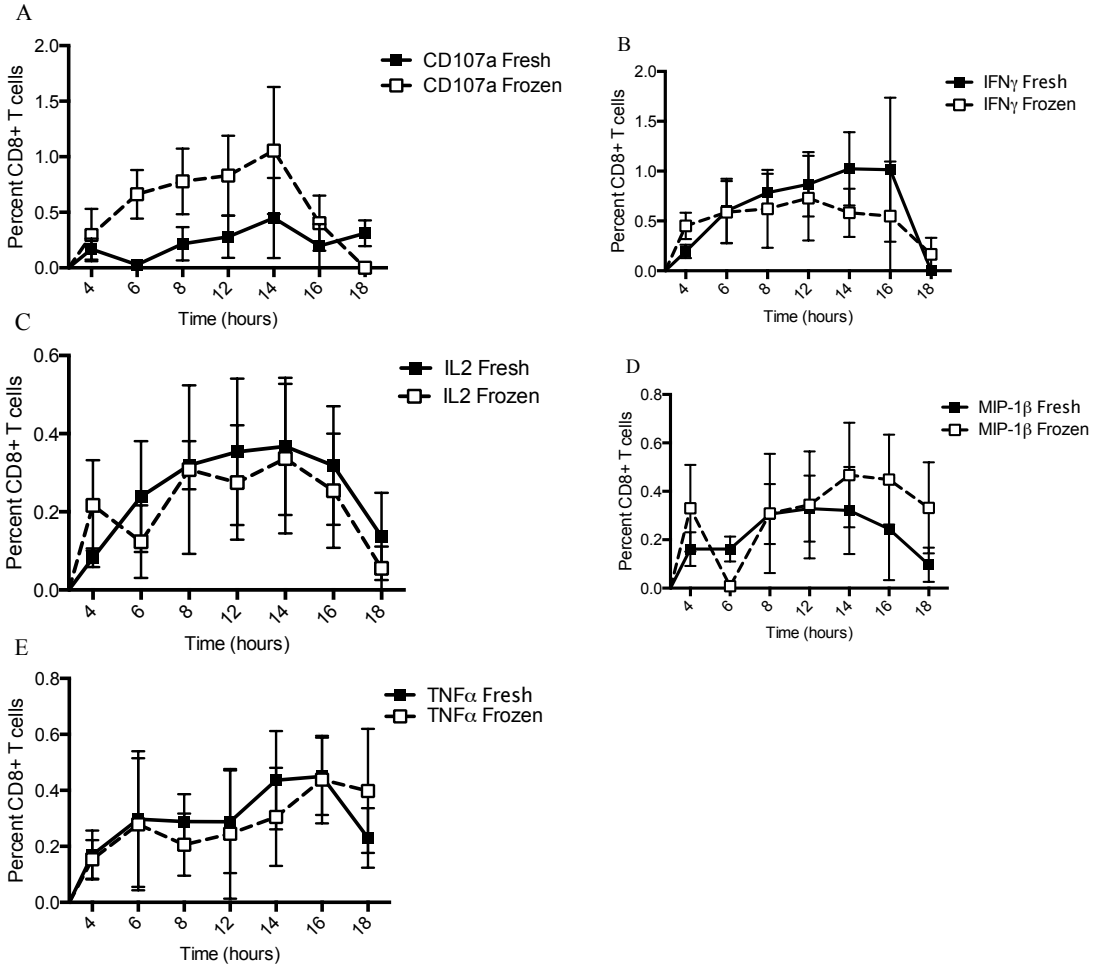

Figure 2.3: Expression levels of multiple CD8+ T cell functions overtime after stimulation with CEF. A) CD107a, B) IFNγ, C) IL-2, D) MIP-1β and E) TNFα. Solid and open squares represent fresh and frozen samples respectively. Error bars indicate variation between individuals. No significant differences were found.

2.2.3.4. Stimulation Protocol

Fresh or rested PBMC were stimulated with peptides or peptide pools (sequence details in Chapters), CEF peptides, SEB or media control overnight for 14 hours. PBMCs were aliquoted into 5ml snap-capped FACS tubes (BD Falcon) and adjusted to 1 x 10^6 cells/ml in 600 ml of complete R10 media.

For intracellular cytokine staining (ICS) a pre-titrated volume (5µl) of Anti-CD107a-PeCy5 was added to PBMCs prior to stimulation. After stimulation cells were incubated at 37^{o}C for 1 hour, followed by the addition of monensin (Golgistop, 1µl/ml; BD Bioscences) and Brefeldin A (Golgiplug, 1µl/ml; BD Biosciences) to prevent secretion, and a further 13 hours incubation at 37^{o}C. For proliferation assays, cells were loaded with carboxyfluoresein diacetate succunimidyl ester dye (CFSE, Molecular Probe, Invitrogen) prior to stimulation than cells were incubated for 6 days.

2.2.4. *Flow Cytometry*

2.2.4.1. Surface staining

Immediately following stimulation, PBMC were washed with PBS containing 2% FCS, and stained for surface marker expression. Stimulated PMBCs were transferred into 96 well v-bottom plates (VWR) and pelleted by centrifugation at 595xg for 5 minutes. The supernatant was aspirated off and cells were re-suspended by vortexing. The cells were then washed with 150µl of FACS wash, centrifuged at 595xg for 5 minutes, followed by removal of supernatant and cells were again re-suspended. A master mix of surface antibodies and LIVE/DEAD stain were prepared according to pre-determined optimal

volumes and added to cells. Cells were incubated for 20 minutes in the dark at room temperature. Cells were than washed with FACS wash and either stained for ICS (section 2.2.4.2) of fixed with 1% PFA. PMBC were transferred back into 5ml FACS tubes for data acquisition.

The LIVE/DEAD viability dye penetrates damaged cell membranes and reacts with free amine groups in the cytoplasm. A bright fluorescent, and stable, product remains in the cytoplasm. Although surface amine groups will also react with the dye, it contributes significantly less fluorescence due to the much lower amount. The contrast between the high and low fluorescence allows the distinction between live and dead cells (315). Lyophilized LIVE/DEAD violet dye (Molecular Probe, Invitrogen) was reconstituted in DMSO at a concentration of 0.5µg/µl, aliquoted and stored at -20°C. Preceding staining, stock LIVE/DEAD violet dye was diluted to 0.005ug/ml (1µl of LIVE/DEAD violet in 99µl PBS). Diluted LIVE/DEAD violet dye was added to the surface staining master mix at a volume of 6.25µl/test. Cells were incubated than washed as described above and either stained for ICS (section 2.2.4.2) or fixed with 1% PFA. The excitation and emission peaks of LIVE/DEAD violet dye are 416nm and 451nm, respectively, resembling those of Pacific Blue. Thus, LIVE/DEAD violet dye was detected by flow cytometry using the channel normally reserved for Pacific Blue detection on the violet (450nm) laser.

2.2.4.2. Intracellular cytokine staining

Following surface staining, cells were washed with 150µl of FACS wash then fixed and permeabilized with 50µl using Cytofix/Cytoperm solution (Fixation/Permeabilization kit, BD PharMingen) for 20 minutes at room temperature. The cells were then washed with 150ml of Perm/Wash solution (Fixation/Permeabilization kit, BD PharMingen) and centrifuged at 595xg for 10 minutes. A master mix of intracellular antibodies (chemokines and cytokines) were prepared according to pre-determined optimal volumes and added to cells. The cells were incubated for 30 minutes at 4^{o}C in the dark. The cells were then washed with 150µl of Perm/Wash and re-suspended in FACS wash, then transferred to 5ml FACS tubes for data acquisition.

2.2.4.3. CFSE dilution proliferation assay

Proliferation assays were performed by CFSE (carboxyfluoresein diacetate succunimidyl ester) dye dilution. Lyophilized CellTrace CFSE (Molecular Probes, Invitrogen) was reconstituted in DMSO (Sigma-Aldrich) at a concentration of 10mM and stored at -20^{o}C. CFSE diffuses into cells and reacts with intracellular amines, forming fluorescent conjugates that can be detected by flow cytometry. As cells divide the fluorescence intensity of progeny cells decreases, allow tracing of proliferating cells by monitoring loss of fluorescent intensity (316). Immediately preceding staining, 10mM of CFSE was diluted in 1ml of PBS (0.5-2µl CFSE/ml PBS). PBS was used twice to wash the cells and remove FBS from media. The cells were then resuspended in PBS at a concentration of 1x10^{7} cells/ml. Diluted CFSE stain was added at equal volume to the cells. Cells were than incubated for 8 minutes at 37°C in the dark. To quench the staining reaction,

subsequent to incubation, an equal volume of cold FBS was added to the cells for one minute. FACS wash was used twice to wash the cells and R10 media was used to resuspend the cells for stimulations. Six days after stimulation, cells were washed with FACS wash and stained for surface and intracellular markers, as described above. Data were acquired by flow cytometry. CFSE has excitation and emission peaks at 492nm and 517nm, respectively. The excitation and emission of CFSE resembles those of fluorescein isothiocyanate (FITC) and thus CFSE was detected on the blue (488nm) laser using the flow cytometry channel typically used for FITC detection.

2.2.4.4. Flow cytometry Compensations

Proper flow cytometry compensation removes fluorescent spill over so that fluorescence values for a parameter reflect only the fluorescence of the antibody or dye of interest. A computer-derived algorithm is applied to the fluorescence intensity measured of each dye individually, to remove spectral overlap between channels. Compensations were performed for each antibody using nanoparticles that bind any rat or mouse κ light-chain bearing antibodies, known as anti-rat or anti-mouse CompBeads (BD Biosciences), respectively. Anti-mouse or anti-rat CompBeads plus negative control CompBeads (naked nanoparticles with no capacity to bind) were mixed then diluted in PBS (one drop of beads per 300µl of PBS). A single compensation FACS tube was set up for each antibody in a multicolour experiment. A volume of 1µl of the antibody was added to 100µl of diluted beads. Mouse-anti-human and rat-anti-human antibodies were mixed with anti-mouse and anti-rat CompBeads, respectively. To allow beads to bind antibody the tubes were incubated for 20 minutes at room temperature in the dark. Beads were fixed with 100µl of 1% PFA.

Aimine reactive beads (Bangs laboratories Inc., 4.27mm) were used for LIVE/DEAD violet dye compensation control. The aimine beads were used at a concentration of $40x10^{-6}$ beads/ml. A volume of 6.25µl LIVE/DEAD violet dye was added to 40µl of amine beads, followed by the addition of 60µl of FACS wash. Dyed beads were incubated for 20 minutes at room temperature in the dark to allow beads to bind free amines. Beads were fixed with 100µl of 1% PFA.

Beads could not be used to prepare compensation tubes for CFSE. Thus, cells were single-stained with CFSE to evaluate spectral overlap. Single-stained CFSE cell, compensation tubes were prepared with other cells prior to stimulations, and acquired six days later.

2.2.4.5. Data acquisition and analysis

Cells were analyzed on a LSRII flow cytometer (BD Biosciences). Between 30,000 and 100,000 events were collected within the lymphocyte gate per sample. Data analyses were performed using FlowJo version 9.2 (TreeStar). Boolean gates were applied to the overnight functions and the polyfunctionality of each response was assessed using SPICE (courtesy of Mario Roederer, Vaccine Research Center, NIAID, NIH).

2.2.4.6. Statistical analysis

CD8+ T cell responses were considered positive for a given parameter if they were ≥2-fold higher than the mean of their respective negative controls. Data are reported after background subtraction. Polyfunctional responses were also background subtracted and a lower threshold corresponding to the 90^{th} percentile of distribution of negative values was built for each cytokine pattern and values below this threshold were set to 0. The change in relative polyfunctionality was analysed via permutation comparisons. Permutation tests are non-parametric tests that use random shuffles of the data to get the correct distribution of a test statistic under a null hypothesis. Ten thousand permutation comparisons were done to increase accuracy of the p value.

3. Chapter 3. Memory phenotypes of HIV-specific CD8+ T cell responses are independent of functional activity as defined by cytokine output and proliferative capacity.

3.1. Rationale

Virus-specific CD8+ T cells are phenotypically and functionally heterogeneous, and a better understanding of specific subsets critical for protective immunity in HIV infection are needed for rational vaccine design and immune monitoring. With the widespread use of polychromatic multi-colour flow cytometry, an increasing number of functional parameters of T cell responses can be assessed simultaneously. These responses comprise a number of functional parameters including the expression of cytokines/chemokines and cytotoxic markers. A complementary approach has been the use of surrogate surface phenotypic markers to help define the functional capacity of antiviral T cells (discussed in section 1.5.7). A common approach measures the expression of the homing marker CCR7, the T cell differentiation marker CD45RA and the co-stimulatory molecule CD27. Typically, CD8+ T cells expressing a CCR7+CD27+CD45RA+ phenotype are considered to be functionally naïve, while CCR7-CD27-CD45RA+/- cells are functionally active, transiently produce IFNγ and represent terminally differentiated (T_{TD}) and T_{EM} cell subsets respectively (178). The phenotype CCR7+CD27+CD45RA- is thought to define a T_{CM} subset, which has more long-term functional responses including IL-2 secretion and proliferative capacity. A transitional memory phenotype (CCR7-CD27+CD45RA-) can also be defined using this model(178).

Although the use of surface markers to directly define the immunological memory of specific T cells *ex vivo* confers practical advantages, accumulating evidence suggests that these memory markers may not necessarily correspond with the functional capacities they are suggested to represent (152, 179, 180). For example, CCR7-expressing CD8+ T cells, normally considered naïve or T_{CM}, have also been described as having immediate effector functions (317, 318). Similarly, CD8+ T cells with proliferative capacity have been found in both CCR7+ and CCR7- subsets, although CCR7- cells are thought to be primarily of the effector phenotype (125). CD45RA kinetics studies reveal that the expression of CD45RA has no correlation to proliferation (319). This suggests that for memory CD8+ T cells, there are no definitive memory markers that can be used to conclusively identify functional subsets (152).

Together, these data imply that the surface markers routinely used to define memory T cells do not necessarily predict the *ex vivo* function of those cells. However, this has not been well defined for the wide range of polyfunctional CD8+ T cell responses that are now routinely measured. To date, most studies have only used IFNγ, IL-2 expression or proliferative capacity to monitor memory T cell functionality, however polyfunctionality, the concurrent expression of more than one cytokine/chemokine, could play an important role in anti-HIV immunity. HIV infected individuals with slow disease progression (LTNP, EC and VC) have been shown to have increased numbers of polyfunctional HIV-specific CD8+ T cells, defined by the concurrent expression of the cytokines IFNγ, IL-2, and TNFα, MIP-1β and CD107a (216, 320, 321). These slow progressors also maintain stronger HIV-specific CD8+ T cell proliferative responses compared to controls (258,

259, 273, 289). This is the first study to look at the association between function and the dominant paradigm for memory markers in the context of multiparametric cytokine expression, and polyfunctionality.

3.2. Hypothesis

We hypothesised that the surface phenotypes defined by CCR7, CD45RA, and CD27 will not accurately identify polyfunctional HIV-specific CD8+ T cells and HIV-specific proliferative capacity.

3.3. Objectives

To examine the relationship of cell surface phenotype and cell function, i.e. polyfunctionality and proliferation.

3.4. Methods

3.4.1. *Subjects*

HIV-infected participants (n=24) were recruited from the Majengo ML cohort (Table 3.1). Ten LTNP were followed for a mean and median of 11.98 years and 14.31 years respectively (range 6.41-17.43 years). The remaining ART naïve participants were normal progressors (NP, n=9) and five subjects were on ARTs. Additionally, five healthy Kenyan controls were included in the study.

Table 3.1. Clinical and demographic data for subjects in this study

Study no.	Status	Age	Years HIV+	CD4 count
890	LTNP	53	16.8	570
1211	LTNP	40	17.43	437
1250	LTNP	49	6.41	468
1287	LTNP	36	6.53	547
1424	LTNP	43	15.55	539
1625	LTNP	43	14.37	418
1647	LTNP	41	13.55	731
1649	LTNP	40	14.31	447
1654	LTNP	36	14.31	433
1725	LTNP	41	10.79	452
1731	ART	42	13.67	228
1771	NP	42	12.74	186
1848	NP	36	10.4	184
1917	ART	37	7.26	456
1932	ART	36	6.42	407
1947	ART	38	6.17	359
1971	ART	45	5.31	168
1974	NP	44	5.29	235
2166	NP	38	4.67	233
2274	NP	37	1.97	921
2522	NP	27	0.74	408
2522	NP	27	0.74	408
2531	NP	48	0.73	256
2560	NP	42	0.7	446
2630	NP	33	0	307
SW7201	HIV neg	41	0	na
SW7202	HIV neg	33	0	na
SW7203	HIV neg	45	0	na
SW7204	HIV neg	29	0	na
SW7209	HIV neg	27	0	na

SW indicates individuals from the Sex Worker Outreach Program in Nairobi, Kenya
na = data not available

3.4.2. *In vitro stimulation*

A peptide library (9 mers overlapping by 8 amino acids) derived from the HIV-1 p24 clade A1 ancestral sequence (discussed in section 2.2.3.1), were pooled in a matrix format using *Deconvolute This!* 1.0 (322) (courtesy of Mario Roederer, Vaccine Research Center, NIAID, NIH). Each peptide was represented twice, resulting in 16 pools with approximately 30 peptides per pool. Epitope-specific mapping is discussed in the next chapter; epitope specificity is not relevant to the data discussed in this section. Peptide pools were used at 2μg/ml/peptide, and stimulations were accompanied by two positive controls (259, 260). CEF peptides and SEB, and duplicate negative controls consisting of media alone. For ICS, cells were incubated for 14 hours as per general stimulation methods (section 2.2.3), while for proliferation assays, cells were incubated for 6 days.

3.4.3. *Flow cytometry*

Immediately following stimulation, PBMCs were washed with PBS containing 2% FCS and stained using the panels described below (Table 3.2 and 4.3) following general surface staining (section 2.2.4.1, these experiments were conducted prior to the inclusion of LIVE/DEAD to our standard protocol), intracellular cytokine staining (section 2.2.4.2) and CFSE dilution assay protocols. Stained cells were immediately analyzed on a LSRII flow cytometer (BD Biosciences) as per general methods section 2.2.4.6.

Table 3.2: Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell memory phenotypes and polyfunctionality

Fluorochrome	Marker	Source	Volume used	Surface/ICS
FITC	IFNγ	BD Biosciences	3µl	ICS
PE	MIP-1β	BD Biosciences	3µl	ICS
PE-Cy5	CD107a	BD Biosciences	5µl	ICS
PE-Cy7	CCR7	BD Biosciences	3µl	Surface
ECD	CD45RA	Beckman Coulter	3µl	Surface
APC	IL-2	BD Biosciences	3µl	ICS
AmCyan	CD3	BD Biosciences	1µl	Surface
AlexaFluor 700	CD27	eBiosciences	3µl	Surface
Pacific Blue	TNFα	eBiosciences	3µl	ICS
APC-Cy7	CD8	BD Biosciences	1µl	Surface

Table 3.3: Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell memory phenotypes and proliferation

Fluorochrome	Marker	Volume used	Source
Blue laser	CFSE	1µl/ml	Invitrogen
PE-Cy7	CCR7	3µl	BD Biosciences
ECD	CD45RA	3µl	Beckman Coulter
AmCyan	CD3	1µl	BD Biosciences
AlexaFluor 700	CD27	3µl	eBiosciences
APC-Cy7	CD8	1µl	BD Biosciences

3.4.4. *Statistical analysis*

Statistical analyses were performed using Graph Pad Prism 5.0 and SPICE 5.1. Phenotype comparisons were determined using Wilcoxon matched-pairs and one-way ANOVA Kruskal-Wallis, Post test Dunn's multiple caparisons were also performed. Functional correlations were performed using Spearman's rank correlation. Due to the large number of correlations involved, we highlighted only the most significant correlations (significance $p>0.001$).

3.5. Results

3.5.1. *Phenotype of HIV- and CEF-specific CD8+ T cell responses:*

A number of memory T cell phenotypes have been described, with the dominant paradigm based on CCR7, CD45RA, and CD27 expression (178, 179, 323). These markers can be used to define eight phenotypes including naïve, central memory, effector memory, transitional memory, and terminally differentiated effector subsets (Table 3.4) as well as undefined combinations of these markers that have not been examined to date. Representative flow cytometry gating shown in figure 3.1 and participant data is shown in table 3.1

Table 3.4. Categories of CD8+ T cells on the basis of surface phenotypic marker expression.

Phenotypic category[a]	CCR7	CD27	CD45RA	HIV+	HIV-
Naïve	+	+	+	15.6%	13.2%
Undefined #1[b]	-	+	+		
Undefined #2	+	-	+		10.4%
Central memory (T_{CM})	+	+	-		
Terminally differentiated (T_{TD})	-	-	+	23.1%	
Transitional memory (T_{TM})	-	+	-	13.8%	
Undefined #3	+	-	-		18.5%
Effector memory (T_{EM})	-	-	-	29.5%	34.9%

[a] (177, 178, 324-326)

[b] No phenotypic category has been ascribed to these combinations of markers

All eight phenotypes were seen in unstimulated CD8+ T cells from HIV infected and uninfected individuals. HIV infected individuals the majority of cells (29.5%) were contained within the T_{EM} category (CCR7-CD27-CD45RA-, Figure 3.2), these were followed, by CCR7-CD27-CD45RA+ ($T_{TD,}$ 23.1%), CCR7+CD27+CD45RA+ (naïve, 15.6%), and CCR7-CD27+CD45RA- ($T_{TM,}$ 13.8%) cells. The remaining four phenotypic categories were less common (<10% of total cells). The predominating phenotypes of uninfected Kenyan participants were T_{EM} (34.9%) followed by Undefined #3 (18.5%, CCR7+CD27-CD45RA-), naïve (13.2%) and Undefined #2 (10.4%, CCR7+CD27-CD45RA+). The remaining four phenotypic categories were less common (<10% of cells). Higher proportions of the T_{TD} phenotype were seen in HIV infected subjects compared to uninfected controls (p=0.0209, Wilcoxon matched-pairs); while there were lower levels of CCR7+CD27-CD45RA- phenotype in HIV infected subjects (p=0.0141, Wilcoxon matched-pairs, Undefined #3).

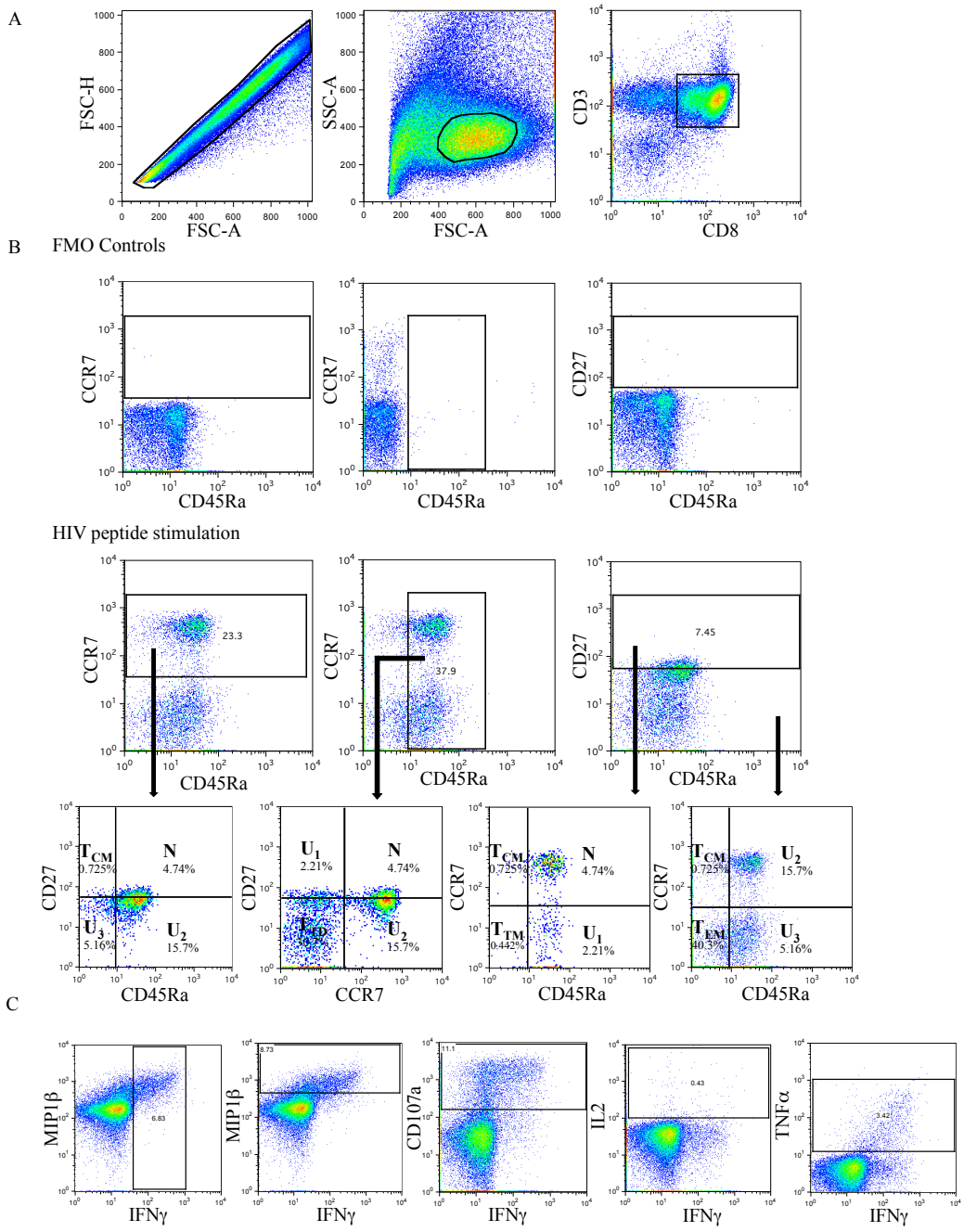

Figure 3.1. Representative flow cytometry data for detection of polyfunctional CD8+ T cell responses and the definition of CD8+ T cell memory phenotypic categories based on surface marker expression. PBMCs were stimulated for 14 hours with HIV p24 peptides. A) Forward scatter area (FSC-A) versus forward scatter height (FSC-H) was used to eliminate doublets. Side scatter area (SSC-A) versus FSC-A was used to identify lymphocytes. B) After gating on CD3+ CD8+ cells, further gates were made for CCR7, CD45RA and CD27 based on florescence minus one staining. Boolean gating was used to identify dual and triple positive phenotypes. C) Boolean surface gates were then applied to cells responding with each functional parameter including IFNγ, MIP-1β, CD107a, TNFα, IL-2 and proliferation.

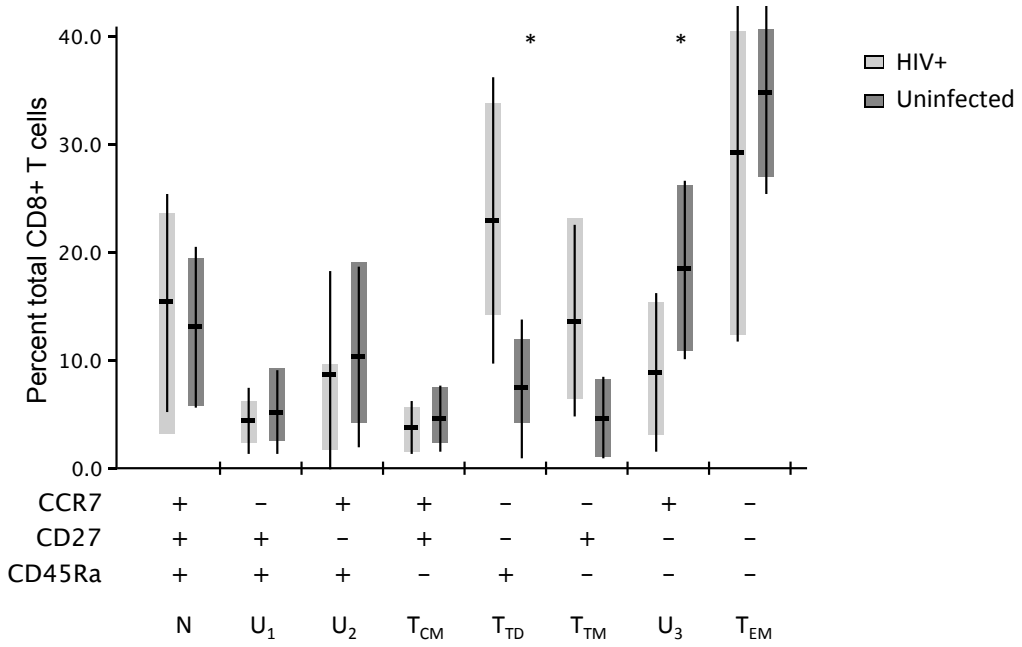

Figure 3.2. **Expression of eight memory phenotypic categories on unstimulated CD8+ T cells from HIV infected and uninfected subjects**. Higher proportions of the T_{TD} phenotype were seen in HIV infected subjects (n=24) compared to uninfected controls (n=5) (p=0.0209, Wilcoxon matched-pairs), while there were lower levels of CCR7+CD27-CD45RA- phenotype in HIV infected subjects (p=0.0141, Wilcoxon matched-pairs, Undefined #3).

HIV p24 and CEF-specific responses were then measured in chronically HIV infected subjects (n=24) using multiple readouts including IFNγ, MIP-1β, TNFα, CD107a, and IL-2. Published previously in Richmond *et. al.* 2012, HIV and CEF-specific responses were detected in the majority of HIV+ subjects (327). Responding cells were assigned to the eight phenotypes defined in Table 3.4 to determine the memory phenotypes of HIV and CEF-specific CD8+ T cells. Overall, HIV and CEF-specific antigen responding CD8+ T cells were evenly distributed between 5 main memory subsets of the 8 total; CCR7+CD27+CD45RA+ (naïve), CCR7-CD27-CD45RA+ (T_{TD}), and CCR7-CD27-CD45RA- (T_{EM}), including a substantial number of responses in two novel phenotypes CCR7+CD27-CD45RA+ (Undefined #2), CCR7+CD27-CD45RA- (Undefined #3), (Range 14.4 - 20.5%, Figure 3.3a) while the remaining three categories CCR7-CD27+CD45RA+ (Undefined #1), CCR7+CD27+CD45RA- (T_{CM}) and CCR7-CD27+CD45RA- (T_{TM}) were observed less frequently (<10%). We then compared the distributions of the various phenotypes between HIV specific and CEF-specific CD8+ T cells. HIV-specific CD8+ T cells were more likely to be CCR7+CD27-CD45RA+ (Undefined #2) compared to CEF-specific cells (p=0.0012, mean 17.27% versus 14.68%, respectively, Wilcoxon matched pairs). These data are in agreement with previous studies that show that different chronic viral infections induce CD8+ T cells with distinct memory phenotypes and functionality (177, 303).

To more clearly understand the phenotypic differences observed between HIV- and CEF-specific CD8+ T cells, we compared the expression of each surface marker individually on responding cells (Figure 3.3b). Our data show that CD27 and CCR7 are relatively equally distributed on HIV- and CEF-specific CD8+ T cells. Both HIV- and CEF-specific cells are typically CD27 negative (67.17 and 67.09%, respectively). There was a modest increase in expression of CD45RA on HIV-specific CD8+ cells compared to CEF-specific CD8+ T cells (54.65 and 50.96%, respectively, p=0.008 Wilcoxon matched-pairs). These data show that depending on the phenotype, CD8+ T cells responding to CEF- and HIV-antigens may have unique memory phenotypes. However, taken together, both categorical and individual marker data show that there is a relatively homogeneous distribution of virus specific T cell responses among all the different phenotypic categories of T cells as defined by CCR7, CD27 and CD45RA, including two previously undefined phenotypes.

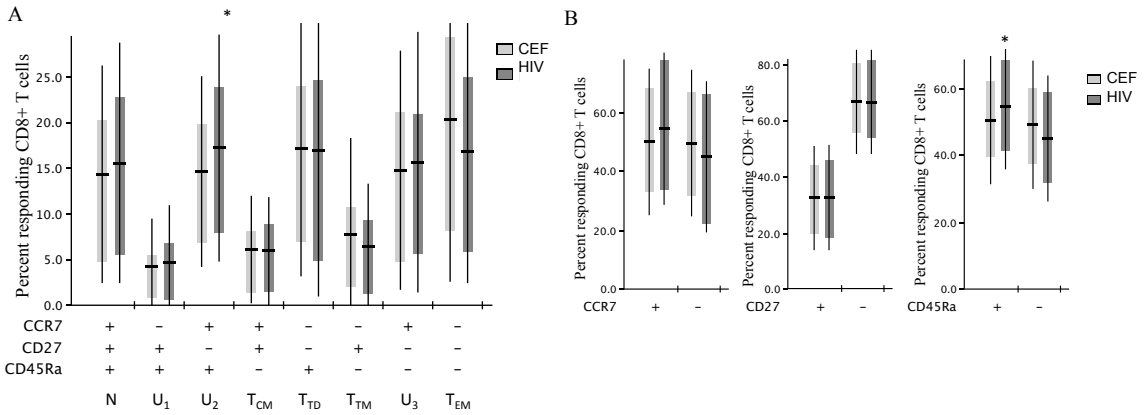

Figure 3.3. Expression of eight memory phenotypic categories on responding CD8+ T cells from HIV-infected subjects. A) Comparison of all subjects' CEF-specific (light gray bars) and HIV-specific (dark gray bars), positive for any single or combination of cytokine/chemokine function. HIV-specific CD8+ T cells were more likely to be CCR7+CD27-CD45RA+ compared to CEF-specific cells (p=0.0012). B) Expression of individual memory surface markers CCR7, CD27 and CD45RA on CD8+ T cells from HIV-infected subjects, comparison of all subjects' CEF-specific (light gray bars) and HIV-specific (dark gray bars) cytokine/chemokine responses. HIV-specific CD8+ T cells were more likely to be CD45RA+ compared to CEF-specific cells (p=0.008). Asterisk indicates statistical significance ($p<0.05$ Wilcoxon matched pairs).

3.5.2. *Antigen-specific proliferating CD8+ T cells are of diverse memory phenotypes*

Proliferative capacity is retained in HIV infected LTNPs and is an attribute of CD8+ T cell responses thought to protect against HIV disease progression (273, 289). While proliferation is a function typically thought to be a property of T_{CM} cells (258, 260, 328), we sought to determine the memory phenotype of proliferating cells as defined by CCR7, CD27 and CD45RA expression in response to HIV-specific antigen stimulation. All responding cells from six-day CFSE labeled stimulation assays were assigned to the eight categories of memory phenotypes defined above (Table 3.1). Interestingly, our data suggests that the majority of proliferating HIV and CEF-specific CD8+ T cells have what can be defined as an effector memory phenotype, CCR7-CD27-CD45RA- (33.8 and 35.4%, respectively, T_{EM}, Figure 3.4). The remainder of responding cells were CCR7+CD27-CD45RA- (22.5 and 21.4%, respectively; Undefined #3) followed CCR7+CD27-CD45RA+ (15.6 and 12.5%, respectively; Undefined #2). The remaining five categories were observed less frequently amongst responding cells (<10%). These data clearly indicate that no single memory phenotype defined by surface marker expression (i.e. T_{CM}) can be used to charcterize proliferative capacity.

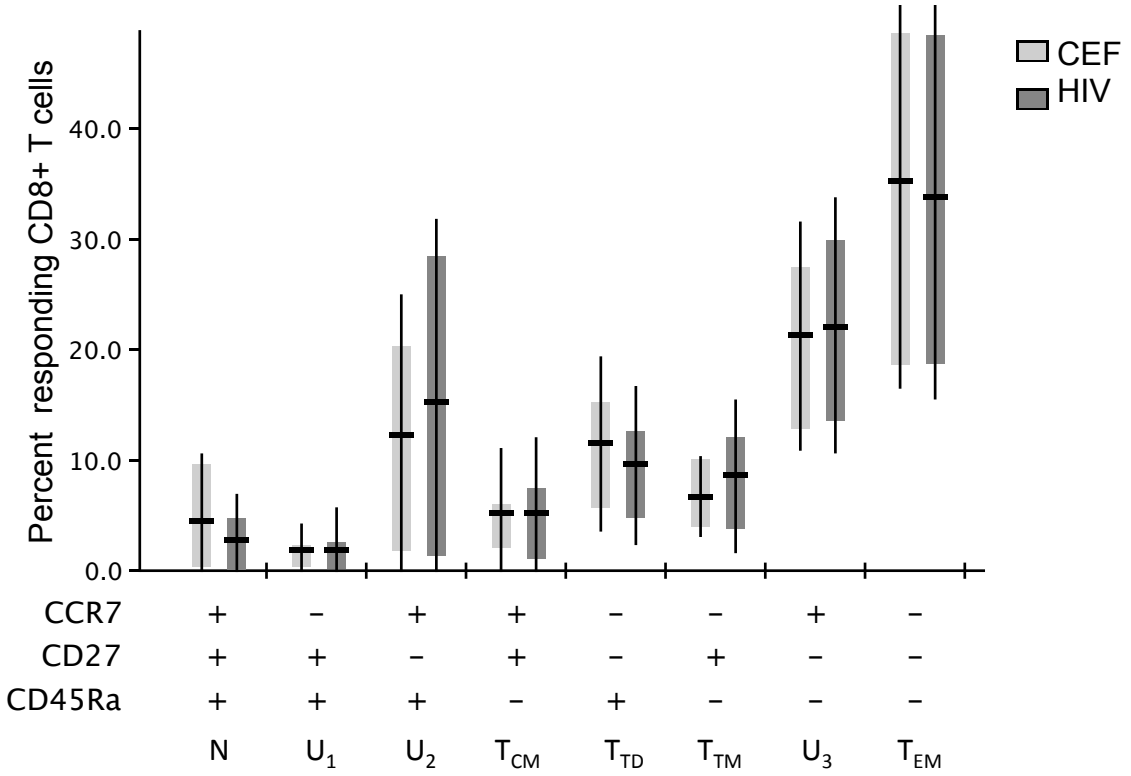

Figure 3.4. **Expression of eight memory phenotypic categories on proliferating CD8+ T cells from HIV infected subjects.** Comparison of all subjects' CEF-specific (light gray bars) and HIV-specific (dark gray bars) day 6 proliferative responses. No significant differences between the two stimulations were observed in the longer assay.

One caveat of our study is that all of these assays were done following *in vitro* stimulation, which could impact the surface phenotypes of antiviral T cell populations. To determine these *in vitro* effects, we compared surface marker expression without stimulation overnight and after 6 days in culture in HIV infected and uninfected participants. An assessment of individual markers showed that CCR7 and CD45RA were significantly higher ($p=0.0001$ and $p=0.0002$ respectively, Wilcoxon matched-pairs) while CD27 was significantly lower ($p=0.0231$, Wilcoxon matched-pairs), in day 6 versus day 1 cells in HIV+ subjects (Figure 3.5). There were no significant differences seen in HIV uninfected subjects suggesting that the instability of these surfaces markers is HIV-dependent. The data suggest that both of CCR7 and CD45RA are re-expressed when removed from constant stimulation in HIV infected individuals. This is in agreement with previous reports which indicate that both CD45RA and CCR7 can be re-expressed depending on stimulation conditions (317, 319, 329). In addition, the data suggest that CD27 expression is lost in the absence of stimulation in HIV infection. These data indicate that the use of surrogate surface markers to identify functionally active CD8+ T cells in HIV infection is inherently complex and that even the absence of stimulation can significantly alter the expression of memory markers.

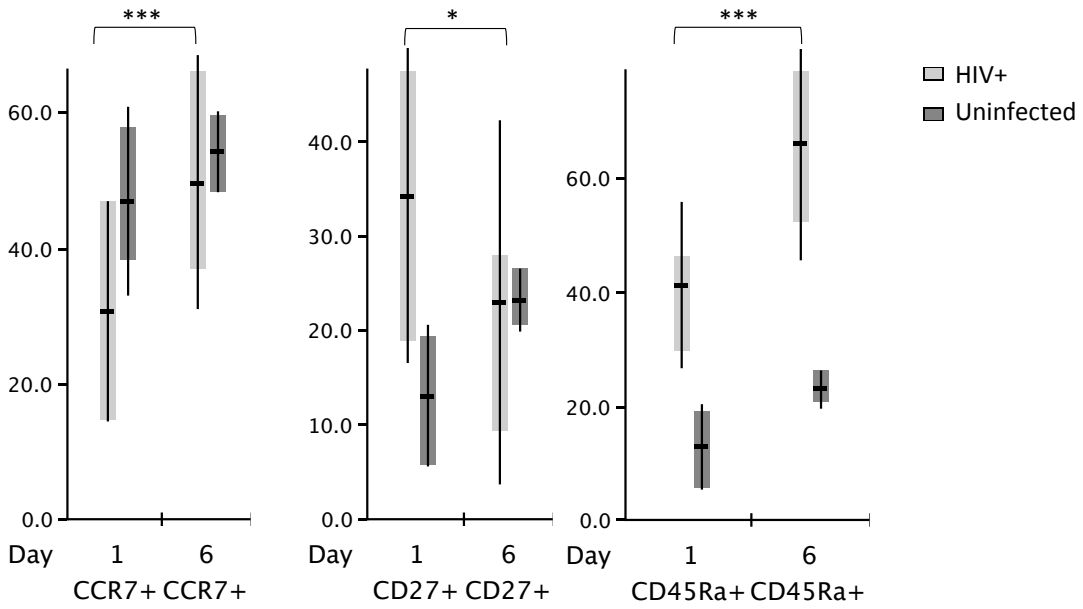

Figure 3.5. Expression of individual memory surface markers CCR7, CD27 and CD45RA on CD8+ T cells from HIV-infected and HIV-negative subjects. In HIV+ individuals, CCR7 and CD45RA were significantly higher (p=0.0001 and p=0.0002, respectively, Wilcoxon matched-pairs) while CD27 was significantly lower (p=0.0231, Wilcoxon matched-pairs), in day 6 versus day 1 cells. No significant differences were observed in HIV uninfected individuals.

3.5.3. *CD8+ T cells expressing different functions have distinct memory phenotypes*

We next determined the surface memory phenotypes of HIV-specific CD8+ T cells expressing each functional parameter individually, independent of expression any other functional readouts (IFNγ, MIP-1β, TNFα, CD107a, IL-2 and proliferation). CD8+ T cells positive for each parameter were stratified across the eight phenotypic categories. Here we observed several functional responses that appear to correlate with the phenotype measured (Figure 3.6). For example, cells defined as T_{EM} were the least likely to be IFNγ positive ($p<0.0001$, Kruskal-Wallis, post test Dunn's multiple comparison). Each of the phenotypes had a particular functional profile associated with it; the majority of "Undefined #2" (CCR7+CD27-CD45RA+) expressed more CD107a and IFNγ , while T_{CM} and Undefined #3 (CCR7+CD27-CD45RA-) were the predominantly expressing IFNγ. The majority of T_{TD} expressed higher MIP-1β, T_{TM} were more often IL-2 positive, and T_{EM} were most likely to be CD107a positive and IL-2 positive. Interestingly, 10-20% of responding cells regardless of function could be phenotypically described as naïve. Thus, effector function as defined by cytokine responses to HIV appear to be completely independent of, and not predicted by, surface memory phenotype.

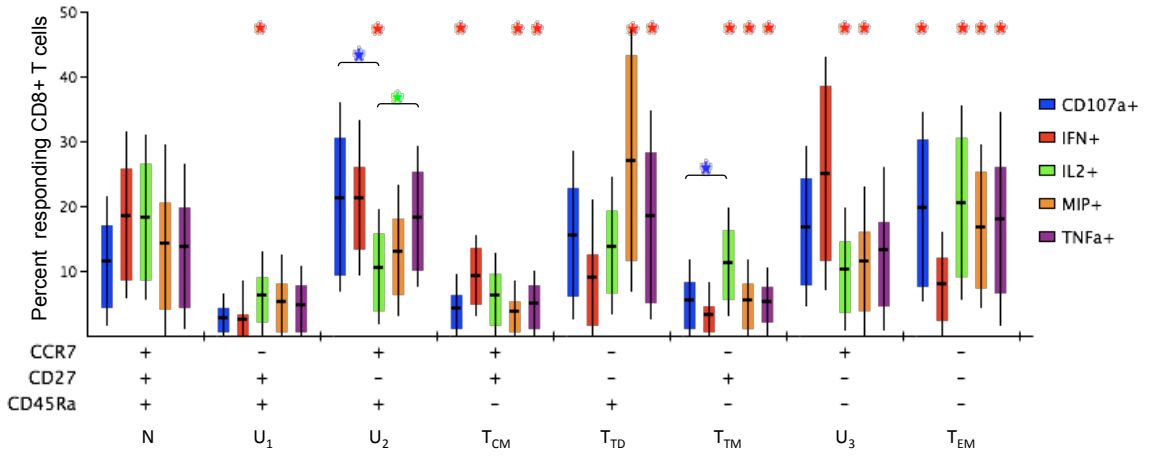

Figure 3.6. Expression of eight memory phenotypic categories on CD8+ T cells from HIV-infected subjects stratified by individual functional readouts. Functional readouts measured after stimulation with HIV p24 peptides include, CD107a (Blue), IFNγ (red), IL-2 (green), MIP-1β (orange) and TNFα (fuchsia). Red asterisks indicate a significant difference relative to INFγ, blue asterisks indicate a significant difference relative to CD107a and the green asterisk indicates a significant difference relative to IL-2 ($p<0.05$, Wilcoxon matched-pairs).

To further explore the link between functional parameters and phenotypic categories, we next sought to determine if any of the functions correlated with a specific surface phenotype. We correlated the percentage of responding cells for each function with the proportion of overall responding cells contained within each of the eight phenotypic categories. All functions (excluding IL-2) correlated with one or more of the eight memory phenotype categories, while proliferating cells did not correlate with any specific memory phenotypes (Table 3.5). The percentage of cells responding with IFNγ and TNFα correlated strongly, which was consistent with our published findings (discussed in chapter 5) that the expression of IFNγ and TNFα are strongly correlated in normal progressing HIV+ subjects (327). Both were positively associated with Undefined #2 and T_{CM} phenotypes (All $p<0.0001$, Spearmen rank), but were inversely correlated to T_{TM}, Undefined #3 and T_{EM} phenotype ($p<0.0001$, $p<0.0001$ and $p=0.0002$, respectively). The percentage of cells responding with CD107a was also inversely correlated to the T_{EM} phenotype ($p<0.001$), but positively correlated with Undefined #1 ($p<0.001$). Conversely, the percentage of cells responding with MIP-1β had no similarity to any of the other functions, only correlating with the Undefined #3 category ($p=0.0005$), again consistent with our published data (discussed in Chapter 5), which found no correlation between MIP-1β responses and other functions (327). These data suggest that while some functional attributes do weakly associate with specific "memory phenotypes", this is parameter-specific. These data suggest that the allocation of antigen-specific cells to functional categories on the basis of surface marker expression is exceedingly complex, and varies greatly depending on the functional attribute examined. Thus, defining CD8+ T cell function by surface phenotype is not predictable using the markers examined here.

Table 3.5: The percentage of cells responding with each function correlated with the proportion of responding cells expressing each of the eight phenotypic categories

	CCR7+CD27+CD45RA+ (Naïve)	CCR7+CD27+CD45RA- (Undefined#1)	CCR7+CD27-CD45RA+ (Undefined#2)	CCR7+CD27-CD45RA- (T_{CM})	CCR7-CD27+CD45RA+ (T_{TD})	CCR7-CD27+CD45RA- (T_{TM})	CCR7-CD27-CD45RA+ (Undefined#3)	CCR7-CD27-CD45RA- (T_{EM})
IFNγ	p=ns	p=ns	r=0.3083 p<0.0001	r=0.2923 p<0.0001	p=ns	r=(-) 0.291 p<0.0001	r=(-)0.2422 p<0.0001	r=(-)0.3385 p<0.0001
CD107a	p=ns	r=0.2071 p<0.0001	p=ns	p=ns	p=ns	p=ns	p=ns	r=(-)0.2168 p<0.0001
IL-2	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns
MIP-1β	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns	r=0.1772 p=0.0005	p=ns
TNFα	p=ns	p=ns	r=0.2973 p<0.0001	r=0.2946 p=<0.0001	r=(-)0.1943 p=0.0001	r=(-)0.2039 p=<0.0001	r=(-)0.2507 p=<0.0001	r=(-)0.1895 p=0.0002
Proliferation	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns

[a] gray and orange shading indicates positive and negative correlations, respectively

3.5.4. *Polyfunctionality does not directly associate with specific memory phenotypes*

The number of functions expressed simultaneously by a single antigen-specific cell is increasingly being considered as a correlate of protective immunity in a number of infections (216, 294, 330-332). To determine the surface phenotypes of polyfunctional cells, we stratified our data by CD8+ T cells concurrently expressing increasing numbers of functions (5+, 4+, 3+, 2+, and 1+). Polyfunctional responses were observed in all eight memory phenotypic categories, regardless of the number of functions expressed. Figure 3.5 shows, for each polyfunctional level, the relative proportion of surface phenotypes that account for the response. The percentage of cells responding with each level of polyfunctionality was correlated with the proportion of responding cells in each of the eight phenotypic categories (Table 3.6). Monofunctional responding cells were associated with phenotypic categories Undefined #1, Undefined #2 and T_{CM} (all $p<0.0001$), but were inversely correlated to T_{TM}, Undefined #3 and T_{EM} (all $p<0.0001$). Similarly, dual-functional responding cells were inversely correlated to T_{TM} and T_{EM} (both $p<0.0001$). Cells expressing 3 and 4 functions were positively correlated with phenotypic category Undefined #3 ($p=0.0004$ and $p=0.0007$, respectively) while cells expressing 4 and 5 functions were correlated to phenotype T_{TM} ($p<0.0001$ and $p=0.0003$, respectively). These data suggest that with increasing expression of multiple functions, the memory phenotype is variable. Although most of our data suggests that memory markers are unable to predict functionally active CD8+ T cells, there was a trend towards polyfunctionality associating with the transitional memory phenotypes, T_{TM} and Undefined #3.

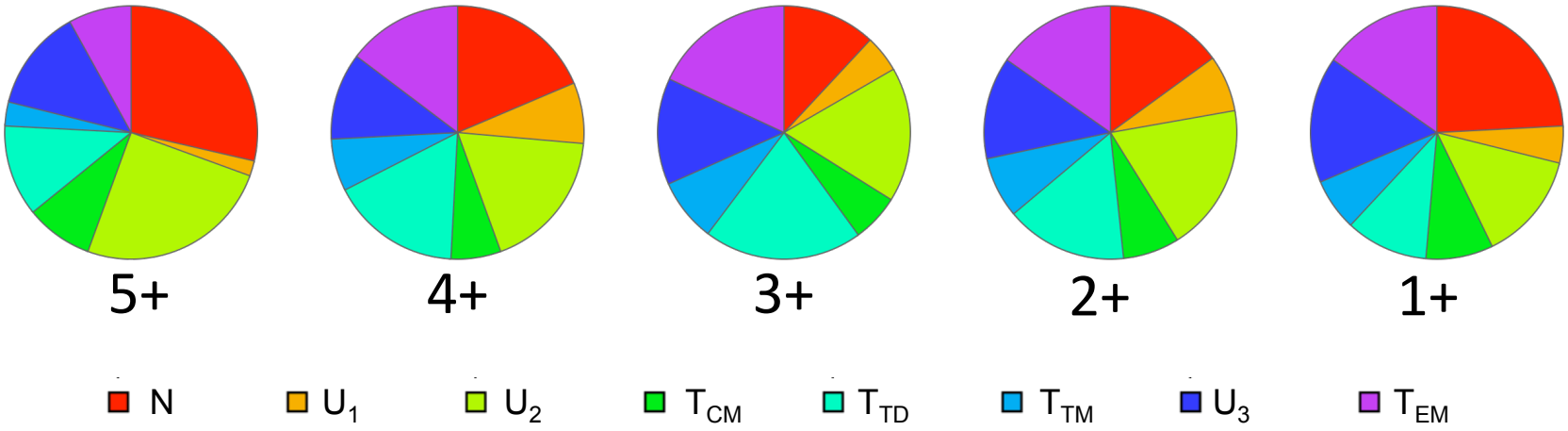

Figure 3.7. Polyfunctional CD8+ T cell responses stratified by surface phenotype. Pies indicated the relative proportion of each surface phenotype that makes up the polyfunctional response.

Table 3.6: The percentage of cells responding with each level of polyfunctionality was correlated with the proportion of responding cells expressing each of the eight phenotypic categories

	CCR7+CD27+CD45RA+ (Naïve)	CCR7+CD27+CD45RA- (Undefined#1)	CCR7+CD27-CD45RA+ (Undefined#2)	CCR7+CD27-CD45RA- (T_{CM})	CCR7-CD27+CD45RA+ (T_{TD})	CCR7-CD27+CD45RA- (T_{TM})	CCR7-CD27-CD45RA+ (Undefined#3)	CCR7-CD27-CD45RA- (T_{EM})
Mono-functional	p=ns	r=0.2698 p<0.0001	r=0.3607 p<0.0001	r=0.3564 p<0.0001	p=ns	r=(-)0.3544 p<0.0001	r=(-)0.3477 p<0.0001	r=(-)0.5155 p<0.0001
2+	p=ns	p=ns	p=ns	p=ns	p=ns	r=(-)0.2535 p<0.0001	p=ns	r=(-)0.2598 p<0.0001
3+	p=ns	p=ns	p=ns	p=ns	p=ns	p=ns	r=0.1816 p=0.0004	p=ns
4+	p=ns	p=ns	p=ns	p=ns	p=ns	r=0.1985 p<0.0001	r=0.1726 p=0.0007	p=ns
5+	p=ns	p=ns	p=ns	p=ns	p=ns	r=0.1847 p=0.0003	p=ns	p=ns

[a] Gray and orange shading indicates positive and negative correlations, respectively

3.5.5. *Phenotype of HIV and CEF-specific CD8+ T cell responses stratified by disease status*

We then determined whether HIV and CEF-specific CD8+ T cells differ in their phenotypic distributions on the basis of HIV disease status in this cohort. Participants were separated into normal progressors (NP), long-term non-progressors (LTNP) and patients on ART to determine phenotypic differences related to disease progression. HIV- and CEF-specific CD8+ T cells were assigned to the eight surface phenotypes. Phenotypic differences were observed in the proportion of responding CD8+ T cells between disease status, with LTNPs having a higher proportion of naïve, and lower proportion of T_{TD} cells, than NPs and ARTs (Figure 3.5ab, HIV, $p<0.0001$ and $p=0.0094$; and CEF, $p=0.0012$ and $p=0.0074$, respectively; Kruskal-Wallis, post test Dunn's multiple comparison). For proliferation, we found no significant differences in the phenotypes of CEF-specific proliferating CD8+ T cells between groups. However, HIV-specific CD8+ T cells from LTNPs had significantly higher proportions of Undefined #2 and lower T_{TM} and T_{EM} than NP and ART (Figure 3.6cd $p=0.0011$, $p=0.0017$, $p=0.0023$, respectively; Kruskal-Wallis, post test Dunn's multiple comparison). These data suggest that phenotypic changes on CD8+ T cells following stimulation also differs between clinical groups and viral-specific populations, perhaps due to different activation/stimulation histories.

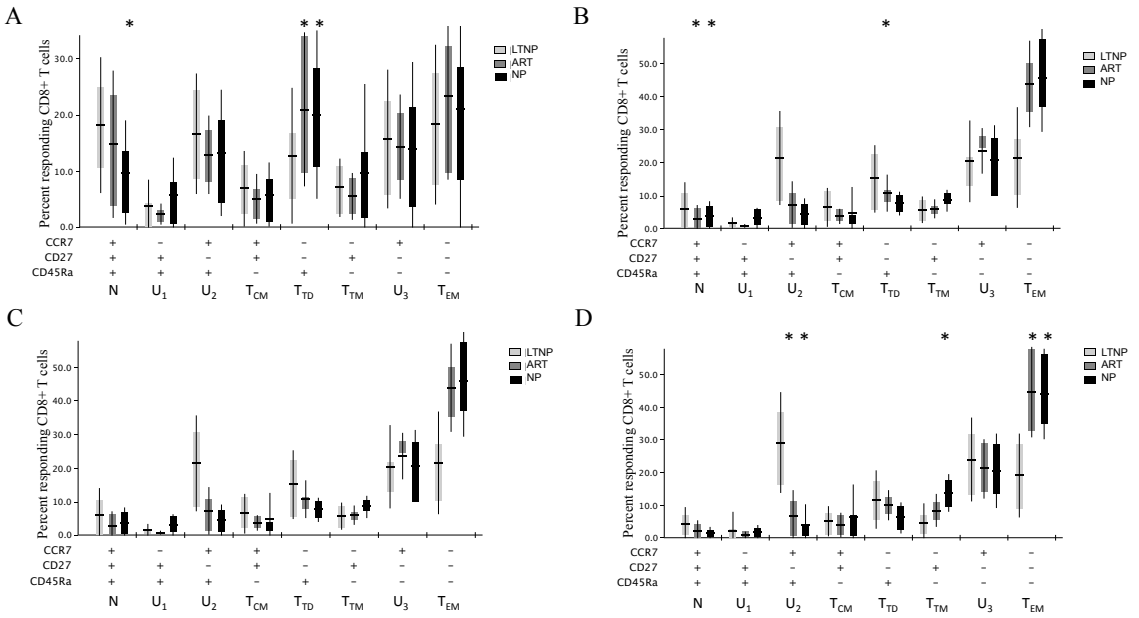

Figure 3.8. Expression of eight memory phenotypic categories on CD8+ T cells from HIV-infected subjects stratified by disease status. A) Comparison of CEF-specific overnight cytokine/chemokine responses stratified by disease status. B) Comparison of all HIV-specific overnight cytokine/chemokine responses stratified by disease status. C) Comparison of CEF-specific day 6 proliferative responses stratified by disease status. D) Comparison of HIV-specific day 6 proliferative responses stratified by disease status: LTNP (light gray bars), ART (dark gray bars) and NP (black bars). An asterisk indicates statistical significance ($p<0.05$, Kruskal-Wallis, post test Dunn's multiple comparison).

3.6. Summary

Here for the first time we present a detailed analysis of the memory phenotypic profiles of HIV and CEF-specific responses and their correlation with specific functional responses in a cohort of chronically infected HIV patients. Chiefly, we find that both HIV and CEF-specific CD8+ T cells responses are observed across all eight phenotypic categories defined by CCR7, CD45RA, and CD27, with naïve, T_{TD}, T_{EM} and two novel phenotypes (CCR7+CD27-CD45RA- (Undefined #2), and CCR7-CD27-CD45RA+ (Undefined #3) predominating. Furthermore, the phenotypic profile differed between HIV and CEF antigen-specific cells, with HIV-specific cells expressing higher levels of CD45RA. Interestingly, we observed several differences in phenotypes of individual functional readouts and polyfunctional cells. Each phenotype had a particular function associated with it. For example, cells with the T_{TD} phenotype, were most likely to express MIP-1β, while cells with the undefined#3 phenotype, were most likely to express IFNγ. Additionally, we found no consistent phenotype to represent the totality of functionally active CD8+ T cells and there were differences between LTNP and NP indicating HIV skews memory phenotypes even further depending on disease status.

3.7. Implications

The implication of these results is that the study of CD8+ T cell responses in HIV infection would be better served if functionality, rather than surrogate surface markers, were used to define effective CD8+ T cell responses. Future CD8+ T cell studies aimed at accessing the quality and specificity of CD8+ T cells in HIV infection should focus on the complexities of CD8+ T cell functionality, with less emphasis on pre-defined memory

cell subsets. Determination of the optimal functional parameters that can be best used to define CD8+ T cells that mediate protective immunity should be a primary goal of HIV vaccine development and evaluation.

4. Chapter 4. Epitope mapping of HIV-specific CD8+ T cell responses reveals distinct specificities defined by function.

Data from chapter is published in the Journal of Virology, Richmond et. al (2011)(327))

4.1. Rationale

As the HIV/AIDS epidemic continues to grow, there is a desperate need for an effective vaccine. Most current HIV vaccine candidates aim, at least in part, to induce HIV-specific CD8+ T cells responses capable of containing viral replication and slowing disease progression. This vaccine concept is based on several lines of evidence suggesting that CD8+ T cell responses can control viral replication (discussed in section 1.6.5). Several large studies have found no correlation between HIV-specific CD8+ T cell IFNγ secretion and viral load, and that high avidity responses to autologous virus can be measured in subjects who are progressing to AIDS (291, 292). HIV-specific CD8+ T cells are often exhausted or functionally inferior in chronic, progressive HIV infection, in some cases lacking perforin expression, cytokine secretion, and proliferative capacity (259, 260). These data suggest that not all CD8+ T cell responses are effective, and responses that better correlate with protection need to be identified.

LTNP provide a valuable model for the study of cell-mediated immune responses that may be capable of controlling HIV. Previous work has demonstrated that these individuals maintain stronger HIV-specific CD8+ T cell proliferation and polyfunctionality compared to progressing controls (discussed in section 1.6.5) (258-260). Although proliferation and polyfunctional responses have previously been associated with LTNP, these studies utilized peptide pools or pre-defined epitopes to measure responses.

Most studies describing epitope-specificity of CD8+ T cell responses in HIV infection have relied extensively on IFNγ Elispot assays (333). While measurement of this antiviral effector molecule facilitates easy screening, using a single readout, especially one that may not accurately describe responses capable of controlling HIV, may miss many effective responses. For example, little is known regarding the specificity of proliferative responses, which have been associated with control of HIV infection (258, 273, 331). Data from our lab examining CD8+ T cell responses to HIV Env using IFNγ Elispot and 6 day CFSE proliferation assays revealed substantial differences in the epitopes recognized with each assay (310). Here, we extend these observations using an unbiased epitope mapping approach by determining the polyfunctional and proliferative specificity of CD8+ T cell responses.

4.2. Hypothesis

We hypothesised that in chronic HIV infection, the specificity of cytokine/chemokine and proliferation responses to HIV epitopes differs and further specific cytokine and chemokine responses will be epitope-dependent.

4.3. Objectives

To conduct fine epitope mapping of short-term (cytokine/chemokine) and long-term (proliferation) CD8+ T cells responses to HIV-1 p24 and to determine whether cytokine/chemokine and proliferation response associate with specific epitopes of HIV-1 p24.

4.4. Methods

4.4.1. *Subjects*

Study participants (n=24, Table 4.1) were all HIV infected and enrolled from the Majengo cohort. Mean and median years of positive follow up of LTNP was 11.98 and 14.31 years, respectively (range 6.41-17.43 years, n=10). Within the NPs (n=14) were 2 subjects who were long-term survivors, i.e. ART naïve subjects with CD4 counts below 400 for over 10 years. Five NP subjects were on antiretroviral therapy (ART) at the time of the study.

Table 4.1: Study subjects' demographic data

Subject ID	Disease Status[a]	Age	Year + follow up	CD4 count	Duration of ART (years)	Viral Load	VL Date	Clade
890	LTNP	53	16.8	570		144	5-8/05	A1
1211	LTNP	40	17.43	437		8512	5-8/05	A1
1250	LTNP	49	6.41	468		226	5-8/05	
1287	LTNP	36	6.53	547				
1424	LTNP	43	15.55	539		481	5-8/05	A1
1625	LTNP	43	14.37	418		2400	19/11/07	A1
1647	LTNP	41	13.55	731				
1649	LTNP	40	14.31	447		1803	11/06	D
1654	LTNP	36	14.31	433				
1725	LTNP	41	10.79	452		1295	5-8/05	A1
1731	ART	42	13.67	228	0	50	5-8/05	A1
1771	NP	42	12.74	186		6700	11/06	
1848	NP	36	10.4	184				
1917	ART	37	7.26	456	1.61	3400	15/4/04	A1
1932	ART	36	6.42	407	2.13	76	5-8/05	A1
1947	ART	38	6.17	359	0.46	479	5-8/05	
1971	ART	45	5.31	168	0.19	3000	28/2/06	
1974	NP	44	5.29	235		8100	6/3/06	D
2166	NP	38	4.67	233				
2274	NP	37	1.97	921				
2522	NP	27	0.74	408				
2531	NP	48	0.73	256				
2560	NP	42	0.7	446				
2630	NP	33	0	307				

[a]LTNP: Long-term non-progressor, NP: Normal progressor, ART: subjects on antiretroviral therapy

4.4.2. *In vitro stimulation*

A peptide library (9 mers overlapping by 8 amino acids) derived from the HIV-1 p24 clade A1 ancestral sequence (discussed in section 2.2.3.1), were pooled in a matrix format using *Deconvolute This!* 1.0 (322) (courtesy of Mario Roederer, Vaccine Research Center, NIAID, NIH). Each peptide was represented twice, resulting in 16 pools with approximately 30 peptides per pool. Peptide pools were used at 2μg/ml/peptide, and stimulations were accompanied by 2 positive controls, CEF peptides and SEB, and duplicate negative controls consisting of media alone. For ICS, cells were incubated for 14 hour as per general stimulation methods (section 2.2.3), while for proliferation assays cells were incubated for 6 days. Unlike the previous chapter where epitope specificity was unneeded, here we looked at epitope-specific responses. Putative responding peptides from within the pools were identified using the pooling matrix and these were subsequently confirmed at later time. For each combination of responding peptide pools the *Deconvolute This!* software narrows the potential responding peptides down to 4 putative epitopes. Positive pool responses were considered 2-fold over background. However, in some subjects, limited cell numbers prevented confirming of all putative epitopes; in these cases the cut-off for a positive pool response was raised to 3-fold over background, and therefore only the strongest peptides were confirmed. Individual peptides were also used at 2μg/ml and accompanied by CEF peptides, SEB and duplicate negative control consisting of media alone.

4.4.3. *Flow cytometry*

Immediately following stimulation, PBMCs were washed with PBS containing 2% FCS, and stained using the panels described below (Table 4.2 and 5.3) following general surface staining (section 2.2.4.1, these experiments were conducted prior to the inclusion of LIVE/DEAD to our standard protocol), intracellular cytokine staining (section 2.2.4.2) and CFSE dilution assay protocols. Stained cells were immediately analyzed on a LSRII flow cytometer (BD Biosciences) as per general methods section 2.2.4.6.

Table 4.2: Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell memory phenotypes and polyfunctionality

Fluorochrome	Marker	Source	Volume used	Surface/ICS
FITC	IFNγ	BD Biosciences	3μl	ICS
PE	MIP-1β	BD Biosciences	3μl	ICS
PE-Cy5	CD107a	BD Biosciences	5μl	ICS
PE-Cy7	CCR7	BD Biosciences	3μl	Surface
ECD	CD45RA	Beckman Coulter	3μl	Surface
APC	IL-2	BD Biosciences	3μl	ICS
AmCyan	CD3	BD Biosciences	1μl	Surface
AlexaFluor 700	CD27	eBiosciences	3μl	Surface
Pacific Blue	TNFα	eBiosciences	3μl	ICS
APC-Cy7	CD8	BD Biosciences	1μl	Surface

Table 4.3: Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell memory phenotypes and proliferation

Fluorochrome	Marker	Volume used	Source
Blue laser	CFSE	1μl/ml	Invitrogen
PE-Cy7	CCR7	3μl	BD Biosciences
ECD	CD45RA	3μl	Beckman Coulter
AmCyan	CD3	1μl	BD Biosciences
AlexaFluor 700	CD27	3μl	eBiosciences
APC-Cy7	CD8	1μl	BD Biosciences

4.4.4. *Statistical analysis*

Statistical analyses were performed using Graph Pad Prism 5.0 and SPICE 4.1. All correlations were determined using Spearman's rank correlation. Breadth and magnitude comparisons between subjects groups were determined using Mann-Whitney tests.

4.5. Results

4.5.1. *Epitope mapping of p24 using parallel measurement of five CD8+ T-cell functions*

HIV-specific CD8+ T cell responses to p24 epitopes were mapped by simultaneously measuring IFNγ, CD107a, MIP-1β, IL-2 and TNFα production following overnight stimulations with 16 peptide pools representing HIV-1 clade A1 p24. Representative intracellular and proliferation responses are shown in Figure 4.1a. Pool-specific CD8+ T cell responses were detectable for all five effector functions and proliferation. The full complexity of the response to all peptide pools was then examined using Boolean gating, yielding 32 unique response combinations for the 5 individual readouts measured (Figure 4.1b).

4.5.2. *LTNPs maintain a higher degree of HIV-specific CD8+ T cell functionality*

Previous work has suggested that LTNPs maintain a higher degree of polyfunctional HIV-specific CD8+ T cell responses when compared to NPs(216) (216). However, this had mostly been examined using pre-defined epitopes or peptide pools and not unbiased epitope screening approaches. After Boolean gating and stratification by disease status and ART use, we confirmed that a higher degree of CD8+ T cell polyfunctionality in LTNPs was observed in our study (Figure 4.1c). HIV-specific CD8+ T cells responses

from LTNPs and ART subjects (indicated by blue and red bars respectively) displayed a higher functional profile when compared to NPs (green bars) ($p=0.0026$ and $p=0.0025$ respectively, Figure 4.1bc). While responses that included all five functions were nearly absent from NPs, they were observed at a low frequency in LTNPs and subjects on ART, presumably due to reconstituted immune responses in the latter. p24-specific responses in LTNPs were distributed mostly in the 2+, 3+ and 4+ categories and are at higher proportion than NPs (Figure 4.1c)

.

4.5.4. *The epitope-specificity of CD8+ T cells responses differs based on functional readout*

To further dissect differences between functional readouts at the epitope level, we confirmed the epitope-specificity of 110 pool-specific responses. Putative responding peptides were identified using the pooling matrix and these were subsequently confirmed at later time points. For 16/24 subjects (the remaining patients could not be assessed due to loss to follow up and one death), 126 putative epitopes were tested (range of 4-21 peptides tested/subject). All 16 subjects responded to ≥1 of their putative epitopes, making a total of 73 responses to 54 epitopes (Table 4.9). There was no difference in the breadth of epitope responses to the select p24 epitopes between LTNPs and NPs (p=0.9423, LTNP: mean 4.7, median 5, range 1-8 peptides, NP: mean 4.5, media 4 range 2-9, data not shown), and similar numbers of putative epitopes were tested between these groups. These data shows that LTNP do not respond to more p24 epitopes than NP, suggesting their better disease outcome is due to the quality rather than the quantity of the response.

Table 4.10: 54 identified epitopes and the responses that were elicited

A1 anc	HXB2 p24 position	Responders N=16	IFNγ	MIP-1β	CD107a	IL-2	TNFα	Proliferation	Polyfunctional
VQNLQGQMV	3-11	LTNP			+		+		Yes[a]
QGQMVHQSL	7-14	LTNP	+	+	+	+	+		Yes
		NP				+			No
HQSLSPRTL	12-20	LTNP				+			No
		NP				+			No
		LTNP	+	+		+	+		Yes
SLSPRTLNA	14-22	ART						+	No
		NP				+	+		Yes
LSPRTLNAW	15-23	LTNP		+		+	+		Yes
SPRTLNAWV	16-24	ART				+			No
LNAWVKVIE	20-28	LTNP			+				No
EEKAFSPEV	28-36	NP		+		+			Yes
		LTNP	+						No
KAFSPEVIP	30-38	ART	+	+		+			Yes
FSPEVIPMF	32-40	LTNP	+						No
		LTNP			+		+		Yes
VIPMFSALS	36-44	LTNP					+		No
PMFSALSEG	38-46	LTNP				+			No
		NP					+		No
		LTNP	+		+				Yes
FSALSEGAT	40-48	LTNP			+		+		Yes
LSEGATPQD	43-51	NP					+		No
GATPQDLNM	46-54	LTNP				+			No
		LTNP	+		+				Yes
		NP		+		+	+		Yes
TPQDLNMML	48-56	ART		+					No

A1 anc	HXB2 p24 position	Responders N=16	IFNγ	MIP-1β	CD107a	IL-2	TNFα	Proliferation	Polyfunctional
LNMMLNIVG	52-60	ART				+			No
		NP	+	+			+		Yes
MMLNIVGGH	54-62	LTNP						+	No
		LTNP			+				No
		NP				+			No
LNIVGGHQA	56-64	LTNP		+	+		+		Yes
		ART					+		No
HQAAMQMLK	62-70	LTNP	+		+				Yes
		NP					+		No
EEAAEWDRL	75-83	LTNP						+	No
EAAEWDRLH	76-84	NP	+	+			+		Yes
AAEWDRLHP	77-85	ART		+					No
RLHPVHAGP	82-90	LTNP		+					No
VHAGPIPPG	086-94	ART	+						No
IPPGQMREP	91-99	LTNP			+				No
PGQMREPRG	93-101	ART			+		+		Yes
MREPRGSDI	96-104	LTNP	+						No
		ART	+		+				Yes
SDIAGTTST	102-110	NP			+		+		Yes
IAGTTSTLQ	104-112	ART						+	No
GTTSTLQEQ	106-114	LTNP	+			+			Yes
TLQEQIGWM	110-118	NP	+				+		Yes
QEQIGWMTG	112-120	LTNP	+	+					Yes
		ART	+	+					Yes
TGNPPIPVG	119-127	LTNP		+	+		+		Yes
KRIILGLN	131-139	LTNP			+				No
WIILGLNKI	133-141	LTNP		+					No

A1 anc	HXB2 p24 position	Responders N=16	IFNγ	MIP-1β	CD107a	IL-2	TNFα	Proliferation	Polyfunctional
LGLNKIVRM	136-144	LTNP	+	+			+		Yes
		ART		+					No
RMYSPVIL	143-151	LTNP		+					No
ILDIRQGPK	150-158	LTNP						+	No
PKEPFRDYV	157-165	LTNP		+		+			Yes
DYVDRFFKT	163-171	NP			+		+		Yes
DRFFKTLRA	166-174	NP				+			No
FKTLRAEQA	169-177	LTNP				+		+	Yes
ETLLVQNAN	187-195	NP	+			+		+	Yes
LVQNANPDC	190-198	NP				+			No
NPDCKSILR	195-203	NP						+	No
DCKSILRAL	197-205	NP		+		+			Yes
RALGPGATL	203-211	NP						+	No
GATLEEMMT	208-216	NP						+	No
LEEMMTACQ	211-219	NP				+	+		Yes
EEMMTACQG	212-220	NP		+				+	Yes
MMTACQGVG	214-222	NP				+	+	+	Yes
		NP						+	No
MTACQGVGG	215-223	NP					+		No
GGPGHKARV	222-230	NP				+			No

[a]Shading indicates epitopes that are considered best defined epitopes (BDE) or can be found within longer, 10 aa -15 aa BDEOverall, there was a further disconnect at the epitope level between IFNγ and proliferative responses. ML1211 (Figure 5.4ab) demonstrates how individual epitopes can preferentially elicit IFNγ and not proliferation (pool 3), and vice versa (pool 10 and 7). Responses to pool 7 in this participant demonstrate that epitope-specificity can differ within the short-term assays, as this pool was CD107a+ in the absence of IFNγ or other overnight parameters. A second representative subject ML1932 (Figure 5.4cd) also demonstrates functional disparity of CD8 responses. This subject responded with monofunctional responses to 3/5 responsive pools (MIP-1β to pool 14 and IL-2 to pools 5 and 11). This subject had polyfunctional responses to pools 7 and 16, with the concurrent expression of CD107a, IFNγ and MIP-1β. In both subjects the immunodominant responses were polyfunctional, pools 3 and 15 in ML1211 and pool 7 and 15 in ML1932.

In general, we observed numerous examples of individual epitopes selectively expressing one or more functional parameter. In ML1211 (Figure 4.4b), peptide 86 elicited a polyfunctional 5+ response, while peptide 68 generated CD107a+ only. Two peptides in this participant (66 and 186) were positive for proliferation but not for any of the short-term parameters. ML1932 (Figure 4.3d) showed similar selectiveness in expression of certain functions, while responses to peptides 202 and 203 were monofunctional and independent from all other parameters (MIP-1β+ and TNFα+, respectively), several polyfunctional responses were also seen. Peptide 208 was CD107a+TNFα+, while peptide 209 was IFNγ+IL-2+, and peptide 210 was 3+ (CD07a, MIP-1β and TNFα). These data further show that the functional readout used to define CD8+ T cell activity can play a major role in determining the epitope-specificity of HIV-specific CD8+ T cells.

In agreement with the data from the peptide pools, non-IFNγ responses were observed in a high proportion (54/73, 74%) of peptide-specific responses. These data clearly show that a substantial portion of immune responses would remain undetected had we measured IFNγ alone. Proliferation was a component of 13/73 (18%) responses, and only one response was proliferation^{+} IFNγ^{+}. Consistent with pool-specific responses the percentages of epitope-specific IFNγ responding cells were inversely correlated to the percentages of proliferating cells ($P<0.0001$, data not shown).

The majority (9/13, 69%) of detected proliferative responses did not have any corresponding responses measured in the overnight assay. Where a corresponding

overnight function was present, it was often IL-2 (3/4, 75%, 1 LTNP and 2 NPs). However, the majority (22/25) of the IL-2 responses did not have a corresponding proliferation response, and the percentage of proliferating cells was inversely correlated to the magnitude of IL-2 response ($p<0.0001$, data not shown).

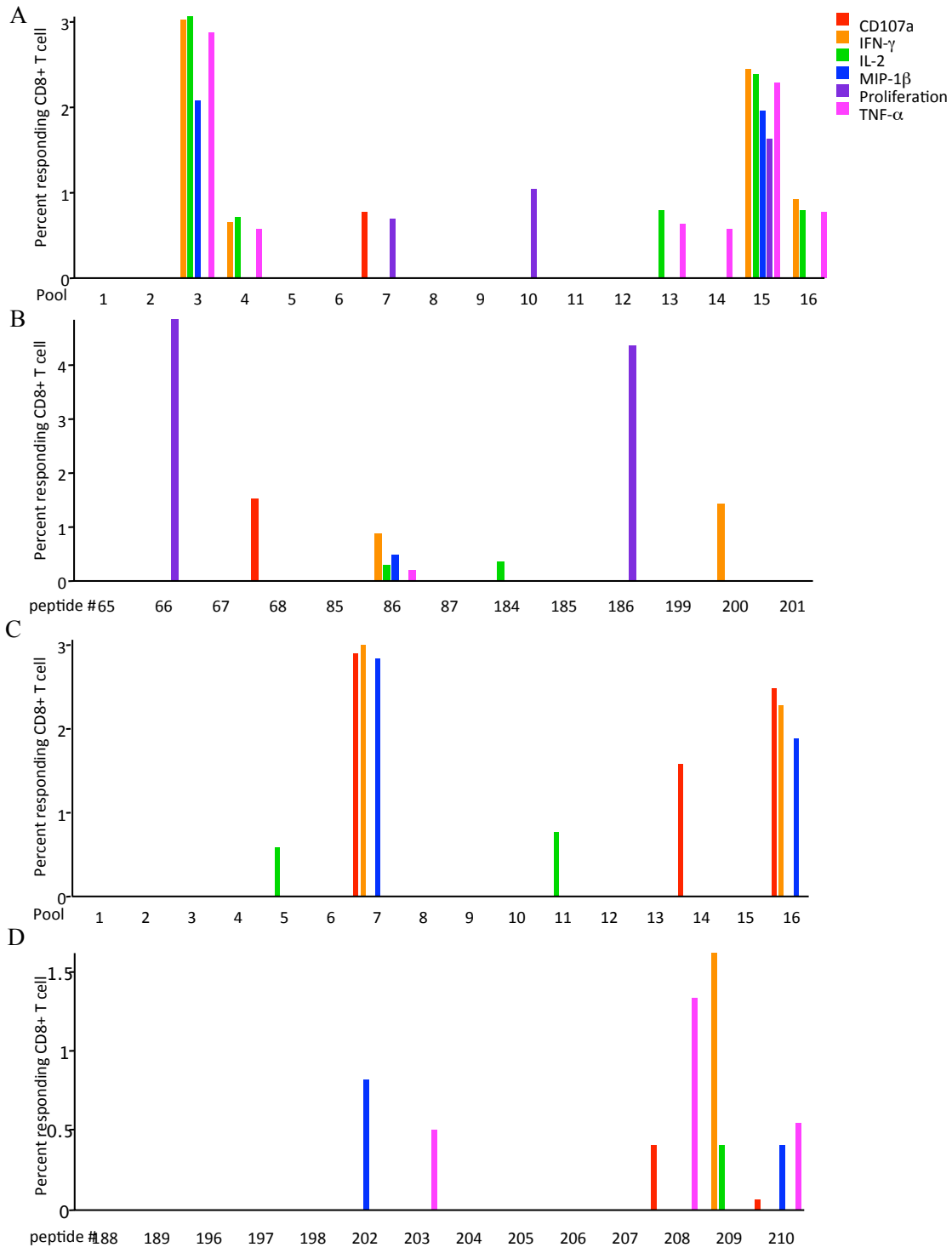

Figure 4.4. HIV-specific CD8+ T cell effector readout is dependent on epitope specificity, as shown in a representative subject. A) Pool responses of subject ML1211. B) Deconvoluted peptide responses of subject ML1211. C) Pool responses of subject ML1932. D) Deconvoluted peptide responses ML1932. CD107a (Red), IFNγ (orange), IL-2 (green), MIP-1β (blue), Proliferation (purple), and TNFα (pink).

Of the 54 epitopes identified in this study, 12 (24%) are considered best defined epitopes (BDE) or can be found within a longer BDE (10-15aa) (334). The remaining epitopes defined here (76%) have not been fully characterized (Table 4.10). We compared the sequence variation between the A1 ancestral sequence used in this study and the B consensus sequence used in previous epitope mapping studies (Table 4.10) (18). Many of the uncharacterized epitopes, 16/42 (38%), had ≥1 amino acid variation between the study sequence and B consensus, possibly accounting for their lack of recognition in previous studies. However, all of the 12 recognized BDE also had ≥1 amino acid variation between the two sequences. Therefore, sequences differences are likely not the reason why these new epitopes have not been previously defined and likely depends on the functional readout examined.

Table 4.11. Comparison of B consensus sequence with recognized peptides from A1 library used in the current study

B cons (top) vs A1 anc (bottom)	HXB2 p24 position	BDE (HLA: Clade)[a]	Sequence variation[b]	Responders N=16
---A----- VQNLQGQMV	3-11	Yes (B13)	1	1
-------AI QGQMVHQSL	7-14	No	2	2
--AI--- HQSLSPRTL	12-20	Yes (B*1510)	2	3
AI------- SLSPRTLNA	14-22	No	2	2
I-------- LSPRTLNAW	15-23	Yes (B*5701,B63)	1	1
--------- SPRTLNAWV	16-24	Yes (B*0702)	0	1
-------V- LNAWVKVIE	20-28	No	1	1
--------- EEKAFSPEV	28-36	Yes	0	2
--------- KAFSPEVIP	30-38	Yes (30-40)[c] (B*5701,B*5703, B63: B)	0	1
--------- FSPEVIPMF	32-40	Yes (B57)	0	2
--------- VIPMFSALS	36-44	No	0	1
--------- PMFSALSEG	38-46	No	0	3
--------- FSALSEGAT	40-48	No	0	1
--------- LSEGATPQD	43-51	No	0	1
--------T GATPQDLNM	46-54	No	1	3
------T-- TPQDLNMML	48-56	Yes (B*0702,B*3910,B *4201,B*8101, Cw*0802: B)	1	1
--T---T-- LNMMLNIVG	52-60	No	2	2
T---T---- MMLNIVGGH	54-62	No	2	3
--T------ LNIVGGHQA	56-64	No	2	2

B cons (top) vs A1 anc (bottom)	HXB2 p24 position	BDE (HLA: Clade)[a]	Sequence variation[b]	Responders N=16
--------- HQAAMQMLK	62-70	No	0	2
--------- EEAAEWDRL	75-83	No	0	1
--------- EAAEWDRLH	76-84	No	0	1
--------- AAEWDRLHP	77-85	No	0	1
--------- RLHPVHAGP	82-90	No	0	1
------A— VHAGPIPPG	86-94	No	1	1
-A------- IPPGQMREP	91-99	No	1	1
--------- PGQMREPRG	93-101	No	0	1
--------- MREPRGSDI	96-104	Yes (94-104) [c] (B13)	0	2
--------- SDIAGTTST	102-110	No	0	1
--------- IAGTTSTLQ	104-112	No	0	1
--------- GTTSTLQEQ	106-114	No	0	1
--------- TLQEQIGWM	110-118	No	0	
--------N QEQIGWMTG	112-120	No	1	2
-N------- TGNPPIPVG	119-127	No	1	1
--------- KRIILGLN	131-139	No	0	1
--------- WIILGLNKI	133-141	No	0	1
--------- LGLNKIVRM	136-144	No	0	2
-----T--- RMYSPVIL	143-151	No	1	1
--------- ILDIRQGPK	150-158	No	0	1
--------- PKEPFRDYV	157-165	No	0	1
------Y-- DYVDRFFKT	163-171	Yes (162-172) [c] (A*2402: A, B4402: B)	1	1

B cons (top) vs A1 anc (bottom)	HXB2 p24 position	BDE (HLA: Clade)[a]	Sequence variation[b]	Responders N=16
---Y----- DRFFKTLRA	166-174	Yes (B*1402: B)	1	1
Y-------- FKTLRAEQA	169-177	No	1	1
--------- ETLLVQNAN	187-195	No	0	1
--------- LVQNANPDC	190-198	No	0	1
-----T--- NPDCKSILR	195-203	No	1	1
---T--K-- DCKSILRAL	197-205	Yes	2	1
K----A--- RALGPGATL	203-211	No	2	1
--------- LEEMMTACQ	211-219	No	0	1
--------- EEMMTACQG	212-220	No	0	1
--------- MMTACQGVG	214-222	No	0	2
--------- MTACQGVGG	215-223	No	0	1
--------- GGPGHKARV	222-230	No	0	1
A-------- GATLEEMMT	208-216	No	1	1

[a]Clade and restricting HLA allele for BDE

[b]Number of amino acid differences between the sequences noted

[c]Position of BDE if responding 9mer was found within a longer epitope.

Interestingly, polyfunctionality (defined as ≥2+ responses) comprised 33/73 (45%) of responses (Table 4.9). Both BDEs and the newly described epitopes frequently stimulated polyfunctional responses (9/17 and 25/56, respectively, p=0.59), and responses in the absence of IFNγ (11/17 and 43/56, respectively, p=0.35). Interestingly, the uncharacterized epitopes were solely responsible for the proliferation responses that were observed (n=13). These data suggest that not only is the measure of multiple functions important for more systematic detection of responses but that performing both short-term and long-term assays is also critical.

4.6. Summary

Here we have extended the functional characterization of HIV-specific CD8+ T cells by using multiple overnight parameters and long-term proliferation assays to map CD8+ T cell epitopes more comprehensively than has been previously described. Critically, over two-thirds of the responses detected in our study were detected in the absence of an IFNγ (54/73) response, and measurement of additional functional parameters expanded the breath of response by almost 4-fold. These data suggest that previous epitope mapping studies and vaccine trials may have missed out on important immune responses by focusing solely on IFNγ, considering that the majority of HIV-specific CD8+ T cell responses observed in our study would have been overlooked by measuring any one response in isolation. The data further reveal that CD8+ T cell responses are complex and that particular epitopes within a given subject will elicit responses with unique functionality.

Additionally, we show that within a given HIV-infected subject, it is possible to identify novel epitopes that induce qualitatively distinct CD8+ T cell responses. Screening for responses by the measurement of six CD8+ T cell functions revealed a greater complexity of CD8+ T cell immunology, and describes a greater breadth of response than would have been derived from measurement of any one response on its own. We have shown that the epitope specificity differs between short and long-term assays, as well as within the short-term assay. We also show that particular epitopes, in any given subject, often tended to elicit one type of CD8+ T cell response in preference to others.

4.7. Implications

The implication of these results is that the quality of CD8+ T cell responses in HIV infection differs substantially depending on the HIV epitope recognized and there is potential to identify epitopes that induce distinct functional responses on a population level. The ability to identify epitopes that elicit functionally superior and protective CD8+ T cell responses could be used in a HIV vaccine and steer the immune system towards a more effective CD8+ T cell response.

5. Chapter 5. Characterization of novel clade A HIV epitopes: measuring polyfunctional responses and proliferative capacity in CD8+ T cells

5.1. Rationale

The limited successes of vaccine trails to date suggest that additional research needs to be done on alternative and unique vaccine formulations that may provide better protection from HIV than traditional vaccine strategies. For example, vaccination with vectors expressing distinct CD8+ T cell epitopes, rather than whole viral proteins, improves immunogenicity (335), which allows the generation of multiple dominant responses (336, 337), and provides the opportunity to focus on beneficial responses and exclude ineffective or even potentially harmful ones (137, 338).

Despite evidence that CD8+ T cell proliferative and polyfunctional responses are associated with control of HIV infection, little is known regarding the specificity of these responses (258, 259, 273). Data from our lab examining CD8+ T cell responses to HIV Env using IFNγ Elispot and 6 day CFSE proliferation assays revealed substantial differences in responses to distinctive epitopes between assays (339). We extended these observations using an unbiased epitope mapping approach to determine the specificity of polyfunctional and proliferative CD8+ T cell responses (Chapter 4). The data revealed that CD8+ T cell responses are complex and that particular epitopes within a given participants will elicit responses with unique functionality. We showed that within a given HIV-infected subject, it is possible to identify novel epitopes that induce qualitatively distinct CD8+ T cell responses.

Therefore, we sought to validate and confirm the ability of distinct epitopes to elicit a proliferative and polyfunctional response by measuring results in a larger sample of the cohort. Identifying and understanding epitopes that elicit polyfunctional and proliferative responses at a cohort level will serve as a valuable resource for successful HIV vaccine design that will presumably induce a wider range of epitope-specific responses and a wider variety of protective responses.

5.2. Hypothesis

We will be able to identify specific epitopes that preferentially elicit polyfunctional and/or proliferative responses at a cohort level and that responses to these epitopes will correlate with a better disease outcome.

5.3. Objectives

To characterize the CD8+ T cell responses specific for epitopes of interest identified during previous study.

To determine if particular epitopes are capable of eliciting different CD8+ T cell responses in a larger sample of the cohort.

To determine if particular epitopes are associated with protection against HIV disease progression.

5.4. Methods

5.4.1. *Subjects*

Study participants (n=83) were all HIV infected and enrolled from the Majengo cohort. Twenty-one of the participants were on Antiretroviral therapy (ART) at the time of sampling, while the remainders (n=62) were ART naïve. The mean and median years of HIV+ follow up was 5.40 years (range 0.35-19.16 years, Median 2.80 years).

5.4.2. *In vitro Stimulation:*

Details of the eleven epitopes in this study can be found in Table 6.3. The peptides were used at 2µg/ml, and stimulations were accompanied by 2 positive controls, CEF and SEB, and duplicate negative controls consisting of media alone. For ICS, cells were incubated for 14 hours as per general stimulation methods (section 2.2.3), while for proliferation assays cells were incubated for 6 days.

5.4.3. *Flow cytometry*

Immediately following stimulation, PBMCs were washed with PBS containing 2% FCS, and stained using the panels described below (Table 6.1 and 6.2) following general surface staining (section 2.2.4.1, with LIVE/DEAD), intracellular cytokine staining (section 2.2.4.2, including MIP-1α) and CFSE dilution assay protocols. Stained cells were immediately analyzed on a LSRII flow cytometer (BD Biosciences) as per general methods section 2.2.4.6.

Table 5.1. Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell polyfunctionality

Fluorochrome	Marker	Source	Volume used	Surface/ICS/Dump Channel
FITC	IL-2	BD Biosciences	3µl	ICS
PE	MIP-1β	BD Biosciences	3µl	ICS
PE-CY5	CD107a	BD Biosciences	5µl	ICS
PE-CY7	IFNγ	BD Biosciences	3µl	ICS
ECD	CD4	Beckman Coulter	1µl	Surface
APC	MIP-1α	BD Biosciences	5µl	ICS
AmCyan	CD3	BD Biosciences	1µl	Surface
AlexaFluor 700	TNFα	BD Biosciences	3µl	ICS
eFlour450	CD14,CD19	eBiosciences	1µl	Dump Channel
Violet laser	Live/Dead	Invitrogen	6.25µl	Dump Channel
APC-H7	CD8	BD Biosciences	1µl	Surface

* MIP-1α was added to the panel for a more thorough analysis of functionality

Table 5.2. Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell memory phenotypes and proliferation

Fluorochrome	Marker	Source	Volume used	Surface/Proliferation/Dump Channel
Blue laser	CFSE	Invitrogen	1µl/ml	Proliferation
PE-Cy7	CCR7	BD Biosciences	3µl	Surface
ECD	CD45RA	Beckman Coulter	3µl	Surface
AmCyan	CD3	BD Biosciences	1µl	Surface
AlexaFluor 700	CD27	eBiosciences	3µl	Surface
eFlour450	CD14,CD19	eBiosciences	1µl	Dump Channel
Violet laser	Live/Dead	Invitrogen	6.25µl	Dump Channel
APC-H7	CD8	BD Biosciences	1µl	Surface

5.4.4. *Statistical analysis*

Statistical analyses were performed using Excel 14.2.5, Graph Pad Prism 5.0 and SAS/STAT 9.2. A Kenward-Roger Mixed Effects Regression analysis was used to evaluate associations between specific peptides, viral load, CD4 count and CD4 slope with monofunctional and dualfunctional responses. While the NLMIXED procedure, a nonlinear mixed model was used to evaluate associations between specific peptides, viral load, CD4 count and CD4 slope with polyfunctional responses expressing 3 or more functions.

5.5. Results

5.5.1. *Selection of Epitopes of interest for characterization*

Previously we conducted an unbiased fine epitope mapping study to assess the epitope specificity of HIV-specific CD8 T cell responses by measuring six CD8+ T cell functions (CD107a, IFNγ, MIP-1β, MIP-1α, TNFα, IL-2 and proliferation) in chronically HIV-infected individuals (327) (Chapter 4). We identified 54 unique epitopes within p24. The majority of identified epitopes (42) have not been fully characterized. Several epitope-specific responses were IFNγ negative (54/73), polyfunctional (33/73) and proliferative (13/73). These data suggested that specificity and function of HIV-specific CD8+ T cell responses differs depending on epitopes recognized. We also showed that the epitope-specificity differs between short and long-term assays, as well as between different short-term assays. We found that particular epitopes, in any given subject, often tended to elicit one type of CD8+ T cell response in preference to others. Our aim was to expand on these findings and determine if these results extended from the individual to the population.

Based on these results we selected eleven of the 54 epitopes for further characterization. We determined the chemokine and cytokine responses induced following stimulation, and the polyfunctional capacity of responding cells. Where possible we attempted to define the putative restricting HLA alleles for these epitopes (Table 5.3, Figure 5.1). The eleven epitopes of interest were selected based on the nature of the responses and number of responding subjects from our previous study. Eight of the selected epitopes have not been fully defined while three were BDE according to the Los Alamos National Laboratory HIV Database and have known HLA restrictions.

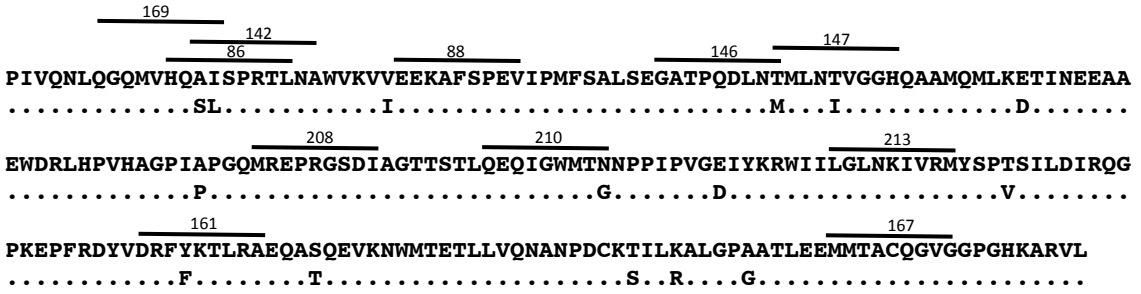

Figure 5.1. HIV p24 Epitope Map. Position of the eleven epitopes. The top sequence is Clade B; the bottom sequence is Clade A. Amino acid differences between the clades are indicated.

Table 5.3. Eleven epitopes studied and HLA associations

Peptide ID	Position (p24)	Epitope sequence	Los Alamos National Database (LAND)	HLA restriction (LAND)	Putative HLA restriction		
					NetMHCpan		Pearson Chi-Squared[b]
					Weak binding	Strong binding	
86	12-20	HQSLSPRTL	BDE	B*1510	B*1402, 1403, 3910, 4016	B*1406, 1503, 1510, 3924, 4805	A*290201
88	28-36	EEKAFSPEV	BDE	B*4415	B*1801, 3701, 4016, 4101, 4403, 4501, 4901		B*4201, 3924, C*0802, 1701
142	14-22	SLSPRTLNA	UN[a]		A*0202		A*0301, 290201, 3002, B*140201
146	46-54	GATPQDLNM	UN		B*1517		A*3601, B*140602,150201,4403, 4901, A*5101, 5701, C*0405, 1602
147	54-62	MMLNIVGGH	UN		A*0301, 2901, 2902, 3402, 8001 B*1503		A*290201, B*130201, 4901, C*0405
161	166-174	DRFFKTLRA	BDE	B*1402	B*7301		A*3009, 6801, B*3924, 8101, 1503
167	214-222	MMTACQGVG	UN				A*0101, B*1403, 8101, C*1801
169	7-14	QGQMVHQSL	UN				B*1503
208	96-104	MREPRGSDI	UN	A*2402, B*4402			
210	112-120	QEQIGWMTG	UN		B*4501		B*1503, C*0210, 030402
213	136-144	LGLNKIVRM	UN				

[a]UN=Uncharacterized

[b] Data based on studies CD8 + T cell responses

5.5.2. *Proliferative capacity of CD8 T cells in response to eleven epitopes of interest*

Our previous data revealed that CD8+ T cells from HIV infected individuals were unlikely to respond to the same epitopes with both a short-term cytokine/chemokine response and a long-term proliferative response. Therefore, we first sought to determine the ability of the eleven epitopes to elicit a proliferative response in a larger sample size. The proliferative capacity of CD8+ T cells following stimulation with each of the eleven HIV-1, clade A1 epitopes of interest was examined in 82 HIV infected individuals (Table 5.4). We performed six-day CSFE dilutions assays (Figure 5.2b) on subjects PBMCs in response to each of the eleven epitopes. Proliferative responses were relatively rare with only 16 of the 82 participants having a proliferative response to the epitopes. Of the 16 participants that had proliferative responses, the frequency of responses ranged from 1-4 responses per participant (mean =1.8 responses per participant). The highest number of responses was to peptide 161 with 6 participants responding, while peptides 146, 147 and 169 did not induce any proliferative responses (Figure 5.3). A proliferative response trended towards being more likely in response to peptide 88, 142 and 161 ($p=0.074$, Chi-Squared Goodness of fit). These data suggest that in addition to proliferative capacity being relatively rare, specific epitopes may be more likely to elicit proliferative responses than others.

Table 5.4. Demographic Data, HLA type and peptide responses of study participants

Subject	ART	CD4 Slope (yr)	CD4 count (cells/ml)	Viral Load (copies/ml)	Peptide Responses	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
199		0.393	258	6,332	88,142,146	02010101	3001	4201	570301	070101	1701
590	Yes	0.415	296	205	86,142,146,147,161,169, 210,213	290201	3002	1503	5301	020204	0405
987		2.034	200	N/A	88,147,208,213		4403	5702			
1054		0.416	521	29,934	88,142,147,161,167,169	03010101	7403	1402	8101	0802	04010101
1230		0.738	255	935	169,208,210,213	6802	7401	4703	5806	0602	070101
1298	Yes	-0.117	303	6,249	161						
1441		0.649	225	4,807	86,88,142,161	290201	3004	4201	4501	0602	1701
1490		0.958	127	N/A	86,146,161	7401	2301	4901	140602	070101	0802
1694		0.888	850	N/A	169,210	02010101	3001	5802	1503	020204	0602
1747		0.531	409	nd	142,167,210,213	0103	03010101	5801	5702	030201	1801
1778		0.213	602	nd	147,169,208	0202	6802	570301	1302	0602	070101
1812	Yes	0.718	266	152,834	88,161	02010101	0205	1503	5801	04010101	070101
1847		0.440	449	10,863	88,161	02010101	24020101	180101	3924	070101	070101
1852	Yes	-0.230	444	nd	146	0102	02010101	570101	5801	030201	070101
1857	Yes	0.317	118	< 40	142,167	02010101	6601	4501	530101	04010101	1601
1862		0.416	473	39,188	86,142	24020101	3004	1503	5301	040401	070101
1880		0.344	422	1,193,940	88,142,161	010101	7401	8101	1503	020204	1801
1904		1.378	225	< 40	161,169,213	2301	6802	140601	1503	07020101	0802
1946		1.327	261	42,066	88	6601	7401	5702	350101	04010101	1801
1973	Yes	0.797	260	1,472	88,142,161,167	0202	3009	5802	180101	0602	070401
1975		0.867	461	N/A	147,161,167	02010101	03010101	4703	1403	070101	0802
2003		0.699	306	N/A	88,167,210	010101	6802	8101	1510	030402	1801
2029	Yes	0.647	267	N/A	147,167	0103	02010101	4901	5702	0708	1801
2142	Yes	0.707	249	N/A	147,161,213	0205	24020101	4901	5801	070101	070101
2151	Yes	2.724	232	44	142,146	010101	03010101	3701	570301	0606	070101
2181		0.524	894	2,886	142	3001	310102	070201	5301	030401	0714
2186		2.118	200	33,057	146	0214	03010101	440301	5301	070101	120301
2203		-0.696	505	nd	88,161,167	010101	680101	070201	8101	07020101	1801
2218		2.667	163	126	142,147,161,167,169	0202	3002	0801	1503	020204	070101
2219		2.153	261	nd	88,146,147,210	3001	6802	4201	5802	0602	1701
2257		1.441	344	3,519	208,210,213			070201	180101	070401	150501
2264		1.708	146	65,748	88,208,210	2301	2301	570301	1503	020204	070101
2269	Yes	-1.998	444	16,042	169,208	03010101	3001	1503	1510	020204	04010101
2326	Yes	-0.557	298	N/A	167	010101	7401	4501	7301		

Subject	ART	CD4 Slope (yr)	CD4 count (cells/ml)	Viral Load (copies/ml)	Peptide Responses	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
2364		3.404	235	5,197	86,142,147,161,210	03010101	68020101	070201	1503		
2377		3.687	805	N/A	86,88,167	300101	3104	4201	440301		
2391		2.574	69	N/A	167,169,208,210	68020101	02010101	3910	1510		
2411		-0.050	255	N/A	142,147,161	02010101	300101	4201	1503		
2455		0.117	532	57,384	208	0202	0202	510101	570301		
2490	Yes	-0.881	334	N/A	208,210,213	02010101	3402	1503	350101		
2498		1.104	506	19,198	86,88,142,147,167,213	290201	300101	15170101	3910		
2509		2.192	1350	106	161,169,208	0102	300101				
2516	Yes	0.508	207	9,496	146	3601	2301	070201	530101		
2527		0.903	459	N/A	86,88,142,146,147,161						
2559	Yes	0.800	241	nd	86,88,161	6601	68020101	3910	3910		
2566		2.189	557	9,100							
2576	Yes	-4.901	413	nd	86,142,167	0214	8001	070201	180101		
2595		1.354	185	39,088	86,146,147,161	021010101	02010101	4016	510101		
2603		-0.448	485	< 40							
2604		3.057	116	N/A	86,88,208,213					0606	1403
2611		3.375	118	287	88,161,167,169,210			1503	1503	0210	0210
2624		1.012	371	46	208,210,213			5801	0709	070101	07020101
2628		1.116	289	nd	86,88,213						
2637		2.287	301	N/A	86,142,161,167	300201	310102	5801	180101	070101	0712
2638		1.993	192	8,179	88,142,161,167,208,210, 213	03010101	300101	3910	130201	06020101	120301
2643	Yes	0.296	356	12,801	213	29010101	2612	440301	4805	150501	04010101
2657	Yes	0.598	288	< 40	213	02010101	0214	1510	080101	030402	030401
2682		1.844	219	351	88,147,169	0205	02010101	5801	530101	070101	04010101
2700		-0.048	262	39,088	88,142,208	3010	0124	440301	1503	0210	06020101
2709	Yes	1.900	250	N/A	142,147,169	300201	02010101	5702	1503	0802	1801
2766		2.135	424	138,579	161			080101	150301		
2778		2.329	557	<40	86,88,208			270502	4016	020202	0802
2779		1.051	528	11,754	86,88,208	0103	2301	070201	140201	07020101	0802
2846		4.102	294	2,408	169			070201	570301	070101	07020101
2871		0.414	377	N/A	86,142,146,147,210			150201	440302		
2895		5.378	325	766	146,161,213			530101	5802		
2906		1.814	471	772	142,169			140201	530101		
2907		0.175	380	nd	86,88,142,213			140201	570301		
2917		4.768	513	nd	210			4703	530101		

Subject	ART	CD4 Slope (yr)	CD4 count (cells/ml)	Viral Load (copies/ml)	Peptide Responses	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
2925		3.004	484	< 40	213			070201	570201		
2927		6.823	309	< 40	88,146,161,210,213			150301	151001		
2948		4.358	380	< 40	161						
2968	Yes	-4.098	252	N/A	146,147,167			490101	5802		
3004		9.938	320	N/A	210,213			350101	4501		
3013		-0.080	326	N/A							
3037	Yes	N/A	236	N/A	86,88,142,147,167,169,213			130201	1803		
3054		3.616	300	N/A							
3073		N/A	1319	< 40	161			151001	3910		
3109		16.326	272	293							
3120		2.890	446	N/A	86,142,147,161,210,213			4501	5802		
3137		10.641	431	N/A	88,146,208			490101	510101		
3165		-0.465	1027	202,003	88,169			530101	5802		
3166	Yes	3.077	199	1,412	88,161,213			4501	5802		
3199		N/A	639	6,332	147,161			130201	5802		

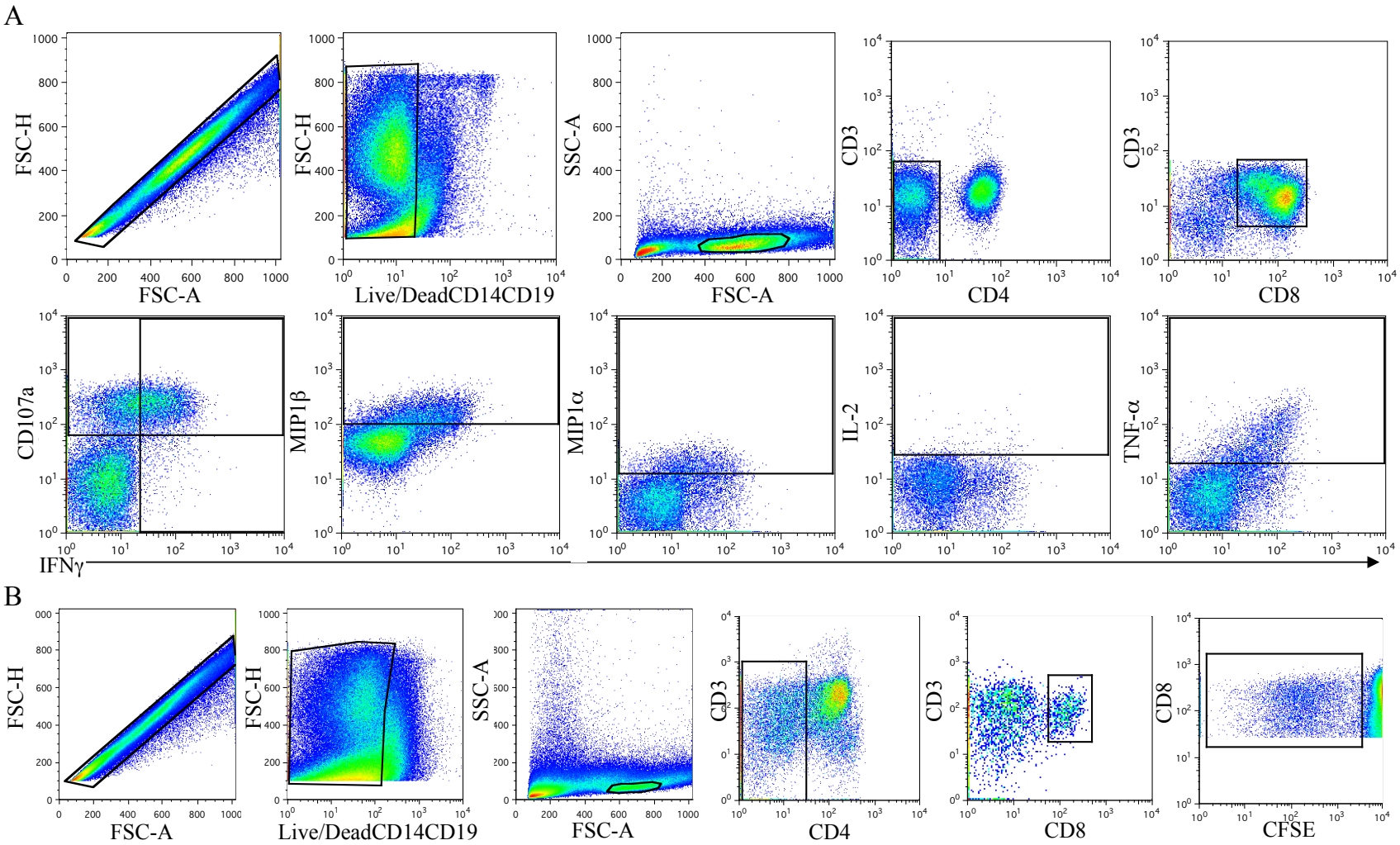
A
FSC-H
FSC-A
FSC-H
Live/DeadCD14CD19
SSC-A
FSC-A
CD3
CD4
CD3
CD8
CD107a
IFNγ
MIP1β
MIP1α
IL-2
TNF-α
B
FSC-H
FSC-A
FSC-H
Live/DeadCD14CD19
SSC-A
FSC-A
CD3
CD4
CD3
CD8
CD8
CFSE

Figure 5.2. Representative polyfunctional intracellular cytokine staining and six day CFSE dilution. A) Initial gating of forward scatter area (FSC-A) versus forward scatter height (FSC-H) was used to eliminated doublets. FSC-H versus pacific blue was used to gate out CD19+, CD14+ and dead cells. Side scatter area (SSC-A) versus FSC-A was used to identify lymphocytes. After gating on CD3+ CD8+ cells, further gates were made for each of the respective functions (IFN-γ, MIP-1β, MIP-1a, CD107a, TNF-α, and IL-2). Boolean gates were applied to the 6 overnight functions resulting in 64 possible CD8+ T cell responses combinations. B) Initial gating of forward scatter area (FSC-A) versus forward scatter height (FSC-H) was used to eliminated doublets. FSC-H versus pacific blue was used to gate out CD19+, CD14+ and dead cells. Side scatter area (SSC-A) versus FSC-A was used to identify lymphocytes. After gating on CD3+ CD8+ cells, proliferating cells were identified by gating on CSFE low cells.

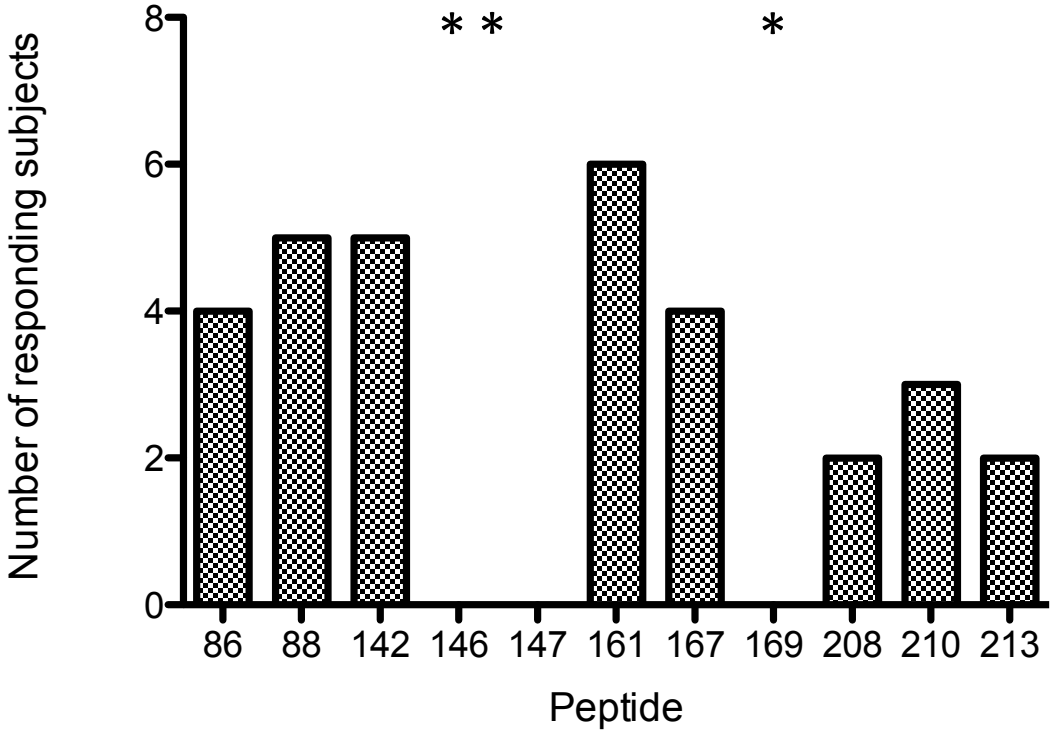

Figure 5.3. Proliferative capacity of CD8+ T cells in response to eleven epitopes of interest. The number of subjects with a CD8+ T cell proliferative response following stimulation with each peptide. No statistical significance was observed. * indicates trend p=0.074, Chi-Squared Goodness of fit.

5.5.3. *Epitope Characterization measuring five CD8+ T cell functions*

We next wanted to determine if specific cytokines or chemokine responses were more likely to be elicited by particular epitopes. HIV-specific CD8+ T cell responses were determined after measuring IFNγ, CD107a, MIP-1β, MIP-1α, IL-2, and TNFα production following overnight stimulations with each of the 11 epitopes. Representative intracellular staining is shown in Figure 5.2a. Seventy-eight of the 82 participants responded to at least one of the eleven epitopes with at least one overnight function. Unlike proliferation responses overnight cytokine and chemokine responses were more frequent with IL-2 and IFNγ responses being most frequent with a total of 77 and 76 responses from the 82 participants, respectively. While 54 participants had CD107a responses, 47 MIP-1β responses, 41 TNFα responses and lastly 40 MIP-1α responses were identified. Of the participants that had a functional response the frequency ranged from 1-7 peptides responses per participant (mean = 2.8 responses per participant). Similar to our previous findings, proliferative capacity was most often observed in the absence of corresponding cytokine or chemokine expression; of the 31 proliferation responses 26 did not have a corresponding cytokine or chemokine response. Of the eleven epitopes, peptide 161 elicited the most responses from 27 of 82 participants, while peptides 146 and 208 each induced 15 out of 82 responses. Across the data set, the mean number of responses was 19.5 (range = 13-27 responses per peptide). When testing if a particular peptide was overrepresented in relation to specific functions no statistically significant differences were seen. For example of the 76 IFNγ responses no peptide was significantly more likely to have induced the response ($p=0.1$, Chi-Squared Goodness of fit, Data from remaining functions not shown). However, when testing if a particular

peptide was more likely to induce specific functions, associations were seen. Of the 19 total cytokine/chemokine responses to peptide 147, 7 were CD107a (35%). Peptide 147 trended toward being more likely than the other epitopes to elicit a CD107a (Figure 5.4a, $p=0.068$, Chi-Squared Goodness of fit). Of the 17 total cytokine/chemokine responses to peptide 169, 10 were IL-2 (53%). Peptide 169 was significantly more likely than the other epitopes to elicit an IL-2 response, than any other function (Figure 5.4b, $p>0.001$, Chi-Squared Goodness of fit). Similarly, of the 31 total cytokine/chemokine responses to peptide 88, 12 were MIP-1β (48%). Peptide 88 was significantly more likely than the other epitopes to elicit an MIP-1β response, than any other function (Figure 5.4c, $p>0.001$, Chi-Squared Goodness of fit). Together, these data suggest that in a large sample size specific epitopes preferentially induce distinct effector functions.

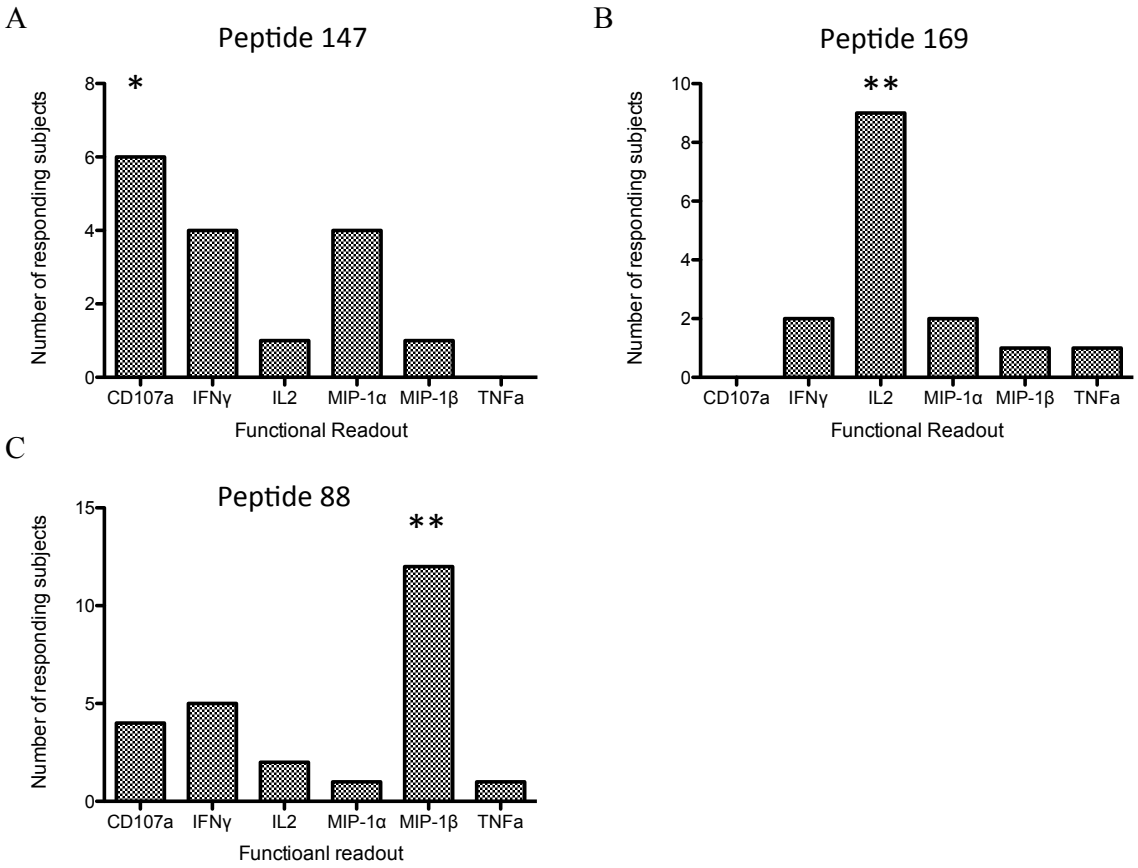

Figure 5.4. Specific epitopes preferentially induce distinct effect functions. The number of subjects responding to peptide A) 147 B) 169 C) 89 with each CD8+ T cell function, CD107a, IFNg, IL2, MIP-1α, MIP-1β and TNF-α. * indicates trend, ** indicates significance, p>0.01, Chi-Squared Goodness of fit).

5.5.4. *Epitope Characterization measuring responding CD8+ T cell Polyfunctionality*

We next wanted to determine if particular epitopes were more likely to elicit multiple cytokine and chemokine responses by examining bulk polyfunctional responses and polyfunctional responses analysed using a Boolean gating approach. First we evaluated bulk polyfunctional responses and secondly we assessed the concurrent polyfunctional expression (Figure 5.5). Seventy-six bulk polyfunctional responses were detected across the data set. Seventeen of these responses were detected after stimulation with the peptide 88 (17/76). Thus, peptide 88 was significantly more likely to elicit a multifunctional response than any of the other epitopes (Figure 5.6, p=0.025, Chi-Squared Goodness of fit). This suggested that distinct epitopes are also more likely to induce potentially protective polyfunctional responses. However, at this stage it is not clear if the polyfunctionality is due to the co-expression by different cells or the concurrent expression by a single cell.

We next wanted to determine the specific polyfunctional nature of the peptide specific responses, using the Boolean gating approach. Boolean gating allows the evaluation of the concurrent expression of multiple cytokines, where one cell is responsible for the secretion of 2 or more effectors (Figure 5.5). With this approach we are able to detect the full array of possible combinations of cells expressing 2, 3, 4, 5 and 6 functions, equating to 64 response combinations when testing 6 functions. The polyfunctional characteristics of peptide 88 are shown (Figure 5.6) as a representative example of the complexity of the CD8+ T cells responses.

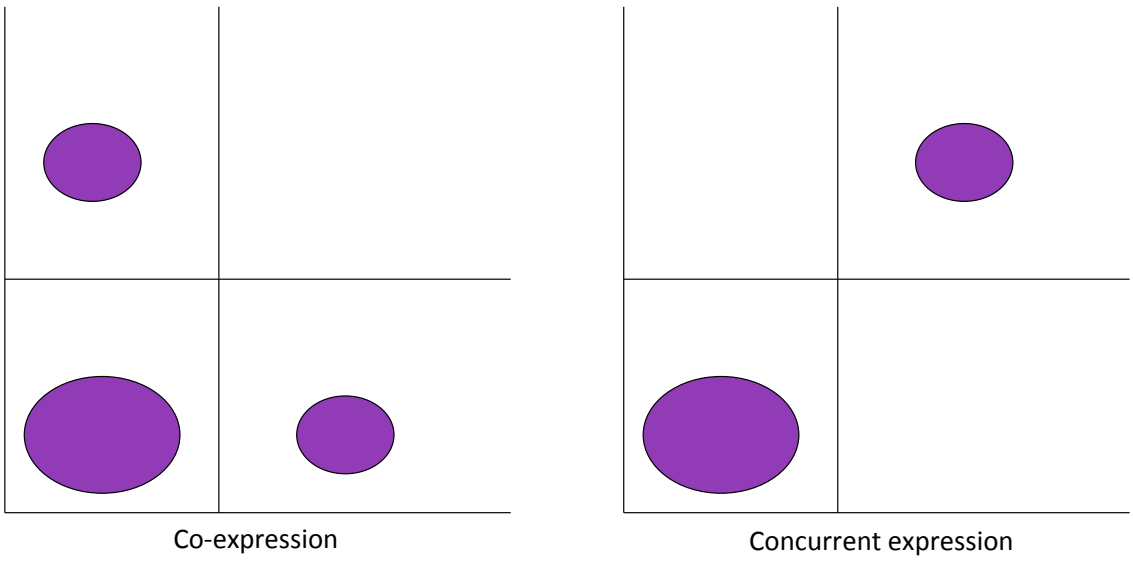

Figure 5.5. Cartoon representing the various analyses of polyfunctionality. Bulk polyfunctional responses include both co-expression, the simultaneous expression of two or more functions by different cells and the concurrent/simultaneous expression of two or more functions by the same cell. Boolean gating is used to demonstrate concurrent polyfunctionality.

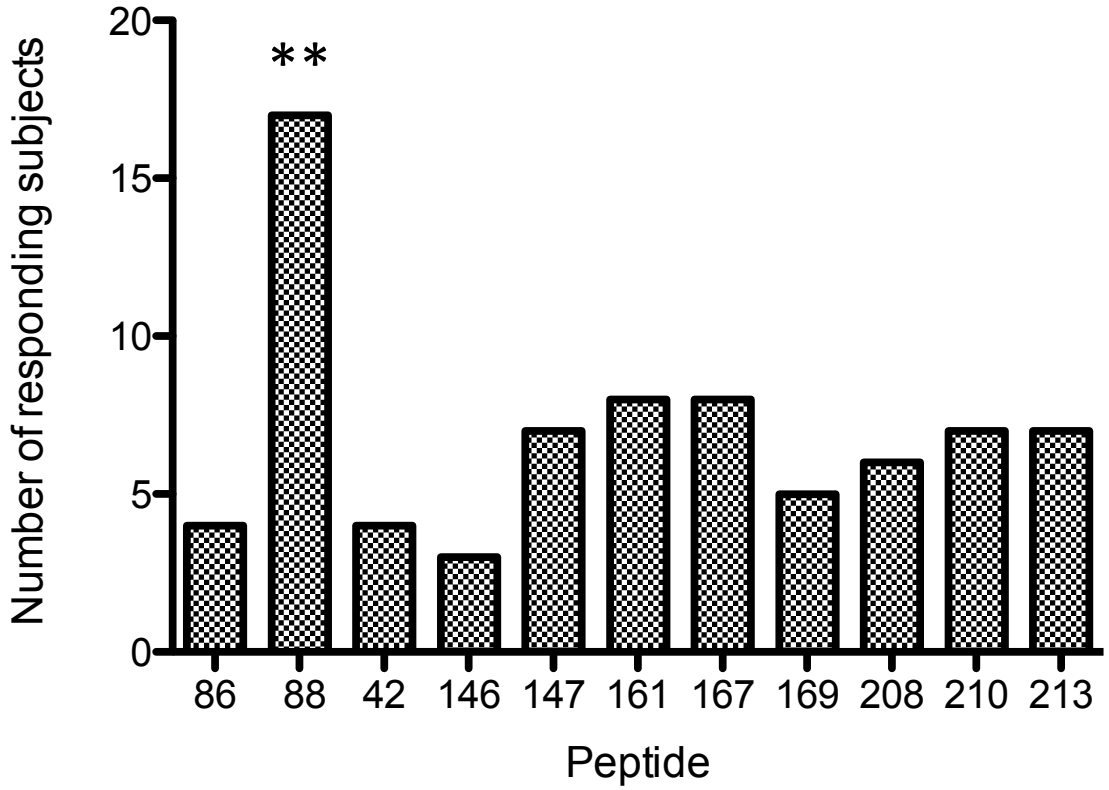

Figure 5.6. Epitope characterization measuring bulk CD8+ T cell polyfunctional responses. Peptide 88 was significantly more likely to elicit a multifunctional response than any of the other epitopes (p=0.025, Chi-Squared Goodness of fit). The number of subjects with a bulk CD8+ T cell polyfunctional response following stimulation with each peptide. * indicates significance, p=0.025, Chi-Squared Goodness of fit.

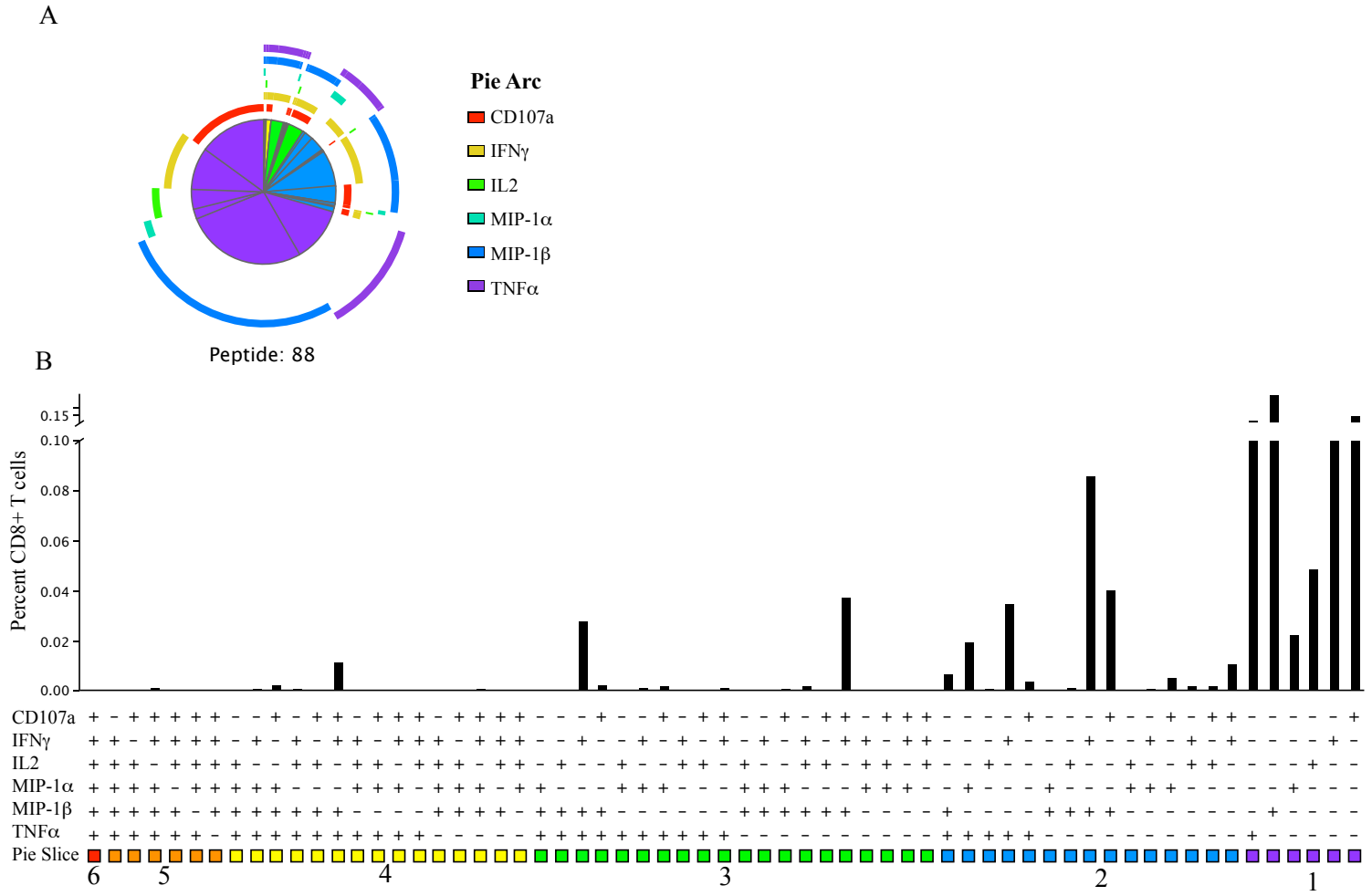

Figure 5.7. Polyfunctional profile of peptide 88. A) Pie chart showing relative percentage of cells responding with each level of polyfunctionality. Arc data indicates functional combination of response. Pie slices indicate 1+ (purple), 2+ (blue), 3+ (green), 4+ (yellow), 5+ (orange) and 6+ (red). Functions measured were CD107a (red), IFNγ (yellow), IL2 (green), MIP-1a (turquoise), MIP-1β (blue) and TNFα (purple). B) Absolute percent of CD8+ T cells responding with each of 32 possible functional combinations.

To simplify the analysis responses were grouped by number of functions, including 1, 2, 3, 4, 5, and 6 functions, regardless of what these functions were. Once the responses were grouped by function we next evaluated if any of the peptides were more likely to elicit a particular type of response. All peptides were equally likely to elicit a mono-functional response (Table 6.5, p=0.3212, Mixed Effects Regression-Kenward-Roger). However, when comparing dual-functional responses with any other responses, significant differences among the peptides were observed (Table 6.5, p=0.0001, Mixed Effects Regression-Kenward-Roger). To determine which peptides were responsible for the differences, post-test analyses were carried out (Table 6.6, Differences of Least Square Means) and revealed that peptide 88 was significantly more likely to elicit a dual-functional response compared to all other peptides.

Table 5.5: Specific peptides are associated with dual-functional CD8+ T cell responses but not mono-functional responses

Response	Effect	p value[a]
1+	**Peptide**	0.3212
2+	**Peptide**	**0.0001**

a Mixed Effects Regression-Kenward-Roger

Table 5.6: Specific peptides are more likely to induce dual-functional CD8+ T cell responses than others

Peptide	Peptide	p value[a]
88	**86**	0.0009
88	**142**	<0.0001
88	**146**	<0.0001
88	**147**	0.0009
88	**161**	0.0366
88	**167**	0.0296
88	**169**	0.0002
88	**208**	<0.0001
88	**210**	0.0139
88	**213**	0.0144
142	**161**	0.0317
142	**167**	0.0391
146	**161**	0.0093
146	**167**	0.0120
146	**210**	0.0255
146	**213**	0.0237
167	**208**	0.0317

[a]Only statistically significant differences are shown; Mixed Effects Regression-Kenward-Roger analysis

We next sought to determine if higher levels of polyfunctionality would also associate with a particular peptide. Higher levels of polyfunctional responses (3+, 4+, and 5+) were rarer and required more complex analysis. First, we used a NLMIXED logistics regression analysis to compare whether one peptide is more likely to have at least one 3+ response compared to any other peptide. This logistic analysis revealed that no peptide was more likely elicit a 3+ response than another peptide (Table 5.7, p=0.9153, NLMIXED Procedure, logistics = 3+ response yes or no). In other words across the entire data set each peptide had at least one 3+ response. However, when we tested whether one peptide was more likely to induce multiple 3+ responses, there were significant differences (Table 5.7, p=0.0065, NLMIXED Procedure, linear = how many 3+ responses per peptide). Again, post-test analysis revealed that peptide 88 was significantly more likely than any of the other peptides to elicit multiple 3+ responses (Table 5.6). No differences were found with higher levels of polyfunctionality (4+ and 5+, 6+ could not be tested due to limited number of responses), likely due to the rarity of these responses.

Table 5.7: Specific peptides are associated with tri-functional CD8+ T cell responses but not 4+ or 5+ responses

Response	Peptide Omnibus	p value[a]
3+	**Logistics**	0.9153
	Linear	**0.0065**
4+	**Logistics**	0.2794
	Linear	0.2093
5+	**Logistics**	0.2419
	Linear	0.4828

[a] NLMIXED logistic regression

Table 5.8: Peptide 88 induced more Tri-functional CD8+ T cell responses than any other peptide

Effect	p value[a]
86	0.0022
142	0.0028
146	0.0002
147	0.0006
161	<0.0001
167	0.0366
169	0.0004
208	0.0002
210	0.0069
213	0.0055

[a] NLMIXED logistic regression

We next evaluated the role of peptide-specific polyfunctionality in disease progression. Again, the analyses of lower and higher levels of CD8+ T cell polyfunctionality were compared using different statistical methods. Using a Mixed Effects Regression-Kenward-Roger procedure, no correlation was observed between either peptide-specific mono-functional or dual-functional CD8+ T cell responses and CD4 counts, CD4 slope, ART or viral loads (data not shown). Similarly, no association was observed between higher peptide-specific polyfunctional CD8+ T cell expressing 3, 4 and 5 functions and CD4 counts, CD4 slope, ART or viral load (NLMIXED procedure, data not shown). The lack of association with these measures of disease progression is likely do to the high variability between individuals' responses to the 11 epitopes (i.e. some epitopes elicit low polyfunctionality while others elicit higher levels of polyfunctionality).

5.5.5. *Characterization of putative restricting HLA*

We next sought to investigate the putative restricting alleles of the eleven epitopes. These data will be important for future charcterization of these epitopes. Initially, HLA peptide binding predictive software analyses was used to identify the potential restricting HLA. The NetMHCpan server (Center for Biological Sequence Analysis, Technical University of Denmark) can be used to predict binding of peptides to any known HLA. This analysis revealed numerous possible HLA restrictions for 7 of the 11 epitopes (Table 5.1). Most of these potential HLAs were predicted to be weak binding, though peptide 86 was linked with a number of HLA alleles (HLA B*1406, 1503, 1510, 3924, 4805) that were predicted to bind strongly to the epitope. One caveat of this approach is that it is not specific for the HLAs that are present within our study population. Therefore, we next performed analysis based on our CD8+ T cell responses and the

participants known HLAs. HLA typing was performed using a high resolution, sequence-based method (312). Pearson chi-squared analysis was performed for each peptide focussing on all HLA alleles associated with detectable functional responses (Table 5.1). Multiple HLA associations were observed, for example peptide 86 was associated with HLA-A*290201 and peptide 169 was associated with HLA-B*1503. These associations, however, must be taken with caution, as our study was not significantly powered for this analysis. This analysis does, however, demonstrate that with higher sample numbers putative restricting HLA alleles could be determined, which will be imperative for future epitope charcterization including tetramer studies.

5.5.6. *Characteristics of Peptide 88*

Our data suggests that peptide 88 may be an important epitope for the immune system to recognize a protective cell-mediated immune response to HIV infection. Peptide 88, or EEKAFSPEV (EV9), is a BDE according to the Los Alamos HIV Database and is restricted by HLA-B*4415. Although the HLA-B*4415 allele is relatively common (but not prevalent in this cohort at a frequency of 2.1%) (340), this allele was not present in these study participants. There were 30 individuals that responded to this epitope and HLA associations from our data suggests that HLA-B*4201, 3924, C*0802, 1701 may be additional restricting alleles ($p<0.05$). The grouped polyfunctional responses to epitope EV9 across the whole data set are shown in Figure 5.6. The high polyfunctional nature of CD8+ T cells responses to epitope 88 can be seen both the 2+ and 3+ categories with responses comprising 11 of the 15 possible 2+ combinations and 7 of the 20 possible 3+ combinations. Unfortunately, neither CD4 counts nor CD4 slope or viral loads associated

with responses to peptide 88 (data not shown), possibly due to sample size. Future characterization of peptide 88 will need to be conducted to determine its association with slower disease progression; however, our data suggests that this epitope is associated with polyfunctional and proliferative CD8+ T cell responses.

5.6. Summary

Studies have demonstrated that HIV+ LTNP maintain a more functional response than those who progress to AIDS (193, 216), but whether these responses differ in specificity remains unknown as is the nature of the epitopes generating these protective responses. Here we have selected eleven HIV-1 Clade A p24 epitopes of interest, which were previously identified during our comprehensive fine epitope mapping study (327), for further characterization using multiple overnight parameters and long-term proliferation assays. Charcterization of these eleven epitopes using multiple cytokines/chemokines and proliferation, extend the evidence from Chapter 4 and further reveal that particular epitopes are significantly more likely to stimulate unique responses at a cohort level.

Screening for responses by the measurement of six CD8+ T cell functions revealed a greater complexity of CD8+ T cell immunology. Our data suggests that peptides 147 and 161 maybe more likely to induce a proliferative CD8+ T cell responses than the other peptides, while peptides 147 and 169 were associated with CD107a and IL-2 expression, respectively. Importantly, peptide 88 was associated with MIP-1β, proliferation and polyfunctional CD8+ T cell responses. These CD8+ T cell responses have all been associated with slower HIV disease progression, suggesting that peptide 88 maybe an important epitope to target for an effective immune response.

5.7. Implications

The implications of these results are that specific epitopes can be identified that can induce distinct functional responses and can be identified on a cohort level. This suggests there is potential to identify specific epitopes that elicit protective polyfunctional and/or proliferative CD8+ T cell responses. Such 'protective' epitopes could be incorporated into vaccine design to express distinct CD8+ T cell epitopes and induce an early and effective CD8+ T cell response. Potentially, these early polyfunctional and proliferative CD8+ T cell responses would steer the immune system towards a more protective response able to control or eliminate viral replication.

6. Chapter 6. Evolution of polyfunctional and proliferative CD8+ T cell responses from early to chronic HIV-1 infection

6.1. Rationale

HIV-specific CD8+ T cells during acute infection play an important role in controlling viral replication leading to a significant reduction in viremia and development of robust CD8+ T cell memory. Unfortunately, in all cases these CD8+ T cells are unable to completely clear the virus and a persistent chronic infection develops. Under chronic antigen pressure HIV-specific CD8+ T cells gradually lose their ability to control infection leading to, or due to, the loss of proliferative capacity and functional capacity. Currently, it is not known how some individuals are able to maintain strong polyfunctional and proliferative capacity well into chronic infection. HIV-infected LTNP and EC maintain stronger HIV-specific CD8+ T cell proliferation and polyfunctionality during chronic infection compared to progressing controls (214, 216). However it is unclear whether these CD8+ T cell response characteristics are responsible for their better disease outcomes or if these CD8+ T cell responses are a consequence of the individuals' high CD4+ T cell counts and low viral loads which has developed as a result of protection by some other mechanism.

Therefore, we performed a cross-sectional and longitudinal analysis of polyfunctional and proliferative CD8+ T cell responses in patients in the early to chronic phase of infection in order to shed light on this question. This is one of the first studies to examine polyfunctional CD8+ T cell response evolution in the same individual from early HIV infection through to chronic infection.

6.2. Hypothesis

We hypothesized that CD8+ T cell responses will initially be highly polyfunctional and proliferative in early infection and as HIV disease progresses, polyfunctionality and proliferative capacity will wane. We also hypothesised that patients that mount and maintain a polyfunctional and proliferative response will have a better disease outcome.

6.3. Objective

To assess the evolution of CD8+ T cell polyfunctional and proliferative responses to HIV-1 p24 peptides, from early to chronic HIV-1 infection.

6.4. Methods

6.4.1. *Subjects*

Study participants (n=20) were all HIV infected and enrolled in the Majengo cohort (Table 6.1). Only participants whose seroconvertion dates could be estimated were enrolled in the study, using the following formula:

$$Days\ post\ infection = Last\ negative\ date + (last\ negative\ date - \frac{first\ positive\ date}{2})$$

Patients were categorised into those in Early (E, n=6, <180 days post-infection), Early-Chronic (EC, n=6, >130 - <365 post-infection) and Chronic (C, n=8, >365 days post-infection) infection. PBMCs from patients in each group were analysed for polyfunctional and proliferative responses as described previously. Additionally, for some patients we were able to obtain samples from multiple time points spanning different stages of infection (Table 6.1). CD4 slope was calculated as follows and is described in the text as decline/year:

$$CD4\ slope\ (\frac{(cells/ml)^2}{year}) = \frac{(CD4\ count\ at\ TPn)^2 - (CD4\ count\ at\ TP1)^2}{TPn - TP1/365}$$

6.4.2. *In vitro stimulation*

Frozen PBMCs were stimulated in a batch to eliminate variation in analysis (although unfortunately a small selection of fresh PBMC samples were stimulated as they were collected prior to the decision to use frozen samples). Peptides were 9mers overlapping by 8aa and pooled into sequential order totalling 6 pools of approximately 30 peptides per pool. Epitope specificity is not evaluated in this study; rather we were interested in the total p24 response. The peptides were used at 2μg/ml, and stimulations were accompanied by 2 positive controls, CEF and SEB, and duplicate negative controls consisting of media alone. For ICS cells were incubated for 14 hours as per general stimulation methods (section 2.2.3), while for proliferation assays cells were incubated for 6 days.

6.4.3. *Flow cytometry*

Immediately following stimulation, PBMCs were washed with PBS containing 2% FCS, and stained using the panels described below (Table 6.2 and 6.3) following general surface staining (section 2.2.4.1, with LIVE/DEAD), intracellular cytokine staining (section 2.2.4.2) and CFSE dilution assay protocols. Stained cells were immediately analyzed on a LSRII flow cytometer (BD Biosciences) as per general methods section 2.2.4.6.

Table 6.1: Subjects' disease status and demographic data

ID		TP 1			TP 2			TP 3			TP 4			TP 5			TP 6			TP 7		
	DS	dpi	T4	VL	dpi	T4	VL	dpi	T4	VL	dpi	T4	VL	dpi	T4	VL	dpi	T4	VL	dpi	T4	VL
1266	E	79	650		299		16042	439	641		590			797	285	33,057	960	339				
2378	E	116	776		446	N/A		1018	1056													
2453	E	117	N/A	9496	205	205																
2552	E	125	N/A		729	650																
2687	E	77	945		305	1116		369			581			801	341	398	976	335		1207	342	351
3317	E	57	727	1937854	497	296	42066															
2039	EC	240	522		966	157	138579															
2487	EC	269	881		421	N/A																
2785	EC	297	446																			
2862	EC	190	979		692	524		745	506	106												
3126	EC	212	120		453 (ARV)	N/A																
3160	EC	189		1472		223	65748															
1529	C	397	611		778	326		543	114													
2008	C	1303	N/A		1518	234		471	296	39088	1958	394	6332									
2114	C	1393	332																			
2139	C	809	467		1195	368		671 (ARV)	259 (ARV)		2023 (AVR)	N/A										
2325	C	616	965	205																		
2484	C	865	555																			
2608	C	474	475		667 (ARV)	293 (ARV)		575 (ARV)	526		1475	1050										
3164	C	490	524	9100	671	408	29934															

Abbreviations: ID= Subject ID, TP= Sampling Time point, DS= Disease status, dpi= Days post infection, T4= CD4+ T cell Count (cells/ml) and VL= Viral load (RNA copies/ml)
ARV indicates subjects that were on treatment at time of sampling

Table 6.2: Panel of fluorochrome-conjugated antibodies to assess CD8+ T cell polyfunctionality

Fluorochrome	Marker	Source	Volume used	Surface/ICS/Dump Channel
FITC	IL-2	BD Biosciences	3µl	ICS
PE	MIP-1β	BD Biosciences	3µl	ICS
PE-CY5	CD107a	BD Biosciences	5µl	ICS
PE-CY7	IFNγ	BD Biosciences	3µl	ICS
ECD	CD4	Beckman Coulter	1µl	Surface
AmCyan	CD3	BD Biosciences	1µl	Surface
AlexaFluor 700	TNFα	BD Biosciences	3µl	ICS
eFlour450	CD14,CD19	eBiosciences	1µl	Dump Channel
Violet laser	Live/Dead	Invitrogen	6.25µl	Dump Channel
APC-H7	CD8	BD Biosciences	1µl	Surface

***** MIP-1α was not included as some early time points were collected and analysed prior to inclusion of this marker into the panel.

Table 6.3: Panel of fluorochrome conjugated antibodies to assess CD8+ T cell proliferation

Fluorochrome	Marker	Source	Volume used	Surface/Proliferation/Dump Channel
Blue laser	CFSE	Invitrogen	1µl/ml	Proliferation
PE-Cy7	CCR7	BD Biosciences	3µl	Surface
ECD	CD45RA	Beckman Coulter	3µl	Surface
AmCyan	CD3	BD Biosciences	1µl	Surface
AlexaFluor 700	CD27	eBiosciences	3µl	Surface
eFlour450	CD14,CD19	eBiosciences	1µl	Dump Channel
Violet laser	Live/Dead	Invitrogen	6.25µl	Dump Channel
APC-H7	CD8	BD Biosciences	1µl	Surface

6.4.4. *Statistical analysis*

Data analysis was performed using FlowJo 9.2 (TreeStar). Statistical analyses were performed using SPICE 6.0, Graph Pad Prism 6.0 and SAS/STAT 9.2. HIV-specific Bulk CD8+ T cell responses had to be at least 2-fold greater than background to be considered a positive response. The change in in relative polyfunctionality was analysed via permutation comparisons while the change in proliferative capacity was analysed via one-way ANOVA.

6.5. Results

6.5.1. *Cross-sectional analysis of CD8+ T cells responses during early, early-chronic and chronic infection reveal a distinct response pattern*

HIV-specific CD8+ T cell responses to p24 peptide pools were evaluated in 20 HIV infected individuals with known seroconversion date (Table 6.1) by simultaneously measuring IFNγ, CD107a, MIP-1β, IL-2 and TNFα production following overnight stimulation and in six day proliferations assays. Pool-specific CD8+ T cell responses were detectable for all five effector functions and proliferation and all participants responded to all pools with at least one overnight functional readout. To evaluate if polyfunctionality changes over the course of HIV infection we initially performed cross-sectional analysis spanning different statuses of HIV disease (Table 6.1). For patients with multiple samples available only the initial sampling point was included in analysis, i.e. TP1. After Boolean gating and stratification by disease phase (Early n=6, Early-Chronic n=6 and Chronic n=8), we observed a higher degree of CD8+ T cell polyfunctionality in subjects in the early and chronic phase of infection compared to the

intermediate early-chronic phase (p=0.0375 and 0.0045 respectively; permutation computation, Figure 6.1a). Overall, early and chronic responses are more polyfunctional regardless of the combination of the type of response. As can be seen in Figure 6.1a, CD8+ T cells from individuals in early and chronic phase of infection respond with higher levels of 2 (green), 3 (yellow), and 4 (orange) responses. Responses with 5 functions are generally rare and differences between the groups were not observed in these figures. When we examined proliferative capacity cross-sectionally between study groups, we found that CD8+ T cells from individuals in the early and chronic phases of infection have lower proliferative capacity than the early-chronic phase (p=0.003, one-way ANOVA, Figure 6.2b). Taken together these data suggest that that something important is occurring in the early-chronic phase of infection.

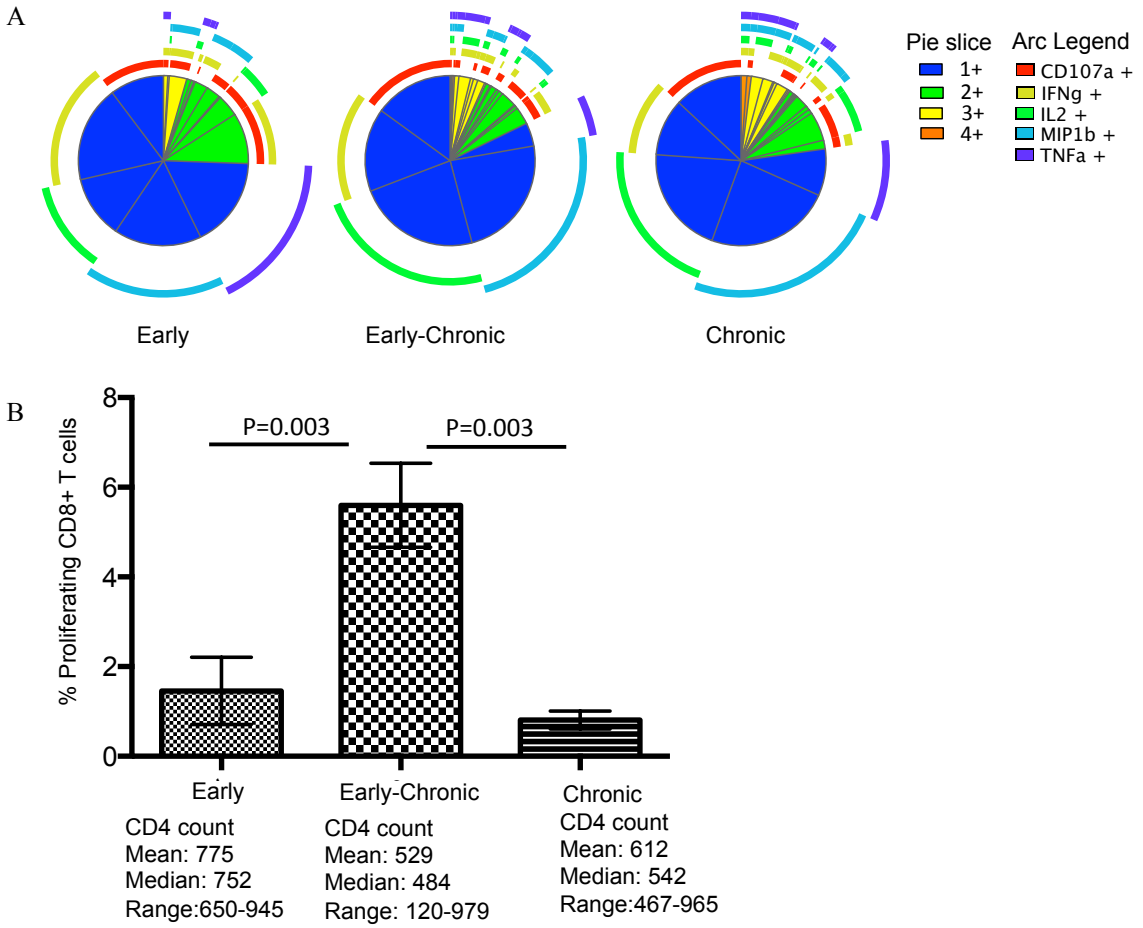

Figure 6.1. Cross-sectional analysis of CD8+ T cell polyfunctional and proliferative responses. A) Polyfunctional CD8+ T cell responses in early (n=6), early-chronic (n=6) and chronic (n=8) infection. Significant differences were observed between CD8+ T cells from participants in early and chronic infection with CD8+ T cells from participants in early-chronic infection (p=0.0375 and 0.0045, respectively; permutation comparisons). Pie slices indicate the level of polyfunctionality and the arc legend indicates specific functionality. B) Proliferative capacity of CD8+ T cell in early, early-chronic and chronic infection. Significant differences were observed between CD8+ T cells from participants in early and chronic infection with CD8+ T cells from participants in early-chronic infection (p=0.003, One-way ANOVA). Each patient was only represented once and only data from their initial sampling (TP1) was included.

We next sought to examine if the nature of CD8+ T cells polyfunctionality during the different stages of infection associated with slower disease progression. Using spearman ranked correlation, we correlated the percentage of CD8+ T cells responding at each level of functionality (1+, 2+, 3+, 4+ 5+ and >2+) from subjects in early, early-chronic and chronic infection, with their CD4 count and CD4 slope. We were unable to see any association between polyfunctionality in early, early-chronic or chronic phase of infection and the CD4 count or CD4 slope. Analysis of proliferative responses in early infection suggests that proliferative capacity is universally low regardless of CD4 count or CD4 slope (Figure 6.2b). Similar findings were obtained when we analysed the percentage of proliferating CD8+ T cells in early-chronic or chronic infection and CD4 count or CD4 slope. It is possible that the lack of association of CD8+ T cell polyfunctionality and proliferation is the result of low sample numbers, rather than a true lack of connection.

Although statistically we did not find any associations with polyfunctionality it was quite evident at the individual level that some individuals with increased polyfunctionality appeared to have a slower CD4+ T cell decline. This was most evident in early infection. For example, participants with higher polyfunctionality (1266 and 2378) in the early phase of infection seemed to have a have slower rate of CD4+ T cell decline (0.776 and 0.969 CD4 decline/year, Figure 6.2a). Conversely, participants with lower or moderate polyfunctionality (2687 and 3317, respectively) seemed to have a more rapid rate of CD4+ T cells decline, 3.406 and 5.249 CD4 decline/year, respectively). These data suggest that early polyfunctionality may be associated with slower CD4+ T cell decline and better disease outcomes, and that larger, appropriately powered studies are needed.

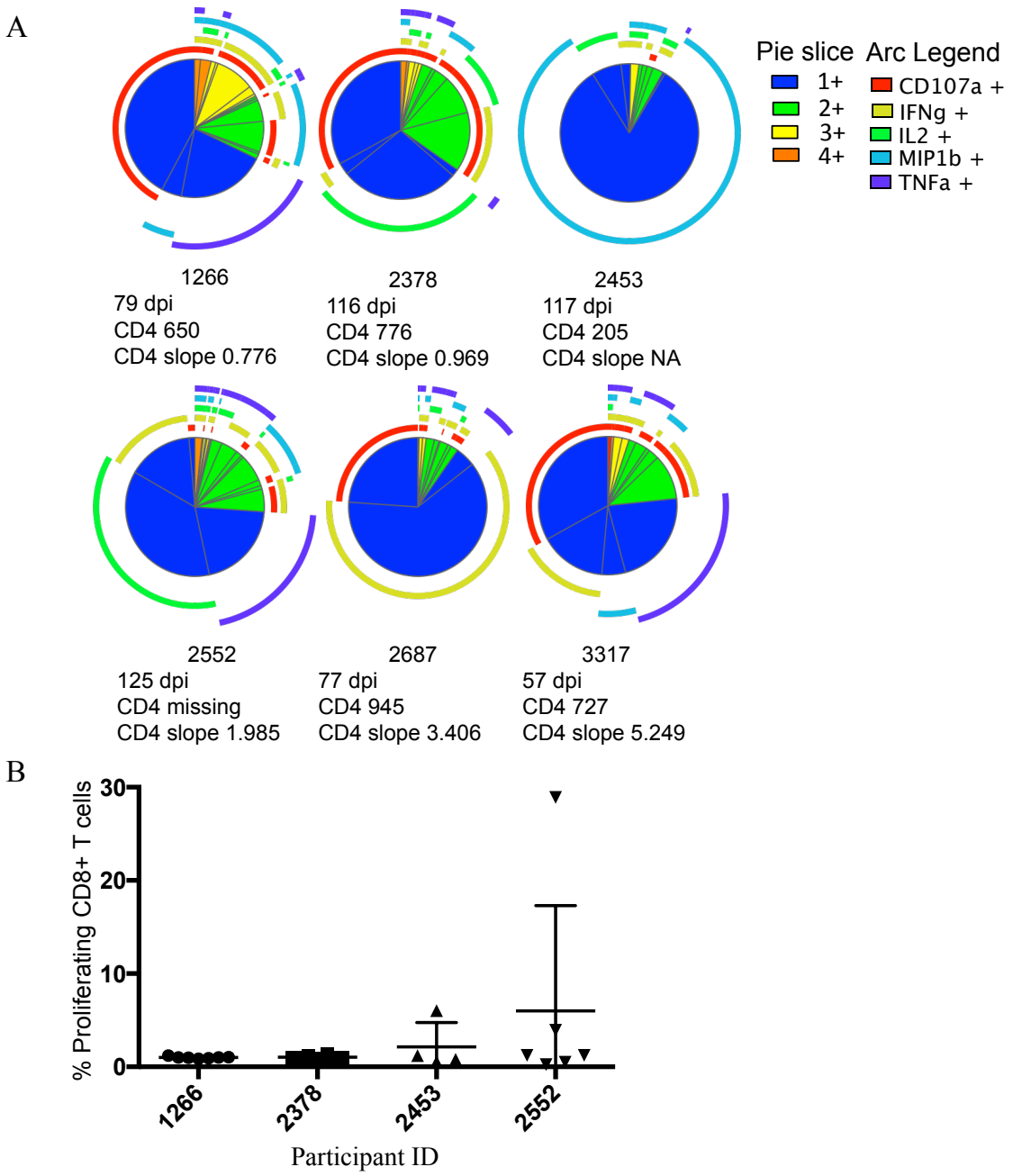

Figure 6.2. Cross-sectional analysis of CD8+ T cells in early infection. A) Polyfunctional profile of CD8+ T cells from individuals in early infection; Days post infection (dpi), CD4 counts, and CD4 slope are shown for each participant. Pie slices indicate level of polyfunctionality and the arc legend indicates specific functionality. B) Proliferative capacity of CD8+ T cell in early infection. The percent of proliferating CD8+ T cells from individuals in early infection. Mean values and Standard Deviation are indicated on the graph.

6.5.2. *Longitudinal analysis of CD8+ T cell responses over time*

We next set out to describe if the polyfunctionality and proliferative CD8+ T cell responses differ longitudinally following seroconversion into chronic infection. The advantage of conducting these kinds of studies in this well-established cohort is that participants keep coming into the clinic, which provides multiple sampling points from the same participant (Table 6.1). Subjects 1266 and 2687 provided a unique opportunity to follow CD8+ T cell responses over time within the same individual. Similar to the data obtained from the cross-sectional analysis, polyfunctionality was highest in patient 1266 during early infection, but declined substantially during the early-chronic phase of infection and appeared to rebound 590 days post-seroconversion, well into the chronic phase of infection (Figure 6.3). This validated the findings from the cross-sectional analysis. Interestingly, subject 1266 had the slowest rate of CD4+ T cell decline of all participants monitored in this study (0.776 CD4 decline/year). Conversely, subject 2687 had the most rapid CD4+ T cell decline (5.249 CD4 decline/year) of all participants monitored in this study (Figure 6.4). Interestingly, her early CD8+ T cell response was one of the least polyfunctional. Taken together these observational data infer that mounting an early polyfunctional response may lead to slower CD4+ T cell decline and better disease outcome, however, further studies are necessary.

Similarly, we observed distinct patterns in the proliferative capacity of CD8+ T cells examined longitudinally. Mirroring the cross-sectional data, both 1266 and 2687 had the highest proliferative response during their early-chronic phase of infection (although limited 2687 cell numbers at TP1 prevented acquisition of proliferation data). In

addition, similar to the cross-sectional data, longitudinal analysis of proliferative capacity suggests that a re-organization of the varieties of CD8+ T cell responses is occurring in the early-chronic phase of infection and may be playing an important role in establishing and balancing the quality of the CD8+ T cell response. This is perhaps due to a rapid expansion of effector CD8+ T cells with varying degrees of polyfunctional capacity, which may be influenced by the polyfunctional nature of CD8+ T cell responses in early infection.

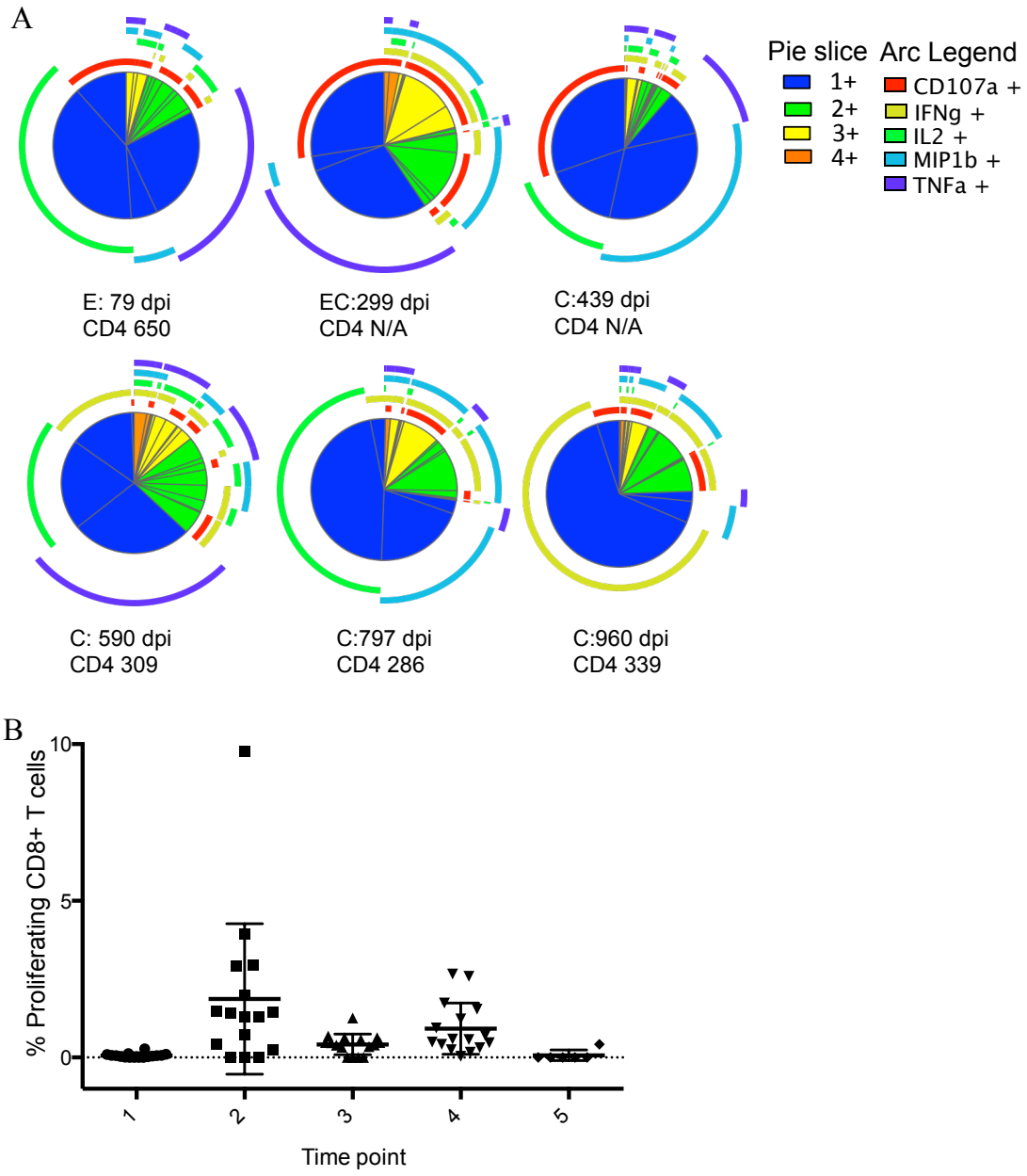

Figure 6.3. Longitudinal analysis of participant 1266 from early to chronic infection. A) Polyfunctional profile of CD8+ T cells from participant 1266. Days post infection (dpi) and CD4 counts for each sampling time point are indicated. Pie slice indicated level of polyfunctionality and Arc legend indicates specific functionality B) Proliferative capacity of CD8+ T cell from participant 1266. The percent for proliferating CD8+ T cells at each sampling time point are shown. Mean and Standard Deviation are indicated.

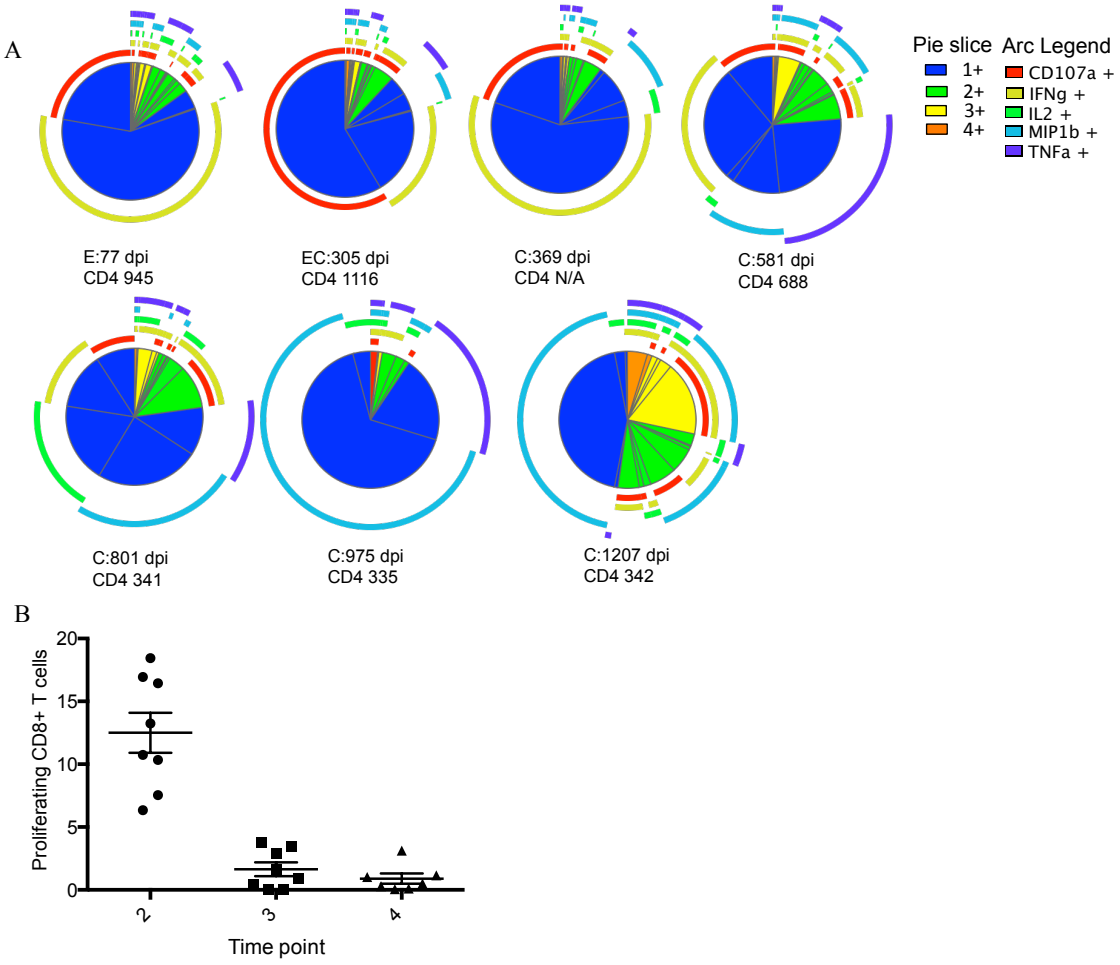

Figure 6.4. Longitudinal analysis of participant 2687 from early to chronic infection. A) Polyfunctional CD8+ T cell responses remain low until late chronic infection. B) Proliferative capacity is highest in early-chronic infection. Pie slice indicated level of polyfunctionality and Arc legend indicates specific functionality.

6.6. Summary

It has been well established that strong CD8+ T cell proliferation and polyfunctionality responses during chronic infection result in a better disease outcome; however it is unclear how particular individuals maintain these protective responses while others do not. Whether their improved disease outcomes are the consequence of these CD8+ T cell characteristics or if these CD8+ T cell responses are merely a consequence of protection by other means remains to be determined. Here, we performed a cross-sectional and longitudinal analysis of polyfunctional and proliferative CD8+ T cell responses from the early to chronic phase of infection. This is one of the first studies to evaluate CD8+ T cell response evolution in the same individual from early HIV infection through to chronic infection.

Our data suggests that the polyfunctional and proliferative capacity of CD8+ T cells follows a distinct pattern from early to chronic infection, even though HIV-specific CD8+ T cell responses vary substantially between individuals. Cross-sectional analysis suggests CD8+ T cells responses in both early and chronic infection have high to moderate polyfunctionality and low proliferative capacity. Our data demonstrates that polyfunctionality is highly variable in the chronic phase of infection. However, overall cross-sectional data suggests that CD8+ T cells in chronic infection are also highly polyfunctional. Our data also demonstrate that during the early-chronic phase of HIV infection, CD8+ T cells responses lose polyfunctionality while gaining proliferative capacity. The early-chronic phase of infection seems to be an important phase of infection in which CD8+ T cells are shifting from a more effector phenotype to a proliferative phenotype.

Longitudinal analysis of individuals CD8+ T responses over time suggests that the pattern of CD8+ T cell response evolution is highly variable between individuals and that this variation may have an impact on disease progression. Individuals with moderate to high polyfunctionality in early infection, exemplified by participant 1266 (0.776 CD4 decline/year), trended towards a slow CD4 decline and went on to maintain healthy CD4+ T cell counts. Although this individual's CD8+ T cells polyfunctionality declined similar to levels observed in the cross-sectional data, her CD8+ T cells rebounded in the later stages of infection and became increasingly polyfunctional. Conversely, individuals with low polyfunctionality in early infection, exemplified by participant 2687 (5.249 CD4 decline/year), trended towards a more rapid CD4 decline. This individual developed poor CD8+ T cell polyfunctionality early in infection, which was maintained well into chronic infection.

Although low patient numbers limited our ability to statistically analyze this data, this is the first study to follow individual HIV infected patients with known seroconversion dates from the early phases of infection into chronic infection with multiple time points. This study gives important insight into the evolution of CD8+ T cell responses, particularly in the case of the intermediate early-chronic phase of infection. Identification of whether polyfunctional responses and strong proliferative capacity is the cause or consequence of HIV control will be needed for the comprehensive evaluation of HIV vaccine candidates and more studies are need.

6.7. Implications

These results imply that there is a skewing in the quality of the CD8+ T cell responses in the early-chronic phase of infection and that changes in the responses at this stage may ultimately affect the course of infection. Additionally, it suggests that polyfunctional CD8+ T cell response in early infection may result in better disease outcomes. If a vaccine can be designed to induce early polyfunctional CD8+ T cell responses, it could be possible to prevent or slow HIV infection.

7. Chapter 7. Discussion

Understanding correlates of protection against HIV infection and progressive HIV disease is a key priority for informing vaccine development, design and evaluation. The aim of the research presented in this thesis is to provide a better understanding of the fine specificity of HIV-specific T_{EM} and T_{CM} responses. We focused on assessing the quality of CD8+ T cell responses using multiparameter flow cytometry, capturing functional attributes of both memory and effector responses in LTNP and HIV+ patients with normal disease progression. Further, we appraised the utility of evaluating CD8+ T cell memory using surrogate surface markers as opposed to functional qualities. We have shown that the epitope-specificity differs between short-term cytokine/chemokine secretion (T_{EM} like) and long-term proliferative (T_{CM} like) assays, as well as within the short-term assay. Moreover, we show that at a cohort level, particular epitopes preferentially elicit specific CD8+ T cell responses in preference to others. We subsequently evaluated the quality and evolution of CD8+ T cell responses over time by following HIV+ patients who are in the early phase of infection through to the chronic phase, finding that early polyfunctional responses may result in better HIV disease outcomes.

This research improves our understanding of HIV pathogenesis and indicates that we can identify specific epitopes that can elicit protective responses and that early polyfunctional responses may slow HIV disease progression. Understanding, the polyfunctional and proliferative capacities of HIV-specific effector and memory cells at various stages of HIV infection is of critical importance to the design of vaccines intended to elicit protective cell-mediated responses.

The complexities of T cell memory and functionality have major implications for understanding protective immunity and candidate vaccine evaluation. In chapter 3 we conducted a detailed analysis of the memory phenotypic profiles of HIV and CEF-specific responses and their correlation with specific functional responses in a cohort of chronically infected HIV patients. Chiefly, we found that both HIV- and CEF-specific CD8+ T cells responses are observed across all eight phenotypic categories defined by CCR7, CD45RA, and CD27, with naïve, T_{TD}, T_{EM} and two novel phenotypes (CCR7+CD27-CD45RA- (Undefined#2), and CCR7-CD27-CD45RA+ (Undefined#3)) predominating (Figure 3.2, Table 3.4). Interestingly, we observed several differences in phenotypes of individual functional readouts and polyfunctional cells. Each phenotype had a particular function associated with it (Figure 3.6). For example, cells with the T_{TD} phenotype, were most likely to express MIP-1β, while cells with the undefined#3 phenotype, were most likely to express IFNγ. Although some functional readouts appear to associate with particular memory phenotypes, we found no consistent phenotype to represent the totality of functionally active CD8+ T cells (Table 3.5). These data suggests that in the context on HIV infection, it is difficult to associate functionality with previously defined memory compartments.

We found that the memory phenotypic profile differed between HIV- and CEF-antigen-specific cells, with HIV-specific cells expressing higher levels of CD45RA (Figure 3.3, 3.5). Theoretically, CD45RA is the first surface memory marker to be lost following antigen stimulation leading to functionally active CD45- effector cells (303). Our data indicates HIV-specific CD8+ T cells are more likely to retain this marker and suggests

that HIV-specific CD8+ T cells are functionally impaired compared to CEF-specific CD8+ T cells. However, the bulk of data presented here suggests that, in the context of HIV infections, the use of surface markers to identify the differentiation and functional state of CD8+ T cell is unreliable.

The unreliability of surface markers to predict functionality was further illustrated, when we examined the correlation between the proportion of memory phenotypes and the percentage of responding cells. Regardless of functional parameter, we observed no specific phenotype that correlated with CD8+ T cell functionality (Table 3.6). These data suggest that it remains important to define antiviral T cells by their functional capacity and that no one phenotype measured by expression of surrogate surface markers can define that capacity. Although there are correlations suggesting a link between phenotypic markers and particular functions or polyfunctionality, the correlations are not absolute and surface markers cannot be used in place of measuring functionality. Interestingly, 10-20% of responding cells, regardless of function, could be phenotypically described as naïve (Figure 3.6, Table 3.3). This is in agreement with Freel et. al. who found a significant proportion of antigen specific CD8+ T cells with a "Naïve" phenotype in vaccinees and HIV virus controllers (321). It could be interpreted that these CD8+ T cells are antigen inexperienced and therefore, functionally inactive. However our data and Freel et. al. do not find any relationship between differentiation state and antiviral activity. This would suggest that vaccine evaluation should focus CD8+ T cells functionally rather than specific surface memory compartments.

A major implication of our data is that surrogate surface markers that have been used to define memory phenotypes may not represent the same antigen-specific T cell populations in all viral infections, populations examined, or even within various disease states. Our data greatly expands previous studies that find inconsistences in the memory surface phenotypes of functional CD8+ T cells (179, 180). CD8+ T cells that have proliferative capacity have been found in both CCR7+ and CCR7- subsets (125). Additionally, CCR7+ and CCR7- memory CD8+ T cells have been described as having immediate effector functions(317, 318). Similarly, no correlation between CD45RA expression and proliferation potential or IL-2 production has been observed (319). Our comprehensive data confirms that the use of memory markers to capture the broader array of CD8+ T cells functions and polyfunctionality may be insufficient, particularly in the context of HIV infection.

Determination of functional and memory phenotypes is particularly critical for understanding the immunology of a chronic infection such as HIV, which skews the naïve/memory compartment and alters many aspects of T cell homeostasis (341). Murine models of chronic viral infection have established that CD8+ T cells are continually recruited as infection progresses (342-344). This is likely the case for HIV as CD8+ T cell specificities can change dramatically between acute and chronic infection (169, 210). Virus-specific CD8+ T cells in chronic infections such as HIV also demonstrates substantial functional exhaustion, which occurs in a hierarchical manner as the infection persists and CD8+ T cells become more antigen experienced (252, 293-295). CD8+ T cells in the final stages of exhaustion have lost their polyfunctional quality. This is

critical information both for understanding immune factors that affect disease progression, but also for defining correlates of protection against HIV disease. By taking into account surface markers less focused on memory and more on function, a better definition of effective CD8+ T cells could be gained. These include activation markers such as CD69, CD38, and HLA-DR (345); exhaustion markers, such as PD-1 and Tim-3 (305, 307, 309); senescence markers such as CD57 (346); and cellular turnover markers such as Bcl-2 (apoptosis marker) and Ki67 (proliferation marker) (331). Moreover, systems biology approaches could be used to identify a number of additional molecules that might better define the phenotypic state of antigen-specific T cells (347, 348). Importantly, many of these markers have functional relevance, as CCR7, CD27, and CD45RA. This is not completely accurate and further skewed by chronic infection with high antigen load. These data strongly suggest that function, rather than surrogate surface markers should be used to define clinically relevant CD8+ T cells. Future studies evaluating the breadth and specificity of CD8+ T cell responses which are able to control HIV progression, need to focus on the functional complexity of CD8+ T cell.

Recent advances in the functional characterization of viral-specific CD8+ T cell responses have expanded our understanding of protective immunity, allowing the opportunity to fully define the breadth and specificity of a large, complex antiviral CD8+ T cell response. For example, acute CD8+ T cell responses to smallpox and yellow fever vaccinations, measured by cellular activation, intracellular cytokine staining and tetramer frequencies, revealed these responses are of much higher magnitude than was previously appreciated (331). While assessment of HIV-specific responses has traditionally relied on assays that measure IFNγ secretion, this cytokine does not always correlate with

protective immunity, nor does it accurately describe the full breadth of HIV-specific CD8+ T cell responses (226, 292, 349). Other studies have demonstrated that HIV+ LTNPs maintain a more functional response than control subjects who experience more rapid progression to AIDS (193, 216), but despite this, few studies have mapped the specificity of these complex responses.

Based on our findings that suggest functionality, rather than surrogate surface markers should be used to evaluate effective CD8+ T cell response. In chapter 4 we have extended this work by using multiple overnight parameters and long-term proliferation assays to map CD8+ T cell epitopes more comprehensively than has been previously described. Critically, over two-thirds of the responses detected in our study were detected in the absence of a detectable IFNγ (54/73) response (Table 4.10), and measurement of additional functional parameters expanded the breath of response by almost four fold. These data suggest that previous epitope mapping studies and vaccine trials may have overlooked important immune responses by focusing solely on IFNγ. The data further reveal that CD8+ T cell responses are complex and that particular epitopes within a given subject will elicit responses with unique functionality (Figure 4.4). Identification of epitopes that are able to elicit polyfunctional and proliferative responses will serve as a valuable resource for vaccine design and ensure wider varieties of protective responses are obtained. By epitope mapping responses to HIV p24, using multiple parameters, we identified 54 epitopes in HIV-1 p24, 42 of which have not yet been fully characterized according to the Los Alamos HIV Immunology database. A number of factors could explain the identification of these novel epitopes. For one, we

used shorter peptides than most prior studies (9mers compared to 15mers), which have an increased sensitivity to detect responses (226, 350-352). Secondly, the database of optimal CD8+ T cell HIV epitopes may be biased towards clade B, while this epitope mapping study was conducted in Kenya, where clade A1 predominates (353-355). As described in the methods section 2.1.2, p24, sequence data from the cohort is near identical to the HIV A1 ancestral sequence. Although the use of the A1 ancestral sequence may be a contributing factor leading to the identification of novel epitopes, we also found that the 12 previously defined epitopes could also be recognized between clades, despite sequence differences. The third, and perhaps most important factor, was the inclusion of a greater number of functional parameters as readouts to define responses. The measurement of five functional parameters (IFNγ, MIP-1β, CD107a, IL-2 and TNFα) plus proliferation, expanded the breath of detected responses by almost four fold. The majority of novel epitopes identified in this study did not include IFNγ (43/56 responses) and 13 responses included a proliferation response (Table 4.10). Interestingly, a number of these previously unidentified epitopes induced polyfunctional (≥2+) responses (25/56), but not necessarily IFNγ. Together, these data indicate that the breadth of HIV-specific immunity may be even greater than previously appreciated, indicating that there is a greater depth of potential HIV targets than previous thought. Currently, several large studies focused on a finite number of best-defined CD8+ T cell epitopes and the CD8+ T cell research community will benefit by exploring the greater breadth of potential HIV epitopes.

There was a striking discordance between responses measured in overnight versus six day proliferation assays, with CD8+ T cells responding to epitopes by either secreting IFNγ or proliferating, but rarely both (Figure 4.2 Table 4.5). This suggests that a given epitope in a subject may preferentially induce either short-term IFNγ or long-term proliferative responses. This observation was not limited to IFNγ but was apparent for all short-term readouts, as proliferation responses were inversely correlated to IFNγ, MIP-1β, CD107α and TNFα (Table 4.5). This was even more pronounced at the epitope level, where the vast majority of proliferation responses (10/13) were completely independent of overnight parameters (Figure 4.10). While an explanation for these data remains unclear, one hypothesis is that the cells that respond in an overnight assay are not equipped to survive for the length of a proliferation assay, which is more likely to identify memory cells that need time to become activated *in vitro*. CD8+ T cell survival may be an important precursor of proliferation, whereas short-term expression of cytokines particularly IL-2 may not require survival to the same extent. Some studies suggest that survival capabilities, measured by exhaustion markers, might be the key attribute of memory cells (178).

Contrary to what has been observed by others, which found that IL-2 and proliferation responses to HIV peptide pools correlated, and that IL-2 responses were rare in progressing subjects(302), our data consistently showed an inverse correlation between these two parameters, which rarely overlapped. A possible explanation for the differences between our results and those of other groups is that our correlations were based on an unbiased epitope screening approach that used IL-2 and proliferation to

define CD8+ T cell specificity, as compared to measuring IL-2+ CD8+ T cells that were also IFNγ+. Since IL-2 and proliferation were both inversely correlated with IFNγ in our cohort, it is possible that by focusing solely on IFNγ responses, the bulk of the IL-2 and proliferation responses were missed in previous studies. A lack of association between proliferation and IL-2 on the bulk of Gag-specific CD8+ T cell has recently been observed in a Chinese cohort, supporting our findings (356).

It has been suggested that functional exhaustion occurs in a hierarchical manner as the infection persists, where IL-2, cytolysis and proliferation are the first to be lost, followed by TNFα, and finally by the loss of IFNγ and anergy/deletion (252, 293-295). Based on this model, the reliance on IFNγ to screen immune responses may overestimate the effectiveness of the immune response by measuring a significant proportion of CD8+ T cells that may be in the final stages of exhaustion. However, our data also challenges this model by finding many CD8+ T cell responses that are independent of IFNγ, including the display of IL-2, CD107a, and proliferation, in isolation from other functions. Many functional divisions in epitope-specificity were seen within the T_{EM} like cell population. Examination of all responses clearly shows that many epitopes stimulate unique combinations of overnight parameters, many of which were expressed in the absence of IFNγ. These data suggest that in a chronic viral infection there exists a complex, heterogeneous antiviral CD8+ T cell response.

In agreement with previous work, our study found that LTNPs possess a more polyfunctional HIV-specific CD8+ T cell response than NP (Figure 4.1) (216). LTNPs also responded with a higher magnitude p24-specific proliferation, TNFα, CD107a, and IL-2 when compared to NPs (Table 4.6). The proliferation data was consistent with other findings, which found that the magnitude of proliferation was significantly lower in progressive chronic infection than primary infection or LTNPs (260). We also found that p24 may be an important target for control of HIV replication (221). In addition, LTNPs demonstrated more consistent associations between functional parameters than progressors, including the rarely observed concordant proliferation and IFNγ responses.

We have shown here that within a given HIV-infected subject, it is possible to identify novel epitopes that induce qualitatively distinct CD8+ T cell responses. Screening for responses by the measurement of six CD8+ T cell functions revealed a greater complexity of CD8+ T cell immunology, and describes a greater breath of response than would have been derived from measurement of any one response on its own. We have shown that the epitope-specificity differs between short and long-term assays, as well as within the short-term assay. We also show that particular epitopes, in any given subject, often tended to elicit one type of CD8+ T cell response in preference to others. Identifying epitopes that preferentially induce more protective polyfunctional and proliferative responses on a population level, will have major implications of future CD8+ T cell studies and vaccine development.

Based upon our data in chapter 4, we then selected eleven HIV-1 Clade A p24 epitopes of interest, previously identified during our comprehensive fine epitope mapping study (327). These 11 epitopes were further characterized using multiple overnight parameters

and long-term proliferation assays to confirm if the functional specificity of epitopes can be seen in a larger sample size. Characterization of these eleven epitopes using multiple cytokines/chemokines and proliferation functions confirmed at the cohort level that particular epitopes are more likely to stimulate unique functional responses. Our data confirms, for example, that proliferative capacity is somewhat rare and particular epitopes could potentially be identified that are more likely to induce a proliferative response, such as peptide 161, 142, and 88; while others are very unlikely to promote proliferative response, such as peptide 146, 147 and 169 (Figure 5.3). Unfortunatly these data did not reach statistical significans and maybe also suggest that proliferative capacity is independent of the epitope recognized. Similarly, some overnight chemokines and cytokine were more likely to be expressed in response to stimulation with particular epitopes including MIP-1β, IL-2 and CD107a in response to peptides 88, 169 and 147 respectively (Figure 5.4). Previously, it has been shown that the expression of specific cytokines and chemokines correlated with protective antiviral cell mediated immune responses (357). Studies have found that MIP-1β production was the dominate response in HIV infected individuals with non-progressing infection and has also been identified following efficacious HBV immunization (216, 296, 358). Betts et. al reported that every major responding HIV-specific CD8+ T-cell population produced MIP-1β, suggesting this chemokine may be the best single indicator of HIV-specific CD8+ T-cell frequency (216). Moreover, IL-2 production is the dominant cytokine in response to efficacious HBV and tetanus immunizations (358). HIV-2-specific CD8+ T cell responses are dominated by the expression MIP-1β, CD107a, and IFNγ with over 60% of responding

cells exhibiting these functions (332). These data suggest that elevated expression of particular functions, mainly MIP-1β, may be critical to an effective immune response. Characterization of the functionality of response to these eleven epitopes by evaluating the co-expression and concurrent expression of multiple cytokines/chemokines revealed that specific epitopes are significantly more likely to stimulate polyfunctional responses. When evaluating bulk responses measuring both co-expression and concurrent expression of multiple cytokines and chemokines, peptide 88 (EV9) was significantly more likely to elicit a multifunctional response than any of the other peptides tested (Figure 5.6). Importantly, when evaluating the concurrent expression of multiple cytokine/chemokines using Boolean gating peptide 88 was again shown to be significantly more likely to elicit polyfunctional responses. Peptide 88 was more likely than all other epitopes to elicit dual-functional and tri-functional functional responses (Table 5.6, 5.8). The importance of inducing polyfunctional virus specific responses in effective vaccines and slowing disease progression has been well established. Our group and others have shown that HIV controllers have more polyfunctional HIV-specific CD8+ T cells, as defined by the concurrent expression of cytokines IFNγ, IL-2, and TNFα, the chemokine MIP-1β, and the degranulation marker CD107a, than normal progressing controls (193, 216, 327, 359). Additionally, the detection of polyfunctional CD8+ T cells in HIV-1 exposed but uninfected subjects, potentially demonstrate that polyfunctional responses may not only slow progressing infection but may, in fact, confer protection against infection (264). These data highlight the importance of developing an HIV vaccine that skews the immune system towards a highly polyfunctional HIV-specific CD8+ T cell response. Importantly, our data suggests that it is possible not only to identify epitopes that induce

distinct cytokines and chemokine profiles in responding CD8+ T cells, but that it is also possible to identify epitopes that preferentially induce polyfunctional responses.

Unfortunately, we were unable to find any association between epitope specific polyfunctionality and disease progression. No correlation was observed between any level of peptide-specific CD8+ T cell polyfunctional responses and CD4 counts, CD4 slope or viral loads (data not shown). The lack of association with these measures of disease progression is likely due to the high variability between individuals' responses to the 11 epitopes. Additionally, these analyses were conducted by examining each level of polyfunctionality (i.e. 1+, 2+, 3+, 4+, 5+) rather than polyfunctionality as a whole. The lack of association is likely due to the analysis of specific levels of polyfunctionality to specific epitopes, diluting out the number of data points tested for each peptide. It is also possible that none of the epitopes tested in this study are important epitopes on a clinical level. Further study would need to be done to identify important epitopes and to evaluate whether targeting peptide 88 is protective.

One caveat of our study, however, is that we are unable to determine the cause/effect relationship in these responses. Is it, in fact, the epitope that is guiding the functional specificity of the response or is it the restricting HLA or TCR clonal type that is guiding the functional specificity? To date, the strongest associations between HIV control and CD8+ T cell responses, is the protective effect observed with certain human leukocyte antigen (HLA)-I alleles. The nature of the association is complex and simply possessing protective alleles does not always guarantee good clinical outcome. Interestingly, individuals with the same HLA allele who respond differently to the same epitopes, result

in vastly different disease outcomes, implying that HLA restriction alone cannot account for protective CD8+ T cell responses (360). CD8+ T cell responses restricted by HLA-B*27 and HLA- B*57 targeting the same epitopes in elite controllers and progressors are distinctly different, which appears to be associated with the specific TCR clonotypes selected during natural infection (245). Similarly, Price et. al. looked at the clonal types of *ex vivo* tetramer-specific CD8+ T cells stimulated with CMV and EBV epitopes. Their major findings regarding CD8+ T cell polyfunctionality were that although multiple clonotypes respond to one epitope, the functional profiles of all eptiope-specific clonotypes were similar and dominated by only a few functional combinations. This is in agreement with our data, which demonstrates distinct epitopes preferentially elicit specific functions and polyfunctional capacity on a cohort level. Together these data suggests that the functional profile of CD8+ T cell responses could at least, in part, be mediated by the restricted epitope.

Conversely, it is equally likely that the nature of the viral epitope may be responsible for the protective nature of the CD8+ T cell responses. Many studies have shown that within the same individual, differed epitopes elicit unique functional responses (294, 361). These studies suggest that the distinct nature of the epitopes may be directing the functional quality of the CD8+ T cell response. Additionally, some studies suggest that small amino acid changes in the same epitope restricted by the same HLA allele, can induce a markedly different response. Previously our group has demonstrated that HIV epitope variants differ dramatically between overnight IFNγ and longer-term proliferation assays (362). Particular epitope variants were favoured in proliferation in the absence of

corresponding overnight IFNγ responses (339). These data suggest that it is possible for the nature of an epitope, whether it is variation in distinct epitopes, and even variation within a single epitope, can guide the functional outcome of a response.

Understanding the nature of CD8+ T cell epitope-specific diversity will be important for HIV vaccines. Based on a recent study in non-human primates which showed that macaques vaccinated with a CMV vector expressing SIV had a robust T_{EM} response, characterized by polyfunctionality, and were less likely to become infected following low-dose challenge (363). However, the authors stressed that the parallel induction of T_{CM} responses, similar to what occurs in many LTNP, may provide a second line of defence in case infection becomes established. These data highlights the importance of developing a vaccine that can specifically induce 'protective' polyfunctional (T_{EM}) and proliferative (T_{CM}) CD8+ T cell responses.

Additionally, vaccination with vectors expressing distinct CD8+ T cell epitopes, rather than whole viral proteins, improves immunogenicity (335), allows the generation of multiple dominant responses (336, 337), and provides the opportunity to focus on beneficial responses and exclude ineffective or even potentially harmful ones (137, 338). Our data suggests that it is possible to identify epitopes that will induce distinct and possibly protective CD8+ T cell responses that could be incorporated into vaccine vectors to selectively elicit CD8+ T cells capable of preventing and controlling infection.

We show that at the individual level and the cohort level, it is possible to identify novel epitopes that induce qualitatively distinct CD8+ T cell responses, which are

polyfunctional or proliferative. Our data suggests it is possible to identify epitopes that are more likely to induce particular cytokines, proliferative capacity and polyfunctional responses. A multiparametric approach to defining epitope-specificity is one that could be used in vaccine evaluation in order to determine the true immunogenicity of a given vaccine target. Moreover, because polyfunctionality and proliferative responses have been associated with attenuated HIV disease course, these data and similarly comprehensive epitope mapping studies, could identify a number of additional epitopes that may serve as useful targets for successful HIV vaccines that will presumably induce protective epitope-specific responses. However, the ability to steer the immune system towards a protective cell-mediated response will largely depend on whether or not early CD8+ T cell responses are able to forecast disease outcomes.

It has been well established that strong CD8+ T cell proliferation and polyfunctionality responses during chronic infection result in better disease outcome, however, it is unclear how particular individuals maintain these protective responses while others do not. In chapter 5, we performed cross-sectional and longitudinal analysis of polyfunctional and proliferative CD8+ T cell responses from the early to the chronic phase of infection. We showed that the polyfunctional and proliferative capacity of CD8+ T cells follows a distinct pattern from early to chronic infection, even though HIV-specific CD8+ T cell responses vary substantially between individuals. Cross-sectional analysis suggests that both early and chronic CD8+ T cells responses have high to moderate polyfunctionality and low proliferative capacity, while early-chronic CD8+ T cells have low polyfunctionality and high proliferative capacity (Figure 6.2). This is in agreement with

other studies that have shown high polyfunctionality in individuals HIV infected for under 6 months (294). Though, acute CD8+ T cell responses are linked with lowering initial peak viremia, which has been repeatedly been associated with better disease outcome, to date no study has been able to demonstrate a clear association between acute or early polyfunctionality and CD8+ T cell mediated protection (364). However data suggests that early CD8+ T cell responses are narrowly directed against a limited number of epitopes and these responses are unique in their ability to efficiently suppress viral replication (210, 211, 271, 365, 366).

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Additionally, our data demonstrates that polyfunctionality is highly variable in the chronic phase of infection, potentially as a result of varying ability to control the virus. However, overall our cross-sectional data suggests that CD8+ T cells in chronic infection are highly polyfunctional (Figure 6.2). Again this is in agreement with previous data that has demonstrated substantial polyfunctionality in chronically infected individuals (216, 367, 368). The variable levels of polyfunctionality in chronic infection are potentially the consequence of both functional exhaustion and epitope escape. Ongoing antigen stimulation during HIV infection places considerable pressure on HIV-specific CD8+ T cells. CD8+ T cells are prone to functional exhaustion during persistent antigen stimulation and it is well established that HIV-specific CD8+ T cells become functionally impaired in chronic infection and lose polyfunctionality (294). Additionally, several studies have demonstrated that CD8+ T cell specificity changes over the course of the infection and the epitopes that are immunodominant in early infection are not the same as those that are immunodominant in chronic infection (369). HIV mutational escape consistently occurs during infection and results in the appearance of new epitopes and therefore, alterations in CD8+T cell specificity. There is potential that the newly developed immunodominant epitope-specific CD8+T cells possess varying levels of

polyfunctionality and therefore, result in a mixture of polyfunctional epitope-specific CD8+T cells against newly emerged epitopes and functionally impaired CD8+ T cells targeting epitopes observed during early HIV infection. Therefore, the consequences of persistent HIV stimulation are ambiguous. It, on the one hand, it could increase the total number of polyfunctional CD8+ T cells and thereby may improve the control of viral replication; alternativelyon the other hand, it may lead to the functional damage to HIV-specific CD8+ T cells due to the viral persistent stimulation. Theoretically, the nature of CD8+ T cell responses established in early infection would influence the ability of chronic CD8+ T cells to withstand continued antigen stimulation.

Currently, there is no data on CD8+ T cells in the transitional phase between early to chronic infection (6 to 12 months). Our data demonstrates that during the early-chronic phase of HIV infection, CD8+ T cell responses lose polyfunctionality while gaining proliferative capacity (Figure 6.2). The early-chronic phase of infection seems to be an important phase of infection in which CD8+ T cells are shifting from a more effector phenotype to a proliferative phenotype. This increased proliferative capacity suggests that something significant is occurring in the early-chronic phase of infection that may be playing a vital role in establishing and balancing the quality of the CD8+ T cell responses. It is possible that during this phase of infection, the strong proliferation response is setting the stage for the subsequent CD8+ T cell response in chronic infection. CD8+ T cell responses in chronic infection were highly variable and these data suggest that during early-chronic infection, CD8+ T cells are rapidly multiplying into either highly polyfunctional or low polyfunctional populations, depending on the

individual. Unfortunately, low sample numbers limited the ability to find associations between proliferation and disease outcomes. It is also possible that, unlike previous studies which find strong association between proliferative capacity and slower disease progression in chronic infection (258-260) , proliferation in earlier stages of infection are not associated with disease outcomes. If this were the case, it would suggest strong proliferation responses in chronic infection are a consequence of viral control rather than a cause.

Longitudinal analysis of individuals' CD8+ T cell responses over time suggests the pattern of CD8+ T cell response evolution is highly variable between individuals and may have an impact on disease progression. Individuals with moderate to high polyfunctionality in early infection, exemplified by participant 1266 (0.776 CD4 decline/year), trended towards slow CD4 decline and went on to maintain healthy CD4+ T cell counts (Figure 6.4). Although this individual's CD8+ T cell polyfunctionality declined similar to cross-sectional data, her CD8+ T cells rebounded in the later stages of infection and became increasingly polyfunctional. Conversely, individuals with low polyfunctionality in early infection, exemplified by participant 2687 (5.249 CD4 decline/year), trended toward more rapid CD4 decline (Figure 6.5). This individual developed poor CD8+ T cell polyfunctionality early in infection, which was maintained well into chronic infection. These observations suggest an association between the development of early CD8+ T cell polyfunctionality and slower disease progression. They also suggest that early CD8 + T cell polyfunctionality may be the cause of, rather than consequence of, better disease outcome.

Unfortunately, our results are contrary to a previous study that suggests early polyfunctionality is unrelated to better disease outcomes, however, our study was underpowered to make any conclusive associations. Turk et. al. argue in favor of the hypothesis that highly polyfunctional CD8+ T cells are a consequence rather than the cause of low viral loads. They found no significant differences in the level of CD8+ T cell polyfunctionality in individuals' infected for less than 6 months when they segregated subject based on rapid (CD4 count >350) or regular (CD4 count <350) progressors. However, it is possible the lack of association between early polyfunctionality and disease progression are the result of the measure of cross-sectional CD4 counts and taken after only 12 months of infection. Had they followed these patients further into chronic infections and used a longitudinal measurement such as CD4 + T cell slope, an association may have been seen.

Although our patient numbers limited the statistical data analysis we could do, this is the first study to follow individual HIV infected patients with known seroconversion dates from the early phases of infection into chronic infection with multiple time points. Our cohort provides a unique opportunity to monitor an individual's disease progression over numerous years and longitudinal analysis. This study gives important insight into the evolution of CD8+ T cell responses, particularly the intermediate early-chronic phase of infection. Identification as to whether polyfunctional responses and strong proliferative capacity is the cause or consequence of HIV control will be needed for the comprehensive evaluation of HIV vaccine candidates. More studies similar to this will

need to be conducted to better answer this question. Future studies would need to analyse a larger sample size and evaluate the nature of the CD8+ T cells within the same individual at more frequent time intervals. Additionally, analysing CD8+ T cells in the acute phase (<30 days) after infection rather than early phase will likely give a more accurate assessment of the potential role polyfunctional CD8+ T cells play in controlling HIV infection.

7.1. Concluding Remarks and Future Directions

7.1.1. *Major findings of thesis*

The data presented in this thesis have major implications for the future of CD8+ T cell research and HIV vaccine design. This thesis focuses on defining the role of CD8+ T cell responses in HIV infection. While various host and viral factors can explain the apparent success of the immune system against HIV, understanding inducible CD8+ T cell responses may offer the best hope for providing protective immunity to HIV infection.

One of the primary hypothesis of this thesis was that the specificity of T_{CM} and T_{EM} responses to HIV epitopes differs in chronic infection and T_{CM} responses will better correlate with protection from HIV disease progression. However, we suspected that the surface phenotypes used to define T_{CM} and T_{EM} would not accurately identify CD8+ T cells with polyfunctional and proliferative capacity and not truly reflect the functional capacity of CD8+ T cells. Although the use of surface markers to define immunological memory directly *ex vivo* presents as a time saving strategy, we found no consistent phenotype to represent the totality of functionally active CD8+ T cells. The implication

of these results is that the study of CD8+ T cell responses in HIV infection would be better served if functionality rather than surrogate surface markers were used to define effective CD8+ T cell responses. Therefore, our future studies aimed at accessing the quality and specificity of CD8+ T cells in HIV infection focused on the complexities of CD8+ T cell functionality, rather than pre-defined memory cell subsets. Previous HIV-specific CD8+ T cell epitope mapping studies identify potential vaccine targets and correlates of CD8+ T cell protective immunity have been characterized exclusively using IFNγ Elispot assays. We mapped CD8+ T cell epitopes much more comprehensively than has been previously described, measuring the specificity of CD8+ T cells using multiple overnight parameters and long-term proliferation assays. Importantly, over two-thirds of the responses detected in our study were detected in the absence of an IFNγ (54/73) response, and measurement of additional functional parameters expanded the breath of response by almost four fold. We also show that particular epitopes, in any given subject, often tended to elicit one type of CD8+ T cell functional response in preference to others. These data confirm our hypothesis and suggests that specificity of T_{CM} and T_{EM} responses to HIV epitopes, when measured based on functionality, differ in chronic infection.

We went on to show that the functional specificity of CD8+ T cell epitopes extends beyond the individual and can be seen at the cohort level. The characterization of eleven epitopes using multiple cytokines/chemokines and proliferation reveal that particular epitopes are significantly more likely to stimulate unique responses at a population level. The implications of these results are that epitopes that induce distinct functional

responses can be identified, and perhaps elicited. It suggests the potential to identify specific epitopes, which elicit protective polyfunctional or proliferative CD8+ T cell responses that associate with viral control. 'Protective' epitopes could be incorporated into a vaccine vector designed to express distinct CD8+ T cell epitopes and perhaps induce early and effective CD8+ T cell responses.

It is unclear, however, whether polyfunctional and proliferative CD8+ T cell responses in LTNP and EC are responsible for their better disease outcomes or if these CD8+ T cell responses are merely a consequence of low viral loads which has developed as a result of protection by other means. Our data suggests that the polyfunctional and proliferative CD8+ T cell responses follow a distinct pattern from early to chronic infection and suggests that polyfunctional CD8+ T cell response in early infection may result in better disease outcomes. Taken together, this thesis suggests that if a vaccine can be designed to induce early polyfunctional CD8+ T cell responses, it may be possible to prevent or slow HIV infection.

7.1.2. *Hypothetical Vaccine design*

Thirty years into the AIDS epidemic, an HIV vaccine that induces CD8+ T cell responses remains elusive to the point that some believe it is not possible. However, the development of an effective vaccine is still the primary goal of the greater HIV research community. The limited successes of previous vaccine trials to date, suggests that additional research needs to be done on alternative and unique vaccine formulations that may provide better protection from HIV, than traditional vaccine strategies. For example, vaccines based on specific CD8+ T cell epitopes represent a rational approach

to generate effective cellular immunity for both prophylactic and therapeutic protection. Vaccination with vectors expressing distinct CD8+ T cell epitopes, rather than whole viral proteins, improves immunogenicity (335), which allows the generation of multiple dominant responses (336, 337), and provides the opportunity to focus on beneficial responses and exclude ineffective or even potentially harmful ones (137, 338). Specific epitopes can be incorporated into the vaccine design with the goal of inducing CD8+ T cells with specific protective functional outcomes. Epitope selection can be biased toward those that are most highly polyfunctional and proliferative. Our data suggests that we can identify "protective" epitopes that elicit beneficial polyfunctional or proliferative responses. By incorporating only "protective" epitopes into a putative vaccine vector, we may be able to eliminate CD8+ T cell responses directed towards ineffective or harmful "bad" epitopes.

A promising vector delivery method for an effective epitope based vaccine may be a CMV vector. A recent study in non-human primates showed that macaques vaccinated with a CMV vector expressing the full SIV_{MAC239} genome had robust T_{EM} responses, which they characterized by polyfunctionality, and were less likely to become infected following low-dose challenge (363). These data suggests that the induction of early polyfunctional responses can actually prevent infection. Similar to our data, which suggests that the induction of early polyfunctional CD8+ T cell can slow disease progression, the CMV vector vaccine also showed therapeutic protection and the establishment of SIV infections reminiscent of EC and LTNP. The authors stressed that the parallel induction of T_{CM}-like responses, similar to what occurs in many LTNP, may

provide a second line of defence in case infection becomes established. These data highlight the importance of developing a vaccine that can specifically induce "protective" polyfunctional (T_{EM}) and proliferative (T_{CM}) CD8+ T cell responses.

Our data and epitope-based vaccine studies suggest that an encouraging HIV vaccine strategy would be to identify "protective" epitopes that elicit polyfunctional or proliferative CD8+ T cell responses and incorporate them into a successful vector such as CMV (Figure 6.1). This strategy will specifically induce only effective CD8+ T cell responses that will prevent infection and/or slow disease progression.

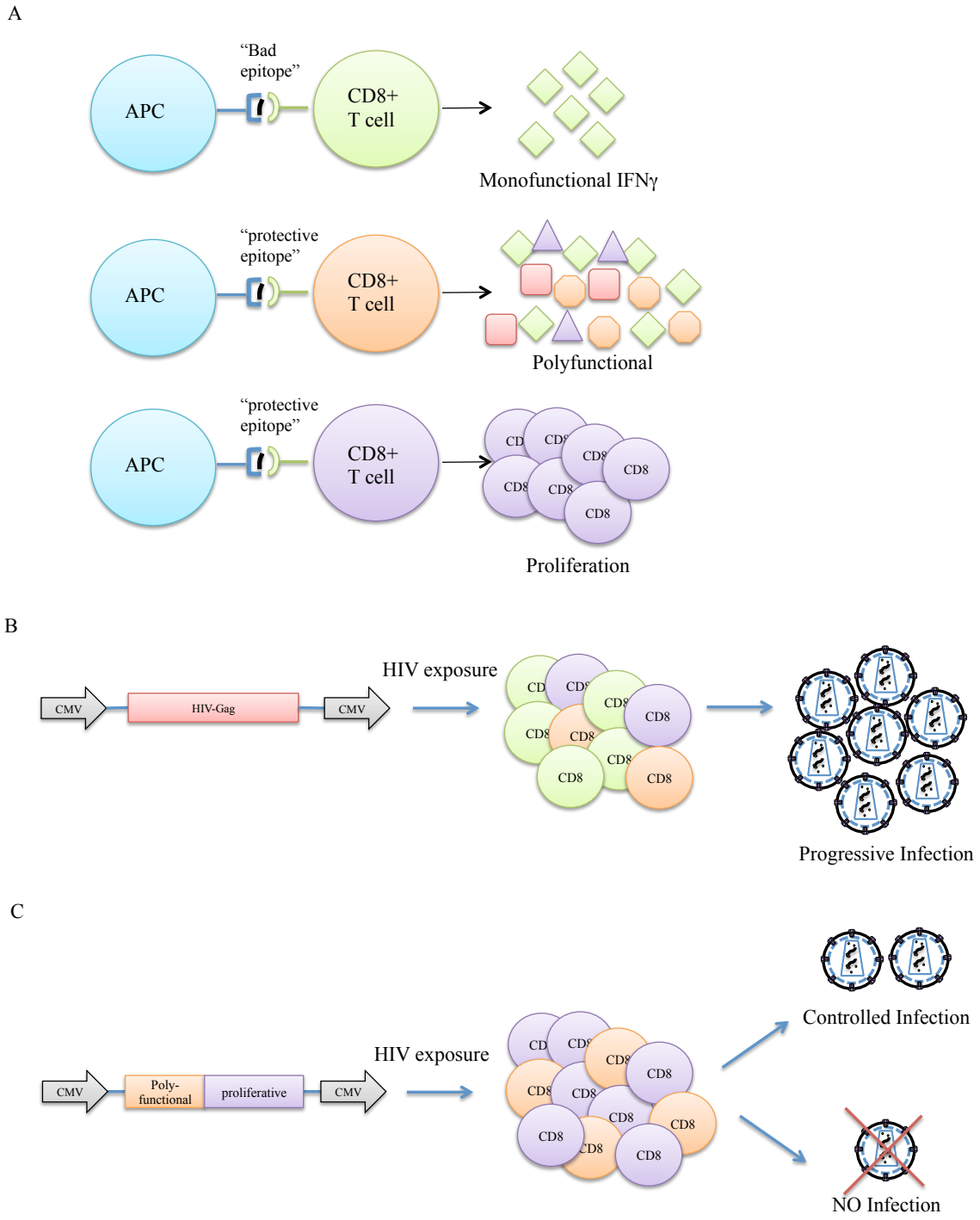

Figure 7.1. Hypothetical epitope-specific vaccine. A) Potential CD8+ T cell responses to "bad" or neutral epitopes and "protective" epitopes. B) Hypothetical vaccine (CMV) vector expressing whole HIV Gag protein. Results in CD8+ T cell responses to both "bad" and "protective" epitopes leading to infection upon HIV exposure. C) Hypothetical vaccine (CMV) vector expressing epitopes that elicit polyfunctional and proliferative responses. Results in CD8+ T cell responses to only "protective" epitopes leading to slow progression or potentially stop infection.

7.1.3. *Limitations and Future Directions*

Although the data presented in this thesis provides valuable understanding of the complexity of HIV-specific CD8+ T cell immunology and has important implications for future vaccine design, it is not without some major limitations. One of the major restraints thoughout the thesis was limitation of study power. This was particularly the case for Chapters 5 and 7. LTNP are rare and the low number of these unique individuals in our cohort was a main constraint in the analysis of chapter 5. Similarly, our clinicians and counsellors have done a wonderful job of lowering the number of new infections in this cohort and therefore, collecting enough seroconverters for statistical power was impossible during the time limit of this PhD. Additional studies of CD8+ T cells in early and acute infection will be imperative. Initially, similar studies to ours following individuals shortly after seroconversion well into chronic HIV infection will be beneficial to elucidate the role of CD8+ T cells in controlling HIV infection. However, a much larger sample size will be crucial to evaluate the clinical benefits of particular CD8+ T cell responses.

In addition to low participant numbers, cell numbers limited nearly all studies in this thesis. The volume of blood permitted to take from each participant per visit often limited the number of immunological tests that could be performed. Intracellular cytokine staining in combination with proliferation assays for multiple peptides or peptide pools required considerable cell numbers. In some cases cell numbers prevented the use of participants' samples thus reducing participant numbers further. Additionally, limited cell numbers prevented the study of additional HIV proteins and our studies were limited to p24 only.

One of the major future directions of this thesis would be to evaluate the breadth and specificity of CD8+ T cell polyfunctionality and proliferation in response to other HIV targets. Additionally, due to cell number constraints, peptides and peptide pools were only used at one concentration and therefore, no data on functional avidity was obtained. Data has shown that peptide concentration has important implications of CD8+ T cell functionality and differences can be seen between low and high avidity peptides. It would be interesting to evaluate the functional avidity of the eleven epitopes characterized in chapter 6.

It would also be beneficial to evaluate the functional specificity of natural epitope variants. Previous and on going work in our lab shows substantial differences in CD8+ T cell functionality when responding to different epitopes variant with one or two amino acid variations. Ideally this analysis would be done using HLA tetramers to insure peptide specificity. Therefore, further studies to elucidate the restricting HLA allele of the eleven epitopes of interest would be of value. The major implications of the data presented in this thesis is that it is possible to identify HIV targets that preferentially elicits protective polyfunctional and proliferative CD8+ T cell responses and expanding this work to more epitopes within p24 and within other HIV proteins would be important for the development of an effective HIV vaccine.

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Appendix A. List of Abbreviations

aa	Amino acids
Ab	Antibody
ABC	Abstinence, Be Faithful, Condoms
Ad	Adenovirus
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cell
ART	ARV Therapy
ARV	Antiretroviral
BCL-2	B cell lymphoma 2
BCR	B cell receptor
BDE	Best defined epitope
BFA	Brefeldin A
C region	Constant region of gp120
C terminus	Carboxy terminus
CCR or CXCR	Chemokine receptor
CD	Cluster of differentiation
CDC	US Centre for Disease Control and Prevention
CDR	Complementarity determining region
CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
CLR	C-type lectin receptors
CMV	Cytomegalovirus
CRF	Circulating recombinant form
CSW	Commercial sex worker
CTL	Cytotoxic T lymphocytes
D	Diversity
DALYs	Disability-adjusted life years
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ddH_20	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of the Congo
dsRNA	Double Stranded RNA
EBV	Epstein-Barr Virus
EC	Elite controller
ECD	Energy-coupled dye
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

ELISpot	enzyme-linked immunosorbent spot
Env	Envelope
EOMES	Eomesodermin
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum associated with antigen processing
FACS	Flow Cytometry Wash Buffer
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FM	Freezing Media
FoxP3	Forkhead box P3
FSW	female sex worker
Gag	Group-specific antigen gene
GALT	Gut-Associated lymphoid tissue
gp	Glycoprotein
HAART	Highly Active ARV Therapy
HBV	Hepatitis B Virus
HESN	HIV-exposed seronegative
HIV	Human Immunodeficiency Virus
HIV-1 or HIV-2	HIV type 1 or HIV type 2
HIV+	HIV-1 positive, or HIV-1 infected
HLA	Human leukocyte antigen
HSV	Herpes Simplex Virus
HSV-2	Herpes Simplex Virus type 2
HTLV	Human T-lymphotropic virus
HTLV-III	Human T-lymphotropic virus type III
HVTN	HIV Vaccine Trails Network
ICS	Intracellular Cytokine Staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IN	Integrase
J	Joining
KIR	Killer Immunoglobulin Receptor
KLRG1	Killer cell lectin-like receptor G1
LAV	lymphadenopathy-associated virus
LCMV	Lymphocytic Choriomeningitis Virus
LN	Lymph node
LPS	Lipopolysaccharide
LTNP	Long-term non-progressor
LTR	Long Terminal Repeat
M	Major

mAb	Monoclonal antibody
mac	Macaque
mDC	Myeloid DC
MEGA	Molecular Evolutionary Genetics Analysis
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
ML	Majengo Sex Worker Cohort
MN	Monensin
MSM	Men who have sex with Men
N	Non major or non-outlier
Nef	Negative factor
NHP	Non-human primate
NIH	National Institutes of Health
NK	Natural killer cell
NKT	Natural Killer T cell
NLR	NOD-like receptor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOD	Nucleotide-binding oligomerization domain
NP	Normal Progressor
NRTI	Nucleoside reverse transcriptase inhibitor
O	Outlier
p value	Probability value
p24	HIV-1 Capsid Protein
PAMPS	Pattern-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCP	Pneumocystis pneumonia
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
pDC	Plasmacytoid DC
PE	Phycoerythrin
PerCp	Peridinin-Chlorophyll-Protein Complex
Perm/Wash	Fixation/Permeabilisation wash
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PMTCT	Prevention of mother to child transmission
Pol	HIV polymerase protein
PrEP	Pre-exposure prophylaxis
PRR	Pattern recognition receptor

R10	RPMI supplemented with FBS and penicillin/streptomycin
Rev	Regulator of virion
RIG	Retinoic acid-inducible gene
RLR	RIG-1-like receptor
RNA	Ribonucleic acid
RORgt	Retinoic-acid-receptor-related orphan receptor-gamma t
RPMI	Roswell Park Memorial Institute media
RT	Reverse transcriptase
SEB	Staphylococcus aureus Enterotoxin B
SIV	Simian Immunodeficiency Virus
ssRNA	Single Stranded RNA
STI	Sexually transmitted infection
T-bet	Th1-specific T box transcription factor
TAP	Transporter associated with antigen processing
Tat	Transactivator of transcription
T_{CM}	Central Memory T cell
TCR	T cell receptor
T_{EM}	Effector Memory T cell
Tfh	Follicular helper T cell
TGF-b	Transforming growth factor-b
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis (TNF)-related apoptosis-inducing ligand
Treg	Regulatory T cell
T_{TD}	Terminally differentiated
T_{TM}	Transitional memory
U_1	Undefined 1
U_2	Undefined 2
U_3	Undefined 3
UNAIDS	United Nations on HIV/AIDS
V	Variable
VC	Viremic controller
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WBC	White Blood Cell
WHO	World Health Organization

Appendix B. Amino Acids

G	Gly	Glycine
A	Ala	Alanine
C	Cys	Cysteine
I	Ile	Isoleucine
L	Leu	Leucine
V	Val	Valine
P	Pro	Proline
M	Met	Methionine
F	Phe	Phenylalanine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
S	Ser	Serine
T	Thr	Threonine
D	Asp	Aspartic acid
R	Arg	Arginine
K	Lys	Lysine
E	Glu	Glutamic acid
H	His	Histidine
N	Asp	Asparagine
Q	Gln	Glutamine