CD8+ T cells in HIV: The impact of responses to consensus HIV epitopes and their natural variants and implications for differential disease progression

by

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Abstract

For three decades, CD8+ T cells have been implicated in the control of HIV, ever since early studies revealed that a temporal correlation exists between the emergence of CD8+ T cells and the decline of viral loads in HIV infection. Subsequently, a large body of research focusing on the impact of CD8+ T cell responses on HIV has been produced. The central aim of this thesis was to investigate the relationship between CD8+ T cells and control of HIV, with a focus on the differences in CD8+ T cell responses to consensus HIV epitopes and their naturally occurring variants, as well as CD8+ T cell-mediated infection inhibition in disease progression groups.

Previous work has indicated that mutations in HIV epitopes of just one or two amino acids can have a drastic impact on the resulting CD8+ T cell response. Considering the extreme genetic diversity of the virus, understanding how CD8+ T cell responses differ to these common natural variants is essential when trying to elucidate what the best targets for an HIV vaccine would be. It was hypothesized that CD8+ T cell responses to consensus HIV epitopes, as a consequence of them being more common in nature, would be more frequent, polyfunctional, and proliferative than responses to their less common variants, as well as being associated with better disease outcomes. After assessing these functional parameters in response to four consensus HIV epitopes and their natural variants, this hypothesis was rejected, and it was determined that the consensus status of an epitope could not reliably dictate the resulting CD8+ T cell response. Rather, it seems more likely that the particular epitope being presented, combined with the HLA allele presenting it and the

particular TCRs binding to it, have a much larger impact on the CD8+ T cell response. In the course of this study, the Gag TL9 T variant epitope was identified as stimulating a CD8+ T cell response that is considered to be beneficial in HIV infection. Responses to this epitope were also associated with higher CD4 counts, which, taken together, suggests that this epitope has potential for further research as an HIV vaccine target.

In the spectrum of HIV infection, there is a significant amount of heterogeneity in disease progression, whereby some individuals progress to disease more slowly, and others, more rapidly. The mechanisms by which this differential disease progression occurs are not completely understood. It was hypothesized that CD8+ T cells from individuals who progress to disease more slowly (long term non-progressors) would be able to inhibit p24 production *in-vitro* to a higher degree than CD8+ T cells from individuals who progress more rapidly or at a normal rate (RP/NP). This hypothesis was confirmed, as CD8+ T cells from LTNP individuals were significantly better at inhibiting both secreted and intracellular p24 levels than CD8+ T cells from RP/NPs in an *in vitro* viral inhibition assay.

Overall, these studies confirm that CD8+ T cells are important in control of HIV, as indicated by an increased capacity to inhibit p24 in LTNP individuals. However, it is also clear from this work that the role that CD8+ T cells play in HIV infection is complex, and the responses to HIV epitopes can vary greatly.

Dedication

This thesis is dedicated to my grandfathers, Wilbert and Leslie. I know you both would have loved to have seen this day, and were so proud of me. I am equally proud to be your granddaughter.

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There are many people who have either directly helped with this project, or supported me in some way through it. If you think you should be on this list and you aren't, please accept my sincerest apologies, and my home address so you can send me as much hate mail as you like.

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Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 The HIV Pandemic

The human immunodeficiency virus-1 (HIV-1) pandemic continues to be one of the largest public health challenges in history, infecting an estimated 35 million people as of 2013[1]. Despite increasing awareness, education, and access to antiretroviral therapy (ART), in 2013, the number of new HIV infections topped 2.1 million, and 1.6 million people died of AIDS related causes[1]. Though this represents an overall decrease since 2001 and 2005 respectively, the enormity of these numbers displays how crucial the need is for advances in prevention and treatment of HIV.

One of the hardest hit regions in the world continues to be sub-Saharan Africa, which, despite comprising only 12% of the world's population, represented a staggering 70% of new HIV infections in 2013[1]. Within this epicenter, heterosexual transmission accounts for the majority of new infections, and women comprise greater than 50% of the infected individuals. Though progress is being made to reduce new infections and provide treatment for more individuals who need it, it is still thought that an HIV vaccine would be the best tool for combating this devastating infection[2]. However, despite decades of investigation into the pathogenesis and immune responses to HIV, an effective vaccine has remained elusive. This failure has highlighted the need for a better understanding of the basic immunology and virology of HIV infection, in order to best inform vaccine design.

1.2 The Kenyan Epidemic

Though HIV has become a pressing health issue worldwide, sub-Saharan Africa has experienced the highest rates of infection on earth. Throughout this region, the adult prevalence currently sits at approximately 4.7%[1]. Kenya has been particularly affected, as at the height of the epidemic in 1995, adult prevalence of HIV in this country was as high as 10.5%[3]. Since then, a gradual decline in prevalence has occurred, and was estimated to be 6.1% in 2012[1]. Though 6.1% is still much higher than the average prevalence worldwide, this represents a 40% decrease in prevalence in Kenya since 1995. Despite this decrease, an estimated 98,000 new infections and 57,000 deaths still occurred there in 2012. HIV continues to disproportionately impact women in this region, whereby 6.9% of all adult women in Kenya are living with HIV compared to 4.2% of men[3]. Though Kenya is now considered to be fighting a generalized epidemic, which has grown to greatly affect the general population rather than only high-risk groups, there are several key populations who continue to be disproportionately infected. For example, 29.3% of commercial sex workers (CSWs) are HIV infected, as well as 18.3% of injection drug users (IDUs) and 18.2% of men who have sex with men (MSM)[3]. Prevention efforts are still being concentrated on these key populations in a continued effort to combat HIV in Kenya. Though there is still a lot of room for improvement in this region, it has been reported recently that there has been success in reducing adult prevalence, reducing mother to child transmission, increasing condom usage, and increasing testing and treatment, which has

illustrated that the prevention and education programs currently in place are having an important impact[3].

1.3 The Origin of HIV

AIDS was first identified in 1981, after it was observed that a large number of gay men in the United States were becoming ill with otherwise rare opportunistic infections[4]. By 1983, HIV had been identified as the causative agent of AIDS, which went on to become the worldwide pandemic we are currently faced with[5]. In the history of HIV infection, four separate primary transmission events have occurred, which have resulted in the four different HIV groups we see today[6]. Group M, which is responsible for the majority of the current HIV pandemic, likely originated from a closely related chimpanzee strain of simian immunodeficiency virus (SIV) in Kinshasa, in the Democratic Republic of the Congo[7], with then spread to the southeastern region of Cameroon[6], [8]. It is thought that the initial transmission event likely occurred through mucosal or cutaneous contact with SIV infected blood through hunting of bush meat in the 1920's[7], [9], [10].

1.4 HIV Transmission

HIV is a blood-borne pathogen, and 80% of adult transmission worldwide occurs as a result of sexual intercourse[1]. This occurs through contact of mucosal surfaces with infected semen or vaginal fluids, either through vaginal or anal sex[11]. Transmission can also occur vertically from mother-to-child (in utero, during delivery or through breast milk), though the risk of infection through this method can be greatly reduced (from ~25-30% to 1-2%) if the HIV-infected mother and

newborn are both given proper care and treatment with antiretroviral therapy (ART)[12]. HIV transmission can also occur percutaneously through the sharing of infected needles by IDUs, and through the use of contaminated blood products via blood transfusions[13]. Since the early 1980's and the discovery of HIV as the causative agent of AIDS, infection via blood transfusion has become a very rare mode of transmission in most countries due to screening of blood products before use[14].

Though the majority of transmission worldwide occurs through heterosexual contact, studies have shown that transmission through unprotected vaginal sex is fairly uncommon, and may be as low as 1 infection per 1000 sexual acts[15]. Further, it has been shown that the likelihood of transmission is close to zero in individuals whose sexual partner has low or undetectable viral loads[16], which is typically the case when individuals are on antiretroviral therapy. Conversely, sexual transmission of HIV has been shown to be highest during primary infection, when there is an unfortunate and dangerous combination of high viral loads and often low awareness of an individual's HIV-infected status[17]. It has also been well established that HIV-negative individuals who have concurrent sexually transmitted infections (STIs) such as Chlamydia, gonorrhea, herpes, bacterial vaginosis and others, are at an increased risk of contracting HIV, due to an increase in inflammation and activation, making HIV more permissible to cellular entry upon exposure[18].

1.5 Treatment

Treatment guidelines for HIV vary by country. The WHO previously recommended that HIV-infected individuals who have CD4 counts below 350 cells/mm^3 should be placed on ART, as this denotes a failure of the immune system to appropriately control the virus, and indicates that the patient may be more susceptible to opportunistic infections[19], [20]. More recently, these guidelines have been modified to state that HIV-infected individuals with CD4 counts below 500 cells/mm^3 should begin ART, in an effort to allow subjects to maintain a relatively healthy immune system rather than attempt to recover from a damaged one[19], [20]. Furthermore, the WHO recommends that anyone co-infected with active tuberculosis (TB) or hepatitis B (HBV) should be on ART regardless of their CD4 count; the same is true for HIV-infected pregnant or breastfeeding women, and HIV-infected individuals in a serodiscordant relationship (where their sexual partner is HIV-negative). These strategies help to ensure that HIV-infected individuals maintain low viral loads and higher CD4 counts, in order to appropriately combat co-infections, prevent mother-to-child transmission, and reduce the likelihood of spreading HIV to their sexual partners[20].

Though effective ART has been a great step forward in the fight against HIV/AIDS, there are many challenges that remain. Access to these life-extending medications can be difficult in some of the hardest hit areas of sub-Saharan Africa due to a lack of supply or lack of access to health centers in rural areas[21], [22]. Side effects and drug toxicity can reduce adherence to treatment regimens, which, importantly, can lead to drug resistance[23], [24]. These challenges underscore the need for easier access to HIV treatment, close adherence to drug regimens to prevent drug

resistance, and the need for new treatment options should the currently available ones become ineffective within individuals and the greater population.

1.6 Prevention

Condoms, when used correctly, are highly effective at preventing sexual transmission of HIV[25]. Though this is common knowledge to many sexually active adults, eliciting behaviour change to increase condom use has been a challenge in many areas[26], [27]. In some cultures, particularly in sub-Saharan Africa, the prospect of condom use within a regular sexual partnership may be suggestive that one partner is not being faithful, which can reduce the likelihood that either partner would suggest such an intervention[28]. As such, the importance of other prevention methods, such as vaginal microbicides containing ARTs, have been the subject of great interest in HIV prevention research.

Recently, a clinical trial testing the effectiveness of a 1% tenofovir gel microbicide showed an ability to reduce infection in women by 39% overall, and by as much as 56% in those who displayed high adherence (>80%) to the application regimen[29]. Similarly, research is being conducted to develop a suitable rectal microbicide to reduce transmission among the MSM populations[30], who still remain a high-risk group around the world.

Prevention of mother to child transmission has seen drastic improvements in recent years, especially in regions where healthcare for pregnant women is easily accessible[31], [32]. Programs have been put in place in many parts of the

developing world in an attempt to reduce mother to child transmission, through increased HIV testing and counseling of pregnant women, as well as increased availability of ARTs to prevent the transmission of HIV in utero or during birth and breastfeeding[12], [33]. Through the implementation of these programs, mother to child transmission of HIV has dropped 43% between 2003 and 2011[34].

In recent years, interest has grown around research into pre-exposure prophylaxis (PrEP) and treatment as prevention programs. The most successful PrEP research to date has used a once-per-day oral tenofovir-emtricitabine pill, which is given to HIV-negative individuals considered to be at high risk of HIV infection[35], [36]. This includes individuals in discordant couples, where one person is HIV-negative but their sexual partner is HIV-infected. In these cases, where adherence to the drug regimen is high, there is a reduction in HIV infection of up to 92%[37]. Since these trials have been completed, PrEP has been licensed for use in the US for high-risk HIV-negative individuals[36], [38]. This has been met with some controversy, as there is concern about drug toxicity and side effects[38]. Also, as adherence to the drug regimen in many of the original studies was quite low, there is concern that individuals may seroconvert while taking PrEP, and unknowingly increase the likelihood of developing drug resistance[38].

Interest has also remained high in treatment as prevention as a viable HIV prevention strategy. HIV-infected individuals are at very low risk of transmitting the virus to others if their viral load is undetectable[39], [40]. By treating all HIV-infected individuals regardless of their CD4 count, it is thought that their viral load

can be maintained at a low or undetectable level, thus reducing the likelihood of spreading the virus to others[39], [40].

Aside from biomedical interventions, the importance of education, awareness, behaviour change, and reduction of stigma for prevention of HIV cannot be ignored. Education programs on the basics of HIV transmission, testing and treatment have been rolled out to key populations and the general public, in an effort to increase awareness of the infection, methods of prevention, and the importance of being tested[41], [42]. The incredible stigma that still exists toward HIV and infected individuals in many parts of the world has hampered some of this behaviour change; in some regions, individuals avoid getting tested for HIV, for fear that their status will become known within their community and that this will have a detrimental impact on their lives[32], [43], [44]. Efforts have been made to begin to reduce this dangerous stigma, and help people to understand how important it is to get tested, and protect themselves and their sexual partners accordingly[45]-[48]. Despite these improvements in treatment and prevention, millions continue to become infected every year[1]. An effective preventative vaccine against HIV, combined with other treatment and prevention strategies, would afford the best chance of slowing the spread of HIV[49].

Even through decades of research, an effective HIV vaccine has remained elusive due to several factors, including its incredible genetic diversity, an incomplete understanding of anti-HIV immunity, and its propensity to destroy CD4+ T cells, which are a crucial component of the immune system[50]-[52]. After greater than

30 years of research, there have been dozens of phase I, II and III HIV vaccine trials, and though some have shown an ability to produce strong anti-HIV immune responses, all but one have shown absolutely no protection from infection in humans[53], [54]. Two vaccine trials of particular interest are the failed Merck STEP trial, and the partially successful Thai RV-144 trial.

The STEP trial used a recombinant adenovirus vector to express the Gag, Pol and Nef genes of HIV[55], [56]. In phase I safety and immunogenicity trials, this vaccine was shown to be safe and to elicit high levels of HIV specific CD8+ T cells capable of producing multiple cytokines, which is thought to be an important aspect of anti-HIV immunity[56]. Previous research displayed the importance of HIV epitope-specific CD8+ T cells against HIV, which instilled confidence that this vaccine would elicit protective CD8+ T cell responses able to prevent infection[57]-[60]. However, in September of 2007, the trial was stopped early, as an interim review found there was no protection against HIV infection, and rather, that individuals who received the vaccine may have been at slightly higher risk of infection than those who did not[55], [56]. This was a huge set back in the HIV vaccine field, and more than anything, shed light on how crucial it is to have a sound understanding of the basic immunology and virology of HIV when trying to inform vaccine design.

The Thai RV-144 trial, which was completed in 2009, tested a prime-boost vaccine consisting of an ALVAC-HIV prime (a canarypox vector), followed by an AIDSVAX boost (a gp120 subunit vaccine). The rate of infection was reduced by 31.2% in the vaccine group compared to the placebo group[61]. Though the protection was

modest, the RV-144 trial represents the first HIV vaccine to show any protection from infection, and has helped to bolster the HIV vaccine field in the years following the results[62]-[65]. Despite this marginal level of success, there is still much more to be done to improve the effectiveness of this or any other vaccine, and as such, the other biomedical and behavioural interventions mentioned above are still of utmost importance in slowing the spread of HIV.

1.7 Basic Innate and Adaptive Immunity

The human immune system consists of two different but equally important arms, termed innate and adaptive immunity. The innate immune system is composed of cells and factors that are always present, before an infection even occurs[66]. This includes physical barriers like the skin and mucosa, as well as cellular components like macrophages, dendritic cells (DCs), natural killer cells (NK cells), neutrophils, basophils, eosinophils and mast cells[67]. Cells of the innate immune system work by recognizing similar patterns common to many pathogens, which triggers the release of cytokines and chemokines. This can help to activate parts of the adaptive immune system, and signal cells to migrate toward the site of infection to help combat it[66].

In contrast to the innate immune system, the adaptive system is specific to the infecting pathogen, and can develop immunological memory, allowing for a much faster and more robust response when the same pathogen is encountered again[67]. In order for a successful adaptive immune response to occur, interaction with the innate immune system is required. Major histocompatibility complexes (MHCs),

also known as human leukocyte epitopes (HLA) in humans, are expressed on the surfaces of cells and are divided into class I and class II[66]. These complexes are used to display and present epitopes to T cells, which can then recognize and bind to them, and produce a downstream immune response. Epitope presenting cells (APCs) endocytose epitopes they have sampled from the environment of the body, and display them using MHC class II molecules, while MHC class I molecules present epitopes from within the cell, including epitopes derived from infecting pathogens[66].

T cells are one of the main cellular components of adaptive immunity, and are responsible for recognizing and binding to the MHC-epitope complexes. This binding occurs due to T cell receptors (TCRs) expressed on the surface of T cells[67]. These receptors go through a great deal of genetic recombination as they are formed, which creates an incredibly diverse repertoire of different TCRs, and as such, a vast array of T cells exist with different specificities for different epitopes[67]. This is what allows them to respond in a far more specific manner than occurs in the innate arm. Once a T cell recognizes and binds to an epitope being presented and the appropriate costimulation is available, a signaling cascade occurs which initiates downstream T cell responses including activation, proliferation, and secretion of cytokines. After recognition of a foreign epitope, the T cells will undergo clonal expansion, which ensures that a high number of T cells specific to the invading pathogen will be present to clear it from circulation in the body. Once the pathogen has been cleared, the T cells will go through a phase of contraction, where the majority of pathogen-specific cells will die, save for a few

that will become long-term memory cells. These cells are a main feature of the adaptive immune response, as their long-term presence within the body will allow for a much more rapid and robust response if that pathogen is encountered in the future[66].

1.8 T Cell Immunity

The adaptive immune system is composed of two main types of T cells: the CD4+ 'helper' T cells, and the CD8+ 'cytotoxic' T cells. CD4+ T cells are a crucial component of the adaptive immune response, and are responsible primarily for helping to direct what type of immune response occurs when foreign epitopes are detected[66]. The release of cytokines by the CD4+ T cells in response to epitopes can assist in directing the immune system to produce either a TH1 (T-helper 1) or TH2 (T-helper 2) response[67], [68]. A TH1 response is considered to be a cellular response, and is characterized by production of cytokines such as IFNγ, TNF and IL-2. TH1 responses are particularly effective against intracellular pathogens such as viruses and intracellular bacteria[66]. The TH2 response is mediated by IL-4 and IL-13, and is protective primarily against parasites and other extracellular pathogens[66]. TH2 is also described as the 'humoral' response, as it generates antibodies when stimulated [66], [69].

CD8+ T cells have important effector functions within the immune system, and are responsible for killing infected cells[67]. When a CD8+ T cell comes into contact with an epitope it recognizes, binding of the TCR to the MHC-epitope complex will occur. There are many sources of variation within this TCR-epitope-MHC binding

complex (Figure 1.1)[67]. Firstly, the TCRs of any particular CD8+ T cell are both diverse and specific. CD8+ T cells can express a vast array of different TCRs, which can recognize multiple epitopes. This diversity allows CD8+ T cells to recognize and bind to many different epitopes from many different pathogens[70]. The second source of variation is the epitope itself. Some epitopes have been discovered to be more immunodominant than others; that is, they are more likely to elicit a response from a higher proportion of CD8+ T cells than other sub-dominant epitopes. The dynamics of this immunodominant/sub-dominant relationship are somewhat complex, as, in some cases, a focused CD8+ T cell response toward an immunodominant epitope may provide a beneficial immune response, whereas in others, targeting of several sub-dominant epitopes may in fact be more beneficial[71]. Furthermore, it is known that changes of one or two amino acids within these epitopes can result in vastly different downstream CD8+ T cell immune responses[72]. This is an important consideration in HIV vaccine research, as it is essential to know not only what kind of immune response would be most beneficial to elicit, but also what particular epitope may be best at stimulating such a response. Considering that HIV is so highly mutable, understanding the impact small changes in the epitopes may have on the resulting CD8+ T cell response is of utmost importance. Lastly, the particular HLA that presents the epitopes can have a huge impact on the resulting response. For example, some HLA alleles have been shown to be associated with more rapid or slow progression to AIDS after HIV infection (discussed below). It is also known that some HLA alleles exhibit better binding to epitopes with specific amino acids at the ends ('anchor' residues), which will impact which epitopes are presented to the CD8+ T cells, as well as impact the overall

immune response[73]. Any modifications within these epitopes can have a profound impact on how well they bind to the HLA, TCR, or both, and as such, impact the downstream immune responses.

One important function of CD8+ T cells is to secrete cytokines and chemokines in response to pathogen-derived epitopes. Some of the main cytokines produced by CD8+ T cells are interferon-γ (IFN γ), tumour necrosis factor (TNF), macrophage inflammatory protein 1-β (MIP1β) and interleukin 2 (IL-2), each of which have their own functions within the immune system[74]. IFNγ plays an important role in immunomodulation of the immune system, and can have direct antiviral properties[75]. TNF is another important mediator of inflammation, and also has antiviral functions when viral infection occurs[76]. MIP1β binds to CCR5, a co-receptor for HIV entry into cells, which reduces the spread of infection, as it works to out-compete the virus for these receptors[77]. IL-2 has an important role in supporting the proliferation and development of T cells[78].

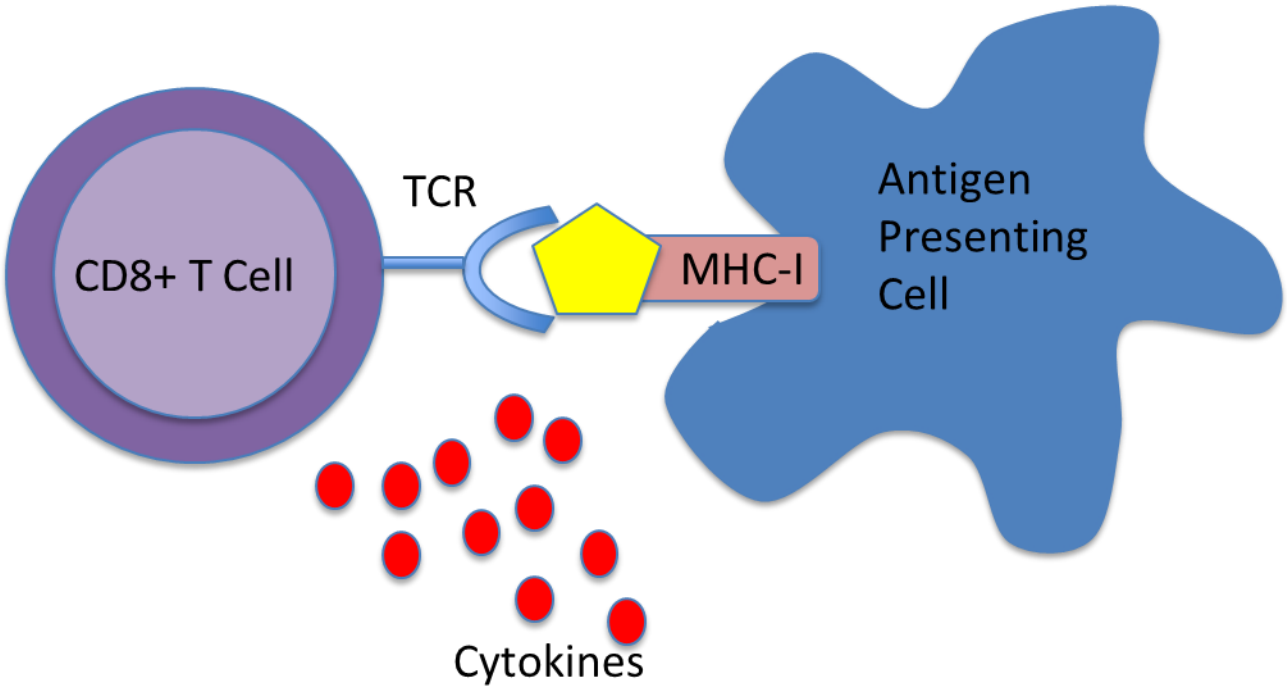


Figure 1.1: Dynamics of CD8+ T cell binding to APCs via the TCR and MHC complex. When an epitope presenting cell encounters a virus, it will present on its surface an epitope from that virus in context with an MHC-I molecule. A CD8+ T cell with a TCR that can recognize that epitope will bind to it, and a cytokine response will occur to help destroy the infection.

CD8+ T cells moderate killing of infected cells through two main mechanisms. In the first, they can release antiviral cytokines like TNF and IFNγ, as well as cytotoxic granules such as perforin and granzymes. Perforin is able to insert into the infected cells, allowing entry of granzymes[79], [80], which then trigger the apoptotic cascade, leading to apoptosis of the infected cell[81]. In the second pathway, CD8+ T cells express Fas ligand (FasL), which binds to Fas, which is expressed on the infected target cells. This binding leads to recruitment of caspases, which also leads to apoptosis of infected cell targets[82].

1.9 TCR Formation and Usage

TCRs are found on the surface of T cells and are responsible for recognizing and binding to epitopes that are presented by APCs. The TCR is a heterodimer composed of alpha and beta chains, each containing variable and constant regions[83]. The constant region is nearest to the cell membrane, while the variable region is that which binds to the HLA-epitope complex[83]. The variable regions of both the alpha and beta chains contain three hypervariable regions called the complementarity determining regions (CDRs), of which CDR3 is primarily responsible for recognition of epitopes[84].

The specificity of the TCRs is determined during their generation, according to the particular structure of the alpha and beta chains, which form the epitope-binding sites. The variable regions are composed of three gene segments, termed the V, D and J segments. Recombination within these gene segments during the generation of the TCR allows for the production of a very diverse TCR repertoire[84].

Epitope-specific CD8+ T cells each have specific TCRs, or TCR clonotypes. Different CD8+ T cells that are specific for the same MHC-epitope complex will express a wide variety of different TCR clonotypes despite their affinity for the same epitope[85]. Interestingly, even when CD8+ T cells express identical TCRs, the resulting CD8+ T cell response can be extremely heterogeneous[49] and dependent on many things, including epitope avidity, dose, and contact time.

1.10 HIV Structure

HIV is an enveloped, single-stranded RNA lentivirus belonging to the family *Retroviridae*[86]. The pandemic M group of HIV is further subdivided into 11 clades, of which clades A1 and D are the most prevalent in Kenya[87]. There are also many recombinant forms of the virus circulating in Kenya, which contribute to the immense diversity of the virus[88]. The genome of HIV contains 9 genes that encode for both structural and accessory proteins, including gag (coding for the viral capsid proteins p17, p24 and p55), env (coding for the envelope glycoproteins gp120 and gp41), pol (coding for reverse transcriptase [RT], integrase and protease), as well as the regulatory genes tat, rev, nef, vpr, vpu and vif[89].

1.11 HIV Life Cycle

In order for HIV to enter human cells, the viral surface gp120 protein must interact with the CD4 receptor on host cell[90]. This occurs most commonly in CD4+ T cells and macrophages, though infection of other cell types such as dendritic cells, langerhans cells and double positive CD8+/CD4+ T cells has been documented[51],

[91]. The interaction between gp120 and CD4 leads to a conformational change, which allows gp120 to bind to chemokine co-receptors, which then permits fusion and viral entry[92]. The two most commonly used chemokine co-receptors are CCR5 and CXCR4, which bind to HIV R5-tropic and X4-tropic viruses respectively[93]. Once the virus has entered the cell, reverse transcription occurs to produce the HIV pre-integration complex, consisting of double stranded DNA, along with integrase, vpr, and RT. The pre-integration complex is transported to the nucleus, where the double stranded DNA is then integrated into the genome of the host cell[94]. Viral replication occurs most efficiently in activated cells, as this activation leads to upregulation of host factors that are required for transcription of the viral genome[94]. After transcription, translation and assembly of new virions occurs in the cytoplasm, and eventually the new viral particles bud from the surface of the host cell[95].

1.12 HIV Diversity

One of the hallmarks of HIV infection, and one reason why vaccine development has been a challenge, is the incredible genetic diversity of the virus. To further complicate the presence of 11 different viral clades within the pandemic M group, there are innumerable different variants and mutated strains (quasispecies) of HIV that may differ by a large number of amino acids, or by as little as one or two[96]. Much of this diversity is due to the error prone RT, which is likely to produce at least one genomic mutation within each new virion produced[8], [97]. Though many of these mutations will result in non-functional or replication deficient viruses, this high mutation rate increases the likelihood that there will be some 'new' viral

strains that survive and are able to thrive within individuals, and potentially within the population as a whole[98]. Viral mutations can also occur as a result of immune pressure on the virus[99], which forces the virus to mutate in order to 'escape' targeting by the immune response. As occurs in random RT-error generated mutations, these escape mutations can sometimes come with a significant fitness cost to the virus[100], [101], which can either result in a less virulent or replication deficient strain, or in additional compensatory mutations in order to repair some of the lost viral fitness[100], [102].

1.13 HIV Pathogenesis and Disease Progression

The typical progression of HIV is described in Figure 1.2. When HIV infection initially occurs, the individual will go through an acute phase of infection that generally lasts 6-12 weeks[103]. During the earliest point of infection, and for the first ~10 days thereafter, the virus typically remains within the draining lymph nodes and mucosa[103]. After this, it spreads quickly systemically and then to other tissues, and in particular to the gut, where it quickly replicates[104]. The gut associated lymphoid tissue (GALT) is an important reservoir of infection, and exponential viral growth occurs at this location[104]. As much as 20% of the resident gut CD4+ T cells become infected, and 80% are depleted during this period[105], [106]. At this point, viral load peaks, which coincides with a noticeable decline in circulating CD4+ T cells[107]. HIV-specific CD8+ T cells are then induced, resulting in a rebound of CD4+ T cells, and a decline in the viral load to a relatively steady state, which is termed the viral set point[108], [109]. The viral set point has been previously shown to be an important indictor of disease progression[107],

[110], whereby individuals with a low viral set point are likely to progress to AIDS more slowly than those whose viral set point is higher. After reaching the set point, the individual progresses into a longer chronic phase of infection, which can last 1-15 years, or more[111]. This period is characterized by low but variable viral loads followed by a gradual decline in CD4+ T cells[111]. Eventually, CD4+ T cells will decline below 200/mm^3 at which point a diagnosis of AIDS has been reached, and the viral load will increase once again. At this point, patients often succumb to opportunistic infections, as their immune systems have been depleted and are unable to effectively respond to and eliminate any invading pathogens[111].

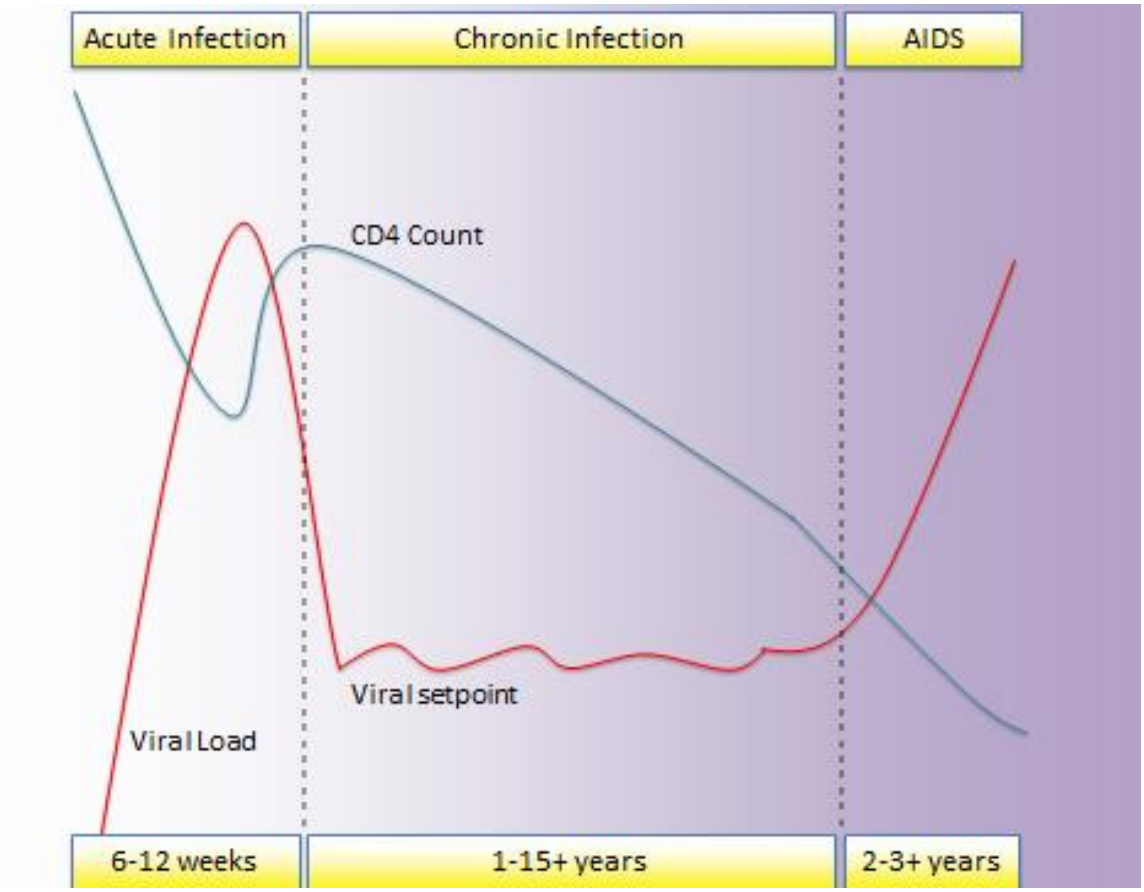


Figure 1.2: HIV disease progression, highlighting key steps in typical HIV infection. In the first 6-12 weeks of HIV infection, viral load (red) will spike sharply, and CD4 cells will begin to decline. At this point, the CD8+ T cell response is established, and leads to a reduction in viral load to a set point, where it will remain during the chronic stage of infection. CD4 cells will typically rebound slightly, but overall decline during the course of chronic infection. Eventually, as an individual's CD4 count dips below 200, a clinical definition of AIDS has been reached, and viral load will increase once more.

HIV-infected individuals can be categorized by the length of time it takes them to progress to disease. Within the Majengo cohort, individuals whose CD4 counts drop below 500 cells/mm^3 in less than 3 years from becoming infected are known as rapid progressors (RP). Others, whose CD4 count falls below 500 cells/mm^3 in 3-5 years are known as normal progressors (NP), as this is the most common course of infection when left untreated in this cohort. Finally, individuals who are able to maintain CD4 counts above 500 cells/mm^3 for 7 years or more are known as long-term non-progressors (LTNP). Other research groups have identified HIV-infected individuals who are able to maintain undetectable viral loads in the absence of treatment, which are termed elite controllers[112]-[115].

Owing to the fact that there is no natural model of recovery from HIV infection for which to base HIV prevention research on, it is important to try to understand why some individuals are better able to control infection to provide a basis from which to work for an effective vaccine or treatment. There have been some mechanisms proposed for why LTNP individuals progress to disease more slowly. Some reports have suggested that LTNPs are infected with strains of HIV that contain deletions or mutations that result in attenuation of the virus, or strains that have reduced replicative capacity, making control of the infection easier[116]-[118]. HLA types that are associated with slower progression to AIDS are consistently over-expressed in LTNP individuals, which may at least partially explain the slower disease progression seen in this group[119]. It is possible that these HLA types are more likely to present HIV epitopes that result in an enhanced ability of the CD8+ T cells to respond effectively in the face of infection[120]. It is likely that these

mechanisms, along with other genetic and immunological factors[121], [122], work in concert to create the advantageous LTNP phenotype[120]. Having a complete understanding of the mechanisms behind this temporary viral control will be greatly beneficial, as a vaccine that can induce a similar mechanism of control may be effective.

1.14 HIV Immunology

HIV is a complex virus, which exhibits a great deal of heterogeneity with respect to disease progression[123]-[125]. Though some individuals may be able to contain the virus for decades without treatment, the vast majority of infected people will eventually lose immune control, resulting in a spike in viral replication and a loss of CD4+ T cells, and causing them to succumb to AIDS[126]. Perhaps the most unique and fundamentally important aspect of HIV is that it targets CD4+ T cells for infection (and, more specifically, activated CD4+ T cells) [127], [128], which, as discussed above, are an essential part of a properly functioning immune system[129]. As the infection progresses, these cells are depleted from the blood, mucosa, and gut tissues of the body, which is what leads to the immune deficiency and the onset of AIDS and AIDS associated opportunistic infections[130].

HIV infection can have other detrimental effects on the immune system, such as structural and functional impairment of the lymph nodes, which can limit the ability of the immune system to regenerate immune cells lost during infection[131]. Infection with HIV can also cause a loss of immune integrity at mucosal surfaces, resulting in microbial translocation from the intestinal lumen to systemic

circulation, which can lead to chronic activation, exacerbating the pressure on the already overworked immune response[132]-[134].

Increased immune activation has been shown to be an important factor in HIV infection and pathogenesis[132], [135]-[137]. Chronic activation can be the result of the presence of a large number of virions within the body, leading to persistent stimulation of T cells attempting to form an effective immune response against constant epitopeic challenge[137]. This leads to a release of inflammatory cytokines, which can enhance activation, leading to more available targets for HIV, which serves to enhance infection even further[137].

Although activated CD4+ T cells are the main targets for HIV infection, the widespread depletion of CD4+ T cells is not only associated with their infection. A large proportion of CD4+ T cells are depleted due to bystander killing and chronic inflammation[138]-[140]. It has been shown that some individuals who maintain low levels of viremia can still progress to clinical AIDS, which suggests that death of uninfected CD4+ T cells during infection is an important determinant in disease progression[138]. Similarly, it has been shown that individuals displaying low immune activation are able to maintain normal CD4 counts, despite having high levels of viremia[141]. This evidence together suggests that immune response mediated bystander death of uninfected CD4+ T cells is an important component of HIV pathogenesis and contributes to disease progression. Somewhat counter-intuitively, this bystander effect may be somewhat beneficial in early infection. The initial decline of viral load in early infection is thought to be due partially to

pressure from epitope specific CD8+ T cells, but also because of a lack of available target cells due to widespread activation induced cell death and apoptosis of CD4+ T cells[105].

An important facet of HIV is its ability to form into latent viral reservoirs within the body[142], [143]. The establishment of these reservoirs is a main reason for HIV's success; though ART is effective at eliminating circulating virus, these latent reservoirs experience a lack of pressure by ART and the immune system, allowing for a chronic, life-long infection[143]. Recently, research has been aimed at attempting to force the virus out of these reservoirs, as a means of fully eradicating it from the body[142], [144]-[146]. The results of this new research remain to be seen.

1.15 CD8+ T Cell Responses in HIV

There has been much evidence since the emergence of HIV that CD8+ T cells are a very important aspect of the immune response against it. Early studies revealed that there was a temporal correlation between the emergence of CD8+ T cells and viral loads, whereby populations of CD8+ T cells expand as the viral load begins to climb in early infection, and contract again once the virus has been contained and control has been re-established[147], [148]. CD8+ T cells are activated and recruited in response to this increase in viral load, and are now known to be actively involved in establishing the viral set point[101], [110], [149], [150]. Early studies in simian models showed that depletion of CD8+ T cells from SIV infected macaques led to a rise in plasma viremia of 1 to 3 orders of magnitude[57], [151], implicating CD8+ T

cells in control of HIV. Despite these observations, CD8+ T cell research in HIV has been hampered by many challenges.

Though many CD8-related correlates of protection in HIV have been identified[125], [152]-[154], the mechanisms behind CD8+ correlates of protection remain incompletely understood, and there are often exceptions found in each case[155]. While the research done has been very informative, the results remain correlative, and there is some level of difficulty in resolving patterns of protection when there are few universal controls available to properly compare such diverse cohorts. Even so, the prevailing theory in the field of CD8+ T cell research in HIV is that a more proliferative, polyfunctional, cytotoxic CD8+ T cell response is most beneficial against HIV[2], [156], [157]. LTNP individuals have enhanced proliferative capabilities, and it is suspected that the increase in cellular turnover prevents T cell exhaustion and allows the cells to maintain high cytotoxic capabilities[120], [125], [155], [158]. It has also been established that an increase in proliferation results in more uniform production of perforin, which aids in the destruction of virally infected cells[80], [159]. LTNP individuals are also more likely to have CD8+ T cells that are highly polyfunctional (produce more than one cytokine per cell) than those individuals who progress to AIDS rapidly[2], [156], [157]. In early assessment of the functionality of CD8+ T cells in HIV, IFNγ ELISPOT assays were often conducted as a test of HIV epitope-specific responses due to their ease of use and the hypothesis that IFNγ was representative of an effective and robust CD8+ T cell response[160]. In time, it was realized that this assessment alone might not be capturing an accurate representation of all epitope-specific CD8+ T cell

responses[161]. Typically, polyfunctionality is measured in terms of an individual's CD8+ T cells to produce IL-2, IFNγ, MIP1β, TNF and CD107α concurrently, and any cells that can produce two or more of these functions is considered to be polyfunctional[155]. One complication of this is that as more functions of CD8+ T cells are discovered, the term 'polyfunctionality' begins to become ambiguous, and the understanding of which functions are truly important or effective against HIV becomes difficult to assess[155]. Despite these cautions, it is reasonable to expect that cells capable of responding with known antiviral factors (MIP1β, IFNγ, TNF, perforin, granzymes, etc), and those which are able to proliferate and hence avoid exhaustion, would be most beneficial in establishing a robust and long lasting anti-HIV response.

The specific targeting of CD8+ T cell responses may play an important role in their ability to control HIV infection. It has been shown that broad targeting of Gag epitopes results in the most beneficial response, while targeting of Env seems to be of little benefit[154], [162]-[164]. A possible reason for this may be related to the dynamics of HIV infection and replication. Studies have indicated that early responses to HIV infection are crucial to retaining CD4+ T cells and achieving a favourable disease outcome[110], [165]. Though Gag is not produced early in the HIV replication cycle, there is such a high amount of Gag within infecting virions that it can be processed directly by APCs without requiring viral replication in the host cell[162], [166]. CD8+ T cells that are able to target it would be able to respond more quickly than those targeting Env, which is not detectable until approximately 20 hours after the recognition of Gag[166]. Another possibility is that Gag is more

conserved than Env, and, as such, immune pressure directed toward this region is less likely to result in escape mutations[154], [162], [164]. As Gag is more conserved, escape mutations that do occur may also be more likely to come at a higher fitness cost to the virus[162], [167], [168]. Mutations within the Gag protein, and the p24 capsid in particular, come with very high fitness costs, as this region is necessary for providing proper structure for the virion[169], [170].

Finally, compelling evidence that suggests a role for CD8+ T cells in control of HIV is the fact that viral escape mutations arise in the course of infection. These viral mutations can occur within the first 4-6 weeks after viral load has peaked in early infection, and occur due to pressure by HIV-specific CD8+ T cells[171]. This adds more weight to the theory that CD8+ T cells are important in control of HIV, as the virus would not be driven to mutate away from immune responses that are ineffective.

1.16 Loss of CD8+ T Cell Function and Viral Escape in HIV

The above presents a strong case for the importance of CD8+ T cells in control of HIV, however, it is well established that this control is eventually lost as disease progression occurs.

Over time and continued exposure to epitopes, CD8+ T cells begin to lose their functions, and eventually succumb to a state of exhaustion[172], [173]. An exhausted phenotype is characterized by a loss of normal functions like secretion of IL-2, TNF and IFNγ, as well as the loss of cytotoxic potential and proliferation[174].

Exhaustion is also enhanced by upregulation of surface markers such as programmed-death 1 (PD-1), CTLA-4, Tim-3 and others, which can negatively regulate the function of T cells[174]. This exhaustion phenotype develops as a consequence of the duration of infection, and in concert with a loss of CD4+ T helper cells[156]. Recently, it has been established that blocking of some exhaustion markers such as PD-1 can lead to a rebound in T cell activity and function[175], [176]. Overall, the chronic stimulation that is endured by the immune system during HIV infection is a clear factor in the resulting exhaustion and loss of function of CD8+ T cells.

In addition to T cell exhaustion, other mechanisms can impair the function of CD8+ T cells in chronic HIV infection. During HIV infection, Nef can downregulate HLA molecules on the surface of cells, which results in an impaired ability for CD8+ T cells to recognize and respond to infected cells[177]. Early in HIV infection, there are typically only a few immunodominant epitopes which the CD8+ T cells respond to, and this narrow response can lead to viral escape[107]. As the virus mutates away from the responding CD8+ T cell response, this will sometimes lead to a reduction in viral fitness, which can function to drive compensatory mutations that are able to circumvent this and allow the virus to thrive once more[167], [178]. These escape mutations can have several different impacts on the CD8+ T cell response. Epitopes that were once efficiently recognized by CD8+ T cells may mutate in such a way that they are no longer able to bind to the HLA or are no longer recognized by their TCR, both of which could result in a deficient CD8+ T cell response[179], [180]. Another common source of escape mutations is those that

result in improper processing of epitopes before presentation on HLA alleles. This results in an inability of the CD8+ T cells to recognize those epitopes that may have been properly presented if not for the escape mutation[179], [180].

1.17 HLA and HIV

Class I HLAs consist of two alleles each of HLA-A, HLA-B, and HLA-C, for which there is a great degree of diversity[71]. As previously stated, HLA alleles are an important component in the determination of disease progression in HIV.

It has been found that within the groups of individuals able to control HIV, certain HLA types are overrepresented when compared to the general population[123]. The HLA types so far described as being associated with slower disease progression include HLA-B*57, HLA-B*58:01, HLA-B*27, HLA-B*14, HLA-B*44, HLA-B*51 and HLA-B*81[181], [182]. There have been several proposed mechanisms for how and why these particular HLA alleles are associated with better disease outcomes. Some of these HLA alleles present epitopes that are found primarily in structurally conserved regions of the viral Gag protein[183]. It has been observed that CD8+ T cell responses to these epitopes, if induced in early infection, can help to reduce viral replication[102]. As research has indicated that broad targeting of Gag epitopes results in a more robust and effective CD8+ T cell response[162], [164], [184], [185], this may explain why the alleles capable of presenting these epitopes are related to better disease outcomes.

Another important aspect of HLA immunity is the heterozygosity and homozygosity of the HLA alleles. Individuals who have different alleles at each or any of the three HLA class I loci will be able to present a broader repertoire of pathogen-derived epitopes to the immune response, resulting in a broader T cell response which can exert more pressure on the infecting pathogen[186]. Previous research has shown that individuals who are homozygous at any of the three HLA class-I loci are likely to progress to AIDS more quickly than those who are heterozygous, with individuals homozygous at all three loci progressing most rapidly of all[186]. An alternate explanation to this phenomenon is that HIV is able to adapt to whatever HLA alleles are most common within a given population, which are the same alleles most likely to be found in individuals who are homozygous at their HLA loci[71]. It is likely a combination of the increased diversity of epitopes presented by heterozygous alleles and the adaptation of HIV to homozygous alleles that accounts for this phenomenon.

HLA-B is the most diverse of the three class I loci, with >2000 identified alleles[71], and is thought to have the most drastic impact on HIV disease progression and HIV specific immune responses. It has been found that variation within HLA-B is associated with viral load in HIV-infected patients[187]. Within these alleles, variation is typically found within genetic regions that code for the peptide binding groove, allowing not only for diversity within the HLA alleles themselves, but for presentation of a huge number of different epitopes[113], [188]. Indeed, a large study of HIV elite controllers has shown that 5 amino acids, all found within the peptide-binding groove of HLA-B, are implicated in either viral control or lack

thereof[113]. This is an important consideration for HIV immune control, since HLA-B is responsible for restriction of most CD8+ T cell responses against HIV. With this in mind, it is intuitive that the HLA class I alleles associated with slow or rapid disease progression most commonly come from HLA-B.

*1.18 HLA-B*57 in HIV Disease Progression*

Of all of the HLA alleles that have been associated with slower HIV disease progression, HLA-B*57 has been one of the most consistent associations, and as such, has been closely studied[119], [187], [189]. It has been found that while the HLA-B*57 allele is present in approximately 5-9% of the general population, 40-85% of HIV controllers possess it[119], [190]. The mechanism behind this relative protection from progression is likely a combination of HLA-B*57 presenting highly conserved Gag epitopes, and epitopes for which escape mutations are rare and/or costly to the virus[190]-[194]. When viral variants from HLA-B*57+ individuals are transmitted to HLA-B*57- individuals, the escape mutations that have managed to form frequently revert back to their wild type, suggesting that these mutations do indeed produce a substantial fitness cost to the virus[193]. Complicating this association, there have also been many HLA-B*57+ individuals identified who progress to AIDS at normal or even enhanced rates, indicating that there are many factors at play, including the HLA type, that determine progression status[190].

CD8+ T cell responses to HLA-B*57 restricted epitopes have also been shown to be more cross-reactive than responses from other HLA alleles, which may help to control the virus even as it mutates, and may play a role in combating viral

escape[195]. Interestingly, it has also been shown recently that CD8+ T cells restricted by HLA-B*57 express low levels of Tim-3, and as such, are resistant to the effects of regulatory T cells, which are known for their suppressive effects on the immune system[196]. Further, it has been demonstrated that CD8+ T cells restricted by HLA-B*57 can also directly kill regulatory T cells, both of which allow them to maintain strong responses even during chronic infection[196].

Project Rationale, Hypotheses and Objectives

Project Rationale

The incredible diversity of HIV has been well documented in previous research, as has the diversity of the resulting CD8+ T cell response. The failure of the STEP trial has underscored the need to better understand the intricacies of CD8+ T cell responses to HIV before trying to inform the design of a T cell based vaccine. Previous work has shown that changes of one or two amino acids in a presented HIV epitope can result in drastically different CD8+ T cell responses[72]. Presently, the differences in CD8+ T cell responses to many consensus and immunodominant HIV epitopes and their natural variants is not completely understood.

Disease progression in HIV is highly heterogeneous, with some individuals progressing to disease more rapidly or slowly than others. Though CD8+ T cells have been implicated as an important factor in slower disease progression, the functional responses thought to be involved in this have been largely correlative in nature. Measuring the capacity of CD8+ T cells from individuals in different progression groups to inhibit viral replication affords the opportunity to directly observe the ability of CD8+ T cells to functionally impact HIV.

Gaps in Knowledge

While it is clear that an effective CD8+ T cell response is essential in a successful anti-HIV immune response, the dynamics of these responses to different HIV epitopes remains incompletely understood. How the CD8+ T cell response varies when it is presented with consensus epitopes compared to natural variants of those epitopes is unclear, along with which may stimulate the most beneficial CD8+ T cell response.

Previously, it has been shown that individuals who are able to control HIV have an enhanced ability to inhibit viral replication *in vitro*. The mechanism of how and why this occurs is not completely understood, nor is it understood if this control exists in HIV-infected sex workers in Kenya.

This thesis investigates the relationship between CD8+ T cells and control of HIV, with a focus on the differences in CD8+ T cell responses to consensus HIV epitopes and their natural variants, and infection inhibition in progression groups.

Hypotheses

There are two major hypotheses explored in this thesis:

1) Consensus HIV epitopes will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their naturally occurring variants. CD8+ T cell responses to consensus epitopes will be more proliferative and

polyfunctional, and be associated with better disease outcomes than responses to natural epitope variants.

2) CD8+ T cells from long-term non-progressors will be able to suppress viral replication more strongly than CD8+ T cells from rapid or normal progressors.

Objectives

To address these hypotheses, the following objectives were undertaken:

1a) Determine the frequency of recognition, proliferative capacity and polyfunctionality of CD8+ T cells in response to HIV epitopes and their natural variants.

1b) Determine the association of CD8+ T cell responses to consensus and variant epitopes with CD4+ T cell counts as a measure of immune control and disease progression.

1c) Assess the changes in cytokine production and proliferative potential in individuals over multiple time points to determine how these factors may change over time with disease progression.

2) Conduct *in-vitro* viral inhibition assays on PBMCs from long-term non-progressors and normal/rapid progressors to determine relative viral suppression capabilities of each group.

Objectives 1a) and 1b) will be addressed in Chapter 3 and Chapter 4. Chapter 3 will characterize two HLA-B*42:01 epitopes and their natural variants, while Chapter 4 will characterize two HLA-B*57:03 epitopes and their natural variants.

Objective 1c) will be addressed in Chapter 4, in the context of HLA-B*57:03 presented epitopes.

Objective 2 will be addressed in Chapter 5

Chapter 2: Methods

2.1 Study Cohorts

This study was conducted primarily on samples from the Majengo female sex worker cohort based in the Pumwani district of Nairobi, Kenya. This cohort was established in 1984 to study HIV infection in female sex workers, and currently has enrolled >4000 participants. When visiting the clinic, participants answer a behavioural questionnaire and provide blood samples, which are used to screen for HIV, to determine viral loads and CD4 counts, and for research purposes. All participants are offered regular medical care for STIs or other illnesses, as well as counseling and education, condoms, and ART if they are HIV-infected and meet the criteria for treatment. Control samples for optimization experiments were accessed from the local donor program in Winnipeg, Manitoba, Canada. All participants in the Majengo sex worker cohort, as well as individuals providing control samples in Winnipeg, gave informed consent to be involved in this research program and provide samples. All studies were approved by the research ethics boards at the University of Manitoba, and Kenyatta National Hospital.

2.2 HIV Testing

Participants in the Majengo sex worker cohort were tested for HIV infection by enzyme-linked immunosorbant assays (ELISA) (Recombigen- Trinity Biotech). All individuals who tested HIV-positive with this first method were provided with a confirmatory test before a diagnosis of HIV positivity was determined. CD4+ T cell counts were determined for all HIV-infected participants, using the Tritest flow

cytometry assay (BD Biosciences) on whole blood samples. CD4 counts are expressed as the number of CD4+ T cells per cubic millimeter of whole blood (mm^3).

2.3 General Reagents

R-10 media: RPMI 1640 media (Hyclone) supplemented with 10% fetal calf serum (FCS) (Hyclone) and 1% penicillin-streptomycin antibiotics (Gibco). FCS was inactivated at 56ºC for 30 minutes prior to use.

Complete media: R-10 media supplemented with 20 international units of IL-2 (NIH AIDS Research and Reference Reagent Program).

FACS wash: PBS supplemented with 2% FCS.

Perm wash: 1x of the stock buffer prepared in H_2O.

Freezing media: 10% dimethyl sulfoxide (DMSO) and 90% FCS.

ELISA Blocking Buffer: PBS with 1% BSA.

ELISA Coating Buffer: 0.015M Na_2CO_3 and 0.035M $NaHCO_3$ in PBS, pH 9.6.

ELISA DEA Buffer: 122mg $MgCL_2$-$6H_20$ dissolved in water, in 117ml diethanolamine, in total 1L PBS. Should be pH 9.8, and kept in the dark.

ELISA Wash Buffer: PBS with 0.05% Tween.

2.4 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using ficoll-hypaque density centrifugation. Blood tubes were centrifuged at 350xg for 7 minutes, and 1ml of plasma was collected and stored at -80ºC. The remaining blood was diluted 1:2 in PBS, and layered over ficoll (Lymphoprep, MJS BioLynx Inc.) at a ratio of 10ml of ficoll per 15ml of diluted blood. Layered tubes were centrifuged at

240xg for 25 minutes with the brake off. The white cell layer was collected and washed once with PBS, and then once with R-10. Cells were then resuspended in 10ml of R-10 and counted using a hemocytometer and Trypan blue exclusion dye (Hyclone).

2.5 Thawing cryopreserved PBMCs

Any cryopreserved PBMCs used in cellular assays were carefully thawed in order to minimize cell stress and death. Vials of cryopreserved PBMCs were placed in a 37ºC water bath until they began to thaw. Then, room temperature FCS was added to each cryovial one drop at a time, in order to minimize cell death due to an improper osmotic balance. Cells were mixed slowly with FCS until the entire vial was thawed, and transferred into a 50ml conical tube containing the remaining 5ml of FCS. Cells were washed twice with room temperature R-10, rested overnight at 37ºC in T25-T75 flasks, and then counted again before being used in any assay.

2.6 Epitopes

This project used several HIV epitopes for stimulation and general study, as well as other mitogens for positive controls.

Epitopes used in this study include 3 from Gag and 1 from Env, along with their associated variants. The HIV epitopes studied are as follows: Env IF9 and two natural variants (IPRRIRQGF, IPRRIRQGA, IPRRIRQGL), Gag TL9 and two natural variants (TPQDLNMML, TPQDLNAML, TPQDLNTML), Gag IW9 and one natural variant (ISPRTLNAW, LSPRTLNAW), and Gag KF11 and one natural variant

(KAFSPEVIPMF, KGFSPEVIPMF). A summary of these epitopes is seen in Table 2.1, with each consensus epitope (the epitope most common in nature) shown in bold, and the variants beneath with the amino acid substitution indicated. These epitopes were purchased from New England Peptide, and were supplied in lyophilized form. They were resuspended in 100% DMSO, and diluted further to working concentrations in sterile PBS.

Epitope	Abbreviation	Source	HLA
IPRRIRQGF	IF9 F	HIV-1 Env	B*4201
IPRRIRQG**A**	IF9 A	HIV-1 Env	B*4201
IPRRIRQG**L**	IF9 L	HIV-1 Env	B*4201
TPQDLNMML	TL9 M	HIV-1 Gag	B*4201
TPQDLN**A**ML	TL9 AG	HIV-1 Gag	B*4201
TPQDLN**T**ML	TL9 T	HIV-1 Gag	B*4201
ISPRTLNAW	IW9	HIV-1 Gag	B*5703
LSPRTLNAW	LW9	HIV-1 Gag	B*5703
KAFSPEVIPMF	KF11	HIV-1 Gag	B*5703
K**G**FSPEVIPMF	KGF	HIV-1 Gag	B*5703

Table 2.1: Epitopes. Two epitopes presented by HLA-B*42:01, and two presented by HLA-B*57:03, along with their natural variants, are assessed in this study. The consensus epitope is shown in bold, with the natural variant/s below, and the amino acid change indicated in red.

For 6-hour intracellular cytokine secretion (ICS) assays, a combination of 25ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) and 500ng/mL ionomycin was used as a positive control. This is a known stimulator of T cell activation, proliferation, and cytokine secretion. However, it can be toxic to cells during long cell culture, so for the 6-day proliferation assays, *Staphylococcus aureus* Enterotoxin B (SEB) was

substituted as a positive control. In the 6-hour ICS experiments, CD28/CD49d (BD FastImmune) was added to all stimulations to act as a co-stimulatory molecule.

2.7 Flow Cytometry

All flow cytometric data was collected on a BD LSR II flow cytometer. Data acquisition was performed using FACS Diva, and analyzed using FlowJo. Polyfunctionality was further assessed using the programs Pestle and SPICE.

2.7.1 Tetramers

Tetramers were created for each epitope of interest seen in Table 2.1, by the NIH Tetramer Core facility. These tetramers consist of multiple biotinylated, soluble MHC molecules that are bound to the epitopes, as well as to a fluorochrome (APC in this case). This combination allows the tetramers to bind to multiple TCRs on the surface of T cells that recognize the epitopes, and binding occurs with a higher avidity than would be seen with monomeric MHC complexes. This allows for detection of epitope-specific CD8+ T cells by flow cytometric analysis[197].

2.7.2 Tetramer/Antibody Titrations

Each tetramer was titrated prior to use in order to identify the concentration that resulted in the highest identification of epitope-specific cells while maintaining low background levels. Patient samples known to be reactive to the epitopes in each tetramer (through previous work) were used for titrations. A final concentration of 0.125ug/ml was used for each tetramer, which resulted in diluting a working stock of 1ul tetramer in 240ul of FACS wash, which was then used at 30ul/sample.

Each antibody used for flow cytometry was also titrated to determine the optimal volume, which would result in high identification and low background staining. This titration was carried out on whatever sample and stimulation conditions were being used in each protocol (whole blood or PBMCs, *ex-vivo* staining or stimulations).

2.7.3 FMOs

For each flow cytometry panel being examined, a fluorescence minus-one panel (FMO) was run. In order to do this, 5ml FACS tubes were set up such that each tube contained all of the antibodies being used in the panel except for one, with each antibody being excluded from one tube. Compared to unstained controls, there is often fluorescent spillover into some channels resulting from the presence of the combination of antibodies in a stained sample. This method ensures that gates are properly set to account for any spillover that may occur due to this combination of antibodies that may not be seen when compared to fully unstained samples.

2.8 Ex-vivo Tetramer Staining

In order to assess the frequency of positive responses to the epitopes in participants in this study, fresh whole blood samples from HIV-infected CSWs in the Majengo cohort were screened using tetramers and flow cytometry.

Whole blood was aliquoted into 96 well plates at 200ul per well (BD Biosciences) for each tetramer being screened, as well as one negative control that received no tetramer. The working concentration of tetramer (1ul +240ul FACS wash) was

added at 30ul per well to each blood sample, and incubated at 4ºC for 20 minutes. After this, the remaining surface antibodies (Table 2.2) were added in a master mix cocktail, at a total of 30ul in FACS wash. The samples were again incubated for 20 minutes at 4ºC. After this incubation step, the samples were transferred into 5ml FACS tubes containing 1ml of 1x Lysis buffer (BD Biosciences), and incubated at 4ºC for 20 minutes in order to lyse the red blood cells. Tubes were then centrifuged for 10 minutes at 350xg, the supernatants discarded, and remaining cells were resuspended. 1ml of FACS wash was added to each tube and mixed well, and centrifuged again at 350xg for 10 minutes. The supernatants were discarded and the cells resuspended in 1% paraformaldehyde (PFA) and FACS wash, to be read on the LSR-II.

Whole Blood Screening Panel		
Marker	Fluorochrome	Source
CD3	AmCyan	BD
CD38	Alexa-700	BD
CD8	APC-Cy7	BD
Tetramer	APC/PE	NIH Tetramer Core
Live/Dead	PacBlue	Life Technologies
HLA-DR	PECy5	BD

Table 2.2: *Ex-vivo* screening panel. The panel as described was used for screening of whole blood samples for reactivity to tetramers specific to each epitope of interest.

2.9 PBMC Surface Staining

The general surface staining protocol used throughout the experiments in this thesis is as follows, with any changes indicated in specific sections.

Antibodies were titrated for staining $5x10^5$ to $1x10^6$ PBMCs, and as such, that was the quantity of cells used for all surface staining experiments. Cells were aliquoted

into 5ml FACS tubes, and washed once with 1ml FACS wash (350xg for 10 minutes). Supernatants were discarded and cells were then resuspended in the residual FACS wash. Tetramer was added at a 1:8 dilution in FACS wash, at 30ul/tube total. Cells were incubated for 30 minutes at 4ºC, protected from light. Next, a master mix of all remaining surface antibodies was created, so that each tube received the pre-titrated amount of each antibody within a total of 30ul in FACS wash. Cells were incubated for 30 minutes at 4ºC, protected from light. FACS wash was added at 1mL to each tube, and they were centrifuged at 350xg for 10 minutes. Supernatants were discarded and the cells were resuspended in a 1% PFA solution to fix the cells for analysis on the LSR-II.

2.10 PBMC Intracellular Staining

In order to conduct intracellular staining of PBMCs, the surface staining protocol outlined above was conducted as stated, but the cells were not fixed with 1% PFA. Rather, after the final washing step, cells were then resuspended in 100ul of Cytofix/Cytoperm (BD Biosciences), in order to both fix and permeabilize the cells for intracellular staining. The cells were incubated for 20 minutes at 4ºC, protected from light. Cells were washed once with 1ml of 1x Perm wash (BD Biosciences) and centrifuged at 350xg for 10 minutes. The supernatants were discarded, and cells resuspended. An intracellular antibody master mix was prepared in a total of 30ul of Perm wash per tube. This stain was added to cells and incubated at 4ºC for 45 minutes. The cells were then washed again in 1ml of Perm wash, for 10 minutes at 350xg. Supernatants were discarded, and cells were resuspended in 300ul of Perm wash to be read on the LSR-II.

2.11 Cell Viability Staining

In order to assess the viability of cells in some assays, a Live/Dead Fixable Dead Cell Stain Kit (Life Technologies) was used in conjunction with flow cytometry panels. This stain is able to permeate the cell membranes of dead cells, allowing them to be visualized in flow cytometry.

To prepare this stain, 50ul of DMSO was added to one vial of dye, and frozen in 10ul aliquots for future use. During staining, 1ul of this stock solution was diluted in 100ul of FACS wash, and added at 6.25ul/sample. This stain was included in the surface staining master mix of the panels it was included in, and the rest of the staining protocol was the same as indicated above.

2.12 CFSE Staining

To assess proliferation, carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) staining was used. CFSE is able to permeate cell membranes and bind to intracellular amines, which ensures it is retained within the cell through the length of the assay. As all cells, proliferating or not, are initially stained with CFSE, the most highly fluorescent cells are those which have not proliferated. During the course of the assay, the finite amount of CFSE in each cell is diluted out during cell division, meaning that the cells that are actively proliferating will have a lower fluorescence than those that are not.

To prepare the CFSE stain, 90ul of DMSO was added to a vial of CFSE. 1790ul of PBS was then added to create a 50uM working concentration, and aliquots were stored at -20ºC until use. During staining, a 50uM vial was thawed, and 10ul of this working stock was added per mL of cells (at $1x10^7$ cells per 1mL of PBS). Cells were incubated at 37ºC for 8 minutes, and then an equal volume of cold FCS was added to the cell suspension in order to quench the cells and remove any unabsorbed CFSE. Cells were then washed twice in PBS, and ready to be stimulated.

2.13 Compensation Controls

For each flow cytometry experiment conducted, compensation controls were created and used in order to control for any fluorescent spillover on the LSR-II. This correction was done using anti-mouse Ig polystyrene microparticles (CompBeads Anti-Mouse, BD Biosciences) as a positive control, and microparticles with no binding capacity (CompBeads Negative, BD Biosciences) as the negative control. The anti-mouse beads bind to any mouse Ig, as is found in the antibodies being used in these panels.

To prepare the compensations, 1 drop of positive anti-mouse beads and 1 drop of negative beads were added per 500ul of FACS wash. This was mixed well and then 100ul was transferred to each positive control (one for each fluorochrome in the panel). The remaining FACS wash/positive/negative bead mixture was left unstained, and used as the negative control. To each positive control, the pre-titrated volume of antibody was added, and tubes were incubated at room

temperature, protected from light, for 20 minutes. After this incubation period, an additional 200ul of FACS wash was added to each tube.

Compensation controls for live/dead viability stain were conducted in a similar fashion, but used ArC Amine reactive beads (Life Technologies), as they are specifically able to bind to the live/dead stain used. For this protocol, 1 drop of the ArC positive beads was added to a 5mL FACS tube and incubated at room temperature for 5 minutes. 1ul of undiluted live/dead stain (Invitrogen) was added to the tube and incubated for 25 minutes at room temperature, protected from light. The beads were then washed twice, using 3ml of FACS wash and centrifuged at 350xg for 5 minutes. Beads were resuspended in 300ul of FACS wash, and one drop of the ArC negative beads was added just prior to being run on the LSR-II.

In order to properly compensate for panels that contained CFSE stain, a CFSE stained tube of PBMCs was used as a positive control. During the set up of 6-day proliferation assays, one tube containing 500,000 cells was set aside with no stimulation and no CFSE staining, until day 6. At this point, it was stained with the CFSE stain only, as per the protocol described above, and used as the CFSE compensation control. Compensation controls were run on the LSR-II and fluorescence overlap was automatically corrected for within the FACS Diva program.

2.14 6-Day Proliferation Assays

6-day proliferation assays were conducted to assess the ability of individual HIV epitopes to elicit cellular proliferation. The epitopes seen in Table 2.1 were used as

stimulants for this assay, as well as SEB for a positive control. The negative control tube was treated the same as the others but contained no epitope or mitogen for stimulation. PBMCs were thawed as described, and rested overnight at 37ºC before being used. Cells were washed once in sterile PBS, and loaded with CFSE as described above. $5x10^5$ cells were added each to 5ml sterile snap-capped tubes, in a total of 500ul of R-10. 5ug/ml of SEB was added to the positive control, and 5ug/ml of each peptide to be tested was added to appropriate tubes. Cells were protected from light and incubated at 37ºC for 6 days. On day 6, cells were removed from the incubator and washed once with 1ml of FACS wash at 350xg for 10 minutes. Cells then underwent surface staining as described above, using the proliferation panel seen in Table 2.3.

Proliferation Flow Cytometry Panel		
Marker	Fluorochrome	Source
CD3	AmCyan	BD
Tetramer	APC	NIH Tetramer Core
CD8	APC-H7	BD
Live/Dead	ECD	Life Technologies
CFSE	FITC	Life Technologies

Table 2.3: 6-day proliferation panel. This panel was used for staining after 6 days of stimulation to determine proliferation levels.

2.15 6-hour ICS Stimulations

PBMCs were thawed as described above, and rested overnight at 37ºC prior to use. The next day, they were aliquoted at $5x10^5$ cells each in sterile 5ml snap-capped tubes, and washed once with 1ml of R-10 for 10 minutes at 350xg. Supernatants were discarded, and cells were stained with a 1:8 dilution of tetramer in 30ul of FACS wash. Cells were incubated for 30 minutes at 4ºC. This step was done prior to stimulation, as it is known that T cell stimulation can lead to MHC downregulation

and, as such, can reduce the binding of tetramers. After this staining step, 5ug/ml of each peptide was added to appropriate tubes along with R-10 to a final volume of 500ul. Cells were incubated at 37ºC for one hour, protected from light. Protein transport inhibitors Golgi Plug (brefeldin A, BD Biosciences) and Golgi Stop (monesin, BD Biosciences) were added to each tube at 1ul each, in order to contain any cytokines being produced in response to stimulation within the cells, for visualization by flow cytometry. Cells were incubated again at 37ºC for 5 hours, protected from light. After this incubation, the surface and intracellular panel seen in Table 2.4 was stained on each sample, as described in the above intracellular staining protocol.

ICS Flow Cytometry Panel		
Marker	Fluorochrome	Source
CD3	AmCyan	BD
CD8	APC-H7	BD
Live/Dead	ECD	Life Technologies
Tetramer	APC	NIH Tetramer Core
CD107a	PECy5	BD
IFNg	V450	BD
TNF	Alexa-700	BD
Mip1B	PE	BD
IL-2	PECy7	BD

Table 2.4: 6 hour ICS panel. The above panel was used for cytokine and surface staining after 6 hours of stimulation.

2.16 Viruses

Infection assays were performed with two strains of HIV. One is documented as ML1956, and is a dual-tropic primary isolate from patient 1956 in the Majengo sex worker cohort in Nairobi. This primary isolate was used for infection assays as it better mimics the strain of virus the study subjects are likely to be exposed to in

nature. The second strain used was IIIB, a commonly used lab-adapted CXCR4-tropic strain of HIV.

2.17 Viral Stock Production

PBMCs were isolated from HIV-uninfected local donor blood samples as described above. In the Containment Level 2 (CL2) lab, cells were resuspended at 2 x 10^6/mL in a T75 flask (BD Biosciences) in complete media, stimulated with 5ug/ml of phytohemagglutinin (PHA) and placed at 37ºC for 3 days. Cells were then washed twice in complete media to remove any residual PHA, and counted. 2x10^7 cells were removed and taken to the Containment Level 3 (CL3) lab for infection. The remaining cells were placed at 2x10^6/mL in a T-75 flask and kept at 37ºC to serve as feeder cells for the infection.

In the CL3 lab, 4 vials of the virus to be amplified were added to the infection-prepped cells, and they were incubated at 37ºC for 4 hours. The infected cells were then transferred to a T25 flask (BD Biosciences) and 10mls of complete media was added, and the flask incubated at 37ºC overnight. The next day (Day 1), the cells were transferred to a 50ml conical tube, washed once, resuspended in 10mls of complete media, and split into two T25 flasks. The flasks were incubated at 37ºC for 72 hours, at which point they were combined into a T75 flask (Day 4). On the same day, the feeder cells left in CL2 were washed and resuspended at 2x10^6 cells/mL. 2x10^7 cells were added to feed the infection along with another 15mls of complete media. The T75 flask was incubated at 37ºC for an additional 72 hours and then

20mls of complete media was added to the infection (Day 7). 48 hours later, all of the media was collected from the T75 flask, without disturbing the cells, and transferred into a 50ml conical tube (Day 9). The virus was spun down once at 320xg for 10 minutes, and the supernatant again collected into a fresh 50ml conical to remove any cells that may have been collected. The supernatant was aliquoted at 500ul per tube into labeled cryovials, and stored at -80ºC.

2.18 TCID50

In order to calculate the infectivity of newly grown viral stocks, a titration assay was conducted to assess the 50% tissue culture infectious dose (TCID50). This assay determines the volume of virus that would be required to infect 50% of the cells in a cell culture. In CL2, PBMCs were isolated from whole blood from an HIV-uninfected local donor. Cells were resuspended at $2x10^6$ cells/ml of R-10 with 5ug/ml of PHA, and incubated at 37ºC for 3 days in a T75 flask. After this incubation, cells were harvested and counted, and washed once in complete media. Cells were resuspended at $2x10^6$/ml in complete media and moved to the CL3 lab for infection.

A 96-well round bottom culture plate (Costar) was used to complete this assay. Of the 12 columns of the plate, column one was used as an uninfected control, with 150ul of media and no virus added to each well. 135ul of complete media was added to the next column in all six rows, and to the remaining wells, 150ul of complete media was added. In the CL3 lab, the virus stock to be tested was thawed, and 65ul of the virus added to each of the six wells containing 135ul of media. The virus was mixed well by pipetting, and 50ul was transferred to the next well

containing 150ul of media. This was carried on across the plate to create quadrupling dilutions of the virus. $1x10^5$ PBMCs were added to each well in 50ul of media, to create a total of 200ul per well of cells and diluted virus. This plate was incubated at 37ºC overnight.

The next day (Day 1), 150ul of supernatant was removed and discarded from the cells without disturbing the cell pellet. 150ul of complete media was added back to each well, and the plate centrifuged at 200xg for 5 minutes. 150ul of supernatant was again removed and discarded, and replaced with 160ul of complete media. The plate was incubated at 37ºC.

Two days later (Day 3), 100ul of supernatant was removed and discarded, and replaced with 110ul of fresh complete media. Three days after this (Day 6), 120ul of supernatant from each well was harvested into a fresh 96 well plate, and 15ul of a 10% TritonX (Sigma) solution added to each well to inactivate the virus. 130ul of complete media was added back to the infection plate, which was incubated at 37ºC for an additional 6 days, at which point the above harvest was repeated (Day 12), and the infection plate discarded. The supernatants collected were assessed using a p24 ELISA (described below) to detect virus production. In order to determine the TCID50 value, calculations were carried out according to the Reed-Muench method, described elsewhere[198]. Briefly, this method determines the viral dilution that was able to infect 50% of the wells, thus serving as the 50% infectious dose value.

2.19 p24 ELISA

Supernatants collected from infection experiments and virus titrations were evaluated for the presence of HIV p24 using a sandwich ELISA. This assay uses a method whereby a monoclonal IgG antibody purified from an HIV p24 hybridoma (NIH AIDS Reagent Program, clone 183-H12-5C) is bound to plates, which then binds to any p24 in the supernatants being tested. The secondary antibody then binds to this complex, and strepavidin alkaline phosphatase (SAAP) and a substrate solution are added which results in a colour change to yellow, the intensity of which is proportional to the amount of p24 bound to each well.

Flat bottom 96-well plates (Nunc) were coated with 100ul/well of coating antibody, and incubated at 4ºC overnight. The antibody was then removed, and the coated plates stored at -80ºC until use. Pre-coated plates were thawed for approximately 30 minutes then washed 6 times with wash buffer on an automated plate washer. 150ul of blocking buffer was added to each well and incubated at 37ºC for 2 hours in a wet box (a plastic container lined with wet paper towels, to avoid evaporation of the plate). Plates were washed again 6 times, and 100ul of infection supernatants, previously inactivated with 10% TritonX, were added to each well. A p24 standard was added to the plate in doubling dilutions in blocking buffer to create a standard curve. The plate was incubated at 4ºC overnight (approximately 16 hours).

Plates were washed 6 times with wash buffer using a plate washer. 100ul of the secondary antibody (Abcam ab20774, 0.75ug/mL) was then added to each well, and the plates incubated at 37ºC for 2 hours in a wet box. Plates were washed again 6 times with wash buffer using a plate washer. 100ul of SAAP (1:13,200 dilution, in

blocking buffer) was added to each well, and the plates incubated at 37ºC for 1 hour in a wet box. Plates were washed again 6 times with wash buffer using a plate washer. 2 phosphatase substrate tablets (Sigma) per plate were added to 10mls DEA buffer, which was protected from light. 100ul of this solution was added to each well, and the plates were incubated for 15 minutes at 37ºC in a wet box. At this point, they were read on a Biotek Synergy H1 plate reader, using GeneX software, which determined the optical density of each well. Plates were read again at 30 and 45 minutes, or longer, until the standard curve reached an r^2 value of at least .98.

2.20 PBMC Expansion for Infection Assays

In order to generate enough effector and target cells (CD8+ and CD4+ respectively) to conduct viral inhibition assays, PBMCs from participants of interest in the Majengo sex worker cohort were stimulated with a combination of IL-2 and bispecific antibodies (NIH AIDS Reagent Program). The CD3/4 antibody contains domains that can bind both CD3 and CD4 on human cells. This binding results in selective killing of the CD4+ T cells, and expansion of the CD8+ T cells[199]. Therefore, this antibody was added to cultures in order to expand the CD8+ T cells. Conversely, the CD3/8 antibody has specificity and binding capacities for CD3 and CD8 receptors. This binding results in selective killing of the CD8+ T cells, and expansion of the CD4+ T cells. Therefore, this antibody was added to cultures in order to expand the CD4+ T cells.

PBMCs from study subjects were thawed as described above. Cells were counted using a hemocytometer and Trypan exclusion dye. For each patient sample, two

T25 flasks were set up and 2-5 million cells were added to each in 2ml of R-10, supplemented with 100 international units of IL-2. One flask was labeled CD3/4 and 1ug of the CD3/4 antibody was added. The other was labeled CD3/8 and 1ug of the CD3/8 antibody was added. Flasks were incubated upright at 37ºC. 3 days later (Day 3), 3mls of R-10 was added to each flask, and they were laid flat, and incubated at 37ºC for an additional 3 days. Then, 5mls of R-10 was added to each flask (Day 6), and the flasks were incubated upright at 37ºC overnight. The following day (Day 7) the cells were counted, and $5x10^5$ cells were removed from each sample for flow cytometric analysis. Staining was conducted as per the PBMC surface staining protocol described above, and cells were evaluated according to the panel in Table 2.5.

Cell Expansion Flow Cytometry Panel		
Marker	Fluorochrome	Source
CD3	AmCyan	BD
CD4	Alexa-700	BD
CD8	APC-H7	BD
Live/Dead	ECD	Life Technologies

Table 2.5: Cell expansion panel. This panel was used to assess cells after 7 days of expansion using bispecific antibodies to produce CD4+ and CD8+ T cells.

2.21 Infection Assay

The newly expanded CD4+ and CD8+ T cells were washed once and then taken into the CL3 lab for infection. CD4+ T cells were centrifuged at 320xg for 10 minutes, the supernatants were discarded, and cell pellets were resuspended in the residual media. IIIB and ML1956 HIV viral strains, grown as described above, were thawed and added to the CD4+ target cells at multiplicities of infection (MOIs) of 0.05 and 0.01. The volume of virus was determined using the following formula:

(# of cells)(desired MOI)/TCID50 of virus = Volume to be used

Cells and virus were incubated at 37ºC for 3 hours, then washed twice in PBS and resuspended at $1x10^6$ cells/ml of complete media.

$5x10^5$ infected CD4+ T cells were added to the appropriate wells of sterile 48 well tissue culture plates (Corning), and $5x10^5$ CD8+ T cells were also added to appropriate wells. For each patient, 10 conditions were assessed (10 wells/patient), composed of 5 pairs that consisted of one well with CD4+ T cells only, and the other with CD4+ T cells and CD8+ T cells at a 1:1 ratio. This method allowed for a comparison of how well the effector CD8+ T cells could restrict viral growth, by comparing the resulting level of p24 among CD4+ T cell only wells, and those in which CD8+ T cells had been added back. Of these 5 pairs, one consisted of uninfected CD4+ T cells, in order to determine if there was substantial background viral growth before exogenous virus was added, as these samples came from HIV-infected individuals. For the remaining 4 pairs, the CD4+ T cells were infected with ML1956 at MOIs of 0.05 and 0.01, and IIIB at MOIs of 0.05 and 0.01.

Once the cells were added to appropriate wells, CD4+ only wells were given an additional 500ul of complete media so that each well contained a total of 1ml of media and cells. The outer ring of wells in the plate was not used for cell culture, and approximately 500ul of sterile PBS was added to each of those wells in order to hydrate the plate and prevent evaporation from the test wells. The plates were incubated at 37ºC. On Day 3, half of the media was discarded from each well and replaced with fresh complete media. On days 6, 9 and 12, 500ul of supernatant was

collected from each well into sterile plates, and replaced with fresh media. The supernatants were assessed for HIV infectivity using a p24 ELISA as described above.

On day 12 of the infection, the remaining cells were harvested from the plates and transferred into 5ml snap cap tubes for staining and flow cytometric analysis. Staining was conducted as described in the intracellular staining protocol outlined above, using the panel seen in Table 2.6.

HIV-1 Infection Flow Cytometry Panel		
Marker	Fluorochrome	Source
CD3	AmCyan	BD
CD4	Alexa-700	BD
CD8	APC-H7	BD
PD-1	PE	BD
CD69	PECy7	BD
CD38	PECy5	BD
Live/Dead	ECD	Life Technologies
KI-67	V450	BD
Perforin	Alexa-647	BD
KC-57	FITC	Coulter

Table 2.6: Infection panel. This panel was used on day 12 of infection assays to monitor p24 levels (KC57) and other surface and intracellular markers.

2.22 Viral Sequencing

gDNA samples (90) from HIV-infected sex workers of the Majengo cohort were sequenced for the IF9 region, to determine if there was any mutation within the cohort away from the consensus IF9 F variant. The IF9 region was first amplified using a nested PCR reaction with two sets of primers, and the products were run via gel eletrophoresis to confirm a band appeared at the 400bp region as expected. The products were then sent for sequencing at the DNA core at the National Microbiology Laboratory.

Chapter 3: Characterization of Responses to HLA-B*42:01 Presented HIV IF9 and TL9 Epitopes and Their Natural Variants

3.1 Rationale:

Data from previous work within the Majengo sex worker cohort has described that vastly different CD8+ T cell responses can result through stimulation with HIV epitopes that differ by as little as a single amino acid. Due to the incredibly high mutation rate of HIV, these small changes in the viral sequence can occur frequently in nature, and individuals are likely to be exposed to many epitope variants. A more thorough understanding of how CD8+ T cells react to both consensus epitopes and their natural variants is crucial to understanding both what immune response would be most beneficial to elicit, and what HIV variants may be best at accomplishing that. For this chapter, the HLA-B*42:01 presented epitopes IF9 F (IPRRIRQGF) and TL9 M (TPQDLNMML) are studied, along with their natural but less common variants. For IF9, the variants are IF9 A (IPRRIRQGA) and IF9 L (IPRRIRQGL), and for TL9, the variants are TL9 T (TPQDLNTML) and TL9 A (TPQDLNAML). Individuals in this study are likely to be more frequently exposed to the consensus epitopes, as they are more common in nature than the variant epitopes. The characterization of immune responses to these epitopes and variants, including cytokine production, polyfunctionality, and proliferation, will allow a better understanding of how CD8+ T cell responses can be altered by stimulation with variant HIV epitopes.

3.2 Hypothesis:

The consensus HIV epitopes IF9 F and TL9 M will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their natural variants.

CD8+ T cell responses to IF9 F and TL9 M epitopes will be more proliferative and polyfunctional, and be associated with better disease outcomes than responses to natural epitope variants.

3.3 Objectives:

1) Determine the frequency of recognition, proliferative capacity and polyfunctionality of CD8+ T cells in response to IF9 and TL9 epitopes and their natural variants.

2) Compare the frequency of CD8+ T cell responses to consensus and variant epitopes with CD4+ T cell counts as a measure of immune control and disease progression.

3.4 Methods:

Ex-vivo screening: A subset of 14 HIV-infected, HLA-B*42:01+ individuals were initially screened for reactivity to tetramers specific to each epitope of interest, using whole blood tetramer staining, as described in section 2.8. The flow cytometry panel used can be found in Table 2.2.

Cytokine production assay: Cryopreserved PBMC samples from 24 HLA-B*42:01+ individuals were thawed as per section 2.5, and stimulated for 6 hours with each

epitope of interest, along with co-stimulatory molecules (outlined in section 2.15). After stimulation, surface, intracellular and viability staining was conducted, as outlined in sections 2.9, 2.10 and 2.11 respectively. This method was used to assess the frequency of epitope-specific CD8+ T cells (via tetramer staining), as well as CD8+ T cell cytokine production and polyfunctionality in response to IF9 and TL9 epitopes and their variants. The flow cytometry panel used in this section can be seen in Table 2.4.

Proliferation assay: Cryopreserved PBMCs from the same 24 HLA-B*42:01+ subjects were also stimulated for 6 days to assess proliferative capacity in response to each epitope, as described in section 2.14. The flow cytometry panel used to assess 6-day proliferation can be seen in Table 2.3.

IF9 sequencing: The IF9 epitope was sequenced in 90 HIV-infected individuals from the Majengo cohort to better understand the prevalence of mutations within this region (described in section 2.22).

Statistical analysis: For all comparisons of frequencies or cytokine production between variants, Friedman tests were conducted, followed by Wilcoxon tests to decipher where any differences lie. A p value of <0.05 was considered significant, and Bonferroni corrections were used to determine the significant p values in Wilcoxon post-tests. For all correlations, Spearman's rank correlation test was used.

3.5 Results:

3.5.1 Study population

All individuals were part of the Majengo commercial sex worker cohort in Nairobi, Kenya. Individuals were enrolled in this part of the study if they were HIV-infected, ART-naïve, and possessed at least one HLA-B*42:01 allele. The women in this study were selected if they had CD4+ T cell counts above 400, to ensure they were generally healthy, and had not progressed to AIDS.

3.5.2 Ex-vivo screening for epitope-specific CD8+ T cells

The epitopes used in this study can be found in Table 2.1, and the flow cytometry panel is described in Table 2.2. In order to determine if CD8+ T cells from the individuals in this study would recognize the epitopes and variants selected for characterization, whole blood surface screening was conducted via flow cytometry using tetramers specific to each epitope. Representative tetramer staining and the gating strategy used can be seen in Figure 3.1. The frequency of epitope-specific CD8+ T cells was displayed as the percentage of CD8+ T cells that were tetramer+. These values ranged from <0.01% to >5% of the total number of CD8+ T cells. Background levels were determined through use of a tetramer-negative staining control. If tetramer staining reached twice the background level, and was >0.01% of total CD8+ T cells, subjects were determined to have a positive response to that tetramer.

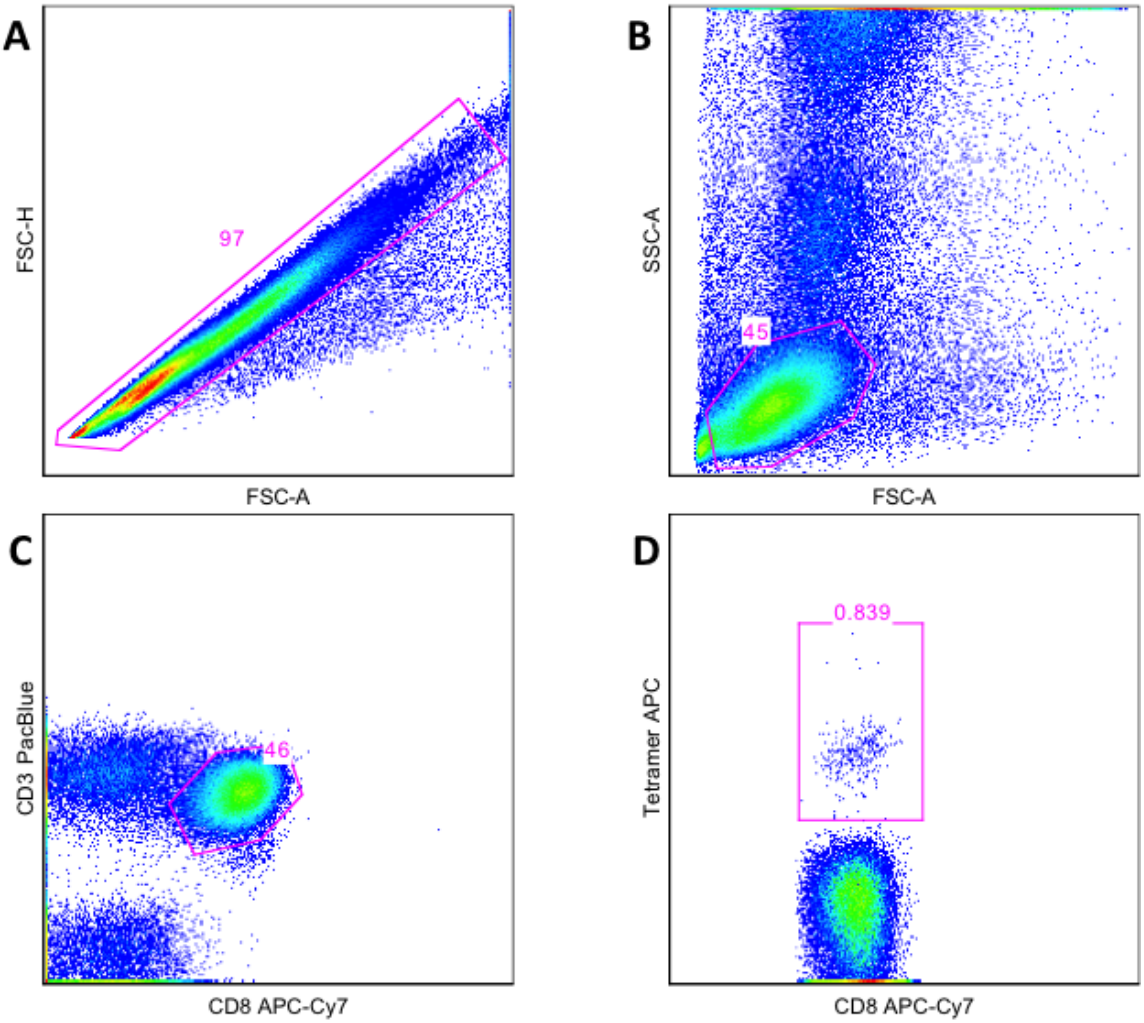


Figure 3.1: *Ex-vivo* tetramer screening gating strategy and representative tetramer staining. Whole blood samples were first gated on FSC-A vs. FSC-H to gate out doublet cells (A), then on FSC-A vs. SSC-A to select the lymphocyte population (B). The CD8+ T cell population was then selected when gated against CD3 (C), and then the tetramer positive population was identified (D). Representative staining is from ML 2312.

The results of this screening can be seen in Table 3.1. Of the 14 individuals screened, a tetramer+ response was found to each epitope in at least 12 of them. No difference was seen in the frequency of CD8+ T cells responding to the IF9 A, F and L variants, however the frequency of the TL9 T variant was significantly higher than TL9 A or the consensus TL9 M (1.933% of CD8+ T cells, compared to 0.272% and 0.249% respectively). As these variants were recognized by at least 85% of tested individuals, these epitopes were determined to be worth examining further in this cohort. The remainder of this chapter focuses on an expansion of these findings in a larger population, using cryopreserved PBMCs.

In summary, *ex-vivo* screening determined that at least 12/14 individuals had CD8+ T cells that recognized the study epitopes, therefore justifying expansion of the examination of these epitopes.

B4201 Tetramer Screening						
Patient	**IF9 F**	**IF9 A**	**IF9 L**	**TL9 M**	**TL9 A**	**TL9 T**
199	0.028	0.011	0.018	0.000	0.018	2.110
900	0.012	0.027	0.071	0.069	0.049	2.970
1430	0.386	0.531	0.532	0.000	0.000	0.408
1500	0.038	0.053	0.013	0.630	0.028	0.035
1535	0.032	0.037	0.000	0.054	0.064	0.077
1957	0.037	0.036	0.118	0.169	0.354	4.540
2135	0.000	0.000	0.304	0.038	0.257	0.011
2147	0.090	0.052	0.421	0.428	0.680	4.030
2169	0.260	0.185	0.801	0.714	0.367	1.040
2312	0.205	0.034	0.049	0.884	1.140	5.010
2411	0.165	0.080	0.072	0.250	0.094	0.111
2415	0.189	0.142	0.127	0.026	0.312	4.630
2493	0.040	0.015	0.276	0.041	0.435	1.850
2542	0.034	0.017	0.000	0.185	0.017	0.246
Average	**0.108**	**0.087**	**0.200**	**0.249**	**0.273**	**1.933**

Table 3.1: Frequency of tetramer staining as a percentage of total CD8+ T cells. Values ranged from <0.01% to >5% of CD8+ T cells, with the highest frequencies observed in CD8+ T cells specific to the TL9 T variant (1.933%). Consensus epitopes are displayed in red.

3.5.3 Frequency of epitope-specific CD8+ T cells from cryopreserved PBMC samples

As it was determined in a subset of subjects in section 3.5.2 that the study epitopes were frequently recognized, the study was expanded. The frequency of CD8+ T cell recognition of the study epitopes was determined in cryopreserved PBMC samples from 24 HLA-B*42:01 individuals, using tetramers specific to each epitope to establish recognition.

Thawed PBMCs were stimulated for 6 hours with each epitope of interest as per the protocol in section 2.15, and analyzed for T cell markers, tetramer reactivity, and cytokine production using flow cytometry (the panel is described in Table 2.4).

After stimulation with each epitope, cells were stained with the matching tetramer to determine the level of CD8+ reactivity to each epitope and variant.

CD8+ T cells recognizing IF9 A were significantly more frequent than those recognizing IF9 F or IF9 L (Figure 3.2, $p<0.0001$, Friedman, Wilcoxon T test). This means that the individuals studied have a larger CD8+ T cell pool specific to IF9 A than IF9 F or IF9 L, despite IF9 F being the consensus epitope. The MFI of CD8+ T cells responding to each variant was also determined within these individuals, in order to assess if the CD8+ T cells bind to each variant with the same or a different number of TCRs. It was found that CD8+ T cells binding to IF9 A had a significantly lower MFI than CD8+ T cells binding to IF9 F (Figure 3.2, $p= 0.0057$, Wilcoxon rank) and trended toward a significantly lower MFI than IF9 L (Figure 3.2, $p= 0.0175$, Wilcoxon rank). This means that though the CD8+ T cell pool specific to IF9 A is larger, the CD8+ T cells specific to IF9 F are able to bind to a higher number of IF9 F epitopes than those responding to IF9 A (Figure 3.2).

PBMCs from the same HLA-B*42:01+ subjects were also stimulated with the TL9 epitopes to assess differences in the number of CD8+ T cells responding to each variant. CD8+ T cells specific to the consensus TL9 M variant were present at a significantly lower frequency than CD8+ T cells specific to either TL9 T or TL9 A (Figure 3.2, Wilcoxon, $p<0.0001$ and $p=0.0028$ respectively), with CD8+ T cells specific to TL9 T having the highest overall frequency. Unlike what was observed with the IF9 variants, the MFI of CD8+ T cells specific to TL9 T was not significantly different than that of TL9 M or TL9 A ($p=0.5693$ and $p= 0.1839$ respectively), though

the MFI of CD8+ T cells specific to TL9 M was significantly higher than those specific to TL9 A ($p=0.0004$). This means that though there are more CD8+ T cells that recognized TL9 T, these cells bind to TL9 T epitopes with similar numbers of TCRs as to TL9 A or TL9 M.

In summary, contrary to the hypothesis, the non-consensus IF9 A and TL9 T epitopes are recognized significantly more frequently than the consensus IF9 F and TL9 M epitopes.

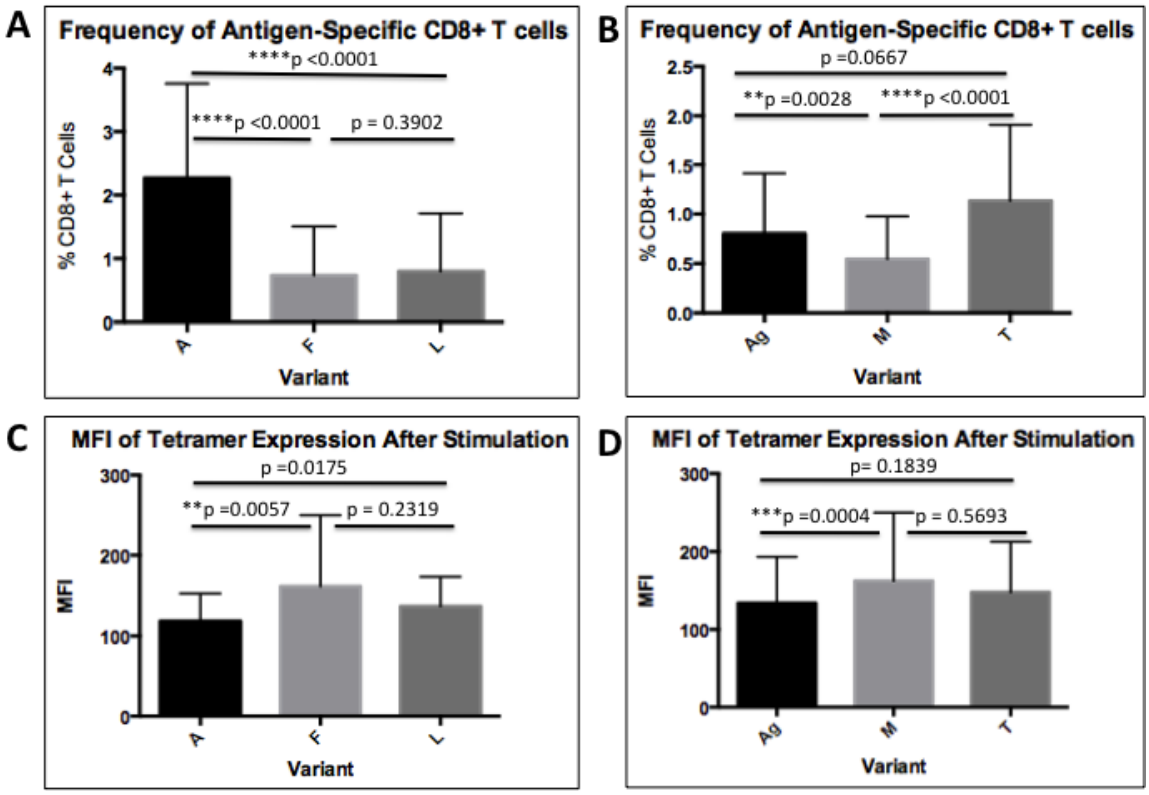


Figure 3.2: Frequency and MFI of epitope-specific CD8+ T cells from PBMCs. Panel (A) shows the frequencies of CD8+ T cells that recognize the IF9 F epitope and its two variants, IF9 A and IF9 L. Panel (B) shows the frequency of CD8+ T cell recognition for each of the TL9 epitopes. The MFI of CD8+ T cells binding to IF9 variants is shown in (C), while panel (D) shows the MFI of CD8+ T cells binding to the TL9 variants. Error bars are displayed as SE.

3.5.4 Correlation of frequency of CD8+ T cell responses to IF9 and TL9 epitopes

In order to understand how the CD8+ T cell responses to each variant may be related to one another, the frequencies of CD8+ T cells that bound to each variant were correlated, and the results are presented in Table 3.2. The frequency of CD8+ T cells recognizing IF9 F and IF9 L correlated very strongly (p=0.0004, Spearman's rank correlation), but the frequency of CD8+ T cells recognizing IF9 A did not correlate at all with that of either IF9 F or IF9 L. This would suggest that there is a disconnect between these epitopes, such that while an increase in frequency of IF9 F responding CD8+ T cells is related to an increase in IF9 L responding CD8+ T cells, no such relationship exists among CD8+ T cell responses to IF9 A. This may also suggest there is a level of cross-reactivity between IF9 F and IF9 L, or at the very least, that there is no cross-reactivity between IF9 A and either IF9 F or IF9 L.

Comparing the TL9 epitopes, strong correlations were observed for the frequency of CD8+ T cell responses to each epitope (Table 3.2, Spearman's rank correlation). The strongest correlations were observed between the frequency of CD8+ T cell recognition of TL9 M with recognition of the other two, which each had a p value of 0.0001. The correlation between CD8+ T cell recognition of TL9 T and TL9 A was still significant, but with a lesser p value of 0.0159.

In summary, there was no correlation between the frequency of CD8+ T cells recognizing IF9 A with that of those recognizing IF9 F or IF9 L, suggesting a lack of cross-reactivity between these epitopes. Strong correlations were found between the frequencies of CD8+ T cell responses to each of the TL9 variants with each other.

IF9	A	F	L
A		ns	ns
F			0.0004
L			
TL9	Ag	M	T
Ag		<0.0001	0.0159
M			0.0001
T			

Table 3.2: The frequency of CD8+ T cell recognition of each IF9 and TL9 variant were correlated to each other. This table displays the p value of each association.

3.5.5 Association of the frequency of CD8+ T cell recognition of each epitope with CD4 count

To understand if recognition of one variant was associated with better clinical outcomes, the frequency of CD8+ T cells recognizing each variant was compared to CD4 count. There were no significant associations between the frequency of CD8+ T cell responses to any of the IF9 or TL9 epitopes with direct CD4 count. To further explore this, individuals were then divided into groups depending on their CD4 count to investigate this further. As previously described, CD4 counts of >500 are considered to be indicative of a healthy, intact immune system, so individuals were divided into groups accordingly (those with CD4 >500 vs. those with CD4 <500). The frequency of CD8+ T cells recognizing any of the IF9 epitopes was not significantly different between individuals with CD4 counts of >500 or < 500, though there was a trend toward significance with recognition of IF9 F (Figure 3.3, p= 0.0698, Mann-Whitney). This trend may suggest that individuals with higher CD4 counts (in the CD4 >500 group) are likely to have more CD8+ T cells that recognize the F variant than those with lower CD4 counts.

The frequency of CD8+ T cells recognizing TL9 T was significantly higher in individuals with CD4 counts >500 (Figure 3.3, p= 0.0099, Mann-Whitney). This suggests that individuals with higher CD4 counts are likely to have more CD8+ T cells that recognize the TL9 T variant than those with lower CD4 counts. This association was not found with recognition of the TL9 A or consensus TL9 M variants.

CD4 ratio, which is a ratio of CD4+ T cells to CD8+ T cells, is used in the context of HIV infection as an indicator of overall immune health, where higher CD4 ratios are indicative of a healthier immune system[200]. When the frequencies of CD8+ T cells recognizing each IF9 variant were correlated with CD4 ratio, it was found that the frequency of CD8+ T cells recognizing IF9 A trended toward a significant correlation (Figure 3.4, p=0.0844, Spearman's rank correlation), while the frequency of CD8+ T cells recognizing IF9 F and IF9 L were not. For the TL9 variants, the frequency of CD8+ T cell recognition of TL9 T was significantly correlated to CD4 ratio (Figure 3.4, p=0.0028, Spearman's rank correlation), while the frequency of CD8+ T cells recognizing TL9 A and TL9 M were not significantly correlated to this function. Overall, this data suggests that a higher frequency of CD8+ T cells recognizing the TL9 T epitope was associated with healthier immune function, as indicated by a relationship to a higher CD4 ratio.

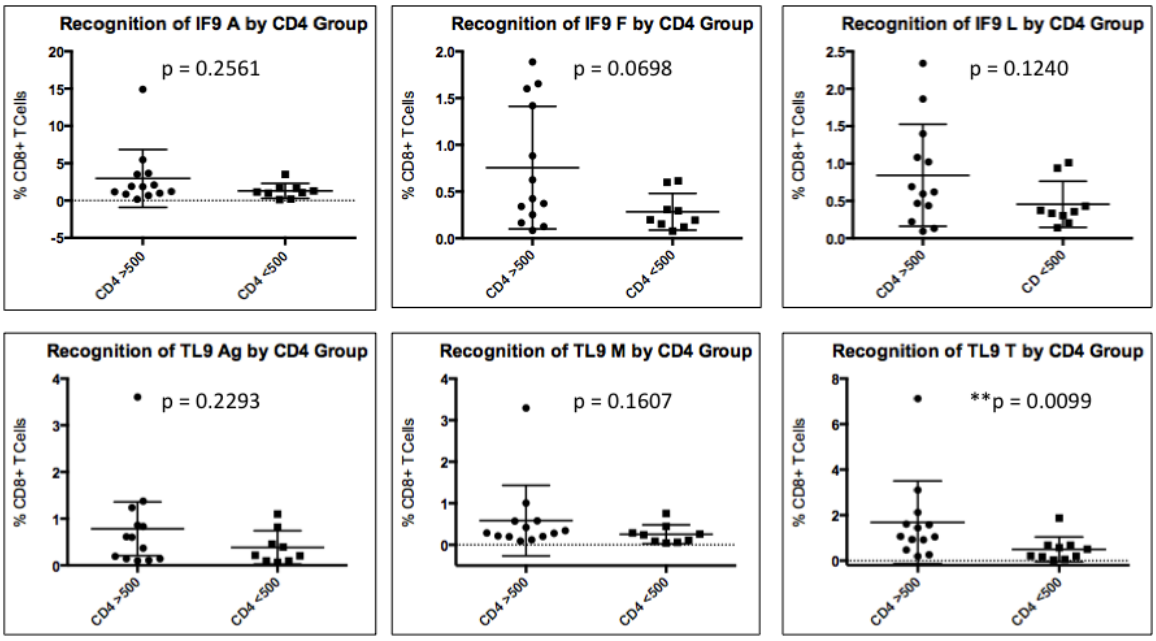


Figure 3.3: Frequency of CD8+ T cells specific for each variant divided by CD4 count. Subjects were divided by their CD4 count, into either >500 or <500 and plotted according to the frequency of each variant. The upper panels show the association between CD4 group and CD8+ T cell recognition of the IW9 variants, and the bottom panel shows the association between CD4 group and CD8+ T cell recognition of the TL9 variants.

After determining that there was a relationship between the frequency of CD8+ T cells specific to the TL9 T epitope and CD4 count, the MFI of these CD8+ T cells was also compared to CD4 counts, to determine if this relationship could be extended to include the number of TCRs binding to each epitope. There was no significant relationship between the MFI of CD8+ T cells responding to the IF9 variants and CD4 count, but there was a significant positive correlation between the MFI of CD8+ T cells recognizing TL9 T to the overall CD4 count (Figure 3.5, p= 0.0324, Spearman's rank correlation). This relationship did not exist with the MFI of C8+ T cell recognition of either TL9 A or the consensus TL9 M. This indicates that individuals whose CD8+ T cells use a higher number of TCRs per cell to engage the TL9 T epitope have higher CD4 counts.

Contrary to the hypothesis that CD8+ T cell recognition of the consensus epitopes would be associated with better clinical outcomes, the frequency of CD8+ T cell responses to the TL9 T variant were the only ones associated with higher CD4 counts and a higher CD4 ratio.

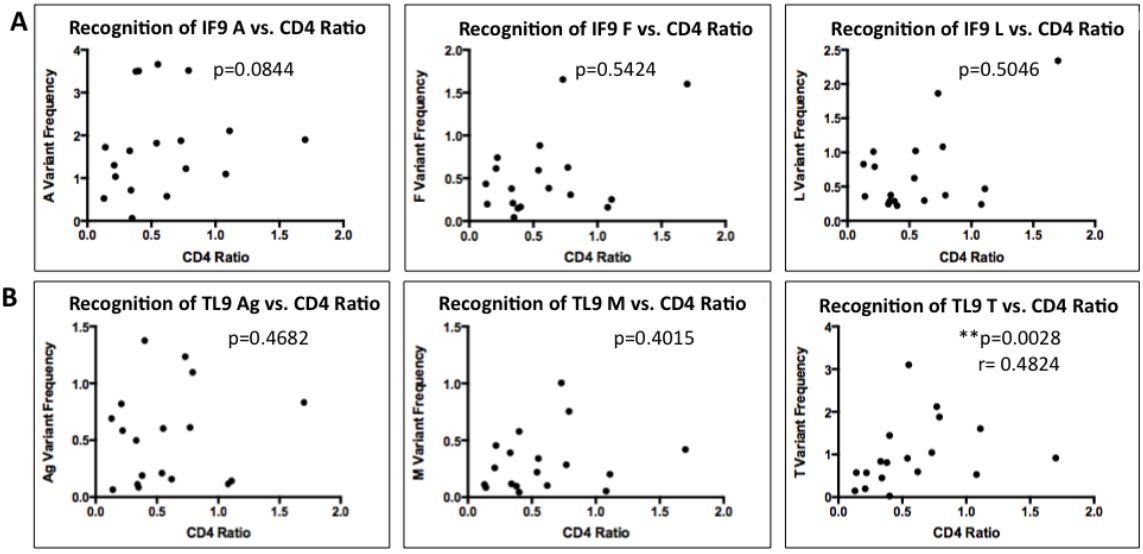


Figure 3.4: Correlation of the frequency of CD8+ T cells specific to each variant with CD4 ratio. In panel (A), the correlation between CD4 ratio and CD8+ T cell recognition of the IF9 variants is shown. Panel (B) shows the correlations between CD4 ratio and CD8+ T cell recognition of the TL9 variants.

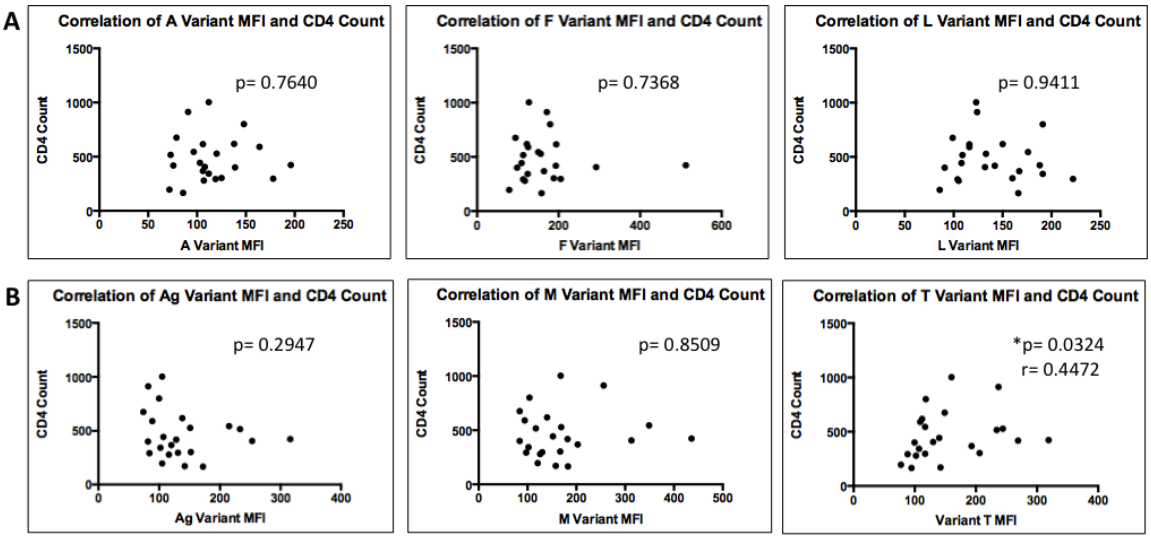


Figure 3.5: Correlation between MFI of epitope-specific CD8+ T cells and CD4 count. Panel (A) shows the correlation between CD4 count and the MFI of CD8+ T cells recognizing the IF9 variants, while panel (B) shows the correlation with the MFI of CD8+ T cells recognizing the TL9 variants.

3.5.6 Post-stimulation cytokine production by all CD8+ T cells

To determine if some cytokines are preferentially produced by bulk CD8+ T cells after stimulation with the study epitopes, intracellular cytokine staining via flow cytometry was performed after 6-hour stimulations with each variant. For this first section, post-stimulation cytokine production was measured as a function of all CD8+ T cells, regardless of what variant was used for stimulation, in order to understand the general cytokine production profile. Overall, MIP1β was produced by the highest frequency of total CD8+ T cells, at an average of 0.591%, followed by IFNγ at an average of 0.259% and then TNF at an average of 0.167% (Figure 3.6, $p<0.0001$ for all combinations, Wilcoxon test). IL-2 producing CD8+ T cells were found at the lowest frequency in all subjects, regardless of the stimulating epitope, ranging between 0.005 and 0.02% of all CD8+ T cells, with an average of 0.031% (Figure 3.6). This indicates that the epitopes tested all appear to have an impaired ability to stimulate production of IL-2, as PMA/ionomycin stimulation was able to elicit an IL-2 response in as high as 0.5% of CD8+ T cells (not shown). This also indicates that stimulation with these epitopes tends to skew the resulting CD8+ T cells to preferentially produce MIP1β rather than IFNγ or TNF.

In summary, after stimulation with any epitope, bulk CD8+ T cells are most likely to produce MIP1β, followed by IFNγ, TNF, and finally IL-2.

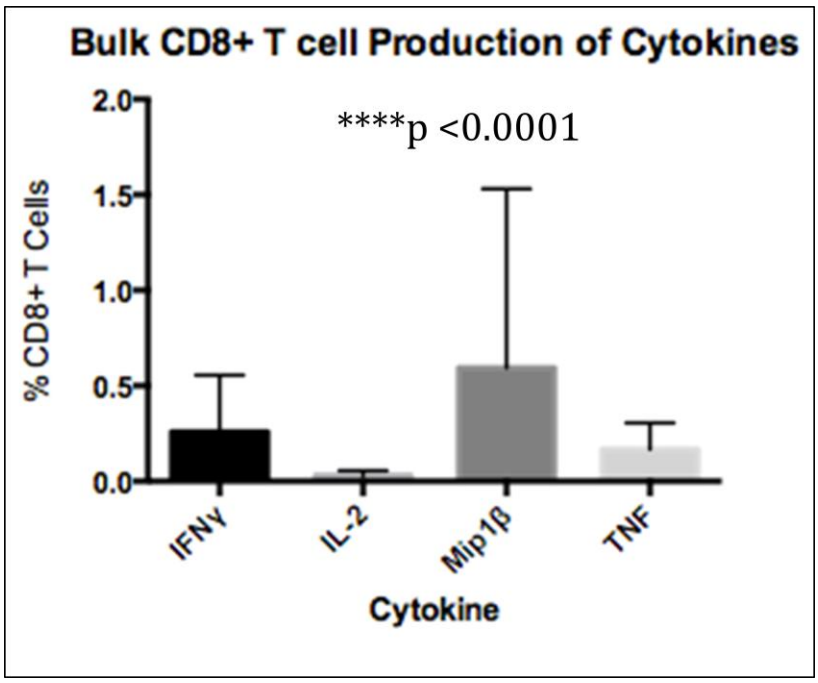


Figure 3.6: Post-stimulation production of cytokines by bulk CD8+ T cells. The percentage of total CD8+ T cells producing IFNγ, IL-2, Mip1β and TNF are shown.

Next, to assess if there were any differences among what cytokines are produced in response to stimulation with each variant individually, the frequency of all CD8+ T cells producing each cytokine was compared. For the IF9 epitopes, stimulation with IF9 A led to significantly higher production of IFNγ by CD8+ T cells than stimulation with IF9 F or IF9 L (Figure 3.7, p=0.0033, p=0.0001 respectively, Wilcoxon rank). Stimulation with IF9 A also led to a higher frequency of CD8+ T cells producing IL-2 than IF9 L (Figure 3.7, p=0.0080, Wilcoxon rank), and a trend to higher IL-2 than when stimulated with IF9 F (Figure 3.7, p= 0.0917, Wilcoxon rank). There was no difference among any variant for production of MIP1β or TNF. There was also no difference in the MFI among cytokines stimulated by any variant, which suggests that regardless of the stimulant, each CD8+ T cell responding was likely producing similar levels of each cytokine (Figure 3.8).

Contrary to the hypothesis, the variant IF9 A, rather than the consensus IF9 F, stimulated the most cytokine producing CD8+ T cells. No differences were observed in the MFI of the cytokine producing CD8+ T cells, indicating that the amount of cytokine produced was roughly the same regardless of the stimulating epitope.

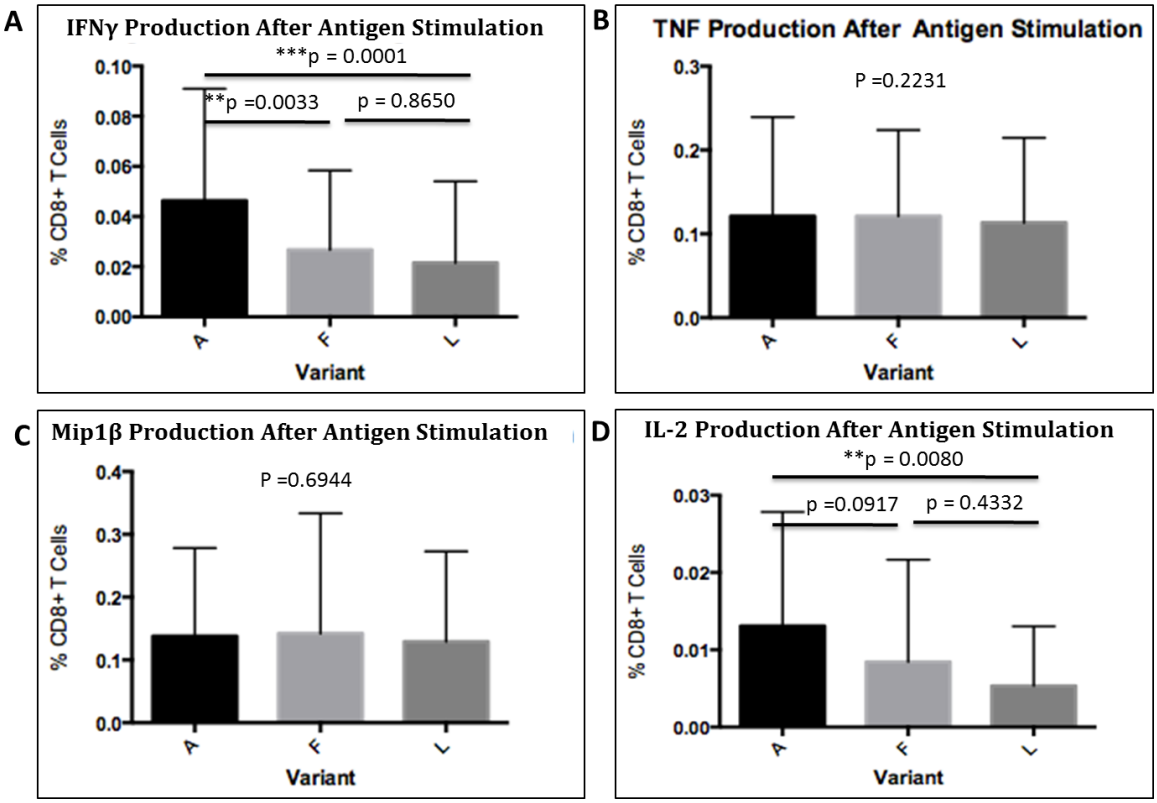


Figure 3.7: Differences in cytokine production by bulk CD8+ T cells after IF9 stimulation. PBMCs stimulated with each IF9 epitope were assessed for the percentage of CD8+ T cells producing each cytokine. Panel (A) shows the percentage of CD8+ T cells producing IFNγ after stimulation with each IF9 epitope, while panel (B) shows TNF production, panel (C) Mip1β production, and panel (D) IL-2 production.

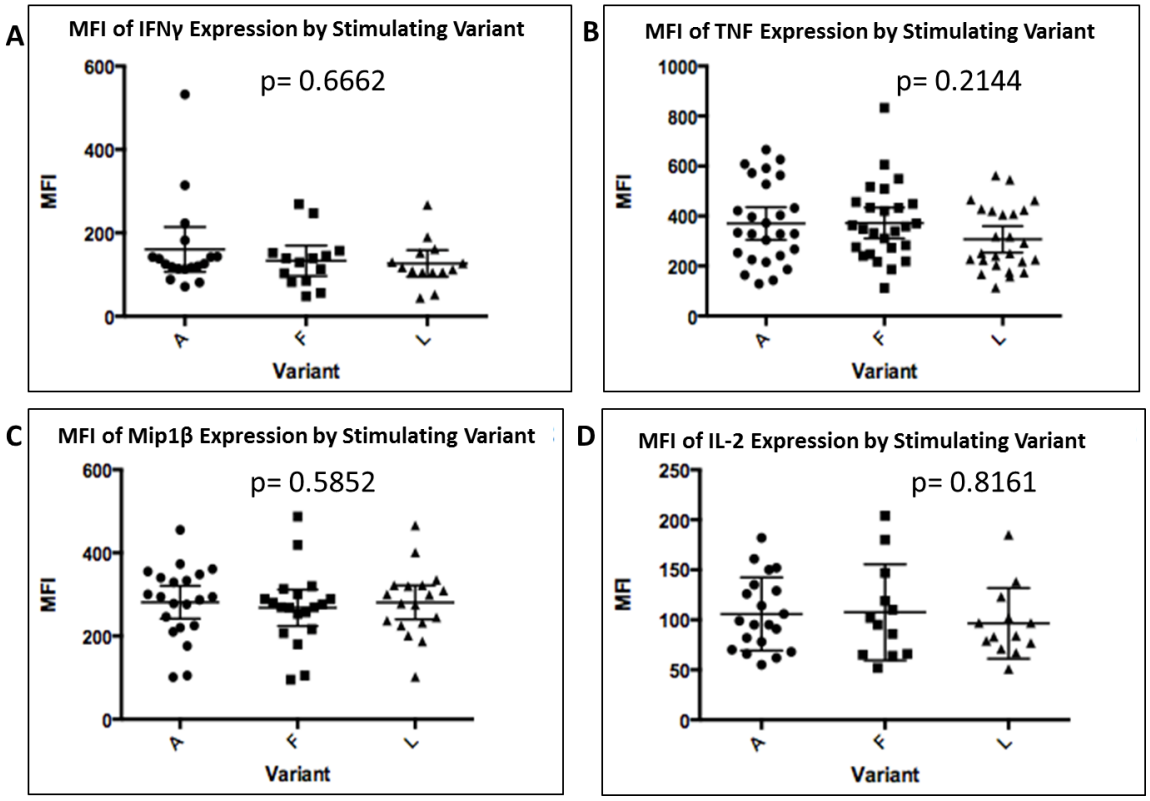


Figure 3.8: Differences in MFI of cytokine producing CD8+ T cells. The mean fluorescence intensity of each cytokine after stimulation with each epitope was determined. Panel (A) shows the MFI of IFNγ after stimulation with each IF9 variant, while panel (B) shows the MFI of TNF expression, panel (C) the MFI of Mip1β expression, and panel (D) the MFI of IL-2 expression.

Examining the TL9 variants, it was found that TL9 T consistently stimulated the highest frequency of cytokine producing CD8+ T cells (Figure 3.9). The number of CD8+ T cells producing TNF was significantly higher in TL9 T stimulated samples than those stimulated with TL9 M or TL9 A (Figure 3.9, $p=0.0056$ and $p=0.0022$ respectively, Wilcoxon rank). TL9 T also stimulated a higher number of CD8+ T cells producing IFNγ and MIP1β than TL9 A (Figure 3.9, $p=0.0019$ and $p=0.0027$, Wilcoxon rank), though the increases compared to stimulation with TL9 M were not significant in these cases. The MFI of CD8+ T cells producing IFNγ was also significantly higher when stimulated by TL9 T or TL9 M than when stimulated by TL9 A (Figure 3.10), but there were no differences among the other cytokines.

In summary, contrary to the hypothesis, the variant TL9 T epitope stimulated significantly more TNF producing CD8+ T cells than the consensus TL9 M epitope, and stimulated the highest number of cytokine producing CD8+ T cells for each of the other cytokines, though these levels were not significantly higher than those stimulated by TL9 M.

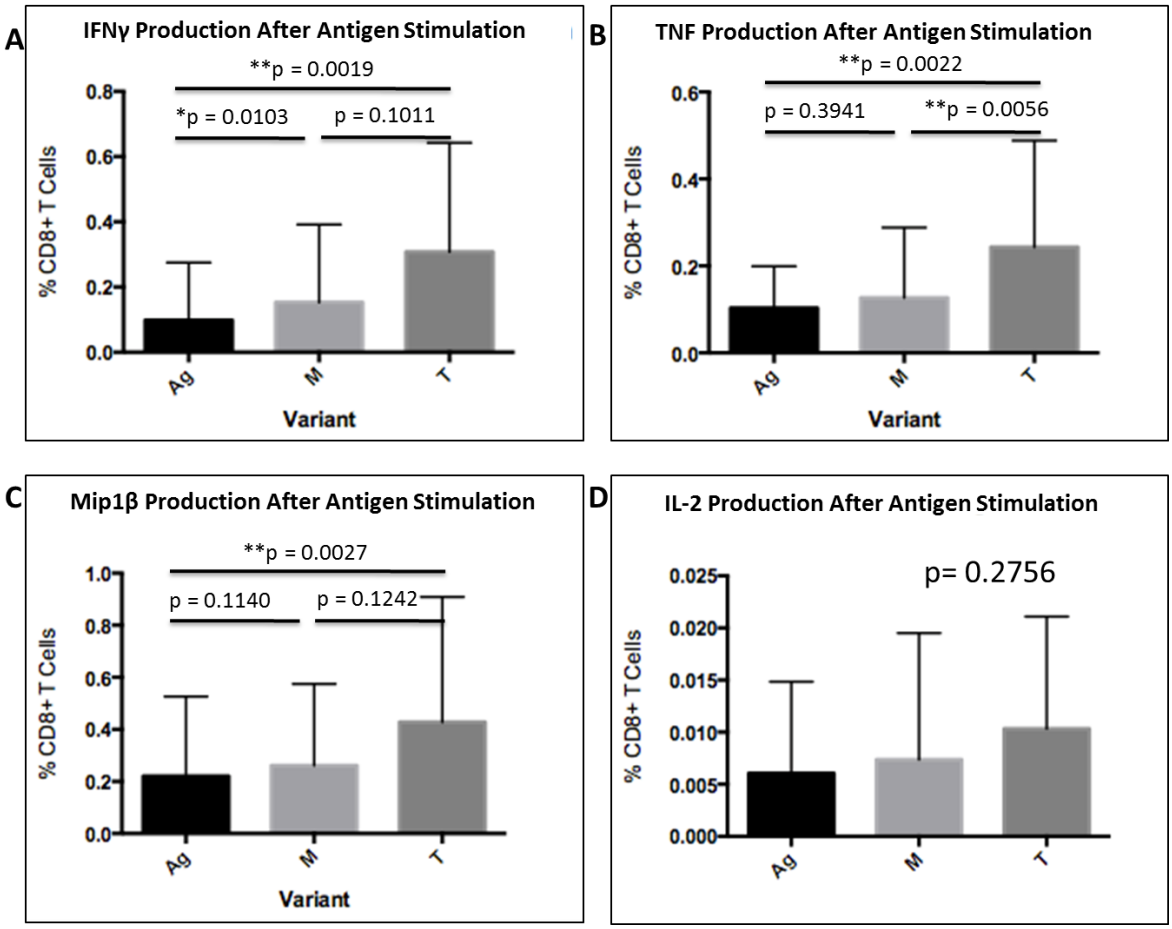


Figure 3.9: Differences in cytokine production by bulk CD8+ T cells after TL9 stimulation. PBMCs stimulated with each TL9 epitope were assessed for the percentage of CD8+ T cells producing each cytokine. Panel (A) shows the percentage of CD8+ T cells producing IFNγ after stimulation with each TL9 epitope, while panel (B) shows TNF production, panel (C) Mip1β production, and panel (D) IL-2 production.

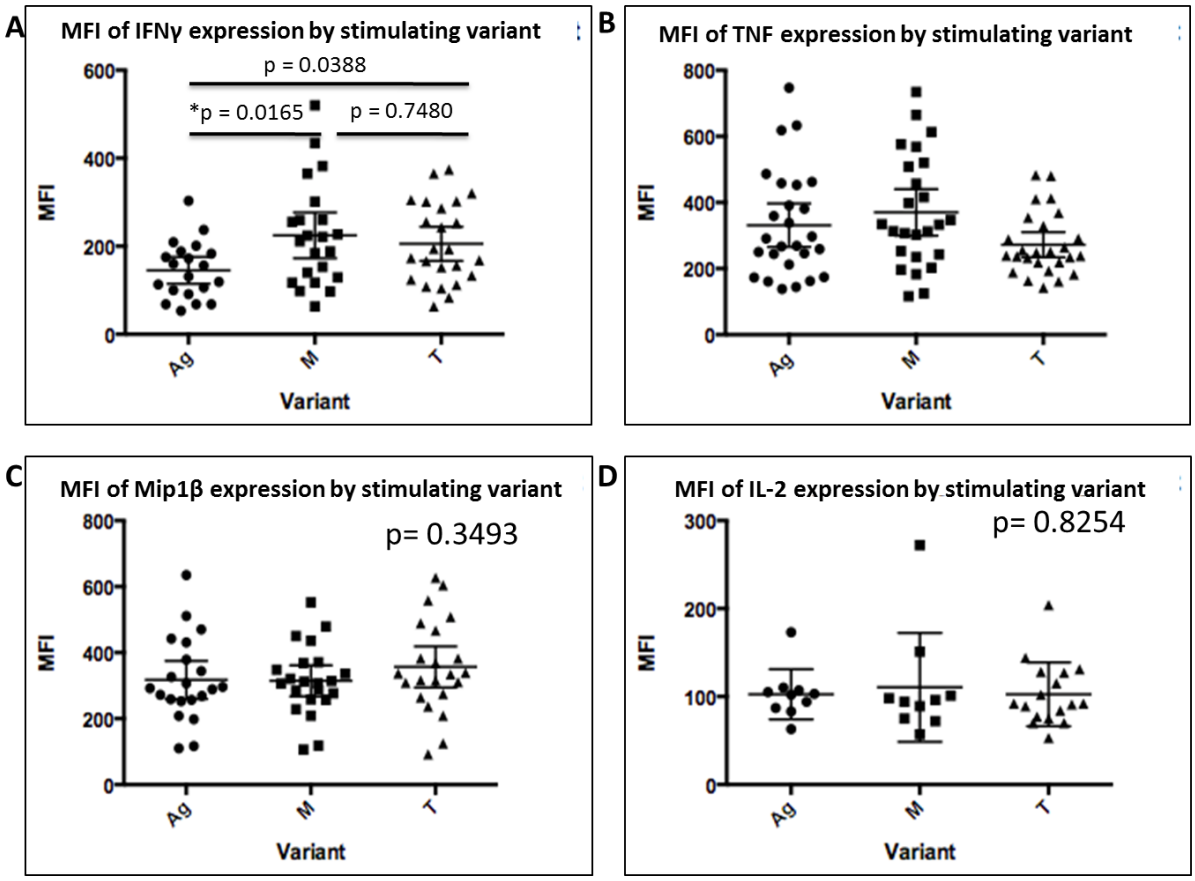


Figure 3.10: Differences in MFI of cytokine producing CD8+ T cells. The mean fluorescence intensity of each cytokine after stimulation with each epitope was determined. Panel (A) shows the MFI of IFNγ after stimulation with each TL9 variant, while panel (B) shows the MFI of TNF expression, panel (C) the MFI of Mip1β expression, and panel (D) the MFI of IL-2 expression.

To identify any relationships between the frequencies of cytokine producing CD8+ T cells after stimulation with each epitope, correlations were conducted (Table 3.3). It was found that the frequency of IL-2 producing CD8+ T cells was not correlated with production of any other cytokines, regardless of the stimulant, which was likely due to the low level of this response. Stimulation with IF9 A led to a highly coordinated immune response, whereby the frequencies of CD8+ T cells producing TNF, IFNγ and MIP1β stimulated by the epitope were highly correlated with one another (Table 3.3, Spearman's rank correlation). Conversely, IF9 F and IF9 L did not stimulate cytokine producing CD8+ T cells that correlated at all with respect to frequency, meaning that stimulation with these epitopes is likely to produce a less coordinated immune response.

Stimulation with the TL9 A, M and T epitopes resulted in strong correlations among all cytokine producing CD8+ T cells for each cytokine observed.

In summary, IF9 A and all three TL9 epitopes stimulated CD8+ T cells that can potentially produce multiple cytokines at once, which was not observed with stimulation by IF9 F or IF9 L.

A

Variant A	TNF	IFNg	IL2
Mip1B	ns	0.001	ns
TNF		0.0044	0.0008
IFNg			ns
Variant F	TNF	IFNg	IL2
Mip1B	ns	ns	ns
TNF		ns	ns
IFNg			ns
Variant L	TNF	IFNg	IL2
Mip1B	ns	ns	ns
TNF		ns	ns
IFNg			ns

B

Variant Ag	TNF	IFNg	IL2
Mip1B	0.0039	0.0001	ns
TNF		0.0001	ns
IFNg			ns
Variant M	TNF	IFNg	IL2
Mip1B	0.0044	0.0001	ns
TNF		0.0001	ns
IFNg			ns
Variant T	TNF	IFNg	IL2
Mip1B	0.0001	0.0001	ns
TNF		0.0001	ns
IFNg			ns

Table 3.3: Correlations in frequency of cytokine producing CD8+ T cells. Correlations were conducted to assess any relationships among post-stimulation cytokine production by CD8+ T cells. Panel (A) shows correlations among CD8+ T cell responses stimulated by the IF9 epitope variants, and panel (B) shows correlations after stimulation with TL9 epitope variants.

3.5.7 Production of cytokines by epitope-specific CD8+ T cells

In the previous section, cytokine production was assessed in all CD8+ T cells after stimulation with each variant. Next, production of cytokines was examined exclusively in CD8+ T cells specific to each epitope, by gating on tetramer+ CD8+ T cells, and then observing the levels of cytokine producing cells. This was done to examine if there were any differences in CD8+ T cells specific to each variant in their

ability to produce each cytokine, as compared to cytokine production by all CD8+ T cells as done in section 3.5.6.

In section 3.5.6, it was observed that, of all CD8+ T cells, those producing MIP1β were most frequent, followed by IFNγ, TNF and finally IL-2 (Figure 3.6). However, when looking at epitope-specific CD8+ T cells (as identified by tetramer staining) it was found that CD8+ T cells producing IFNγ were actually the most common, followed by TNF, MIP1β, and IL-2 (Figure 3.11, $p<0.0001$, Wilcoxon rank), regardless of the epitope used for stimulation. This indicates that, after stimulation, MIP1β is produced by most CD8+ T cells compared to the other cytokines, but when looking only at epitope-specific CD8+ T cells, IFNγ is actually produced with the highest frequency.

In summary, while stimulation with the study epitopes resulted in a cytokine response dominated by MIP1β when considering all CD8+ T cells, epitope-specific CD8+ T cells primarily produced IFNγ.

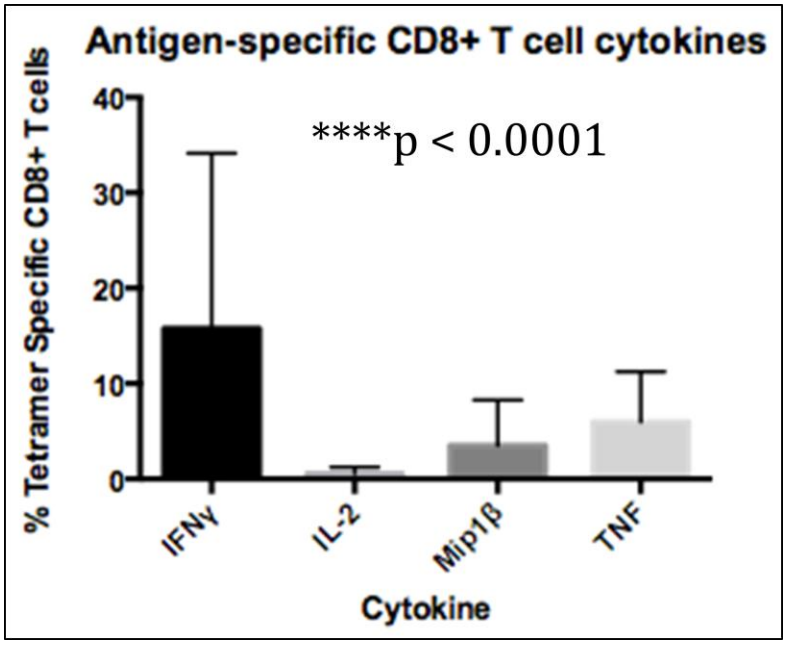


Figure 3.11: Frequency of expression of cytokines by CD8+ T cells specific to study epitopes. CD8+ T cells identified by positive tetramer staining to be specific to epitopes in this study were assessed for the frequency that they produce IFNγ IL-2, Mip1β and TNF.

After identifying that IFNγ was the most frequently produced cytokine by epitope-specific CD8+ T cells regardless of the stimulating epitope, cytokine production was then assessed in response to each epitope individually. Interestingly, though the IF9 A epitope stimulated more IFNγ production in bulk CD8+ T cells than the IF9 F or IF9 L epitopes (Figure 3.6), it was found that cytokine producing CD8+ T cells specific to IF9 A, as identified by tetramers, were significantly less frequent than those specific to either IF9 F or IF9 L (Figure 3.12, Wilcoxon rank). This indicates that though IF9 A could stimulate bulk CD8+ T cells to produce IFNγ, it is not necessarily epitope-specific stimulation, as indicated by a lower frequency of IF9 A-specific cytokine producing CD8+ T cells than IF9 F or IF9 L specific cells. Overall, cytokine producing CD8+ T cells specific to the IF9 F variant were most frequent compared to those specific to either IF9 A or IF9 L.

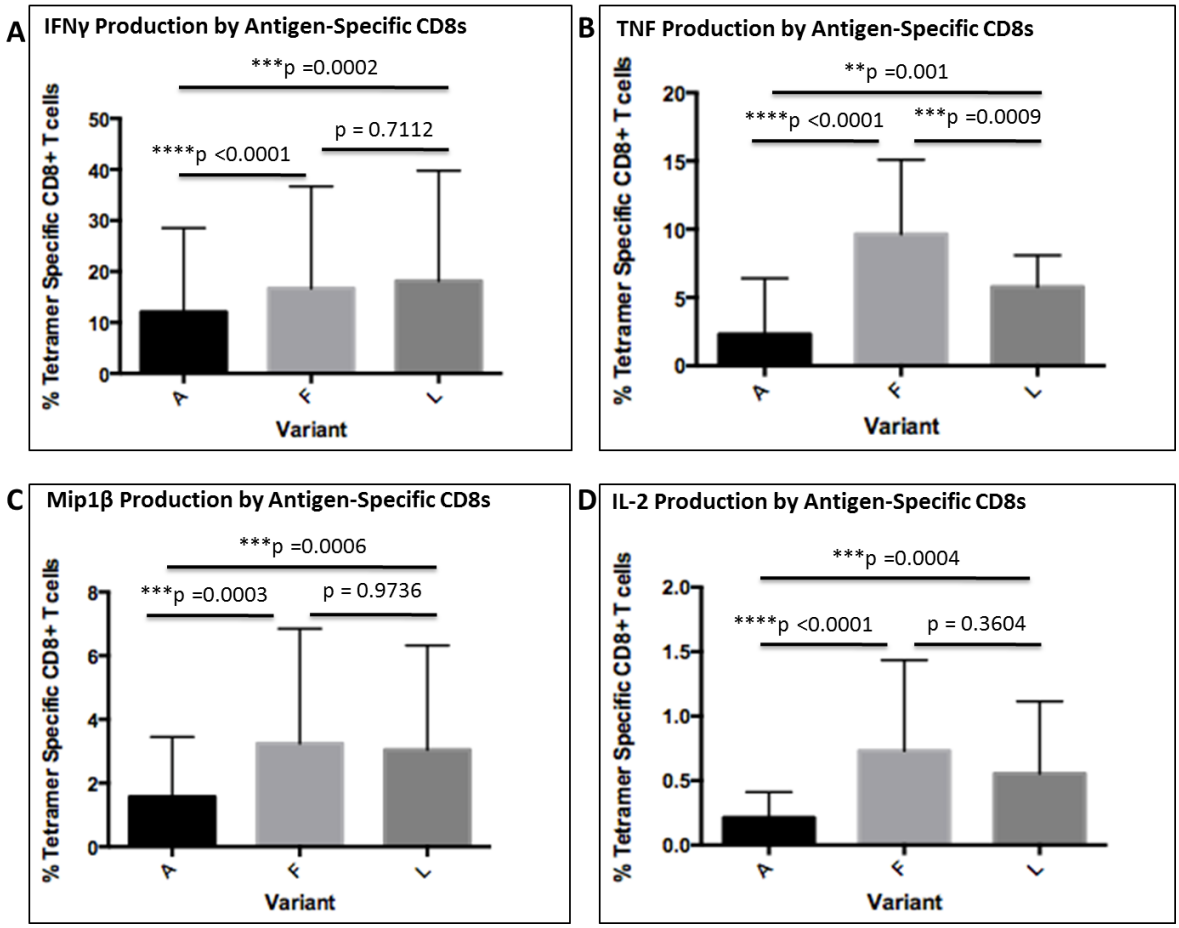


Figure 3.12: Production of cytokines by epitope-specific CD8+ T cells. CD8+ T cells specific to each IF9 epitope, as identified by tetramer staining, were compared for how frequently they produced each cytokine. In panel (A), the percentage of IFNγ-producing CD8+ T cells specific to each IF9 epitope are compared. In panel (B), TNF production is compared, and in panel (C), Mip1β production is compared, and finally in panel (D), IL-2 production is compared.

Examination of the TL9 A, M and T variants revealed that there were significantly fewer TL9 A-specific IFNγ producing CD8+ T cells than those specific to TL9 M (Figure 3.13, p= 0.0088, Wilcoxon rank). It was also identified that there were significantly fewer TL9 T-specific and TL9 A-specific CD8+ T cells producing TNF than TL9 M-specific cells (Figure 3.13, p= 0.0004 and p=0.001 respectively, Wilcoxon rank). Otherwise, there were no differences observed between the frequencies of epitope-specific cytokine-producing CD8+ T cells. In Figure 3.9, it was identified that the TL9 T variant was able to stimulate the most cytokine producing bulk CD8+ T cells, while it appears from this data that TL9 M is actually able to stimulate the most epitope-specific cytokine-producing CD8+ T cells. This may suggest that, though the TL9 T variant can stimulate the most cytokine producing bulk CD8+ T cells, the TL9 M consensus epitope is actually better able to stimulate an epitope-specific response, as seen in Figure 3.13.

In summary, despite stimulating the most cytokine-producing bulk CD8+ T cells, IF9 A-specific cytokine producing CD8+ T cells were significantly less frequent than those specific to IF9 F or IF9 L, suggesting stimulation with IF9 A results in a non-specific CD8+ T cell response. Similarly, though the TL9 T variant is able to stimulate the most cytokine producing bulk CD8+ T cells, the TL9 M consensus epitope actually stimulates the strongest epitope-specific CD8+ T cell cytokine response.

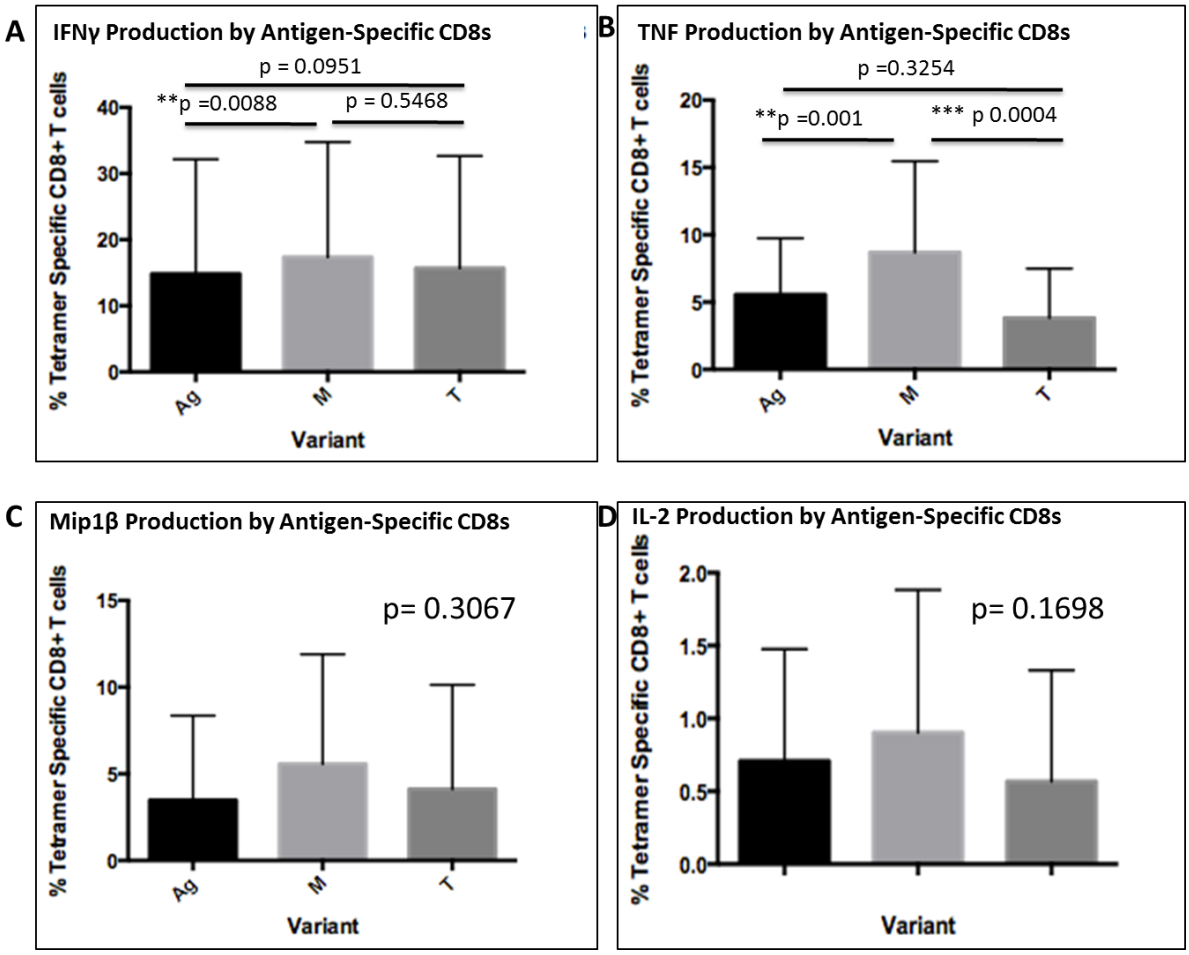


Figure 3.13: Production of cytokines by epitope-specific CD8+ T cells. CD8+ T cells specific to each TL9 epitope, as identified by tetramer staining, were compared for how frequently they produced each cytokine. In panel (A), the percentage of IFNγ-producing CD8+ T cells specific to each TL9 epitope are compared. In panel (B), TNF production is compared, and in panel (C), Mip1β production is compared, and finally in panel (D), IL-2 production is compared.

3.5.8 Polyfunctionality of cytokine responses by stimulating variant

As previously described, an important measure of a beneficial CD8+ T cell response in HIV is the ability of each cell to produce more than one cytokine at a time[120], [152]. As such, the polyfunctionality of CD8+ T cells after stimulation with each epitope was assessed. For the IF9 A, F and L epitopes, it was found that stimulation with IF9 A led to the highest frequency of 3+ CD8+ T cell polyfunctional responses (CD8+ T cells producing 3 or more cytokines simultaneously), which was significant when compared to stimulation with IF9 L (Figure 3.14, p=0.0001, Wilcoxon rank), and trended toward significance compared to stimulation with IF9 F (Figure 3.14, p=0.0671, Wilcoxon rank). Similarly, 2+ CD8+ T cell polyfunctional responses were significantly more frequent after stimulation with IF9 A than either IF9 F or IF9 L stimulation (p=0.019 and p=0.0004 respectively, Wilcoxon rank).

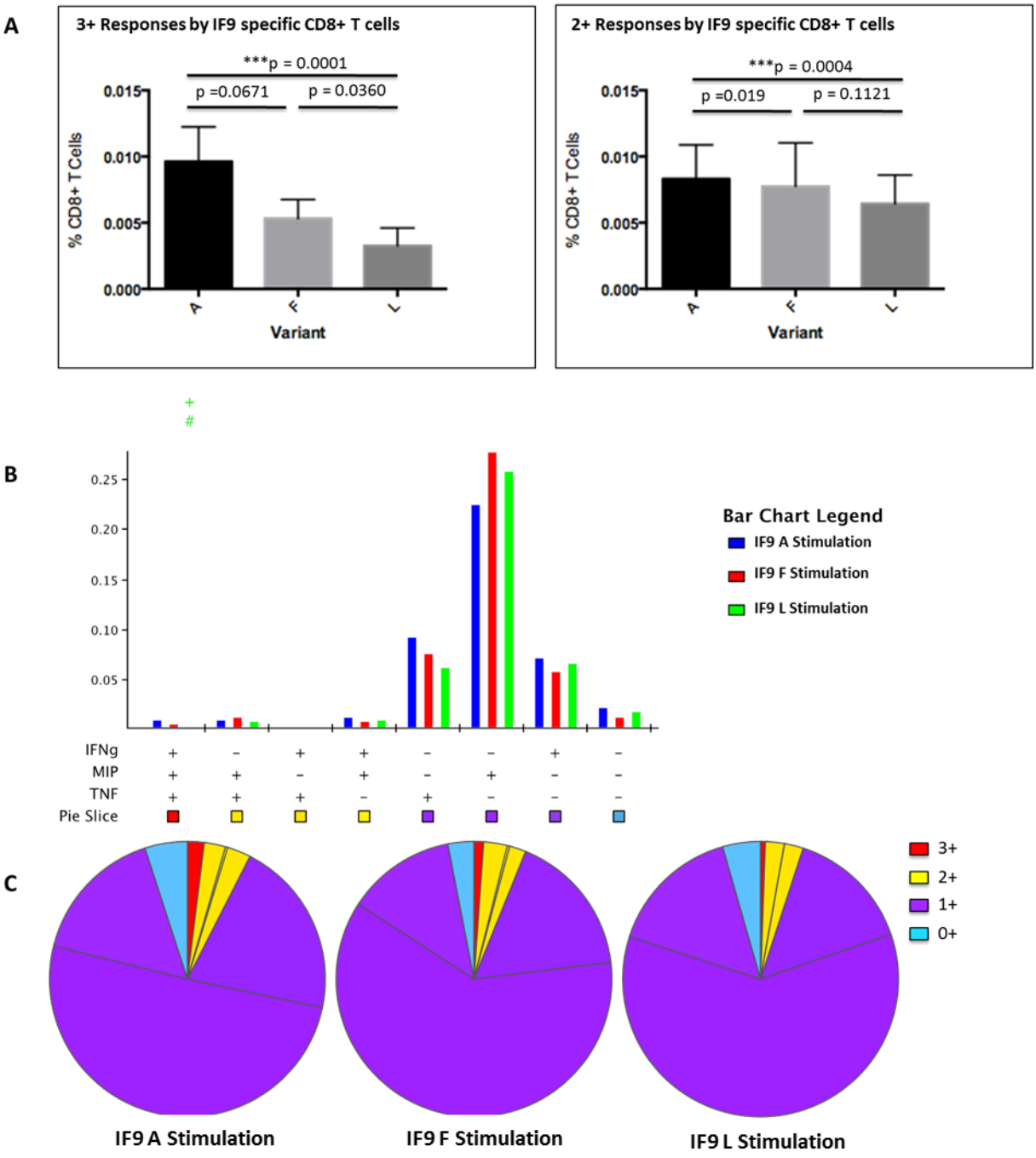


Figure 3.14: Polyfunctionality of responses to IF9 A, F, and L variants.
Panel (A) shows the differences in frequency of 3+ and 2+ polyfunctional CD8+ T cells after stimulation with each IF9 variant. The specific cytokines making up each response are shown in panel (B) and panel (C) shows the summary of the combined polyfunctional responses.

Examining polyfunctionality after stimulation with the TL9 A, M and T variants, TL9 T produced the strongest polyfunctional response in the resulting CD8+ T cells (Figure 3.15). TL9 T stimulated a significantly higher 3+ polyfunctional response than TL9 A (Figure 3.15, $p< 0.0001$, Wilcoxon rank), while there was no significant increase over stimulation with TL9 M. TL9 T also stimulated the most frequent 2+ polyfunctional response, with a significantly higher frequency compared to TL9 A (Figure 3.15, $p= 0.0017$, Wilcoxon rank) and a trend toward significance compared to M (Figure 3.15, $p= 0.0337$, Wilcoxon rank).

In summary, contrary to the hypothesis, the strongest polyfunctional CD8+ T cell responses were stimulated by the variant epitopes IF9 A and TL9 T, rather than the consensus epitopes IF9 F and TL9 M.

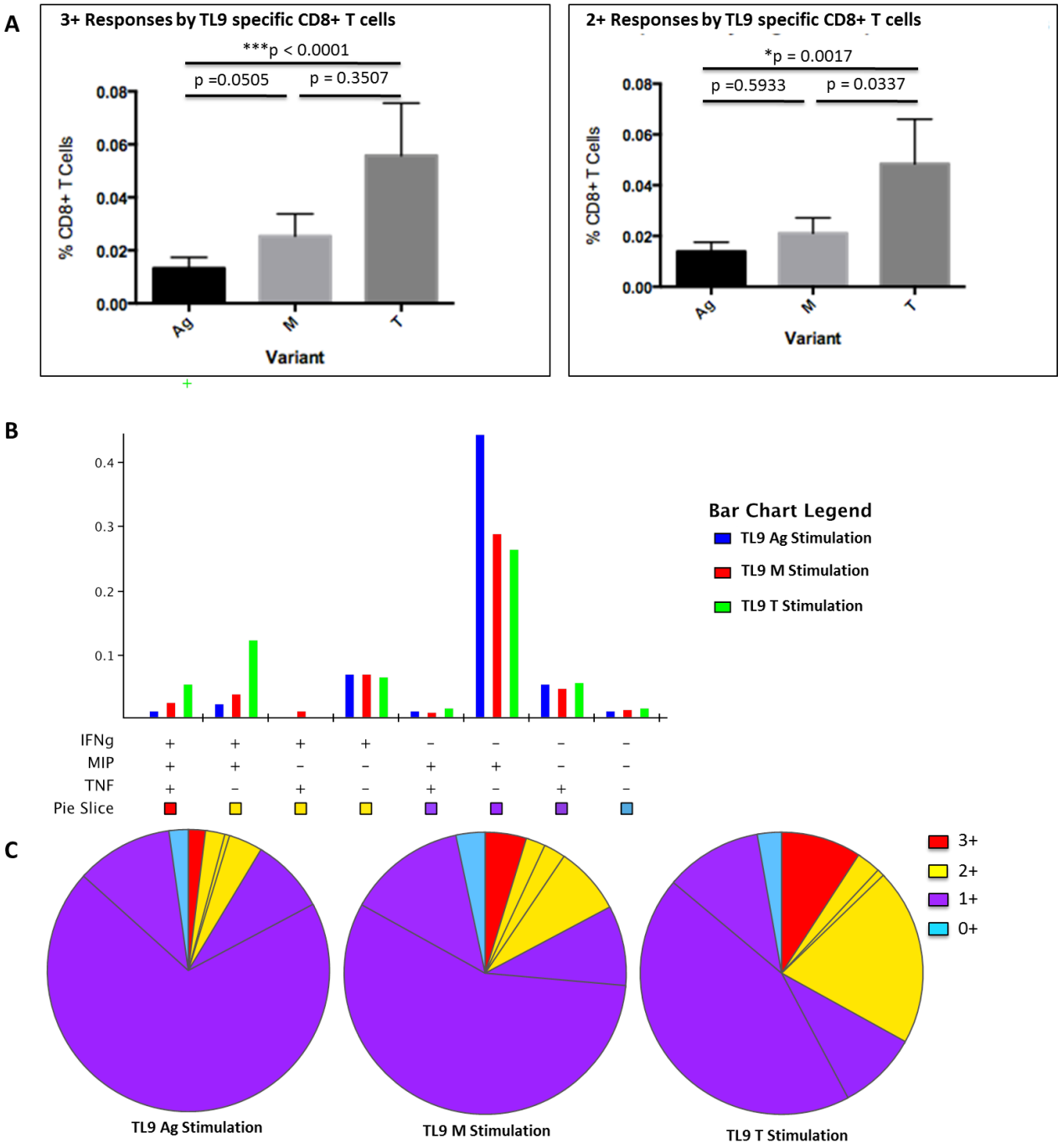


Figure 3.15: Polyfunctionality of responses to TL9 A, M and T variants.
Panel (A) shows the differences in frequency of 3+ and 2+ polyfunctional CD8+ T cells after stimulation with each TL9 variant. The specific cytokines making up each response are shown in panel (B) and panel (C) shows the summary of the combined polyfunctional responses.

3.5.9 Proliferative responses by CD8+ T cells after variant stimulation

Another important measure of an effective CD8+ T cell response to HIV is the ability of these cells to proliferate in response to stimulation. In order to understand if one variant is better or worse than the others at stimulating a proliferative response in CD8+ T cells, 6-day stimulations with each variant and CFSE proliferation dye were conducted. Overall, proliferative responses were very infrequent, despite the cells responding well to stimulation with SEB as a positive control. However, of the individuals that did produce a strong proliferative response, four were in response to TL9 T only, and one individual responded with strong proliferation to each of the IF9 A, IF9 F, TL9 M and TL9 T variants (representative proliferative staining can be seen in Figure 3.16). This suggests that, though the proliferative responses to these epitopes are infrequent, they are possible, and are most likely to occur after stimulation with the TL9 T variant than any of the others.

Contrary to the hypothesis, proliferation was infrequent, but most commonly observed in response to the TL9 T variant epitope rather than the consensus TL9 M.

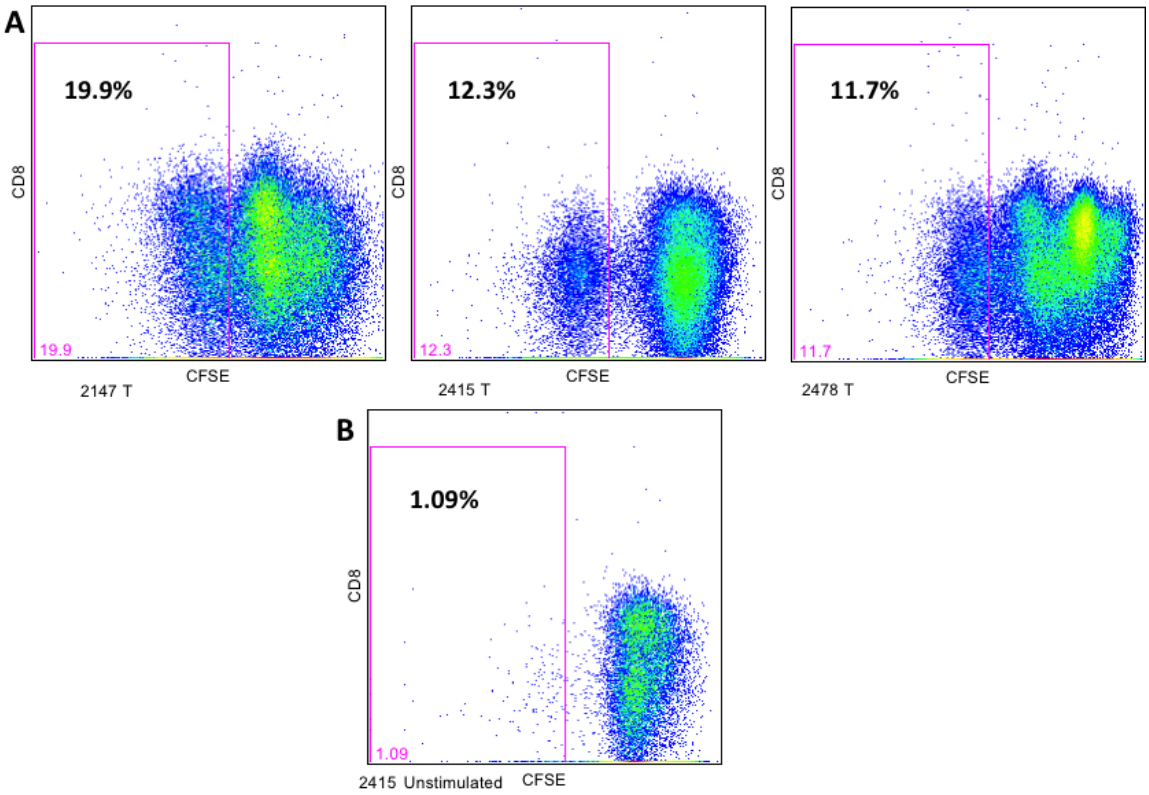


Figure 3.16: Representative staining of positive proliferative responses. Plots are gated on lymphocytes and represented as CD8+ vs. CFSE. Above panel shows three representative subjects with positive proliferative responses to the TL9 T epitope (A). Shown below is representative proliferative staining of an unstimulated control (B).

3.5.10 Viral sequencing of IF9

Considering the extreme genetic diversity within HIV Env and this region in particular, 90 HIV-infected individuals from this cohort were sequenced to confirm the presence of the IF9 F epitope as the consensus in this population, and to assess the relative abundance of the other IF9 epitopes. Overall, it was found that the most common outcome was a sequence that differed completely from the IF9 consensus, at several different positions (Figure 3.17). IF9 F was found in 28/90 subjects, and among the A, F and L variants of IF9, was identified most frequently. IF9 L was the next most common, sequenced in 21/90 individuals, and IF9 A was only present in 2/90 tested individuals.

In summary, IF9 sequencing revealed that the most common sequence observed was IF9 F, though it was only found in 28 of 90 individuals.

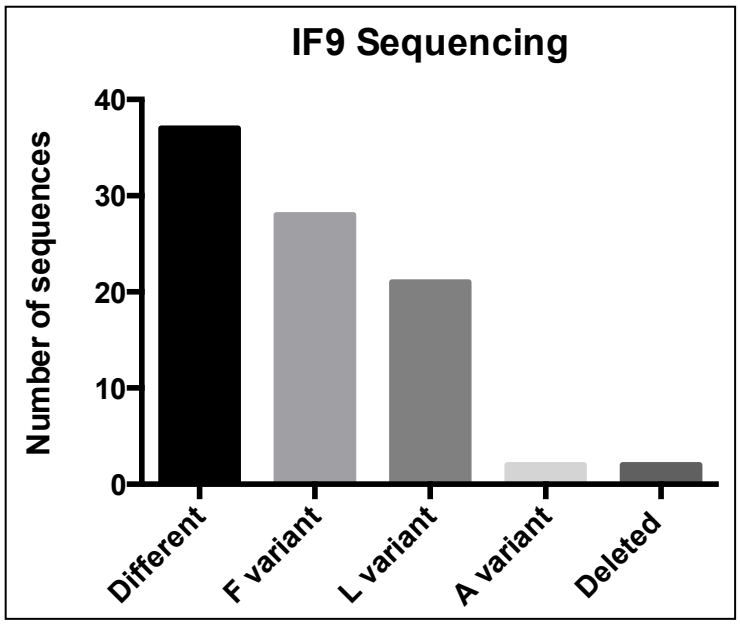


Figure 3.17: IF9 sequencing of 90 HIV-infected subjects within the Majengo cohort. The sequences were identified and displayed as either the F, L or A variants, something different than these three, or deleted entirely.

3.6 Conclusions

It was hypothesized that HIV consensus epitopes would be more frequently recognized by CD8+ T cells by virtue of being more common in circulation, and would produce a more proliferative, polyfunctional, and overall stronger cytokine response. This hypothesis was not confirmed in the IF9 A, F and L variants, whereby CD8+ T cell responses to the consensus IF9 F epitope were found to be less frequent than responses to the IF9 A variant. The IF9 A variant was also significantly more likely to elicit a cytokine response when looking at all CD8+ T cells than the IF9 F or IF9 L variants, but, interestingly, when looking at cytokine responses in epitope-specific CD8+ T cells, the frequency of IF9 A-specific cytokine-producing CD8+ T cells was significantly lower in all cases compared to IF9 F-specific or IF9 L-specific CD8+ T cells. Also contrary to the hypothesis, the variant IF9 A also stimulated the most polyfunctional response in CD8+ T cells. For these IF9 epitopes, there were no significant associations with any measures of CD4. Overall, and contrary to the hypothesis, it was determined that the non-consensus IF9 A epitope was actually more likely to be associated with more frequent CD8+ T cell recognition and cytokine production than the consensus IF9 F.

Similarly, the hypothesis was not confirmed in the case of the TL9 A, M and T epitopes. Here, the non-consensus TL9 T epitope was most frequently recognized by CD8+ T cells, was the only one able to stimulate a proliferative response, consistently stimulated the highest level of cytokines, and led to a more polyfunctional response in CD8+ T cells than its related TL9 A and consensus TL9 M epitopes. Interestingly, the frequency of CD8+ T cell recognition of TL9 T was also

correlated with several beneficial measures of CD4, suggesting this epitope may be related to slower disease progression, especially when compared to the TL9 A and TL9 M variants, for which there were no significant correlations observed with measures of CD4.

The next chapter will continue this exploration of epitope and variant stimulated CD8+ T cell responses, and characterize CD8+ T cell responses to two immunodominant consensus Gag epitopes and their variants, presented by the beneficial HLA-B*57:03 allele.

Chapter 4: Cross-Sectional and Longitudinal CD8+ T cell Responses to HIV IW9 and KF11 Epitopes and Their Natural Variants in HLA-B*57:03 Subjects

4.1 Rationale:

Mutations in the Gag epitopes ISPRTLNAW (IW9) and KAFSPEVIPMF (KF11) exist in nature[191], however, for both epitopes and IW9 in particular, the reversion rate from a mutated form back to the consensus is high[191], which means this mutation likely comes with a high fitness cost to the virus. Within the Majengo sex worker cohort, mutations within both of these epitopes have been observed. The IW9 and KF11 epitopes are known to be immunodominant[162], which would suggest that individuals will exhibit strong immune responses to them. This data also suggests that responses would be less frequent to their common variants, LW9 and KGF, especially considering that these mutations have a tendency to revert back to the immunodominant forms whenever possible, meaning exposure to them is ultimately likely lower within a given population. Further, a stronger CD8+ T cell response against these immunodominant epitopes may be associated with better disease outcomes, as it may force the virus to mutate into a less fit form if the immune pressure remains strong. Considering that these mutations are very common, having an understanding of how the CD8+ T cell responses against them differs will be important for our overall understanding of how the immune system handles this pathogen under changing circumstances.

4.2 Hypotheses:

1) The consensus and immunodominant IW9 and KF11 epitopes will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their natural variants. The IW9 and KF11 epitopes will elicit CD8+ T cell responses that are more proliferative and polyfunctional than their natural variant epitopes.

2) CD8+ T cell responses to IW9 and KF11 will be associated with slower disease progression, determined by CD4 count. Beneficial CD8+ T cell responses including proliferation and polyfunctionality will be preserved in individuals who maintain healthy CD4 counts longitudinally.

4.3 Objectives:

1) Determine the frequency of recognition, proliferative capacity and polyfunctionality of CD8+ T cells in response to IW9 and KW11 and their natural variants, LW9 and KGF.

2) Determine the association of the frequency of CD8+ T cell responses to IW9 and KF11 and their natural variants with CD4+ T cell counts as a measure of immune control and disease progression.

3) Assess the changes in cytokine production and proliferative potential in individuals over multiple time points to determine how these factors may change over time with disease progression.

4.4 Methods:

Ex-vivo screening: 16 HIV-infected individuals from the Majengo CSW cohort who possessed at least one HLA-B*57:03 allele were screened for recognition of tetramers to each epitope, using *ex-vivo* whole blood staining, as outlined in section 2.8. The panel used for staining can be found in Table 2.2.

Cytokine production assay: Cryopreserved PBMCs from 31 HIV-infected, ART-naïve, HLA-B*57:03+ individuals were thawed as per section 2.5, and stimulated for 6 hours with the HLA-B*57:03 epitopes, as outlined in section 2.15. Surface and intracellular staining for cytokine production was done as described in sections 2.9-2.11. Table 2.4 shows the flow cytometry panel used for this section.

Proliferation assay: PBMCs from the same individuals were also assessed for proliferative potential via a 6-day stimulation assay, as described in section 2.14. Table 2.3 shows the proliferation flow cytometry panel used.

Statistical analysis: A p value of <0.05 was used as a cut off for significance. For all group comparisons with 3+ groups (examining bulk cytokine responses), Friedman tests were used as an initial test of significance. If this test was significant, Bonferroni corrected p values were used with Wilcoxon T tests to decipher where the differences were found. For comparisons of just two groups, Mann-Whitney tests were used. For all correlations, Spearman's rank correlations were used.

4.5 Results:

4.5.1 Study population and epitopes

HIV-infected individuals from the Majengo sex worker cohort in Nairobi, Kenya were enrolled in this study if they were HLA-B*57:03+, ART-naïve, and had CD4 counts >400 at the time of sampling in order to follow some individuals longitudinally, before they progressed to AIDS and were placed on ART. Whole blood samples from 16 subjects were screened *ex-vivo* for responses to the study epitopes and PBMC samples from 31 individuals were obtained for the remaining experiments. The epitopes for this study were IW9 (ISPRTLNAW) and one natural variant, LW9 (LSPRTLNAW), and KF11 (KAFSPEVIPMF) and one natural variant, KGF (KGFSPEVIPMF). These epitopes are described in Table 2.1.

4.5.2 Ex-vivo screening for epitope-specific CD8+ T cells

In order to determine if there was a high level of recognition of these epitopes in the study cohort, individuals were screened for epitope-specific CD8+ T cells to the study epitopes, by *ex-vivo* whole blood staining. A representative flow cytometry gating strategy and staining is displayed in Figure 4.1. A total of 16 subjects were screened, and all 16 had IW9 and LW9 specific CD8+ T cells, as evidenced by positive CD8+ tetramer binding. The recognition of the KF11 and KGF epitopes were somewhat less reliable, with 9/16 having KF11-specific CD8+ T cells and 6/16 for KGF-specific CD8+ T cells. It was determined from this *ex-vivo* staining that as the majority of individuals screened were able to recognize at least one of these epitopes, they were reasonable candidates for more intensive study within this cohort.

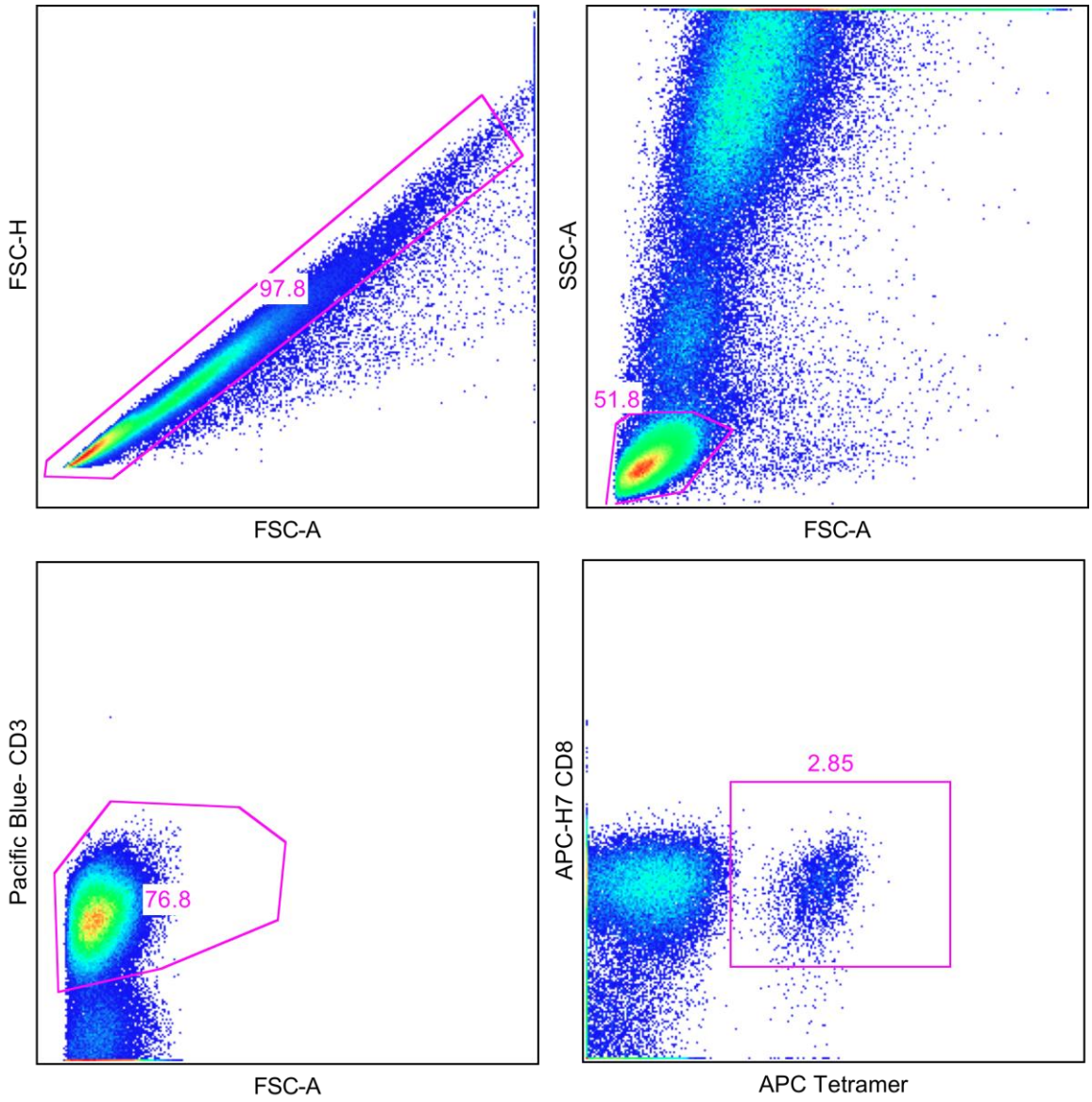


Figure 4.1: *Ex-vivo* tetramer screening gating strategy and representative tetramer staining. Whole blood samples were first gated on FSC-A vs. FSC-H to gate out doublet cells, then on FSC-A vs. SSC-A, and then the lymphocyte population was selected. The CD8+ T cell population was then selected when gated against CD3, and the tetramer positive population was visualized. Representative staining is from ML 1778.

4.5.3 Frequency of CD8+ T cells recognizing each variant

After *ex-vivo* screening revealed that recognition of the study epitopes were common in this cohort, cryopreserved PBMCs were stimulated with each epitope, to determine the frequency of CD8+ T cells recognizing each variant.

It was found that after stimulation, CD8+ T cells recognized the consensus IW9 epitope significantly more frequently than the LW9 variant (Figure 4.2, $p < 0.0001$, Mann-Whitney). Also, the MFI of the CD8+ T cells recognizing the IW9 variant was significantly higher than the MFI of those responding to the LW9 variant (Figure 4.2, $p = 0.0089$, Mann-Whitney). This suggests both that there is a larger pool of CD8+ T cells capable of recognizing the consensus IW9 epitope than the LW9 variant, and also that those CD8+ T cells specific to IW9 are binding to it with a higher number of TCRs than CD8+ T cells binding to LW9. Conversely, despite the KF11 epitope being the consensus sequence, there was no significant difference between the frequency of CD8+ T cells responding to KF11 vs. its common variant KGF (Figure 4.2, $p = 0.9423$, Mann-Whitney), nor in their relative MFIs (Figure 4.2, $p = 0.7460$, Mann-Whitney).

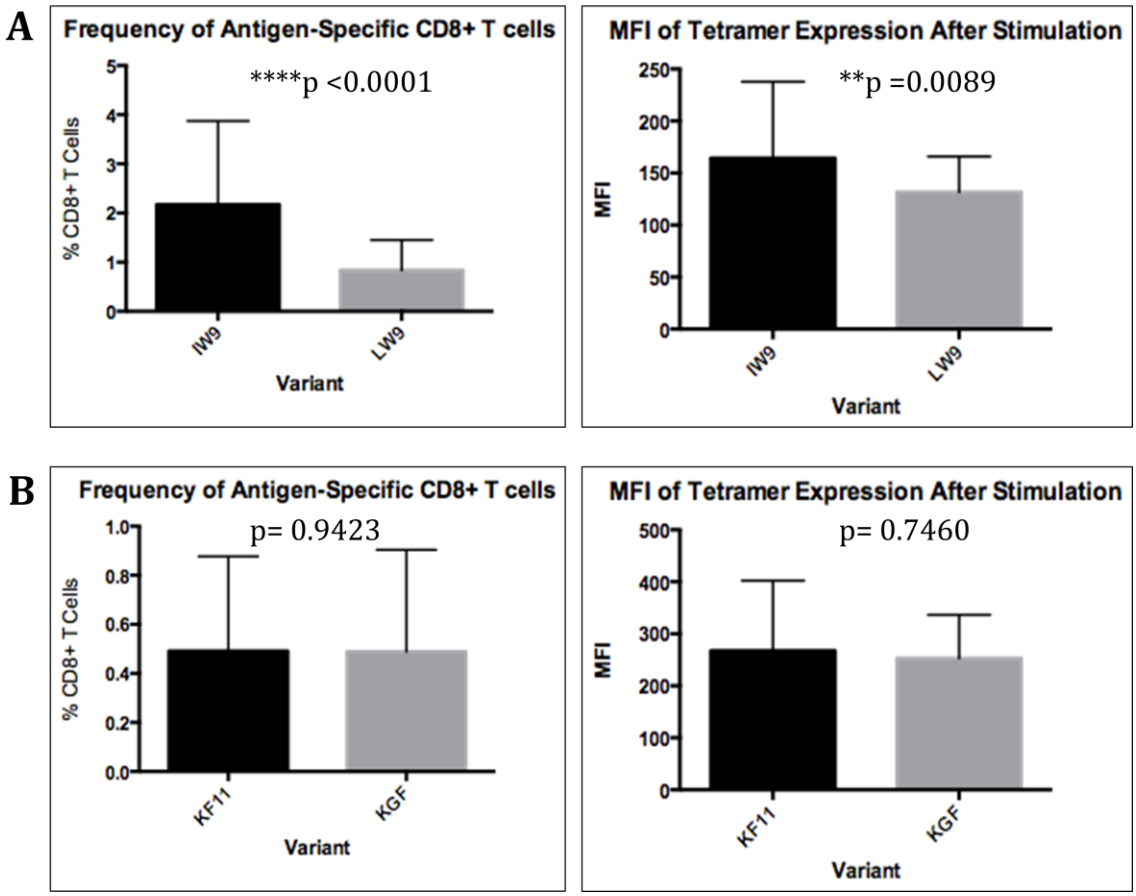


Figure 4.2: Frequency and MFI of epitope-specific CD8+ T cells from PBMCs. After 6-hour stimulation with matched peptides, the frequency of epitope-specific CD8+ T cells was determined using tetramers for each variant. The frequency and MFI of CD8+ T cell responses to the IW9 and LW9 epitopes are shown in figure (A), and those to the KF11 and KGF epitopes shown in figure (B).

Next, correlations were conducted between the frequency of CD8+ T cell recognition of each epitope, to determine if the magnitude of recognition was related among each epitope. It was observed that the frequency of CD8+ T cells recognizing each variant was very strongly correlated to each other variant, as was the MFI (Table 4.1, Spearman's rank correlation). In other words, individuals whose CD8+ T cells recognize one epitope with a high frequency are likely to have CD8+ T cells that recognize all epitopes with a high frequency, though the relative size of the epitope-specific CD8+ T cell pool may still be significantly different (as in the case of IW9 and LW9 from Figure 4.2). This linkage exists not just for the related epitopes (IW9 vs. LW9 and KF11 vs. KGF), but also the unrelated ones (IW9 vs. KF11, IW9 vs. KGF, etc). This would suggest not only that cross-reactivity may exist between the related epitopes, but perhaps also that the seemingly unrelated but immunodominant IW9 and KF11 epitopes may be linked as well.

In summary, CD8+ T cell recognition of the consensus IW9 epitope was significantly higher than that of the LW9 epitope, while there was no difference in the frequency of CD8+ T cell recognition of the consensus KF11 epitope or the KGF variant. The magnitude of CD8+ T cell recognition of all epitopes were strongly correlated.

A

Variant	IW9	LW9	KF11	KGF
IW9		<0.0001	0.0073	0.0055
LW9			0.0128	0.0108
KF11				<0.0001
KGF				

B

Variant	IW9	LW9	KF11	KGF
IW9		0.0583	0.0189	<0.0001
LW9			0.0005	<0.0001
KF11				<0.0001
KGF				

Table 4.1: Correlations between frequency and MFI of CD8+ T cell responses to stimulating variants. In panel (A), the frequency of CD8+ T cells responding to each of the four variants were correlated. Panel (B) displayed the correlations among the MFI of CD8+ T cells responding to each variant.

4.5.4 Association of the frequency of CD8+ T cell recognition of each variant with measures of CD4 count

In order to establish if there was a relationship between CD8+ T cell recognition and disease status, the frequency and MFI of CD8+ T cell recognition of each epitope were correlated with patient CD4 count at the time of sampling. There was a significant positive correlation between the frequency of CD8+ T cell recognition of the KF11 epitope and CD4 count, while the rest showed no significant correlations (Figure 4.3, p= 0.0171, Spearman's rank correlation). This data suggests that possession of a larger pool of CD8+ T cells that recognize the KF11 epitope was related to higher CD4 counts, an association not seen with the less common KGF variant, or with either of the IW9 or LW9 epitopes.

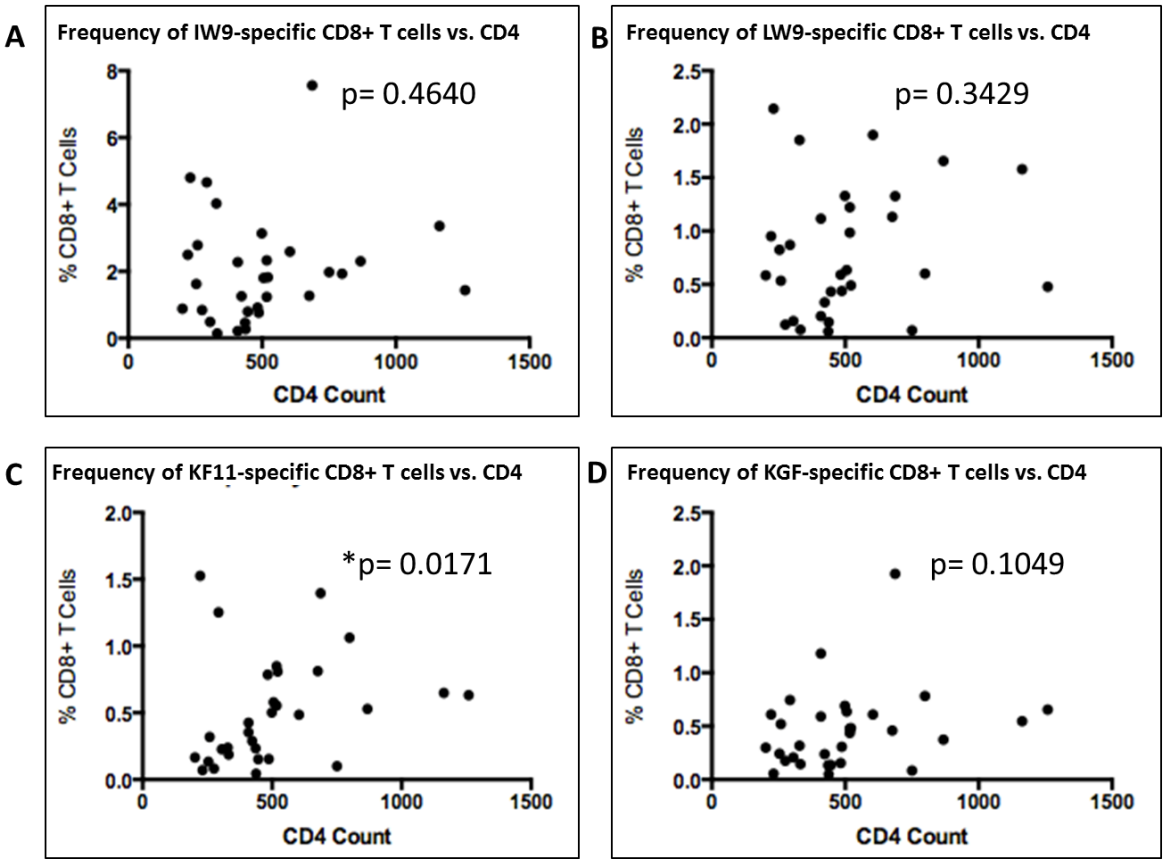


Figure 4.3: Correlation of the frequency of CD8+ T cells responding to each variant with CD4 count at the time of sampling. In panel (A), the percentage of CD8+ T cells responding to the IF9 epitope was correlated with CD4 count. In panel (B), the same was done for CD8+ T cells specific to LW9, in (C) with cells specific to KF11, and in (D), cells specific to KGF.

To investigate this relationship further, subjects were divided into groups according to their CD4 count at the time of sampling. They were categorized into those with CD4 counts >500 or those with CD4 <500. It was found that individuals with CD4 counts of >500 are more likely to have a significantly higher frequency of CD8+ T cells recognizing the KF11 and KGF epitopes than individuals with CD4 counts <500 (Figure 4.4, p= 0.0037 and p= 0.0391 respectively, Mann-Whitney). There was a similar trend with the frequency of CD8+ T cells responding to the IW9 and LW9 variants, though this did not reach significance in either case (Figure 4.4, p= 0.1007 and p= 0.0773 respectively, Mann-Whitney). This data suggests again that having a larger pool of CD8+ T cells that recognize the KF11 and KGF epitopes is associated with having a higher CD4 count, and may therefore be beneficial in combating infection with HIV.

Next, the frequency of CD8+T cells recognizing each epitope was also correlated to CD4 ratio. There was a significant positive correlation observed between the frequency of CD8+ T cells recognizing both KF11 and KGF with CD4 ratio (Figure 4.5, p= 0.0015 and p= 0.0057 respectively, Spearman's rank correlation), while there were no significant correlations found with CD8+ T cell recognition of either IW9 or LW9. This data suggests that individuals with a higher frequency of CD8+ T cells specific for either KF11 or KGF are likely to have higher CD4 ratios, and therefore, an overall healthier immune system.

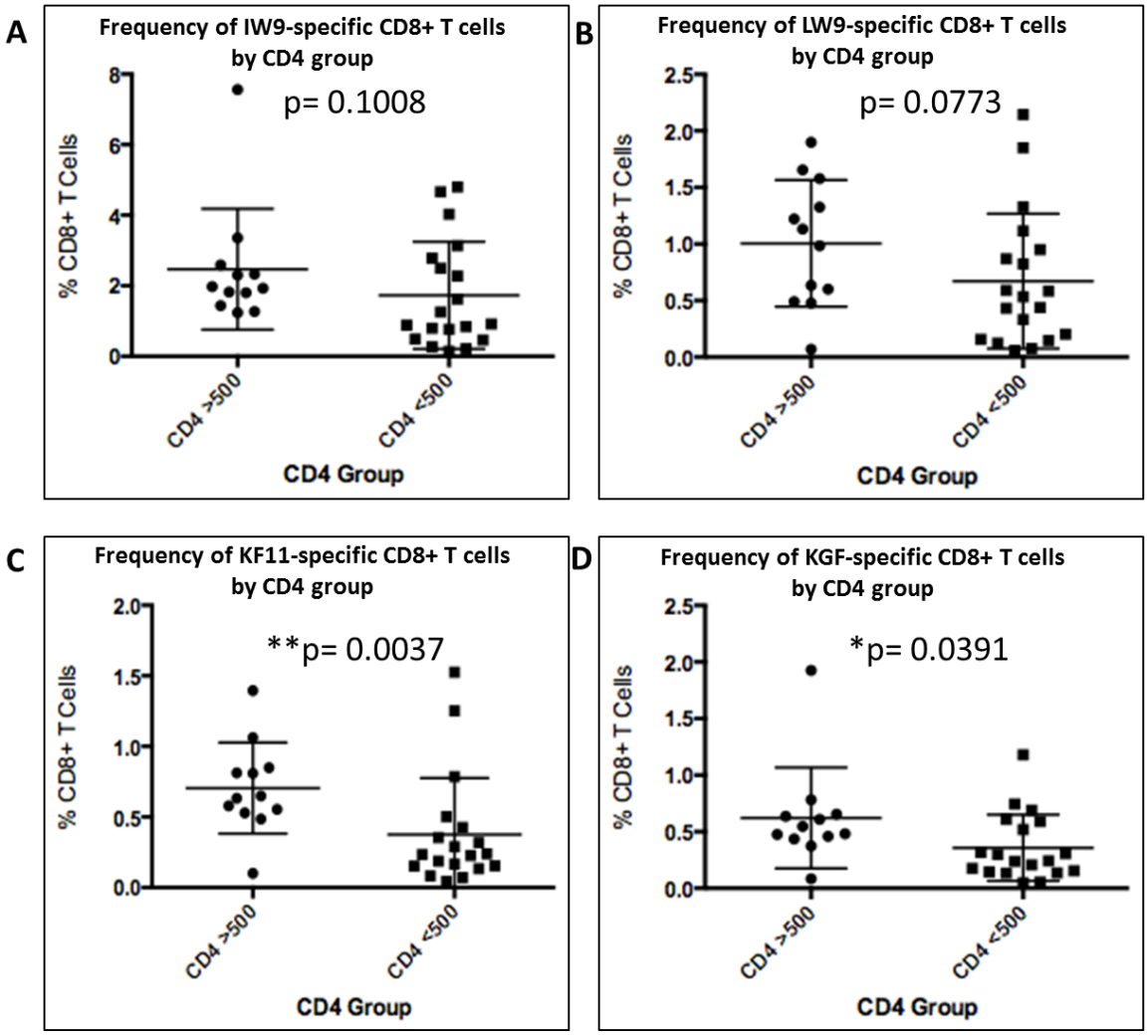


Figure 4.4: Frequency of CD8+ T cells specific for each variant stratified by CD4 count. Subjects were divided by their CD4 count, into either >500 or <500 and plotted according to the frequency of CD8+ T cell recognition of each variant. The frequency of CD8+ T cell recognition of the IW9 and LW9 variants are shown in plots (A) and (B), while KF11 and KGF are shown in (C) and (D) respectively.

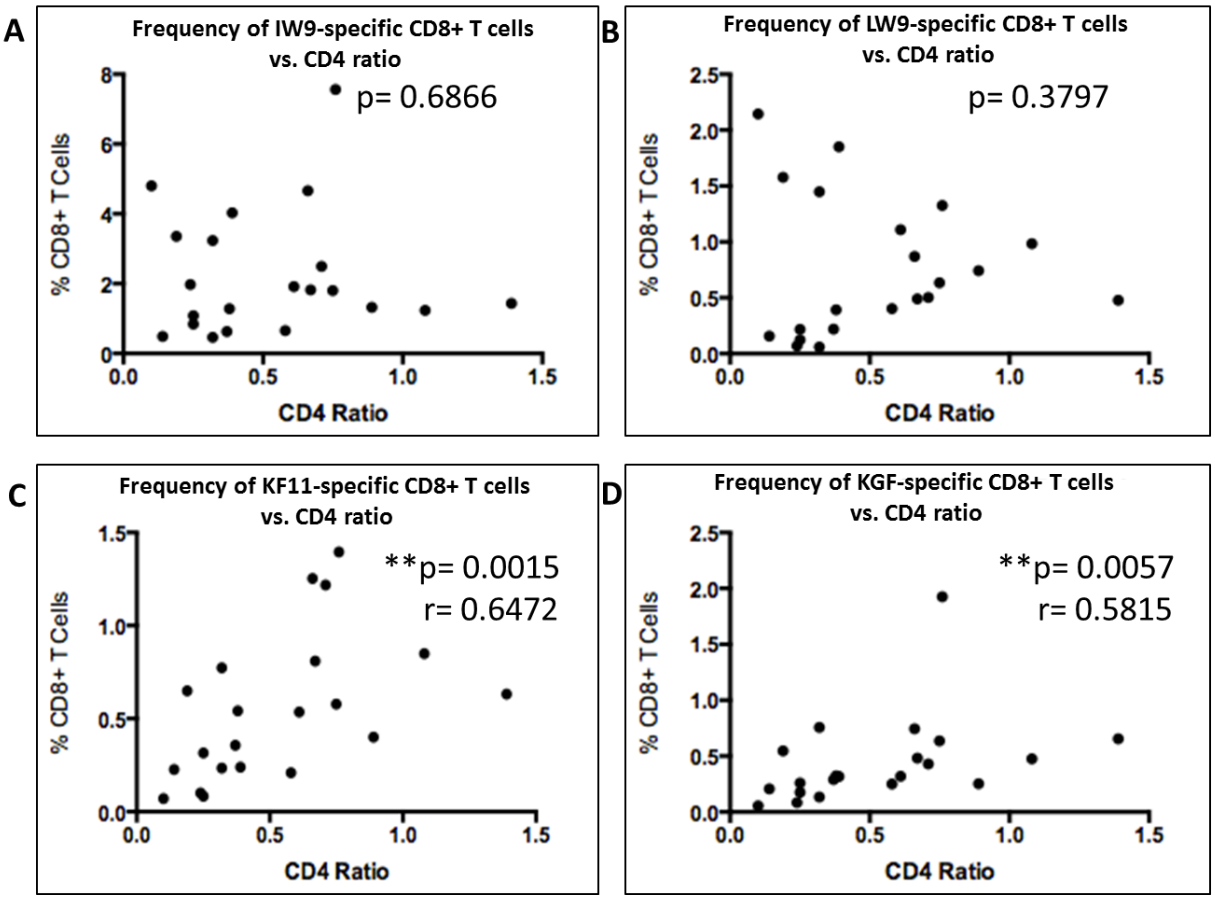


Figure 4.5: Correlation of the frequency of CD8+ T cells recognizing each variant with CD4 ratio. Panels (A) and (B) show the correlation between CD4 ratio and the percentage of CD8+ T cells recognizing IW9 and LW9 respectively; panels (C) and (D) show the correlation with CD8+ T cells recognizing KF11 and KGF respectively.

Next, the frequency of CD8+ T cell responses to each epitope was compared to the nadir CD4 count, which is the lowest recorded CD4 count a patient has encountered. A low nadir CD4 count has been associated with a higher likelihood of disease progression and is thus an important indictor of immune control in HIV infection[201]. In this analysis, a significant positive correlation was found between the frequency of CD8+ T cells recognizing both KF11 and KGF with the nadir count (Figure 4.6, p= 0.0056 and p= 0.0185 respectively, Spearman's rank correlation). No significant correlation was observed with the level of CD8+ T cell recognition of either IW9 or LW9 and the nadir count (Figure 4.6, Spearman's rank correlation). Again, this data suggests that more frequent CD8+ T cells recognition of the KF11 and KGF epitopes is beneficial in HIV, as evidenced by the relation with a higher nadir count, which, as previously stated, is an indicator of immune health in HIV infection.

In summary, more frequent recognition of KF11 and KGF epitopes by CD8+ T cells was associated with a higher CD4 count, higher CD4 ratio, and higher nadir count. This relationship was not observed with the frequency of CD8+ T cells recognizing IW9 or LW9.

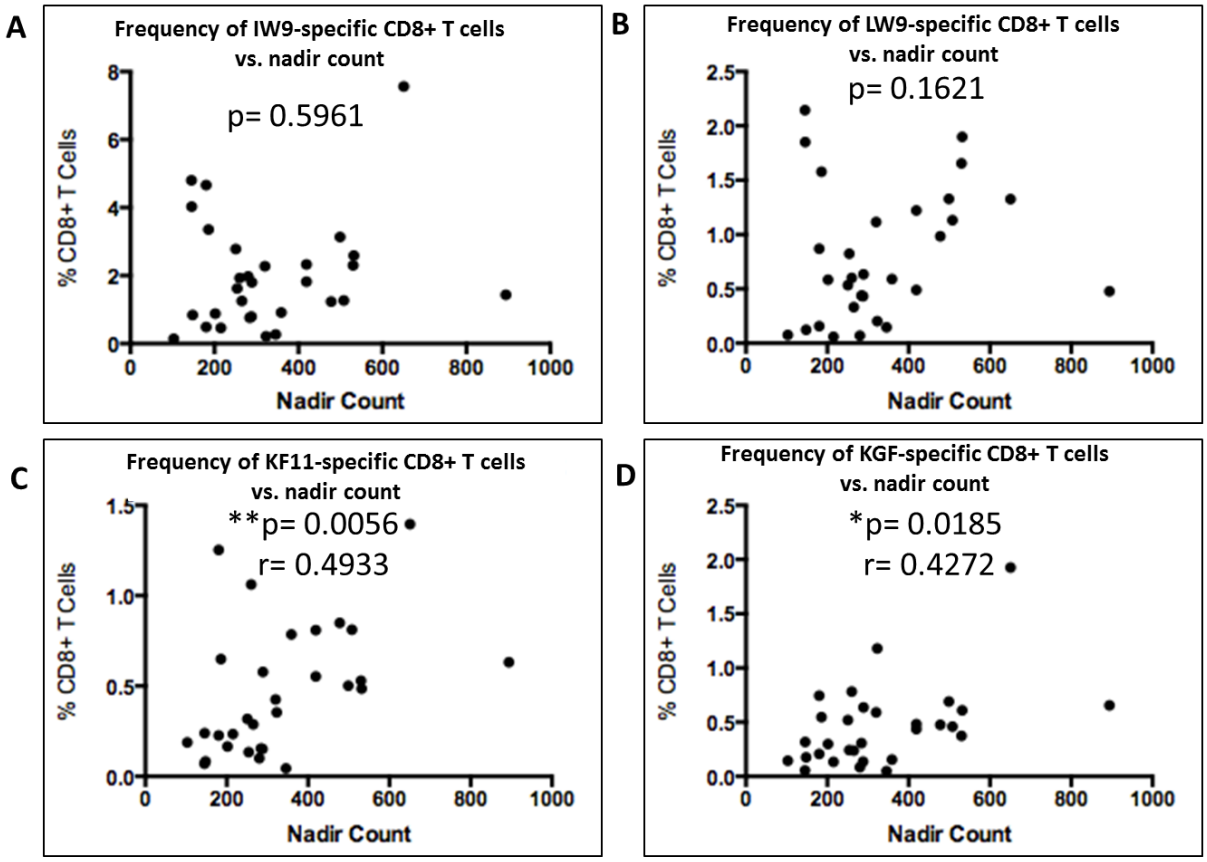


Figure 4.6: Correlation between the frequency of CD8+ T cells recognizing each variant and the nadir CD4 count. Panels (A) and (B) show the correlation between nadir count and the percentage of CD8+ T cells recognizing IW9 and LW9 respectively; panels (C) and (D) show the correlation with CD8+ T cells recognizing KF11 and KGF respectively.

4.5.5 Cytokine production by bulk CD8+ T cells after epitope stimulation

An enhanced ability to produce cytokines suggests a better overall functional capacity of the CD8+ T cells responding to infection[202]. In order to determine if there were differences in the ability of each epitope to stimulate cytokine production in bulk CD8+ T cells, PBMCs were stimulated for 6 hours with each epitope and the resulting cytokines were measured through ICS. To better understand the general flavour of the cytokine response to stimulation in bulk CD8+ T cells, all results were combined regardless of the stimulating epitope. There was no difference in the number of CD8+ T cells producing TNF, MIP1β and IFNγ (Figure 4.7, Wilcoxon rank), however the production of IL-2 was significantly lower than each of the other cytokines (Figure 4.7, $p< 0.0001$ for all, Wilcoxon rank). This suggests that, regardless of the stimulating epitope, fewer CD8+ T cells are likely to produce IL-2, though the number of CD8+ T cells producing each of the other cytokines is similar. This is the same cytokine production profile that was observed in bulk CD8+ T cells after stimulation with IF9 and TL9 epitopes in Chapter 3.

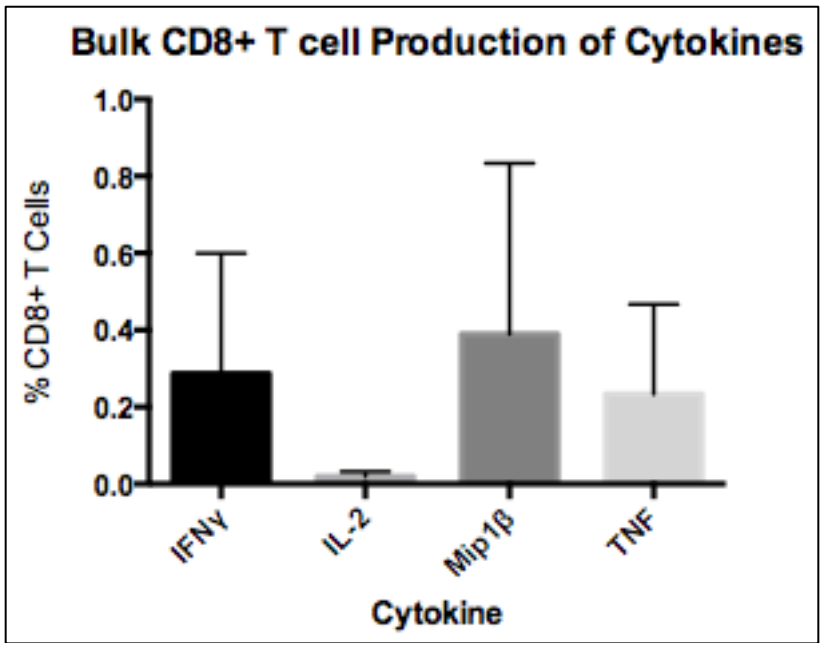


Figure 4.7: Post-stimulation production of cytokines by bulk CD8+ T cells, regardless of stimulating epitope. Cytokine production was observed among all CD8+ T cells stimulated by any of the four variants.

Each epitope was then examined individually to assess if one was able to stimulate the production of cytokines in more bulk CD8+ T cells than the others. Overall there were no significant differences between any of the variants with respect to production of IFNγ, TNF, MIP1β or IL-2 in bulk CD8+ T cells (Figures 4.8 & 4.9, Mann-Whitney). Similarly, there were no significant differences in the MFI of any of the cytokine-producing bulk CD8+ T cells after stimulation with any of the epitopes (Figures 4.10 & 4.11, Mann-Whitney), meaning the CD8+ T cells specific to each epitope bound to them with a similar number of TCRs.

In summary, contrary to the hypothesis, the consensus epitopes, IW9 and KF11, were not able to stimulate significantly more cytokine-producing bulk CD8+ T cells.

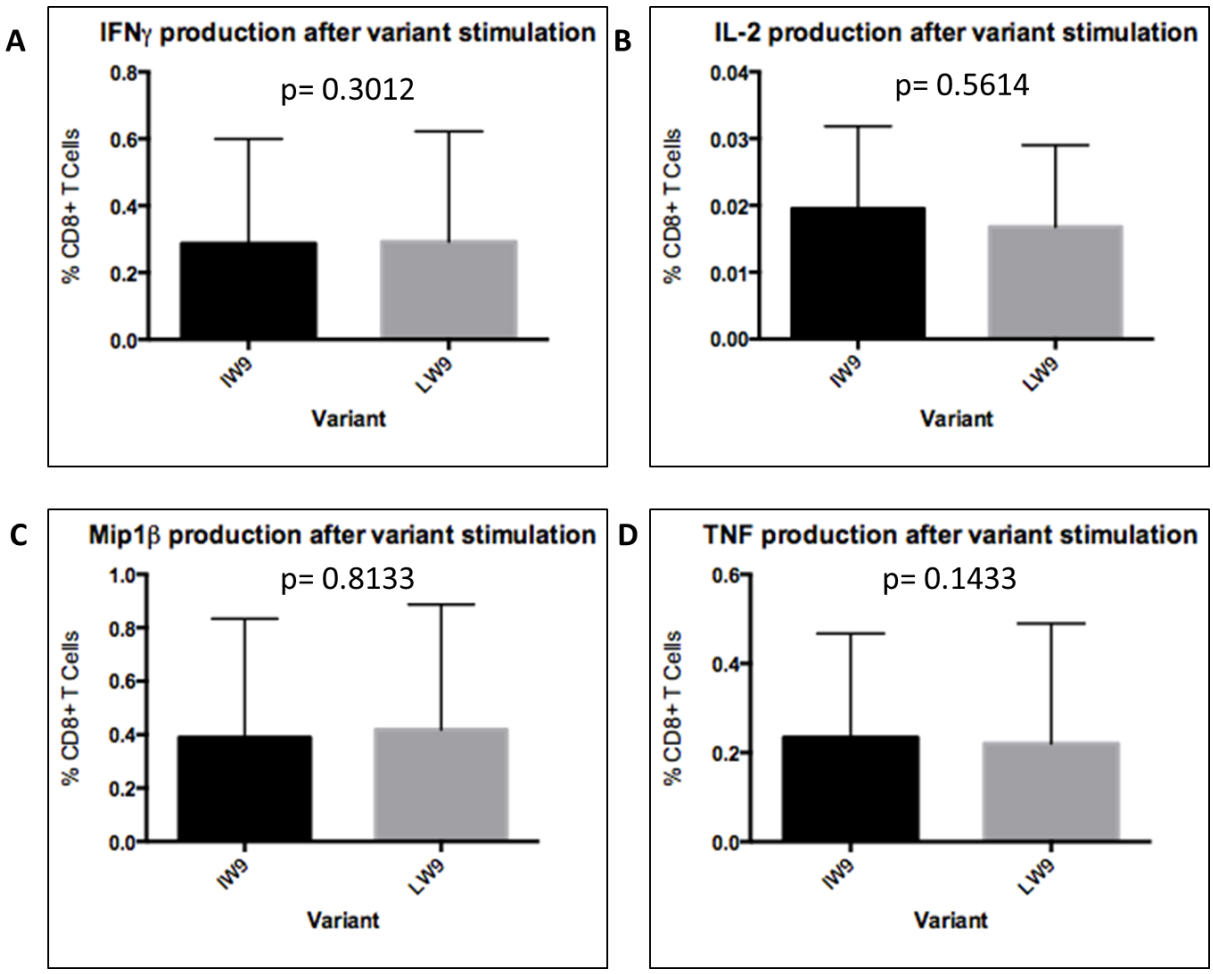


Figure 4.8: Cytokine production by bulk CD8+ T cells after stimulation with IW9 and LW9. After 6-hour stimulations with each epitope, the production of each cytokine by CD8+ T cells was assessed and compared among the stimulating variants. Panel (A) shows the comparison of production of IFNγ by all CD8+ T cells after stimulation with IW9 and LW9. Panels (B), (C), and (D) show the same, for IL-2, Mip1β and TNF production respectively.

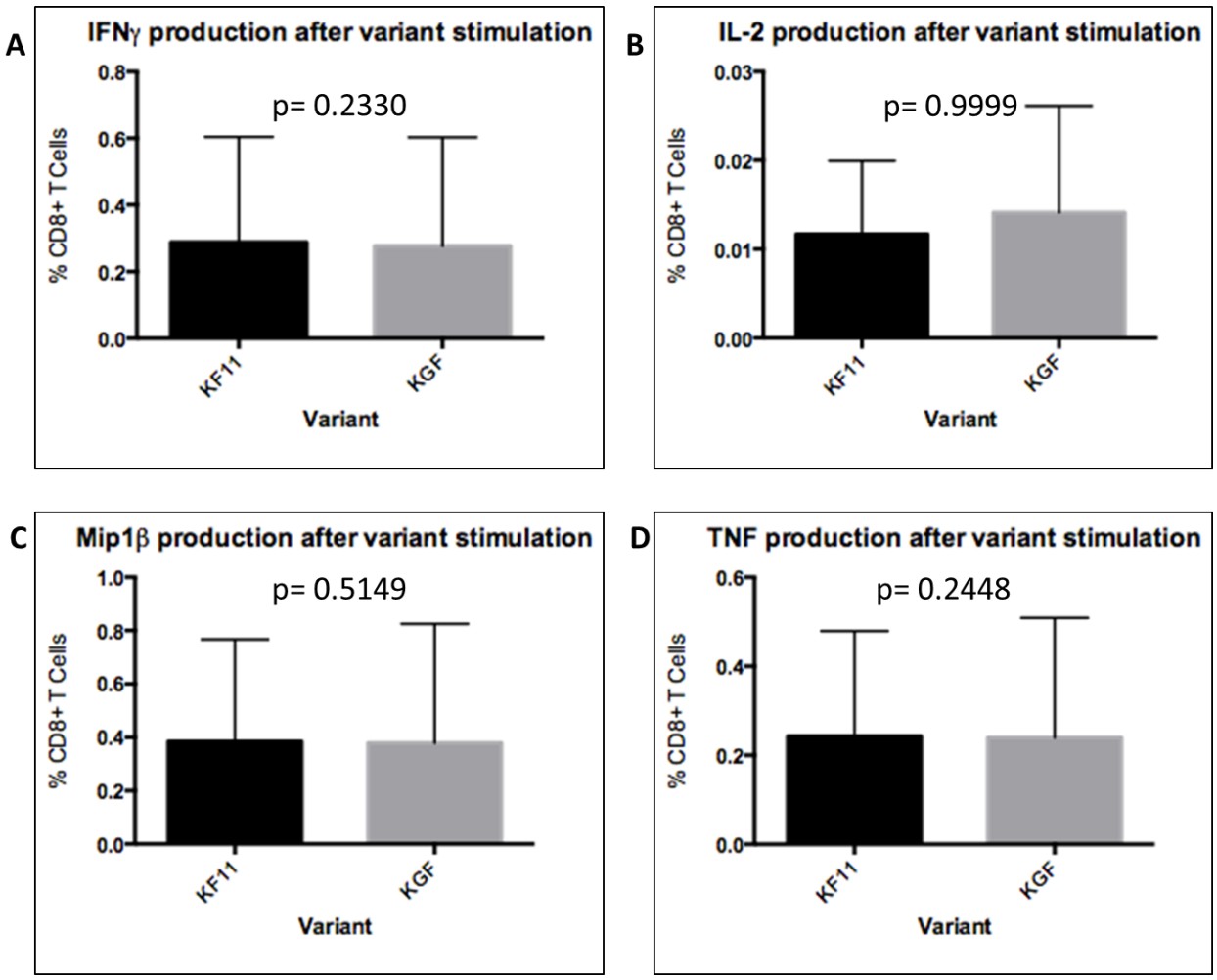


Figure 4.9: Cytokine production by bulk CD8+ T cells after stimulation with KF11 and KGF. After 6-hour stimulations with each epitope, the production of each cytokine by CD8+ T cells was assessed and compared among the stimulating variants. Panel (A) shows the comparison of production of IFNγ by all CD8+ T cells after stimulation with KF11 and KGF. Panels (B), (C), and (D) show the same, for IL-2, Mip1β and TNF production respectively.

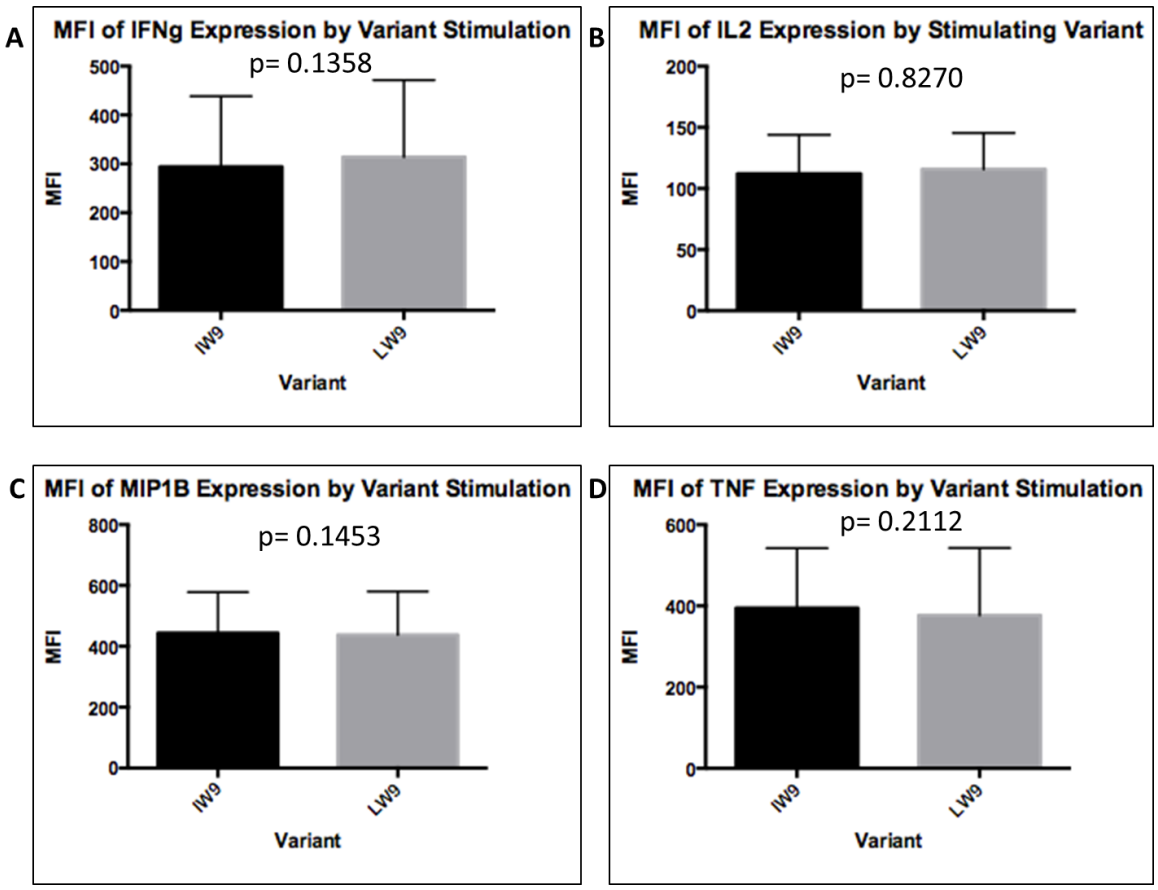


Figure 4.10: MFI of cytokine responses by bulk CD8+ T cells after stimulation with IW9 and LW9 epitopes. Panel (A) shows the comparison of MFI of IFNγ producing CD8+ T cells after stimulation with IW9 and LW9. Panels (B), (C), and (D) show the same, for MFI of IL-2, Mip1β and TNF production respectively.

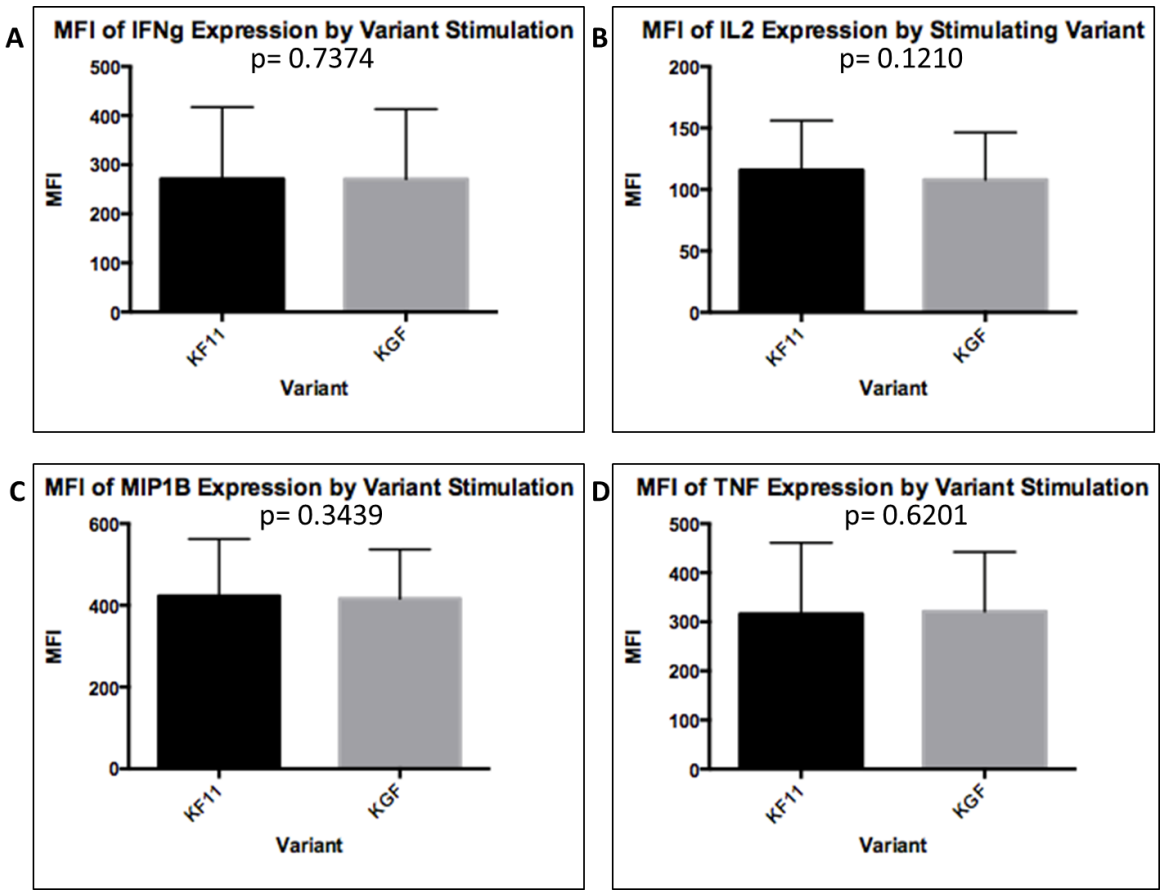


Figure 4.11: MFI of cytokine responses by bulk CD8+ T cells after stimulation with KF11 and KGF epitopes. Panel (A) shows the comparison of MFI of IFNγ producing CD8+ T cells after stimulation with KF11 and KGF. Panels (B), (C), and (D) show the same, for MFI of IL-2, Mip1β and TNF production respectively.

4.5.6 Post-stimulation cytokine production by epitope-specific CD8+ T cells

After assessing that there were no differences in the frequencies of cytokine-producing bulk CD8+ T cells post-stimulation, cytokine production by epitope-specific CD8+ T cells were evaluated to determine if those epitope-specific cells in particular were more or less likely to produce cytokines after epitope stimulation. To do this, tetramer+ cells specific to each epitope were first identified, and then the number of CD8+ T cells producing cytokines within this group were observed. When observing all epitope-specific CD8+ T cells, regardless of what epitope they were specific to, it was found that these epitope-specific CD8+ T cells were significantly more likely to produce TNF, then MIP1β, followed by IFNγ, and then finally IL-2 (Figure 4.12, all comparisons $p < 0.0001$, except between TNF and MIP1β, where $p = 0.0068$, Wilcoxon rank). This is in contrast to cytokine production by bulk CD8+ T cells (Figure 4.7), where MIP1β, TNF and IFNγ were all produced at similar levels. This suggests that, though no differences were observed in the frequencies of cytokines produced when considering production by bulk CD8+ T cells (except for lower levels of IL-2), epitope-specific CD8+ T cells are significantly more likely to produce TNF than anything else.

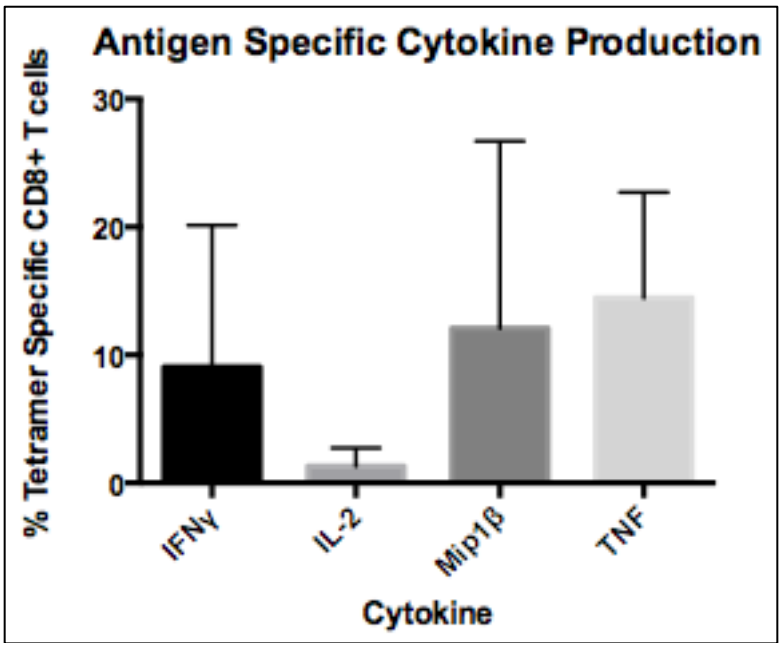


Figure 4.12: Cytokine production by epitope-specific CD8+ T cells. The percentage of epitope-specific CD8+ T cells producing each cytokine was compared.

After assessing the differences in cytokine producing capacities of the epitope-specific CD8+ T cell population as a whole, these responses were evaluated in more detail by looking at the CD8+ T cell pool specific to each epitope individually. It was observed that CD8+ T cells specific to the LW9 variant (as identified by their LW9 tetramer binding capacity) were more likely to produce IFNγ and MIP1β than cells specific to IW9 (Figure 4.13, $p< 0.0001$ for both, Mann-Whitney), and trended toward a significant increase in production of IL-2 (Figure 4.13, $p= 0.0585$, Mann-Whitney). This suggests that though the LW9 epitope is not the consensus, the CD8+ T cells specific to it are actually better able to produce cytokines than those specific to the consensus IW9 epitope. It was also found that CD8+ T cells specific to the KF11 variant were more likely to produce TNF than cells specific to the KGF epitope (Figure 4.14, $p= 0.0270$, Mann-Whitney). CD8+ T cells specific to the KF11 epitope also trended toward significantly higher production of MIP1β (Figure 4.14, $p= 0.0636$, Mann-Whitney), but there was no significant difference in the number of CD8+ T cells producing IL-2 or IFNγ. Therefore, CD8+ T cells responding to the KF11 epitope, which is the consensus, are more likely to produce cytokines than the KGF variant.

In summary, contrary to the hypothesis, stimulation with the variant LW9 epitope was able to stimulate significantly more epitope-specific cytokine producing CD8+ T cells than the consensus IW9 epitope. Conversely, the consensus KF11 epitope stimulated epitope-specific CD8+ T cells significantly more likely to produce TNF than CD8+ T cells specific to the KGF variant.

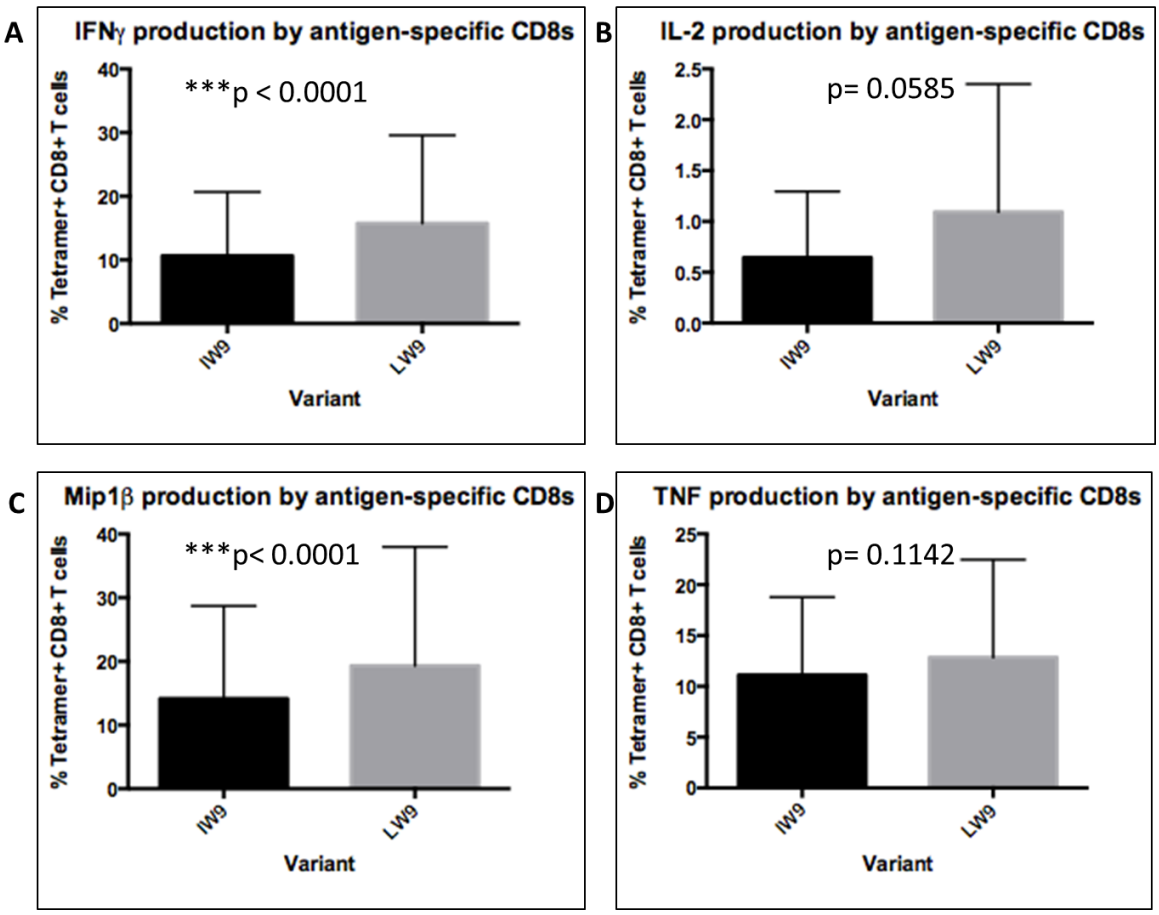


Figure 4.13: Comparison of production of cytokines by epitope-specific CD8+ T cells. Shown above is a comparison of the ability of IW9 and LW9 to stimulate cytokine production. Panel (A) shows IFNγ producing CD8+ T cells after stimulation with IW9 and LW9. Panels (B), (C), and (D) show the same, for epitope-specific production of IL-2, Mip1β and TNF production respectively.

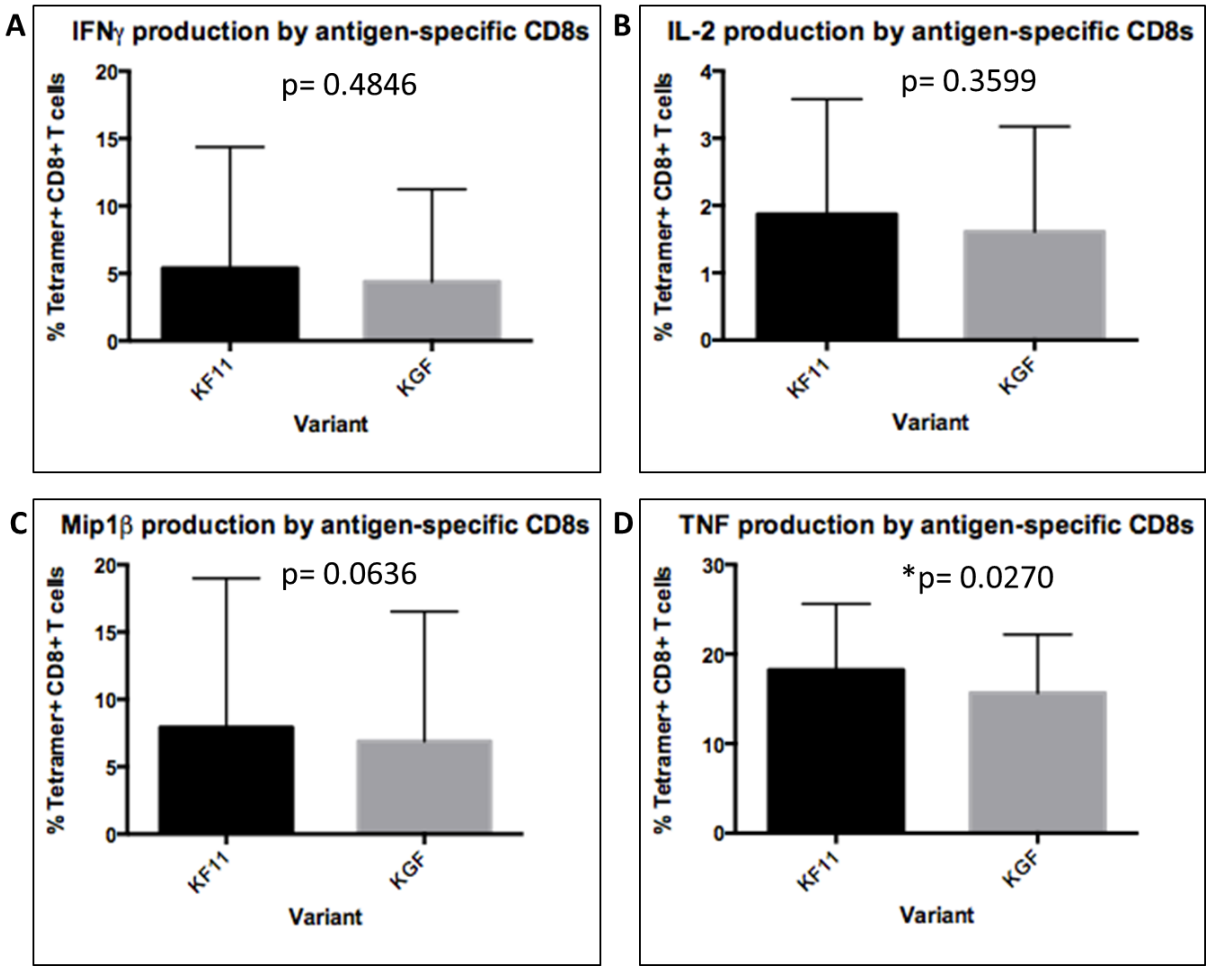


Figure 4.14: Comparison of production of cytokines by epitope-specific CD8+ T cells. Shown above is a comparison of the ability of KF11 and KGF to stimulate cytokine production. Panel (A) shows IFNγ producing CD8+ T cells after stimulation with KF11 and KGF. Panels (B), (C), and (D) show the same, for epitope-specific production of IL-2, Mip1β and TNF production respectively.

4.5.7 Polyfunctionality of cytokine responses after epitope stimulation

It has been shown repeatedly in HIV research that the ability of CD8+ T cells to produce more than one cytokine on a per-cell basis is linked to better disease outcomes[155], [202]. For this reason, polyfunctionality was assessed in study subjects in response to each of the HLA-B*57:03 epitopes. It was observed that stimulation with the IW9 epitope lead to a stronger 2+ CD8+ T cell polyfunctional response than stimulation with the LW9 epitope (Figure 4.15, p= 0.0016, Mann-Whitney), but that there was no difference between the ability of these variants to stimulate a 3+ polyfunctional response. This suggests that the consensus IW9 epitope can stimulate a more frequent polyfunctional response in the responding CD8+ T cells than the variant LW9. Similarly, stimulation with the KF11 epitope resulted in a significantly higher 3+ polyfunctional response by responding CD8+ T cells than stimulation with the KGF epitope (Figure 4.16, p= 0.0065, Mann-Whitney), though no difference was observed in the production of 2+ cytokine polyfunctionality. So, although in section 4.5.6 it was shown that LW9 is able to stimulate CD8+ T cells that are more likely to produce single cytokines, in fact IW9 is better able to stimulate a polyfunctional response. Conversely, the KF11 epitope was better able to stimulate CD8+ T cells that produce both of single cytokines (in section 4.5.6) as well as a polyfunctional response.

In summary, the consensus IW9 and KF11 epitopes were able to stimulate significantly more polyfunctional CD8+ T cells than their variant epitopes.

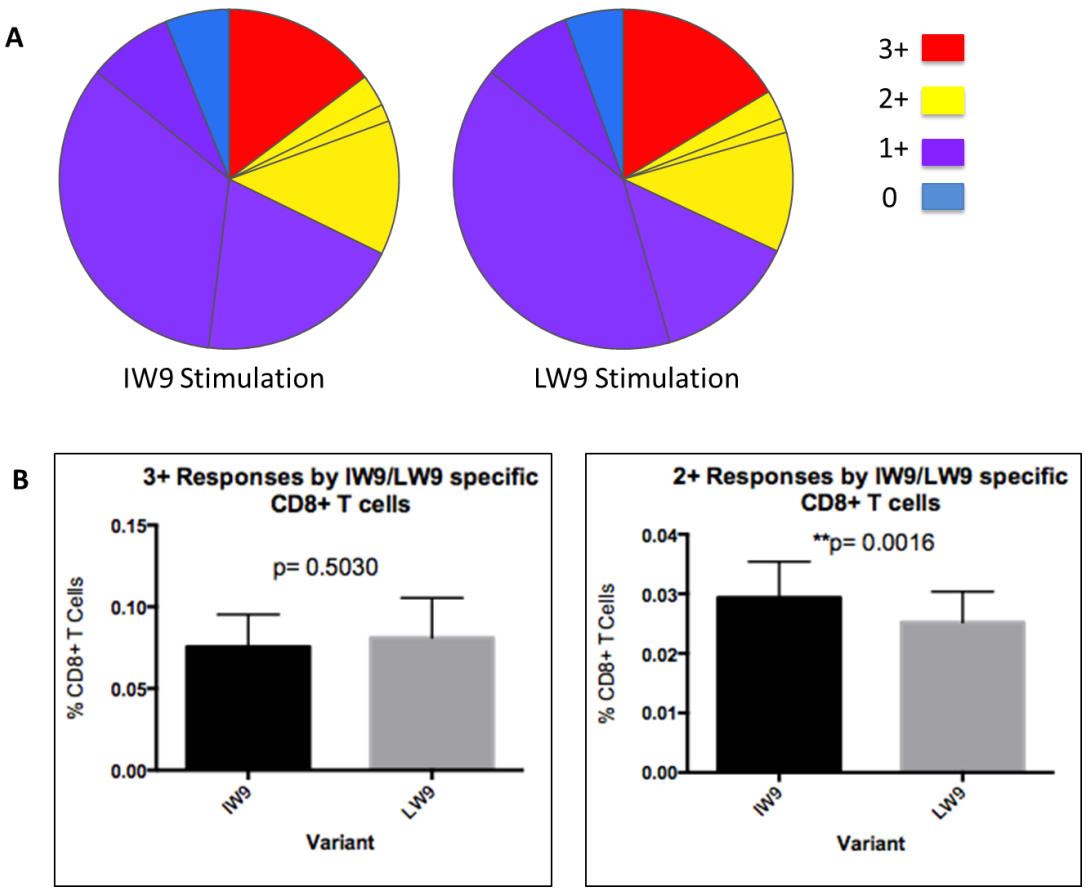


Figure 4.15: Polyfunctionality of CD8+ T cell responses to stimulation with IW9 and LW9 epitopes. Panel (A) shows the differences after stimulation with IW9 and LW9 in polyfunctional responses. Panel (B) displays the differences in 3+ and 2+ polyfunctionality after stimulation with IW9 and LW9.

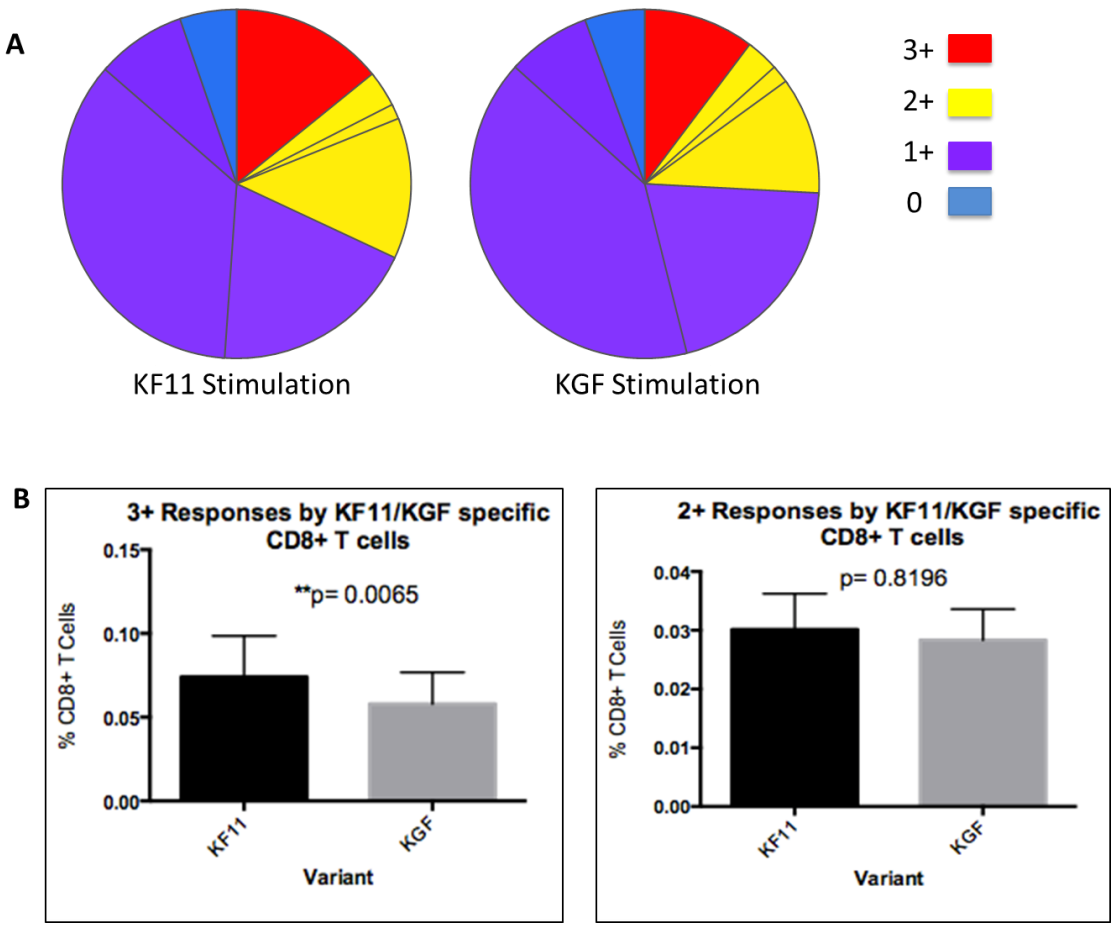


Figure 4.16: Polyfunctionality of CD8+ T cell responses to stimulation with KF11 and KGF variants. Panel (A) shows the differences after stimulation with KF11 and KGF in polyfunctional responses. Panel (B) displays the differences in 3+ and 2+ polyfunctionality after stimulation with KF11 and KGF.

4.5.8 Proliferation of CD8+T cells after epitope stimulation

A high proliferative capacity has been recognized as an important factor of CD8+ T cell control of HIV, as cellular turnover is necessary for a sustained, effective T cell response[202]. After 6 days of stimulation with the HLA-B*57:03 epitopes, only 2 of the 31 individuals assayed displayed any proliferative potential that was higher than background levels. These positive responses were to the IW9 epitope in one case, and the KF11 epitope in the other (Figure 4.17).

In summary, proliferative responses were observed in only two individuals, in response to the consensus IW9 and KF11 epitopes.

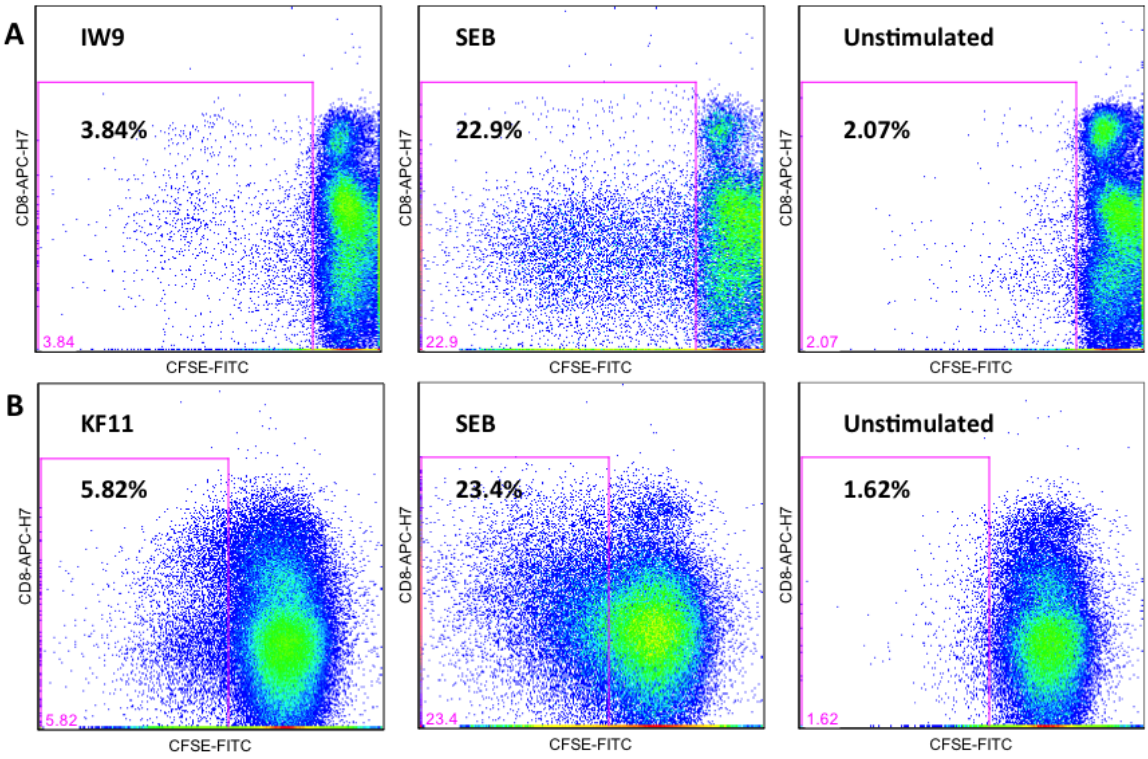


Figure 4.17: Representative proliferation staining. In (A), the proliferative response to IW9, then SEB, and unstimulated cells respectively is shown. In (B), the proliferative response to KF11, then SEB, and unstimulated cells respectively is shown.

4.5.9 Longitudinal assessment of cytokines and polyfunctional CD8+ T cell responses

To better understand how cytokine production and polyfunctionality of CD8+ T cells may differ over time, these factors were observed in a subset of individuals over multiple time points. Two study subjects (ML1778 and ML1514) who fit the criteria for long-term non-progression (HIV-infected and ART- for >7 years with CD4 counts >500) were selected to represent the data, as they characterized two distinctly difference situations for this longitudinal assessment. For ML 1778, cytokine production after stimulation with the HIV epitopes increased over time, being highest in the most recent sample, while for ML 1514, it slightly decreased (Figure 4.18). In both individuals, the number of IL-2 producing CD8+ T cells was very low, in agreement with earlier findings in this chapter (Figures 4.7 & 4.12). The remaining cytokines were each produced by a similar level of CD8+ T cells in each individual, with cytokine-producing CD8+ T cells increasing slightly in ML 1778 over time, and decreasing slightly in ML 1514 over time. Similarly, the polyfunctionality of CD8+ T cells increased over time in ML 1778 (Figure 4.19), with significant increases in 2+, 3+ and 4+ polyfunctionality. Conversely, in ML 1514, polyfunctionality of responding CD8+ T cells decreased over time, with fewer CD8+ T cells displaying 2+ and 3+ polyfunctional responses, and with 4+ polyfunctional responses being almost entirely absent (Figure 4.20). The decrease in polyfunctionality and decrease overall in cytokine production observed in ML 1514 over time may seem indicative of a loss of viral control, but despite this, both individuals maintained CD4 counts >500 for all sampling points and remained ART naïve. This highlights that heterogeneity in CD8+ T cell responses can exist even within groups of individuals that have similar clinical characteristics. Though

polyfunctionality has been associated with a better clinical outcome, it is clear that this characteristic does not exist within all LTNP individuals, and also, that this ability can be lost despite the individuals maintaining high CD4 counts.

In summary, contrary to the hypothesis, although both ML 1778 and ML 1514 maintained high CD4 counts over time, cytokine production and polyfunctionality increased in ML 1778, while decreasing in ML 1514, indicating that not all LTNP individuals continuously display the typical beneficial CD8+T cell responses.

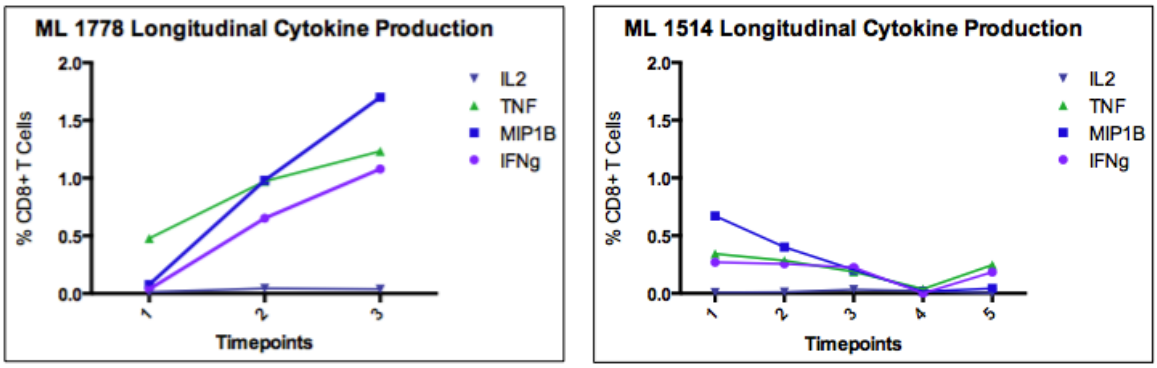
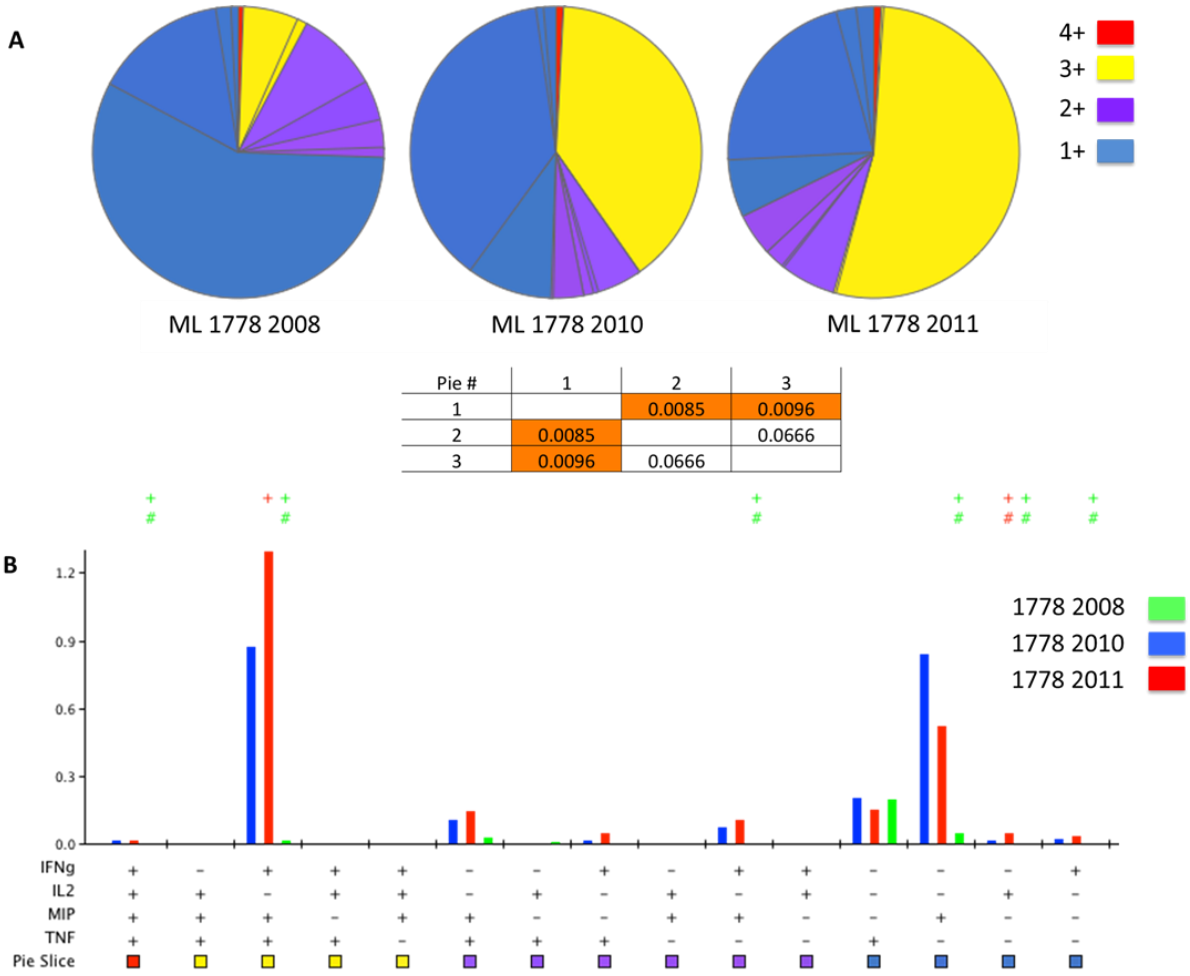


Figure 4.18: Longitudinal assessment of cytokine production by two long-term non-progressors. In the first panel, cytokine production by CD8+ T cells from ML 1778 is shown over three time points. The second panel shows cytokine production by CD8+ T cells from ML 1514 over five time points.



Pie #	1	2	3
1		0.0085	0.0096
2	0.0085		0.0666
3	0.0096	0.0666	

Figure 4.19: Polyfunctionality of ML 1778 over three time points. The polyfunctionality overall of CD8+ T cells in ML 1778 is displayed comparing 2+, 3+ and 4+ responses between the first time point and the last two (A). Panel (B) shows the breakdown of which cytokines are being produced at each time point.

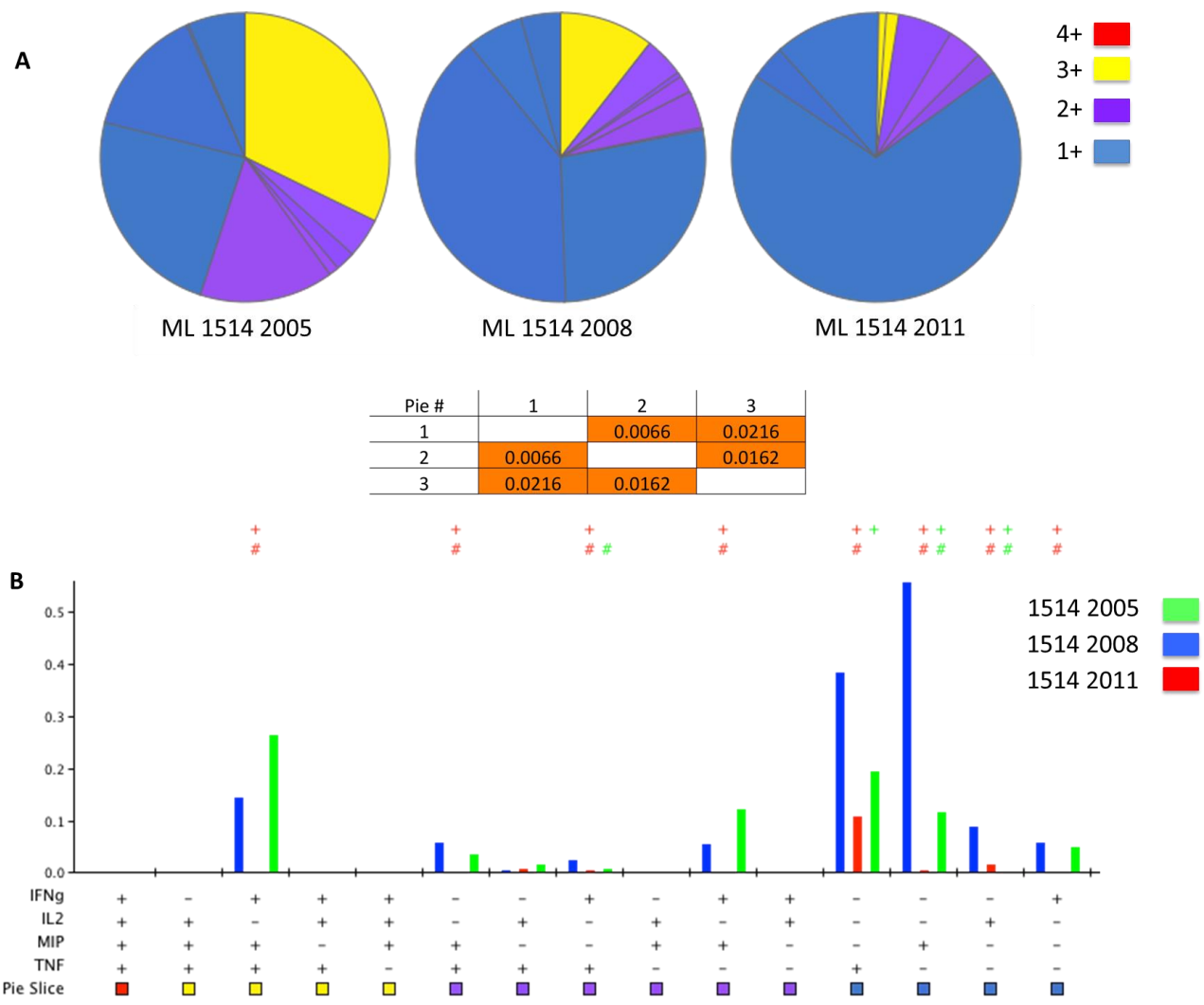


Figure 4.20: Polyfunctionality of ML 1514 over three time points. The polyfunctionality overall of CD8+ T cells in ML 1514 is displayed comparing 2+, 3+ and 4+ responses between the first time point and the last two (A). Panel (B) shows the breakdown of which cytokines are being produced at each time point.

4.6 Conclusions

In this chapter, it was hypothesized that the immunodominant, consensus IW9 and KF11 epitopes, due to being present more frequently in nature, would be more frequently recognized by CD8+ T cells than their LW9 and KGF variants. It was also hypothesized that CD8+ T cell responses to the consensus epitopes would be more proliferative and polyfunctional than responses to their variants.

While the consensus IW9 was recognized at a much higher frequency by CD8+ T cells than its LW9 variant, contrary to the first hypothesis, the CD8+ T cells responding to the LW9 variant were more likely to produce individual cytokines. Also contrary to the first hypothesis, the consensus KF11 and its variant KGF epitope were recognized by a similar number of CD8+ T cells, and there were no differences in the level of individual cytokines stimulated by either of them. Conversely, and supporting the hypothesis, the consensus IW9 and KF11 epitopes were associated with the ability to stimulate slightly more frequent polyfunctional responses than their variant epitopes. Proliferative responses were nearly absent after stimulation with any of the epitopes, which was in opposition to the hypothesis. Taken together, this conflicting data suggests that the immunodominance or consensus status of an epitope is unlikely to reliably dictate how frequently it is recognized by CD8+ T cells, or the type of resulting CD8+ T cell response.

It was also hypothesized that CD8+ T cell responses to the consensus epitopes would be associated with better disease outcomes than responses to their variants, as determined by CD4 count, and that beneficial CD8+ T cell responses would be maintained over time in individuals with stable CD4 counts.

The second hypothesis was also not confirmed. CD8+ T cell responses to the consensus IW9 epitope were not associated with higher CD4 counts than those to the variant LW9 epitope. Furthermore, though CD8+ T cell responses to the consensus KF11 epitope were associated with higher CD4 counts, this was also seen in response to the variant KGF epitope. It was also found that despite the fact that some individuals could maintain high CD4 counts, not all of them were able to maintain high cytokine production or polyfunctionality in response to HIV epitopes. This data suggests that the consensus status of an epitope is unlikely to determine whether or not CD8+ T cell responses to it will be related to better disease progression, and also that individuals do not necessarily always display beneficial CD8+ T cell responses despite maintaining viral control.

In the next chapter, CD8+ T cell function will be addressed in the context of disease progression patient groups, and their differential ability to inhibit p24 production in an *in-vitro* infection assay.

Chapter 5: CD8+ T cell Mediated Inhibition of HIV Production in Long-Term Non-Progressors and Normal/Rapid Progressors

5.1 Rationale:

It is known that in HIV infection, progression to a clinical AIDS diagnosis is very heterogeneous, whereby some individuals progress within a normal time frame (3-5 years), some progress more slowly (long-term non-progressors, 7+ years), and others progress more quickly (rapid progressors, <3 years). There are many factors that influence this varying progression, and CD8+ T cells are thought to play a major role in this [155], [202], [203]. Individuals able to maintain highly proliferative, polyfunctional, non-exhausted CD8+ T cell responses are associated with better disease outcomes[124]. However, these measures are merely correlative, and it is difficult to assess if they are truly impacting viral production in HIV-infected individuals. Using an *in-vitro* viral inhibition assay affords the opportunity to observe the direct anti-viral capacity of CD8+ T cells, without having to rely on correlative evidence to determine their anti-HIV activity.

5.2 Hypothesis:

CD8+ T cells from long-term non-progressors will be able to suppress viral replication more strongly than CD8+ T cells from rapid or normal progressors.

5.3 Objective:

Conduct *in-vitro* viral inhibition assays on PBMCs from long-term non-progressors and normal/rapid progressors to determine if the CD8+ T cells from one group are better able to suppress viral replication.

5.4 Methods:

Viral stock production: ML1956 and IIIB virus stocks were grown from previous lab stocks as outlined in section 2.17, and titred for level of infectivity using a TCID50 assay, as described in section 2.18.

PBMC expansion: In order to generate enough cells from cryopreserved PBMC stocks to carry out the infection inhibition assays, target (CD4+) and effector (CD8+) populations were expanded, using bispecific CD3/4 and CD3/8 antibodies and exogenous IL-2, as outlined in section 2.20.

Inhibition assay: The general outline of this experiment can be seen in Figure 5.2. Infections were carried out at MOIs of 0.01 and 0.05, as per previous optimization experiments. Wells containing infected CD4+ T cells only were compared to wells containing both infected CD4+ T cells and CD8+ T cells in order to measure p24 inhibition by CD8+ T cells. The details of this methodology are outlined in section 2.21.

Statistical analysis: A p value <0.05 was used in all tests as a cut off for statistical significance. All correlations were analyzed using Spearman's rank correlations. Comparisons between patient groups were analyzed for significance using Mann-Whitney tests.

5.5 Results

5.5.1 Study population

PBMCs for this study were acquired from 25 long-term non-progressors and 18 normal or rapidly progressing individuals from the Majengo Sex Worker Cohort in Nairobi. All individuals were ART-naïve at the time of sampling. Long-term non-progressors all maintained CD4 counts >500 for >7 years. Individuals in the normal/rapid progressor group had variable CD4 counts.

5.5.2 Cell growth for infection assays

In order to generate enough cells to conduct the infection inhibition assays, bispecific CD3/4 and CD3/8 antibodies were added to PBMC cultures to expand CD8+ and CD4+ T cells respectively, as described in section 2.20. After expansion, each sample was stained and run on an LSR-II to ensure proper cell expansion had occurred. A representative gating strategy and representative cell expansion can be seen in Figure 5.1. For all samples, stimulation with IL-2 and the CD3/4 antibody resulted in a population of cells comprised of >75% CD8+ T cells and <10% CD4+ T cells; stimulation with IL-2 and the CD3/8 antibody resulted in a population of cells consisting of >85% CD4+ T cells and <2% CD8+ T cells.

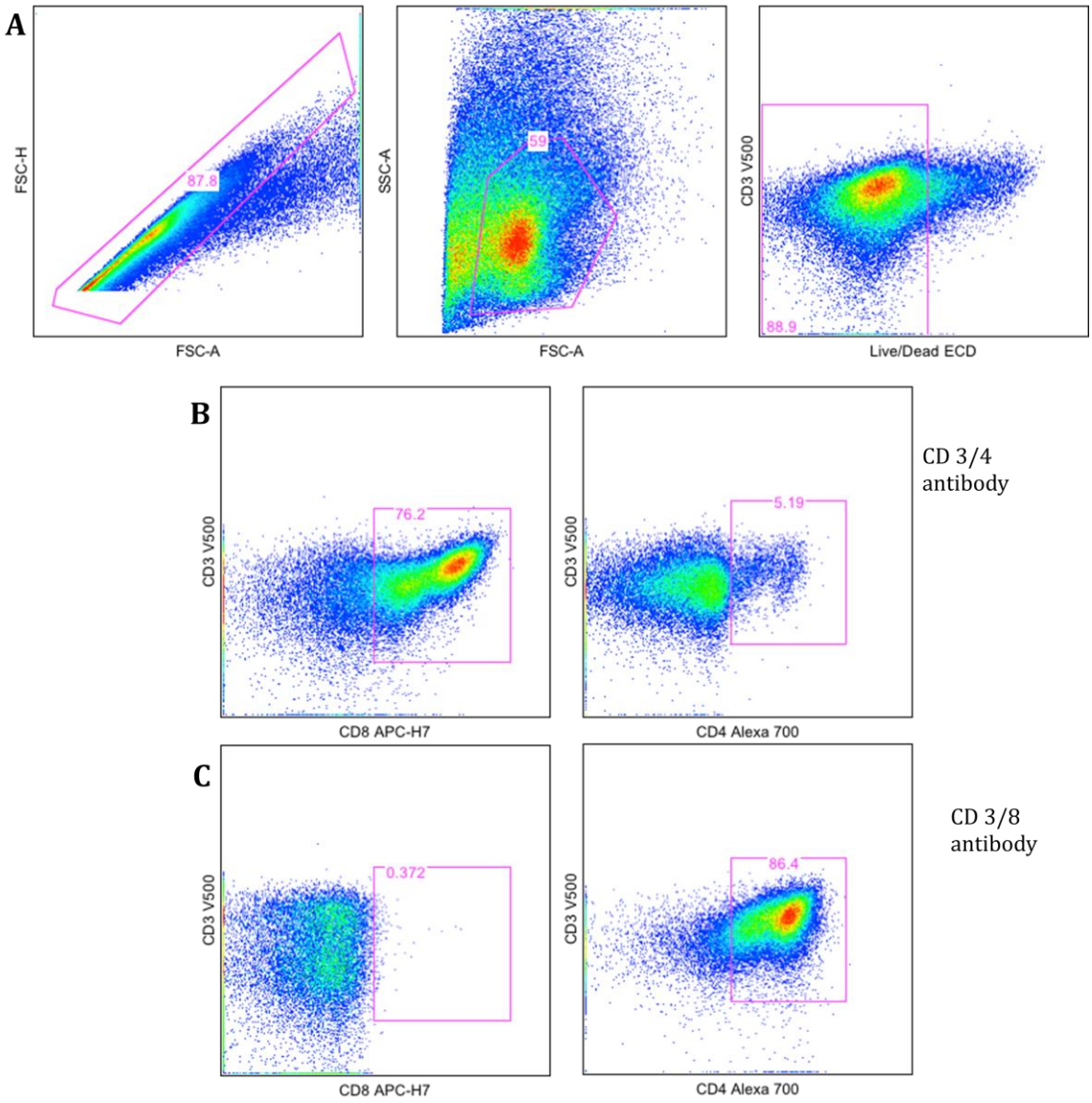


Figure 5.1: Representative gating strategy and expansion of CD4+ and CD8+ T cells for infection assays. (A) The gating strategy used for the staining in this study, first gating out doublets, then gating on lymphocytes, and finally gating out dead cells. Expansion of CD8+ T cells with the CD3/4 antibody is shown in (B). Similarly, expansion of CD4+ T cells with the CD3/8 antibody is shown in (C).

5.5.3 Measurement of secreted p24 in LTNP and RP/NP by p24 ELISA

To understand if CD8+ T cells from long-term non-progressors are better able to suppress *in-vitro* infection than those from rapid/normal progressors, 12-day infection assays were carried out, as displayed in Figure 5.2. Supernatants collected at days 6, 9 and 12 were collected to measure secreted p24.

The overall level of infection in wells containing CD4+ T cells only was compared between LTNPs and RP/NPs to establish if there were any differences in the capability of the cells to be infected, and to serve as a baseline to be sure the inhibition results could be reasonably compared between groups. Examining days 6, 9 and 12 individually, or by combining them, there were no significant differences in the baseline secreted p24 between LTNP and RP/NP individuals(Figure 5.3, Mann-Whitney).

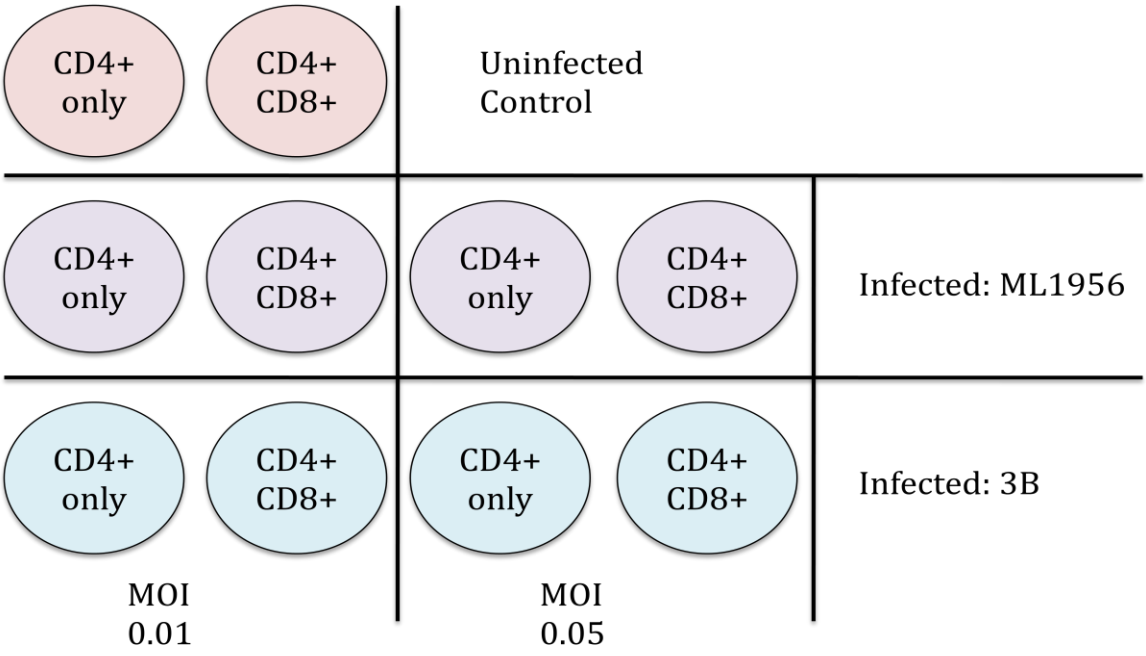


Figure 5.2: Experimental design. For each individual, ten conditions were used.

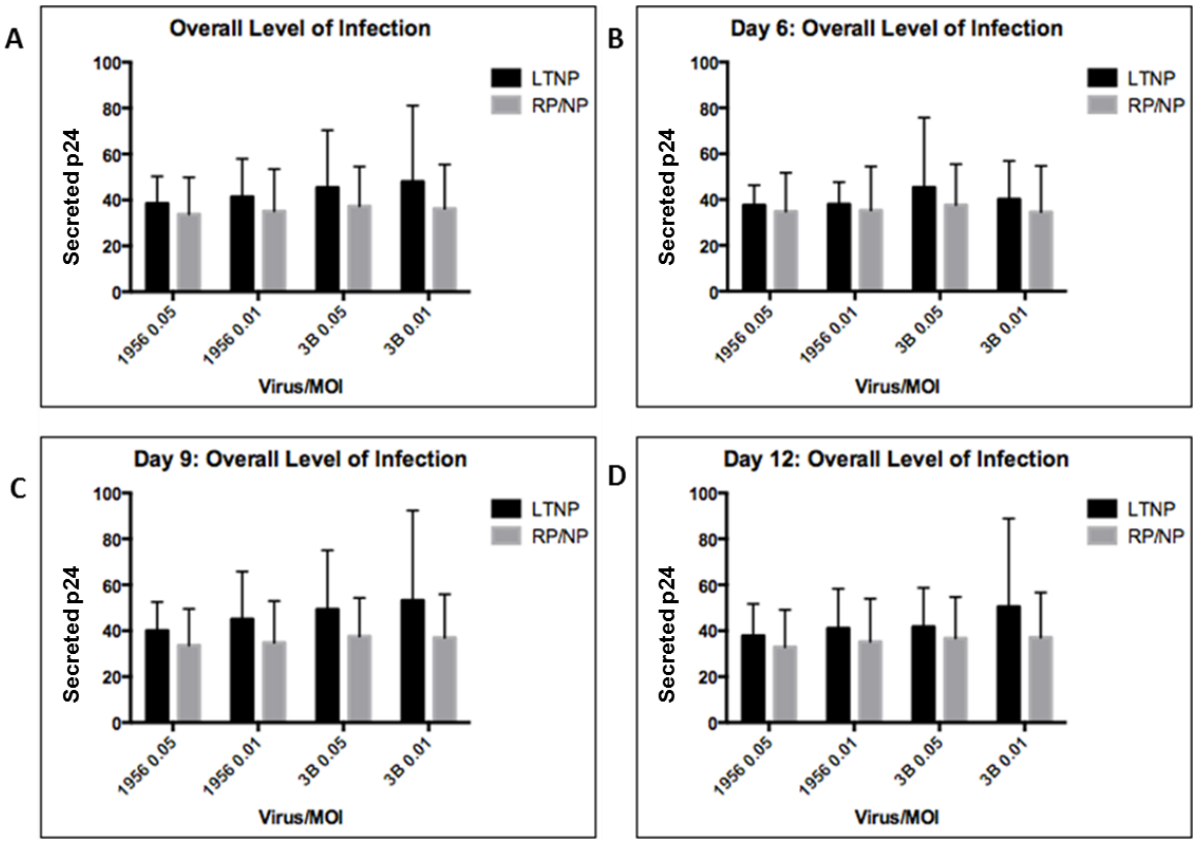


Figure 5.3: Comparison of overall secreted p24 levels in LTNP vs. RP/NP groups. Baseline secreted p24 was compared among target cells only for LTNP and RP/NP groups overall (A), on day 6 (B), day 9 (C) and day 12 (D).

To assess the ability of CD8+ T cells to inhibit viral production, the level of secreted p24 was compared in wells with CD4+ T cells only, and those that had CD8+ T cells added back. It was observed that CD8+ T cells from both the LTNP and RP/NP groups were able to significantly inhibit production of intracellular p24 (Figure 5.4, $p< 0.0001$ for all combinations, Mann-Whitney). Interestingly, some stratification was seen within the RP/NP group with respect to inhibition of HIV (Figure 5.5) that was not seen in the LTNP group. The RP/NP group appears to be divided into two fairly distinct and even groups, composed of those who are unable to inhibit infection, or do so only to a very low level, and those who inhibit infection very well. Investigating this further, the RP/NP individuals who display strong inhibition of p24 had a significantly higher average CD4 count than those who exhibit low p24 inhibition (Table 5.1, Figure 5.6, $p= 0.0012$, Mann-Whitney).

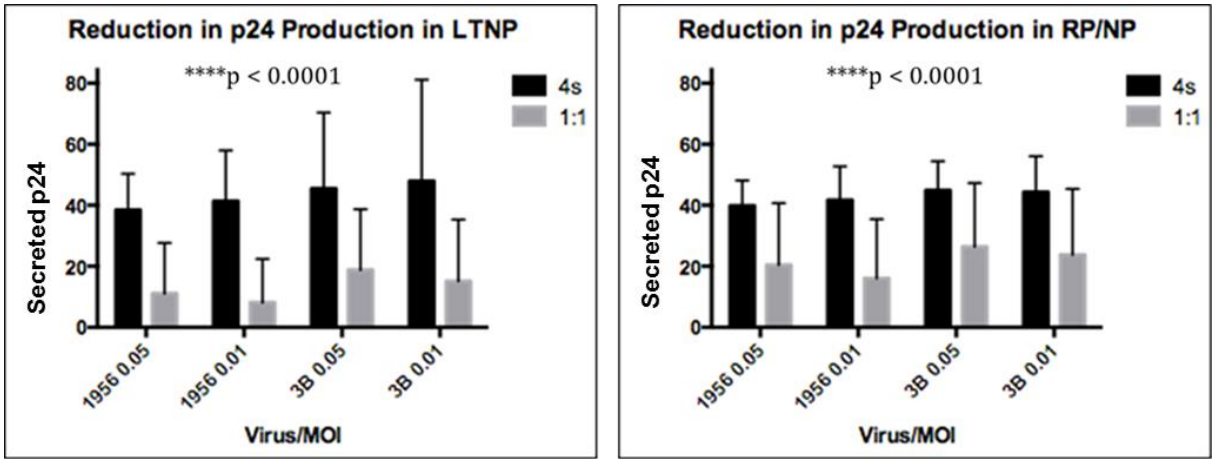


Figure 5.4: Reduction in secreted p24 production in LTNP and RP/NP patient groups. Seen above is a comparison is the level of secreted p24 in wells with CD4+ target cells only and cultures where CD8+ T cells were added back at a 1:1 ratio, among LTNP in the first panel, and RP/NP in the second. Representative data from day 6.

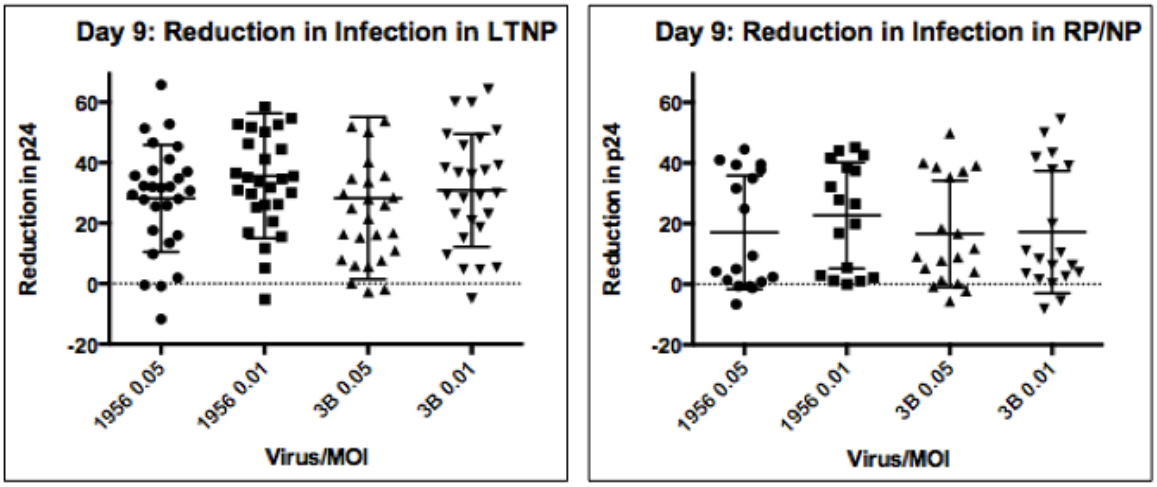


Figure 5.5: Percent reduction in secreted p24 levels in LTNP and RP/NP on Day 9. Displayed above is the overall reduction in secreted p24 after CD8+ effector cells were added to infected target cultures. The left panel is of LTNP, and the right of RP/NP. Representative data from day 9.

High Inhibition		Low Inhibition	
Patient	**CD4**	**Patient**	**CD4**
2364	382.00	2139	430.00
2612	396.00	2449	354.00
2719	525.00	768	319.00
2122	505.00	2095	320.00
2726	443.00	2033	338.00
2732	523.00	2123	360.00
2259	380.00	2141	360.00
2452	466.00	2746	345.00
		58	213.00
		2322	442.00
Average CD4:	**452.50**	**Average CD4:**	**348.10**

Table 5.1: CD4 counts of RP/NP subjects with high p24 inhibitory capacities and low inhibitory capacities.

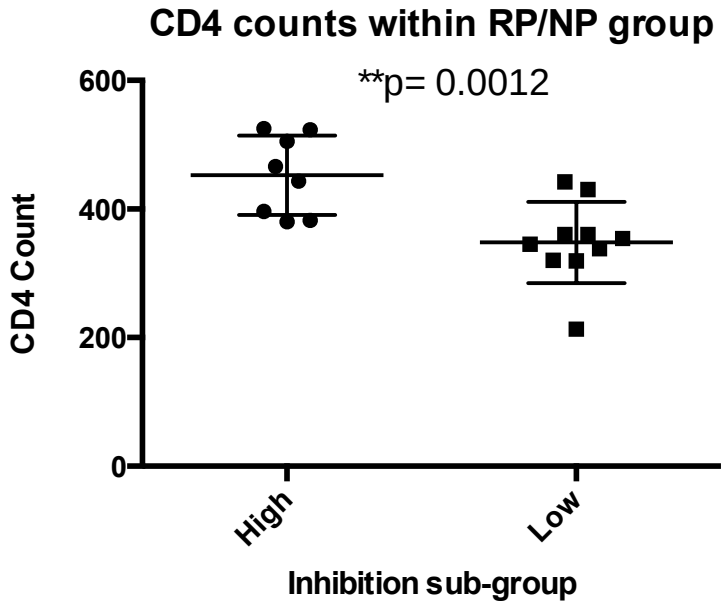


Figure 5.6: CD4+ T cell counts among RP/NP individuals who inhibit p24 to a high degree vs those who inhibit to a low degree.

To better understand the relative capacity of LTNP and RP/NP individuals to inhibit p24 production, the level of inhibition for each virus/MOI pair was compared between patient groups. Though both groups were able to significantly reduce p24 levels (Figure 5.4), the level of p24 inhibition was significantly higher in the LTNP group than in the RP/NP group for each virus/MOI pairing (Figure 5.7, p= 0.0065, p= 0.0189, p= 0.0218, p= 0.0006 for ML1956 0.05 and 0.01, IIIB 0.05 and 0.01, respectively, Mann-Whitney).

In summary, although CD8+ T cells from both LTNP and RP/NP groups were capable of some level of secreted p24 inhibition, CD8+ T cells from the LTNP patient group were able to inhibit p24 to a significantly higher level than CD8+ T cells from the RP/NP group.

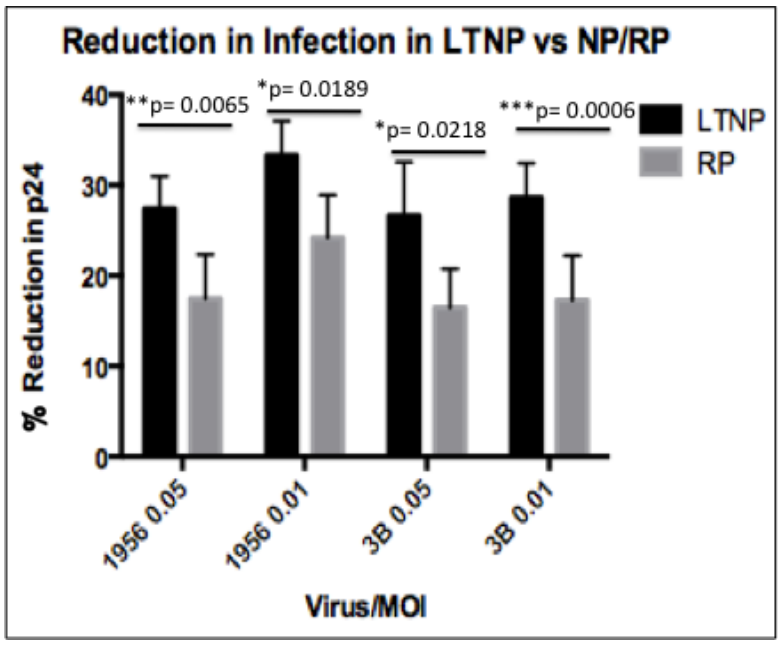


Figure 5.7: Comparison in overall ability of progression groups to inhibit secreted p24. The percentage of reduction in secreted p24 after CD8+ effectors were added to infection cultures was compared among LTNP and RP/NP individuals.

5.5.4 Comparison of inhibition of intracellular p24 in LTNP and RP/NP

In the previous section, inhibition of secreted p24 was measured using p24 ELISAs on infection supernatants collected at days 6, 9 and 12 of the infection assay. In order to corroborate and validate these findings, intracellular p24 was also measured through intracellular p24 staining of the cells on day 12 of the assay. Representative p24 staining can be seen in Figure 5.8.

Again, the overall level of p24 production in CD4+ T cell only wells was evaluated to determine if there was any difference between LTNP and RP/NP individuals in the susceptibility of their amplified CD4+ target cells to be infected (Figure 5.9, Mann-Whitney). Overall, there were no significant differences between LTNP and RP/NP individuals in the level of intracellular p24 in their CD4+ T cells, in either of the MOIs or viruses tested.

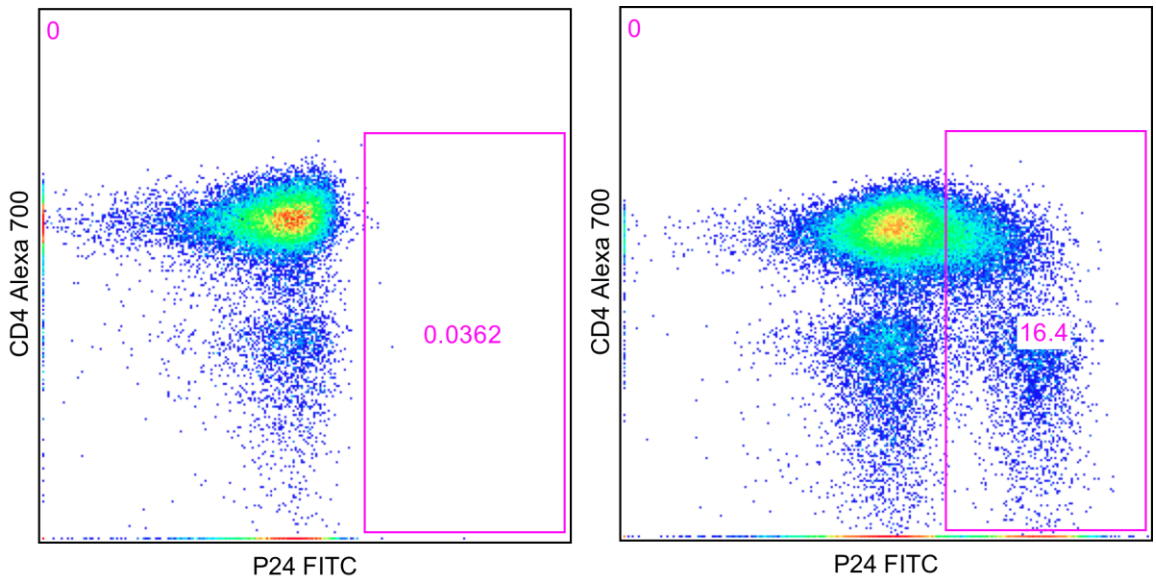


Figure 5.8: Representative intracellular p24 staining. The left panel shows an uninfected sample, with CD4 on the x axis and p24 on the y axis. The right panel shows a sample that has been infected with ML1956 virus, after 12 days of culture.

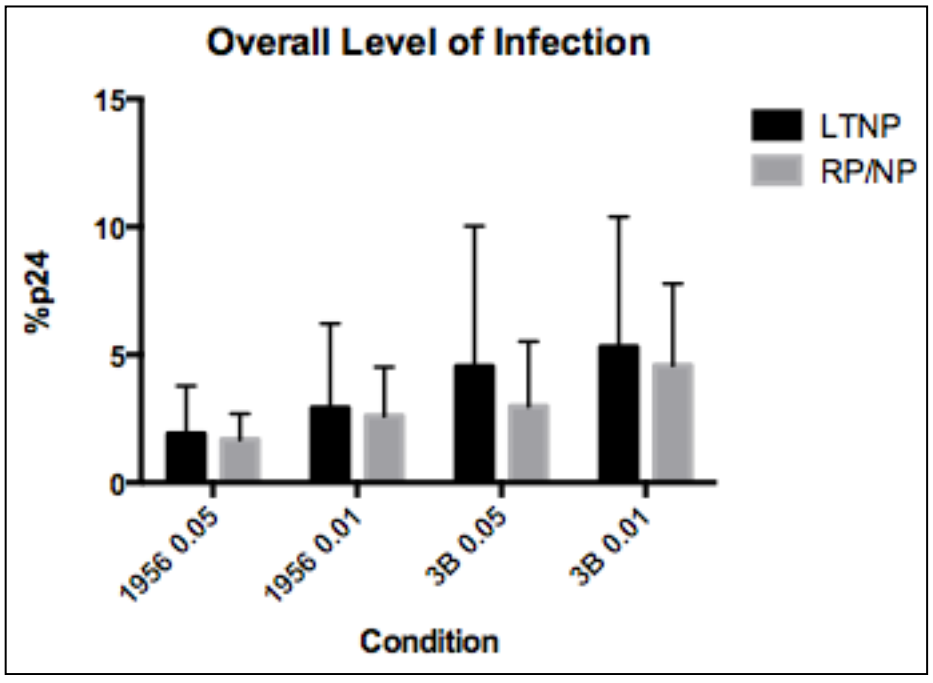


Figure 5.9: Overall intracellular p24 in CD4+ wells only for each condition for RP/NP and LTNP individuals. Intracellular p24 was measured on day 12 by intracellular flow cytometry.

Next, to determine if CD8+ T cells from LTNP were better able to inhibit intracellular p24 than those from RP/NP individuals through a CD8+ dependent mechanism, intracellular p24 levels were assessed both in wells containing only CD4+ T cells, and those in which CD8+ T cells had been added back. In the RP/NP group, there was no significant difference in the level of intracellular p24 between wells containing only infected CD4+ T cells and those with CD8+ T cells added back, at any MOI or with either virus (Figure 5.10, Mann-Whitney). This indicated that CD8+ T cells from the RP/NP group were unable to inhibit intracellular p24 production. Similarly, CD8+ T cells from HIV-negative individuals were unable to reduce the level of intracellular p24. However, in LTNP individuals, there was a significant reduction in intracellular p24 levels between infected CD4+ T cell only wells and those wells with CD8+ T cells added back (Figure 5.10, Mann-Whitney).

In summary, the ability of CD8+ T cells to reduce the level of intracellular p24 in autologous CD4+ T cells was observed in LTNP individuals only.

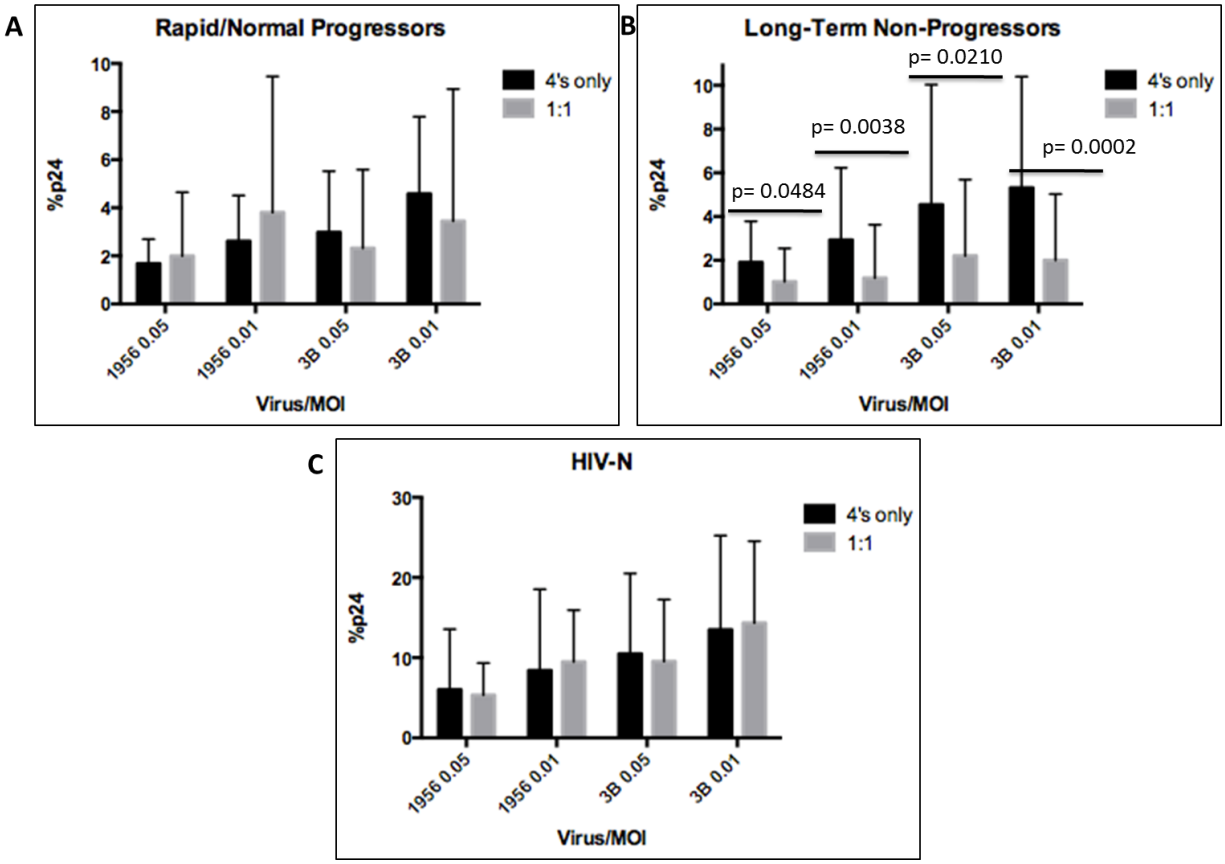


Figure 5.10: Reduction in the level of intracellular p24 via flow cytometry. The percentage of T cells producing intracellular p24 in target cell only conditions vs. with CD8+ effectors added back were compared in RP/NP (A), LTNP (B) and HIV-uninfected controls (C).

5.5.5 Expression of surface markers in relation to p24 production

In order to assess how activation (CD69, CD38), T cell turnover (Ki67), T cell function (perforin) and exhaustion (PD-1) may be related to infection or infection inhibition, each of those markers were included in the day 12 infection flow cytometry panel (seen in Table 2.6). Perforin was examined as it is known to be produced by CD8+ T cells in response to HIV, and is has been correlated in the past with better control of HIV[79]. The levels of perforin production were not significantly different between LTNP and RP/NP groups (Figure 5.11, $p= 0.4977$, Mann-Whitney). To investigate further if there was any relationship between perforin production and patient groups, correlations were conducted between perforin production and intracellular p24 levels. In the LTNP group, there was a significant positive correlation observed between the level of perforin and intracellular p24 production (Figure 5.11, $p< 0.0001$, Spearman's rank correlation). This relationship did not exist in RP/NP individuals, and in fact, there was a trend toward a negative correlation (Figure 5.11, $p= 0.0591$, Spearman's rank correlation).

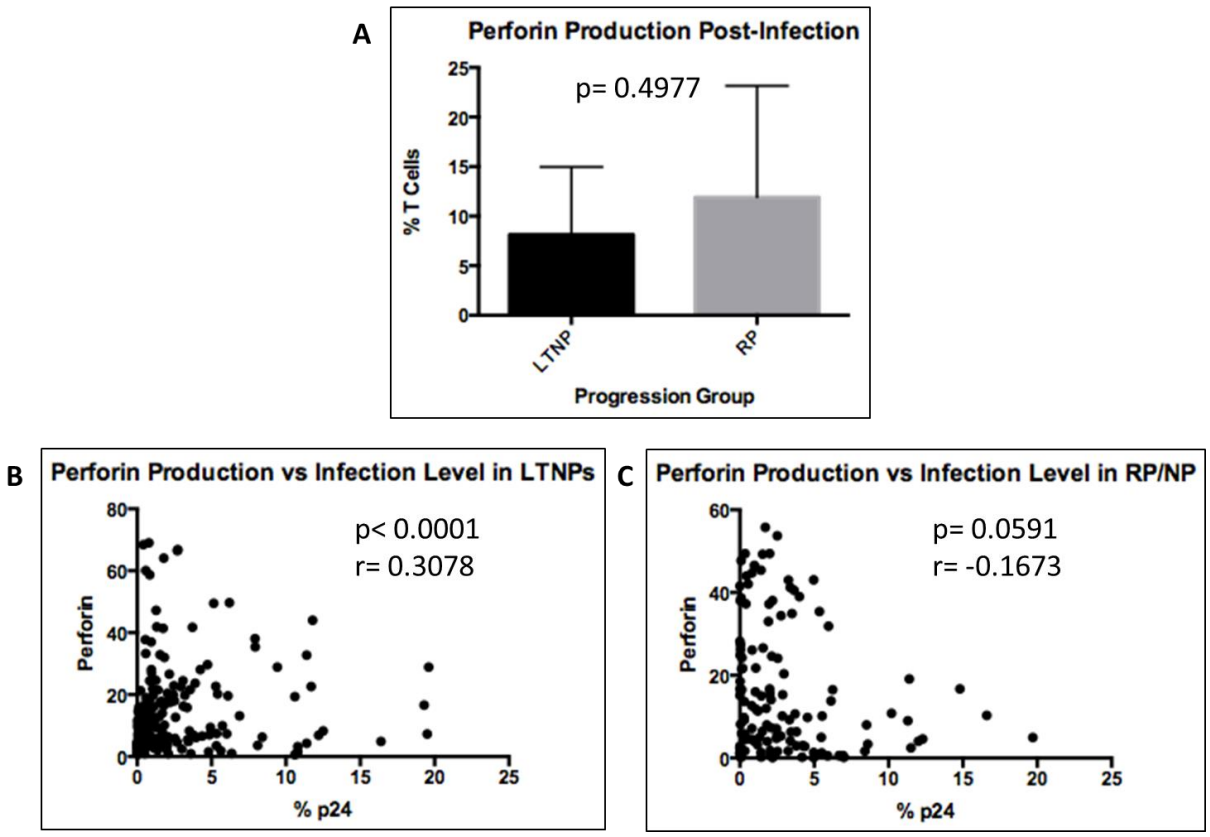


Figure 5.11: Perforin production in LTNP and RP/NP. The percentage of CD8+ T cells producing perforin was compared among LTNP and RP/NP individuals after infection (A). Perforin production was also correlated to the percentage of intracellular p24 in LTNPs (B) and RP/NPs (C).

Expression of PD-1 was also examined, as it is a marker of T cell exhaustion, and has been shown to be upregulated on infected cells[175]. There was no significant difference in the level of PD-1 expression on T cells between LTNP and RP/NP (Figure 5.12, $p= 0.4931$, Mann-Whitney). To further investigate if there was any relationship between this exhaustion marker with progression groups in the context of HIV infection, intracellular p24 levels were correlated with PD-1 expression. There was a significant negative correlation observed in LTNP between PD-1 expression and infection level (Figure 5.12, $p< 0.0001$, Spearman's rank correlation), a relationship that did not exist for RP/NP individuals.

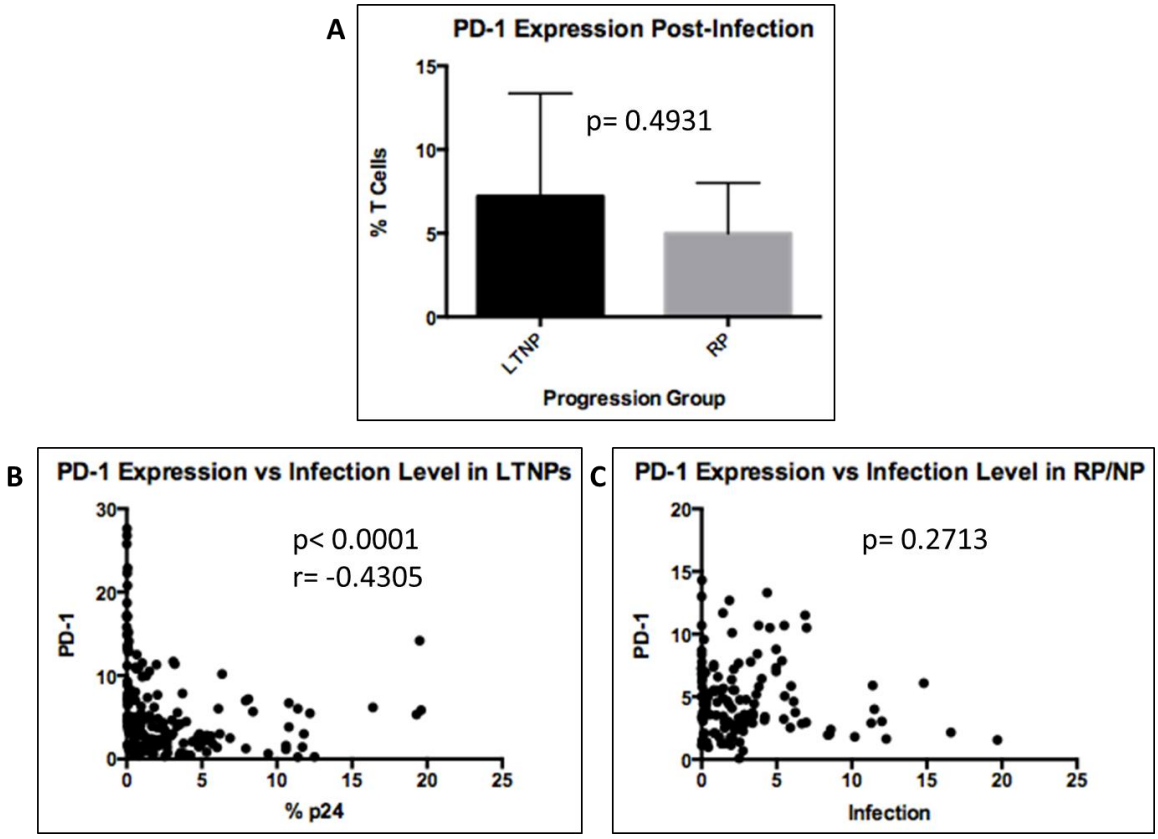


Figure 5.12: PD-1 expression in LTNP and RP/NP. The percentage of CD8+ T cells expressing PD-1 was compared among LTNP and RP/NP individuals after infection (A). PD-1 expression was also correlated to the percentage of intracellular p24 in LTNPs (B) and RP/NPs (C).

Expression of the marker Ki67 is associated with cellular activation and proliferation, as it is not expressed when cells are at rest[137]. Examining the relationship between Ki67 and progression groups, there was no significant difference in the level of Ki67 expressed on T cells in LTNP compared to RP/NP individuals (Figure 5.13, $p= 0.1122$, Mann-Whitney). Ki67 expression levels were then correlated with intracellular p24, to further investigate if any relationship exists. There was a significant positive correlation observed between Ki67 and intracellular p24 in T cells from LTNP individuals (Figure 5.13, $p< 0.0001$, Spearman's rank correlation), which was a trend not observed in RP/NP individuals (Figure 5.13, $p= 0.3846$, Spearman's rank correlation).

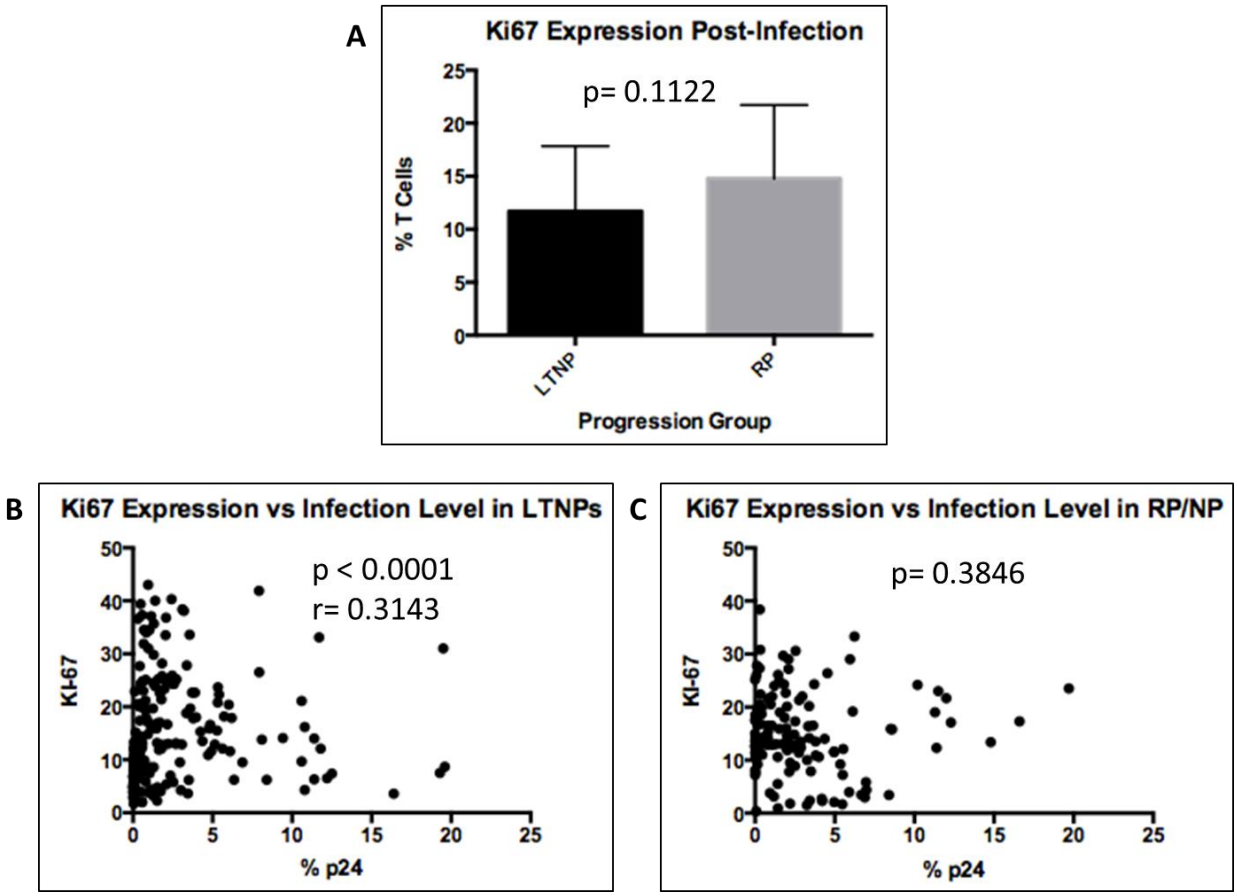


Figure 5.13: Expression of Ki67 in LTNP and RP/NP groups. The percentage of CD8+ T cells expressing Ki67 was compared among LTNP and RP/NP individuals after infection (A). Ki67 expression was also correlated to the percentage of intracellular p24 in LTNPs (B) and RP/NPs (C).

The surface markers CD38 and CD69 are often used to denote activation on T cells[137], and were included in this panel to assess any differences in cellular activation among patient groups. There was no significant difference between LTNP and RP/NP groups with respect to CD38 expression (Figure 5.14, p= 0.9639, Mann-Whitney). Correlations were again conducted, between CD38 and intracellular p24, to continue examining this relationship. No correlations were observed between CD38 and infection level in either patient group (Figure 5.14, Spearman's rank correlation). Conversely, there was a trend toward a significant increase in CD69 expression on T cells from RP/NP patient group compared to those from LTNP (Figure 5.15, p= 0.0666, Mann-Whitney). CD69 expression was also positively correlated with intracellular p24 levels in LTNP, but negatively correlated with intracellular p24 in RP/NP (Figure 5.15, p= 0.0012 and p= 0.0084 respectively, Spearman's rank correlation).

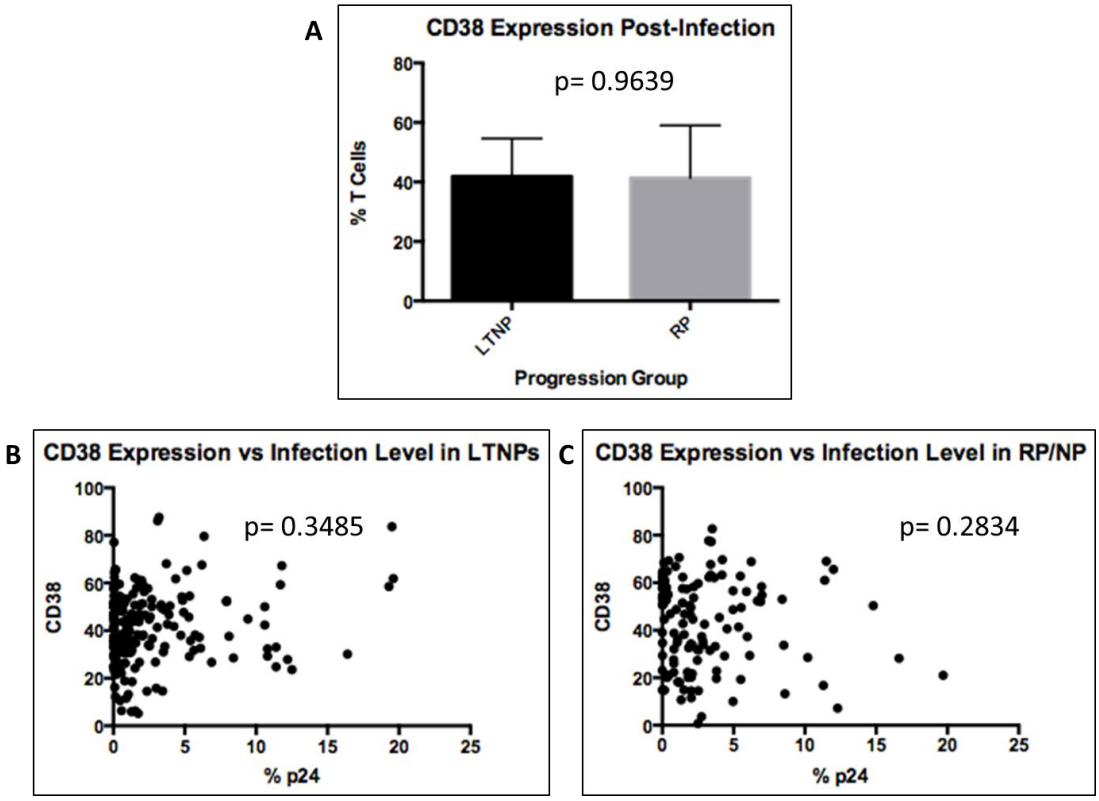


Figure 5.14: Expression of CD38 on CD8+ T cells of LTNP and RP/NP. The percentage of CD8+ T cells expressing CD38 was compared among LTNP and RP/NP individuals after infection (A). CD38 expression was also correlated to the percentage of intracellular p24 in LTNPs (B) and RP/NPs (C).

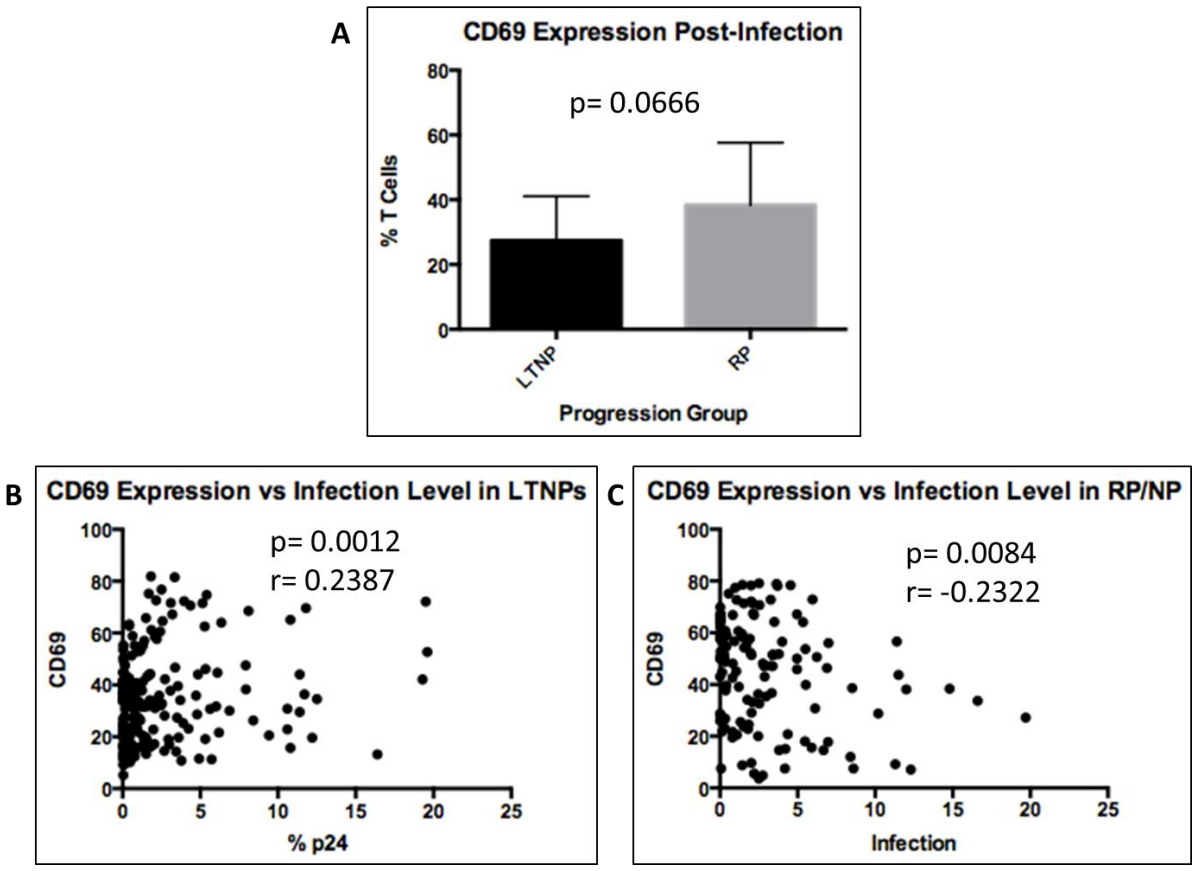


Figure 5.15: Expression of CD69 on CD8+ T cells from LTNP vs. RP/NP. The percentage of CD8+ T cells expressing CD69 was compared among LTNP and RP/NP individuals after infection (A). CD69 expression was also correlated to the percentage of intracellular p24 in LTNPs (B) and RP/NPs (C).

To better understand the differences between the co-expression of each marker in LTNP vs. RP/NP patient groups, correlation analysis between each marker was conducted. Table 5.2 describes the positive and negative correlations associated with each pairing. In the LTNP group, PD-1, which is an exhaustion marker, was significantly negatively correlated with perforin and Ki67, and positively correlated with both activation markers. However, within the RP/NP group, PD-1 expression was only negatively correlated with perforin expression; there was no significant correlation with Ki67, CD38 or CD69 in this group. This may indicate that there is a dysregulation within CD8+ T cell responses from RP/NP individuals.

In summary, intracellular p24 levels in LTNP individuals were positively correlated with perforin, Ki67, and CD69, and negatively correlated with PD-1, none of which were observed in RP/NP individuals. This suggests that LTNP are able to respond to increased intracellular p24 levels with more antiviral factors, higher cellular turnover, higher activation, and lower cellular exhaustion, perhaps aiding in their ability to control infection.

A

LTNP	PD-1	Perforin	Ki67	CD38	CD69
PD-1		p < 0.0001 r= -0.3212	p= 0.0005 r= -0.2195	p < 0.0001 r= 0.3246	p= 0.0472 r= 0.1571
Perforin			p < 0.0001 r= 0.4036	p= 0.0008 r= -0.2103	p= 0.0001 r= 0.2408
Ki67				p= 0.0073 r= 0.1693	p= 0.0001 r= 0.2426
CD38					p < 0.0001 r= 0.3248
CD69					

B

RP/NP	PD-1	Perforin	Ki67	CD38	CD69
PD-1		p =0.0149 r= -0.1922	ns	ns	ns
Perforin			p < 0.0001 r= 0.3493	p= 0.0116 r= 0.1990	p< 0.0001 r= 0.5768
Ki67				p= 0.0067 r= -0.2135	p< 0.0001 r= 0.3175
CD38					p < 0.0001 r= 0.3931
CD69					

No Correlation	Positive Correlation	Negative Correlation

Table 5.2: Correlations between expression of surface and intracellular markers. Panel A shows the significant positive and negative correlations in markers from LTNP, while panel B shows the correlations in RP/NP.

5.6 Conclusions:

It was hypothesized in this chapter that CD8+ T cells from long-term non-progressors would be able to suppress viral replication more strongly than CD8+ T cells from rapid or normal progressors. This hypothesis was confirmed using an infection inhibition assay, and assessing both secreted and intracellular p24 inhibition by CD8+ T cells from LTNP and RP/NP individuals.

When evaluating the secreted p24 by ELISA, it was observed that both the LTNP and RP/NP groups were able to significantly inhibit secreted p24 upon addition of CD8+ T cells, but the LTNP group was able to inhibit p24 to a significantly higher level than the RP/NP group. When the RP/NP group was observed more closely, it was found that they stratified into two groups, of which one appeared to inhibit p24 well, and the other did not. Of the group that was able to inhibit p24, the average CD4 count at the time of sampling was significantly higher than that of those individuals not able to inhibit p24. This may suggest that the ability of CD8+ T cells to inhibit HIV infection may be lost as progression occurs and CD4 counts drop.

The intracellular p24 staining on day 12 of the infection assay displayed that only CD8+ T cells from the LTNP group, and not the RP/NP group, were able to significantly inhibit intracellular p24, suggesting that these individuals may have an enhanced ability to inhibit p24 production.

Looking at markers of activation and function, a significant positive correlation was found between intracellular p24 and perforin production, Ki67 expression, and CD69 expression, and a negative correlation with PD-1, within the LTNP group only. This could suggest that CD8+ T cells from LTNP are better able to respond to increases in p24 with secretion of more anti-viral factors, an increase in cellular turnover, higher activation, and lower T cell exhaustion. These correlations were not seen in the RP/NP group.

Chapter 6: Discussion

As there are no examples of natural recovery from HIV infection, the research community has relied heavily on other models of protection to better understand how best to fight infection. Within the CD8+ T cell field, this has included studying immune responses correlated with better disease outcomes, such as polyfunctionality and proliferation, as well as observing groups like long-term non-progressors to understand the role of CD8+ T cells in disease progression. The aim of this work was to provide clarity to the intricacies of the CD8+ T cell response to HIV infection, in an effort to better understand what epitopes would be useful targets, how similar or different the responses to their common variants are, and to observe differences in inhibition of infection among clinical progression groups.

Two major hypotheses were explored in this thesis:

1) Consensus HIV epitopes will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their naturally occurring variants. CD8+ T cell responses to consensus epitopes will be more proliferative and polyfunctional, and be associated with better disease outcomes than responses to natural epitope variants.

2) CD8+ T cells from long-term non-progressors will be able to suppress viral replication more strongly than CD8+ T cells from rapid or normal progressors.

The first hypothesis was explored in Chapters 3 and 4 of this thesis, while the second hypothesis was addressed in Chapter 5.

*6.1: Characterization of Responses to HLA-B*42:01 Presented HIV IF9 and TL9 Epitopes and Their Natural Variants*

The extreme genetic diversity of HIV has posed a challenge in the creation of an effective vaccine. As the virus mutates, different epitopes are presented to CD8+ T cells, and the resulting responses of these cells can vary drastically[204]. Currently, there is an incomplete understanding of how CD8+ T cell responses to these epitope variants differ, and what the best target would be for an effective anti-HIV response. Consensus epitopes are more common in HIV strains circulating in nature, and, as such, it was hypothesized there would be larger CD8+ T cell pools available to respond to these epitopes over less common variants. 24 HLA-B*42:01+ subjects were studied for responses to two consensus HIV epitopes and their natural variants, to understand how these small changes in the stimulating epitope may alter the resulting CD8+ T cell response. The hypothesis for this chapter was: **The consensus HIV epitopes TL9 M and IF9 F will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their natural variants. CD8+ T cell responses to TL9 M and IF9 F epitopes will be more proliferative and polyfunctional, and be associated with better disease outcomes than responses to their natural epitope variants.**

6.1.1: Stimulation with consensus epitopes does not reliably result in a more highly functional and beneficial CD8+ T cell response

It was suspected that consensus epitopes, as they are more common in nature, would be more frequently recognized by CD8+ T cells[205], and by virtue of this recognition, elicit an overall stronger functional response characterized by production of more cytokines, higher polyfunctionality, and more proliferation. The IF9 epitope and its variants (IF9 A and IF9 L), and the TL9 epitope and its two variants (TL9 T and TL9 A), were assessed to explore this hypothesis in Chapter 3. The results obtained from the study of these epitopes and variants did not confirm the hypothesis, but rather, suggested that there are other factors more important than the consensus status of a presented epitope that are more likely to dictate the resulting CD8+ T cell response.

IF9 F is the most commonly circulating IF9 sequence in Kenya, but it was the variant IF9 A that was recognized most frequently by CD8+ T cells (Figure 3.2). IF9 F was the next most commonly recognized variant, and IF9 L was recognized at a low level in HIV-infected individuals from this cohort. The IF9 epitope has been studied extensively in the context of HLA-B*0702 presentation in clade B and HLA-B*42:01 in clade C infections[109], [206]-[208] (KLOVERPRIS 2012***), in which the IF9 L variant is frequently recognized by CD8+ T cells, whereas the IF9 F and IF9 A variants are rarely reported in the literature in association with these clades or HLA alleles. In fact, in a clade C infected South African cohort, it has been reported that less than 5% of HLA-B*42:01+ individuals are able to recognize the IF9 F epitope (KLOVERPRIS), despite this epitope being frequently recognized in clade A1/D

infections (MCKINNON). In the present study, ***% of individuals examined possessed CD8+ T cells able to recognize the IF9 F epitope, which suggests there may be some important differences in this particular epitope among different viral clades. Furthermore, despite the high level of recognition of IF9 L in other studies, in this study, recognition of IF9 L was significantly lower than that of IF9 A, and was no different than recognition of the consensus IF9 F. This may suggest that presentation of the IF9 L epitope is impaired in the context of presentation by HLA-B*42:01 alleles, or that this epitope is simply present at a lower frequency in clade A infections, resulting in less recognition by CD8+ T cells. Sequencing data from this cohort (Figure 3.17) confirmed that the IF9 L variant is present in infecting strains within this cohort almost as frequently as the consensus IF9 F epitope, which may lend support to the speculation that HLA-B*42:01 presentation of IF9 L is somehow impaired.

There was an interesting disconnect between CD8+ T cell recognition of IF9 A and the IF9 F and IF9 L variants (Table 3.2). Though IF9 A was recognized by a significantly higher frequency of CD8+ T cells than either of the other two, correlation analysis revealed that the frequency of CD8+ T cell recognition of IF9 A was not correlated to the frequency of CD8+ T cell recognition of either IF9 F or IF9 L. Conversely, the frequency of recognition of IF9 F and IF9 L correlated strongly with one another. It may be possible that IF9 F and IF9 L display some level of cross-reactivity, by which the same pool of CD8+ T cells recognize them and hence drives this correlation, while IF9 A is recognized by a different CD8+ T cell pool. Indeed, in a study of HLA-B*07 humanized mice, strong cross-reactivity was

observed with IF9 F when mice were immunized with strains containing IF9 L[109]. This same cross-reactivity was also observed in individuals infected with HIV strains from clades A1 and B, when presented by HLA-B*0702[209]. This lends support to the possibility that, when presented by HLA-B*42:01 in clade A1/D infected individuals, cross-reactivity between the IF9 F and IF9 L variants may also exist. If this cross-reactivity is indeed specific to the presented epitopes, and occurs across clades and HLA alleles, it is possible that epitopes could be identified that, despite frequent mutations, are able to maintain the same CD8+ T cell responses when recognized in HIV infection. If these epitopes elicited a beneficial CD8+ T cell response, and were cross-reactive with their common variants, they could make good targets for a potential vaccine.

It has been well established that mutations occur frequently within the HIV Env gene, from which the IF9 epitope is derived[162], [210], [211]. The higher recognition of IF9 A could potentially be explained by a higher number of IF9 A variants within the infecting strains in the study cohort. However, viral sequence analysis of 90 individuals from this cohort revealed that IF9 A was only found in 2% of the sequences examined (Figure 3.17). Another possible explanation could be related to the strength of the cellular response, as indicated by the MFI of CD8+ T cells responding to it. The MFI of CD8+ T cells specific to IF9 F was significantly higher than those specific to IF9 A or IF9 L. Therefore, although there are fewer CD8+ T cells recognizing the consensus IF9 F epitope, the ones that do are able to bind a higher number of virions per cell than the CD8+ T cells specific to IF9 A.

When considering the nature of the total CD8+ T cell response as indicated by cytokine production, IF9 A was more likely to stimulate bulk CD8+ T cells that produced IFNγ and IL-2 than IF9 F or IF9 L, and there were no differences among production of TNF or MIP1β (Figure 3.7). When looking more closely at epitope-specific CD8+ T cells only, the cytokine production profile was much different than when considering the entire CD8+ T cell compartment (Figure 3.12). After stimulation, CD8+ T cells specific to IF9 A produced significantly lower levels of all four cytokines than CD8+ T cells specific to IF9 F and IF9 L. This may suggest that IF9 A is able to stimulate an immune response in a non-specific way, whereby the overall pool of CD8+ T cells responds with more IFNγ and IL-2 production, but the cells specific to that variant do not. It is also possible that the CD8+ T cells specific to IF9 A have simply become exhausted, and, as such, are less able to produce a strong functional response. Another explanation for this data could be that the IF9 A epitope is cross-reactive with another epitope. Though it seems unlikely that this cross-reactivity occurs with either IF9 F or IF9 L considering the lack of correlation of CD8+ T cell responses to them seen in Table 3.2, it may well be cross-reactive with another, untested epitope. To tease out this information, more work would need to be done looking at cross-reactivity and exhaustion markers in the context of variant stimulation. Of interest, IF9 A was able to stimulate a significantly stronger 3+ and 2+ polyfunctional response than IF9 L, and trended to significance compared to IF9 F (Figure 3.14). As polyfunctionality has been associated in the past with better disease outcomes[156], this may suggest that IF9 A is more likely to be associated with beneficial CD8+ T cell responses than IF9 F or IF9 L. However, as the IF9 epitope as a whole is known to be incredibly variable, it is unlikely that even

with further research any of these variants would be considered as a reasonable vaccine target.

The TL9 epitope is derived from the gag gene, which is known to be a far more conserved region than Env [154], [166], and overall a better target for anti-HIV immunity[110], [162]. Within the study cohort in Kenya, >90% of individuals are infected with clade A1 or clade D viruses, with A1 being approximately 3 times more common than clade D in these individuals[212]. The TL9 T epitope is the consensus in most HIV clades, but TL9 M has been found to be most common within clade A viral strains[204]. As such, it is difficult to know for certain which TL9 variant is the consensus in the individuals studied, but it can be assumed that the majority are infected with clade A1 viruses as per previous work, and would therefore have TL9 M as a consensus. The rest are likely infected with clade D viruses, and would therefore have TL9 T as their consensus epitope. In the work presented here, TL9 T was most frequently recognized by CD8+ T cells, followed by TL9 A, and TL9 M was recognized by the smallest pool of CD8+ T cells (Figure 3.2). The hypothesis that the consensus epitope would be most frequently recognized was therefore not confirmed in this epitope. It is possible that there was a larger number of CD8+ T cells specific to TL9 T due to mutation of the consensus M epitope to the less common T variant, however, it has been previously reported that escape within the TL9 epitope occurs rarely when presented by HLA-B*42:01[213], [214]. Even so, previous work has shown that mutation at this epitope from M to T or vice versa can occur without a substantial fitness cost to the virus, so it is not unreasonable to suggest that mutation may be occurring here[204]. Interestingly, it has been

observed that there is a significant amount of cross-reactivity between the M and T variants within TL9[204]. Though cross-reactivity was not directly addressed in this study, it may explain why such strong responses were observed to TL9 T, despite the fact that TL9 M is likely more common in infecting strains within this population. This, combined with the fact that approximately 25% of individuals in this cohort are likely infected with clade D viruses possessing the TL9 T epitope as consensus could explain why TL9 T was more frequently recognized by CD8+ T cells.

When looking at the functional responses to these epitopes, it was found that TL9 T stimulated significantly more bulk CD8+ T cells that responded with production of IFNγ, TNF and MIP1β than TL9 A (Figure 3.9). Compared to the consensus TL9 M, TL9 T only stimulated more CD8+ T cells producing TNF. Considering the known cross-reactivity of the TL9 T and TL9 M variants, it is unsurprising that one did not stimulate a better cytokine response than the other for most of the cytokines measured. Though TL9 T did stimulate more bulk CD8+ T cells that produced TNF, there was a trend toward a significantly higher MFI of CD8+ T cells producing this cytokine when stimulated with TL9 M. This would suggest that, though TL9 T stimulation led to more CD8+ T cells producing TNF, those CD8+ T cells stimulated with TL9 M could in fact produce more TNF on a per-cell basis, which may have effectively reduced the overall difference in the total output of TNF. Interestingly, though TL9 T stimulated a higher number of total CD8+ T cells to produce TNF, TL9 M actually stimulated a stronger TNF+ epitope-specific CD8+ T cell response (Figure 3.13). This could suggest that, though TL9 T appears more effective at stimulating a

TNF response in bulk CD8+ T cells, TL9 M may stimulate production of this cytokine in a more epitope-specific manner. It is also unsurprising that TL9 A stimulated the smallest cytokine response, both in the number of responding CD8+ T cells and the amount of cytokine produced, as this epitope has not been identified in previous work as being a particularly common variant[204], [214].

Similar to stimulation with the IF9 variants, none of the TL9 variants were able to stimulate a strong IL-2 or proliferative response. This may suggest that the individuals in this study had already reached a point at which the quality of their epitope-specific CD8+ T cell response had begun to decline, which is characterized by a loss of proliferative capacity and IL-2 production initially[158], [215]. Positive control samples, stimulated with PMA/ionomycin, were often able to stimulate relatively strong production of IL-2, which suggests it is indeed the epitopes that are unable to stimulate production of IL-2 rather than some deficiency in the capabilities of the cells. Of the few individuals who were able to mount a significant proliferative response, all of them were in response to stimulation with the TL9 T epitope. The TL9 T epitope was also able to stimulate the most frequent 2+ and 3+ polyfunctional cytokine response (Figure 3.15), which has been correlated with better disease outcomes in previous studies[156], [216]. Considering this, along with the ability of the TL9 T epitope to stimulate the strongest cytokine response and a proliferative response in some individuals, may suggest that it is beneficial to mount a CD8+ T cell response to this epitope in HIV infection.

*6.1.2: Recognition of the non-consensus TL9 T epitope is associated with better CD4 counts in HLA-B*42:01+ individuals*

Interestingly, CD8+ T cell responses to the TL9 T epitope appeared to have an association with higher CD4 counts that was not seen in response to the other epitopes. When individuals were divided into groups based upon their CD4 count (CD4 <500 and CD4 >500), there was an association whereby individuals with CD4 counts >500 were more likely to have CD8+ T cells that recognized TL9 T with a higher frequency than those individuals with CD4s of <500 (Figure 3.3). Effectively, this means that those individuals who have a larger pool of CD8+ T cells that respond to TL9 T are likely to have higher CD4 counts, which would indicate a better clinical outcome. The frequency of recognition of TL9 T was also associated with CD4 ratio (Figure 3.4), whereby individuals whose CD8+ T cells recognized TL9 T with a higher frequency are likely to have a higher CD4 ratio. The CD4 ratio is considered to be an indicator of the overall immune health in HIV-infected individuals, and a low CD4 ratio has been associated with immune irregularities, including a higher number of terminally differentiated CD8+ T cells[217]. Lastly, the MFI of CD8+ T cells specific to TL9 T was positively correlated with CD4 count, meaning that individuals were more likely to have higher CD4 counts if the CD8+ T cells specific to TL9 T were able to bind to multiple TL9 T epitopes at once (Figure 3.5). None of these associations were present with responses to the consensus TL9 M or TL9 A epitopes, and taken together, provide an interesting case that CD8+ T cell responses to TL9 T may be associated with better disease outcomes. Though it has been shown previously that the frequency of CD8+ T cells responding to any

epitope is not necessarily the most important factor[156], in this case it may well be playing a role.

6.1.3: Conclusions

It has become clear from this research that, contrary to the first hypothesis of this thesis, the consensus or variant status of an epitope does not reliably dictate if it will be able to stimulate a beneficial response to infection, or even any response at all. From this work, it seems likely that the importance lies with what the specific epitope is, along with what MHC presents it, TCR usage by the responding CD8+ T cells, and how it interacts with both the MHC and TCRs. HLA-B*42:01 restricted presentation of the TL9 epitope has been shown previously to result in lower IFNγ production, and to comprise a significantly lower proportion of the total CD8+ T cell response, compared to when this epitope is presented by either HLA-B*8101 or HLA-B*3910[214]. This suggests that the presenting MHC is an important determinant of what the overall immune response is. Previous work has also shown that the same epitope, when presented by different HLA alleles, can exhibit distinctly different TCR clonotype usage, which represents yet another mechanism to explain these differential CD8+ T cell responses[214]. The work presented here would suggest that the specific epitope being presented is of particular importance, as seen with the differences in the CD8+ T cell response to the TL9 epitope and its variants. Despite the fact that the hypothesis of this chapter was not confirmed, interesting associations of the TL9 T variant with better CD4-dictated clinical outcomes, cytokine production, and polyfunctionality suggest that this epitope may be a good target to produce a desirable CD8+ T cell response against HIV.

6.1.4: Limitations and Future Directions

There are some limitations to this study that should be addressed. The infecting HIV sequence for each patient is not known, which likely has an important impact on the resulting pool of CD8+ T cells in each patient. Further, it would have been interesting to know in general what the consensus TL9 epitope was for this cohort; though it can be estimated that the majority of individuals would have TL9 M as their consensus epitope, it would have been ideal to sequence this region in a subset of the cohort participants to be certain. Viral loads were also not available for the subjects in this study, which would have been an interesting clinical measure to use for comparison along with CD4 count.

There are many questions surrounding this work that have been left unanswered, and which could benefit from additional study. While it was found that the TL9 T variant was associated with what would be considered a 'beneficial' CD8+ T cell response, there are many aspects of the CD8+ T cell response to this epitope that remain unclear, including the functional avidity, cross-reactivity with its common variants, TCR usage, and viral inhibition capacity. All of these factors are essential to characterizing CD8+ T cell responses both to this 'beneficial' epitope, and others which may well produce a less beneficial CD8+ T cell response. These additional assays could also be conducted more thoroughly for the IF9 epitope. Further, accessing viral load data and sequencing to determine the infecting strains would provide a great deal of insight into how these responses are related to clinical

outcomes, and how the infecting epitope can impact the resulting CD8+ T cell response after stimulation.

With regard to the fine specificity of CD8+ T cell immunity, variations can occur in responses due to the presenting MHC, the epitope being presented, and the TCR usage by the responding CD8+ T cells, or some combination of the three. Future work could help to better understand this interaction, by looking at these same epitopes when presented by different HLA alleles. In particular, the IF9 epitope is also presented by HLA-B*0702, which is an HLA allele known to be associated with more rapid disease progression[214]. When presented by HLA-B*0702, the frequency of responses are very low (~5%), especially when compared to presentation with HLA-B*42:01 (~90%)[214]. Understanding what role the IF9 and other epitopes may play in this low level of recognition and response within HLA-B*0702 presentation would be interesting for future study. Some of these studies have already been conducted, but largely in the context of clade B and clade C infection, rather than clade A and D infection as presented here.

*6.2: Cross-Sectional and Longitudinal CD8+ T cell Responses to HIV IW9 and KF11 Epitopes and Their Natural Variants in HLA-B*57:03 Subjects*

Though the IW9 and KF11 epitopes have been observed to be immunodominant in nature, mutations to their variant LW9 and KGF epitopes have also been observed, with varying frequency depending on the infecting clade. It is thought that these mutations are due to a high level of immune pressure, as they tend to revert back to the IW9 and KF11 forms when immune pressure is removed[191]. This indicates

that the LW9 and KF11 mutations come with a fitness cost to the virus[121], owing to high reversion rates. However, these escape mutations remain incompletely understood with respect to the impact they have on the immune response, how these responses differ from one another, and what impact they have clinically within clade A and D infection in Kenya. The hypotheses for this chapter were:

1) The consensus and immunodominant IW9 and KF11 epitopes will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their natural variants. The IW9 and KF11 epitopes will elicit CD8+ T cell responses that are more proliferative and polyfunctional than their natural variant epitopes.

2) CD8+ T cell responses to IW9 and KF11 will be associated with slower disease progression, determined by CD4 count. Beneficial CD8+ T cell responses including proliferation and polyfunctionality will be preserved in individuals who maintain healthy CD4 counts longitudinally.

6.2.1: Immunodominance and consensus status of an epitope does not account for all of the functional differences in resulting CD8+ T cells

As the IW9 and KF11 epitopes are known to be immunodominant and the consensus epitopes in this population, it was suspected that CD8+ T cell responses to these would be more frequent, polyfunctional, and proliferative than CD8+ T cell responses to their less dominant natural variants. However, this first hypothesis was not confirmed, but rather, the data displayed that the CD8+ T cell responses to these immunodominant, consensus epitopes and their variants are extremely variable, and certainly do not fit to a rigid definition depending on their consensus status within the population.

The consensus IW9 epitope was recognized with a much higher frequency than the LW9 variant, and was also found to have a significantly higher MFI (Figure 4.2). This would suggest that not only is there a larger CD8+ T cell pool responding to it, but that on a per-cell basis, CD8+ T cells specific to IW9 are able to bind to more epitopes at once than those specific to LW9. Despite this, and contrary to the second hypothesis, there was no association with either epitope and any measure of CD4 (direct CD4 count, CD4 ratio, nadir count, or when divided into CD4 >500 and CD4 <500) (Figures 4.3 to 4.6).

There was no significant difference between IW9 and LW9 in their ability to stimulate a cytokine response, when measuring any of the four cytokines (IFNγ, MIP1β, TNF, IL-2) (Figure 4.8). This is not surprising, considering it has been reported that IW9 specific CD8+ T cells are cross-reactive and able to recognize LW9 epitopes[180]. It is possible that both epitopes are in fact stimulating the same pool of CD8+ T cells, which would explain why the number of CD8+ T cells producing each of the cytokines does not differ when stimulated with either IW9 or LW9. The IW9 epitope has been reported as an immunodominant epitope within Gag[218], however, the lack of association with any measure of CD4 count may indicate a case in which targeting of immunodominant epitopes is not necessarily ideal in terms of clinical outcomes. On the other hand, it has been reported in at least one study that early targeting of the IW9 epitope is associated with better outcomes[190], so it is also possible that the lack of association with good clinical outcomes is due to individuals in this study being sampled at a later stage of

infection. Proliferation and polyfunctionality were also assessed in this study, both of which are associated with better control of infection. However, very little proliferative capacity was seen in any individual, and there were no differences depending on which epitope was used for stimulation. Similarly, there were no differences in 3+ polyfunctional responses, though there was a slight increase in 2+ polyfunctionality associated with the IW9 epitope compared to LW9 (Figure 4.15). Taken together, this data may also suggest that few differences exist between these two epitopes, potentially due to a high level of cross-reactivity between them, and that overall, the data presented within this epitope does not support the first hypothesis.

KF11 is also known to be an immunodominant Gag derived epitope[190], [218], presented by the HLA-B*57:03 allele. In this case, there was no significant difference in the frequency of CD8+ T cell responses to KF11 and its variant KGF, or the MFI of these responses (Figure 4.2). The fact that there was no difference in the level of CD8+ T cell recognition of the consensus KF11 and the variant KGF may indicate there is a high level of mutation to the less common KGF variant within this cohort. It is also possible that there is a significant level of cross-reactivity between these epitopes, which would explain the lack of more frequent CD8+ T cell recognition of one epitope over the other. Indeed, it has been previously reported that cross-reactivity exists to a significant degree between KF11 and KGF[219], and that mutation rates when presented specifically by HLA-B*57:03, which was the allele tested in this cohort, may be higher than when presented with HLA-B*5701[220].

There was a significant correlation found between the frequency of CD8+ T cell recognition of the KF11 epitope and CD4 count (Figure 4.3), which was not significant with the KGF epitope, though it was nearly trending with a p value of 0.1049. When the subjects were divided into groups based upon if their CD4 count was >500 or <500, the frequency of KF11 and KGF specific CD8+ T cell recognition was significantly higher in individuals with CD4 counts >500 (Figure 4.4). When considering CD4 ratio and nadir count, both were significantly positively correlated with CD8+ T cell recognition of both KF11 and KGF (Figures 4.5 and 4.6). This data together suggests that, contrary to the second hypothesis, individuals who mount strong CD8+ T cell responses to either the consensus KF11 or the variant KGF epitope have better clinical outcomes, suggesting that this is not a function of the consensus status of the epitope.

In the work presented here, stimulation with both the consensus KF11 and variant KGF epitopes resulted in similar numbers of bulk CD8+ T cells producing each cytokine (Figure 4.9). This same trend was observed when looking specifically at KF11 or KGF specific CD8+ T cells (Figure 4.13), though KF11-specific CD8+ T cells were more likely to produce TNF than those specific to KGF. A high level of cross-reactivity has been observed in functional assays between KF11 and its natural variants when presented by the HLA-B*5701 HLA allele, which may serve to explain why few differences were found in functional assays stimulated by these epitopes here[99], [219]. The work presented here suggests that, though cross-reactivity is known to exist between KF11 and its natural variants, the CD8+ T cell pools

stimulated by each epitope do not completely overlap, as the CD8+ T cells specific to KF11 are more likely to produce TNF (Figure 4.13). There was also no difference observed in the ability of KF11 or KGF to stimulate proliferation, which also supports the theory that there is a high level of cross-reactivity between these epitopes. Lastly, the KF11 epitope was able to stimulate a significantly more frequent 3+ polyfunctional response than KGF (Figure 4.16). If both epitopes are largely stimulating the same pool of CD8+ T cells, it would be expected that the functional outcomes would not differ greatly. However, since there were some minor differences observed, with KF11-specific cells more likely to produce TNF, and KF11 stimulation more likely to produce a 3+ polyfunctional response, it would seem likely that the cross-reactivity is incomplete, and the KF11 epitope is still stimulating some CD8+ T cells that are not accessed by the KGF epitope.

Overall, there was a striking correlation seen between the frequency of CD8+ T cell responses to each epitope with one another (Table 4.1). In other words, individuals who have a large pool of CD8+ T cells recognizing one epitope will also likely recognize the others with a high frequency. In the context of each epitope and variant (IW9 and LW9, and KF11 and KGF) this makes sense, due to the reported high level of cross-reactivity. However, more interesting is the correlation found between CD8+ T cell responses to seemingly unrelated epitopes (IW9 and KF11, for example). This correlation may be suggestive of a linked anti-HIV immune response, linked by the fact that both epitopes are known to be immunodominant, derived from Gag, and also presented by HLA-B*57:03 alleles.

A comparison of cytokine production on the whole, regardless of stimulating epitope, revealed some interesting differences between cytokine production in total CD8+ T cells compared to CD8+ T cells specific to each epitope. When considering the cytokine production of all CD8+ T cells, MIP1β was produced most frequently, followed by IFNγ, TNF, and finally, IL-2, though these differences were not statistically significant. However, when looking at cytokine production by epitope-specific CD8+ T cells only (as identified by tetramers), TNF was more frequently produced than MIP1β or IFNγ. This may suggest that there are important functional differences between responses from bulk CD8+ T cells and those CD8+ T cells that are specific to these immunodominant epitopes, such that the cytokine response is skewed away from a MIP1β centric response to a TNF response.

6.2.2: Longitudinal analysis reveals individuals who maintain control of the virus despite losing beneficial CD8+ T cell functionality

In section 4.5.9, the second hypothesis of chapter 4 was explored further, looking at functional responses of two long-term non-progressing individuals longitudinally. A polyfunctional cytokine response has been associated with better clinical outcomes in HIV, and as such, the hypothesis was that, over time, LTNP individuals would maintain strong cytokine production and polyfunctionality while their CD4 counts remained high. However, this hypothesis was not confirmed, as drastic differences were observed in the functional responses of individuals in this cohort when observed over time. Two individuals, ML 1778 and ML 1514, were selected to represent this data, as both of these individuals were HLA-B*57:03+ and maintained CD4 counts above 500 for the duration of each sampling point, but displayed

polarized functional responses longitudinally. ML 1778 displayed the sort of functional responses that were expected, where, over 3 time points in 4 years, the number of CD8+ T cells producing cytokines either remained steady or increased (Figure 4.18). Conversely, the number of CD8+ T cells producing cytokines in ML 1514 declined slightly over the 3 time points. The polyfunctionality of these responses increased dramatically over time in ML 1778 (Figure 4.19), with significantly higher 3+ and 2+ polyfunctionality with each time point. There were striking differences when compared to ML 1514, which displayed a significant reduction in 3+ and 2+ polyfunctionality over time (Figure 4.20). Interestingly, the CD4 counts for each of these two subjects were maintained at >500 over the entirety of the sampling period, despite the drastic differences in functional outcomes.

The mechanism and dynamics of these beneficial functional responses in LTNP individuals in HIV have not been well described[221]. While it has been identified in many studies that CD8+ T cells from LTNP individuals exhibit an enhanced ability to produce a polyfunctional cytokine response[190], [216], [222], it has not been conclusively shown if this functional response is a cause or an effect of viral control. By observing that some individuals in this cohort apparently maintain control of the virus while losing the ability of their CD8+ T cells to produce a polyfunctional response, it may lend support to the theory that a more polyfunctional response may be a consequence of viral control rather than a cause. That being said, there are many factors that impact the control of viral replication that were not assessed in this study, including production of other anti-viral factors such as granzymes and perforin. It is possible that the dynamics of this response are in fact a combination

of situations, where in some individuals, a polyfunctional response is helpful in combating the virus, while in others, it is simply a function of an overall healthier immune response mediated by some other factor.

6.2.3: Conclusions

The HLA-B*57:03 allele has been linked to better clinical outcomes in several studies[162], [192], [214]. Though the IW9 and KF11 epitopes have been described as immunodominant, and two of the three most frequently targeted epitopes within Gag p24, how CD8+ T cell responses to these epitopes differ from their common viral variants within the context of clade A1 and D infections was unknown. The data presented in this chapter displayed that few differences existed among the consensus IW9 and KF11 epitopes and their variants in their ability to stimulate cytokine production, which does not support the hypothesis that consensus epitopes would be associated with more beneficial CD8+ T cell responses than their variants. Rather, this suggests that there may be a high level of cross-reactivity between these epitopes.

The second hypothesis was that recognition of consensus epitopes would be related to better clinical outcomes as measured by CD4 counts, and that individuals who maintain high CD4 counts over time would also maintain strong anti-HIV CD8+ T cell responses. However, CD8+ T cell responses to neither the consensus IW9 nor the LW9 variant were associated with higher CD4 counts, and also, responses to both the consensus KF11 epitope and its variant KGF were associated with higher CD4 counts, both of which do not support this hypothesis. Further, it was observed

that though some individuals are able to maintain healthy CD4 counts above 500, this does not necessarily mean they will also maintain beneficial CD8+ T cell responses, as indicated by the polarizing data from ML 1778 and ML 1514.

6.2.4: Limitations and Future Directions

An important limitation of this work is not knowing the infecting strain of the subjects studied, which would have provided insight into the resulting CD8+ T cell responses. Also, an important component of the CD8+ T cell response is the particular T cell receptors being used, which was not assessed in this study, but would have provided a more complete picture of the interactions at hand. Viral loads were also not assessed, which would have provided another excellent measure of clinical outcomes with which to compare this data.

Several future directions are possible for this work. For all epitopes investigated, an expanded analysis could be conducted to include functional avidity and TCR usage, as well as sequencing of the infecting strains. The sequencing will allow a better understanding of how and why individuals are responding to each epitope, and would help to explain how and when mutations occur within this cohort. As it was determined that cross-reactivity may be playing a role with the epitopes studied in this section, assessing the level of cross-reactivity between the study epitopes would be of great interest.

Here, we were able to display that there is a high degree of heterogeneity within individuals who control HIV with respect to their CD8+ T cell responses. By

observing the same individuals over time, and conducting these same assays from the point of infection through to progression to AIDS, it would provide a more thorough understanding of the dynamics of the T cell responses that occur and help provide evidence to clear up the question of if these responses are a cause or effect of slow disease progression. Some individuals would undoubtedly progress more quickly than others, allowing an interesting comparison of these factors. Further, as HLA-B*57:03 is known to be associated with better clinical outcomes, it would be interesting to identify HLA-B*57:03+ individuals who progress at normal or rapid rates within this cohort and conduct the same studies on them, to understand how they may differ from their slow progressing counterparts, and to understand how and when LTNP individuals lose control of the infection.

6.3: CD8+ T cell Mediated Inhibition of HIV Production in Long-Term Non-Progressors and Normal/Rapid Progressors

Though a lot of research has been conducted within HIV disease progression groups, it has not been clear exactly how or why some individuals are better able to control the infection than others. As such, it was explored in Chapter 5 of this thesis if CD8+ T cells from LTNP individuals were better able to inhibit HIV infection than cells from RP/NP individuals. The hypothesis of this chapter was **CD8+ T cells from long-term non-progressors will be able to suppress viral replication more strongly than CD8+ T cells from rapid or normal progressors.**

6.3.1: CD8+ T cells from long-term non-progressors display an enhanced ability to inhibit secreted p24 production in autologous CD4+ T cells

The results from section 5.5.3 revealed that CD8+ T cells from both LTNP and RP/NP individuals were able to inhibit secreted p24 (Figure 5.4), but that overall, the CD8+ T cells from the LTNP group had a greater capacity to inhibit secreted p24 than those from the RP/NP group (Figure 5.7). The enhanced ability of CD8+ T cells from LTNP to inhibit secretion of p24 may indicate that they are functioning at a higher level or are producing more anti-viral factors to cause a more significant reduction in the production of p24 than CD8+ T cells from the RP/NP group. Though CD8+ T cells from LTNP were better able to reduce secreted p24, CD8+ T cells from the RP/NP were still able to significantly inhibit secreted p24 when examined on their own. Upon closer examination, it was observed that within the RP/NP group there exists some stratification in the ability to inhibit p24 production (Figure 5.5), whereby one group appears to almost completely lack inhibitory capacity, and the other produces inhibition to a level similar to that of the LTNP group. Comparatively, the LTNP group does not possess this stratification. This indicates that within the RP/NP group there exists some heterogeneity in their ability to inhibit HIV production, which could be due to a number of factors, such as the overall health of their immune systems. Comparing CD4 counts within the RP/NP group at the time of sampling, the sub-group who were able to successfully inhibit HIV had a significantly higher CD4 count than those who could not (Figure 5.6, Table 5.1), so this could be an indication that some within this patient group have already lost control of the virus, while others, at the time of sampling, had still maintained a relative level of viral control, allowing them to inhibit p24 secretion.

This would indicate that though RP/NP individuals progress to AIDS at an increased rate when compared to LTNP, their ability to inhibit HIV remains intact before this progression has occurred. In this case, it would seem there is some other factor which dictates how quickly they progress to AIDS, and their ability to inhibit and/or control the virus is simply a result of their declining immunity.

6.3.2: CD8+ T cells from LTNP individuals are capable of inhibiting intracellular p24 production, while CD8+ T cells from RP/NP individuals are not

To complement the secreted p24 data, cells from day 12 were harvested and stained for intracellular p24 using flow cytometry. When the progression groups were assessed individually, it was found that CD8+ T cells from LTNP are significantly able to inhibit intracellular p24, while CD8+ T cells from the RP/NP group are not (Figure 5.10). Upon closer inspection, the same stratification in inhibitory capacity was observed in the RP/NP group in the intracellular p24 assay as was observed in the secreted p24 data. The same individuals who showed strong inhibition of secreted p24 also show strong inhibition of the intracellular p24, and vice versa. While an assessment of epitope-specific CD8+ T cells was not done in this study, there are some conclusions that can be drawn about the presence of these cells in LTNP vs. RP/NP groups. Looking at HIV-negative individuals in the same assay, it was observed that they were unable to inhibit HIV infection at any time point, unlike the HIV-infected LTNP group (Figure 5.10). This could indicate that the inhibition in p24 is at least partially due to an HIV epitope-specific CD8+ T cell response, which is absent or very low in HIV-negative individuals.

6.3.3: Level of infection in LTNP, but not RP/NP, is correlated with cellular markers

To better understand what was occurring at a cellular level in this infection assay, markers of activation (CD69 and CD38), cellular turnover (Ki67), exhaustion (PD-1) and cellular function (perforin) were examined on day 12 along with intracellular p24 levels. It was found that there were no significant differences in the expression or production of any of these markers between LTNP and RP/NP individuals.

Though perforin levels were not significantly different among the patient groups (Figure 5.11), production of perforin was significantly positively correlated with infection levels in LTNP, but not RP/NP. This may be suggestive that individuals in the LTNP group are better able to respond to infection, and as such, as p24 levels are higher, they are able to produce more perforin, which is a known anti-viral factor. Conversely, in the RP/NP group, there was no significant association with infection level and perforin production, but there was a trend toward a negative correlation, suggesting that within this group, the ability to produce perforin may be impaired as p24 levels increase and the immune system loses control of the virus (Figure 5.11).

When looking at T cell exhaustion with the PD-1 marker, again no differences were found overall between the LTNP and RP/NP group, but a significant negative correlation was observed between PD-1 expression and intracellular p24 in the LTNP group only (Figure 5.12). In this group, as p24 levels increase, the level of PD-1 on cells appears to decrease, which may be an indication that, as p24 levels increase, there is a higher level of cellular turnover, preventing cells from becoming exhausted and ineffective at controlling the virus. Indeed, a significant positive

correlation was found between Ki67 and the level of intracellular p24 within the LTNP group only (Figure 5.13), which supports the notion that higher cellular turnover in the face of higher levels of p24 may be preventing cellular exhaustion as indicated by PD-1 levels. The associations between PD-1 and Ki67 with intracellular p24 were only observed in LTNP, which may indicate an impaired functionality and lack of necessary cellular turnover in the RP/NP group.

Activation was assessed in this infection assay by staining for CD38, which is a chronic activation marker, and CD69, which denotes early activation in T cells. There were no differences in the expression of CD38 between LTNP and the RP/NP group (Figure 5.14), suggesting that both groups have a similar number of chronically activated CD8+ T cells. Conversely, there was a trend to a higher number of CD8+ T cells expressing CD69 in the RP/NP group than the LTNP group (Figure 5.15). Previous work has shown that a high level of cellular activation can make individuals more susceptible to HIV infection[138], [223], so it makes sense that CD69 may be elevated in the RP/NP group. In the LTNP group, CD69 expression was significantly positively correlated with the level of p24 expression, while in the RP/NP group, there was a significant negative correlation (Figure 5.15). This could suggest that in the LTNP group, individuals are likely to have a higher level of activation as p24 levels rise, perhaps suggesting that their CD8+ T cells are better able to react to the higher levels of infection. This is in contrast to the RP/NP group, who have lower activation levels as p24 levels increase, which may suggest their CD8+ T cells are less functional in response to increasing levels of infection, leaving them unable to control the virus.

6.3.4: Conclusions

Overall, these results confirm the hypothesis, and indicate that CD8+ T cells from LTNP are better able to control and inhibit p24 production than CD8+ T cells from RP/NP. This is evidenced by an enhanced ability to inhibit p24 both in its secreted and intracellular forms in *in-vitro* infection assays. CD8+ T cells from LTNP also appear to have a better functional capacity in the face of increasing p24 levels compared to RP/NP, as shown by an increase in perforin, an increase in cellular turnover, and a decrease in cellular exhaustion as p24 levels are elevated. These associations are not present in the RP/NP group, which indicates they may have an impaired CD8+ T cell functional response to infection, which could be driving their inability to control p24 levels to the same degree as LTNP.

In previous work in LTNP groups, it has been suggested that the enhanced capacity of LTNPs to inhibit HIV may be due to them being infected with replication incompetent strains, or strains which replicated more slowly[118]. One benefit of this in vitro infection assay is that it eliminates this possibility, as both patient groups are infected with the same, known viral strains. Also, as this work was conducted in individuals who all possessed HLA-B*57:03 alleles, is removes the association with this protective HLA allele as a potential mechanism for better p24 inhibition in LTNPs in this assay.

6.3.5: Limitations and future directions

There were some limitations of this section that should be addressed. Viral loads were not available, which would have been a useful baseline control to possess. However, since intracellular p24 staining was conducted on CD4+ T cells from each subject that were left uninfected, any residual p24 production from the individual's own viral infection would have been noted. As individuals in this study had to be ART-naïve in order to be included, sample choices were limited, especially in the RP/NP group, as there is a small window between diagnosis of infection and progression to the point of requiring ART. This small sample size meant it was not possible to correct for age or other potentially confounding factors.

There are many future directions that could be undertaken to add to the observations presented here. In this study, it was found that RP/NP individuals, when their CD4 counts remain relatively high, are still able to inhibit HIV to a level similar to that of LTNP individuals. In order to better understand how and when individuals in different progression groups lose the ability to inhibit HIV, it would be interesting to assess this ability again looking at previous LTNPs who have gone on to develop AIDS. It would be expected that even LTNP individuals, once their CD4s begin to decline and they progress to AIDS, would lose their ability to control the virus in a similar *in-vitro* assay.

This assay could be conducted again with some minor modifications, to allow the inclusion of a trans-well system. This system would allow for the physical separation of infected CD4+ T cells and effector CD8+ T cells, while still allowing

cytokines and chemokines to permeate the barrier between them. This would allow a better understanding of the CD8+ T cell mechanisms used to inhibit p24 in this case, whether they are related to anti-viral cytokine production or direct contact via a Fas-FasL pathway to induce apoptosis. Supernatants from these assays could also be tested for a panel of cytokines using a multiplex-based array, to gain an in-depth understanding of how these progression groups are different functionally while faced with infection of their CD4+ T cells.

An interesting future direction combines the two main parts of this thesis. While it was discovered in this work that CD8+ T cells from LTNP individuals inhibit p24 to a significantly higher degree than those from RP/NP individuals, it would be very interesting to assess how well CD8+ T cells specific to each epitope and variant differ in their inhibitory capabilities. This would require the expansion of large numbers of epitope-specific CD8+ T cells, and then a comparison of inhibitory capabilities in a similar assay as outlined in Chapter 5, which was unfortunately outside the scope of this work.

6.4 Overall Conclusions

The main objective of this thesis was to gain a better understanding of the complexities of the CD8+ T cell response in human HIV infection. The two overall hypotheses of this thesis were:

1) Consensus HIV epitopes will be more frequently recognized by CD8+ T cells in HIV-infected individuals than their naturally occurring variants. CD8+ T cell responses to consensus epitopes will be more proliferative and

polyfunctional, and be associated with better disease outcomes than responses to natural epitope variants.

2) CD8+ T cells from long-term non-progressors will be able to suppress viral replication more strongly than CD8+ T cells from rapid or normal progressors.

The first hypothesis of this thesis was rejected. It is clear from the studies done on the IF9 and TL9 epitopes in Chapter 3, and the IW9 and KF11 epitopes in Chapter 4, that the consensus status of an epitope does not reliably predict the resulting CD8+ T cell response. Furthermore, though CD8+ T cell responses to some of the studied epitopes were associated with higher CD4 counts, this was not predicated on whether or not the epitope in question was the consensus sequence or immunodominant.

The overall conclusion from the studies of these epitopes and their variants is that there are many factors that come together to impact the resulting CD8+ T cell response, including the TCR usage and presenting MHC, but also the strength of binding of the epitope to both the TCR and MHC, and, importantly, the specific epitope in question. Though it is known that the TCR and MHC and binding of the epitope to these sites is important in guiding the resulting CD8+ T cell response[125], [155], [162], this study highlights the importance of the specific epitope being presented in impacting the CD8+ T cell response. Through each epitope studied, except the KF11/KGF epitopes, it was observed that the CD8+ T cell responses were vastly different. The presenting MHC was the same for each set of epitopes, which controlled for that possible cause for the different responses. The

vast majority of diversity seen within HLA alleles is due to differences in the residues of the peptide binding grooves, as this is the region that is specific to the epitopes each HLA allele can present[162]. These differences can be crucial for the resulting CD8+ T cell response. For example, the HLA-B*58:01 allele has been found to associate with a low viral load, whereas HLA-B*5802 is associated with a high viral load, despite these alleles only differing by three amino acids[71]. This underscores the importance of HLA diversity, whereby even closely related HLA types can have vastly different peptide binding capacities, and associate with vastly different CD8+ T cell responses[162]. It is possible that the TCRs being used are different and having an impact, which is something that would need to be addressed in future work. Overall the CD8+ T cell responses to these epitopes and their variants were very diverse, which can be at least partially attributed to the small changes in amino acid content within each variant.

For a T cell vaccine to enjoy any success against HIV, it would need to target regions of the virus that are conserved and mutate infrequently, or that, upon mutation, render a large fitness cost to the virus[167]. Though some variable epitopes can be highly immunogenic, it is possible that these epitopes act as a sort of decoy. Despite their immunogenicity, this is no guarantee that the immune response directed against these epitopes will be beneficial against the virus, and may indeed serve only to redirect the immune response away from epitopes that may lead to a better anti-HIV response[167]. As such, it is important not only to understand the level of conservation and mutation of potential vaccine targets, but also to understand the specifics of the T cell response against them. Some epitopes may be better targets

while being subdominant, by virtue of being more conserved, and more likely to stimulate a proliferative, polyfunctional, cross-reactive, anti-viral CD8+ T cell response. The work presented in this thesis has highlighted the importance of appreciating how very similar epitopes can stimulate very different CD8+ T cell responses, and how this information and understanding is crucial before selecting epitopes as T cell vaccine targets.

The second hypothesis of this thesis was confirmed. CD8+ T cells form LTNP individuals within this cohort were better able to inhibit both secreted p24 and intracellular p24 than RP/NP individuals. Interestingly, while CD8+ T cells from LTNP were able to inhibit p24 both in its secreted and intracellular forms, the RP/NP group were able to inhibit p24 in its secreted form only. This may indicate that the mechanism of inhibition of p24 may differ between LTNPs and RP/NPs, whereby CD8+ T cells from LTNPs can inhibit p24 production when circulating and intracellular, the RP/NPs are only able to eliminate or reduce p24 that has already been secreted.

The major conclusions arising from this thesis that have added to the body of knowledge of HIV are as follows:

1) The consensus or immunodominance status of HIV epitopes does not appear to be an important factor that dictates the resulting CD8+ T cell response. Rather, the particular epitope being presented, the HLA allele presenting it, and the TCRs used to bind to it, may be of more importance.

2) Though the Gag TL9 M epitope is the consensus within clade A1 HIV strains, which make up the majority of infections in Kenya, the variant TL9 T epitope was more frequently recognized by CD8+ T cells, and associated with more frequent cytokine production, polyfunctionality, and proliferation than the consensus TL9 M epitope. Also, responses to TL9 T were associated with higher CD4 counts, which, combined with the known cross-reactivity to the TL9 M epitope, may suggest that this is an attractive epitope to investigate further as a potential vaccine target. However, before this epitope could be considered for use in any kind of vaccine or treatment as a target, there are many things that still need to be considered and identified, including its functional avidity, the rate and mechanism of mutations to the epitope variants studied here and others common in nature, the TCR usage, and it would also be interesting to identify any other HLA types that may present this epitope, and how immune responses differ in that context.

3) It was identified that some HIV-infected individuals who maintain high CD4 counts do not necessarily maintain beneficial functional CD8+ T cell responses. This lends support to the theory that some beneficial CD8+ T cell responses to HIV may be an effect, rather than a cause, of viral control.

4) CD8+ T cells from LTNP individuals in this cohort are better able to inhibit p24 *in-vitro* than CD8+ T cells from RP/NP individuals. This may be related to the ability of CD8+ T cells from LTNPs to respond to increases in p24 with higher secretion of antiviral factors, better cellular turnover, and lower levels of T cell exhaustion.

Overall, the research outlined in this thesis has expanded our appreciation for the importance of understanding CD8+ T cell responses to both consensus and variant HIV epitopes, as well as how progression groups play a role in HIV inhibition.

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Chapter 8: Appendices

List of Abbreviations

HIV-1: Human Immunodeficiency Virus-1
ART: Antiretroviral therapy
AIDS: Acquired Immune Deficiency Syndrome
CSWs: Commercial sex workers
IDUs: Injection drug users
STIs: Sexually transmitted infections
MSM: Men who have sex with men
SIV: Simian Immunodeficiency Virus
PrEP: Pre-exposure prophylaxis
TB: Tuberculosis
RT: Reverse transcriptase
RP: Rapid progressor
NP: Normal progressor
LTNP: Long-term non-progressor
EC: Elite controller
DCs: Dendritic cells
NK cells: Natural killer cells
APCs: Epitope presenting cells
MHC: Major histocompatibility complex
IFNγ: Interferon gamma
TNF: Tumour necrosis factor
IL-2: Interleukin 2
MIP1β: Macrophage inflammatory protein β
HLA: Human leukocyte epitope
TH1: T-helper type 1
TH2: T-helper type 2
GALT: Gut associated lymph tissue
PD-1: Programmed death 1
ELISAs: Enzyme-linked immunosorbant assay
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PHA: Phytohemagglutanin
DMSO: Dimethyl sulfoxide
CL2: Containment level 2
CL3: Containment level 3
TCID50: Tissue culture infectious dose 50
SAAP: Strept-avidin alkaline phosphatase
MOI: Multiplicity of infection
PMA: Phorbol 12-myristate 13-acetate
SEB: Staphylococcal enterotoxin B
ICS: Intracellular cytokine staining

FMO: Fluorescence minus one
PFA: Paraformaldehyde
CFSE: Carboxyfluorescein succinimidyl ester