The Exploration of the ‘Vaccinal’ Effect in HIV Infection and Remission



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A thesis submitted for the degree of

Doctor of Philosophy

Michaelmas 2024

Acknowledgements

The work submitted herein is a testament to the hard work on my behalf, but also numerous people who have provided me with invaluable inspiration and expertise. This body of work would not have been possible without the immense support afforded to me by both members of the Medawar and beyond.

I would like to thank my supervisors, John Frater, Philip Goulder and Ane Ogbe. I would also like to thank Susie Dunachie and Ellie Barnes for acting as both my transfer and confirmation of status assessors and providing me with constructive feedback on the progress of my DPhil project. I also want to thank all the principal investigators, staff and participants involved in the PITCH and SPARTAC trials, the data of which have formed a significant part of my thesis. Most importantly, I would like to specifically thank all the investigators, staff and participants of the RIO clinical trial, as well as Sarah Fidler and her team at Imperial College London who have been extremely supportive and encouraging. This amazing work would not have been possible without your mentorship for which I am eternally grateful. Thank you especially to Ming Lee for your mentorship support and helping with everything regarding the RIO trial.

Thank you also to everyone in the Frater lab for their support and advice: Mohammed Altaf, Dan Coneyworth, Penny Zacharopoulou, Lucia Parolini, Helen Brown, Nicola Robinson, Kahlio Mader, Nina Hardy, Devinder Srai, Carla Nel, Chanice Knight, Callum Beck, as well as previous members Matthew Jones, Matthew Pace and especially Ane Ogbe for making me a more rigorous scientist.

I would also like to thank everyone in the Medawar (current and previous) who has either helped me or been a positive beacon of light during my time here: Carl-Philipp Hackstein, Cecilia Jay, Jeremy Ratcliff, Nick Lim and Nick Herbert, Emily Adland, Daniel Hambidge, Thiruni Adikari, Cathy de Lara, Anna Csala, Priyanka Abraham, Sandra Adele, Mohammed Ali, Isabel Neale, Isana, Patpong Rongkard, Martha Zewdie, Barbara Kronsteiner-Dobramysl, Visa Nurmi, Julien Andreani, Andreas Huysman.

Thank you also to my mentors from The Chinese University of Hong Kong who have been supporting me since medical school, especially Prof. Justin Wu and Prof. Sunny Wong as well as the Esther Yewpick Lee and Diana Lee Wong Pui Yue Scholarships that have enabled me to study at such a world leading institution. A huge thank you to Sammi Lee, Nowell Wong, Kassia Cheung, Fish Chan, Delphine, Sandy Ming and Daphne Lai for handling all my scholarship related questions.

I would also like to thank my good friends and family for their unwavering support during the ups and downs of this DPhil, including George Tipoe and Emily Liong, Allan Lui, Andrew Kwok, Phil Xie, Christien Li, Kevin Lui as well as Beatrice Wong, Gabriel Sit, Tai Tsz Long and Jim Wing Hong for their spiritual support, and also Liam Carrick for your moral support over the last year.

Finally, thank you to my sister Eileen Tipoe for bearing with me for four and a half years; I will miss you dearly when I return to Hong Kong to continue pursuing my dreams of becoming a clinical researcher.

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Abstract

Treatment interruptions (ATIs) have been extensively used by studies to investigate therapeutic interventions that could induce an ART-free remission. Early studies suggested that sequential ATIs could induce an "autovaccination" effect on T cells leading to durable viral control off ART, however evidence for this is controversial and examining the immunological impact of ATIs on HIV-specific T cell immunity has not been fully explored. More recently, the passive transfer of broadly neutralising antibodies (bNAbs) in both animal and human studies have been associated with prolonged viral suppression during ATI as well as increased HIV-specific T cell immunity. However, it is unclear whether bNAbs exert a "vaccinal" effect on T cells and whether viral control is being mediated by T cells.

To explore the immunological impact of ATIs, we analysed HIV-specific T cell responses via interferon-gamma ELISpot in individuals with primary HIV infection from two cohorts that received different durations of ART: one cohort received greater than 2 years of ART (PITCH, n=7), while the other cohort received either 48 weeks of ART or were ART-naïve (SPARTAC). In SPARTAC, participants treated in PHI for 48 weeks made significantly more novel Gag responses between Weeks 24 and 60 in both number ($p=0.0137$) and proportion ($p=0.0001$). On the contrary, new responses in untreated participants off ART became less Gag-directed in both number ($p=0.0198$) and proportion ($p=0.0126$). In the more-intensively followed PITCH study, 6/7 participants showed dominant Gag and/or Pol-specific responses post-TI compared to pre-TI. Interestingly, one PITCH participant who remained virally suppressed throughout TI showed stepwise increases in Gag-specific responses until ART re-initiation.

Then, we analysed participants from the RIO clinical trial that received bNAbs followed by an analytical treatment interruption (ATI). We demonstrated that combination bNabs were associated with increases in Gag-specific T cell immunity in both Arm A and B participants, and these were observed up to Week 24 post-bNAbs in some cases. Additionally, we found significantly positive inter-assay correlations between different functional T-cell assays and showed that Gag CD4 proliferative responses at Week 24 in virally suppressed Arm A participants were associated with a reduced likelihood of time to viral rebound. Finally, we analysed selected Arm B participants that achieved lower viral load set points during the 2nd ATI, and showed that viral control coincided with enhanced Gag-specific T cell immunity. Notably, one participant achieved complete and spontaneous viral suppression during the 2nd ATI post-bNabs, which was accompanied by increases in Gag and Nef-specific T cell immunity; this was confirmed via subsequent epitope mapping.

In summary, this thesis demonstrates that ART treatment positively influences the potential to make novel Gag responses during ATI, and that combination bNabs are associated with favourable shifts towards Gag-specific T cell immunity that may be responsible for prolonged viral suppression. Further investigation is required to elucidate the mechanisms underlying this control, and how these could be harnessed to achieve durable immune-mediated control in the absence of ART.

Chapter 1-

Introduction

1.1 Introduction

The adaptive immune response plays a paramount role in defending the human body from viral pathogens and infection. Extensive research has been performed on the functions of T and B cells in host defense, and to understand immunological defects that arise in diseases such as human immunodeficiency virus (HIV).

The discovery of HIV, now known as the virus that causes acquired immunodeficiency syndrome (AIDS), first received public attention in June 1981 when the Centre for Disease Control and Prevention (CDC) published a report of five healthy men who had contracted pneumocystis pneumonia (PCP), an infection that usually manifests in severely immunocompromised individuals. All five individuals were men who had sex with men (MSM) and had abnormally low T cell counts (1,2). Following this incident, further reports emerged in July 1981 that documented 26 other cases of MSM who also presented with Kaposi sarcoma (KS), PCP or other opportunistic infections. As the incidence and mortality rate of this deadly disease increased, the CDC officially announced a new illness in September 1982, which is now known as AIDS (1).

At around the same time, scientists speculated that AIDS could have been caused by an infectious agent, such as retroviruses. This was suspected as retroviruses had been previously discovered in other immunocompromised patients with leukaemia, who also had similar impairments in T cell function. Using established T cell culture techniques, scientists isolated from T cells in the lymph nodes of patients with symptoms preceding AIDS (such as enlarged lymph nodes) and demonstrated that these retroviruses could infect T cells but not other cells (such as B cells or fibroblasts). Thus in 1986, scientists officially termed the retrovirus HIV-1, which is characterised by a preferential infection and progressive depletion

of T cells, the emergence of opportunistic infections and certain malignancies, as well as progression to AIDS (2).

Since its identification three decades ago, HIV-1 has continued to become widespread globally and has claimed over 39 million deaths to date. The main pandemic form of HIV-1 is known as the main (M) group, which has infected over 60 million people since its discovery (3). The spread of HIV-1 is primarily through three main routes: sexual, perinatal and intravenous, and it is estimated that around 80% of adults acquire HIV via mucosal exposure (4,5). Extensive research has been conducted to understand the origins and spread of HIV. The discovery of a similar but different virus in Western Africa in 1986 provided early clues about the origin of HIV. (6). Notably, the virus (now known as HIV-2), was found to be closely related to a simian virus that caused immunodeficiency in sooty mangabeys. Other simian immunodeficiency viruses (SIVs) were also discovered in various primates in sub-Saharan Africa such as chimpanzees and sooty mangabeys (7,8). Interestingly, these SIVs clustered together in a single phylogenetic lineage along with human and simian AIDS viruses, although they appeared to be relatively nonpathogenic in their hosts. Moreover, further phylogenetic studies reported that chimpanzees and sooty mangabeys harboured close simian relatives of HIV-1 and HIV-2 (9,10). Thus, it was established that SIV was in fact the primate precursor of HIV-1 and that HIV-1 and HIV-2 were caused by zoonotic transfers of viruses infecting primates in Africa, as well as cross-species infections with lentiviruses of different primates (3,11).

1.1.1 The Pathogenesis of HIV and Course of Infection

HIV is a retrovirus that requires its viral genome to be transcribed from RNA to DNA via the reverse transcriptase enzyme to be integrated into the host genome and subsequently undergo viral replication. HIV virions (or viral particles) are spherical and are around 60 times smaller than the T cells that it infects.

Within the viral capsid lies two copies of an RNA genome and viral enzymes that are required for establishment of infection in the cellular host (**Figure 1.1**)(12).

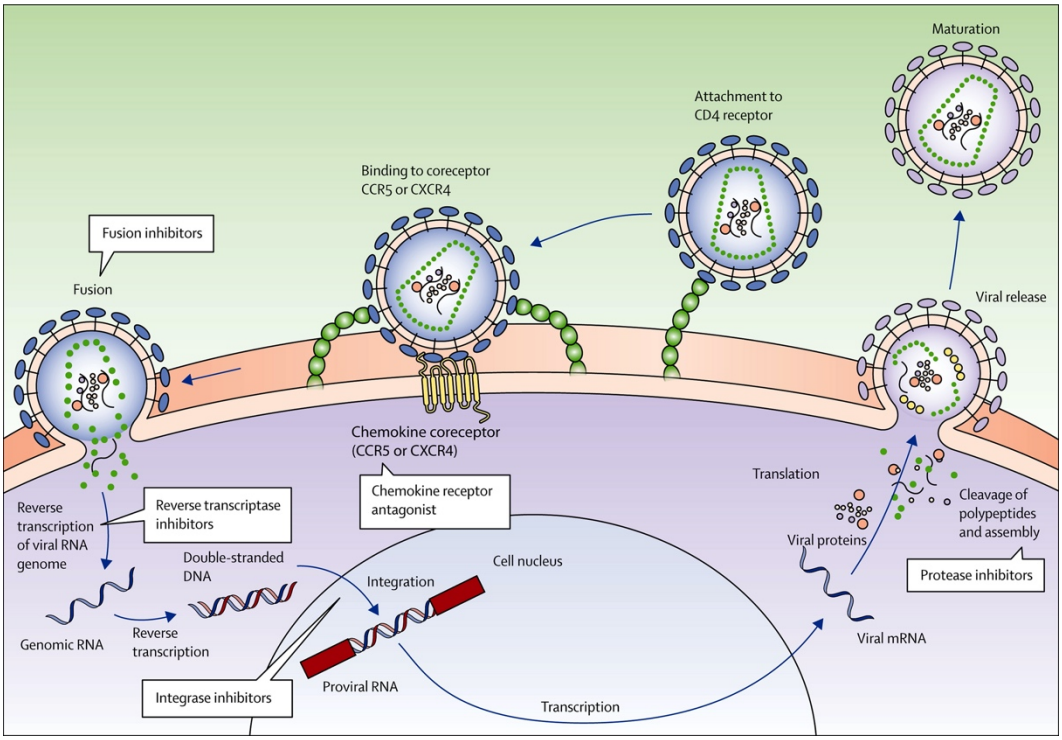


Figure 1.1. The pathogenesis of HIV entry and release from cells (adapted from (13))

HIV typically infects and replicates within activated cells of the immune system. The cells that it can infect are largely determined by its cellular tropism: the expression of specific receptors for the virus on cell surfaces. The major co-receptors required for HIV entry into cells are CCR5 and CXCR4; CCR5 is predominantly expressed on effector memory CD4 T cells, dendritic cells and macrophages, while CXCR4 is expressed mainly on naïve and central memory CD4 T cells. Thus, CD4 T cells are the most targeted cell for viral replication. HIV entry into cells relies on two viral glycoproteins, gp120 and gp41, which form trimers in the viral envelope. When gp120 binds to CD4 and its co-receptor, it induces a conformational change that exposes a high affinity site bound by the co-receptor which allows gp41 to unfold and fuse the viral envelope with cell membrane; this releases the viral core into the cytoplasm of the cell (12).

Subsequently, the viral core releases the RNA genome and reverse transcription of the viral RNA into complementary DNA (cDNA) occurs. This migrates to the nucleus where it integrates into the host genome with the help of viral integrase. The integrated cDNA is then called a provirus (12). As CD4 T cells are very long-lived in comparison to dendritic cells and macrophages, HIV can establish a reservoir of latent HIV provirus for years after initial infection (12). When CD4 T cells become activated, it induces expression of the transcription factors NFkB and NFAT, which bind to the proviral long terminal repeats (LTRs) and initiate transcription of the HIV genome. RNA transcripts are multiply spliced which allows translation of the early genes Tat and Rev. While Tat enhances proviral transcription and stabilizes RNA transcripts, the Rev gene binds these transcripts and transports them to the cytoplasm. Finally, the late proteins Gag, Pol and Env are translated and assembled into virus particles, which form infective HIV virions that bud off from the cell (12).

The first phase of HIV infection is the acute phase, which is characterised by an acute flu-like illness occurring in the first few weeks. In this phase, there is rapid viral replication occurring primarily in CCR5-expressing CD4 T cells. This is accompanied by high levels of virus circulating in the blood (viraemia) and rapid decreases in the number of CCR5-expressing CD4 T cells (12). This is followed by an adaptive immune response, which consists of cytolytic CD8 T cells that recognise viral antigens and kill HIV-infected cells, as well as the generation of virus-specific antibodies that become detectable in the serum of infected people (seroconversion). The cytolytic response subsequently results in a drop in viral titres and partial restoration of CD4 T cell counts; however, the virus remains uneradicated (12). At this juncture, the disease transitions to a latent, or asymptomatic, phase which is usually characterised by lower level viraemia (compared to primary HIV infection) and a slow decline in CD4 T cell numbers, which occurs over several years. During this period, the process of ongoing viral replication continues but is controlled by HIV-specific CD8 T cells and antibodies (12). After some years, the decline in CD4 T cell count is followed

by an increased incidence of opportunistic infections and symptoms. This is accompanied by a rise in viral load and eventual progression to AIDS, which is defined as a CD4 T cell count of less than 200 cells/ul (12).

1.1.2 Introduction of HAART and discovery of the latent viral reservoir

As the HIV pandemic intensified in the 1980s, scientists urgently sought a drug to treat the disease. The first breakthrough treatment was, in fact, a failed cancer therapeutic medication which had been marketed in the 1960s and was known as azidothymidine (AZT). This medication had previously been shown to be effective against the Friend virus, a retrovirus that causes leukaemia in rodents (1). Subsequent testing in animal studies showed it was able to potently inhibit reverse transcriptase activity in HIV, and was the first antiretroviral therapy to be approved in 1987 (1). However, clinical trials soon showed that AZT had numerous shortcomings, including side effects such as liver dysfunction, low blood counts, antiviral drug resistance due to viral mutations, and being incredibly costly (2). Later studies including the Concorde trial in 1993 also showed that AZT did not significantly affect clinical outcome – such as disease progression to AIDS – compared to placebo (14). A few years after the published findings, scientists discovered other more effective means of targeting the HIV virus; for example, by using the protease inhibitor saquinavir which interferes with the cleavage of viral polyproteins by viral protease, followed by another class of antivirals known as non-nucleoside reverse transcriptase inhibitors. Collectively, discovery of these drugs led to a new era of therapeutics known as highly active antiretroviral therapy (HAART) which officially became the standard of care for HIV in 1996 (15). This therapy involves treating HIV with a combination or “cocktail” of drugs that inhibit and interfere with the HIV viral life cycle. The striking benefits of giving HAART were evidenced by the rapid declines in viraemia after treatment, enabling infected individuals to maintain HIV RNA levels that were below the limits of detection (typically less than 20 copies/ml) for long periods of time while on therapy (1). Since then, HAART has drastically

reduced morbidity and mortality, as well as dramatically improved life expectancy of people living with HIV (1).

1.2 The latent viral reservoir

Despite being a revolutionary treatment for HIV, HAART also had numerous issues such as frequent side effects, drug related toxicities and being costly especially for individuals in low-income countries (16–18) . More concerningly, HAART was not a curative treatment. In early published studies, scientists reported that despite receiving HAART and maintaining undetectable plasma viraemia, purified CD4+ T cells that were isolated from people living with HIV still showed evidence of integrated proviral DNA (19). More importantly, in vitro activation of these cells rendered them capable of producing infectious virions (19). Soon after, other papers also reported that HIV-1 patients on HAART had resting CD4+ T lymphocytes that carried "replication-competent" virus; in other words, virus that is capable of reactivation, replication and production of infectious virions (20). This phenomenon later became widely known as the latent HIV reservoir, and despite the successes of HAART this reservoir of quiescent CD4+ T cells carrying proviral DNA remains an insurmountable barrier to an HIV cure to this day (21); termination of HAART almost always leads to rapid viral replication and rebound, and thus people living with HIV must remain on HAART indefinitely or for life (22).

Despite decades of research, the latent viral reservoir has not yet been fully characterised, and uncertainties remain on how it persists and what strategies could effectively reduce or eliminate it. Early studies demonstrated that, despite prolonged administration of HAART (21), the mean half-life of the viral reservoir was very long (43.9 months) and that eradication of this reservoir could take decades at the predicted rates of viral decay (21,23). Some initial studies hypothesised whether HAART could be

terminated in individuals with non-evolving latent viruses, citing evidence such as the study by Finzi et al. that showed that replication competent virus remained at stable and low frequencies in HAART-treated individuals (0.2-16.4 cells per million resting CD4 T cells); moreover, the recovered viruses did not show evidence of anti-viral drug resistance (24) However, this hypothesis was quickly disproven as subsequent studies showed that, even with prolonged viral suppression and more than 1 year on HAART, rapid viral rebound occurred within 2-3 weeks of ART discontinuation (22). Although very early initiation of HAART is unable to prevent the development of this pool of latently-infected cells (25), this still has a crucial influence on the size and stability of viral reservoirs and is an important intervention needed to achieve the goal of a functional cure (26) (see later sections).

1.2.1 Persistence of the viral reservoir

Since the discovery of the viral reservoir, researchers have attempted to characterise the dynamics of viral reservoir persistence. One key factor contributing to this persistence is that latent HIV-1 proviruses often reside in long-lived resting central memory CD4+ T cells , and these cells undergo a mechanism known as clonal expansion which is the major driving factor for HIV persistence (19,27). Recent studies have demonstrated that these expanded clones make up around 50-60% of the reservoir at any given time (28–30). An earlier study suggested that central memory and transitional memory T cells form the main cellular reservoirs of HIV but have divergent mechanisms of ensuring viral persistence. While central memory T cells maintain viral persistence via low-level antigen driven proliferation, IL-7 mediated homestatic proliferation drives the persistence of HIV in transitional memory T cells *in vivo* (31). This finding was also reported in similar studies looking at the production of HIV-1 in clonally expanded CD4 T cells (32). In addition, it was also suggested that co-infection with viruses such as EBV and CMV may result in antigen-driven proliferation of CD4+ T cells harbouring HIV-1 DNA (33). Recently, it has been agreed that to successfully reduce or eliminate the reservoir, the focus should be to target cells that harbour replication-

competent proviruses (29). Initially, it was reported that most cells undergoing clonal expansion carry defective proviruses rather than replication competent ones (34). However, later studies subsequently demonstrated the presence of clonally expanded cells that contained replication-competent proviruses, which meant that reservoir persistence was at least partially due to the proliferation of expanded clones (31–33,35). Notably, several studies have also confirmed that cells containing replication-competent proviruses may have different susceptibilities to induction than those with defective proviruses (28,35). This has been attributed to factors such as the chromatin environment of individual proviruses or the transcriptional and metabolic state of the CD4+ T cell subset harbouring latent virus (36). In fact, some studies showed that defective proviruses can be transcribed and translated, and may be preferentially targeted by cytotoxic T lymphocytes; thus, defective proviruses could still play a major role in shaping the proviral landscape (37).

1.2.2 Quantifying the viral reservoir

Extensive study has been done to characterise the cellular and anatomical reservoirs of HIV, which would be crucial for virus eradication. After undergoing local replication at the site of viral entry, HIV disseminates to the lymph nodes within a few days, and eventually the bloodstream after a couple of weeks (5). After penetrating the bloodstream, individual HIV virions become widespread across the entire body. While the lymphoid tissues – such as the spleen, thymus, lymph nodes and gut-associated lymphoid tissue) are the most important sites of viral replication in the context of active infection, HIV can also be found in many other organs, including the brain, lungs, kidneys and the bone marrow (38,39). Within these organs, HIV usually resides within T cells and macrophages, as well as other organ-specific cell types. With regards to the cellular reservoir, it is well established knowledge that HIV preferentially infects CD4+ T cells, and these reservoirs have been well characterised in virally suppressed patients on HAART (38,40,41). Of these CD4+ T cells, central memory CD4 T cells are thought to be the main source of the

HIV reservoir (40,41). Interestingly, different T cell subsets have been reported to harbour HIV to various extents and support viral persistence via different mechanisms (27,31). For example, while activated CD4 T cells are good targets for harbouring productive HIV infection, they are often short-lived due to cytotoxic T lymphocyte-mediated killing or cytopathic effects of the virus (42). Notably, the distribution of HIV-1 reservoirs in different compartments appears to influence the degree of natural control of the virus. For example, in patients who naturally control HIV to undetectable levels (elite controllers) and those that can maintain viral suppression after stopping ART (post-treatment controllers), skewed distributions were observed towards shorter-lived T cell populations such as transitional memory (TM) or effector memory (EM) cells. Some studies also reported that early ART initiation results in protection of certain T cell memory subsets such as central memory T cells, however evidence is fairly conflicting and this finding has not been consistently established by further studies (43–46). Aside from T cells, macrophages are a known target for HIV infection as they are not killed by viral cytopathic effects *in vitro* (38–41,47,48). Dendritic cells are also known to harbour HIV, given their role in presenting intact virions to T cells and subsequently becoming infected by them in the process (49–52).

Although considerable progress has been achieved on the characterisation of the latent reservoir, it is still hard to define due to the heterogeneity of patient populations used to study the reservoir (26). Moreover, there are difficulties in establishing the best method for quantifying the replication competent component of the reservoir. For many years, the gold standard for measuring the HIV reservoir was the quantitative viral outgrowth assay (QVOA). This involves quantifying the amount of infectious virus that is released from one round of activating latently-infected T cells (53). A statistical approach is then applied to estimate the number of latently-infected T cells in the original sample. While this was a highly sensitive method and had the advantage of detecting replication-competent virus, this method greatly underestimates the reservoir size as not all latently-infected T cells become reactivated within one round

of T cell activation. This method of quantification is also very costly and time-consuming (35). On the other hand, simple molecular quantitive PCR (qPCR) techniques for measuring HIV DNA are rapid and cost-effective; however, the main drawback is that this method is unable to distinguish between intact and defective proviruses (35,54). More recently, newer assays have been developed to accurately measure the intact proviruses and distinguish these from defective ones (54). In particular, the intact proviral DNA assay (IPDA) harnesses the use droplet digital PCRs with strategically placed probes in the packaging site and envelope regions to accurately distinguish between intact and defective proviruses (54). In addition to this, Gaebler et al. recently published a method that could accurately determine intact provirus. This utilises four qPCR probes covering the gag, pol, env and packaging signal region, followed by next generation sequencing of sequences that showed positive signals for at least two of the four probes (55). Overall, there is still no consensus on the best assay for measuring the HIV reservoir; however, it is likely that the most scalable strategy would likely involve a combination of proviral quantitation and sequencing data.

1.3 Approaches to an HIV Cure

1.3.1 Targeting the viral reservoir for an HIV Cure

Due to persistence of the latent reservoir, numerous attempts have been made to determine the best way to reduce, eliminate or eradicate this pool of latently-infected cells to achieve an HIV cure. One popular hypothesis in earlier years was known as the “shock and kill” approach; the theory was that latently infected cells remain transcriptionally silent, which allows them to evade immune recognition and clearance; thus, reactivation of latent virus via special latency reversing agents (LRAs) could make them susceptible to virus-induced cytolysis or clearance by immune cells (56,57). There are various LRAs that can increase viral gene expression *in vitro;* well-known examples include epigenetic modifiers such as

histone deacetylase inhibitors (HDACs) and toll like receptor (TLR) agonists that act on innate immune receptors. Of the HDACs, vorinostat, panabinostat and romidepsin have all been studied in clinical trials (58–62). The main issue with LRAs, however, is the inability to clear or "kill" infected cells once they have become reactivated. To date, most clinical trials involving HDACs have led to increased HIV RNA transcription, but no subsequent decline in HIV-infected cells (58,60–64), apart from one trial which combined HDAC administration with a T-cell vaccine and observed reductions in HIV DNA (via unexplored mechanisms) (65) . In addition, the more recent "RIVER" study failed to observe significant differences in mean total HIV DNA between groups receiving ART plus vorinostat and a T cell vaccine versus ART only (66). Thus, it appears that kick and kill approaches are unable to confer significant benefits over ART with regards to reservoir size. The failure to "kill" infected cells after the initial "shock" has been attributed to several possible reasons, including potential inhibition of CD8 T cell and natural killer (NK) cell function *in vitro* by HDACi's, expression of pro-survival proteins such as Bcl-2 in *in vitro* models, and persistent immune dysfunction and T cell exhaustion which impairs T-cell mediated clearance (67–70). Currently, some studies are investigating the use of combination LRAs from multiple mechanistic classes to achieve a more effective "shock" component (71–73).

In contrast to the "shock and kill" strategy, another strategy that has emerged is known as the 'block and lock' approach (74). This involves transcriptionally silencing all HIV proviruses to achieve a state of deep latency. HIV transcription can be regulated via a few different mechanisms; for example, the transactivator of transcription (Tat) protein plays an important role in facilitating transcriptional elongation and promoting open chromatin (75,76). For example, studies using involving mouse latency showed that a Tat inhibitor, didehydro-corstatin A (dCA), was able to delay viral rebound post-ART interruption compared to control mice (77). While these results suggest that inhibiting Tat-dependent transcription can prevent HIV reactivation, these findings have not yet been replicated in human trials.

Other factors affecting HIV transcription have also been discussed, including the regulation of transcription factors such as nuclear factor kappa-B (Nf-Kb) (78), as well as three-dimensional organization of chromatin and epigenetic factors such as DNA methylation in the long terminal repeat promoter region (79–82).

Despite the findings presented, clinical data is still lacking and none of the strategies have been able to achieve long term viral suppression in cell and/or animal models to date. Other problems with this strategy are also apparent, such as its scalability and being able to silence all replication-competent proviruses. In the near future, it is likely that a combination block-and-lock treatment would be more effective; also, this approach provides useful insights on how the translation-competent reservoir could be targeted, as defective proviruses still produce viral proteins and can induce significant chronic immune activation (83,84). Other cure approaches have included gene modification via CRISPR gene editing, however these cure strategies are still under investigation and have not yet entered clinical trials (85). To date, the only cases of successful HIV cure have resulted from haematopoietic stem cell transplantation using cells from donors that were homozygous for the CCR5 delta-gene mutation, thereby blocking HIV entry into cells (86–88). For the purposes of this thesis, however, this thesis will focus on the role of T cells in achieving an HIV cure.

1.3.2 The role of T cells for an HIV Cure

CD8 T cell responses to HIV

CD4 and CD8 T cells play important roles in maintaining the immune response to HIV, although in either case control of viraemia is not achieved apart from a few exceptional phenotypes. Previous research has suggested that early events occurring during acute HIV infection strongly predict disease progression and

has thus warranted further investigation into whether early CTL responses are indeed effective. In the early stages of infection (e.g Fiebig I and II), peak viraemia occurs and this can often be as high as 1-5 million viral copies/ml (89). This is followed an acute phase CD8 T cell response and subsequent decline in viraemia, usually to around 30,000 copies/ml and results in achieving what is known as the viral set point (89). Evidence shows that this response is usually narrowly directed and studies using autologous virus have shown that initial detectable responses tend to target between 1-3 epitopes, usually towards the Env or Nef region of HIV: these are followed by later waves of T cell responses to more conserved Gag p24 and Pol proteins (90–92). However, this CD8 T cell-mediated response is only partially effective, and this immune pressure leads to viral evolution and formation of escape variants (93,94). Evidence shows that CD8 T cell responses during primary HIV infection are major determinants of viral set point, and this is lost during the transition to chronic infection (95).

After the initial viraemic decline seen during acute HIV infection, CD8 T cell responses tend to broaden during the transition towards chronic infection (96), with the average individual targeting up to 14 epitopes simultaneously as evidenced from studies using reference peptides (although this number may be even 20-30% higher in the case of autologous ones) (90,97). Patients with progressive infection tend to exhibit higher magnitude and breadth of CD8 T cell responses than those with controlled infection (98). Regarding the specificity of responses, studies done in patients with chronic infection have shown that Gag targeting is associated with a lower viral load, and in people with clade C infection, a broader Gag and Env-specific response was associated with lower and higher viral loads respectively (99–102). Notably, human leukocyte antigen (HLA) molecules play crucial roles in recognizing and presenting HIV antigens to CD8 T cells (see later sections), and studies show that protective HLAs target relatively conserved regions in the Gag protein (103); subsequent mutations in this Gag p24 region can affect assembly of the viral nucleocapsid, and incur high fitness costs. While these “protective” CD8 T cell responses may induce loss

of viral fitness and subsequent viral control in the short term, the virus inevitably restores its virulence via compensatory mutations (93,103–106).

CD4 T cell responses to HIV

CD4 T cell responses provide integral help to facilitate CD8 T cell responses. However, CD4 T cells are depleted early on in infection, either by direct targeting by the HIV virus or due to bystander activation-induced cell death(107–109). Within memory CD4 T cell populations, HIV-specific ones tend to be preferentially infected and destroyed (110). Early studies have shown that CD4 T cell antigen-specific proliferative responses are reduced in both early and late infection, although HIV-specific CD4 T cell numbers are normally higher in early infection (111). In conjunction with this finding, very early administration of antiretroviral drugs may rescue CD4 T cell responses to HIV, and individuals with strong CD4 T cell responses make a slower progression to AIDS as well (112,113). With regards to epitope targeting, CD4 T cells seem to target more epitopes in Gag and Nef; however, far fewer epitopes have been defined in comparison to CD8 T cell responses (114). In addition, the less restrictive binding site of HLA-class II molecule allows for more promiscuity in the binding of peptides. In contrast to HLA-class I molecules, mutational escape is much rarer or even absent, and this has been potentially attributed to the fact that CD4s do not exert direct cytotoxic effects on HIV-infected cells and tend to interact with dendritic cells; however, the reasons for this are still unclear and additional studies are warranted to confirm this (115–117).

1.3.3 T cells and viral control in HIV

The role of human leukocyte-antigen (HLA) class I in HIV

Researchers have long studied the significant role of genetic factors in HIV disease progression. In particular, numerous studies have documented that certain HLA-restricted responses, especially HLA-class I, confer significant advantages in CD8-mediated pathogen defence. The protection conferred by HLAs is largely attributed to the extensive allelic polymorphism in the amino acid positions that determine specificity for foreign peptides, which are presented via HLA molecules to T-cell receptors (118,119). This diversity at specific peptide binding residues is thought to have arisen from natural selection over time, which ensures that the human species can resist an extensive variety of pathogens (120,121). In particular, HIV-infected individuals that are heterozygous at HLA class I loci are thought to present a greater variety of antigenic peptides to T cells than homozygotes, and subsequently produce a more effective immune response (122,123). This hypothesis was tested in one study that looked at 498 HIV-1 infected individuals with known dates of seroconversion, to determine whether HLA class I homozygosity was associated with delayed progression to AIDS in both Caucasians and African Americans. They showed that all three class I loci contributed independently to the association, and that the effects of homozygosity on delaying AIDS progression were more pronounced in one, two and three loci (124). These results were confirmed by a more recent analysis on 1060 individuals on the same AIDS cohort studies, as well as another study done in Dutch homosexual men and 202 Rwandan heterosexual women living with HIV-1 (125). This suggests that heterozygosity may confer an immune advantage by presenting a broader range of peptides, which subsequently delays the formation of escape variants.

Although forming genetic associations with HIV-1 disease has proven difficult due to the heterogeneity observed in the clinical course of AIDS progression, as well as the differential incidence of HIV-1 infection

in exposed individuals among different risk groups, data replicated in independent cohort studies have shown that certain class I alleles confer protection or susceptibility to AIDS progression (126–130). Most of the protective alleles are HLA-B restricted, which may be due to ability to resist Nef-mediated downregulation (131). Among all the HLA-class I alleles, numerous studies have reported consistent genetic and epidemiological associations with HLA-B*57 (132–136). While no mechanistic reason has been demonstrated, studies have reported that HLA-B*57 molecules can exhibit plasticity in binding by targeting multiple HIV-1 peptides, as well as having cross-reactivity to common and rare variants of dominant Gag epitopes (137). Further B*57-restricted peptides were primarily targeted as opposed to other HLA types in studies looking at long-term non progressors and slow progressors to AIDS (136,138). Another well-known protective HLA-class I allele is HLA-B*27. One study showed that some HLA-B*27 restricted responses target conserved regions in the p24 Gag protein, and that mutations in this region reduce B27 binding and accelerate progression to AIDS (139–141). Moreover, compensatory mutations in this region may be required to maintain viral replicative fitness. In addition, individuals with HLA-B*27 and HLA-B*57 have been shown to respond more robustly to vaccines against HIV-1, such as the ALVAC-HIV recombinant canarypox vaccine (although whether this confers protection or resistance against HIV infection is still uncertain). In addition, Pereyra et al. performed a genome-wide association analysis on a multi-ethnic cohort of elite controllers and non-progressors and found that the HLA type and viral interaction were major determinants of durable virological control (142). More importantly, persons restricted to HLA-B*27 had more polyfunctional CD8+ T cell responses.

On the contrary, other HLA-class I alleles – in particular, HLA-B*35 – has been consistently implicated in susceptibility to AIDS progression (124,143–145). In fact, studies involving Caucasians and African Americans demonstrated that rapid progression to AIDS is influenced by peptide specificity within specific subtypes of HLA-B*35 (132). For instance, the HLA-B*3501 and B*3503 molecules differ by a single amino

acid at position 116, and that substitutions occurring at this position have been previously associated with increased risks for transplant-related deaths (which demonstrates the clinical importance of amino acid variation at this site) (146–149). In terms of resistance to infection, there is less definitive research in this area due to difficulties in quantifying HIV exposure; however, some evidence has arisen from investigation of HLA supertypes: groupings of HLA molecules based on their structural similarity, peptide binding specificity and evolutionary affinity (150–152). For instance, the A2/A6802 supertype was shown to confer decreased perinatal HIV infection risk in a Nairobi mother-infant cohort (153). Another study done in highly exposed seronegative prostitutes in Gambia revealed that these individuals exhibited HLA-restricted CD8 responses to conserved epitopes, and that these associated HLA types were linked with resistance to infection in these women (154,155).

1.3.4 How T cells contribute to viral control in special phenotypes

Elite controllers (ECs)

Another key evidence for the relationship between HLA-class I alleles and viral control has been demonstrated in elite controllers (ECs) – individuals with HIV that are able to achieve an undetectable viral load in the absence of antiretroviral therapy. This phenotype of people who can “control” HIV is albeit quite rare and makes up less than 1% of HIV patients. Several studies indicate that the source of control in ECs is due to superior polyfunctionality of CD8 responses compared to progressors, including an efficient degranulation process and ability to release perforin, granzyme B and other cytokines such as

interferon-gamma and tumor necrosis factor (156,157). In addition, elite controllers have been shown to have enhanced proliferative capacities and expanded CD8+ T cell populations *in vitro*.

With regards to HLA alleles, studies have shown that ECs are particularly enriched in the protective alleles HLA-B*27 and HLA-B*57, and that these HLA-restricted responses are similar in both the blood and mucosal tissue (158,159). In both compartments, ECs showed a higher magnitude and skewed breadth towards Gag-specific responses, compared to progressors that had an even distribution among different epitopes (Gag, Env and Nef) (158). While protective HLA class I alleles are present in many ECs, it is not a necessary nor sufficient condition for viral control, and in fact one comparative study found that HLA-B*57/27 negative ECs could not be distinguished from HLA-B*57/27 progressors based on targeted epitopes alone. However, HLA-B*57/27 negative ECs still had higher levels of cytotoxicity than progressors regardless of HLA type, which likely demonstrates the driving reason for durable immune control in this subset of individuals. In addition, proliferative CD8 responses in ECs have been shown to preferentially target HIV epitopes that contain amino acids from highly interconnected regions of viral protein structure, regardless of HLA type. These topologically important regions of the viral proteome are constrained from mutation due to high viral fitness costs (160). Moreover, most ECs have extremely small proviral replication competent reservoirs with low sequence diversity, which suggests suppression of the functional reservoir (161). Further analysis of these features in ECs would be useful for developing subsequent HIV cure strategies and elucidating factors that strongly influence durable immune control.

Post-treatment controllers (PTCs)

In addition to spontaneous HIV control, some individuals that are treated with ART during primary HIV infection can maintain long term viral suppression for months or even years after ART interruption: these are known as post-treatment controllers (PTCs). Compared to ECs, the prevalence of PTCs are much higher

than ECs and comprise around 10-15% of the all people infected with HIV (162). Extensive work has been done to characterise the features of PTCs in comparison to ECs; for instance, the VISCONTI cohort which followed up 14 patients exhibiting PTC found that PTCs often had disease-susceptible HLA alleles compared to ECs which were enriched in protective ones (162). PTCs also had slightly higher viral load set points and poorer CD8 T cell responses than ECs and maintain very small reservoirs that consist mainly of transition memory T cell subsets. Other immunological features of PTCs include lower levels of CD4 and CD8+ T cell activation and exhaustion, as well as more robust Gag-specific CD4+ and natural killer (NK) cell responses (163). Virologically, PTCs have also been explored in the CHAMP study where they found that PTCs were likely to have transient viral rebound preceding subsequent viral control, with a heterogeneous durability of viral remission (164). Overall, the occurrence of PTCs is still fairly uncommon; however, understanding the mechanisms behind the capacity of PTCs to maintain durable viral control poses important implications on future HIV cure research (165).

1.4 Measuring the HIV-specific T cell response to HIV

T cell activation can be measured by a number of different functional T cell assays, depending on which readout is preferred for analysis. For example, one can measure T cell proliferation via a CellTrace violet proliferation assay, the upregulation of activation markers (such as CD25) using the activation-induced marker (AIM) assay and the production of effector cytokines via conventional interferon gamma (IFN-γ) enzyme linked immunosorbent (ELISpot) assays (1). Each assay has its own advantages and disadvantages. The advantage of the CTV assay is it provides a semi-quantitative readout which can differentiate between memory CD4+ and CD8+ proliferative responses, with a skew towards detecting CD4+ T cells which are more proliferative than CD8+ T cells. In addition, the longer incubation time (7 days in our protocol) allows for the measurement of measurement of very low-magnitude responses (167). The potential drawbacks,

however, are that clonal expansion of small subsets of cells may occur early on in the assay protocol, making the assay vulnerable to stochastic results.

In contrast, IFN-y ELISpot assays detect the secretion of IFN-y cytokines, and has long been regarded as a sensitive and validated method of quantifying HIV-specific T cell responses in HIV cure and vaccine studies (168,169). Another advantage of ELISpot assays is the ability to deconvolute positive responses using a matrix-based assay which has been described in previous literature. These assays have been previously used for measuring HIV-specific T cell responses in the setting of limited PBMC cell numbers, and enable a comprehensive analysis of T cell immunity while conserving the use of PBMCs for other functional T cell assays (170). Using the positive responses observed in the matrix-based assay, further deconvolution of specific peptide responses can be performed via ELISpots using single peptides (96,170). However, this assay also has drawbacks; due to cytokine-specific detection, it is biased towards CD4 T helper responses, which tends to underestimate the true CD4 response by missing key responses from cells such as T-follicular helper (Tfh) cells that are critical for the production of effective antibody responses (171,172).

To address limitations in these assays, the newer “AIM” assay has garnered significant interest. This is a predominantly CD4-based assay which overcomes the limitations of classical cytokine based assays by measuring the expression of TCR-stimulation rather than the production of cytokine. This makes it helpful in detecting cell subsets such as Tfh and Treg cells that are hard to detect via traditional cytokine-based methods such as intracellular cytokine staining (ICS) (168,173). Recent, a number of publications have described the use of validated AIM assays for the sensitive detection of antigen-specific CD4 T cells by gating on multiple AIM pairs (for example, OX40, CD137, CD69 and CD40-L and other various AIM markers) followed by application of a Boolean gating strategy to analyse the percentage of antigen-specific CD4 and CD8 T cells (174,175). With regards to the choice of AIM markers, there are variations in the markers

used for HIV-specific T cell responses; however, most published works have included a combination of either double positive CD4+/CD25+, CD137+ and/or CD69+, which are all well-described markers of T cell activation (175). For CD8 responses, however, the AIM assay is less widely used as a standard method; in our AIM assay, we incorporated the measurement of antigen-specific CD8 responses via a Boolean gating method which involves combination gating on double and triple positive CD25+, CD137+ and CD69+, so as to increase the sensitivity and specificity of detection. To reduce potential biases from individual assays, data presented in this thesis (where possible) incorporates results from all three assays in combination, to provide more functional readouts for interpretation.

In addition to functional T-cell assays, this thesis has also incorporated the use of immune phenotyping to assess expression of memory T-cell subsets, as well as specific activation and exhaustion markers that are commonly upregulated in HIV. Activated T-cells, when defined by the expression of both CD38 and HLA-DR, have been implicated in HIV pathogenesis and consistently correlate with HIV disease progression to AIDS (176,177) (both of these have been included in our phenotyping of activated T cells). Previous literature has also described that during primary HIV infection, there is an expansion of activated CD4 and CD8 T cells that are skewed towards an effector memory (EM) phenotype, and this is accompanied by increases in immune checkpoint receptor expression including markers such as programmed cell death protein (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) (69,178–184). In addition, the expression of (PD-1), Tim-3 and TIGIT during primary HIV infection (PHI) also correlates with disease progression, and despite ART initiation during PHI, this altered T-cell phenotype is only partially restored to a phenotype seen in healthy controls (185). In addition to gating on these exhaustion markers, further gating can be performed on memory T cell subsets using the transcription factor markers T-box transcription factor TBX21 (T-bet) and eomesodermin (EOMES). According to existing literature, the transcription factor T-bet is crucial in

directing Th1 lineage commitment and controls expression of interferon-gamma by Th1 cells. T-bet expression also correlates positively with interferon-gamma expression in Th1 and NK cells (186). With regards to EOMES, it is a paralogue of T-bet that is induced in CD8+ T cells and is responsible for driving differentiation of effector CD8+ T cells (187). In murine models involving chronic lymphocytic choriomeningitis virus clone 13 (LCMV-13) infection, T-cell exhaustion was shown to be associated with an inverse relationship with T-bet and EOMES (188,189). Moreover, recent evidence in human studies revealed that long-term non progressors exhibited high levels of T-bet expression (190). In addition, in chronic HIV infection it was found that persistent EOMES expression after ART initiation was longitudinally related to a transitional and exhausted CD8+ T cell phenotype, and that $Tbet^{dim}Eomes^{hi}$ virus-specific CD8 T cells exhibited poor polyfunctionality/effector functions (51). Thus, measuring these activation and exhaustion markers can provide useful readouts and clues regarding the phenotypic changes in T cell subsets over time.

1.5 The Limitations of T cells for an HIV Cure

Preventative T-cell vaccines

While (HIV-specific) CD4 and CD8 T cells in many cases have demonstrable benefits on viral control, other factors hinder the successful control of HIV such as the mutational diversity of the HIV genome. It is estimated that aligned HIV protein sequences can vary in about 10 to 40 percent of amino acid positions, even in the proteins Gag and Pol which are already relatively conserved at the epitope level (160,192–194). During acute HIV infection, cytotoxic T lymphocyte (CTL) responses play an important role in controlling viral replication (68); however, the strong selection pressure exerted by CTLs results in immune escape by the virus in an attempt to escape CTL recognition (90,139,195–199). The accumulation of escape mutations prevents the CTL response from controlling the infection, resulting in progression to

AIDS if left untreated with HAART (68). Another factor that makes T cells less effective is the development of immune exhaustion (90,196,197); in the context of chronic immune activation in HIV "progressors", for example, there is strong *ex vivo* activation of CD8+ T cells (193); however, in the context of chronic infection, there is an upregulation of surface exhaustion markers such as programmed death 1 (PD-1), which impairs the cytolytic and proliferative capacity of CD8+ T cells and leads to subsequent immune dysfunction (70,193,200,201). Evidence shows that blockade of specific exhaustion markers such as PD-1 can potentially reverse this immune dysregulation (201). In addition, administering early ART could potentially abrogate this decline in immune dysfunction, although recent studies demonstrated that this only partially normalises immune checkpoint receptor (ICR) expression (185).

Over the past few decades, numerous vaccine trials in HIV have been performed to investigate whether CD8 T cell responses could be harnessed for both preventative and therapeutic settings (202–211). The theory was that CD8 T cell-directed vaccines would effectively induce CTL responses to conserved regions in HIV where mutational escape is associated with high fitness costs, resulting in more durable viral control. While existing trials showed evidence of vaccine-induced T cell responses, few if any showed subsequent associations between these responses and vaccine efficacy. To date, the only HIV vaccine shown to have a modest efficacy against HIV prevention (31.2%) was shown in the Thai RV144 trial (212). In this trial, heterosexual individuals at risk of HIV received four priming injections of a recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]), followed by two additional booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E). In this study, robust humoral responses and high HIV-Env antibody titres were reported in vaccinees with higher IL-2 producing CD4+ T cells (213). In addition, most participants had highly polyfunctional CD4+ effector memory T cells that expressed CD107a, although these *ex vivo* T cell responses were much lower than the humoral response observed. Epitope mapping of the CD4+ T cell response demonstrated targeting of the V2 loop in the envelope protein as

opposed to Gag, and that this epitope has also been reported in other vaccine trials (214). Interestingly, the relative boosting of *ex vivo* CD4 T cell responses as opposed to CD8+ T cell responses has been reported in a number of studies involving DNA prime followed by poxvirus or adenovirus boost (215–219). An analogous regimen using HIV-1 subtype C strains was developed and was also shown to induce strong cellular and humoral responses (220,221). Vaccine-induced CD4+ T cell responses have also been correlated with virus control in SHIV vaccine challenge studies using ALVAC *gag-pol-env* prime/gp120 boost vaccine regimens (222).

Despite the modest successes of the RV144 vaccine trial in showing efficacy against HIV prevention, further phase II and III trials were unsuccessful in preventing HIV-1 acquisition. As a follow up of the ALVAC-gp120 vaccine regimen used in the RV144 trial, a larger phase IIb-III trial was undertaken to further determine vaccine efficacy in a South African epidemic setting; unfortunately, trial results did not demonstrate significant prevention of HIV compared to placebo, despite previous evidence of immunogenicity (220). Furthermore, another highly anticipated STEP study in 2008 demonstrated no significant reduction in HIV acquisition in the group receiving the Merck Ad5/HIV trivalent subtype B vaccine compared to placebo (223,224). Moreover, the T cell vaccine regimen failed to lower viral set point post-infection, despite eliciting strong HIV-specific CD8+ T cell responses (225). Other phase IIb vaccine trials such as HVTN505 used a DNA prime–recombinant adenovirus type 5 boost (DNA/rAd5) vaccine regimen, and also showed no overall efficacy in the prevention of HIV or lowering of viral set point in at-risk individuals (226). More recently, the phase IIb “Imbokodo” trial which incorporated the use of adenoviral vector mosaic HIV vaccine also showed a lack of preventative efficacy in young women in Sub-Saharan Africa.

Therapeutic T-cell vaccines

In recent years, several studies have examined T-cell vaccines as a therapeutic means of curing HIV, mostly by enhancing adaptive immune responses towards HIV antigen with or without reactivation of the latent reservoir. With regards to reduction of the latent reservoir, the recent RIVER study – which was the first randomised control study testing the kick and kill strategy - demonstrated that the administration of T-cell vaccines with vorinostat (an HDACi) was associated with increases in vaccine-induced, HIV-specific CD4 and CD8 responses; however, these did not significantly reduce HIV DNA or RNA in CD4+ T cells compared to the placebo arm (66).

Despite the reported shortcomings in HIV T cell vaccines, several key features have emerged as potential drivers of viral control in these studies which may require further investigation. For instance, high Env-specific CD8 T cell responses were associated with a reduced infection risk in a subset analysis of the HVTN505 trial (192,227,228), and while the STEP trial failed to demonstrate overall vaccine efficacy, there was evidence from follow up analysis that individuals making three or more Gag responses were associated with lower viral set points (although this was only observed in 5 of 72 recruited participants) (192,227). Furthermore, NHP challenge studies involving SHIV have consistently associated Gag-specific CD8 T cell responses with better control and survival (229–235). Another important factor to consider is ensuring adequate epitope diversity coverage by improving CD8 T cell vaccine antigens, as seen in the development of newer “mosaic” vaccines that use full length mosaic proteins in order to maximise epitope coverage and increase presentation of potential T-cell epitopes (PTEs) relative to natural proteins (202,236–238). Other vaccine approaches currently being tested include conserved-region vaccines which incorporate larger stretches of relatively conserved protein regions, or more recently the use of dendritic-cell based therapeutic vaccine strategies (202,236,237,237,239–242).

1.6 Inducing protective immunity – the autovaccination hypothesis

ART is not a functional cure, and treatment is associated with numerous issues such as ART-related toxicities, side effects including ART-associated drug resistance in some cases, and difficulties with adherence to therapy. Historically speaking, there was much difficulty in determining how to balance the adverse effects of ART combination therapy with its benefits. Questions were also raised as to whether ART should be given continuously or intermittently, and whether non-viral biomarkers could predict reservoir reduction or viral control as a major endpoint (243). Thus, analytical antiretroviral treatment interruptions (ATIs) were introduced to assess the in-vivo efficacy of therapeutic interventions, as well changes in host immunity, with the aim of achieving an ART-free remission (244). While researchers hypothesised that ATIs could reduce ART toxicity and enhance HIV-specific immune responses, this was quickly disproved by the SMART study which showed increased morbidity, mortality and disease progression in HIV-infected individuals that interrupted ART; in addition, these effects were largely attributed to differences in CD4 T cell counts and HIV RNA levels (245,246). Subsequently, there was a recommendation towards continuous ART administration with early initiation of treatment, as well extensive investigation into the safety of performing ATIs. In systematic reviews by Stecher and Lau et al., both meta-analyses showed that the incidence of treatment interrupted adverse events (AEs) was low and did not cause irreversible harm to study participants. Some of the factors associated with reduced risk of AEs were: baseline CD4+ T cell count >500, shorter periods off ART (<14 days had the lowest risk), narrower monitoring intervals and more conservative thresholds for ART re-initiation (245,247). These findings thus indicated that ATIs were a safe strategy to use in HIV cure trials if participants were closely monitored for viral rebound during ART interruption.

1.6.1 Studies on the autovaccination hypothesis

Since the introduction of treatment interruption studies, there has been a historical interest as to whether ATIs could in itself induce viral control after ART withdrawal, known as the autovaccination hypothesis. This was the theory that ART interruptions cause a re-exposure to HIV antigens, which stimulates an immune response that leads to lower levels of viraemia upon subsequent withdrawal of ART (248). To determine whether this hypothesis was true, several studies investigated the effect of consecutive cycles of HAART followed by treatment interruption in ART-naïve or ART-treated individuals with primary HIV infection. While it was demonstrated that STIs were safe and did not induce drug resistance, none of the studies showed a correlation between response and control of viral replication; rather, HIV-specific CD8 responses correlated with the degree of antigen exposure as evidenced by viral load (248). In a small study by Lori et al., two of the three ART-naïve participants that interrupted treatment had lower viral set points that were maintained for several months. However, the sample size was extremely small and the remaining participant did not show any evidence of persistent immune control (249). Other studies done in ART-treated participants with primary HIV infection also had similar results; for instance, Kaufmann et al. reported in an intention-to-treat analysis that only 8 of 14 participants treated during acute infection were able to sustain viral control under 5000 copies/ml for more than 180 days, and that the magnitude of HIV-specific responses did not predict duration of control (250). Similarly, Colby et al. found that in 8 participants that been treated during Fiebig stage I infection and remained on ART for a median of 2.8 years, all participants experienced rapid viral rebound of >20 copies/ml with the longest participant controlling for only 7 weeks (251).

Although structured treatment interruptions have been ineffective in achieving long term viral control in most cases, early and prolonged ART during primary HIV infection has been associated with a higher likelihood of viral control after treatment interruption, and as well as other beneficial effects on HIV-

specific T cell immunity. It has been demonstrated that early administration of ART (during primary HIV infection and soon after seroconversion) preserves the physical and functional properties of HIV-specific CD8 T cells as well as T-helper cell responses, which are usually damaged if left untreated (252). Evidence for this has been shown in studies such as Rosenberg et al. who reported that early-treated participants exhibited increased Gag-specific T-helper cell response, and that this was linked with viraemic control post-ATI (around 5000 copies/ml). Similarly, treatment-induced control of viraemia was found to be linked with development of strong T-helper cell responses and more narrowly directed CTL responses, and that these responses persisted up to 1-year post-ATI despite having undetectable viral loads (112,113).

1.7 Broadly neutralising antibodies (bNAbs) and the antibody response against HIV

The envelope glycoprotein (Env), which consists of a trimer made up of cleaved gp120-gp41 heterodimers, is the only antigen exposed on the surface of HIV-1 located on the surface of the HIV-1 virus and is thus a key target for HIV-specific neutralising antibodies (253). While HIV-specific antibodies are produced, antibodies that can neutralise autologous virus take roughly 12 weeks to develop after HIV transmission (254–256). Years after infection, only around 20% of individuals with HIV develop some degree of neutralising antibodies against heterologous virus (254,255,257). Historical studies have been performed to analyse early B cell responses to the transmitted virus in plasma donors; after the first few weeks of HIV infection, antibodies against the transmitted are detected in the form of antibody-immune complexes amidst increasing plasma viraemia (**Figure 1.2**).

Around 13 days after initial infection, Env-gp41 specific antibodies are detected in the plasma, followed by the appearance of Env-gp120 specific and non-neutralising Env-specific antibodies around 2 weeks

after (196). This autologous virus neutralising antibody response can exert initial immune control on the virus quasispecies present in the plasma; however, the narrowness of the response results in viral escape and formation of escape mutants (255,256). Moreover, the envelope protein has cleverly evolved to evade immune recognition via different mechanisms such extensive glycosylation, high variability, and steric occlusion of key epitopes (258). Unfortunately, induction of broadly neutralising antibodies (BNabs) towards conserved regions of the HIV-1 envelope – for example, the CD4 binding site and membrane proximal external region (MPER) – are very rare and, when they do occur, only develop after 20-30 months of infection (254,256,257,259). The reason for this is that bNAbs are required to possess unusual traits in order to successfully neutralise the envelope protein, such as long heavy chain complementarity determining region 3's (HCDR3), as well as undergo multiple rounds of extensive somatic hypermutation to select for improbable antibody mutations (as determined by structural and functional analysis) (260–264). It is thought that such traits are hindered by the lack of CD4+ T cell help, as well as B cell regulatory mechanisms such as self-tolerance (260). These obstacles have important implications on what constitutes a protective antibody response in HIV; in other words, priming of an early antibody response with broad specificity and neutralising capacity that results in effective control of early viral expansion.

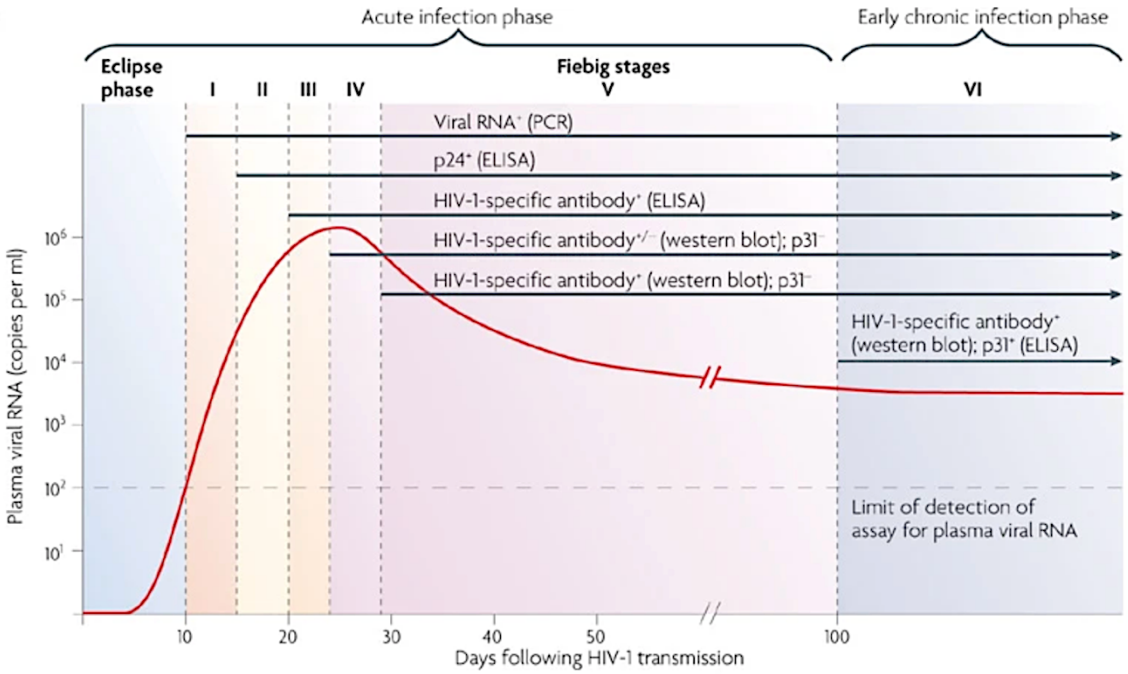


Figure 1.2. The different stages of acute and chronic HIV infection (adapted from (260))

Despite this, scientists have observed that a subset of individuals after primary HIV infection subsequently develop broadly cross neutralising responses which are able to recognise heterologous HIV-1 variants (254,262,265–270) . It is unclear why certain individuals develop these responses, however it may be attributed in part to persistent viral stimulation over time, as well the duration of infection and viral levels (269–272). Some studies suggest that an immunological hierarchy of neutralising antibody responses exist, whereby immunodominance is shaped by the emergence and fluctuation of escape variants (273). Additionally, it is thought that the epitope immunodominance of these escape variants changes over time, resulting in the exposure of more conserved regions and the subsequent induction of broadly cross-neutralising antibody responses (273). In fact, recent studies have identified several cohorts of individuals that possess these responses, which indeed suggests that this unique antibody response is likely crucial in controlling HIV-1 infection (257).

Currently, there is no exact definition for what is classed as a broadly neutralising antibody, and what distinguishes these from monoclonal antibodies (mAbs). However, studies have suggested several features that set bNAbs apart from mAbs. For example, bNabs undergo extensive somatic hypermutation during B cell development, which allows them to achieve a high neutralisation breadth by targeting highly conserved epitopes on the envelope protein (253). In addition, many of them exhibit polyreactivity to autologous molecules and have long CDR3 regions as previously mentioned (274). Also, they can penetrate the heavily glycosylated glycan shield in the envelope protein and bind to inaccessible epitopes that are otherwise unreached by normal mAbs. Compared to normal mAbs, bNAbs are effective against most circulating strains and can neutralise the more resistant "tier 2" and "tier 3" viruses (253,275). While the induction of autologous bNabs has been largely unsuccessful, advancement in single B cell cloning techniques have allowed scientists to isolate bNabs from rare subsets of individuals with HIV. The first generation bNAbs were isolated with the use of phage display technology in the 1990s. The potency and breadth of these early bNabs were not ideal and showed only moderate viral suppression with rapid immune escape in early clinical trials (207,276–278); however, newer generation bNAbs isolated from single cell cloning techniques have had a much higher breadth and neutralisation activity, and the safety, efficacy and immunogenicity of these are currently being assessed in clinical trials (279,280).

1.7.1 Mechanisms of action and the "vaccinal" effect

bNabs, like other antigen-specific antibodies generated from B cell clones, contain two important components: one is the antigen binding fragment (Fab) which binds to conserved epitopes on the HIV-1 envelope protein and prevents binding of HIV to the cell membrane. The other is the Fc region, which plays important roles in Fc-mediated effector functions (281–284); for instance, bNabs can opsonise viruses or viral antigens via their Fc domains, enabling their recognition by complement and Fc-gamma (Fcy) receptors expressed on innate immune cells such as NK cells. These Fcy-receptors can recognise

opsonised viruses and eliminate infected cells via complement, and antibody dependent cellular phagocytosis (ADCP) and antibody dependent cellular cytotoxicity (ADCC) (285–292). The role of FcyRs in clearing infected cells has been mentioned by several studies; for example, Lu et al. reported that in humanised mice models, mice treated with combination bNabs 3BNC117 and 10-1074 showed reduction in HIV-infected cell frequencies compared to mice receiving isotype control antibodies (293). When bNAb-treated mice were simultaneously administered an FcyR monoclonal antibody, the levels of HIV-infected cells were not significantly different from control mice, suggested that FcyRs had essential roles in clearing infected cells. Other human studies also showed that bNab-treated individuals showed evidence of increased ADCP, and spontaneous HIV controllers were found to be enriched with polyfunctional antibodies with enhanced capacities to drive ADCC (although whether this was the mechanism for viral control remains unclear) (294).

In addition to complement-mediated pathways, the crucial theory is that bNAbs can bind directly to viral antigen expressed on HIV-infected cells and form bNab-Env immune complexes (295). This attracts FcyRs that are expressed on antigen presenting cells, such as dendritic cells, which enhances antigen uptake and presentation and results in the elicitation of stronger and higher magnitude HIV-specific CD8+ T cell responses with higher cytotoxic activity (288). It is theorised that in this way, bNAbs can harness these immune mediated mechanisms to clear cell-free virions as well as stimulate antiviral immune responses, which is known as the “vaccinal effect” (164,248,250). This effect on HIV-specific T cells is thought to confer long term viral remission in the absence of ART, in comparison to ART interruption without the presence of bNabs which historically results in rapid viral rebound, viral persistence and subsequent ART re-initiation. However, this mechanism remains unproven and remains controversial.

1.7.2 Evidence for the "vaccinal" effect

The potential ability of bNabs to enhance HIV-specific T cell responses and induce subsequent T cell-mediated control has been shown in several animal studies involving SHIV-infected rhesus macaques. For example, in rhesus macaques with chronic SHIV that were given cocktails of HIV-specific monoclonal antibodies (PGT121, 3BNC117 and b12), host virus-specific neutralising antibody responses increased as well as the functionality of Gag-specific CD8 T cell responses as measured by CD8 T-cell dependent viral suppression assays (296). More evidence was reported in the landmark study by Nishimura et al.; in the study, 13 SHIV-infected rhesus macaques were given three-weekly intravenous infusions of the combination bNAbs 3BNC117 (targeting the CD4 binding site) and 10-1074 (targeting the V3 loop) on day 3 after challenge with SHIV. While the macaques showed varying levels of viral control, 6 of the macaques suppressed viraemia to undetectable levels. However, when a CD8-depleting mAb was administered to the 6 "controller" animals between weeks 48-131 after SHIV infection, rapid bursts of viraemia were documented in 5 of the 6 animals. Finally, an anti-CD8beta antibody (specific for CD8+ T cells) was administered to 3 of the controllers between weeks 136 and 150, which again resulted in immediate increases in plasma viraemia. In comparison to the "controller" animals, the control macaques treated with 15 weeks of ART became persistently viraemic upon ART interruption and none of the macaques achieved controller status (297). In an extension of this study by the same author, two more groups of SHIV-infected rhesus macaques received either bi-weekly combination bNAb therapy or bNAb therapy plus ART at 2 weeks post-infection to determine whether receiving bNAbs at a more clinically relevant time would be more effective (6 in each group). They reported that 7 of the 12 macaques achieved undetectable viral loads ("controller" status) which was followed by bursts of viraemia and loss of control upon the infusion of anti-CD8alpha mAbs (260). Taken together, this data suggests that passive combination bNAb immunotherapy, when initiated during acute infection, can induce potent CD8+ T cell immunity that is capable of durably suppressing viral replication.

Several papers have reported similar effects of bNAbs on viral control in HIV infection. In particular, the same combination of bNabs, 3BNC117 (which targets the CD4 binding site) and 10-1074 (which targets the V3 loop), have been tested in human studies. In the study by Mendoza et al, 9 HIV-positive, ART-treated individuals with antibody-sensitive HIV strains were given three infusions of combination bNAb therapy just before ART interruption, as well as at 0,3 and 6 weeks. Subsequently, the 9 enrolled individuals maintained viral suppression for a median of 21 weeks, and no individuals developed resistance to either antibody (299). Even more remarkably, some studies have demonstrated that combination bNabs (3BNC117 and 10-1074) are associated with the induction of HIV-specific T cell responses. In one study, Niessl et al. administered combination bNAbs to 9 ART-treated individuals at 0-, 3- and 6-weeks post-treatment interruption. They reported an increase in Gag-specific CD8 T cell responses between week 0 and 6/7, with increased polyfunctionality of CD4 cells expressing cytokines such as IFN-y and TNF-alpha. In addition, 4 out of the 6 participants with detectable IFN-y ELISpot responses had broader responses to Gag that were highly conserved and not seen at week 0 (173). In another study done in 59 ART-naïve individuals with HIV, the bNAb 3BNC117 was given with or without romidepsin (a histone deacetylase inhibitor and latency reversing agent), or romidepsin alone, after ART initiation. This was followed by an optional ATI. They found associated increases in Gag and Pol-specific CD8+ T cells in the group that received 3BNC117 at 3 and 12 months post-ART initiation, compared to the control group that received ART only. Furthermore, Gag-interferon gamma release was also elevated at 12 months after ART initiation, and this was shown to predict ART-free virologic control (300). Combined, these studies provide evidence that bNabs could enhance HIV-specific T cell responses and subsequently induce durable immune control in the absence of ART.

1.8 Aims of this Thesis

Via the use of peripheral blood mononuclear cells (PBMCs) collected from individuals that are recruited from the PITCH and SPARTAC cohorts, as well as peripheral blood samples obtained from individuals recruited from the RIO clinical trial, this thesis aims to:

1. Explore the effects of treatment interruption alone on HIV-specific T cell immunity in both ART-treated and ART-naïve primary HIV infection (Chapter 3)

2. Investigate the effects of bNAbs on HIV-specific T cell responses during treatment interruption in a placebo-controlled study in the context of ART-treated primary HIV infection (Chapter 4)

3. Examine the immune mechanisms underlying spontaneous viral control post-bNabs in selected ART-treated participants with primary HIV infection (Chapter 5)

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Chapter 2-

Methods

2.1 Sample Processing

Whole blood samples were transported from participant recruitment sites to an academic laboratory and processed on the same day. Peripheral blood mononuclear cells (PBMCs) were isolated as described similarly elsewhere (1). Briefly, whole blood samples were spun down via high density gradient centrifugation using Lymphoprep (1.077 g/mL, Stem Cell Technologies, Canada). Plasma was collected and stored at -80 for further use. PBMCs were spun down twice using R0 (RPMI 1640 [Sigma, USA] 2mM L-glutamine, and 1mM penicillin/streptomycin [Sigma, USA]). Cells were resuspended in R0 and counted using the Bio-RAD automated cell counter (Bio-RAD, USA). After counting, PBMCs were frozen down at an estimated 10 million cells per cryovial, using freezer mix containing 90% heat inactivated foetal calf serum (FCS) and 10% DMSO (Sigma, USA) and stored in -80 overnight. PBMCs were cryopreserved in liquid nitrogen the following day. For experimental use, PBMCs were thawed in a 37°C water bath and transferred into 30ml of pre-warmed R10 (RPMI 1640 [Sigma, USA] containing 10% heat inactivated foetal calf serum [FCS], 2mM L-glutamine, and 1mM penicillin/streptomycin [Sigma, USA]), then spun down and resuspended in 2ml of R10 for counting.

2.2 ELISpot assays

2.2.1 Peptide pool ELISpot assays – PITCH Cohort

Cryopreserved PBMCs were used for analysis via ex vivo Interferon gamma (IFN)-y enzyme-linked immunosorbent spot (ELISPOT) assays. Peptide pool ELISpots were performed for PITCH cohort participants only. HIV-specific T cell immunity to peptide pools spanning the whole HIV genome [47]- Gag, Polymerase, Accessory, Envelope and Nef (NIH Reagent Program)- was assessed as described below **(Table 2.1).** Cells stimulated with peptide pools were plated at 200,000 cells per well, as well as the negative controls. This concentration was used to maximise the responses observed at the peptide pool level as an

initial benchmark (as responses were robust, further downstream ELISpot assays were subsequently plated at 50,000 cells per well). In order to conserve cells, positive control wells were plated at a concentration of 50,000 cells per well for the peptide pool ELISpots. The concentration of DMSO (Sigma, USA) used was the same as the peptide-stimulated wells to account for DMSO-specific background activation **(Figure 2.1).**

Peptide pools (2ug/ml per peptide)	**Positive controls**	**Negative controls**
200,000 cells/well	50,000 cells/well	200,000 cells/well
○ Gag ○ Pol ○ Acc (Vif + Vpr + Tat) ○ Env and Nef	○ Phytohaemagluttinin (PHA) (Sigma, USA) 5ug/ml ○ CMV lysate (Virusys, USA) 2ug/ml ○ FECT (peptide pool containing flu, EBV, CMV and tetanus) (GenScript, UK) 2ug/ml	○ DMSO (Sigma) 0.2% ○ Negative (cells + RAB10, no peptide added) ○ RAB10 media only

Table 2.1. Description of reagents used for ELISpots using peptide pools, including cell numbers per well and use of positive and negative controls respectively.

	Timepoint #1				Timepoint #2				Timepoint #3			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Gag			DMSO	Gag			DMSO	Gag			DMSO
B	Pol			DMSO	Pol			DMSO	Pol			DMSO
C	Acc			DMSO	Acc			DMSO	Acc			DMSO
D	Env			Neg	Env			Neg	Env			Neg
E	Nef			Neg	Nef			Neg	Nef			Neg
F	PHA			Neg	PHA			Neg	PHA			Neg
G	FECT			RAB10 only	FECT			RAB10 only	FECT			RAB10 only
H	CMV			RAB10 only	CMV			RAB10 only	CMV			RAB10 only

Figure 2.1. Example ELISpot plate layout using peptide pools. Different colours represent different conditions tested including positive (PHA, FECT, CMV) and negative (DMSO, Neg, and RAB10) controls respectively.

ELISpot protocol- Peptide pools

Briefly, 96-well Multiscreen-I plates (Merck, UK) were coated overnight with 100ul of 10 µg/ml anti-human IFN-γ (Mabtech AB, Sweden) at 4 degrees. For peptide pool ELISpots, cryopreserved PBMCs were added in triplicate wells at 2 x 10^5 cells in 100µl per well and stimulated with 60µl of HIV peptide pools (2 µg/ml per peptide) as indicated in the figure legends and controls. Filtered RAB10 (RPMI [Sigma, USA] supplemented with 10% heat inactivated human Ab serum [Sigma, USA]) with DMSO (final concentration 0.2%, Sigma USA) was used as the negative control and the following reagents were used as positive controls: FECT peptide pool (2 µg/ml final concentration, GenScript UK), CMV lysate (Virusys, USA) and Phytohaemagluttinin A (5 µg/ml final concentration, Sigma USA). Cells were incubated for 18 hrs at 37 °C, 5% CO_2, and 95% humidity. After incubation, cells were removed and secreted IFN-γ was detected by adding 50ul of 1 µg/ml anti-IFN-γ biotinylated mAb (7-B6-1-biotin, Mabtech Ab Sweden) for 1.5 hours, followed by 50ul of 1 µg/ml streptavidin alkaline phosphatase for 1 hour (Mabtech Ab, Sweden). The plates were then developed using 50ul of BCIP/NBT substrate (Mabtech Ab, Sweden) according to the manufacturer's instructions and scanned using a plate reader (CTL Immunospot, USA) using the same machine settings each time (diffuse processing normal, spot separation = 3, background balance = 80). Results were background subtracted using the mean spot-forming units (SFU) from the DMSO wells and reported as SFU per million PBMC. The threshold for background in DMSO-stimulated wells was set at 50 SFU/10^6 PBMC; any plate with background higher than this value was repeated, subject to sample availability.

2.2.2 ELISPOT Screening/Matrix-based assays

Matrix Design- PITCH study and RIO clinical trial

Matrix-based ELISpot assays were performed to investigate the immune-dominance of HIV-specific responses at a minipool level. To design the megamatrix, 742 individual peptides (15-mers length with 11 amino acid overlap) spanning the entire HIV Clade B genome were used (Gag, Pol, Env, and Nef), apart from Vpr, Tat, Vpu and Vif which were ordered as separate pools (NIH reagent program, USA). The decision to include the accessory proteins as separate pools was made on the basis that accessory protein responses have been less well-documented in existing literature, as well as limited cell availability. More importantly, Gag and/or Pol-specific responses have been frequently associated with viral control in previous literature so a focus on these peptide pools was more important and relevant for the aims of this thesis (2–5). However, this data was still included as pools on the plate design. With the exception of Vpr, Tat, Vpu and Vif, HIV overlapping peptides were combined in pools consisting of 11 to 13 peptides/pool. Each individual peptide was represented in two separate pools, such that a positive response in two corresponding pools indicated a response to a specific peptide **(Tables 2.2-2.4, Figure 2.2),** and also functions as an internal positive control. This peptide would then be taken forward for further ELISpots on single peptides.

In addition to the peptide stimulated wells, the following control wells were plated in duplicate: PHA (Sigma, USA) was used as a positive control and CMVpp65 (NIH Reagent Program, USA) and FECT (Genscript, UK) were used as antigen TCR controls respectively, and DMSO was used as a negative control. Notably, the FECT peptide pool was only used for the separate leukocyte cone control. To provide a healthy negative control for the matrix-based assays, a leukocyte cone was taken from a HIV-negative anonymised blood donor. PBMCs from this leukocyte cone were run along with HIV-infected PBMCs in

each experiment, using the controls listed above. To generate a benchmark mean response, the leukocyte cone control was run with positive controls PHA, FECT and CMV in ten independent experiments. Thus, if the response from the leukocyte cone control in any assay was greater or less than 30% of the benchmark mean response, the ELISpot assay was repeated for consistency.

Protein	Peptides	Format
Env	211	Megamatrix
Gag	123	Megamatrix
Pol	249	Megamatrix
Nef	49	Megamatrix
Vpr	22	Pool
Tat	23	Pool
Vpu	19	Pool
Vif	46	Pool

Table 2.2. HIV clade B peptide pools used. Pools containing Gag, Pol, Env and Nef were included in a megamatrix pool format, whereas Vpr, Tat, Vpu and Vif were included as separate peptide pools respectively.

POOLSET	NUMBER OF POOLS		Protein
POOLSET 1	22 pools	Pools 1-22	ENV 1-121
POOLSET 2	22 pools	Pools 23-44	ENV 122-211; GAG 1-31
POOLSET 3	22 pools	Pools 45-66	GAG 32-123; NEF 1-29
POOLSET 4	22 pools	Pools 67-88	NEF 30-49; POL 1-101
POOLSET 5	24 pools	Pools 89-112	POL 102-249

Table 2.3. HIV clade B peptide pools used with poolset numbers and corresponding protein

Pools:	1	2	3	4	5	6	7	8	9	10	11	Pools:
	ENV 1	ENV 2	ENV 3	ENV 4	ENV 5	ENV 6	ENV 7	ENV 8	ENV 9	ENV 10	ENV 11	12
	ENV 12	ENV 13	ENV 14	ENV 15	ENV 16	ENV 17	ENV 18	ENV 19	ENV 20	ENV 21	ENV 22	13
	ENV 23	ENV 24	ENV 25	ENV 26	ENV 27	ENV 28	ENV 29	ENV 30	ENV 31	ENV 32	ENV 33	14
	ENV 34	ENV 35	ENV 36	ENV 37	ENV 38	ENV 39	ENV 40	ENV 41	ENV 42	ENV 43	ENV 44	15
	ENV 45	ENV 46	ENV 47	ENV 48	ENV 49	ENV 50	ENV 51	ENV 52	ENV 53	ENV 54	ENV 55	16
	ENV 56	ENV 57	ENV 58	ENV 59	ENV 60	ENV 61	ENV 62	ENV 63	ENV 64	ENV 65	ENV 66	17
	ENV 67	ENV 68	ENV 69	ENV 70	ENV 71	ENV 72	ENV 73	ENV 74	ENV 75	ENV 76	ENV 77	18
	ENV 78	ENV 79	ENV 80	ENV 81	ENV 82	ENV 83	ENV 84	ENV 85	ENV 86	ENV 87	ENV 88	19
	ENV 89	ENV 90	ENV 91	ENV 92	ENV 93	ENV 94	ENV 95	ENV 96	ENV 97	ENV 98	ENV 99	20
	ENV 100	ENV 101	ENV 102	ENV 103	ENV 104	ENV 105	ENV 106	ENV 107	ENV 108	ENV 109	ENV 110	21
	ENV 111	ENV 112	ENV 113	ENV 114	ENV 115	ENV 116	ENV 117	ENV 118	ENV 119	ENV 120	ENV 121	22

Table 2.4. Example layout of peptide megamatrix used for Env. Each individual peptide is represented in two pools. For example, a response in pools 1 and 12 in the above matrix would suggest a response to ENV 1 region in the envelope protein. Each code (e.g ENV 1) corresponds to a specific amino acid sequence provided by the NIH reagent program database and allows identification of the specific peptide response.

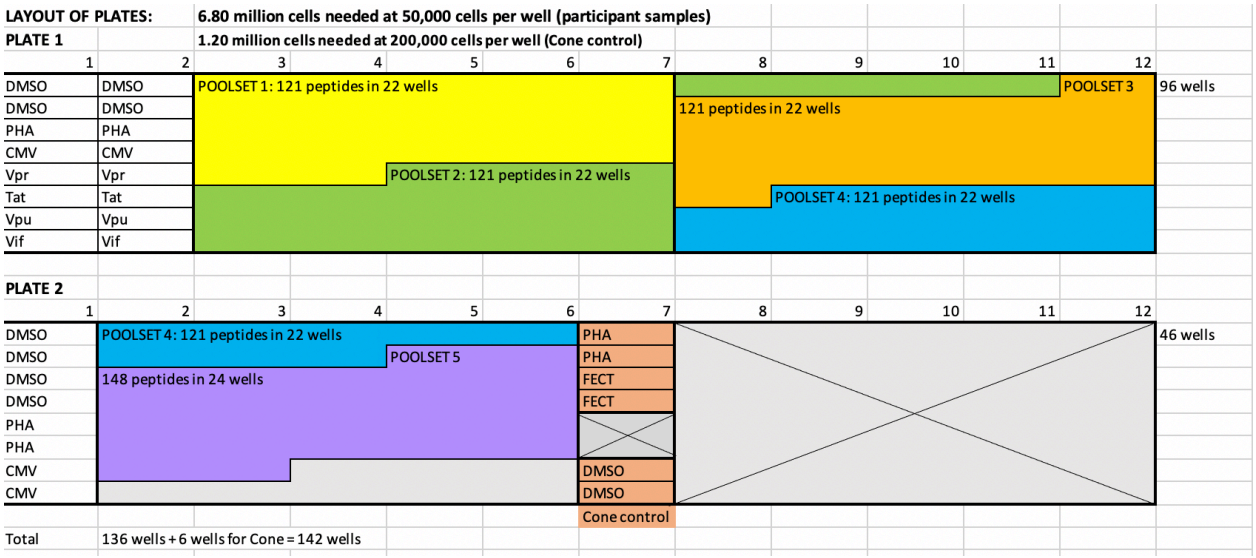


Figure 2.2. 13 peptides per pool were included in the matrix. Each peptide was represented in two separate pools which allows for the deconvolution of single overlapping peptide responses in a separate ELISpot assay. Positive (PHA, CMV) and negative (DMSO) controls were included in both plates. In addition, plate 2 included a reference control obtained from leukocyte cones as a measure of inter-assay variability; positive (PHA, FECT) and negative (DMSO) controls were tested on this reference control.

Matrix Design- SPARTAC trial

The data acquired from ELISpot assays for SPARTAC were collected prior this thesis back in 2013. Acknowledgments of laboratory staff who performed these assays are listed at the beginning of this chapter.

To design the megamatrix, 181 subtype B optimal peptides were used covering the Gag (n = 67), Pol (n = 47), Nef (n = 30) and Env (n = 37) proteins. These were derived from the "best-defined 'A list' CTL epitope" database (Los Alamos HIV Immunology Database). In addition to this, 123 Gag overlapping peptides covering the HIV consensus B sequence were also tested in a separate matrix layout. Each peptide was represented in two different pools as seen in the PITCH and RIO matrix-based assay protocols **(Figure 2.3-2.4).**

	PLATE LAYOUT:											
	OLP POOLS				**OPTIMAL POOLS**					**VARIANT POOLS**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	A	I	Q	Y	B1	B9	C8	D7	E5			
B	B	J	R	Z	B2	C1	C9	D8	E6			
C	C	K	S	AA	B3	C2	D1	D9	E7			
D	D	L	T	AB	B4	C3	D2	D10	E8			
E	E	M	U	AC	B5	C4	D3	E1	E9			
F	F	N	V	AD	B6	C5	D4	E2	E10			
G	G	O	W	AE	B7	C6	D5	E3	PHA CTRL			
H	H	P	X	AF	B8	C7	D6	E4	PHA CTRL	NEG CTRL	NEG CTRL	NEG CTRL

Figure 2.3. SPARTAC mega-matrix layout.. Each pool contains 8-9 peptides, consisting of either overlapping or optimal peptides. PHA and DMSO were used as positive and negative controls respectively.

INTERPRETATION:

OLPS:

POOL	I	J	K	L	M	N	O	P	POOL	Y	Z	AA	AB	AC	AD	AE	AF
A	1	9	17	25	33	41	49	57	Q	65	73	81	89	97	105	113	121
B	2	10	18	26	34	42	50	58	R	66	74	82	90	98	106	114	122
C	3	11	19	27	35	43	51	59	S	67	75	83	91	99	107	115	123
D	4	12	20	28	36	44	52	60	T	68	76	84	92	100	108	116	
E	5	13	21	29	37	45	53	61	U	69	77	85	93	101	109	117	
F	6	14	22	30	38	46	54	62	V	70	78	86	94	102	110	118	
G	7	15	23	31	39	47	55	63	W	71	79	87	95	103	111	119	
H	8	16	24	32	40	48	56	64	X	72	80	88	96	104	112	120	

OPTIMALS:

POOL	C1	C2	C3	C4	C5	C6	C7	C8	C9
B1	GAG1	GAG10	GAG19	GAG28	GAG37	GAG46	GAG55	GAG64	POL6
B2	GAG2	GAG11	GAG20	GAG29	GAG38	GAG47	GAG56	GAG65	POL7
B3	GAG3	GAG12	GAG21	GAG30	GAG39	GAG48	GAG57	GAG66	POL8
B4	GAG4	GAG13	GAG22	GAG31	GAG40	GAG49	GAG58	GAG67	POL9
B5	GAG5	GAG14	GAG23	GAG32	GAG41	GAG50	GAG59	POL1	POL10
B6	GAG6	GAG15	GAG24	GAG33	GAG42	GAG51	GAG60	POL2	POL11
B7	GAG7	GAG16	GAG25	GAG34	GAG43	GAG52	GAG61	POL3	POL12
B8	GAG8	GAG17	GAG26	GAG35	GAG44	GAG53	GAG62	POL4	POL13
B9	GAG9	GAG18	GAG27	GAG36	GAG45	GAG54	GAG63	POL5	POL14

POOL	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
D1	POL15	POL25	POL35	POL45	ENV8	ENV18	ENV28	NEF1	NEF11	NEF21
D2	POL16	POL26	POL36	POL46	ENV9	ENV19	ENV29	NEF2	NEF12	NEF22
D3	POL17	POL27	POL37	POL47	ENV10	ENV20	ENV30	NEF3	NEF13	NEF23
D4	POL18	POL28	POL38	ENV1	ENV11	ENV21	ENV31	NEF4	NEF14	NEF24
D5	POL19	POL29	POL39	ENV2	ENV12	ENV22	ENV32	NEF5	NEF15	NEF25
D6	POL20	POL30	POL40	ENV3	ENV13	ENV23	ENV33	NEF6	NEF16	NEF26
D7	POL21	POL31	POL41	ENV4	ENV14	ENV24	ENV34	NEF7	NEF17	NEF27
D8	POL22	POL32	POL42	ENV5	ENV15	ENV25	ENV35	NEF8	NEF18	NEF28
D9	POL23	POL33	POL43	ENV6	ENV16	ENV26	ENV36	NEF9	NEF19	NEF29
D10	POL24	POL34	POL44	ENV7	ENV17	ENV27	ENV37	NEF10	NEF20	NEF30

Figure 2.4. SPARTAC Matrix pool design. Each pool contains 8-9 peptides (overlapping or optimal peptides) and were added to both vertical or horizontal pools. Each peptide is represented in two pools enabling identification of the respective peptide-specific response; for example, a response in pools B5 and C4 indicates a likely response to GAG 32, which corresponds to a specific region in Gag as described in NIH database.

ELISpot protocol- Matrix-based assay

For screening ex vivo ELISpots in the PITCH and RIO studies, cryopreserved PBMCs were added at 0.5 x 10^5 cells in 100ul per well and stimulated with 60µl of HIV peptide pools (2 µg/ml per peptide). Filtered RAB10 with DMSO (final concentration 0.07%, Sigma USA) was used as a negative control and the following were used as positive controls: FECT peptide pool (2 µg/ml, Genscript UK), CMVpp65 (NIH

Reagent Program, USA) and Phytohaemagluttinin A (5 µg/ml final concentration, Sigma USA). For the screening ex vivo ELISpots in SPARTAC, cryopreserved PBMCs were added at 0.5 x 10^5 cells in 50ul with 50ul of 4ug/ml HIV overlapping or optimal peptide pools (final concentration 2ug/ml per peptide). Other steps were performed identically to the ELISpot protocol described earlier.

2.2.3 Single peptide "confirmatory" ELISpot assays

For the PITCH and RIO clinical trials, responses seen at screening were subsequently tested at a single peptide level using HIV-1 consensus B OLPs. The rationale for performing confirmatory ELISpots was to validate positive responses seen at screening; positive responses seen at confirmation would potentially be used for designing custom peptides to deduce optimal peptide responses. In order to increase the sensitivity of responses observed at a single peptide level, a decision was made to use 100,000 cells per well and 5ug/ml per overlapping peptide.

A positive response was recorded if the peptide-stimulated wells had spots per million PBMCs (after background subtraction) = >3 SD of overall mean DMSO at any given timepoint, and a response greater than 50 spots per million PBMCs. If a large number of positive responses were recorded using the above criteria (>=30), threshold was increased by multiples of 100 spots per million PBMCs (for example, 100, 200, 300 etc) in order to select a positive response. The reason for doing this is based on existing literature

that most individuals in acute HIV infection usually produce dominant responses to 1-3 epitopes, and also for feasibility of performing the assay and for conserving limited cell numbers (6–8) **(Figure 2.5).**

	ART STOP						ART RESTART					
PSM04	1	2	3	4	5	6	7	8	9	10	11	12
A	GAG 7		ENV 85		POL 20		GAG 7		ENV 85		POL 20	
B	GAG 33		ENV 96		POL 196		GAG 33		ENV 96		POL 196	
C	GAG 55		ENV 130		NEF 9		GAG 55		ENV 130		NEF 9	
D	GAG 66		ENV 163		NEF 36		GAG 66		ENV 163		NEF 36	
E	GAG 110						GAG 110					
F												
G	PHA	PHA	DMSO	DMSO			PHA	PHA	DMSO	DMSO		
H	PHA	PHA	DMSO	DMSO			PHA	PHA	DMSO	DMSO		

Figure 2.5. Example layout for participant PSM04 used for confirmation ELISpot assays. Each individual OLP is run in duplicate (as shown above) with 100,000 PBMCs/well. 4 wells each of positive (PHA) and negative (DMSO) controls are run for each timepoint. A positive response was defined as mean background subtracted value of 50 spots/million PBMCs or greater.

ELISpot protocol- Single peptide "confirmatory" ELISpot assay

Briefly, cryopreserved PBMCs were added at 1 x 10^5 cells in 100ul per well in duplicate and stimulated with 60µl of HIV OLPs (5 µg/ml final concentration per peptide). Filtered RAB10 with DMSO (final concentration 0.03%, Sigma USA) was used as a negative control and Phytohaemagluttinin A (5 µg/ml final concentration, Sigma USA) was used as a positive control. A decision was made to exclude CMVpp65 and FECT peptide pools, as these responses have already been recorded at the screening level and doing so would also conserve cell numbers which were limited in some cases. The ELISpot protocol used was otherwise identical to the ELISpot matrix-based assay described previously.

2.2.4 ELISpots using HLA-restricted peptides to determine "optimal" responses

Based on the positive OLP responses seen at confirmation, optimal peptide sequences were deduced and tested on timepoints of selected RIO participants to determine optimal peptides with the highest binding affinity at each timepoint. Optimal peptide sequences were deduced using a combination of the Los

Alamos HIV database and matching of participant HLA class-I types with the HLA handbook respectively (9). The Los Alamos HIV database lists previously defined cytotoxic lymphocyte (CTL) epitopes. The epitope location finder (ELF) tool (Los Alamos Database, USA) was used and configured to determine optimal peptides of 8-10 mers length that had the highest binding affinities, while taking into account the participant's HLA class I type **(Figure 2.6-2.7)** (10). The predicted binding affinity tool was based on the NetMHC4.0 pan software which has been tested and validated, and is being updated regularly with new epitopes (11). In addition, the epitope map from the Los Alamos database was used to align confirmatory peptide sequences to HLA-class I alleles (shown below); this was done to determine if there were any additional HLA-class I alleles that were restricted to specific peptide sequences (12). Also, participant HLA class I types were matched with data from the HLA handbook to determine anchoring residues and retrieve optimal epitopes that have been previously reported and described in the literature (9). In addition to matching known HLA-restricted epitopes from the Los Alamos database, variant epitopes were also selected for analysis based on known amino acid sequences from Gag and/or Nef regions of participants, at specific timepoints during their treatment interruption.

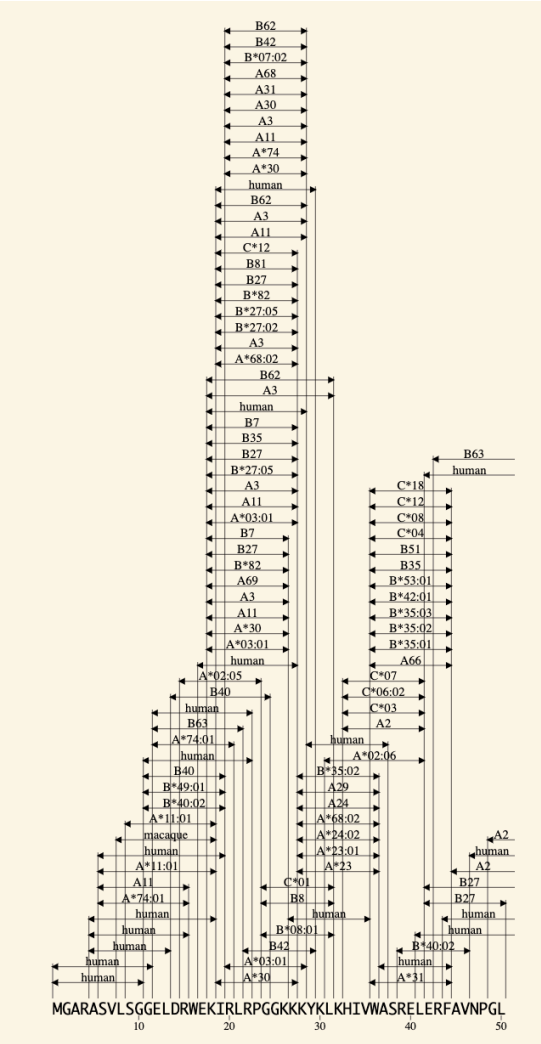


Figure 2.6. Epitope location finder (ELF) software used from the Los Alamos HIV Database website. The overlapping peptide sequence was inserted followed by the HLA-class I alleles of each participant. Based on this information, a list of known and/or potential epitopes with predicted binding affinities was generated and this was used to determine optimal peptides to test for downstream ELISpot assays.

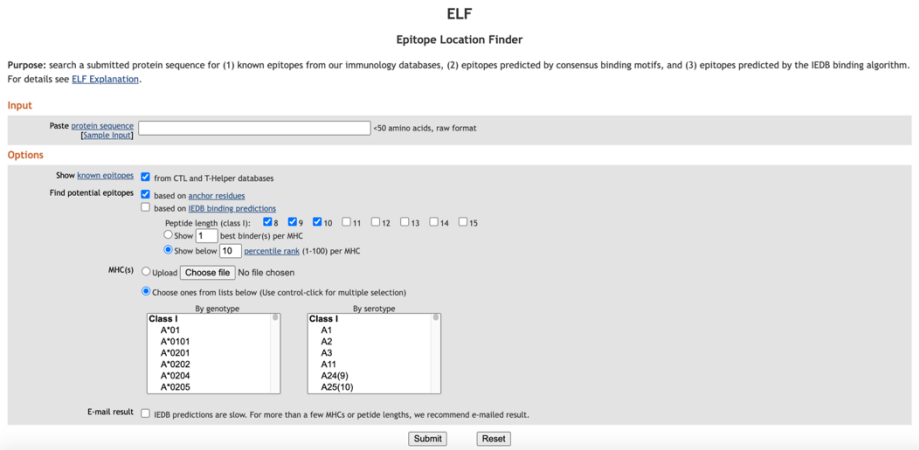


Figure 2.7. Example of epitope map for Gag (amino acid positions 0-50). The overlapping peptide response was aligned with the HIV-1 consensus B sequence (as shown) and reported HLA-class I restrictions were compared to participant HLA alleles to choose optimal peptides.

Based on data from all of the above tools, the most likely optimal peptides were deduced and chosen for further ELISpot assays. Approximately 5-10 HLA-restricted peptides were chosen for testing for each participant. For consistency, optimal peptides were chosen and designed according to the HIV-specific clade of the participant analysed.

Due to time and feasibility constraints, and in the interest of selecting for participants that made clear dominant or potentially protective responses, three participants from the RIO clinical trial were selected for further ELISpots using optimal peptides (001-003, 002-001 and 003-001). These participants were selected for analysis because they showed enhanced dominant Gag and/or Nef responses during their second treatment interruption after receiving bNabs compared to their first treatment interruption, while achieving a lower viral set point during their second ATI (see Chapter 5).

ELISpot Protocol- for assays using "optimal" HLA-restricted peptides

Briefly, cryopreserved PBMCs were added at 1 x 10^5 cells in 100ul per well in duplicate and stimulated with 60µl of HLA-restricted peptides (Mimotopes, Australia). Peptides were tested in a serial dilution, with the highest concentration of 5ug/ml followed by 5-fold dilutions down to 0.008ug/ml (table below). The set of serial dilution concentrations were optimised depending on the titration curve observed; in some participants, the response failed to taper off at the minimum peptide concentration used, and thus a decision was made to repeat the experiment using a steeper 10-fold dilution (highest concentration 5ug/ml followed by 10-fold dilutions down to 5 x 10^{-7} ug/ml).

Filtered RAB10 with DMSO (final concentration 0.03%, Sigma) was used as a negative control; this was also serially diluted in the same fashion to account for potential variations in background T-cell activation at each dilution. Phytohaemagluttinin A (5 µg/ml final concentration, Sigma) was used as a positive control.

A decision was made to exclude CMVpp65 and FECT peptide pools, as these responses have already been recorded at the screening level and doing so would also conserve cell numbers which were limited in some cases. Other aspects of the remaining ELISpot protocol used were otherwise identical to the ELISpot matrix-based assay described previously. Responses from duplicate wells were averaged to get the mean response in spots per million PBMCs. The peptide that tapered the least at lower concentrations was defined as the "optimal" epitope for that participant.

2.3 Nested PCRs and Next Generation Sequencing of HIV-specific regions

Rationale

Participant responses were tested against overlapping peptides spanning the HIV-1 consensus B genome; however, for each peptide it is uncertain where the exact optimal epitope lies within the particular amino acid sequence. More importantly, it is not possible to infer whether the response was made to a mutated or variant amino acid sequence. This information is important as it would directly affect the chosen peptides for downstream ELISpot assays, and would allow analysis of potential immune escape between analytical treatment interruptions in the participants.

Nested PCRs

Gag and Nef Primers

To determine the HIV sequences at relevant timepoints, nested PCRs were performed using outer and inner primers flanking the Gag and Nef regions of the HIV genome (Sigma-Aldrich, USA). Primer sets used for Gag and Nef respectively are shown as follows **(Tables 2.5-2.14).**

Table 2.5: GAG				
Oligo Name	Batch #	Oligo #	Sequence (5'-3')	Amplified region in genome
GagC5OP	HA16717128	8820597142-60/0	CTCTAGCAGTGGCGCCCGAA	627→646
GagC3OP	HA16717129	8820597142-70/0	TCCTTTCCACATTTCCAACAGCC	2023←2045 (reverse complement)
GagC5IP	HA16717130	8820597142-80/0	ACTCGGCTTGCTGAAGTGC	696→714
GagC3IP	HA16717131	8820597142-90/0	CAATTTCTGGCTATGTGCCC	1984←2003 (reverse complement)

Table 2.6: NEF				
Oligo Name	Batch #	Oligo #	Sequence (5'-3')	Amplified region in genome
BEF1	HA16906269	8820863754-50/0	TAATAGCAATAGTTGTGTGG	6111→6130
BNR1	HA16906270	8820863754-60/0	AGCTCCCAGGCTCAGATCT	9559←9577 (reverse complement)
BNF1	HA16906271	8820863754-70/0	CTGGCTGTGGAAAGATACCT	7965→7984
BNR2	HA16906272	8820863754-80/0	ATCTGAGGGCTCGCCACT	9489←9506 (reverse complement)
*UNINEF	HA17425043	8821609742-60/0	GCACTCAAGGCAAGCTTTATTGAGGCTT	9605←9632
*Nefyn05	HA17425046	8821609742-90/0	GTGTGTAGTTCTGCCAATCAGGGAA	9157←9181
*ENVoutF1	HA17425044	8821609742-70/0	AGARGAYAGATGGAACAAGCCCCAG	5550←5574

*ENVinF1	HA17425045	8821609742-80/0	TGGAAGCATCCRGGAAGTCAG CCT	5861←5884

Tables 2.5-2.6. Primers (Gag and Nef) used for both primary and secondary nested PCRs. The primary reaction was a one-step reaction (reverse transcriptase followed by DNA amplification), followed by a secondary PCR using primers flanking the inner regions of the primary amplified product.

Gag primers used were previously optimised in the lab and reported previously. Nef primers were chosen based on previous literature that reported successful amplification of the Nef gene (13,14). To confirm binding positions of primers used, primers were aligned to the HIV consensus B genome using "quickalign" software from the Los Alamos HIV database (15). All primers were diluted to 100 uM and then to 10uM working stock in double distilled water (ddH2O) to use in subsequent nested PCR experiments. Nested PCRs for both Gag and Nef were subsequently performed using a one-step reverse transcriptase (RT)-PCR reaction (Invitrogen, USA). Optimised protocols used for Gag and Nef are shown below.

PCR- One Step RT-PCR (Gag)

Master Mix	ul (for 1 reaction)
2x platinum superfidelity	25
Forward outer primer (Gag C5OP)	2.5
Reverse outer primer (Gag C3OP)	2.5
Superscript IV Reverse Transcriptase	0.5
Template RNA	10
Nuclease free H2O	9.5
Total volume	50

Table 2.7. Master mix protocol for the One Step RT-PCR (Gag).

PCR Cycle Settings – First Round

	Degrees	Time (minutes)	
cDNA amplification	48	10:00	
Denaturation	98	2:00	
Denaturation	98	0:10	35 cycles
Annealing	55	0:10	35 cycles
Extension	72	1:00	35 cycles
Extension	72	5:00	

Table 2.8. PCR reaction settings for the One Step RT-PCR (Gag).

Amplified DNA was stored at 4 degrees for further use until required for second round PCRs. 2ul of primary PCR product was used in the reaction as follows:

***Second round PCR – Gag**

Master Mix	ul (for 1 reaction)
H20	38
10x PCR buffer	5
MgCl2	1.5
dNTP's	1
Gag C5IP	1
Gag C3IP	1
Taq polymerase	0.5
Template RNA	2
Total Volume	50

Table 2.9. Master mix protocol for the nested PCR using inner primers (Gag).

PCR Cycle Settings – Second Round

	Degrees	Time (minutes)	
cDNA amplification	94	2:00	X1 cycle
Initial Denaturation	94	0:30	40 cycles
Denaturation	55	0:30	40 cycles
Annealing	72	1:30	40 cycles
Extension	72	5:00	X1 cycle

Table 2.10. PCR reaction settings for the nested PCR using inner primers (Gag).

PCR- One Step RT-PCR (Nef)

Master Mix	ul (for 1 reaction)
2x platinum superfidelity	25
Forward primer BEF1	2.5
Reverse primer BNR1	2.5
Superscript IV RT	0.5
Template	10
Nuclease free H20	9.5
Total volume	50

Table 2.11. Master mix protocol for the One step RT-PCR (Nef).

PCR Cycle Settings- First Round

	Degrees	Time (minutes)	
cDNA amplification	48	10:00	
Initial denaturation	98	2:00	
Denaturation	98	0:10	X 40

Annealing	56	0:30	X 40
Extension	72	4:00	X 40
Extension	72	10:00	

Table 2.12. PCR Reaction Settings for the One step RT-PCR (Nef).

***Second round PCR- Nef**

Master Mix	ul (for 1 reaction)
Nuclease free H2O	38
10x buffer	5
MgCl2	1.5
dNTP	1
BNF1	1
BNR2	1
Taq	0.5
Template	2
Total volume	50

Table 2.13. Master mix protocol for the nested PCR using inner primers (Nef).

PCR Cycle Settings- Second Round

	Degrees	Time (minutes)	
cDNA amplification	94	3:00	
Initial denaturation	94	0:30	
Denaturation	58	0:30	X 40
Annealing	72	2:00	X 40
Extension	72	10:00	X 40

Table 2.14. PCR reaction settings for the nested PCR using inner primers (Nef).

Amplified DNA from second round PCRs was stored at 4 degrees prior to gel electrophoresis.

Nested PCRs- 003-001 TK Gag and Nef

Due to difficulties in amplifying Gag and Nef regions for participant 003-001 (TK timepoint), additional primer pairs reported in the literature were used for nested PCRs. In contrast to the one-step RT-PCR used for other participant timepoints, the RT-PCR was performed in a separate step to synthesise complementary DNA (Superscript IV First Strand Synthesis, ThermoFisher, USA). Protocol for cDNA synthesis was performed according to manufacturer's instructions; protocols and PCR reaction settings are shown below. Following cDNA synthesis, two rounds of PCR were performed using gene-specific outer and inner primers. For Gag, these were the same primers used for other reactions (denoted by asterisks); for Nef, these were separate primers that have been previously documented in the literature (14). The PCR master mix protocols were identical to those used in the second round PCRs as previously described (see asterisks). PCR reaction settings used for these are shown below in **Tables 2.15-2.18.**

cDNA synthesis Reverse-transcriptase reaction set-up (ThermoFisher Scientific, USA)

	20ul reaction (ul)
DEPC-treated water	to 20
5x superscript IV buffer	4
10mM dNTP mix	1
100mM DTT	1
Ribonuclease inhibitor (40U/ul)	1
50uM oligod$(T)_{20}$ primer	1
Template RNA	2

Table 2.15. Master mix protocol for the cDNA synthesis RT-PCR setup (Gag and Nef).

cDNA synthesis- PCR reaction settings

Step	Degrees	Time (minutes)
Initial denaturation	94	2:00
Denature	94	0:15 (x 35 cycles)
Annealing	55	0:30 (x 35 cycles)
Extension	68	1:00 (x 35 cycles)
Hold	4	Forever

Table 2.16. PCR reaction settings for the cDNA synthesis RT-PCR setup (Gag and Nef).

PCR Settings- First and second round (Gag)

	Degrees	Time (minutes)	
cDNA amplification	94	2:00	
Initial denaturation	94	0:30	
Denaturation	60	0:30	X 35
Annealing	72	4:00	X 35
Extension	72	7:00	X 35

Table 2.17. PCR reaction settings for the DNA amplification using Gag primers.

PCR Settings- First and second round (Nef)

	Degrees	Time (minutes)	
cDNA amplification	94	2:00	
Initial denaturation	94	0:30	
Denaturation	56	0:30	X 35
Annealing	72	1:30	X 35
Extension	72	5:00	X 35

Table 2.18. PCR reaction settings for the DNA amplification using Nef primers.

2.4 Protocol- Gel Electrophoresis

A 1% agarose gel was made using agarose, 1x TBE buffer, and 7ul of Sybersafe dye and left to harden for 45 minutes. Next, 5ul of amplified DNA was added to 2ul of DNA loading buffer. This was loaded onto the agarose gel. 5ul of 100bp and 1kb DNA ladder respectively was loaded onto the gel (Invitrogen, USA). 1x TBE buffer was added to submerse the gel. The gel was run using a voltage power pack at 70 volts for up to 45 minutes. After 45 minutes, the power pack was switched off and excess TBE buffer was washed off. The gel was read using an automated gel reader.

An example gel (samples containing Gag amplified DNA) is shown (**Figure 2.8**).

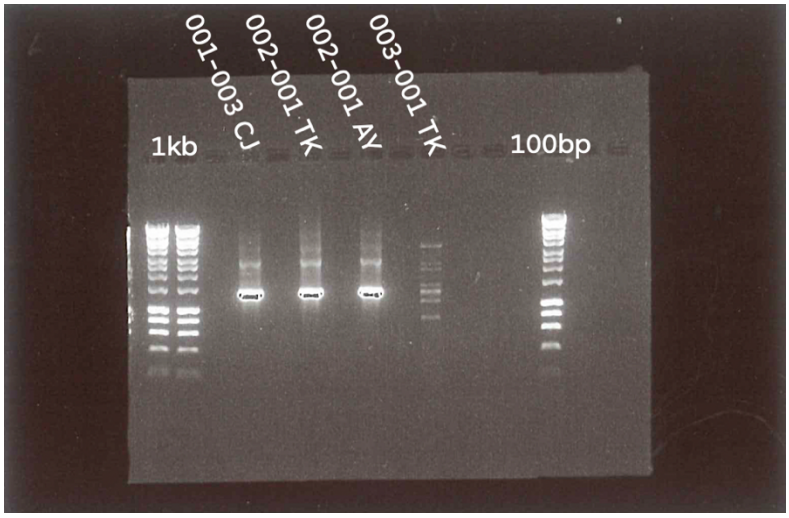


Figure 2.8. Example gel for participants 001-003, 002-001 and 003-001. Each column represents one sample, with two 1 kb and one 100bp DNA ladders on either side respectively as shown.

2.5 Sequencing library preparation

Sequencing library preparation was performed by Panagiota Zacharopoulou. Extracted and amplified DNA (PCR product) was prepared for sequencing using a Nextera DNA Library Preparation Kit (Illumina, USA) according to the manufacturer's instructions.

2.6 Other Functional T-cell assays

2.6.1 HIV-specific T cell proliferation assays

Cellular proliferation assays were performed using cryopreserved PBMCs from samples in the RIO clinical trial. Due to limited cell numbers, this assay was not performed on samples from the PITCH or SPARTAC cohorts. Moreover, the analysis of PITCH and SPARTAC focused primarily on the induction and shifts of immune-dominance during and/or after treatment interruption, so the use of ELISpot assays to determine peptide-specific responses was a more appropriate and feasible choice.

Protocol- Celltrace Violet (CTV) Proliferation Assays

PBMCs from cryopreserved samples were washed twice with PBS and stained using CellTrace® Violet (CTV, Life Technologies) at a final concentration of 2.5 µM (0.5ul of CTV dye per 1ml of PBS) for 10 minutes at room temperature. The reaction was then quenched by adding cold filtered heat inactivated foetal calf serum (FCS). CTV-labelled PBMCs in RPMI containing 10% human AB serum (Merck), 1 mM Pen/Strep and 2mM L-Glut were subsequently plated in 96-well round-bottom plates at 250,000 cells/190ul respectively.

Cells were stimulated with peptide pools from Gag, Pol, Env, Nef, Vpr, Tat and Vif (1 µg/ml final concentration per peptide). Media containing 0.1% DMSO (Sigma) representing the DMSO content in

peptide pools was used as a negative control and phytohemagglutinin L (5ug/ml final concentration, PHA-L, Sigma) was used as a positive control. Cells were then incubated at 37 °C, 5% CO_2, 95% humidity for 7 days with a media change of 100ul of RAB10 on Day 4. On Day 7 at the end of incubation, PBMCs were resuspended in cell staining buffer (Biolegend) and cells were incubated with fluorochrome-conjugated primary human-specific antibodies for CD3, CD4, CD8 (Biolegend) and near-infrared live/dead stain (Invitrogen) in cell staining buffer containing 10% FCS for 30 minutes at 4 °C. After incubation, cells were washed with cell staining buffer, fixed with 4% paraformaldehyde (PFA, Sigma) and stored at 4 °C in the dark until data acquisition. Data was acquired on a MACSquant analyser 10 (Miltenyi Biotech) flow cytometer and analysis was performed with FlowJo Version 10.7.1 (BD Biosciences). Responses above 1% were considered true positive. To determine the magnitude of the total response to structural and accessory proteins, the average number of cells proliferating in response to any of the peptide pools (Gag, Pol, Env, Nef, Vpr, Tat, Vif) was obtained as a function of their respective $CD4^+$ or $CD8^+$ T cell population and then expressed as a percentage. The background from DMSO/unstimulated wells was then subtracted from the total response for each participant timepoint. Assay-specific gating strategies and antibody panels used are shown below **(Table 2.20, Figures 2.8-2.9).**

Table 2.20- Celltrace Violet (CTV) assay staining panel

Antibody	Fluorochrome	Volume per 50ul
CD3-FITC	FITC	1
CD4-APC	APC	0.25
CD8 PE-CY7	PE-Cy7	0.25
Live dead near infrared	BV510	20

Table 2.20. Staining panel used for the Celltrace Violet (CTV) assay. Volumes stated are per condition (50ul per well). The remaining volume is made up with staining buffer (i.e staining buffer volume = 50 – master mix total volume).

Celltrace Violet (CTV) assay gating strategy

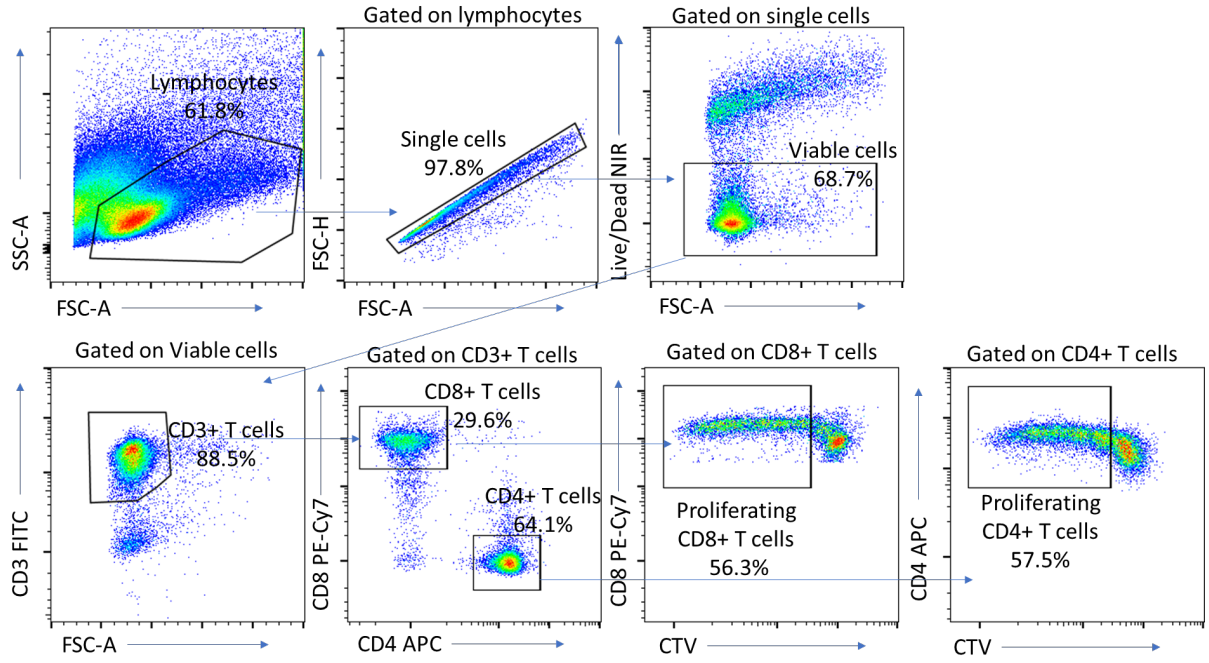


Figure 2.8. Gating strategy for Celltrace Violet (CTV) proliferation assay. Gates were set on lymphocytes and then single cells (to exclude doublets), followed by gating on viable CD3+ cells. Subsequent gating was performed to isolate CD4 and CD8+ cells. Of these, CTV+ cells were gated on which represent actively proliferating cells in response to either PHA, CMV, FECT, DMSO or HIV peptide pools.

Representative FlowJo software plot (example participant)

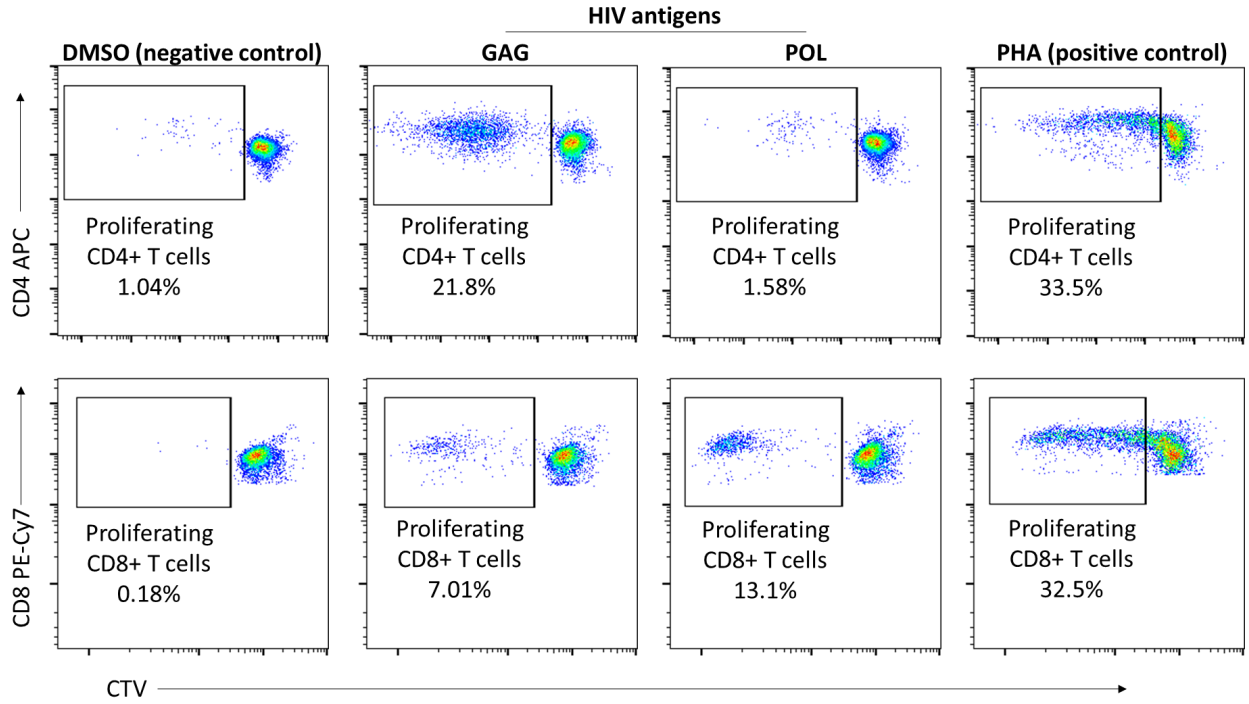


Figure 2.9. FlowJo software plot showing HIV-specific proliferating cells in response to Gag and Pol respectively in both CD4 and CD8 compartments. PHA and DMSO were used as positive and negative controls respectively. FACS plot kindly provided by Dr. Mohammed Altaf.

2.6.2 HIV-specific Activation-Induced Marker (AIM) assays

Protocol

Cryopreserved PBMCs were used for activation induced marker (AIM) assays. Briefly, PBMCs were thawed in R10 (RPMI + 10% FCS, 1% Pen/strep and 1% L-glutamine). Cells were washed, counted, and rested for 3 hours in IMDM-10 (Iscove's Modified Dulbecco's Medium - Sigma, I3390 + 10% Human AB serum, 1% Pen/strep and 1% L-glutamine) and 1ul/ml of benzonase nucleases (Merck, 70746-3). Following rest, 100ul of cells at a concentration of 1.5 x10^6 cells/100ul were plated at in a 96 well round bottom plate, along with 75ul RAB10, 5ul CXCR3 APC (BD Biosciences, USA) and 20ul of HIV peptides (2ug/ml final concentration). Cells were then incubated for 24 hours at 37ºC and 5% CO2. After stimulation, cells were stained with 25ul of FC Receptor blocker (Biolegend, USA) and 20ul of Live-dead aqua (Invitrogen, USA) for 10 minutes each at room temperature, and then with the antibody panel at 37 degrees for 15 minutes.

Following staining, cells were fixed in 4% PFA, and resuspended in 200ul PBS prior to acquisition on a BD LSR II flow cytometer. The data was analysed using FlowJo version 10 and Prism version 9. Assay specific gating strategies and antibody panels shown are shown below.

As previously used in other studies, data from AIM markers was analysed via a Boolean gating strategy to increase the sensitivity and specificity of detecting HIV-specific responses (16–18). For both CD4s and CD8s, we included CD25 which is a well-described marker of T cell activation (19,20). For CD4 T cells, we used the additional markers 41BB and CD69 and gated on cells using a Boolean analysis. For CD8 T cells, gating was done on cells that were single positive for CD25, 41BB and CD69, followed by a separate Boolean analysis that was performed using combination gating to produce an overall percentage of "AIM-positive CD8+ T cells" **(see Figure 2.10, Table 2.21).**

Activation-induced marker (AIM) assay gating strategy

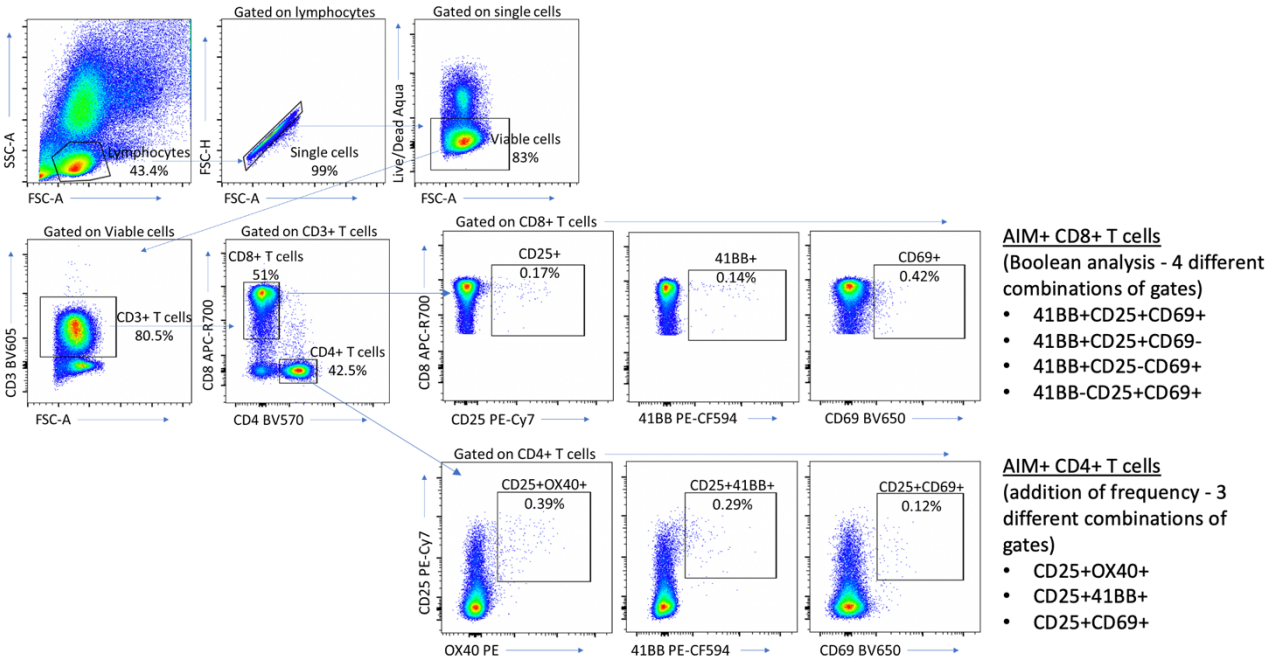


Figure 2.10. Gating strategy for AIM assay. Gating was performed on lymphocytes followed by singlets and CD3+ cells. Subsequent gating was performed on CD4+ and CD8+ cells. For CD4 T cells, a Boolean gating analysis was performed using a combination of the AIM markers CD25, CD69 and 41BB. For CD8 T cells, gating was performed on double positive CD25/41BB/CD69 cells, followed by a Boolean analysis which involved adding the total frequency of all double positive cells. Adapted from Dr. Mohammed Altaf.

Table 2.21- Activation-induced marker (AIM) assay staining panel

Marker	Fluorochrome	Clone	volume per 50ul
CD134 (OX40)	PE	L106	1.25
CXCR5	BB515	RF8B2	1
CCR6	APC-CY7	G034E3	1
PD-1	BV421	EH12.2H7	1
CD137	PECF-594	4B4-1	1
CD4	BV570	RPA-T4	1
CD39	PERCP-EFLUOR710	eBioA1	1
CD25	PE-CY7	2A3	1
CD69	BV650	FN50	0.5
CD8	APCR700	RPA-T8	0.5
CD3	BV605	UCHT1	0.5
CD19	BV510	HIB19	0.25
CD14	BV510	M5E2	0.25

Table 2.21. Staining panel used for the activation-induced marker (AIM) assay. Volumes stated are per condition (50ul per well). The remaining volume is made up with staining buffer (i.e staining buffer volume = 50 – master mix total volume).

2.6.3 Immune phenotyping using activation and exhaustion markers

In addition to measuring HIV-specific CD4 and CD8 T cell responses via ELISpot, AIM and cellular proliferation assays, immune phenotyping of T cells was also performed on RIO clinical trial participants. In contrast to the other assays, this method involved the analysis of unstimulated peripheral blood mononuclear cells (PBMCs) via specifically chosen activation and exhaustion markers to determine if there were any phenotypic changes in T cell subsets post-bNab infusion and throughout periods of analytical treatment interruption.

Explanation of markers and gating strategy

For analysis of phenotypic changes in T cells over time, the gating strategy focused on the T cell memory subsets as we hypothesised that these subsets may exhibit changes post-bNab infusion. First, forward and

side scatter were used to gate on the lymphocyte population, followed by gating on single cells to exclude doublets. Then, a live-dead amine-reactive dye (live-dead aqua, Invitrogen, USA) along with surface markers CD19 (for B-cells) and CD14 (for monocytes) (21) were used as a dump channel to exclude dead cells and cells other than T cells. Subsequently, the lineage marker CD3 was used to gate on CD3+ T cells, followed by gating of CD4+ and CD8+ T cells respectively. Using the CD4+ T cell population, these were further divided by gating on CD25+/CD127lo cells which helps categorise the Treg cell population. Following this, memory T cell subsets were gated on using a combination of the markers CCR7 and CD45RA, which are two surface molecules that allow the division of human T cells into four distinct subsets: $CD45RA^{+}CCR7^{+}$ naive (TN), $CD45RA^{-}CCR7^{+}$ central memory (TCM), $CD45RA^{-}CCR7^{-}$ effector memory (TEM), and $CD45RA^{+}CCR7^{-}$ effector memory re-expressing CD45RA (TEMRA) T cells (22–24).

For CD4+ T cell memory subsets, these were further gated into CXCR5+ cells which are known as T follicular helper (Tfh) cells; for the CXCR5- cells, these were further divided using the markers CCR6 and CXCR3 to distinguish separate T-helper populations, namely Th1, Th2 and Th17 and Th1/Th17 cells. This combination of markers has been well described in existing literature to define T-helper cell phenotypes (25–27). In addition to gating on CXCR5+ cells, the double positive PD1+/CXCR5+ cells (which are also markers of Tfh cells) from the total memory CD4+ T cell subsets were also characterised. Finally, the expression of activation markers HLA-DR and CD38 was measured on all memory T cell subsets, as well as T-helper cell subsets. Both HLA-DR and CD38 are prominent activation markers that have been shown in the literature to be associated with HIV disease progression (28,28–30), and higher levels of pre-ART activation via measurement of CD38 have been correlated with higher HIV DNA levels (31). In addition, T cell activation levels have also been correlated with reservoir size (32–35). However, CD38 is known to be constitutively expressed on CD8 T cells, and thus measurement of T cell activation via a single activation marker may cloud the true extent of T-cell activation. Thus, we have decided to use a combination of both

HLA-DR and CD38 for measurement of T-cell activation. The markers used in the activation marker panel (full and fluorescence minus one (FMO)) as well as the gating strategy are shown in **Figure 2.11.**

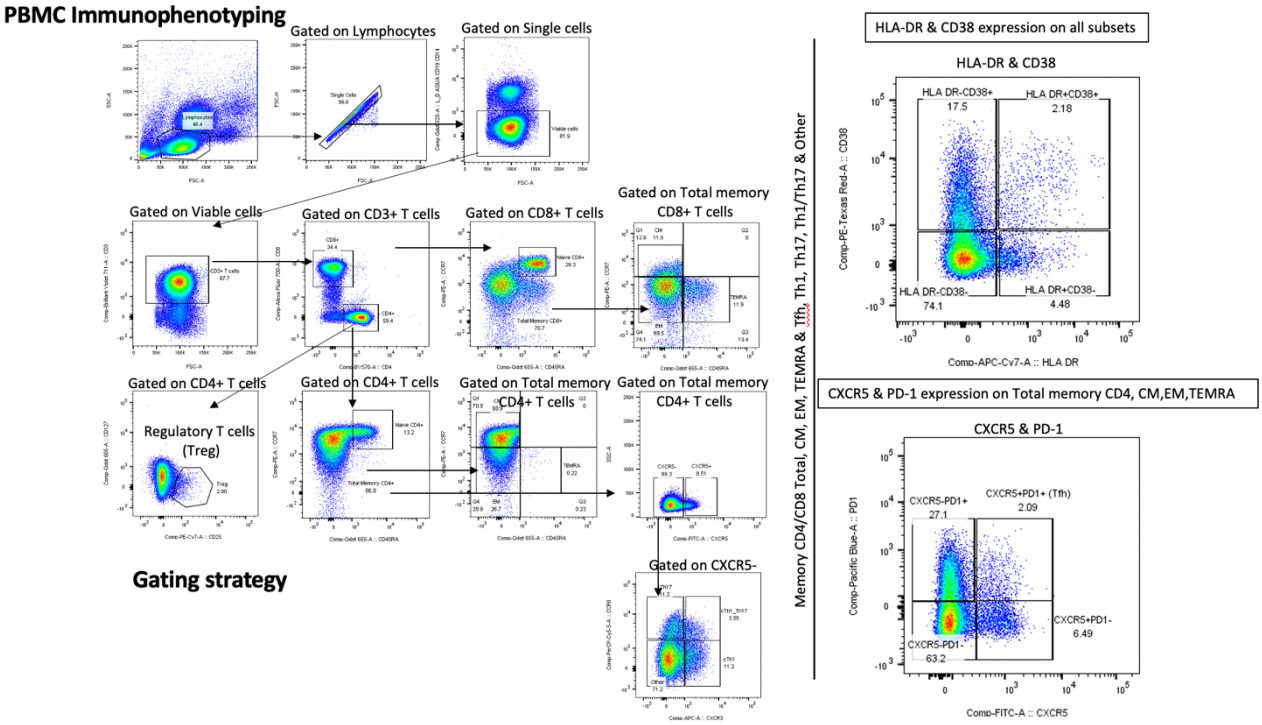


Figure 2.11. Gating strategy for immunophenotyping using activation markers. After gating on viable CD3+ singlet lymphocytes, gating was performed to isolate CD4+ and CD8+ cells. For CD4+ T cells, gating was on bulk CD4+ T cells to isolate regulatory T cells (CD25+CD127lo). Using total memory CD4+ T cells, further gating was performed to isolate single and double positive CXCR5/PD-1 (Tfh) cells, as well as T-helper cell subsets (Th17/Th1/Th2). For both memory CD4 and CD8 T cells, single and double positive HLA-DR/CD38 cells were isolated.

Protocol- Activation and exhaustion marker staining

Sample processing was performed as previously described. Staining of cells for activation and exhaustion markers were performed simultaneously; however, the protocols are written separately for the purposes of clarity.

Cryopreserved PBMCs were used for surface staining of activation markers. For experimental use, PBMCs were thawed in a 37°C water bath and transferred into 30ml of pre-warmed R10 (RPMI 1640 [Sigma, USA] containing 10% heat inactivated foetal calf serum [FCS], 2mM L-glutamine, and 1mM

penicillin/streptomycin [Sigma, USA]), then spun down at 1500rpm for 5 minutes and resuspended in 2ml of R10 for counting using the Bio-RAD automated cell counter (Bio-RAD, USA). Cells were then rested for 3 hours in an incubator at 37°C, 95% humidity and 5% CO2. After resting, cells were spun down at 1500rpm for 5 minutes and resuspended in 500ul of cell staining buffer (Biolegend, USA). For both the activation and exhaustion marker staining, 200ul and 50ul were used for the "full" stain and "FMO" stain respectively.

Next, cells were spun down and non-specific binding to cells was blocked using 25ul Fc receptor blocker (25ul of a 1:100 dilution with PBS) and left for 10 minutes at room temperature. Then, 20ul of a live-dead stain was used ((live-dead aqua, Invitrogen, USA) 1:200 dilution with PBS) and left in the dark for 10 minutes at room temperature. Following incubation, 150ul of cell staining buffer was added and cells were spun down at 1800rpm for 3 minutes. During the centrifugation process, a master mix for both the full and FMO stains for both panels were prepared as shown in the staining panel below. The remaining volume was topped up with cell staining buffer to make a total of 50ul per condition. Following centrifugation, excess supernatant was discarded and 50ul of staining master mix (full or FMO) was added to cells and incubated at 37°C, 95% humidity and 5% CO2 for 15 minutes. Following incubation, separate protocols were followed for the activation and exhaustion marker panels.

Activation marker staining

Following incubation with the staining master mix, cells were fixed by adding 100ul of 4% formaldehyde (Sigma-Aldrich, USA) and incubated at 4 degrees Celsius for 10 minutes. Then, 100ul of PBS was added and cells were washed at 1800rpm for 3 minutes. Following washing, supernatant was discarded and cells were resuspended in 200ul PBS and wrapped in foil at 4 degrees Celsius. Sample acquisition was done via a flow cytometer (BD, LSR-II) on the same or following day **(Tables 2.22-2.23).**

Table 2.22- Activation marker phenotyping- staining panel (full)

Marker	Fluorochrome	Clone	volume per 50ul
CXCR3	APC	IC6/CXCR3	10
CCR7	PE	G043H7	2
CD127	BV605	A019D5	1.5
HLA-DR	APC-Cy7	L243	1.25
CD3	BV711	UCHT1	1
CD4	BV570	RPA-T4	1
CXCR5	BB515	RF8B2	1
PD-1	BV421	EH12.2H7	1
CCR6	BB700	11A9	1
CD45RA	BV650	HI100	1
CD8	APC-R700	RPA-T8	1
CD25	PE-Cy7	2A3	1
CD38	PETR	HIT2	0.5
CD19	BV510	HIB19	0.25
CD14	BV510	M5E2	0.25

Table 2.23- Activation marker phenotyping- staining panel (fluorescence-minus-one)

Marker	Fluorochrome	Clone	volume per 50ul
CXCR3	APC	IC6/CXCR3	10
CCR7	PE	G043H7	2
CXCR5	BB515	RF8B2	1
CCR6	BB700	11A9	1
CD45RA	BV650	HI100	1
CD3	BV711	UCHT1	1
CD4	BV570	RPA-T4	1
CD8	APC-R700	RPA-T8	1
CD19	BV510	HIB19	0.25
CD14	BV510	M5E2	0.25

Tables 2.22-2.23. Activation marker staining panel (full and fluorescence minus one (FMO)) respectively. The volume per 50ul indicates the volume required per sample. After addition of all antibodies into the master mix, the remaining volume was added in the form of staining buffer (Biolegend, USA) such that the final volume totalled 50ul.

Exhaustion marker staining

As seen in the activation marker panel, the phenotypic changes in T-cell exhaustion were primarily focused on the memory T cell subsets. First, forward and side scatter were used to gate on the lymphocyte population, followed by gating on single cells to exclude doublets. Then, a live-dead amine-reactive dye (live-dead aqua, Invitrogen, USA) along with surface markers CD19 (for B-cells) and CD14 (for monocytes) (21) were used as a dump channel to exclude dead cells and cells other than T cells. Subsequently, the lineage marker CD3 was used to gate on CD3+ T cells, followed by gating of CD4+ and CD8+ T cells respectively. Next, we gated on the total memory CD4+ and CD8+ T cell subsets, followed by subsequent gating on the single positive PD-1, Tim-3, Lag-3 and TIGIT populations which are also known as immune checkpoint receptors (ICRs). Subsequently, we then gated on the transcription factors T-bet and EOMES using the memory CD4 and CD8 T cell subsets. To determine whether we could observe similar differences in our cohort, we decided to gate on Tbet^{dim}Eomes^{hi} and Tbet^{hi}Eomes^{lo} populations; these gating strategies have been outlined in previous studies involving exhaustion marker phenotyping (36), predominantly gating on CD8 T cells. Gating strategies are shown in **Figure 2.12-2.13.**

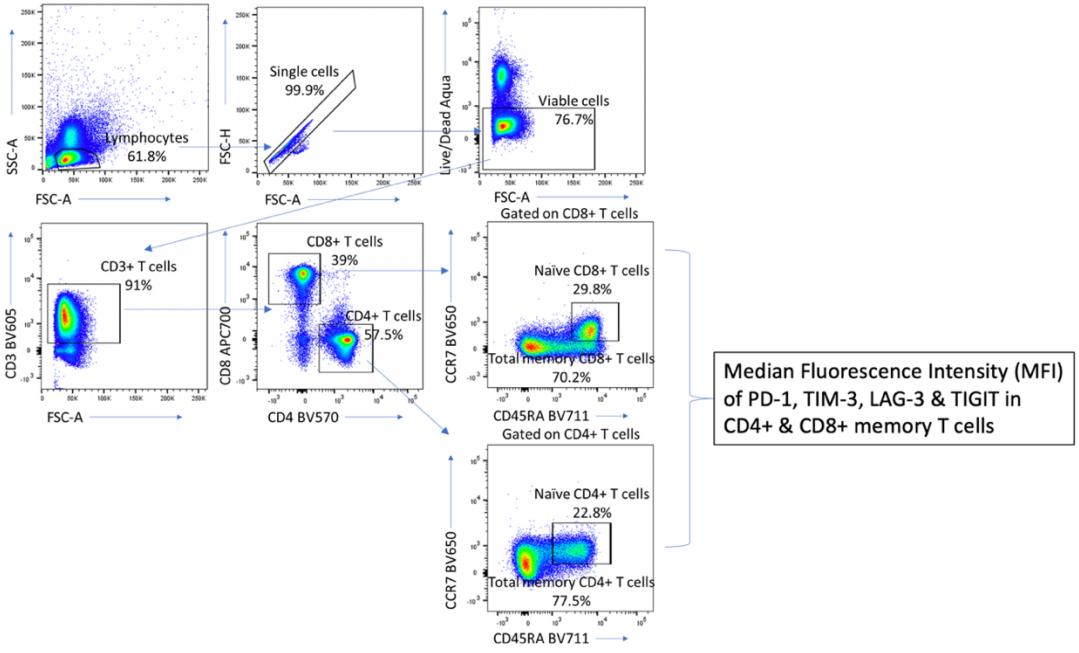


Figure 2.12. Gating strategy for immunophenotyping using exhaustion markers. After gating on viable CD3+ singlet lymphocytes, further gating was performed to isolate CD4+ and CD8+ T cell subsets. Then, using the total memory CD4+ and CD8+ compartments, the median fluorescence intensity of exhaustion markers PD-1, Tim-3, Lag-3 and TIGIT were measured.

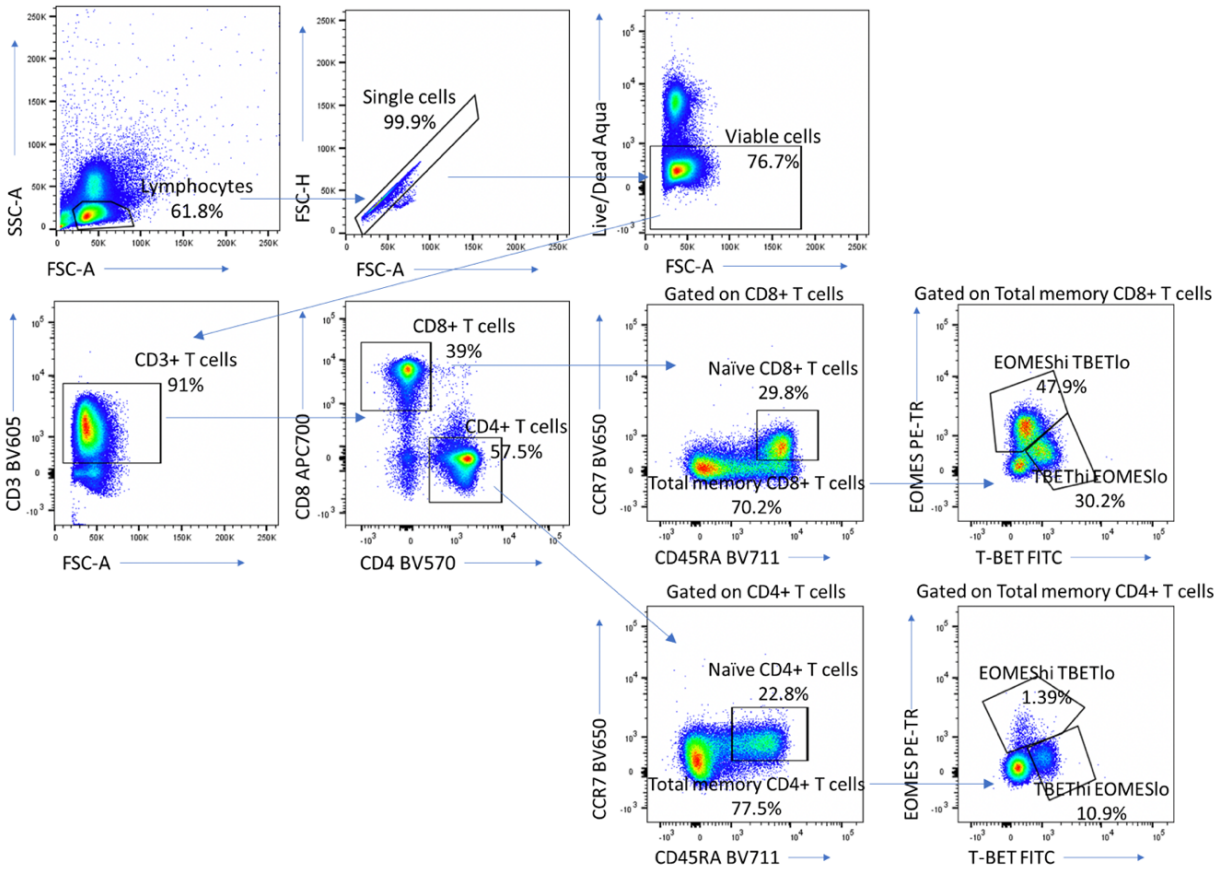


Figure 2.13. Gating strategy for transcription factor markers T-bet and EOMES. Initial gating of CD3+ CD4/CD8 T cells was done identically as previously described above. Using the memory CD4 and CD8 compartments, further gating was performed to isolate $Tbet^{lo}EOMES^{hi}$ and $Tbet^{hi}EOMES^{lo}$ cell subsets.

Exhaustion marker staining

Staining for exhaustion markers was performed in conjunction with activation markers as described previously. After surface staining cells at 37°C, 95% humidity and 5% CO2 for 15 minutes, cells were washed with 150ul of staining buffer at 1800rpm for 3 minutes. Next, cells were fixed using 100ul of fixation buffer (Biolegend USA, diluted 1 part fixation buffer to 3 parts fixation diluent according to manufacturer's instructions) and incubated for 30 minutes at room temperature. Next, cells were washed

twice using permeabilisation buffer (Biolegend USA, diluted 1 part permeabilisation buffer to 9 parts double distilled water according to manufacturer's instructions), the first and second wash using 100ul and 200ul of permeabilisation buffer respectively at 1800rpm for 3 minutes. After washing, cells were stained using 50ul of a staining master mix including T-bet and EOMES (see staining panel below) at room temperature for 30 minutes. Following staining, cells were washed with 150ul of permeabilisation buffer at 1800rpm for 3 minutes. Finally, excess supernatant was discarded and cells were resuspended in 200ul of PBS. Cells were wrapped in foil and stored at 4 degrees celsius; samples were acquired via flow cytometry (BD LSR-II) on the same or following day. Antibody panels used for exhaustion marker phenotyping are shown in **Tables 2.24-2.26.**

Table 2.24- Exhaustion marker phenotyping- staining panel (full)

Marker	Fluorochrome	Clone	volume per 50ul
TIGIT	PerCP-EF710	GIGD7	2.5
CCR7	BV650	G043H7	2.5
LAG-3	PE-Cy7	11C3C65	2
TIM-3	PE	344823	1.25
CD4	BV570	RPA-T4	1
PD-1	BV421	EH12.2H7	1
CD45RA	BV711	HI100	0.625
CD3	BV605	UCHT1	0.5
CD8	APC700	RPA-T8	0.5
CXCR5	AF647	RF8B2	0.25
CD19	BV510	HIB19	0.25
CD14	BV510	M5E2	0.25

Table 2.25- Exhaustion marker phenotyping- staining panel (fluorescence-minus-one)

Marker	Fluorochrome	Clone	volume per 50ul
CCR7	BV650	G043H7	2.5
CD4	BV570	RPA-T4	1
CD45RA	BV711	HI100	0.625
CD3	BV605	UCHT1	0.5
CD8	APC-R700	RPA-T8	0.5
CD19	BV510	HIB19	0.25
CD14	BV510	M5E2	0.25
CXCR5	AF647	RF8B2	0.25

Tables 2.24-2.25. Staining panel for the full and fluorescence minus one (FMO) stains using exhaustion markers. Volumes shown are per 50ul condition; the remaining volume was made up using staining buffer (Biolegend) so that the total volume per well was 50ul.

Table 2.26 Transcription factor phenotyping (after fixation and permeabilisation)- staining panel

Marker	Fluorochrome	Clone	volume per 50ul
EOMES	PE Texas Red	WD1928	1.25
TBET	FITC	4B10	1.25

Table 2.26. Staining panel for transcription factor immunophenotyping. Cells were stained for transcription factor markers following fixation and permeabilisation steps in the protocol.

2.6.4 MHC-class I tetramer assays

MHC-class I tetramer assays were performed using cryopreserved PBMCs from samples in the RIO clinical trial. This was only performed on selected participants to confirm and quantify antigen-specific T cell populations based on dominant peptide responses that were observed in ELISpot assays. At the time of writing this thesis, only preliminary tetramer staining using MHC-class I gag tetramers were performed; further investigation is underway to define other antigen-specific T cell populations using other tetramers.

Protocol

Cryopreserved PBMCs were thawed, counted and rested as previously described (see Protocol- Activation and exhaustion marker staining). Then, cells were plated at a concentration of 200,000 cells per 100ul of PBS-2% FCS and washed with 200ul of PBS-2% FCS (cell staining buffer) followed by centrifugation at 1000g for 2 minutes. Next, cells were spun down and non-specific binding to cells was blocked using 25ul Fc receptor blocker (25ul of a 1:100 dilution with PBS) and left for 10 minutes at room temperature. Then, cells were stained for live/dead cells using 25ul of live-dead aqua (Invitrogen, USA, 1:200 dilution with PBS) and incubated for 10 minutes in the dark. Then, cells were washed with 150ul of cell staining buffer and incubated with 50ul of staining mix (consisting of a 1:80 dilution of HLA-B*42:01-GPGHKARVL (in APC) in cell staining buffer) at 37 degrees for 15 minutes.

After tetramer staining, cells were washed at 1000g for 2 minutes and surface stained with 50ul of the following staining panel (**Table 2.27**) in cell staining buffer at 37 degrees for 15 minutes. Following staining, cells were washed at 1000g for 2 minutes and fixed with 100ul of formaldehyde (Sigma-Aldrich, USA) at 4 degrees for 10 minutes. Finally, cells were washed with 100ul of cell staining buffer, washed and then resuspended in 200ul of cell staining buffer. Cells were wrapped in foil and stored at 4 degrees celsius; samples were acquired via flow cytometry (BD LSR-II) on the same or following day. The gating strategy for quantifying tetramer positive CD8+ cells is shown in **Figure 2.14**.

Table 2.27- MHC-class I tetramer staining panel

Marker	Fluorochrome	Volume per 50ul
CD3	FITC	1
CD4	BV570	0.5
CD8	PE-Cy7	0.5
Tetramer	APC	0.625 (1:80) stained separately
Live/Dead	Aqua	0.1
CD14	BV510	0.25
CD19	BV510	0.25

Table 2.27. Tetramer staining panel. Cells were stained for tetramer separately prior to surface staining.

Figure 2.14- Gating strategy for tetramer positive CD8+ cells

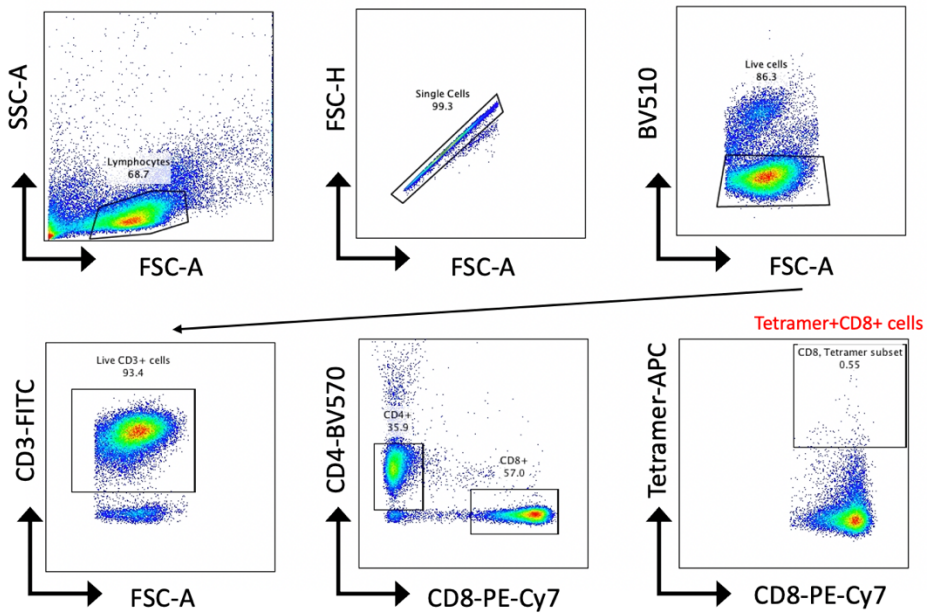


Figure 2.14. Tetramer staining panel. Cells were stained for tetramer separately prior to surface staining.

Chapter 2: References

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Chapter 3

The impact of antiretroviral treatment interruption on T cell responses in primary HIV infection

Contributions to this chapter

I would like to express my thanks to all the participants and staff that made the SPARTAC trial and PITCH pilot study possible; this includes the SPARTAC trial and PITCH pilot study research investigators, as well as the clinicians and research staff at the respective clinical recruitment sites.

Content from this chapter has been published previously:

- Tipoe T, Ogbe A, Lee M, Brown H, Robinson N, Hall R, et al. Impact of antiretroviral therapy during primary HIV infection on T-cell immunity after treatment interruption. European Journal of Immunology. n/a(n/a):2451200.

Sample collection and processing for the SPARTAC clinical trial and PITCH pilot study were performed by Helen Brown and Nicola Robinson. Peptide pool dilutions and Interferon-gamma ELISpot assays for the SPARTAC clinical trial were performed by Helen Brown and Nicola Robinson, and both staff also assisted where necessary with results acquisition and assay optimisation. Ane Ogbe assisted with the PITCH pilot study assay sampling design for the ELISpots and experimental expertise. Additional ELISpot raw data for the SPARTAC clinical trial was kindly provided by Jacob Hurst. Detailed acknowledgements for both the SPARTAC clinical trial and PITCH pilot study are described in the publication above.

Aims of this chapter

In this chapter, we aimed to investigate the impact of treatment interruption on HIV-specific T cell responses in participants with primary HIV infection (PHI). Moreover, we determined whether there were differences in HIV-specific T cell responses in participants with PHI that received varying durations of ART (2 years versus 48 weeks), and how this compares with ART-naïve participants with PHI.

3.1 Introduction

While ART has dramatically improved the quality of life in people living with HIV, it is not curative and latently infected cells (the HIV reservoir) remains as a significant challenge in the search for a functional cure. Patient adherence to ART is crucial but often hindered by factors like medication non-adherence, logistical difficulties, and psychosocial issues such as fear of stigmatisation (1,2). ART regimens also have numerous side effects and failure to adhere to ART can lead to ART-related drug resistance. Moreover, treatment options are severely restricted in people with conditions such as renal and hepatic impairment (3–5).

Due to difficulties and risks posed by ART administration at the time, early studies investigated whether withdrawal of therapy for brief periods, known as treatment interruption, was more beneficial compared to continuous therapy. In 1999, a notable case report was published by Lisziewicz et al. about an anonymous “Berlin” patient (6). In this report, the patient received combination ART 10 days prior to complete seroconversion, followed by a temporary suspension of treatment. After viral rebound, the patient was restarted on ART for 176 days, followed by a second round of treatment interruption. During the second interruption, however, the patient maintained viral suppression to less than 500 copies/ml of HIV RNA, despite the withdrawal of combination ART for more than two years without a single viral blip

(6). This landmark case of "post-treatment control" prompted other researchers to investigate the reproducibility of these findings, and test the feasibility of structured treatment interruptions in prospective studies.

Based on the ART regimen chosen for the Berlin patient, Lori et al. tested in three patients that were treated at various times following seroconversion and showed heterogeneity in viral load (range 16130-719,000 copies/ml) (7). Patients received 3 months of structured treatment interruptions, which included cycles consisting of 3 weeks ART followed by 1 week off ART. Remarkably, two out of three participants achieved lower viral set points over several months, and all three participants did not show emergence of ART-related drug resistance despite multiple rounds of ATI (7). However, one participant did not suppress viraemia off ART, meaning that structured treatment interruptions were more successful in some patients while not so much in others (7). In 2006, the large scale "SMART" study (which compared the effectiveness and feasibility of intermittent versus continuous ART in 5000 participants) concluded that while intermittent ART had an overall low risk of adverse events, those receiving intermittent ART had almost double the risk of morbidity and mortality versus compared to receiving continuous ART (8). Furthermore, intermittent ART was also associated with higher risk of opportunistic infections, HIV disease progression and death (9,10). Other risks with structured treatment interruptions were also raised, such as a potentially diminished response to future interventions involving cure, as well as the risks of expanding viral reservoirs and increased chronic inflammation which persists despite the re-introduction of ART (9).

Since then, analytical treatment interruptions (ATIs) have been limited to clinical trial settings to investigate the efficacy of therapeutic interventions to achieve an ART-free remission in HIV. A recent review of ATI studies by Stecher and Lau et al found that ATIs were safe to perform as long as they included more frequent viral load monitoring, with conservative ART-reinitiation thresholds and shorter durations

of ATI (11,12). While ART-treated individuals are unable to maintain viral suppression during treatment interruption, early ATI studies have explored the possibility of the "auto-vaccination" hypothesis (13,14). They suggested that ART-treated individuals with chronic HIV infection remain virally suppressed without any remarkable circulation of HIV antigen; however, upon interrupting therapy, there is a "burst" - or expansion of - viraemia and accompanying antigen which subsequently activates a HIV-specific T cell response that is capable of inducing and maintaining viral suppression in the absence of ART (14,15).

Evidence for the role of T-cells in maintaining viral control off ART has been reported in some studies. In the case of the Berlin patient, HIV-specific T cell responses were examined during viral suppression post TI. Interestingly, the study found evidence of increased HIV-specific T helper cell responses, as well as potent responses to a p17 Gag epitope on both a tetrameric-complex and enzyme linked immunosorbent assay which could explain control off ART (6). In another paper by Rosenberg et al., individuals with acute HIV infection that maintained viral control (less than 500 copies/ml) for several months post-TI exhibited elevated HIV-specific cytotoxic and T-helper cell responses, and these responses one year post-TI were narrowly directed towards a few epitopes (16). However, viral suppression in these participants was incomplete and all participants experienced viral rebound. Other studies done in untreated acute and chronic HIV infection have also shown that Gag-specific CD8 responses negatively correlate with viral load suggesting that targeting the Gag protein is crucial for controlling viraemia off ART (17,18).

More recently, the concept of "post-treatment control" (PTC) and factors affecting the likelihood of PTC have been explored. Post-treatment controllers are rare individuals that start ART, but maintain viral suppression after ART interruption for months or even years. These individuals are different from elite controllers (ECs) that are able to maintain undetectable viral loads for at least 12 months despite not having started ART. Phenotypic characteristics in both groups are distinct in a number of aspects. For

example, PTCs have been shown to have smaller viral reservoirs with an enrichment of transitional memory T cells (VISCONTI), with more robust Gag-specific CD4+ responses as shown in the recent CHAMP study (14). However, the role of HLA-class I alleles in PTC is less certain; for example, the VISCONTI cohort showed that PTCs were actually enriched with disease-susceptible or unfavourable alleles (19). In contrast to PTCs, ECs tend to have strong polyfunctional CD8 responses directed towards Gag and are often enriched in well-known protective alleles (19–22). While HLA-class I alleles are major determinants of peptide presentation and subsequent immune control in HIV, they are not a necessary nor sufficient mechanism for viral control (23), and the role of HLA-class I restricted CD8 responses in PTCs are less clear. Thus, other factors that affect the likelihood of PTC need further exploration.

Apart from HLA-class I alleles, current evidence indicates that initiating ART early during primary HIV infection is linked with an increased likelihood of post-treatment control. For example, data from the CHAMP study on PTCs showed that the frequency of PTC was higher in individuals treated during early HIV infection versus chronic infection. Moreover, the Thai RV254/SEARCH 010 study demonstrated that treatment during Fiebig stage I/II infection dramatically lowered viral load set point as well as total HIV DNA levels compared to the untreated group (14,24,25). Also, data from the SPARTAC trial revealed that 14% of patients treated with 48 weeks of ART during primary HIV infection were able to maintain undetectable viral loads after stopping therapy, compared to 3% in those who received 12 weeks of ART (26). More interestingly, the famous Mississippi baby study demonstrated that administering extremely early ART (within 30 months of birth) can lead to prolonged viral control off ART (although viral remission was only transient in this case). These findings demonstrate that the duration of ART and, in some instances, the timing of ART initiation, are critical factors in achieving PTC.

With regards to early ART, it is known that treatment with ART during primary HIV infection better preserves HIV-specific CD8 T cells and HIV-specific CD4 T cell helper responses, which are lost with delayed ART initiation (27). While epitope-specific responses in early treatment before seroconversion are often weaker, they are more narrowly directed and persist even during viral suppression after ART administration (28). Studies have also described other immunological and virological parameters that may significantly contribute to viral control post-ATI, including higher CD4 T cell counts, lower HIV DNA and lower viral loads during primary HIV infection prior to ART initiation (29). Finally, Hurst et al. also showed that certain exhaustion markers, such as PD-1, Tim-3 and Lag-3, strongly predicted return of viraemia (30).

Despite characterisation of PTC by other studies, the immunological mechanisms that drive PTC remain unclear. More research is required to identify the immune correlates of post-treatment control, and to understand the dynamics of HIV-specific T cells during treatment interruption. With the current use of treatment interruptions in interventional studies involving an HIV cure, it is also important to understand the immunological impact of treatment interruption alone on HIV-specific T cell responses. It is also unclear whether the duration of ART prior to treatment interruption has significant effects on shifts in immune dominance during and post-ATI. A comprehensive evaluation of HIV-specific T cell immunity through treatment interruption studies - in the absence of therapeutic intervention - could significantly inform the design of interventional strategies for individuals with primary HIV infection. Moreover, such studies may facilitate the deconvolution of epitopic responses that are instrumental in achieving immune control.

Based on these existing findings, this chapter aims to explore the impact of treatment interruption - without any intervention - on HIV-specific T-cell immunity in individuals with primary HIV infection. In addition to this, we will also examine whether the duration of ART prior to ATI affects these responses,

and compare these findings to ART-naïve participants in primary HIV infection. To address these questions, data will be examined from the PITCH pilot study and SPARTAC clinical trials respectively. The following hypotheses will be examined:

3.2 Hypotheses:

1. Treatment interruption is associated with shifts in immune dominance in individuals with PHI that receive ART for more than 2 years
2. Participants in PHI that receive 48 weeks of ART demonstrate shifts in immune dominance that are not observed in ART-naive participants with PHI

3.3 Results

3.3.1 Description of Cohorts

PITCH- Rationale

In the PITCH pilot study, participants were recruited from the HEATHER cohort where participants received ART with a maximum of 6 months from seroconversion and remained on therapy for at least two years prior to treatment interruption. These participants received ART very early, which means that their viral reservoir sizes are likely very small and potentially less likely to expand upon withdrawal of therapy. Analysing this cohort would enable investigation into the possible immune mechanisms of post-treatment control, and determine factors that affect long term remission in the absence of ART. PITCH was initially designed as a pilot study to investigate the sustainability, frequency and mechanisms of PTC with the aim of aiding design of future HIV cure studies, and also to determine the feasibility of stopping ART. Unfortunately, funding was withdrawn in early 2020 due to the COVID-19 pandemic; however, examining

this cohort is still useful to examine the effect of treatment interruption (in the absence of immunological interventions) on HIV-specific T cell responses and/or PTC.

Description of PITCH cohort

The 'Prospective Interruption of Therapy Towards a Cure for HIV' (PITCH) cohort was a prospective open-label study of individuals with confirmed PHI. All participants enrolled to the PITCH pilot study were recruited from the HEATHER cohort (('HIV Reservoir targeting with Early Antiretroviral Therapy'). HEATHER is a programme of work describing a cohort study of HIV-infected adults treated during primary HIV infection with suppressive ART. Participants were recruited from June 2018 and were followed up to 5 years of the last participant's follow-up visit. The HEATHER cohort consists of two groups: HEATHER A, which comprises participants that enrolled at the time of PHI and received ART within 3 months of HIV diagnosis, and HEATHER B, which comprises participants that are on ART and remain viral suppressed since initiating ART within 3 months of HIV diagnosis. All participants recruited via HEATHER provided written consent and approval was obtained from the National Research Ethics Service (NRES Committee West Midlands – South Birmingham reference number 14/WM/1104). All PITCH participants were existing HEATHER participants, so the ethics approval was covered under the same reference number.

Eligible participants to PITCH pilot were individuals with primary HIV infection (PHI) that have fulfilled the following criteria: confirmed evidence of PHI, commenced on ART within 3 months of HIV diagnosis in accordance with the British HIV Association Guidelines, and who subsequently remain virally suppressed (<50 copies HIV RNA/ml) on ART for at least two years with HIV DNA levels <=3.25 log copies/million CD4 cells and either a CD4 count >500 or a CD4:8 ratio>1. The rationale for this HIV DNA cutoff is based on a study by Williams et al. where they showed that individuals exceeding this cutoff experienced viral rebound within 12 weeks (31). Full inclusion and exclusion criteria are listed in **Table 3.1**. Samples were

collected from October 2018 to March 2020 from recruited participants across two clinical sites (St. Mary's Hospital and Guys and St. Thomas's Hospital, London).

Eleven participants were screened for PITCH during the study period. Two were excluded as baseline HIV DNA levels exceeded the inclusion criteria, and another two participants chose not to participate despite meeting all inclusion criteria. The remaining seven participants successfully completed an Intensively Monitored Treatment Interruption. A ninth was about to commence screening and their IMTI, when funding was withdrawn due to the SARS-COV-2 pandemic. The seven participants were all on INSTI-based regimes comprising Triumeq (3), Genvoya (1), Descovy/Dolutegravir (1), Truvada/Raltegravir (1) Truvada/Rezolsta (1).

Time to ART start following diagnosis ranged from 4 to 43 days (median 19 days). CD4 counts at baseline ranged from 479 to 917 (median 792 cells/ul) and CD4:CD8 ratio at baseline ranged from 0.75 to 2.3 (median 1.4). Participants had been on ART for a median of 1481 days at the time of stopping ART (range 736 to 1814 days). Baseline DNA at screening was 2.99 log copies/million CD4 T cells (range: 'not detected' to 3.08). In terms of monitoring, viral load measurements were taken using the GeneXpert viral load assay (32) (which has been adapted for point of care use as well as formal viral load assays) weekly between Weeks 0 to 24 of the intensively monitored treatment interruption (IMIT), and fortnightly between Weeks 24-52. At the patients request, viral load measurements could be reduced to fortnightly measurements between Weeks 12 to 24. Research bloods were taken at screening and baseline prior to IMIT, and every two weeks during the IMIT period. For statistical purposes undetectable HIV DNA values are given the LLD value of log 2. Further details of participants are shown in **Table 3.2** below.

Inclusion Criteria	Exclusion Criteria

- HIV-1 positive males
- Aged 18 years or above.
- Signed Informed Consent
- Patient aware of and understands their HIV status
- Initiated on ART (any standard ART regimen in accordance BHIVA guidelines) within 3 months of HIV diagnosis (defined as date of confirmed positive HIV test)
- Have confirmed evidence of PHI according to one of the following criteria:
  ○ HIV positive antibody test within 6 months of a HIV negative antibody test
  ○ HIV antibody negative with positive PCR (or positive p24 Ag or viral load detectable)
  ○ RITA (recent incident assay test algorithm) assay result consistent with recent infection
  ○ Equivocal HIV antibody test supported by a repeat test within 2 weeks showing a rising optical density
  ○ Having clinical manifestations of symptomatic HIV seroconversion illness supported by antigen positivity and <4 bands positive in western blot
- On ART for >2 years
- Total HIV-1 DNA levels ≤3.25 log copies/million CD4 cells
- CD4 count >500 or CD4:8 ratio > 1
	- HIV-1 positive females*
	- Unable or unwilling to give informed consent
	- Absence of definition of PHI according to the criteria above
	- Persistent Anaemia Hb level <10g/dL (on two or more consecutive occasions)
	- Hepatitis B and/or Hepatitis C co-infection at time of enrolment
	- Not willing or able to participate in the monitoring study visits
	- If a participant has had a rectal or terminal ileal biopsy for other reasons apart from PITCH or the HEATHER study within 6 months of screening, they will be excluded from the gut biopsy section of the protocol.
	- If a participant has had a venesection of >250mls for reasons apart from PITCH within 3 months of screening.
	- Previous significant cardiovascular disease, previous MI, CVA, untreated hypertension
	- Previous malignancy

Table 3.1. Inclusion and Exclusion Criteria for PITCH pilot study participants.

*HIV-1 positive females were excluded to minimise the risk of vertical transmission during ATI for women who could get pregnant.

	Dob	Rx	Date of dx	Date ART start	Days to ART start	PHI Criteria	CD4 count at baseline	CD4/8 ratio at baseline	DNA (log10 copies)	Pre-ART viral load (copies/ml)
PSM01	11/03/1993	TRIUMEQ	15/09/2014	28/10/2014	43	Ab-ve P24+	809	2.3	3.00	1155656
PSM02	02/06/1965						Withdrew		3.30	
PSM03	24/02/1986						Withdrew		2.90	
PSM04	27/06/1980	TRIUMEQ	31/08/2017	04/09/2017	4	Ab-ve P24 +	792	1.6	ND	N/A
PST01	05/09/1988						Withdrew		3.09	
PST02	13/11/1979						Withdrew		3.71	
PST03	27/10/1983	TRIUMEQ	28/02/2015	09/04/2015	40	RITA	917	1.6	2.65	1,000,000
PST04	09/09/1971	GENVOYA	31/05/2014	10/06/2014	10	Ab-ve P24+	792	1.2	2.99	999,999
PST05	16/07/1957	DESCOVY/ DOLUTEGRAVIR	04/07/2014	05/08/2014	32	6 months from neg test	654	0.75	3.08	N/A

PST06	17/12/1961	TRUVADA/ RALTEGRAVIR	24/04/2014	13/05/2014	19	Ab-ve P24+	776	1.3	2.99	132786
PST07	24/06/1977	TRUVADA/ REZOLSTA	15/05/2015	21/05/2015	6	RITA and 6 months from neg	479	1.4	3.02	3,972,955
PST08	20/01/1988	GENVOYA	28/07/2015	30/07/2015	2	6 months from neg test	500	0.7	ND	

Table 3.2. Demographic characteristics of recruited PITCH pilot study participants (total n=7) that underwent an analytical treatment interruption. Pre-ART viral loads are shown where available; for some participants, pre-ART viral loads could not be retrieved due to restrictions regarding hospital records.

SPARTAC- Rationale

The SPARTAC clinical trial was designed to determine the feasibility of administering shorter as opposed to longer durations of ART, and whether early ART administration resulted in preserved HIV-specific CD4 responses and correlated with clinical outcomes (26). The primary endpoint of the study was re-initiation of ART and CD4 T cell count declining below 350 cells/ul, with three separate arms of the trial: either 48 weeks or 12 weeks of ART followed by treatment interruption, or treatment-naive for 60 weeks. While data concerning viral loads and HIV-specific CD4 responses from the trial has been previously published (33), no previous analysis of the HIV-specific CD8 responses during the treatment interruption (or treatment naïve) period has been reported; thus, investigation of this data would provide useful insights into the effects of early ART (or no ART) on the immunodominance of HIV-specific T cell responses during treatment interruption. Due to the data availability as a historical cohort, only the 48 week groups and treatment-naïve (No ART) groups will be compared. Data will also be compared to the PITCH pilot study to examine whether a longer duration of ART has differential effects on shifts in T-cell immunodominance.

Description of SPARTAC Cohort

Short Pulse AntiRetroviral Therapy At HIV seroconversion ("SPARTAC") was an open-label, randomised controlled trial that aimed to compare three different therapeutic strategies in HIV-1 infection; either long course combination antiretroviral therapy (LCART) for 48 weeks or short course combination antiretroviral therapy (SCART) for 12 weeks followed by treatment interruption, or no ART (which was the standard of care at the time of performing the study) (SPARTAC Controlled-Trials.com number ISRCTN76742797 and EudraCT number 2004-000446-20). The primary endpoint of the trial was focused on the rate of CD4 T cell decline and time to long term ART initiation (26).

Information regarding recruitment and randomisation of the SPARTAC cohort has been previously described elsewhere (see "Methods" chapter). Recruitment, randomisation and follow up of SPARTAC participants has been previously published and described (26). A total of 371 participants across eight different countries (South Africa, Australia, Italy, Uganda, Brazil, Spain, UK and Ireland) underwent initial randomisation. Of these, 5 met major ineligibility criteria and were excluded. Criteria used are shown in **Table 3.3.** Subsequently, a total of 366 participants underwent randomisation to either LCART (n=123), SCART (n=120) or no ART (n=123). Clinic visits were scheduled at weeks 0, 4, 12, 16, and 24, and every 12 weeks thereafter; anti-HIV treatment was given according to local guidelines, and all participants provided written informed consent with ethical approval in line with local guidelines. The trial started in August 2003 and continued until the 360th participant had completed 3.5 years of follow-up, which was in November 2010. Peripheral blood samples for immunology analysis were taken at weeks 12, 16, 24, 48, 52 and 60.

Out of the 366 participants that were randomised to each of the three arms (48 weeks ART, 12 weeks ART and no ART), 209 participants reached the primary endpoint. **Figure 3.1a-b** shows an example of viral load

dynamics in the SPARTAC cohort and the participant demographics in the two arms that were analysed for this thesis. In the ART-48 group, participants received suppressive ART for 48 weeks from baseline, followed by treatment interruption from Week 48 onwards. In comparison, the No ART group did not receive any suppressive ART and remained off therapy for 60 weeks. Of the 209 participants that reached the primary endpoint, 61 participants with available T-cell ELISpot data from the 48-week ART group (ART-48, n = 24) and the no ART group (No ART, n= 37), were selected for analysis.

Inclusion Criteria	Exclusion Criteria

• Have reached the age of consent in their country for participating in a clinical study
 	
Are confirmed PHI by at least one of following criteria:
 	
• HIV positive antibody test within 6-months of an HIV negative antibody test
  ○ (randomisation must take place within 6 months of previous negative test)
• HIV antibody negative with positive RT-PCR
• Test "incident" at low level (<0.6) using detuned assay (must be subtype B) (there
  ○ is a cap of one-third of randomised patients under this criterion)
• Equivocal HIV antibody test supported by a repeat test within a 2-week period
  ○ showing a rising optical density
• Have clinical manifestations of symptomatic HIV seroconversion illness supported
  ○ by antigen positivity and <4 bands positive on Western Blot
 	
Able and willing to give written informed consent
	• Pregnant
	• Unlikely to comply with protocol, and in particular adhere to therapeutic regimen
	• Likely to use narcotics during the study period
	• Antiretroviral therapy is indicated
	• Antiretroviral therapy is contraindicated.

Table 3.3. Inclusion and exclusion criteria for SPARTAC clinical trial participants

a)

	ART-48 (n=24)	No ART (n=37)
Male/Female	24/0	36/1
Age-yrs (in 2013)	42 (40-48)	42 (37-48)
Ethnicity		
White British	22	32
White Australian	1	3
Italian	1	0
Brazilian	0	2
CD4 count baseline	510 (386-758)	534 (412-573)
Mean p24 antigen (pg/ml) Baseline	41 (14-127)	47 (10-140)
Median HIV RNA level- copies/ml		
Week 0	80080 (41675-186835)	98878 (26300-202778)
Week 24	<50 (50-50)	48929 (8951-103754)
Week 60	10423 (1401-79226)	17464 (268-40095)

b)

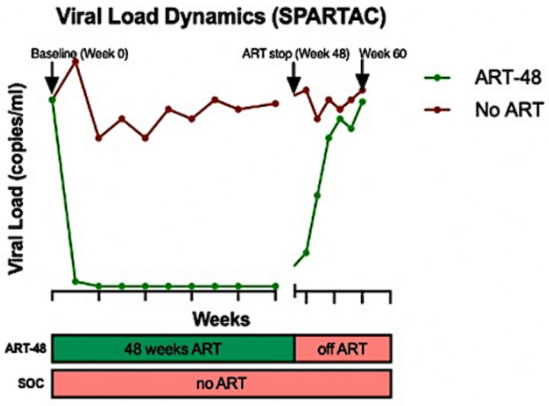


Figure 3.1a. Participant demographics for the SPARTAC cohort (ART-48 and No ART arms).

Figure 3.1b. Viral Load dynamics – Timings of sampling for ELISpot assays in the SPARTAC cohort. Different colour lines represent example viral load dynamics for SPARTAC groups ART-48 (green) and No ART (red). The x axis denotes time in weeks followed by the intervention received (ART or no ART), as well as the periods of analytical treatment interruption. Samples for SPARTAC were analysed at Weeks 0, 24 and 60 for both arms.

3.4 PITCH

Clinical Outcomes

The PITCH viral load dynamics and timepoints sampled are shown in **Figure 3.3a**. Of the seven participants who agreed to undergo a TI, all participants experienced eventual viral rebound and restarted ART and achieved fully suppressed plasma viral loads. The median time to viral rebound after TI was 36 days, with time to viral rebound defined as the first sustained detectable viral load above 50 copies/ml. The criteria for restarting ART was based on a combination of the degree and duration of viraemia, and was a joint decision between recruitment site clinicians and enrolled participants **(Figure 3.2).** Following a protocol amendment, certain participants were permitted to continue TI after rebound viraemia, according to certain parameters. **Figure 3.3b** shows the rebound dynamics for all enrolled PITCH participants. Both PSM01 and PST03 experienced rapid viral rebound and recommenced ART at peak viraemia. While PST07

maintained high levels of viraemia until ART re-initiation (34032 copies/ml at ART re-initiation), participants PST04, PST05 and PST06 demonstrated spontaneous – although moderate - reductions in viral load after reaching peak viraemia (3981, 4534, and 1877 copies/ml at ART re-initiation respectively). Participant PSM04 maintained undetectable viraemia (<20 copies HIV RNA/ml) for 170 days apart from three viral blips (all <150 copies/ml), and chose to restart ART at the third viral blip of 129 copies/ml (**Figure 3.3**).

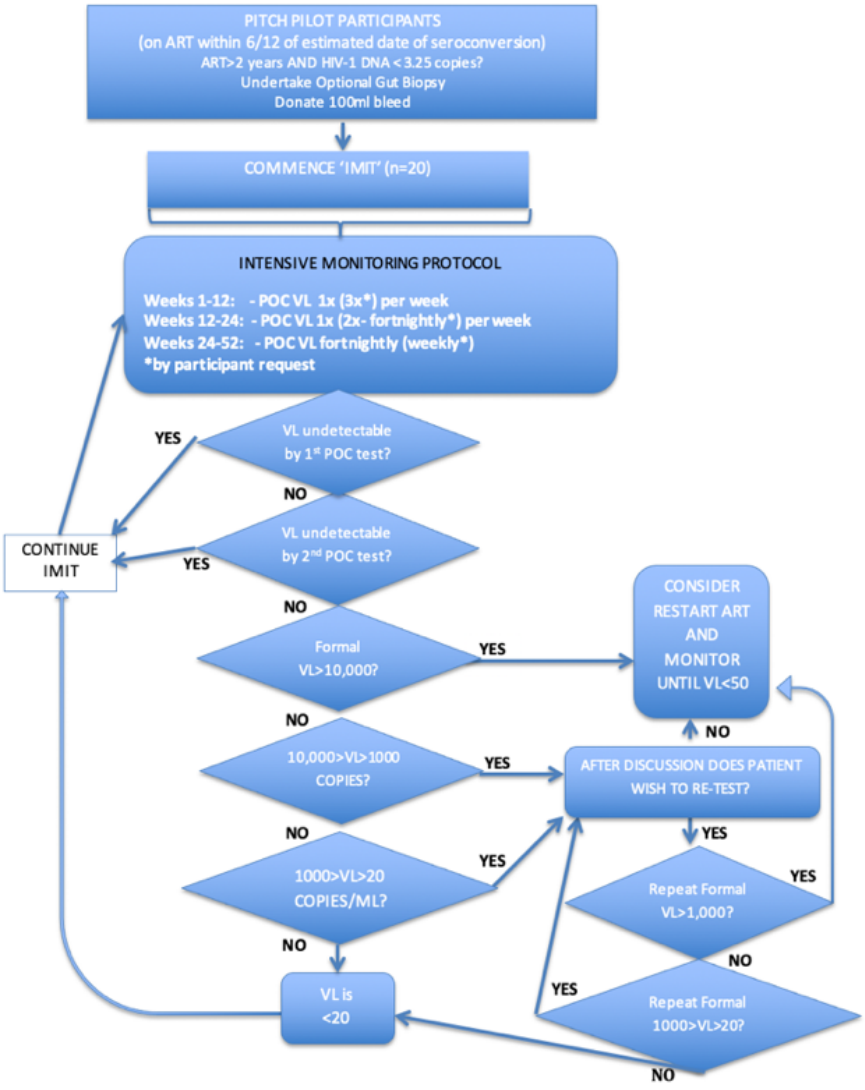


Figure 3.2. Algorithm used to determine ART restart criteria. Consideration to restart ART was given if 1. viral load (VL) as determined by a point of care (POC) test was greater than 10,000 copies, or 2. VL>1000 copies/ml but retesting was not desired by participant, or 3. Two consecutive repeat POC tests revealed a VL>1000 copies/ml.

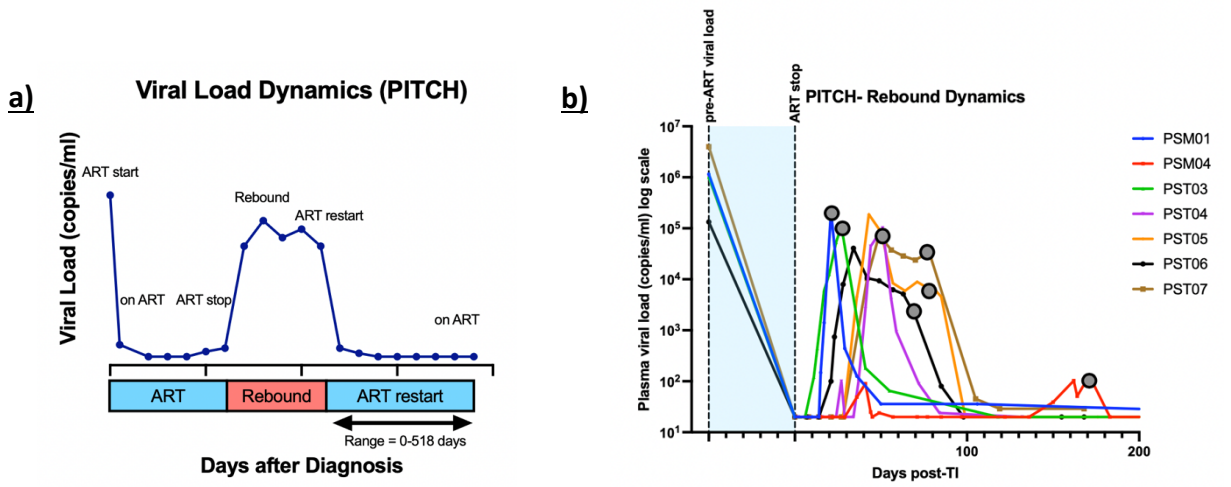


Figure 3.3a. Viral load dynamics for participants in PITCH study. Changes in viral load in copies/ml of PITCH participants from Day 0 (start of TI) to last follow-up date. The blue coloured line represents an example viral load dynamics pattern for PITCH participants; blue dots represent timepoints where blood was taken (where available) and analysed for HIV-specific T cell immunity (namely ART start, on ART, ART stop, rebound, ART restart, on ART). The last post-TI timepoint in analysed participants ranged from 0-518 days.

Figure 3.3b. PITCH viral load rebound dynamics in copies/ml of PITCH participants from Day 0 (start of TI) to the last follow-up date. Black circles represent the date of ART resumption; each coloured line represents a participant while solid-coloured dots represent viral load measurements. Where available, pre-ART viral loads are shown representatively for each participant prior to ATI.

3.4.1 **Hypothesis 1:** Treatment interruption is associated with shifts in immune dominance in individuals with PHI that receive ART for more than 2 years

We first examined whether there were observed shifts in T-cell immunodominance in individuals with PHI that have been treated for ART for more than 2 years. In the PITCH pilot study, participants received ART with a maximum of 6 months from seroconversion and remained on therapy for at least two years prior to treatment interruption. Because these participants received very early ART (and for a longer duration), this may have preserved T-cell responses to a greater extent, enabling these individuals to potentially make more beneficial responses after the withdrawal of ART.

3.4.2 Methods

To determine the impact of treatment interruption on HIV-specific T cell immunity, participants from the PITCH pilot study were examined. Due to cell availability and heterogeneity in the blood sampling schedule between follow-ups, relevant timepoints ranging from pre-ART, during TI and post-TI were selected for analysis. As such, ELISpot assays (where samples permitted) were performed at the initial pre-ART viraemic timepoint following diagnosis ("ART start"; n=4/7); when fully suppressed on ART prior (3/7; "On ART"); at viral rebound following TI ("rebound"; n=7/7); at ART restart following rebound ("ART re-start; n=4/7) and when fully suppressed again after ART re-start ("re-suppressed"; n= 6/7) **(Figure 3.3a).** As the 'rebound' and 'ART restart' samples were both viraemic timepoints and close, they were pooled to simplify subsequent analyses.

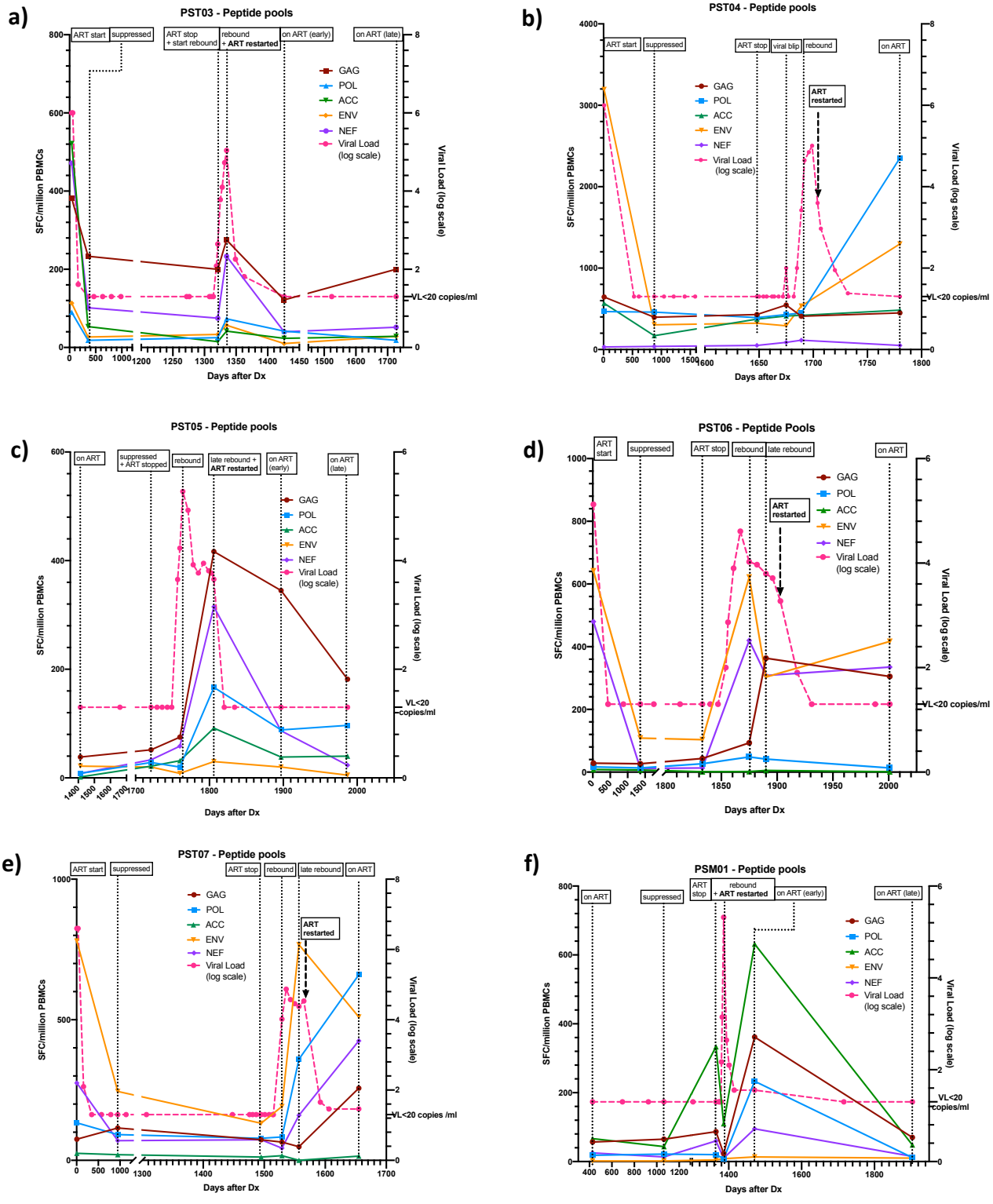
In the first instance, responses were tested to peptide pools covering the major HIV proteins (Gag, Pol, Env, Nef and Accessory) in the HIV-1 consensus B genome to obtain a broader view of pan-genome responses (total 742 overlapping peptides). We then deconvoluted the peptide responses seen at the peptide pool level using single 15-mer overlapping peptides. Data using peptide pools will be presented first, followed by single peptide analyses. Due to the small sample size of the pilot study, only qualitative and comparative analysis was performed on the data.

3.4.3 Treatment interruption in ART-treated individuals induces HIV-specific responses that persist after ART re-initiation

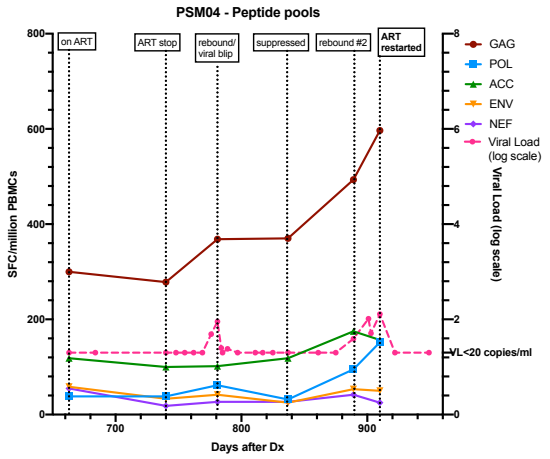
- **Peptide pool level**

Using peptide pools, HIV-specific responses were compared between aviraemic pre-TI and post-TI timepoints. This was done as viral loads at these timepoints were suppressed (less than 20 copies/ml), thus excluding viraemia as a confounder for changes in HIV-specific responses. Dominant responses were classified as the HIV protein that produced the highest response at each timepoint (for example, dominant Gag responses). If there were many high magnitude responses, the top three were classified as dominant responses. In participant PST03, dominant responses were made to Gag, Accessory and Nef responses at the point of ART initiation; however, during post-TI Gag responses dominated **(Figure 3.4a)**. For participant PST04, dominant responses were made to Env at the point of ART initiation; however, both Pol and Env responses dominated post-TI **(Figure 3.4b)**. In PST05, no responses dominated pre-TI; however, Gag and Pol-specific responses dominated post-TI **(Figure 3.4c)**. In PST06, Env and Nef responses dominated pre-TI; however, Gag, Pol, Env and Nef responses dominated post-TI **(Figure 3.4d)**. In PST07, Env and Nef-specific responses dominated at ART initiation; however, these were accompanied by dominant Gag and Pol responses post-TI and persisted a few months after ART initiation **(Figure 3.4e).** In PSM01, there were no dominant responses pre-TI; while there were reactive dominant responses to Accessory, Gag, Pol and Nef a few months post-TI, these waned over time **(Figure 3.4f)**. Most interestingly, participant PSM04 demonstrated evidence of Gag-dominant responses while remaining virally suppressed on ART pre-TI. The participant then maintained viral suppression for 170 days during treatment interruption; in addition, stepwise increases in Gag-specific responses were observed at all viral blips during the TI period, which increased up to the point where the participant chose to resume ART **(Figure 3.4g).** We also compared the response against viral load for all HIV proteins in each participant, but did

not observe any significant correlations **(Figure 3.5)**; however, it is important to note that the viral load measurements used were not viral load set points, as the ART restart criteria used meant that participants resumed ART before the set point was achieved. This may explain the lack of correlations.



g)



Figures 3.4a-g. ELISpots using HIV consensus B peptide pools in PITCH participants a) PST03, b) PST04, c) PST05, d) PST06, e) PST07, f) PSM04 and g) PSM01 at six selected timepoints, which included but were not restricted to: ART start, on ART, ART stop, rebound, ART restart, and resuppressed depending on sample availability. Each coloured line represents a timepoint where responses to peptide pools (Gag, Pol, Env, Nef or Accessory) were measured, expressed in spots per million PBMCs. Viral loads are plotted as pink dotted lines and expressed in copies per ml.

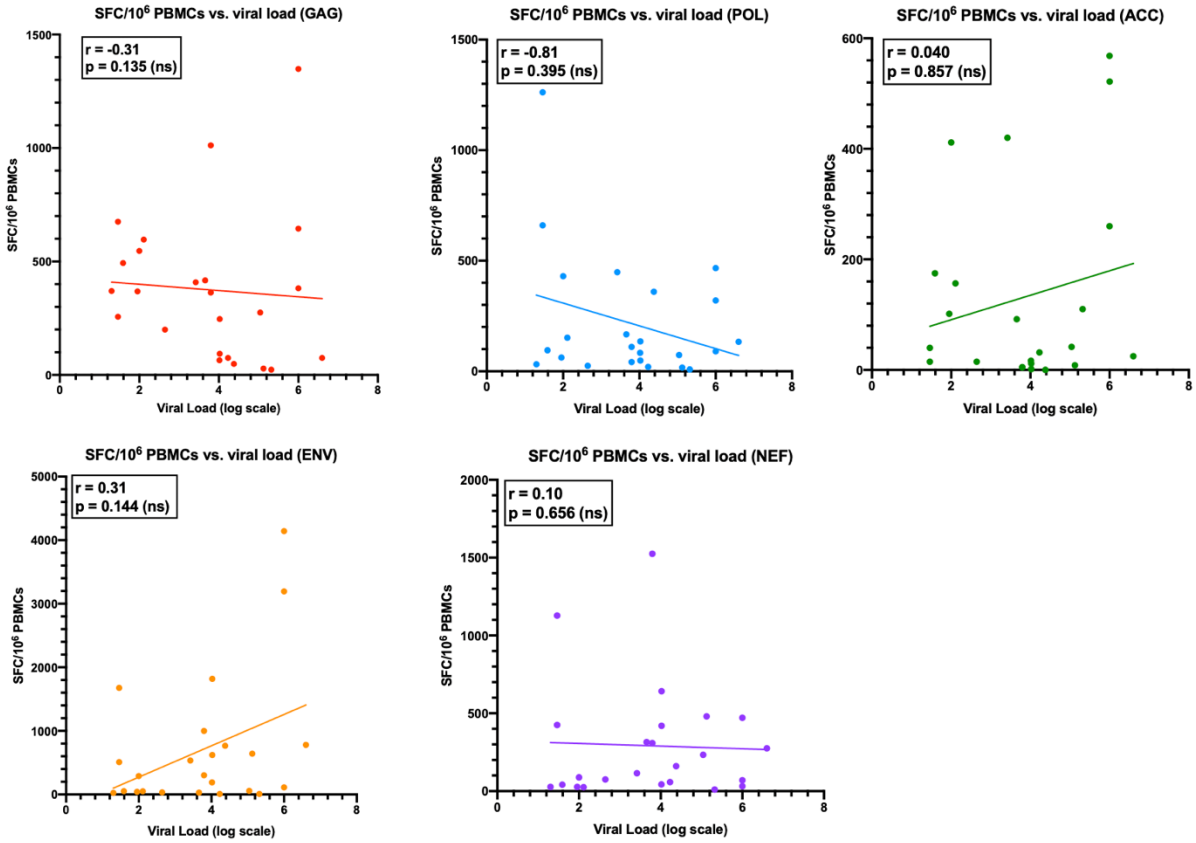


Figure 3.5. Correlations between SFC per million PBMCs and viral load in copies/ml for all data points for Gag, Pol, Acc, Env and Nef. The line in each graph represents a simple linear regression with r values shown. P values were calculated using Spearman correlations.

- **Single peptide level**

Next, we performed a comprehensive screening of HIV-specific T cell responses using "screening" matrix-based ELISpot assays, which have been previously described as a method to deconvolute individual peptide responses that are observed at the peptide pool level (see Chapter 2). Based on the positive response from these assays, we performed further ELISpots on the same participants using single overlapping peptides (OLPs) to "confirm" the responses observed at screening. Out of the seven participants who underwent treatment interruption, only five were chosen for further confirmation single peptide ELISpot assays; two of the participants were not taken forward for further analysis as there was no availability of PBMC samples left at these timepoints. If there were responses to two adjacent overlapping peptides in the matrix, the OLP responses were matched with the most likely HLA class I-matched "optimal" epitope retrieved from the Los Alamos database. In the cases where cell numbers restricted the number of OLP responses to test, priority testing was given to regions or epitopes that have been well documented in the literature.

Figures 3.6-3.7 shows results from the single peptide ELISpots performed for the five participants taken forward for "confirmatory" assays. For all participants, timepoints were selected pre, during and post-TI where cell availability permitted. The first participant, PST03, made a single Gag response to VDRFYKTLRAEQASQ pre-TI; however, this response failed to surpass the "positive response" threshold post-TI **(Figure 3.6).**

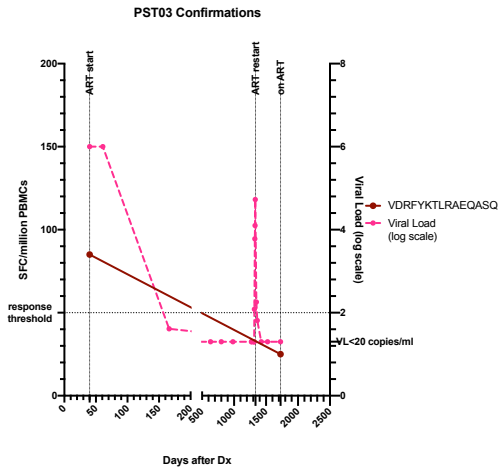
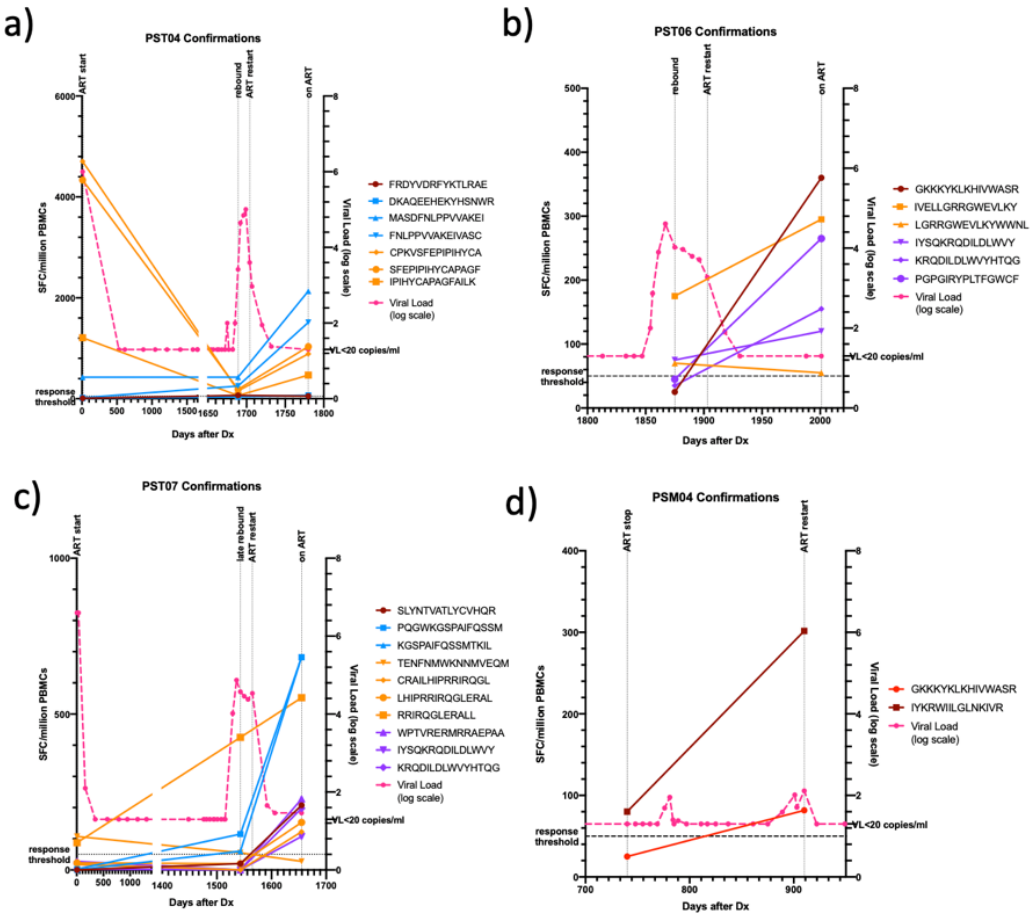


Figure 3.6. Single peptide ELISpots for PITCH participant PST03 at selected timepoints between ART start, rebound, and ART resumption/on ART. Each coloured line represents a response to an individual HIV peptide (Gag, Pol, Env, or Nef) expressed in spots per million PBMCs. Viral loads are plotted as pink dotted lines.



Figures 3.7a-d. HIV-specific T cell ELISpot responses in relation to treatment interruption.Single peptide ELISpots for PITCH participants a) PST04, b) PST06, c) PST07 and d) PSM04 at selected timepoints between ART start, rebound, and ART resumption/on ART. Each coloured line represents a response to an individual HIV overlapping peptide (Gag, Pol, Env, or Nef) expressed in spots per million PBMCs. Viral loads are plotted as pink dotted lines.

The remaining four out of five participants tested, however, showed demonstrable shifts in immune dominance towards novel Gag and/or Pol-specific OLPs that were not targeted prior to treatment interruption. To begin with, PST04 who rebounded 14 days after TI made dominant responses pre-TI, to CPKVSFEPIPIHYCA/SFEPIPIHYCAPAGF and IPIHYCAPAGFAILK in Env, and to MASDFNLPPVVAKEI in Pol. These Env and Pol-specific responses were maintained 91 days after ART re-initiation, with additional dominant Pol responses to MASDFNLPPVVAKEI/FNLPPVVAKEIVASC **(Figure 3.7a).**

For PST06, pre-TI samples were not available, thus only samples at rebound and post-ART re-initiation could be analysed. At the rebound timepoint, dominant responses were made to IVELLGRRGWEVLKY/ LGRRGWEVLKYWWNL in Env and IYSQKRQDILDLWVY in Nef; 4 months post-ART re-initiation, these pre-existing responses were enhanced (from 175 to 295 spots/million for IVELLGRRGWEVLKY and from 75 to 120 spots/million for IYSQKRQDILDLWVY) with an additional new Gag response to GGKKKYKLKHIVWASR that was not targeted during rebound **(Figure 3.7b).** For participant PST07, weak dominant Env responses were made to TENFNMWKNNMVEQM and RRIRQGLERALL at the point of ART initiation. Three months after ART re-initiation following TI, responses to RRIRQGLERALL were enhanced (from 87.5 to 552.5 spots/million) with new dominant responses toward Gag (SLYNTVATLYCVHQR), Pol (PQGWKGSPAIFQSSM /KGSPAIFQSSMTKIL) and Nef (WPTVRERMRRAEPAA, IYSQKRQDILDLWVY/KRQDILDLWVYHTQG) **(Figure 3.7c).**

Participant PSM04 remained virally suppressed for 180 days off therapy. At the point of TI, one dominant response to Gag was made (IYKRWIILGLNKIVR). During TI and up to the point of ART re-initiation, the response to IYKRWIILGLNKIVR increased and an additional new response to GKKKYKLKHIVWASR was also recorded at ART re-initiation (**Figure 3.7d).** The dominant peptide responses and likely HLA-class I matched “optimal” epitopes are for each participant are summarised in **Table 3.4**.

Summary Table – Dominant Peptide Responses	HLA Type (Class I)	ART start/on ART (Pre-TI)		ART re-initiation/on ART (Post-TI)	
		Peptide pools	Confirmations (Likely corresponding optimal)	Peptide pools	Confirmations (Likely corresponding optimal)
PST03	A 01:01/33:01 B 08:01/14:02 C 07:01/08:02	Gag Nef Acc.	DRFYKLTRA	Gag	N/A
PST04	A 02:01/29:02 B 38:01/51:01 C 07:01/12:03	Env	SFEPIPIHY IPIHYCAPA	Env Pol	SFEPIPIHY IPIHYCAPA LPPVVAKEI
PST05	A 03:01/11:01 B 07:05/35:01 C 04:01/15:05	None	N/A	Gag Pol	N/A
PST06	A 01:01/24:02 B 08:01/40:02 C 03:02/07:01	Env Nef	RRGWEVLKY KRQDILDLWVY	Gag Env Nef	GGKKKYKLKHIV RRGWEVLKY KRQDILDLWVY GPGIRYPLTFGWCF
PST07	A 01:01/02:01 B 07:02/08:01 C 07:01/07:02	Env	VTENFNMWKN RQGLERALL	Gag Pol Env Nef	SLYNTVATLY SPAIFQSSM IPRRIRQGL RQGLERALL WPTVRERM KRQDILDLWVY
PSM01	A 02:01/02:01 B 18:01/38:01 C 07:01/12:03	None	N/A	None	N/A
PSM04	A 02:01/24:02 B 15:01/27:05 C 01:02/03:03	Gag	KRWIILGLNK	Gag	KRWIILGLNK GKKKYKLKHIV

Table 3.4. Dominant OLP responses before (ART start/on ART) and after (ART re-initiation/on ART) TI. Each participant is on a separate row; corresponding HLA types and dominant responses seen at the peptide pool and confirmation level are shown in separate columns. The corresponding NIH peptide sequence derived from the HIV Los Alamos Database is shown for each dominant response at confirmation. Note that for PST06 confirmation responses pre-TI, the rebound timepoint was analysed instead due to lack of sample availability.

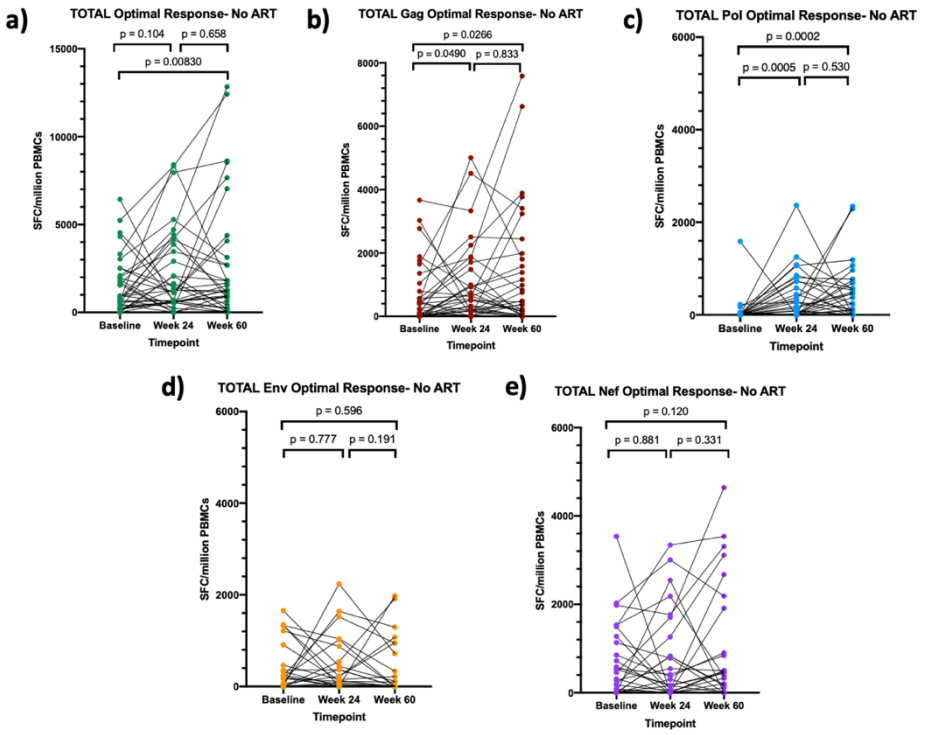
3.5 SPARTAC

3.5.1 **Hypothesis 2:** Participants in PHI that receive 48 weeks of ART demonstrate shifts in immune dominance that are not observed in ART-naive participants with PHI

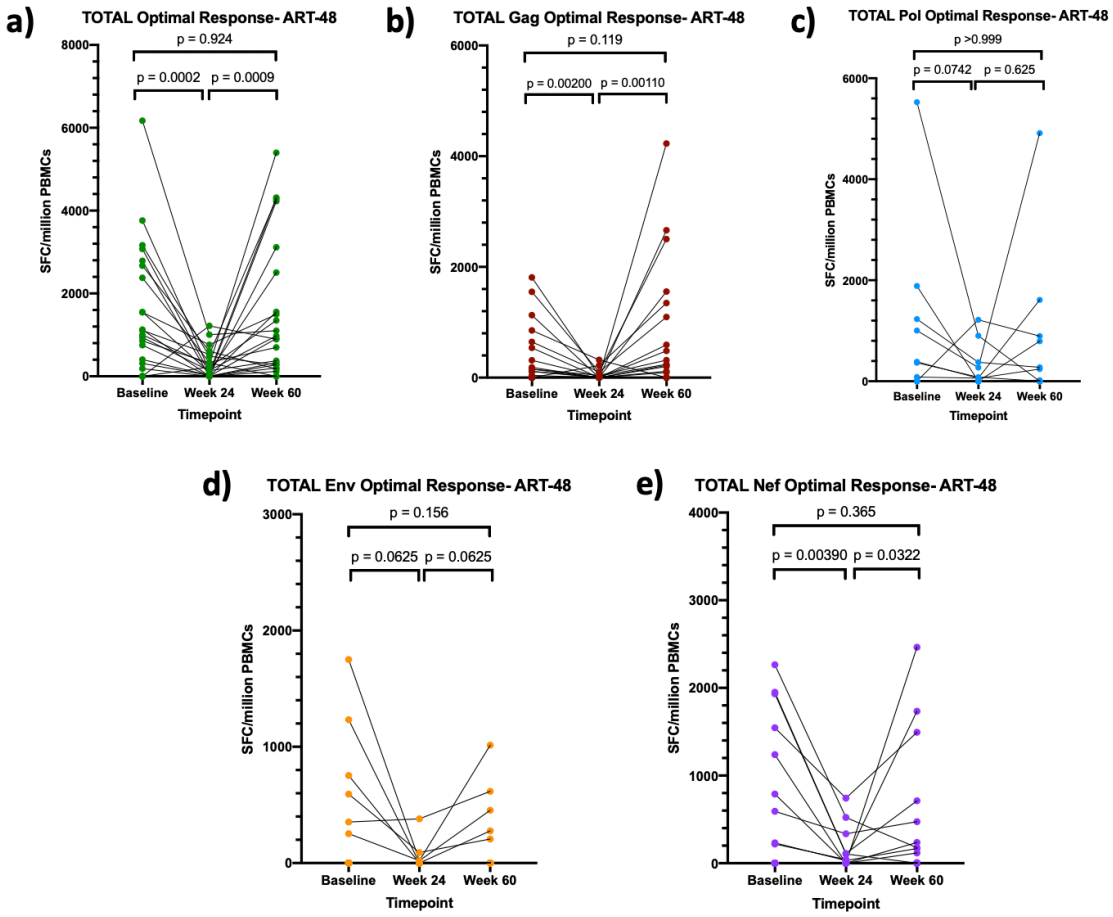
Next, we determined whether HIV-specific T cell responses were different in participants in PHI that received 48 weeks of ART versus no ART. We examined this using two arms of the SPARTAC cohort. We hypothesised that 48 week-treated participants would make differential shifts in immune dominance compared to ART-naïve participants, as we anticipated that ART would be associated with an increased production of beneficial responses.

3.5.2 Magnitude of T cell ELISPOT responses 60 weeks after PHI diagnosis is not significantly impacted by 48 weeks of ART

First, we examined the magnitude of HIV-specific T cell responses in both participant groups at Weeks 0, (during untreated PHI, within an estimated 6 months of seroconversion), 24 and 60 **(Figures 3.8a-e)**. For No ART participants, there were significant increases in total (p=0.0083), Gag (p=0.027) and Pol optimal responses (p=0.0002) from baseline to Week 60 **(Figures 3.8a-c),** which is consistent with an immune response to persistent viraemia and was likely driven by increases in viral load in the first 24 weeks. On the contrary, similar increases in responses between baseline and Week 60 were not observed for Env (p=0.596) and Nef (p=0.12). Interestingly, there were significant increases in Gag (p=0.049) and Pol (p=0.0005) optimal responses between baseline and Week 24; however, these responses lost statistical significance beyond Week 24 and did not further increase up to Week 60 (p=0.833 and p=0.530 for Gag and Pol respectively) **(Figure 3.8b-c)**.



Figures 3.8a-e. Line graphs showing the total response magnitude towards total, Gag, Pol, Env and Nef optimals at baseline, Week 24 and Week 60 timepoints for No ART participants. A total "optimal" response is defined as a response towards optimal peptides as chosen from the best-defined "A list CTL Epitope" database. Magnitude of response is shown as SFC per million PBMCs. P values were calculated using a Wilcoxon-matched pairs signed rank test.

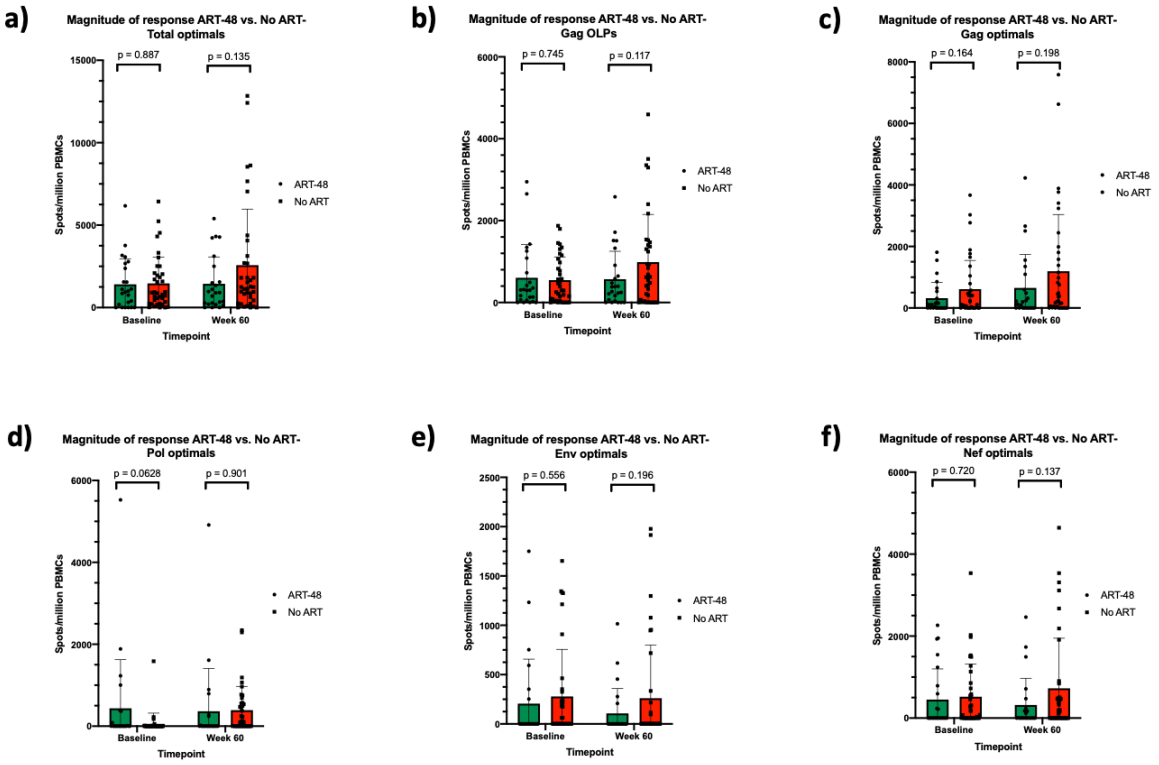


Figures 3.9a-e. Line graphs showing the total response magnitude towards total, Gag, Pol, Env and Nef optimals at baseline, Week 24 and Week 60 timepoints for ART-48 participants. A total "optimal" response is defined as a response towards optimal peptides as chosen from the best-defined "A list CTL Epitope" database. Magnitude of response is shown as SFC per million PBMCs. P values were calculated using a Wilcoxon-matched pairs signed rank test.

Next, responses in ART-48 participants were examined to determine whether similar changes in magnitude were observed **(Figures 3.9a-e).** In comparison, responses in ART-48 participants declined significantly by Week 24 for total (p=0.0002), Gag (p=0.002) and Nef (p=0.0039); however, after 12 weeks

of treatment interruption between Weeks 48 and 60, these responses subsequently rebounded towards baseline values.

Next, the magnitude of responses was compared between the No ART and ART-48 groups across all HIV proteins between baseline and Week 60. There was ultimately no significant difference between the groups at both baseline and Week 60 **(see Figure 3.10a-f).** This suggests that responses were rapidly restored in the ART-48 group after Week 48, and that participants were able to achieve a similar magnitude of response at Week 60 compared to those that had remained off ART for 60 weeks. The insignificant result at baseline is also reassuring, as it suggests that the sample selection (despite the availability of T-cell data) was sufficiently unbiased.



Figures 3.10a-f. Bar graphs comparing the response magnitude (in SFC/million PBMCs) between ART-48 (green) and No ART (red) participants at baseline (Week 0) and Week 60. Each bar represents the mean response magnitude and error bars indicate the standard deviation from the mean response. P values were calculated using non-parametric tests of significance.

Finally, the responses between the ART-48 and No ART groups between baseline and Week 60 could have been confounded by differences in viral loads; however, subsequent analysis demonstrated a lack of significance between the mean viral loads at Weeks 0 (p=0.6) and 60 (p=0.5) between both groups, which excludes viral load as a potential confounder **(Figure 3.11).**

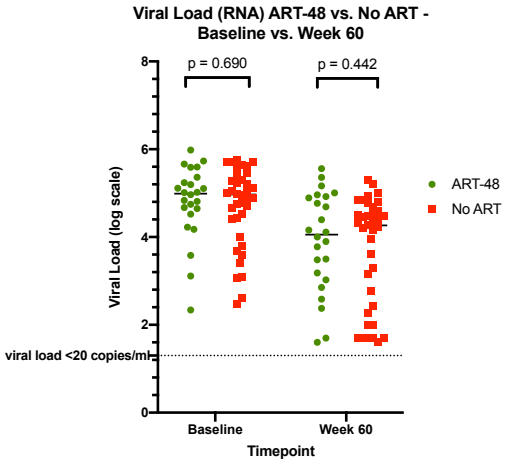
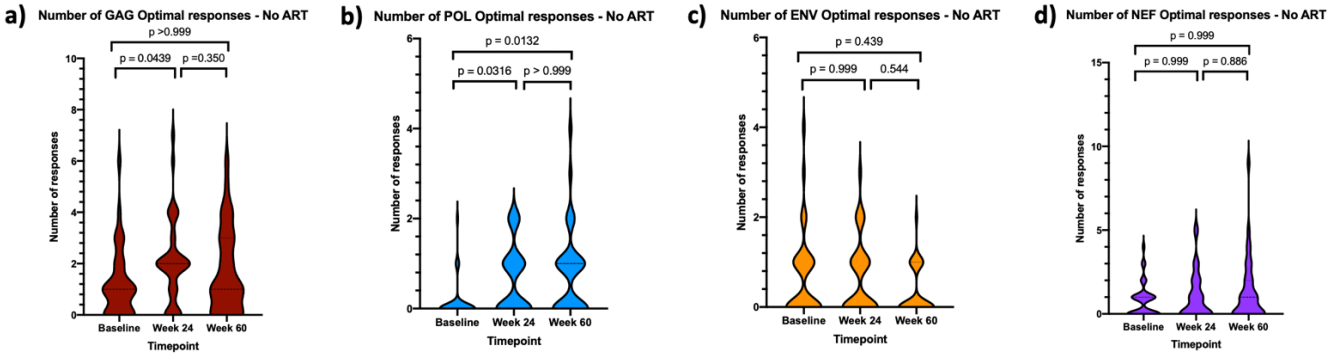


Figure 3.11. Graph showing viral load (RNA) of ART-48 (shown in green) and No ART (shown in red) participants at baseline (Week 0) and Week 60. P values were calculated using non-parametric tests of significance.

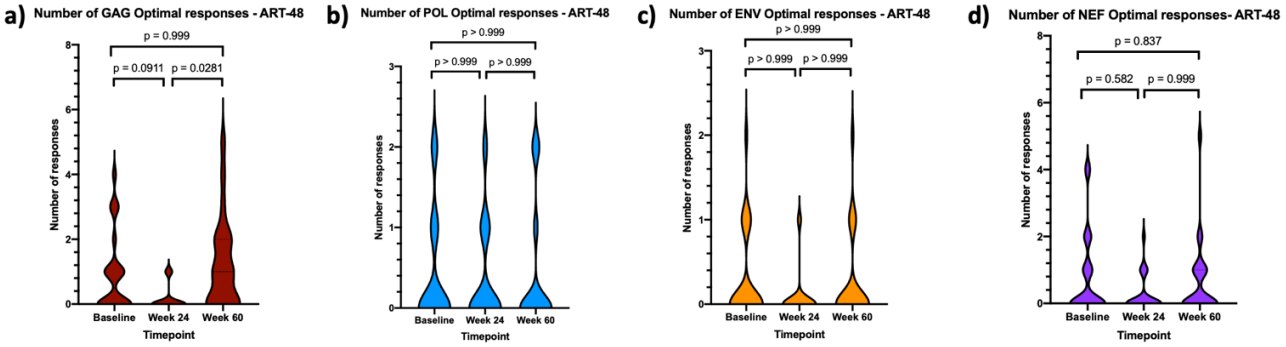
3.5.3 The absence of ART does not result in broader HIV-specific T cell responses by Week 60

In addition to response magnitude, we also examined changes in the breadth of response at Weeks 0 and 60 for ART-48 and No ART participants. This was performed by comparing the number of optimal peptide responses at baseline, Week 24 and 60. In No ART participants, responses to Gag (1.19 +/-1.35 -> 2.03 +/-1.66) p=0.0439) and Pol (0.14 +/-0.42 -> 0.65 +/-0.75) (p =0.0316) optimals increased significantly between Weeks 0 and 24 (**Figure 3.12a-b).** This was likely attributed to viraemia while remaining off ART. However, these increased responses were short-lived as these did not significantly expand further between Weeks 24 and 60 (2.03 +/-1.66 -> 1.65 +/-1.64, p=0.350 and 0.65 +/- 0.75 -> 0.78 +/-0.95, p>0.999 for Gag and Pol respectively) **(Figure 3.12a-b)** which echoes the findings observed in the previous analysis of response magnitude.



Figures 3.12a-d. Violin plots showing the change in number of responses to Gag (red), Pol (blue), Env (orange) and Nef (purple) optimal peptides between baseline and Week 60 for no ART participants. Bolded dotted lines indicate the mean response while faint dotted lines represent 1 standard deviation from the mean. P values were calculated using non-parametric tests to determine significance.

In comparison with No ART participants, no significant change in breadth was observed for ART-48 participants over the full 60 weeks **(Figure 3.13a);** changes in Gag optimal responses were observed between Weeks 0 and 24 (0.92 +/-1.21, -> 0.17 +/-0.38, p=0.0911) and between Weeks 24 and 60 (0.17 +/-0.38 -> 1.17 +/-1.37, p=0.0281), however these response dynamics were likely due to ART introduction and withdrawal during this period. Despite the transient impact of ART in the ART-48 participants, the final number of Gag optimal responses at Week 60 was not significantly different between ART-48 and No ART participants (1.17 +/-1.37 versus 1.65 +/- 1.64, p = 0.995) **(Figure 3.14),** suggesting that longer periods of viraemia do not confer stronger T cell responses.



Figures 3.13a-d. Violin plots showing the change in number of responses to a) Gag (red), b) Pol (blue), c) Env (orange) and d) Nef (purple) optimal peptides between baseline and Week 60 for ART-48 participants. Bolded dotted lines indicate the mean response while faint dotted lines represent 1 standard deviation from the mean. P values were calculated using non-parametric tests to determine significance.

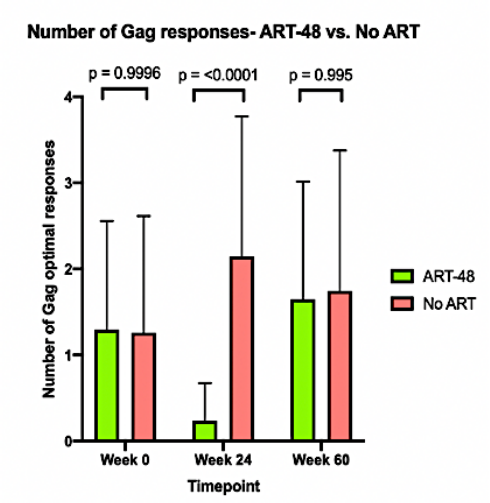


Figure 3.14. Bar graph showing the number of Gag optimal responses made by ART-48 vs. no No ART arms at Week 0, 24 and 60. Bars are plotted as mean values with error bars indicating the SD. P values were calculated using a two-way ANOVA to determine significance.

As HIV Gag specific T cell responses have been associated with reduction in viraemia (including in bNAb interventional studies (17,34)), a focused qualitative analysis was performed on the changes in the number of Gag optimal responses in No ART individuals between Weeks 0 and 60. This analysis focused on the dynamics of response versus the absolute responses made at each timepoint. While most No ART participants made increases in Gag optimal responses by Week 24 (69%, n=24/35), the majority lost at least one Gag response by Week 60 (71%, n=25/35) and only 29% (n=10/35) of No ART participants gained and/or increased pre-existing Gag optimal responses between Weeks 24 and Week 60 **(Table 3.5a-b).** On

the contrary, most participants in the ART-48 group lost Gag-specific responses at Week 24. This was likely due to the suppressive effects of ART on circulating antigen. Following ART withdrawal at Week 48, most ART-48 participants gained and/or increased pre-existing Gag optimal responses by Week 60 (65%, n=11/17), having only been off ART for 12 weeks prior to TI **(Table 3.5a).** This data demonstrates that ART-48 participants required a shorter period of viraemia (12 weeks) to achieve a similar breadth of response as No ART participants (60 weeks) **(Figure 3.14).**

a)

ART-48	Week 24	Week 60
A1		
A2		
A3		
A4		
A5		
A6		
A7		
A8		
A9		
A10		
A11		
A12		
A13		
A14		
A15		
A16		
A17		

KEY
gained new/increased existing response
shift
same/lost/no response

b)

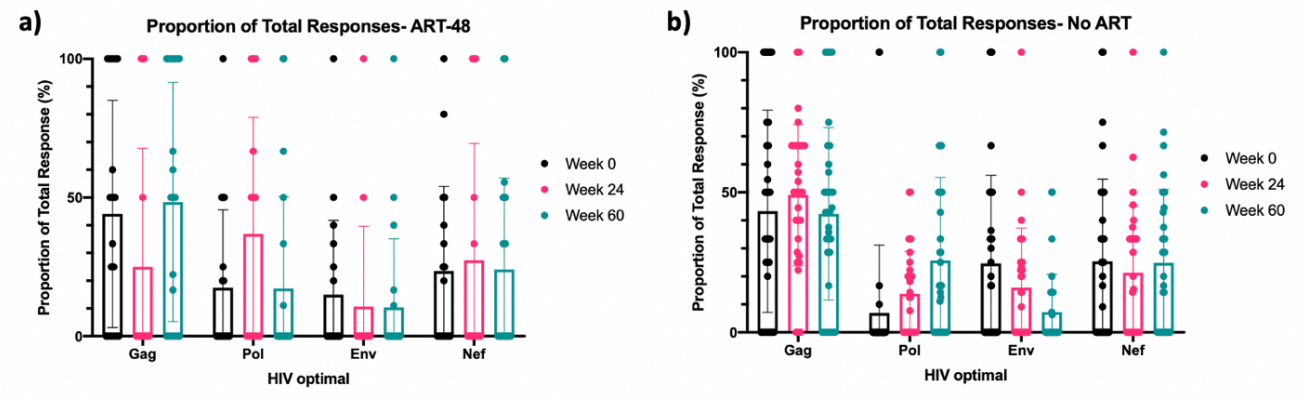
No ART	Week 24	Week 60
B1		
B2		
B3		
B4		
B5		
B6		
B7		
B8		
B9		
B10		
B11		
B12		
B13		
B14		
B15		
B16		
B17		
B18		
B19		
B20		
B21		
B22		
B23		
B24		
B25		
B26		
B27		
B28		
B29		
B30		
B31		
B32		
B33		
B34		
B35		

Tables 3.5a-b. Changes in Gag optimal responses between Week 24 and 60 for the a) ART-48 and b) No ART arm. Bars represent participants that gained or enhanced pre-existing Gag responses (green), gained and lost Gag responses (yellow) or made the same/lost/made no responses (red).

3.5.4 "ART-48" individuals make more Gag-directed responses by Week 60 compared to "No ART" individuals that makes less Gag-directed responses by Week 60

Next, we investigated shifts in immunodominance across different HIV proteins in ART-48 and No ART participants. We first examined whether the percentage of total optimal responses differed between both

participant groups between Weeks 0, 24 and 60. In both groups, there was no significant difference in the percentage of total responses **(Figure 3.15).** In addition, there was no difference in the number of Gag optimal responses made at Week 60 between both groups (p=0.995, **Figure 3.14**).



Figures 3.15a-b. Percentage of total responses to Gag, Pol, Env and Nef optimals by each participant in both a) ART-48 and b) No ART arm at Week 0 (black), 24 (pink) and 60 (green). Bar graphs indicate the mean proportion while error bars indicate the standard deviation from the mean.

Next, the changes in the percentage of novel responses were examined; a novel response was defined as a response to an epitope that was not previously seen at either baseline (Week 0) or Week 24. Upon analysis, the results showed that ART-48 participants made marginally more novel Gag responses (p=0.0694) and significantly less Pol-specific ones (p=0.0287) at Week 60 **(Figure 3.16)**. To determine whether shifts in immune dominance were present prior to Week 60 in both groups, the change in the percentages of novel responses across different HIV proteins between Weeks 24 and 60 was also examined. In the ART-48 group, the number (p=0.0137) and percentage (p=0.0001) of novel Gag responses significantly increased between Week 24 and 60 **(Figure 3.17a-b)**. In comparison, participants in the No ART group made a smaller number (p=0.0198) and percentage of Gag responses (p=0.0126) between Weeks 24 and 60 **(Figure 3.17c-d).**

The next question was whether the changes in optimal responses between Weeks 0, 24 and 60 were due to changes in dominant epitopic responses. Again, the analysis was particularly focused on Gag, as the quantity and quality of Gag-specific responses have been previously associated with reductions in viraemia (17). The qualitative analysis shows that, in both groups, a significant percentage of participants maintained the same dominant responses to Gag across Weeks 24 to 60. Also, many participants in each group maintained the same dominant Gag response across all timepoints **(Table 3.6a-b).** To verify whether these "dominant" Gag responses were HLA-restricted, the dominant Gag responses in both the ART-48 and No ART participants were compared to the Los Alamos Database and matched with their respective HLA-alleles. The results showed that the key immunodominant Gag optimal responses were indeed HLA-class I restricted, which supports their validity. Notably, the majority of No ART participants exhibited changes in dominant Gag responses between Week 24 and 60; however, this was not reflected in the mean viral loads when compared to the ART-48 participants **(see Figure 3.11 previously).** Detailed HLA types and immunodominant Gag responses are in **Tables 3.7a-b**. Finally, a subset analysis was performed based on existing available data from SPARTAC to determine whether the shifts in dominant responses were accompanied by changes in CD4+/CD8+ T-cell activation or exhaustion by Week 60. The results showed no significant difference in CD4+/CD8+ single positive PD-1, Tim-3 and Lag-3 expression between the ART-48 and No ART groups **(Figures 3.18a-g)**. In addition, the subset of participants examined had no significant difference in mean viral load at Week 60 as well ($p>0.999$) **(Figure 3.18g).** The demographics of analysed participants are shown in **Table 3.8**.

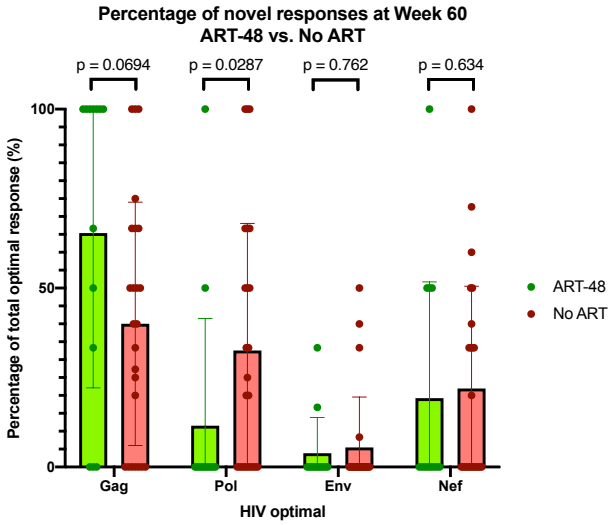
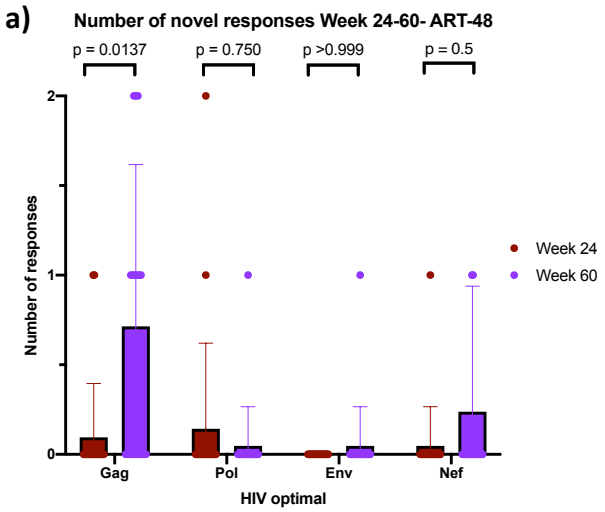
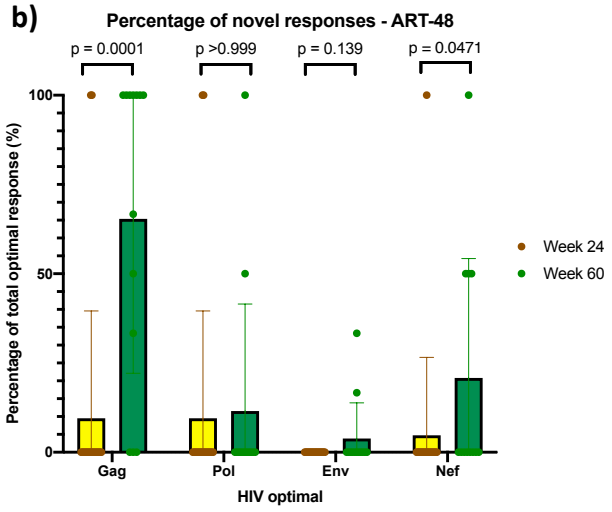
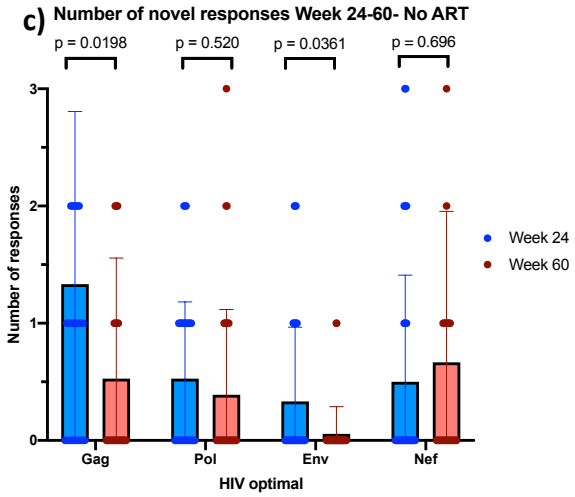
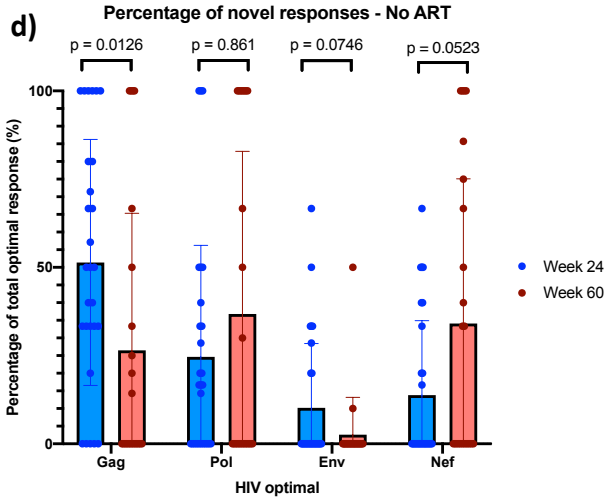


Figure 3.16. Percentage of novel responses to Gag, Pol, Env and Nef optimals in the ART-48 and No ART arms at Week 60. Bar graphs indicate the mean percentage of response, while error bars indicate the SD. A novel response was defined as a new epitopic response that was made by Week 60 and not previously seen at Week 24. P values were calculated using non-parametric tests to determine significance.









Figures 3.17a-d. Number and percentage of novel responses to Gag, Pol, Env and Nef in ART-48 and No ART arm between Week 24 and 60. Bars show the mean values and error bars indicate the SD. Percentage of response was calculated by dividing the number of HIV optimal responses by the total number of optimal responses at each timepoint. P values were calculated using non-parametric tests to determine significance.

a)

ART-48	Week 0	Week 60
SUC044011	none	NANPDCKTI
SUA038008	KIRLRPGGKK	PPIPVGDIY
SUJ036035	KRWIILGLNK	none
SUP036041	RMYSPTSI	RMYSPTSI
SUN036050	HQAISPRTL	RLRPGGKKK
SUC214013	KRWIILGLNK	KRWIILGLNK
SUA036054	KIRLRPGGKK	RLRPGGKKKY
SUK033005	HPVHAGPIA	none
SUR054005	GEIYKRWII	GEIYKRWII
SUT054004	GEIYKRWII	GEIYKRWII
SUX214024	KRWIILGLNK	KRWIILGLNK
SUY038009	RQGLERALL	GEIYKRWII
SIN039002	FLGKIWPSYK	none
SUX044005	none	IEIKDTKEAL
SUE036062	none	GEIYKRWII
SUQ036069	none	ACQGVGGPGHK
SUE033007	none	LYNTVATLY

b)

No ART	Week 0	Week 24	Week 60
SUT036022	GPGHKARVL	GPGHKARVL	TPQDLNTML
SUC036029	none	GEIYKRWII	GEIYKRWII
SUQ036032	none	GEIYKRWII	none
SUB044003	none	RSLYNTVATLY	GLNKIVRMY
SUF214003	KYKLKHIVW	KYKLKHIVW	KRWIILGLNK
SUT033001	TSTLQEQIGW	TSTLQEQIGW	TSTLQEQIGW
SUP033003	none	SEGATPQDL	SEGATPQDL
SUB036055	KRWIILGLNK	GHQAAMQMLKE	KRWIILGLNK
SUV214008	none	EVIPMFSAL	none
SUT036059	IEIKDTKEAL	IEIKDTKEAL	IEIKDTKEAL
SUA214014	RLRPGGKKKY	KIRLRPGGKK	RLRPGGKKKY
SUC036063	KYKLKHIVW	IEIKDTKEAL	KYKLKHIVW
SUS036068	GEIYKRWII	GEIYKRWII	GEIYKRWII
SUP054006	KYKLKHIVW	KYKLKHIVW	KELYPLTSL
SUP036078	GEIYKRWII	IEIKDTKEAL	AEQASQDVKNW
SUY036082	none	GLNKIVRMY	none
SAU010002	none	AEQASQDVKNW	SLYNTVATL
SAV032002	none	RQANFLGKI	none
SRC002017	none	SEGATPQDL	RSLYNTVATLY
SUY036048	GEIYKRWII	GEIYKRWII	GEIYKRWII
SUV054003	RLRPGGKKK	RLRPGGKKK	none
SUK038004	TSTLQEQIGW	TSTLQEQIGW	TSTLQEQIGW
SAX032001	KIRLRPGGKK	RLRPGGKKKY	RLRPGGKKKY
SRS002002	SEGATPQDL	SEGATPQDL/AEQASQDVKNW	SEGATPQDL
SUK036043	TSTLQEQIGW	GEIYKRWII	KAFSPEVIPMF
SUF214020	GEIYKRWII	GEIYKRWII	none
SUD214004	FLGKIWPSYK	FLGKIWPSYK	none
SUU036067	IEIKDTKEAL	IEIKDTKEAL	
SUD036071	HPVHAGPIA	none	none
SUF036070	VLAEAMSQV	none	none
SUM036079	AEQASQDVKNW	GEIYKRWII/EVKDTKEAL	GEIYKRWII
SUC036080	FLGKIWPSYK	none	GEIYKRWII
SUW036083	AEQASQDVKNW	AEQASQDVKNW	AEQASQDVKNW
SUC036046	KRWIILGLNK	none	KRWIILGLNK
SUU214017	none	none	RDYVDRFYKTL

Tables 3.6a-b. Tables showing Gag optimal responses made by each participant at Week 0, 24 and 60 in the a) ART-48 arm and b) No ART arm respectively. The dominant response at each timepoint was defined as the response that had the highest response magnitude; in some cases, two peptides were equally dominant and both are shown. If a participant did not make any responses or all responses were equally dominant, this was classified as “none”. Yellow highlighted rows indicate when the same dominant response was seen at two or more timepoints.

a)

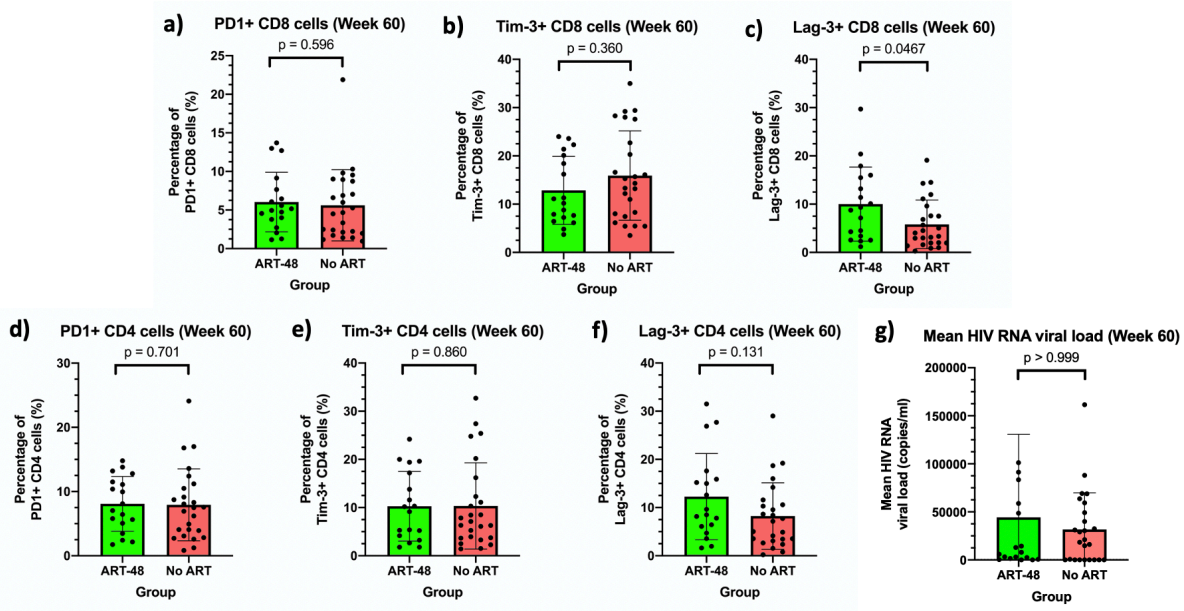
ART-48	HLA-A		HLA-B		Week 0				Week 24			Week 60			
					GEIYKRWII	KRWIILGLNK	FLGKIWPSYK	KIRLRPGGKK	KRWIILGLNK	KIRLRPGGKK	NANPDCKTI	GEIYKRWII	KIRLRPGGKK	FLGKIWPSYK	KRWIILGLNK
A1	0201	2301	1537	5101			YES				YES			YES	
A2	0301	2402	0801	3501	YES	YES		YES	YES	YES	YES	YES	YES		YES
A3	3101	3201	1501	2705		YES		YES	YES	YES			YES		YES
A4	3101	2407	3501	5201		YES		YES	YES	YES			YES		YES
A5	0201	0301	0702	3901		YES	YES	YES	YES	YES			YES	YES	YES
A6	0201	3001	2705	4402		YES	YES	YES	YES	YES			YES	YES	YES
A7	0201		4001	0702	YES		YES	YES		YES		YES	YES	YES	
A8	0101	0201	1501	3503			YES	YES		YES			YES	YES	
A9	0101	0201	0801	2705	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
A10	0101	0201	0801	1501	YES		YES				YES	YES		YES	
A11	0201	2404	0801	2705	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
A12	0101		0801	1501	YES						YES	YES			
A13	0201	0201	4402	5101			YES				YES			YES	
A14	0201	2402	4001	1501	YES	YES	YES		YES			YES		YES	YES
A15	0101		0801	3701	YES						YES	YES			
A16	0201	1101	0702	3501			YES	YES		YES			YES	YES	
A17	0201		1501	4501			YES							YES	

b)

No ART	HLA-A		HLA-B		Week 0		Week 24					Week 60		
					GEIYKRWII	AEQASQDVKNW	GEIYKRWII	FLGKIWPSYK	EVKDTKEAL	SLYNTVATL	GGKKKYKLK	AEQASQDVKNW	GEIYKRWII	EVKDTKEAL
B1	0201	2402	1402	0702				YES		YES				
B2	0101	2901	0801	5001	YES		YES	YES	YES		YES		YES	YES
B3	0101	2601	0801	5201	YES		YES	YES	YES		YES		YES	YES
B4	0201		1501	4402		YES		YES		YES		YES		
B5	0201	2403	2705	3801				YES		YES				
B6	0101	0201	0702	5701		YES		YES		YES		YES		
B7	2301	6601	4403	5201		YES						YES		
B8	0201	3201	2705	3801				YES		YES				
B9	2601	2402	1503	3801		YES						YES		
B10	0101	0201	0801	4001	YES		YES	YES	YES	YES	YES		YES	YES
B11	0301	2402	0702	3504										
B12	2402	3002	1501	4001	YES		YES				YES		YES	
B13	0101	0201	0801	1402	YES		YES	YES	YES	YES	YES		YES	YES
B14	2402	2902	4001	4403	YES	YES	YES	YES				YES	YES	
B15	0101	2402	0801	1507	YES		YES		YES		YES		YES	YES
B16	0101	3101	1501	5501										
B17	0101	0201	0702	4402		YES		YES		YES		YES		
B18	1101	3001	1302	3802							YES			
B19	0201	0301	3906	4403		YES		YES		YES		YES		
B20	0101	0201	0801	0702	YES		YES	YES	YES	YES	YES		YES	YES
B21	0201	0301	0702	4001	YES		YES	YES		YES			YES	
B22	0101	0301	1501	5701		YES				YES		YES		
B23	0301	0301	0702	0702										
B24	2608	3201	0702	4405		YES						YES		
B25	0101		0801	5701	YES	YES	YES		YES		YES	YES	YES	YES
B26	0101	2402	0801	3501	YES	YES	YES		YES		YES	YES	YES	YES
B27	2301	0201	1518	4403		YES		YES		YES		YES		
B28	0101		3508	4001	YES		YES						YES	
B29	0201	3201	1402	5501				YES		YES				
B30	0201		4402			YES		YES		YES		YES		
B31	0201	1101	0801	4403	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
B32	0201		0702	0801	YES		YES	YES	YES	YES	YES		YES	YES
B33	0101	6801	3901	5301						YES				
B34	0201	2902	2705	4402		YES		YES		YES		YES		
B35	0201	6801	4001	4402	YES	YES	YES	YES		YES		YES	YES	

Tables 3.7a-b. Tables showing HLA types for participants in both arms with HLA-matched dominant Gag responses between Weeks 0 and 60.

Dominant peptide responses are shown at corresponding timepoints in separate columns, and each row represents a separate participant. A dominant response was defined as a response that was recognised by >=10% of participants in each arm. These were matched with participant HLA-types and the Los Alamos epitope database; if the corresponding peptide was theoretically matched with a participant's HLA-type, this was written as "YES". An additional green bar represents a participant that made an actual response to that peptide at a given timepoint (thus it is clear that a significant proportion of participants at any given timepoint made responses to a dominant HLA-specific Gag peptide).



Figures 3.18a-g. Bar graphs showing CD8+ cells that were single positive for a) PD1, b) Tim-3 and c) Lag-3 respectively at Week 60 for ART-48 and No ART participants selected for analysis. P values were calculated using Mann Whitney tests and errors bars represent one standard deviation from the mean. Figure 18g shows the mean HIV RNA viral load (copies/ml) between the ART-48 and No ART participant subsets. P values were calculated via Mann Whitney tests and error bars represent one SD from the mean.

Participant Subset Demographics	ART-48	No ART
Male/Female	18/0	24/1
Age (yrs) Median-IQR	34.9	35.2
Mean viral RNA Level (copies/ml) at Week 60	50025	34848

Table 3.8. Demographics of participants analysed for ART-48 and "No ART" groups. Only participants with complete datasets for CD4/+CD8+ single positive PD-1, Tim-3, and Lag-3 were included in analysis.

3.6 Discussion

Despite existing evidence on post-treatment control, few studies have properly characterised HIV-specific T-cell responses during and after treatment interruption in individuals with primary HIV infection. Given the numerous interventional studies on HIV cure that incorporate treatment interruptions, as well as evidence on the potential role of CD8 T cells in favouring long term viral control, it is therefore important to determine the immunological impact of TI alone. It is also important to determine how the duration, or absence of, ART affects these responses. In this chapter, we hypothesised that treatment interruption in individuals treated during primary HIV infection would induce shifts in immune dominance that could favour viral control. We also determined whether these shifts were potentially restricted to specific HLA-class I alleles.

To examine this hypothesis, we characterised the HIV-specific T cell responses amongst individuals with primary HIV infection that received different lengths of ART or no ART. In the PITCH cohort, individuals were given ART during primary HIV infection and remained on ART for at least two years prior to TI; the rationale for doing this was that a longer duration of ART would reduce the size of the viral reservoir, and thus increase the likelihood of viral control upon interrupting treatment (35,36). In the SPARTAC cohort, individuals were randomised to receive either 48 weeks of 12 weeks ART followed by a treatment interruption, or no ART. The primary endpoint of the trial was determined by CD4 T cell counts and/or long-term ART initiation, and to determine if 48 weeks of ART would delay disease progression as opposed to short course (12 weeks) ART. Previous publication of the SPARTAC trial proved this hypothesis (26), however the changes in HIV-specific CD8 T cell responses between ART-treated versus ART-naïve groups have yet to be characterised from this cohort. Here, we showed that ART-induced viral suppression is associated with changes in the magnitude and dominance of HIV-specific T cell responses, and that TI

itself is associated with new Gag- and/or Pol-specific responses that are sustained, in some cases, up to 24 weeks after ART initiation (as seen in the PITCH cohort).

With regards to the methods used to examine HIV-specific T cell responses, the SPARTAC cohort used standard CD8 ELISpot assays to characterise responses to the HIV optimals within the HIV consensus B genome, as well as to Gag overlapping peptides. To maintain consistency and for the purposes of comparison to data from PITCH, similar ELISpot assays were performed for PITCH participants. ELISpots were an appropriate method to use, as they have been a clinically validated method for the robust detection and quantification of antigen-specific T cell responses via the measurement of cytokine-secreting cells as previously described (37–39). While ELISpots have specific drawbacks such as being biased toward Th1 helper responses and the detection of other non-T cell derived production of cytokines, this method was chosen because it has been extensively used by notable papers in the deconvolution of antigen-specific T cell responses via the use of matrix-based and/ or optimal peptide assays (40–42). As this analysis was aimed at characterising shifts in immune dominance between different HIV proteins, this method was a suitable choice to use.

In our analysis of PITCH, participants receiving >2 years of ART had shifts in immunodominance towards Gag and/or Pol-specific responses post-TI. In the SPARTAC cohort, receiving 48 weeks of ART was associated with a significant increase in new Gag-specific responses between Weeks 24-60, compared to untreated (No ART) participants that made significantly less Gag-specific responses during the same period. This was also observed in the percentage change of responses between Weeks 24-60. This suggests that treatment with ART may increase the potential to make new Gag responses over time compared to no ART. However, we did not observe differences in immunodominant Gag epitopes targeted between untreated and treated participants. In both groups, there were a number of frequently

targeted epitopes (e.g GEIYKRWII and EVKDTEAL), however the immunodominance of these epitopes is likely attributed to their high frequency of recognition by common HLA class I alleles, which were enriched in both participant groups.

Despite our analysis of immune dominance via ELISpot assays in both cohorts, there were some limitations. Firstly, the SPARTAC cohort focused on time to viral rebound as the primary endpoint; thus, this limited the analysis to examining changes in HIV-specific T cell immunity as opposed to correlating responses with viral control. Similarly for PITCH, all participants rebounded rapidly and as such, it cannot be concluded whether HIV-specific T cell immunity in either context, or "auto-vaccination", has a demonstrable clinical benefit. However, the data presented still provides useful insights into the immune response dynamics occurring during treatment interruption. With exception of one participant (PSM04) in the PITCH cohort, shifts in immunodominance were all confounded by bursts of viraemia and circulating viral antigen during TI, and it is therefore difficult to determine whether these responses were truly beneficial or simply produced in response to viraemia. This would only be possible to determine if the period of analytical treatment interruptions were extended to allow a sufficient window of opportunity for HIV-specific T cell responses to control viral replication; however, in the PITCH cohort, ART re-initiation was a joint decision between the participant and the recruitment site clinician and therefore varied for each participant. Furthermore, the algorithim used for ART-reinitiation was based on existing evidence from previous TI studies that closely monitored treatment interruptions would not increase the risk of HIV disease progression, morbidity or mortality (11). In the PITCH cohort, timepoints were only available up to 4 months following ART re-initiation; for future studies, it would be interesting to observe the durability of these HIV-specific T cell responses, and whether these responses could be potential candidates for inducing durable viral control and/or protection in the absence of ART. Nonetheless, it is worth noting that one participant, PSM04, remained virally suppressed during TI apart from three viral blips, and at the

same time made persistent and dominant Gag-specific responses while off ART. According to the peptide pool data for PSM04, these responses increased in a stepwise fashion after each viral blip until the point of ART re-initiation, while was a joint decision between the participant and clinician rather than meeting the ART-restart criteria. These increases were further validated by the single peptide responses which showed increases to targeted Gag epitopes GKKKYKLKHIVWASR and IYKRWIILGLNKIVR. While this was only an n of 1, PSM04 is an interesting case study because the participant showed evidence of viral suppression with accompanying increases in HIV specific T cell immunity. Likewise, this has been observed in previous studies that have investigated Gag-specific responses in relation to durable immune control (18,43,44).

Apart from PST04, we noted that all participants that demonstrated at least partial viral control prior to ART resumption showed evidence of increased Gag-specific responses at the peptide pool level during viral rebound or after ART resumption. In addition to this, the majority of PITCH participants appeared to make enhanced responses towards non-Gag epitopes, such as Env and Nef; for example, PST04 made detectable responses to three Env overlapping peptides pre-TI, while PST06 made two Env and three Nef responses recorded at viral rebound and these subsequently increased in magnitude after ART resumption. However, these responses were present pre-TI and while these responses were enhanced post-TI, they are likely "passenger" responses (45–47) and reflect viraemia rather than contributing significantly to viral control. In addition, this evidence does not conflict with the findings surrounding the development of new Gag responses post-TI.

Moving forward, future studies should focus on examining HIV-specific T cell immunity in participants that remain virally suppressed in the absence of ART (which will also be explored in subsequent chapters). In addition, this analysis would benefit from a larger sample size, which was unfortunately infeasible as

funding was withdrawn due to the COVID-19 pandemic. The initial aim of the PITCH pilot study was to determine the feasibility of undertaking an intensively monitored interruption of treatment (IMIT), with the aim of scaling up to a larger treatment interruption study. Despite the small sample size used, 4 out of 5 participants demonstrated shifts in immune dominance towards Gag and Pol-specific responses after restarting ART; this phenomenon would benefit from larger scaled prospective studies with IMIT to confirm these findings in other cohorts.

With regards to the SPARTAC cohort, there were a few caveats regarding the comparison of the treated (ART-48) and untreated (No ART) groups. In the comparisons made, the ART-48 and No ART groups were off therapy for different durations (12 weeks versus 60 weeks respectively) so it is uncertain whether ART-treated individuals would continue to produce Gag-biased responses beyond 12 weeks of treatment interruption. It is possible that the "new" Gag responses made by the ART-48 group could be lost over time; however, going against this argument is the fact that untreated participants did not show significant declines in Gag responses between Weeks 24 and 60 ($p=0.35$). The only method to formally test this is to analyse relevant timepoints up to 60 weeks post-TI in the ART-48 group; however, the SPARTAC trial was completed over 10 years ago and no samples were available for further analysis. This prevented a more in-depth longitudinal comparison of HIV-specific T cell responses. In addition, while we examined shifts in immune dominance between ART-48 and No ART groups, it is hard to determine whether the T cell responses made were protective responses that could potentially be driving immune control, or whether these responses were simply "passenger" responses produced because of viraemia. While this is possible, against this argument is the fact that viral load was excluded as a confounder between both groups at Week 0 and 60. Moreover, it is also important to note that the primary aim of SPARTAC was to determine whether 48 weeks of ART was more effective in delaying disease progression to the primary endpoints (CD4 count less than 350 and/or re-initiation of ART) than 12 weeks of ART or no ART, which was the

standard of care at the time of the trial publication. As such, our analysis of HIV-specific T cell immunity was a secondary outcome of this dataset and the main aim was to determine differences in immune dominance between both groups. Nevertheless, the findings presented demonstrate that, in untreated participants, a longer duration off therapy does not result in an increased production of Gag-specific responses, and in fact skews HIV-specific T cell immunity towards non-Gag ones.

Although we could not demonstrate a correlation between these responses and control, Gag-specific responses have been consistently associated with viral load in several previously published studies and thus these findings pose significant implications on the effects of prolonged time off therapy (17,18,47,48). Another limitation of this study was that we were unable to characterise HIV-specific T cell responses using other functional T-cell assays, which includes but are not limited to HIV-specific proliferation and/or HIV-specific T cell activation, exhaustion or immune phenotyping. However, current ongoing and future studies involving TI should incorporate additional functional T cell assays to further investigate phenotypic and functional changes in HIV-specific T cells. In addition, a deeper characterisation of T-cell functionality will be explored in subsequent chapters of this thesis. Notably, in the context of mechanistic work around clinical trials, the ELISpot is still one of the most reproducible and quantitative assays available, and while not giving the additional functional data associated with cytometry-based approaches does give a robust measure of antigen-specific responses (48,49). Additionally, ELISpot assays permit the deconvolution of epitope-specific responses to analyse shifts in immune dominance over time.

Overall, the findings presented provide evidence that treatment interruption, in both ART-treated and untreated individuals with primary HIV infection, causes shifts in immunodominance of HIV-specific T cell responses. Analysis of the SPARTAC cohort enabled a longitudinal comparison of HIV-specific T cell immunity in untreated individuals compared to 48-week treated ones, while the PITCH cohort provided a

more in-depth deconvolution of individual epitope-specific responses. However, our understanding of the clinical significance of this shift and the contribution of specific epitopic responses on immune control is still incomplete. Despite this, the data demonstrates that ART given during primary HIV infection induces shifts in immunodominance towards Gag and sometimes Pol, and these responses are likely to be beneficial towards achieving immune control. Moving forward, examining the durability of these responses post-ART resumption, as well as the characterisation of HIV-specific responses in ART-treated individuals receiving novel immunotherapeutic interventions who remain virally suppressed during TI, will be instrumental for understanding the role of HIV-specific T cell immunity in achieving post-treatment control and ultimately an ART-free remission. **Figure 3.19** below summarises the findings presented in this chapter.

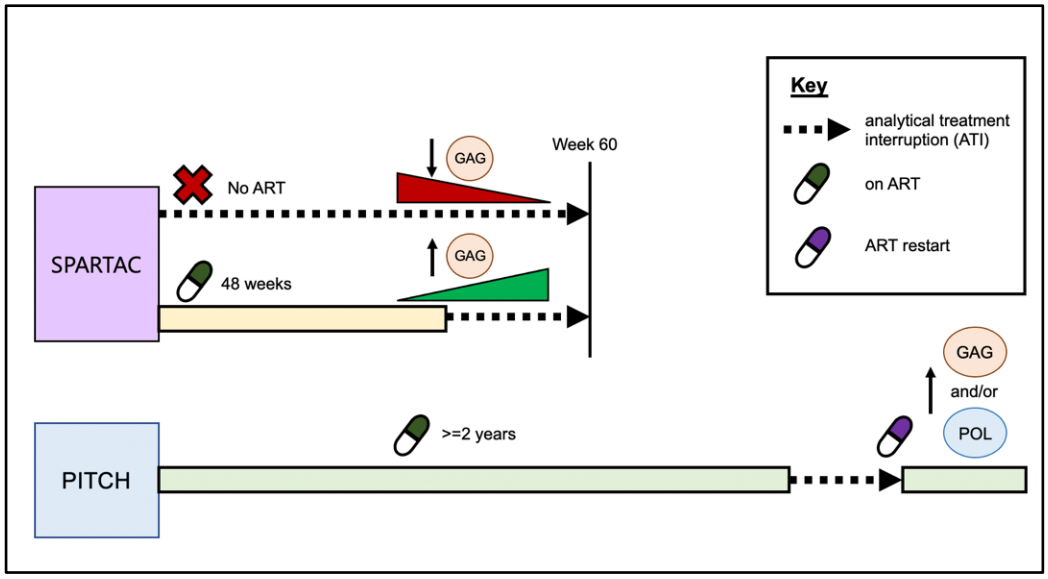


Figure 3.19. Summary of Findings in Chapter 3

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Chapter 4

The impact of broadly neutralising antibodies (BNAbs) and treatment interruption on T cell responses in primary HIV infection

Contributions to this chapter

I would like to express my thanks to all the participants and staff that made the RIO clinical trial possible; this includes the RIO pilot study research investigators, clinicians and research staff at the respective clinical recruitment sites, as well as the trial steering committee.

Sample collection and processing for the RIO clinical trial were performed by members of the Prof. Graham Taylor's lab at Imperial College London, UK. Peptide pool dilutions and interferon-gamma ELISpot assays were performed by Helen Brown and Nicola Robinson, and both staff also assisted where necessary with results acquisition and assay optimisation. Dr. Ane Ogbe and Dr. Ming Lee assisted with the RIO trial study design and initial setup as well with experimental expertise. Dr. Ming Lee provided pharmacokinetic modelling data. The T-cell data presented here have been produced in collaboration with Dr. Mohammed Altaf and Carla Nel who helped with T-cell assay design, optimisation and results acquisition for the ELISpot, proliferation, immune phenotyping and AIM assays. Penny Zacharopoulou provided data on bNAb sensitivity at baseline.

NB: Due to the ongoing blinded status of the study at the time of submission, we used a combination of real-time unblinding and available pharmacokinetic data to infer treatment arms of participants. Although the results presented here are preliminary and do not reflect the final formal report, we expect them to be highly comparable.

4.1 Introduction

Aim: To investigate the effects of treatment interruption with or without bNabs on HIV-specific T cell immunity in ART-treated primary HIV infection.

The majority of individuals living with HIV mount an antibody response to the envelope glycoprotein of the virus; in fact, antibodies against autologous virus take approximately 12 weeks to develop after HIV transmission (1–3). However, antibodies against HIV are often "non-neutralising" and mostly ineffective, which is partly attributed to the emergence of immune escape mutations in the HIV "Env" gene (2,3). Furthermore, HIV-specific antibodies take years to develop and only around 20% of individuals with HIV develop some degree of neutralising antibodies against heterologous virus (1,2,4). Moreover, broadly neutralising antibodies (bNAbs) – antibodies which can neutralise up to 90% of circulating strains in some cases- are very rarely produced by individuals with HIV infection. These bNAbs only develop approximately 20-30 months after infection (5) owing to infrequent B cell priming and the requirement for extensive antibody somatic hypermutation (5). In early years, standard immunisation strategies prevented the production of *ex vivo* bNabs to adequate levels for use in clinical studies. However, recent advancement in single cell cloning techniques have enabled the isolation of bNabs from bNAb-producing individuals to facilitate the production of high bNab titres (6). In addition, the *in vivo* half-life of bNabs has been dramatically extended via the LS variant, which involves the modification of two amino acids (M428L and N434S) in the Fc region (7). These engineered Fc regions have an increased binding affinity to neonatal Fc-receptors (FcRn) which extends the half-life of serum antibody levels, and have been demonstrated in non-human primate models and human studies (8–11). Moreover, recent clinical trials have shown that

this enhanced FcRn binding did not significantly impair Fc-mediated effector functions such as ADCC (12,13). This has enabled even longer periods of virological suppression in the absence of ART.

Regarding the action of bNAbs, the Fc domain on the antibody is thought to exhibit multiple Fc-mediated effector functions which can attract Fcy receptors on immune cells such as NK cells, macrophage and complement proteins to initiate antibody-dependent cellular cytotoxicity, cellular phagocytosis and complement dependent cytolysis respectively (14–20) **(Figure 4.1).** The role of Fcy-receptors in the clearance of infected cells has been described in humanised murine models (21), and the significance of the Fc region in bNabs has been reported in spontaneous HIV controllers that have demonstrated enrichment for polyfunctional antibodies in the absence of ART and an enhanced capacity to drive ADCC (7,21). In addition to complement-mediated pathways, bNAbs are hypothesised to bind directly to conserved regions on the HIV envelope protein and form immune complexes that are recognised by Fcy-receptors on antigen presenting cells such as dendritic cells (DCs). This results in enhanced antigen presentation and boosted HIV-specific cytotoxic T cell responses (the "vaccinal" effect) (22–24). Although the link between bNab-Env immune complex formation and induction of the vaccinal effect has not been mechanistically proven, other studies investigating autoimmune diseases have used immune complex assays to quantify complexed antibody. Additionally, recent studies have used assays incorporating immobilised antigen to investigate the ability of virus-specific IgG to trigger Fc-receptor signalling (25–27). Likewise, these assays could be applied in future studies to detect bNab-Env immune complex formation and Fc-mediated interactions with HIV antigen.

The bNAb-driven "vaccinal" effect has been associated in several studies with the production of Gag-specific responses. While bNabs primarily target conserved sites on the envelope protein, these regions are often highly variable and poorly immunogenic, resulting in frequent immune escape and ineffective

targeting by most neutralising antibodies (4). There is currently no mechanistic proof to explain why bNAbs preferentially induce Gag-specific T cell responses. However, existing literature has shown that targeting Gag is more effective due to its highly conserved nature and inability to mutate without incurring high viral fitness costs (28); thus, the production of Gag-specific responses has a clear immune advantage. While Env-specific responses are being concurrently produced, they are likely being rendered ineffective due to the rapid mutational rate of epitopes in the envelope protein. Consequently, a vicious cycle ensues where new responses rapidly lose neutralising capacity in the context of a highly variable Env protein.

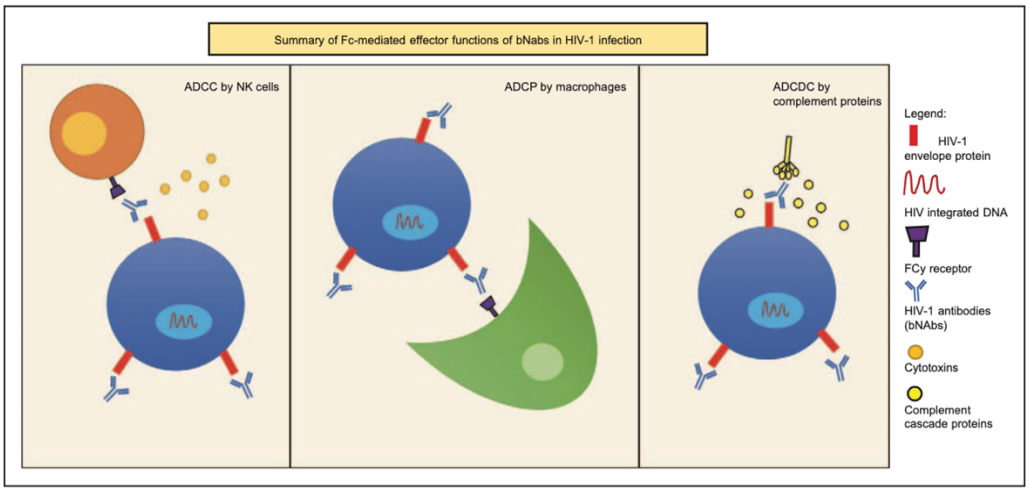


Figure 4.1. Schematic summary of the Fc-mediated effector functions of bNabs in HIV-1 infection (adapted from Tipoe et al ((29)).

The epitope targets of bNabs on the HIV envelope protein are relatively conserved and accessible regions, and become transiently exposed during cell attachment and viral entry which enables binding of bNabs to these sites (30). While some bNAbs target epitopes in the V3 loop (10-1074), others target the highly conserved CD4 binding site (VRC01 and 3BNC117) (31). Antibodies that target other sites, such as the V1/V2 loop and the membrane proximal external region (MPER) have also entered clinical trials, and

gp120-gp41 interface bNAbs have also been described in the literature (32,33) **(Figure 4.2, also see Appendix).**

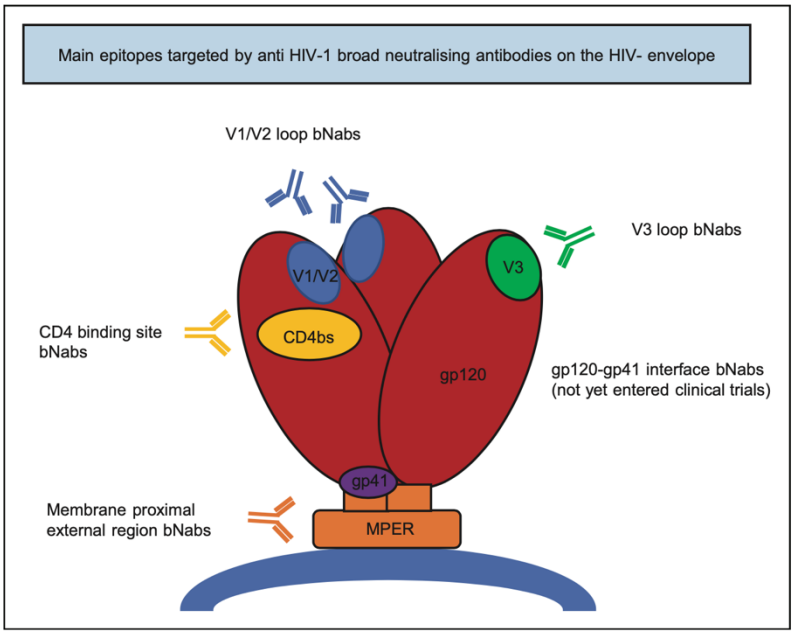


Figure 4.2. Main epitopes targeted by HIV-1 bNAbs on the HIV envelope (adapted from Tipoe et al((29)).

BNabs have previously been tested in proof of concept studies using animal models. The landmark study by Nishimura et al. describes SHIV-infected rhesus macaques that were treated with a combination of the short acting bNAbs 3BNC117 (which targets the CD4 binding site) and 10-1074 (which targets the V3 loop) (34). In their study, 6 out of 13 SHIV-infected rhesus macaques that were given three-weekly intravenous courses of combination bNAbs suppressed viraemia to undetectable levels. This viraemic control persisted below the subtherapeutic levels of bNabs. In comparison, macaques that were only treated with 15 weeks of ART rebounded rapidly upon ART interruption without any evidence of prolonged viral control. In the "controller" macaques, administration of a CD8-depleting antibody resulted in rapid return of viraemia (34). In an extension of this study by Nishimura, 12 SHIV-infected rhesus macaques were given either bi-weekly combination bNAb therapy or bNAb therapy plus ART at 2 weeks post-infection; subsequently, 7 out of 12 macaques achieved undetectable viral loads ("controller" status), which was again reversed via CD8 T cell depletion as shown in their previous study (35). These findings indicate that passive

combination bNAb therapy using 3BNC117 and 10-1074 can effectively induce durable CD8 T cell-mediated viral control in animal models, which is subsequently aborted upon CD8 depletion.

To extend these findings, Mendoza et al. tested this combination of bNabs in a human study with 9 individuals, all with primary HIV infection on ART. They received three three-weekly infusions of the combination bNAbs 3BNC117 and 10-1074, before and during treatment interruption (36). Subsequently, all 9 enrolled individuals with antibody-sensitive virus demonstrated prolonged viral suppression for a median of 21 weeks without evidence of developed antibody resistance. Additionally, two of the individuals exhibited viral control for an impressive 11 months. More interestingly, recent evidence in human studies has shown that bNAbs are associated with increased HIV-specific T cell responses. For example, the landmark paper by Niessl et al. looked at 9 aviraemic individuals on ART with primary HIV infection, and showed that individuals receiving short-acting combination bNab therapy made significantly higher CD8 T cell responses to Gag after 6 weeks of ART interruption compared to controls receiving ART only, as well as increased polyfunctionality of cytokine producing CD4 T cells (37). Furthermore, they demonstrated that 4 out of 6 participants with detectable interferon-gamma ELISpot responses produced broader Gag responses that were highly conserved and not present at Week 0 (37).

In a slightly different setting another study administered 3BNC117 monotherapy with or without romidepsin to ART-naïve individuals at the time of ART initiation during viraemia (38), rather than during viral suppression on ART. They hypothesised that administering 3BNC117 at ART initiation in the context of high plasma viraemia would maximise exposure to viral antigens and subsequent antibody-mediated immune enhancement. Interestingly, their study showed that individuals receiving 3BNC117 plus ART (with or without romidepsin) that were sensitive to 3BNC117 made increased Gag and Pol-specific CD8 T cell responses at 3- and 12-weeks post-ART initiation, versus those that received ART only (38). Moreover,

Gag-interferon gamma release was also increased at 12 weeks post-ART initiation compared to the ART-only group, and was shown to be a significant predictor of ART-free virological control (38). Taken together, these data suggest that bNabs could induce potent CD8 T cell immunity which could result in a T-cell mediated control of HIV.

Despite existing evidence from pre-clinical and clinical studies, the underlying immune mechanisms of these bNAbs remain undetermined. The current (although highly controversial) hypothesis, is that bNabs exert a "vaccine-like" effect by forming bNAb-Env immune complexes, which enhances antigen presentation and subsequently boosts the adaptive immune response sufficiently enough to maintain prolonged viral suppression (38). However, there has not yet been a clinical study that is statistically powered to answer this research question. Furthermore, while evidence of the effects of bNAbs on T cells has been proven in previous studies, a more detailed investigation into the precise immune mechanisms occurring during treatment interruption in ART-treated individuals with primary HIV infection that have received bNAbs is required. Existing studies have only used very small sample sizes, and it is imperative that these results are confirmed in larger sample size with a control arm. In addition, existing studies using combination bNAb therapy have used short-acting bNabs, and have subsequently shown that this combination can induce sustained viral suppression in the absence of ART. To extend these findings, the RIO clinical trial uses dual long-acting "LS" variants of these antibodies to determine whether this combination can achieve prolonged viral suppression in the absence of ART.

Given the current gaps in the literature regarding bNAbs, the RIO clinical trial aims to test these hypotheses via its primary, secondary and exploratory endpoints. The RIO is a phase IIb randomised clinical trial examining the effects of ART plus dual long-acting HIV-specific broadly neutralising antibodies (bNAbs) vs ART plus placebo in treated primary HIV infection on viral control off ART (39). The main

objective of RIO is to test whether dual long-acting bNabs, 3BNC117 and 10-1074, can induce a period of virological remission in ART-treated individuals in primary HIV infection when ART is stopped, compared to participants that receive ART plus placebo. In this chapter, the following hypotheses will be examined:

4.2 Hypotheses:

1. Dual long-acting bNabs (3BNC117-LS and 10-1074-LS) are associated with increases in HIV-specific T cell immunity in Arm A participants who remain virally suppressed and off ART
2. Arm B participants who receive dual long-acting bNabs during viraemia show evidence of increases in HIV-specific T cell immunity
3. Significant inter-assay correlations are observed between T-cell assays that measure HIV-specific T cell immunity, and some of these are predictive immune correlates for viral rebound

4.2.1 Description of RIO Cohort

Table 4.1 describes the participants who were analysed in each arm across all T-cell assays. Participants received either 1 or 2 bNab doses (except for one Arm B participant who has not yet rebounded and has thus not received any bNabs to date). The median age in both groups was similar and both groups were only composed of male participants. Most participants in both groups were white English and HIV clade B. Otherwise, there were no significant differences between both groups.

	Arm A (n=23)	Arm B (n=25)
Number of bNAb doses	1 (8) 2 (15)	0 (1) 1 (4) 2 (19)
Age (years)	36 (33-41.5)	44 (31.5-50.5)
Gender (M/F)	M (23) F (0)	M (25) F (0)
Ethnicity	Asian Indian 8.7% Asian Chinese 4.4% Black 4.4% White British 17.4% White English 43.5% White other 21.7%	Asian Indian 0% Asian Chinese 0% Black 8% White British 12% White English 40% White other 36% Other 4%
HIV Clade	A 0% AG 4.4% B 69.6% C 4.35% F 4.35% Unknown 17.4%	A 12% AG 4% B 64% C 4% F 4% Unknown 12%
Years of ART	6 (4.5-7)	5.5 (4-7)
CD4 count (Week 0)	679 (607.5-952.5)	733 (621-932.5)
Screening weight (kg)	71.8 (69.7-82.8)	78 (71.2-82.3)
Screening BMI	22.8 (22.1-25.87)	24 (22.7-27.3)
Screening creatinine (umol/l)	82 (74-88.5)	89 (77.5-96.5)

Table 4.1. Participant demographics for Arm A and B participants in the RIO trial that were analysed in this chapter. Numbers represent the median value, while brackets indicate the interquartile range.

4.3 Hypothesis 1: Dual long-acting bNabs (3BNC117-LS and 10-1074-LS) are associated with increases in HIV-specific T cell immunity in Arm A participants that remain virally suppressed and off ART

First, we determined if dual long-acting bNAbs (3BNC117-LS and 10-1074-LS) could enhance HIV-specific T cell immunity in ART-treated individuals with primary HIV infection who subsequently interrupt therapy; if so, which T cell functions were being enhanced. We hypothesised that administering dual long-acting bNAb therapy to ART-treated individuals with primary HIV infection induces a “vaccine-like” effect on T

cells via the formation of immune complexes, which results in more effective antigen processing and presentation and prolonged viral suppression off ART. This hypothesis has been tested by previous studies using short-acting bNAbs (37,38), but needs to be tested in a larger sample size, with the usage of long-acting combination bNAbs to determine whether similar effects on HIV-specific T cell immunity are observed.

4.3.1 Methods

To determine whether there was evidence of a "vaccinal" effect during ATI post-bNabs, specific Arm A participants were selected for analysis at Week 0, Week 12 and Week 24 timepoints post-bNab infusion. At the time of submitting this thesis, the trial remained blinded. We determined treatment arm assignments for the analyses presented using two methods: (1) unblinding participants as they completed the first stage of the trial, and (2) using pharmacokinetic (PK) data to infer treatment group based on drug levels. To ensure that observed increases in HIV-specific T cell immunity were not confounded by viraemia at specific timepoints, selected participants had to be virally suppressed: virally suppressed timepoints were defined as any timepoint with a viral load of less than or equal to 200 copies/ml. We chose this cut-off as some participants experienced viral blips above 20 copies/ml, but subsequently re-suppressed viral load and hence did not meet the criteria for viral rebound. Secondly, participants had to be off ART at these timepoints (i.e undergoing ATI), which excludes the protective effects of antiretroviral therapy as a potential confounder of viral suppression. Using these criteria, any increases in HIV-specific T cell immunity between these timepoints could be attributed to the effects of combination bNabs rather than viraemia.

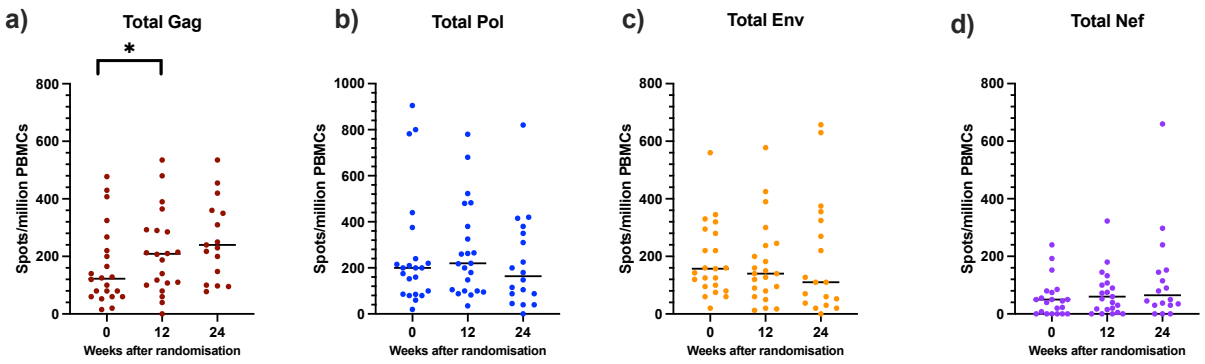
To analyse HIV-specific T cell immunity, PBMCs of participants were sampled at the timepoints mentioned above using T cell functional assays, including HIV-specific interferon-gamma ELISpot, cellular

proliferation, and T-cell activation via measurement of upregulated activation markers. For interferon-gamma ELISpot assays, we also predicted the likely HLA-class I restricted responses at specific timepoints. Detailed methods of these assays are described in Chapter 2. Statistical analyses were performed using Graphpad prism Version 10. To compare responses at different timepoints, Wilcoxon matched-pairs signed rank tests and repeated measures ANOVA were performed to determine significance.

4.3.2 Results- Arm A

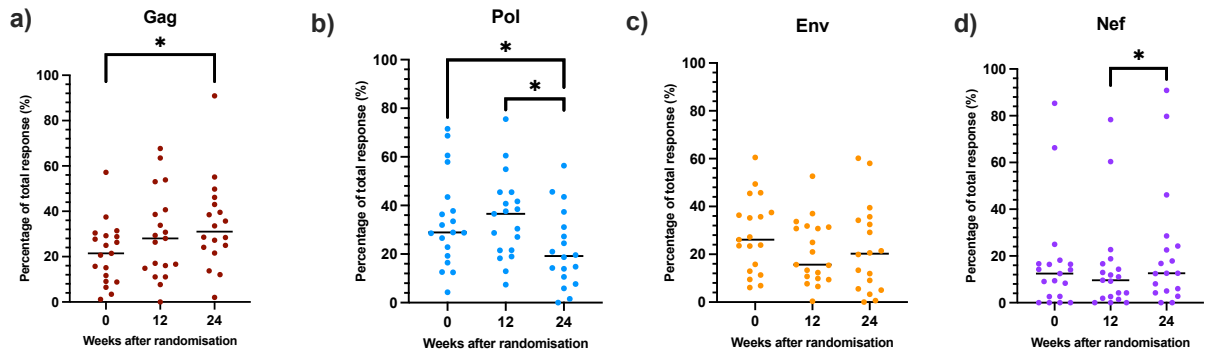
Combination bNAbs are associated with increases in Gag-specific interferon-gamma secretion in Arm A "virally suppressed" individuals off ART

Firstly, we examined whether Arm A participants who were virally suppressed off ART showed any changes in HIV-specific interferon-gamma secretion over time. To achieve this, we performed a general analysis of the "pooled" responses to each HIV protein to determine the total response at each timepoint (detailed methods are described in Chapter 2). We observed significant increases in Gag-specific responses between Weeks 0 and 12 (mean diff. 95.7 spots, p=0.0135) **(Figure 4.3a);** however, responses did not increase further between Weeks 12 and 24 (mean diff. 132 spots, p=0.106). There was also a significant increase in Gag responses between Weeks 0-24 using a univariable Wilcoxon matched-pairs signed rank test (164 -> 260 spots, p=0.0242), this was no longer significant when using a repeated measures ANOVA. There were no significant changes in HIV-specific T cell immunity observed between Weeks 0, 12 and 24 for Pol, Env and Nef respectively **(Figure 4.3b-d).**

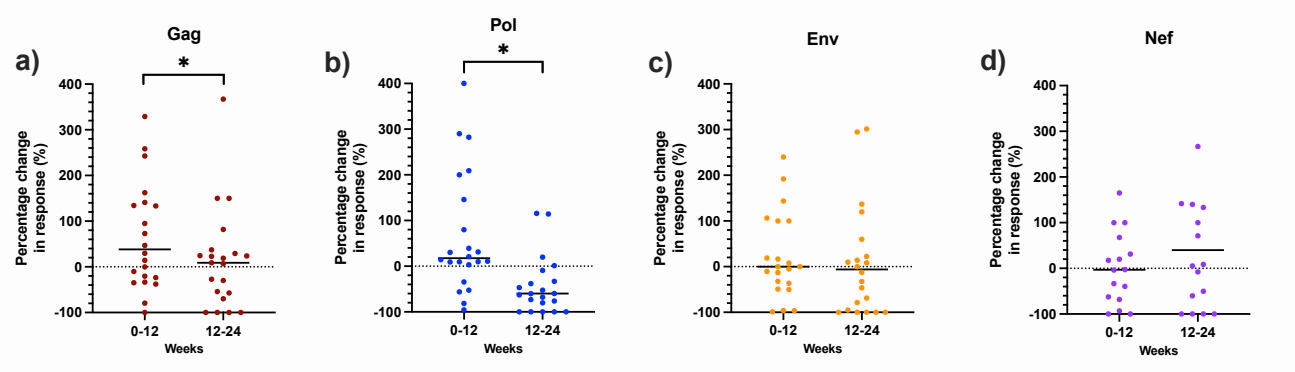


Figures 4.3a-d. Graphs showing the total a) Gag, b) Pol, c) Env and d) Nef "pooled" responses in Arm A participants that were virally suppressed off ART at Weeks 0,12 and 24 (n=23). Each coloured dot represents a separate data point expressed in spots per million PBMCs. Statistical significance was calculated using a repeated measures ANOVA; $p<0.05$ indicates significance and is marked by asterisks (*). Only significant p values are shown.

In addition to total response, we also analysed changes in the percentage of response between Weeks 0 and 24 to further assess shifts in immune dominance (as previously described in Tipoe et al. (40). For this analysis, only participants that experienced viral rebound after 20 weeks were included to ensure that observed shifts would be clinically relevant for viral control. Interestingly, there was a shift towards Gag-specific responses between Weeks 0-24 (21.4 -> 34.1%, p=0.0125) **(Figure 4.4a),** but not between Weeks 0-12 (21 -> 29%, p=0.118) or Weeks 12-24 (29 -> 34%, p=0.275). The percentage of Nef responses also increased between Weeks 12-24, but this was not significantly higher than at Week 0 (16.7% -> 21.7%, p=0.129). Additionally, the percentage of Pol responses decreased between Weeks 0-24 (33.8 -> 21.9%, p=0.0054) and 12-24 (34.9 -> 21.9%, p=0.0034) respectively. As another metric to evaluate changes in response between Weeks 0 and 24, we also calculated the percentage change in responses between Weeks 0-12, and Weeks 12-24 respectively. We observed significant decreases in the percentage change of response for Gag (p=0.0263) and Pol (p=0.0190) **(Figures 4.5a-b).** No significant increases were observed for Env or Nef **(Figures 4.5c-d).**



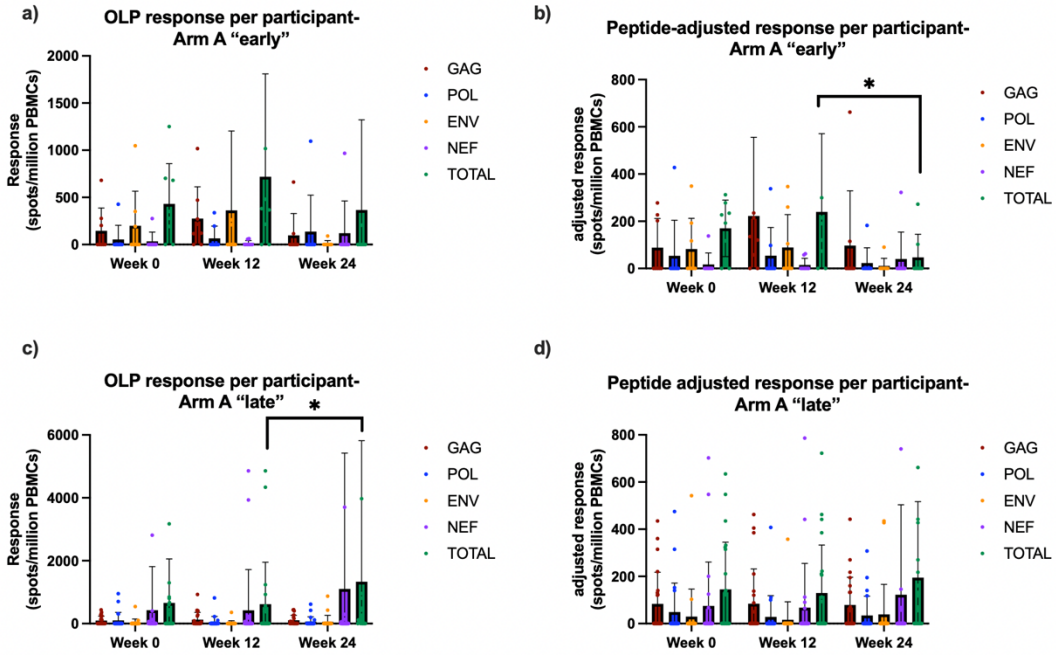
Figures 4.4a-d. Graphs showing the percentage of responses towards a) Gag, b) Pol, c) Env and d) Nef "pooled" responses in Arm A participants (n=19) between Weeks 0-24. Only participants that rebounded beyond 20 weeks were included in the analysis. Percentage response was calculated by dividing the response to individual proteins Gag, Pol, Env and Nef over total response (%). Statistical significance was calculated using a repeated measures ANOVA; p<0.05 indicates significance and is denoted by asterisks (*).



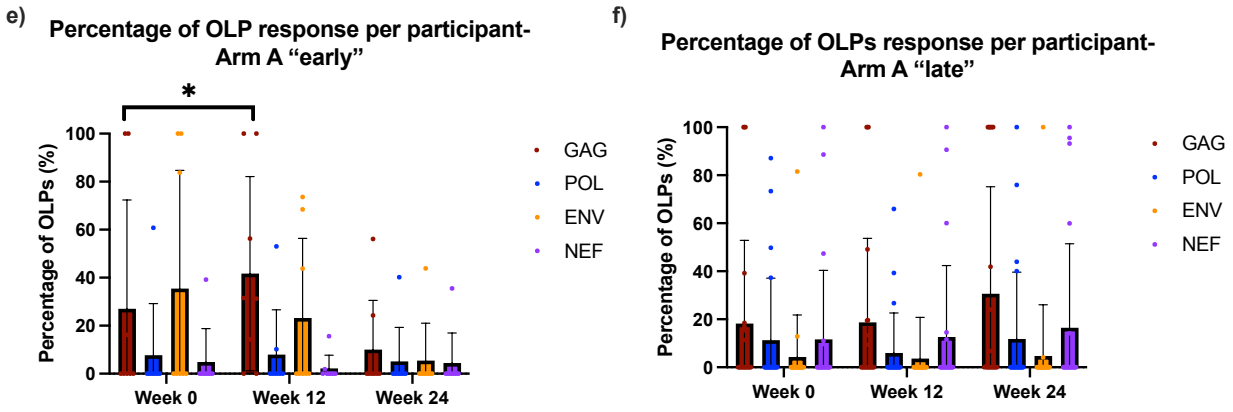
Figures 4.5a-d. Graphs showing the difference in percentage change of response for a) Gag, b) Pol, c) Env and d) Nef "pooled" responses in Arm A participants (n=22) that are virally suppressed off ART between Weeks 0-12 and 12-24. Percentage change in response was performed by calculating the difference in responses between timepoints and dividing this by the response at that timepoint; for example, percentage change in Gag response between Weeks 0-12 = (Week 12 Gag response – Week 0 Gag response)/Week 0 Gag response)) x 100. Each coloured dot represents a separate data point expressed as a percentage change in response (%). Statistical significance was calculated using a Wilcoxon matched pairs signed rank test; P<0.05 indicates significance.

To further investigate whether there were differences observed in Arm A participants that were virally suppressed during ATI versus Arm A participants that experienced rapid rebound, we categorised participants into "early" and "late" rebounders. Early rebounders were defined as participants that rebounded before week 20 post-ATI; conversely, late rebounders had to remain virally suppressed for at least 20 weeks post-ATI (all the participants in the late rebounder category rebounded beyond Week 24 for consistent analysis). Then, we analysed the changes in response to all overlapping peptides (OLPs)

tested at Weeks 0, 12 and 24, as well as the number of OLPs targeted. To account for the number of OLPs targeted in relation to the responses observed, we also calculated a peptide-adjusted response as an additional metric. A similar but slightly different metric to this was previously used in Altfeld et al., where they calculated the average magnitude (in spots/million PBMCs) response to different HLA-restricted peptides (41). To calculate the peptide-adjusted response, the total response towards one HIV protein in one participant was divided by the number of times the protein was recognised (for example, a total response of 1000 spots to 4 recognised peptides would give an adjusted response of 250). In the "early" Arm A participants, we observed a decrease in "peptide-adjusted" total response between weeks 12-24 **(Figure 4.6b)** (p=0.039). In comparison, we observed an increase in absolute response between weeks 12-24 in "late" Arm A participants (p=0.047) (although this was not observed after peptide adjustment) (**Figure 4.6c)**. When we examined the percentage of OLP responses, Arm A "early" participants showed increases in Gag response between Weeks 0-12 (p=0.047), although this was probably due to viraemia as 6/8 participants in this group had already rebounded by Week 12 **(Figure 4.6e)**.

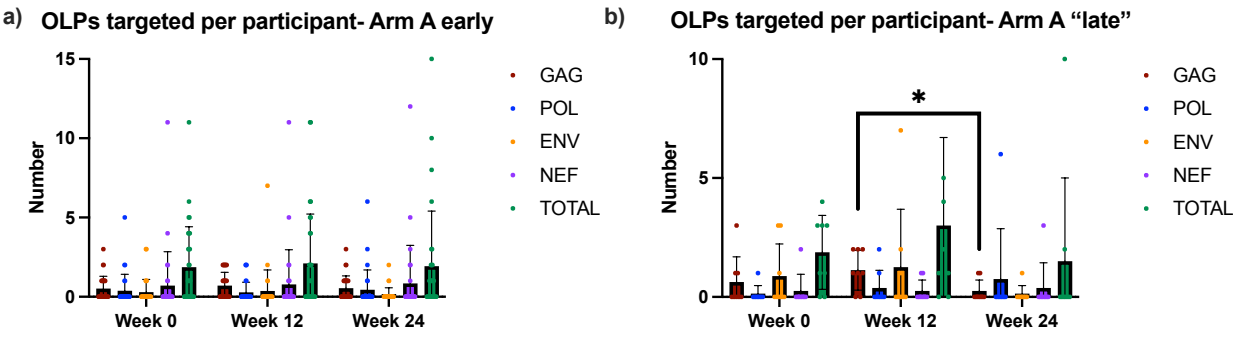


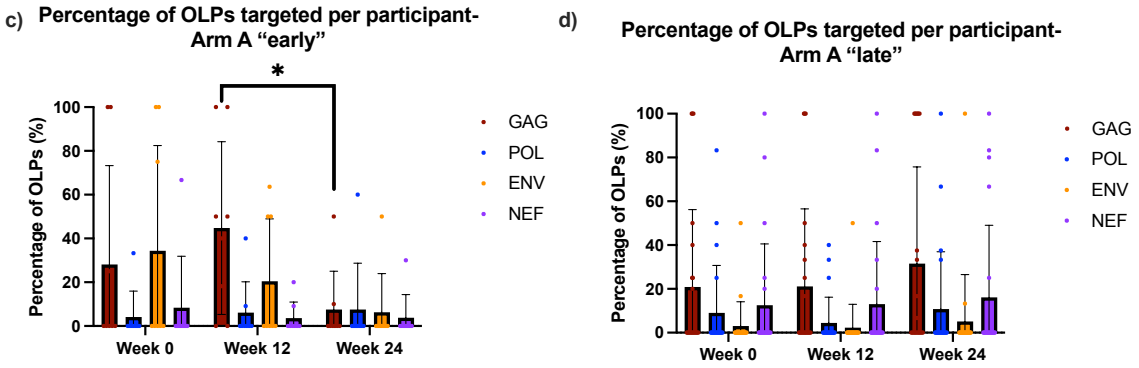
Figures 4.6a-d. Bar graphs showing changes in absolute OLP and peptide-adjusted responses for Arm A "early" (n=8) and "late" participants (n=22) between Weeks 0, 12 and 24. Different colours represent responses to either Gag, Pol, Env, Nef or total proteins. P values were calculated using repeated measures ANOVA; p values <0.05 indicate significance.



Figures 4.6e-f. Bar graphs showing change in the percentage of OLP responses for Arm A "early" (n=8) and "late" participants (n=22) between Weeks 0, 12 and 24. Different colours represent responses to either Gag, Pol, Env, Nef or total proteins. P values were calculated using repeated measures ANOVA; p values <0.05 indicates significance.

Next, we examined whether there were changes in the number and percentage of OLPs targeted per participant in each of the two Arm A subsets. Notably, Arm A "late" participants showed decreases in the number of Gag OLPs targeted between Weeks 12-24 (p=0.041), although the percentage of Gag OLPs appeared to slightly increase over time (but was not significant) **(Figure 4.7b)**. We also observed decreases in the percentage of Gag OLPs in Arm A "early" participants (p=0.035) **(Figure 4.7c)**.





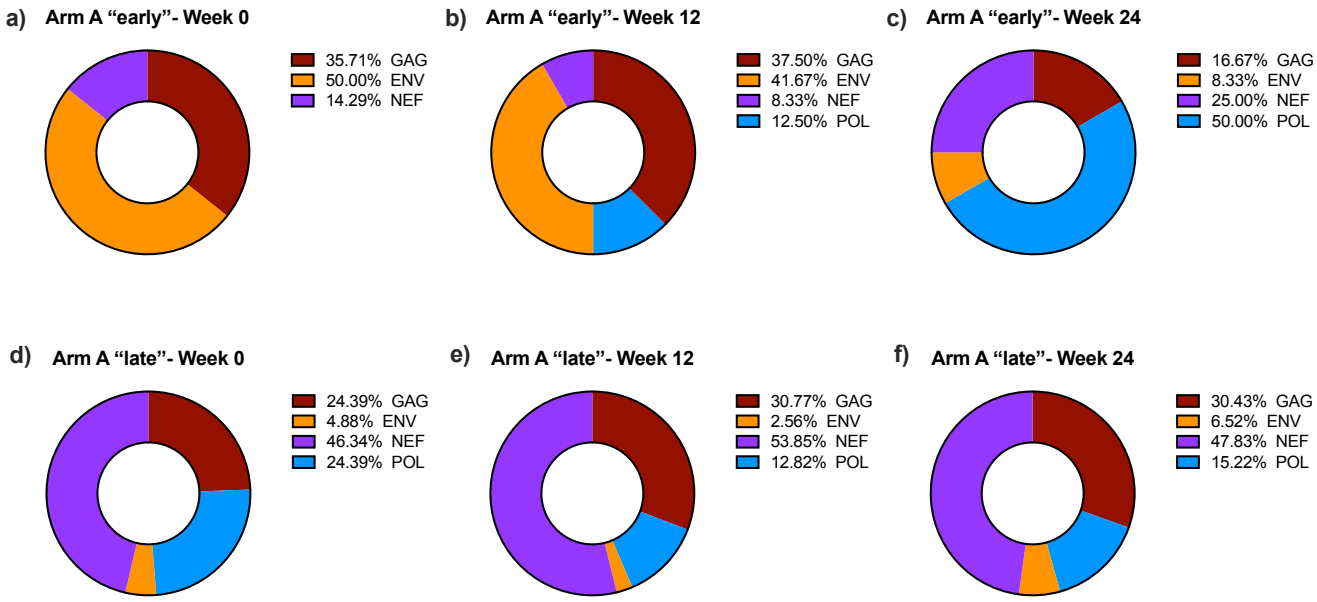
Figures 4.7a-d. Bar graphs showing change in the number (a-b) and percentage (c-d) of OLPs targeted per participant for Arm A "early" (n=8) and "late" participants (n=22) between Weeks 0, 12 and 24. Different colours represent responses to either Gag, Pol, Env, Nef or total proteins. P values were calculated using repeated measures ANOVA; p values <0.05 indicates significance.

To condense the analysis, we examined the number of OLPs targeted by all Arm A participants and expressed this as a percentage in relation to all HIV proteins targeted at each timepoint ("early" or "late" rebounders). In the Arm A "early" group, participants targeted less Gag OLPs over time (35.7% -> 16.67% at Week 24) and more Pol (0% -> 50% at Week 24) **(Figures 4.8a-c)**. Conversely, Arm A "late" participants showed small increases in targeted Gag OLPs over time (24.4% at Week 0 to 30.43% at Week 24) **(Figures 4.8d-f)**.

Next, we examined the recognition of OLPs at Weeks 0 and 24 in both Arm A "early" and "late" rebounders to determine if there were different trends in peptide recognition over time. As the "early" rebounder group only had an n=8, we could not draw any significant conclusions apart from a descriptive analyses; however, results showed that multiple OLPs were recognised across all HIV proteins (although no single region came out as dominant). At Week 0, responses to peptide GAG 66 (IYKRWIILGLNKIVR) dominated in both early and late rebounders (**Figure 4.9a**). In the late rebounders at Week 0, regions in Gag, Pol and Nef were targeted. At Week 24, both the "early" and "late" rebounders exhibited dominant responses towards two Gag peptides, GAG 5 EKIRLRPGGKKKYKL and GAG 60 AGTTSTLQEQIGWMT and one Nef peptide (NEF 29 WVYHTQGYFPDWQNY) **(Figure 4.9b).** In comparison, the "early" rebounders made

significantly more responses to Pol OLPs, while the "late" rebounders made significantly more responses to Nef OLPs (**see Appendix Table 4.1**).

Within each group, Arm A "early" rebounders made persistent responses to GAG 5 (EKIRLRPGGKKKYKL) at both Weeks 0 and 24, while Arm A "late" rebounders made persistent responses to GAG 60 (AGTTSTLQEQIGWMT), 66 (IYKRWIILGLNKIVR), and NEF 32 (QNYTPGPGIRYPLTF) and 33 (PGPGIRYPLTFGWCF) respectively. The overall peptide recognition in all Arm A participants is shown in **Figure 4.10** and **Appendix Table 4.1** for reference.



Figures 4.8a-f.Pie charts showing the percentages of OLPs targeted in relation to other HIV proteins in all Arm A "early" (n=8) and "late" participants (n=22) between Weeks 0, 12 and 24. Percentages were calculated by dividing the number of OLPs targeted in each HIV protein by all participants by the total number of OLPs targeted at each timepoint. Different colours represent responses to different HIV proteins.

a)

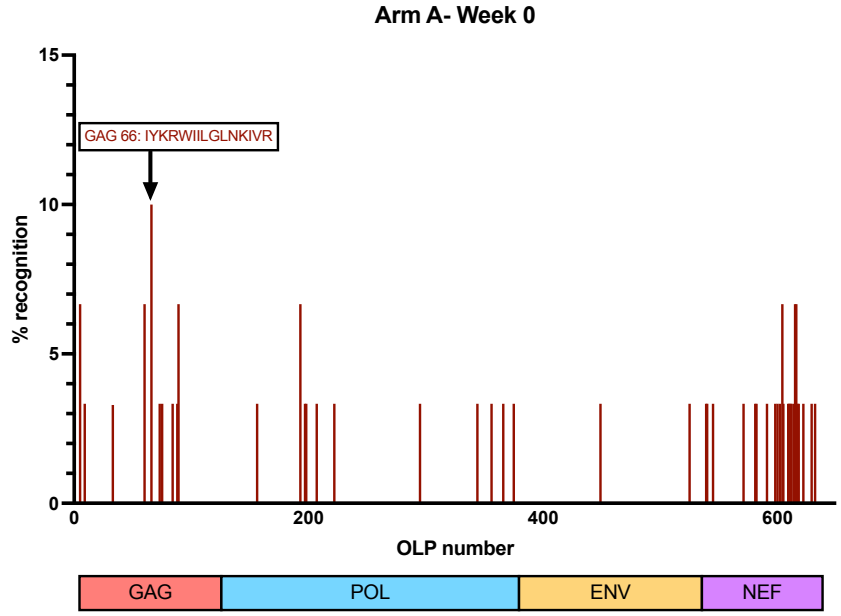


Figure 4.9a. Bar graphs showing the frequency of recognised OLPs at a) Week 0 and b) Week 24 in all Arm A participants. Each line indicates the percent recognition of each OLP. Dominant response across all HIV regions are labelled with arrows and their respective OLP sequence.

b)

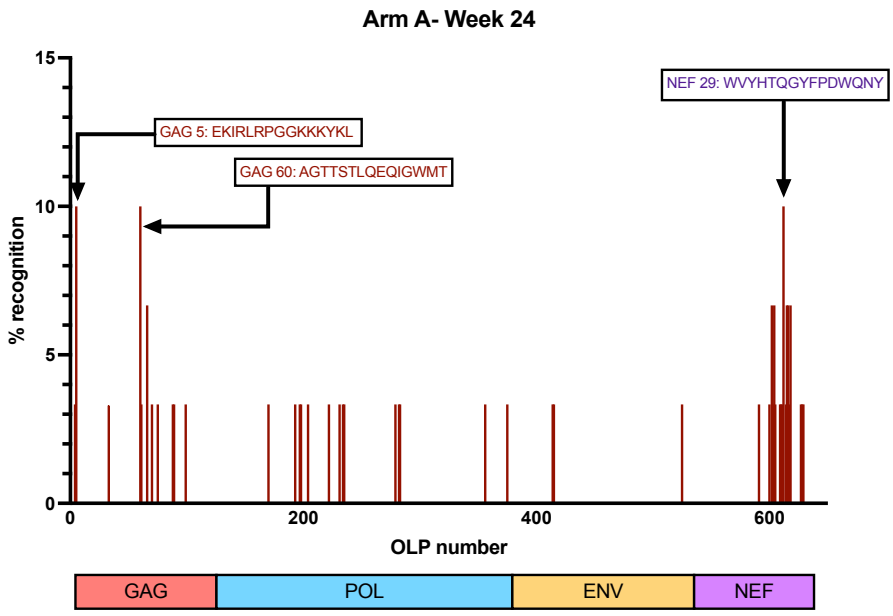


Figure 4.9b. Bar graphs showing the frequency of recognised OLPs at a) Week 0 and b) Week 24 in all Arm A participants. Each line indicates the percent recognition of each OLP. Dominant response across all HIV regions are labelled with arrows and their respective OLP sequence.

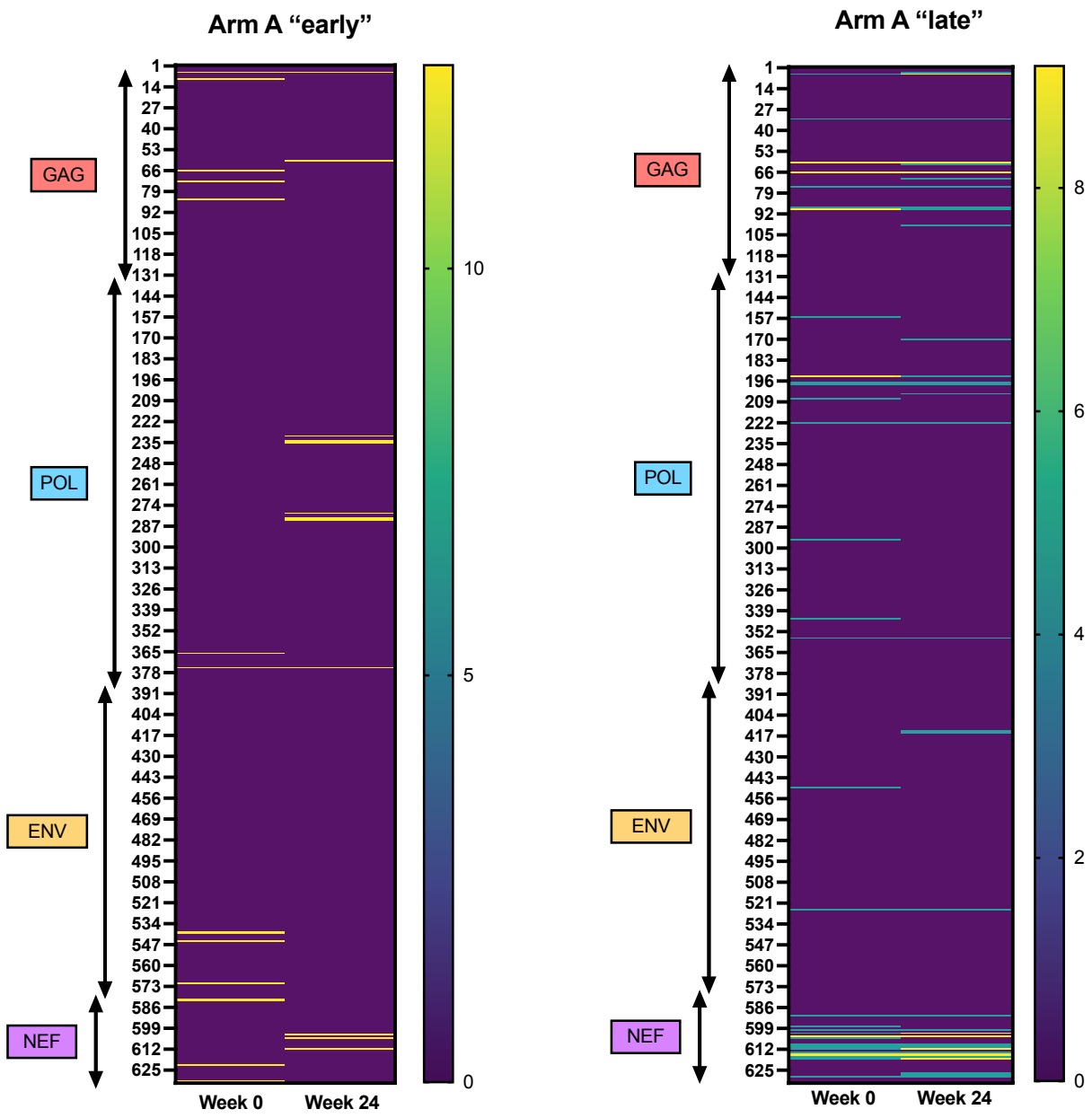


Figure 4.10. Heat maps showing percentage recognition of OLPs at Weeks 0 and 24 in Arm A "early" (n=8) and "late" participants (n=22). Each numbered OLP corresponds to an amino acid sequence in Gag, Pol, Env and Nef respectively. The coloured bar represents the percentage recognition; for example, yellow bars indicates a high recognition whereas dark purple indicates a low recognition.

Finally, we sought to determine whether there were differences in the HLA-restricted responses for Arm A "early" and "late" rebounders. First, we categorised participant HLA-class I alleles into protective, disease-susceptible and neutral based on existing data in the literature (42,43) (**see Figure 4.11**). Between

Arm A and B participants, we observed that Arm B participants had slightly more disease-susceptible (38.99% versus 27.97%) and less protective HLA-class I alleles (15.09% versus 28.67%) than Arm A, although the percentage of neutral alleles was roughly the same **(Figures 4.12a-d)**. Between the Arm A "early" and "late" rebounders, both groups had similar numbers of protective and disease-susceptible alleles.

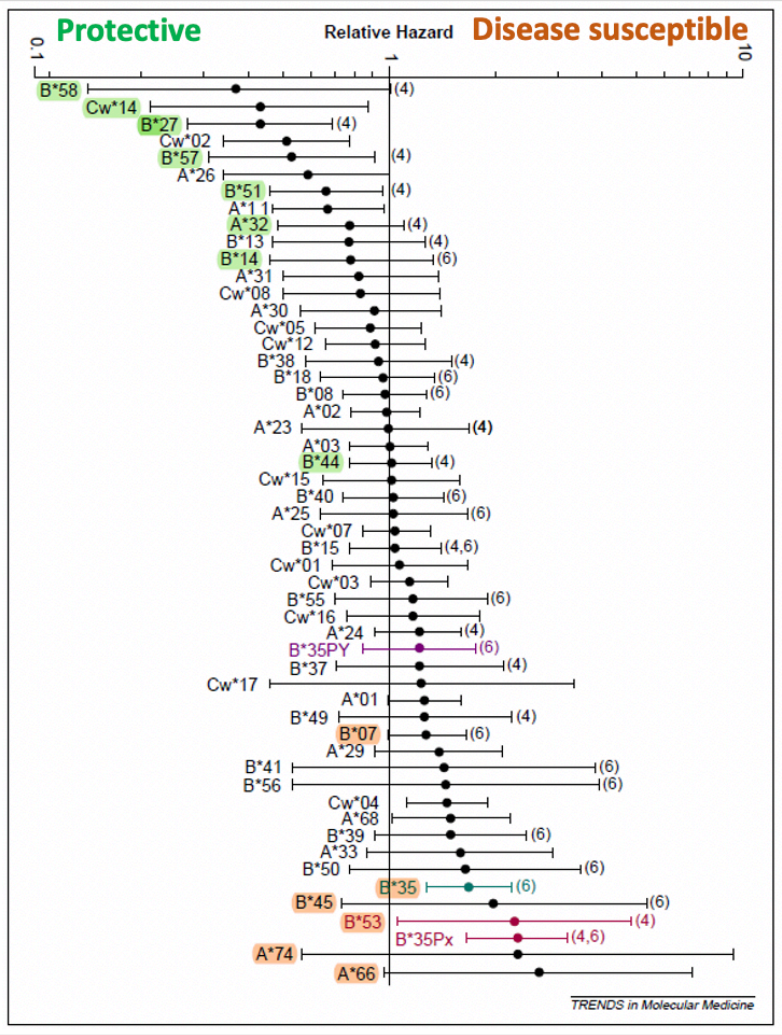
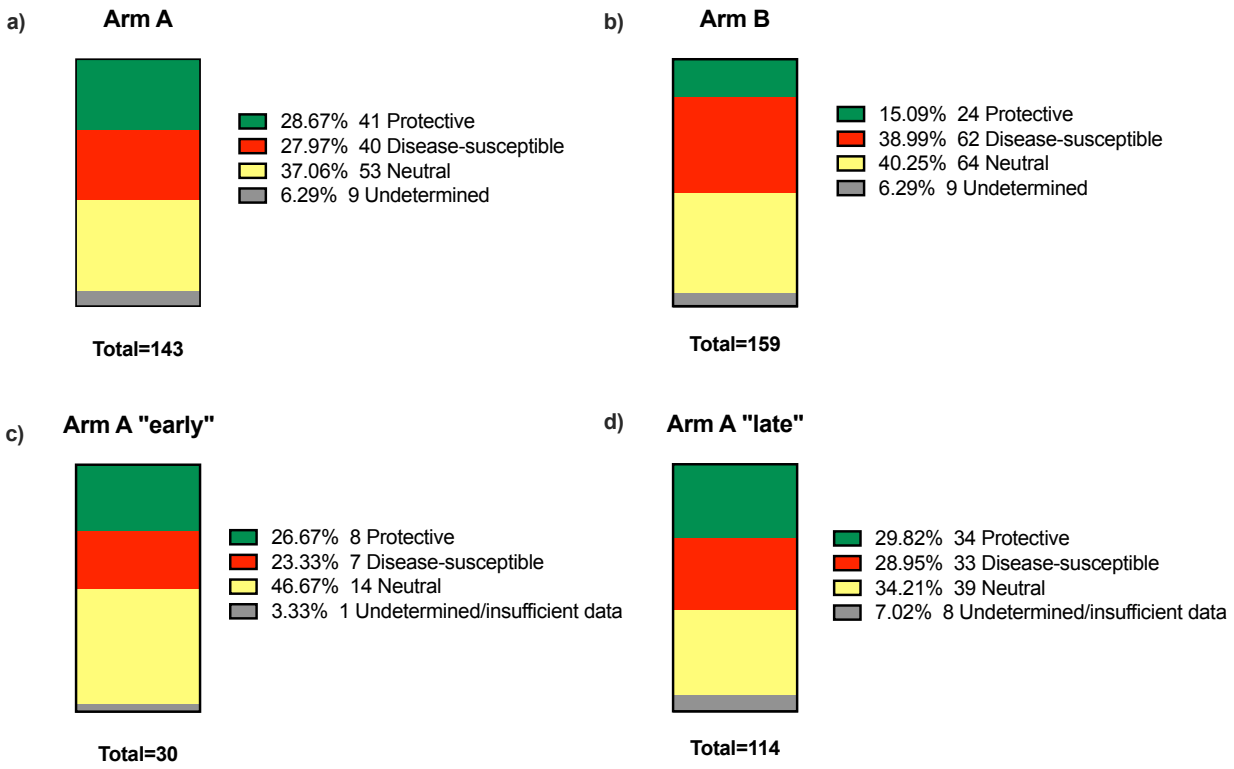
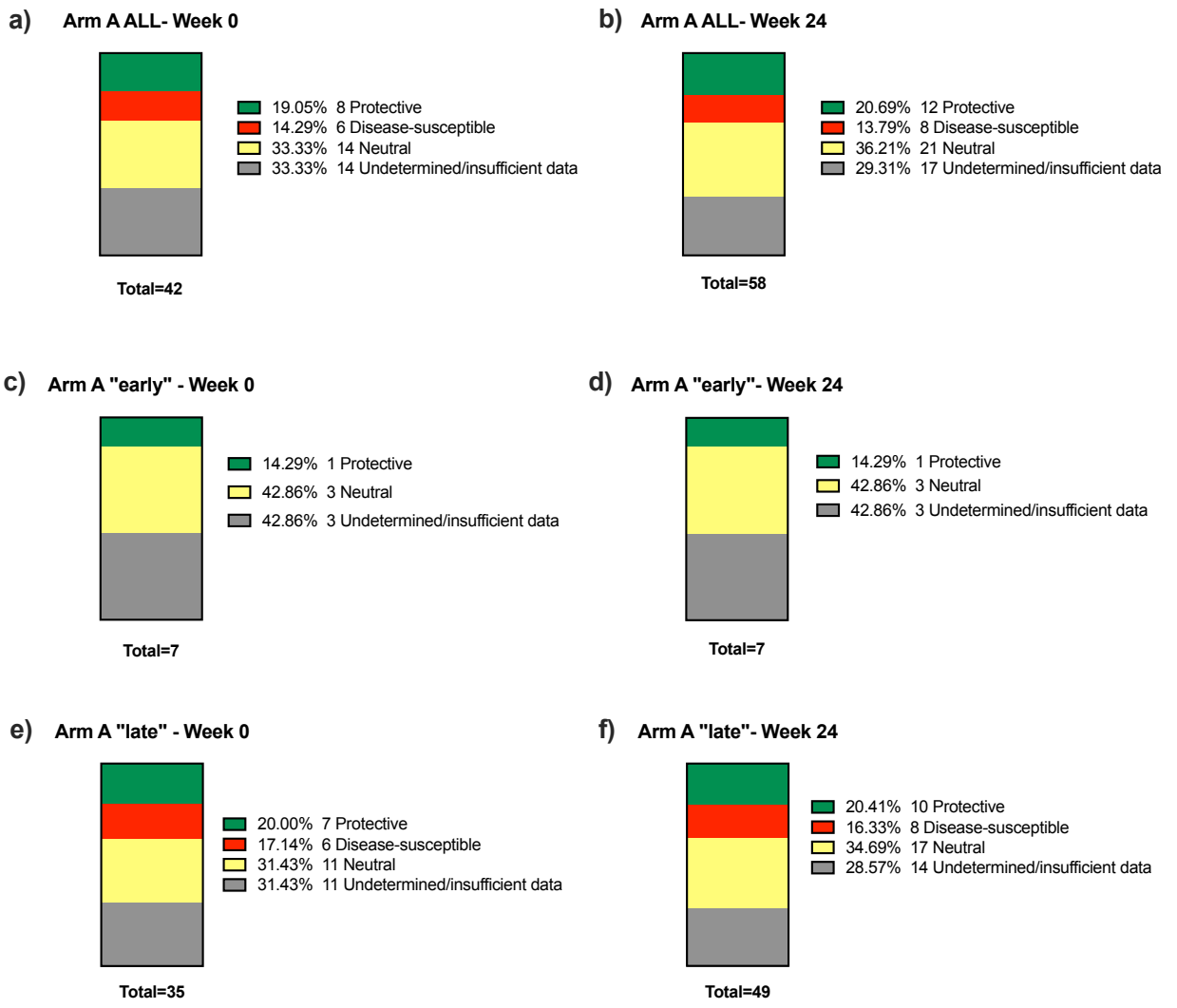


Figure 4.11 adapted from O'Brien et al. (42). Relative hazards of different HLA-class I alleles. If the hazard ratio was either >1 or <1, this was defined as a disease susceptible or protective allele respectively. If the hazard ratio dot intersected 1, this was classed as a neutral allele for analysis purposes.



Figures 4.12a-d. Bar plots showing the percentage of protective, disease-susceptible, neutral and undetermined HLA-class I alleles for a) Arm A, b) Arm B, c) Arm A "early" and d) Arm A "late participants respectively. Colours represent different allele types. If no match was found, the allele was classified as undetermined/insufficient data. All six HLA-class I (A, B and C) alleles of each arm were categorically assigned using existing data on the protectiveness/disease-susceptibility of HLA-class I alleles.

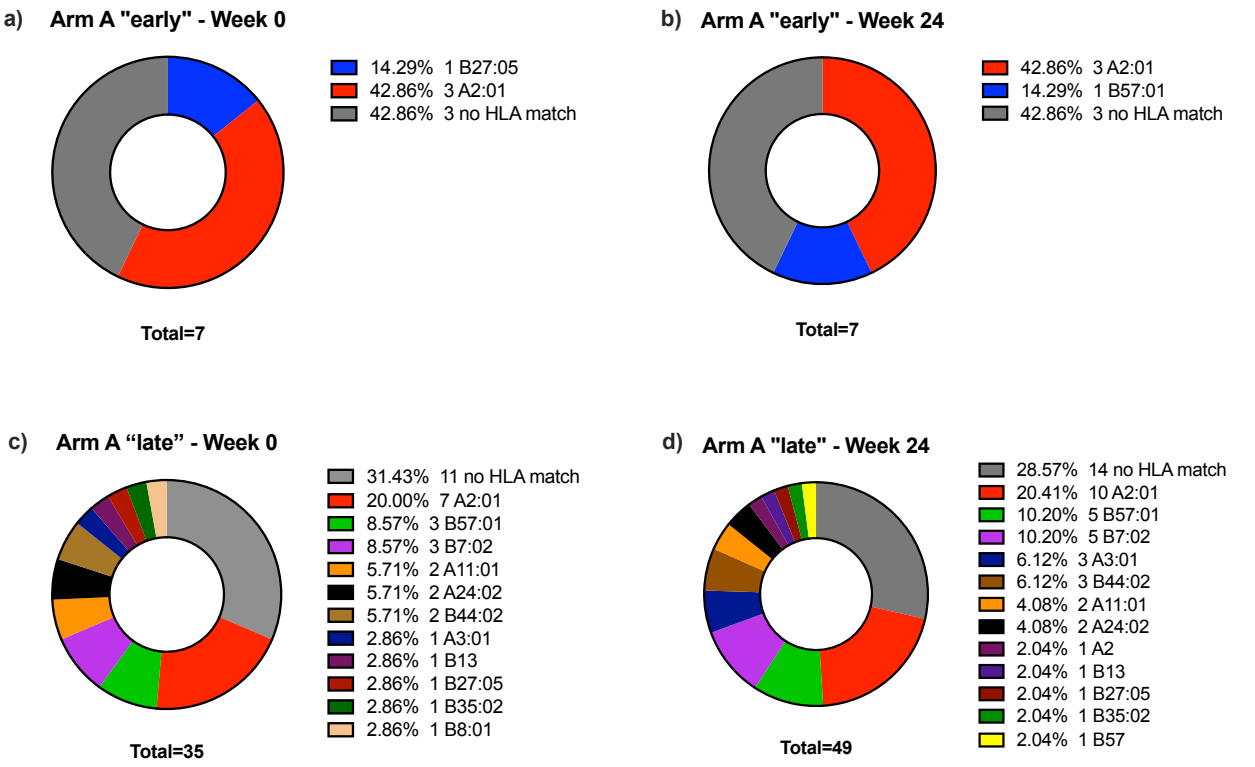
Next, we examined the HLA-class I restrictions of OLP responses at each timepoint in Arm A participants to determine whether there were significant differences in HLA-class I restricted responses during ATI. As we used OLPs to screen participants, the HLA-class I restrictions were matched with OLP responses using epitope information from the Los Alamos HIV database to infer predicted epitopes within OLPs; these predicted optimals have been associated with HLA-class I alleles in previous studies, and/or match the relevant binding motifs. In Arm A participants as a whole, there were no major differences in the percentage of protective/disease susceptible allele-restricted responses at Week 0 and 24. Similarly, there were no obvious differences in the HLA-class I restrictions of OLP responses made in both the "early" and "late" rebounder groups (**Figures 4.13a-f**).



Figures 4.13a-f. Bar plots showing the percentage of protective, disease-susceptible, neutral and undetermined HLA-class I restricted responses for Arm A participants (a-b), Arm A "early" (c-d), and Arm A "late" (e-f) participants respectively. Colours represent different allele types. If no match was found, the allele was classified as undetermined/insufficient data.

In addition to deconvoluting the risk associations with HLA-class I restricted responses, we also analysed the most likely inferred HLA-class I restrictions of OLP responses at Week 0 and 24 in both Arm A "early" and "late" participants to determine whether there were any HLA-class I restricted responses that were particularly enriched in either group. Notably, the sample size for Arm A "early" participants was small (n=8), thus we could not make any significant conclusions; we observed A2:01 and B57:01-restricted responses at Week 0 and 24. For Arm A "late" participants (n=22), a significant percentage of responses

at Week 0 and 24 did not have a HLA-match. Aside from unmatched responses, the top three HLA-restrictions remained consistent between the Week 0 and 24 timepoints (A2:01, B57:01 and B7:02), and more responses were observed at Week 24 as well (**Figures 4.14a-d**).

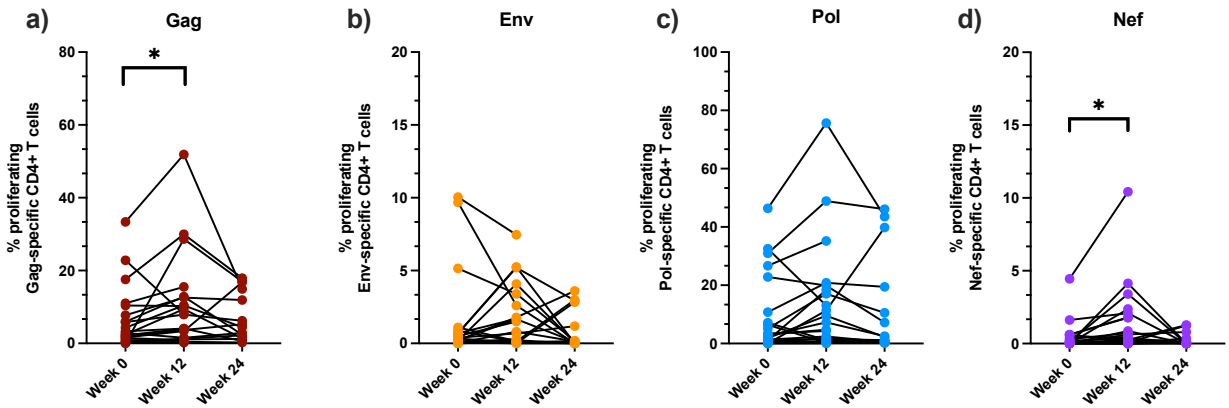


Figures 4.14a-d. Pie charts showing the HLA-class I restrictions of OLP responses at Week 0 and 24 in Arm A “early” (a-b) and “late” (c-d) participants respectively. Each colour represents responses that are restricted to a particular HLA-class I allele. If no match was found, this was written as “no HLA match”.

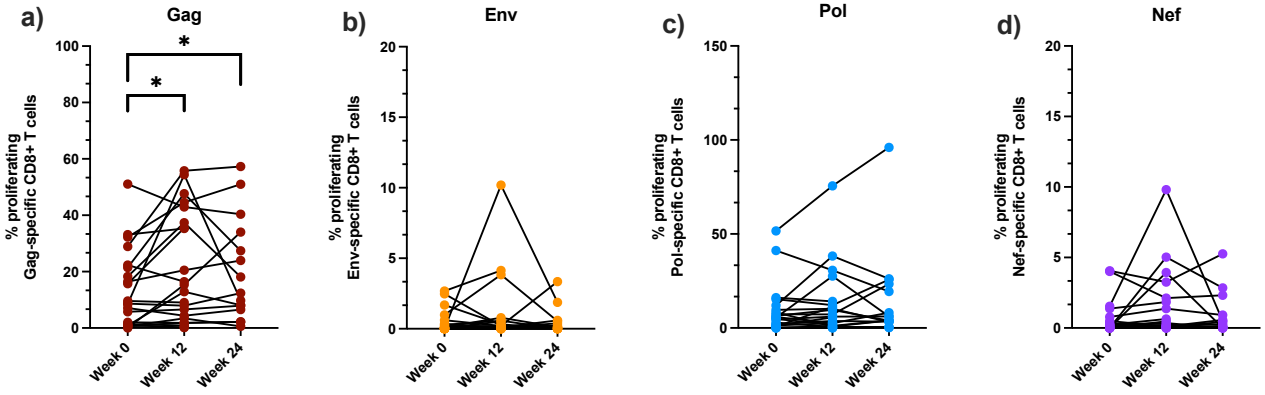
Combination bNAbs are associated with increases in HIV-specific T cell proliferation in Arm A "virally suppressed" individuals off ART

The data from this section was in joint collaboration with Dr. Mohammed Altaf, a postdoctoral researcher in our laboratory who assisted in acquiring and analysing the T-cell data.

Next, we determined whether combination bNAbs had any effect on HIV-specific T cell proliferation in Arm A participants that were virally suppressed off ART. Based on the selection criteria, 21 participants were included in the analysis. Timepoints at Week 0, 12 and 24 (where available) were analysed. For Gag-specific CD4+ proliferation, we observed significant increases between Weeks 0-12 only (p=0.0426), however this was not significant after correction for multiple comparisons using a repeated measures ANOVA. We also observed increases in Nef-specific CD4+ proliferation between Weeks 0 and 12 (p=0.0124) **(Figure 4.15d);** while Pol-specific CD4+ proliferation was insignificant on a repeated measures ANOVA, we did observe significant increases between Weeks 0-12 using a Wilcoxon matched-pairs signed rank test (p=0.0275)**.** For HIV-specific CD8+ proliferation, however, significant increases were observed for Gag-specific CD8+ proliferation between Weeks 0 and 12 (p=0.0266) **(Figures 4.16a-d).** While there was no further significant increase from Weeks 12 to 24, Gag proliferative responses at Week 24 were significantly higher than at Week 0 (p=0.0496) **(Figure 4.16a).** Apart from Gag, none of the other HIV-specific CD8 responses showed significant changes from Weeks 0 to 24.



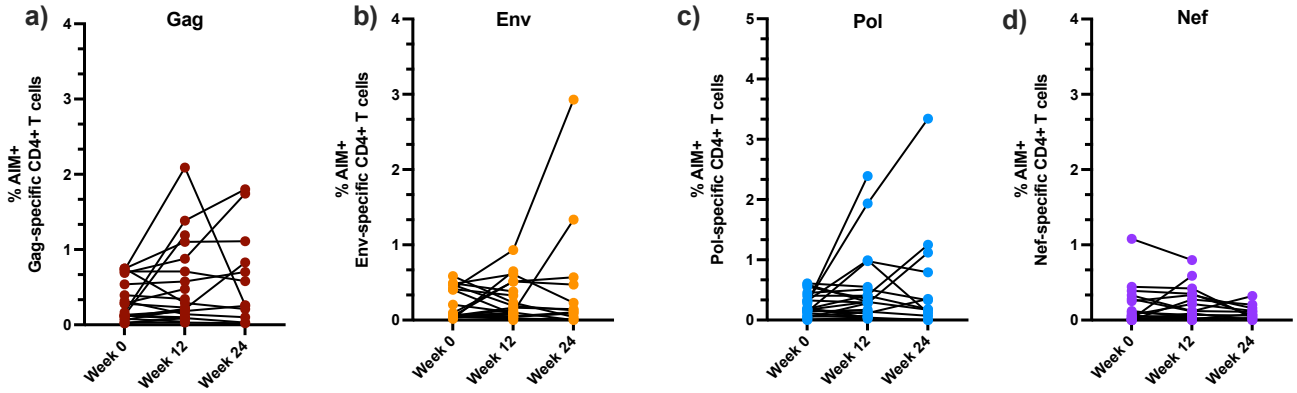
Figures 4.15a-d. Line graphs showing HIV-specific CD4+ cell proliferation to a) Gag, b) Env, c) Pol and d) Nef respectively between Weeks 0, 12 and 24 for Arm A (BNab-treated) participants that were virally suppressed and off ART (n=22). Each data point represents the percentage of background-subtracted proliferating CD4+ T cells as a percentage of all T cells. Repeated measures ANOVA were used to determine significance; P<0.05 indicates significance and are marked by asterisks (*).



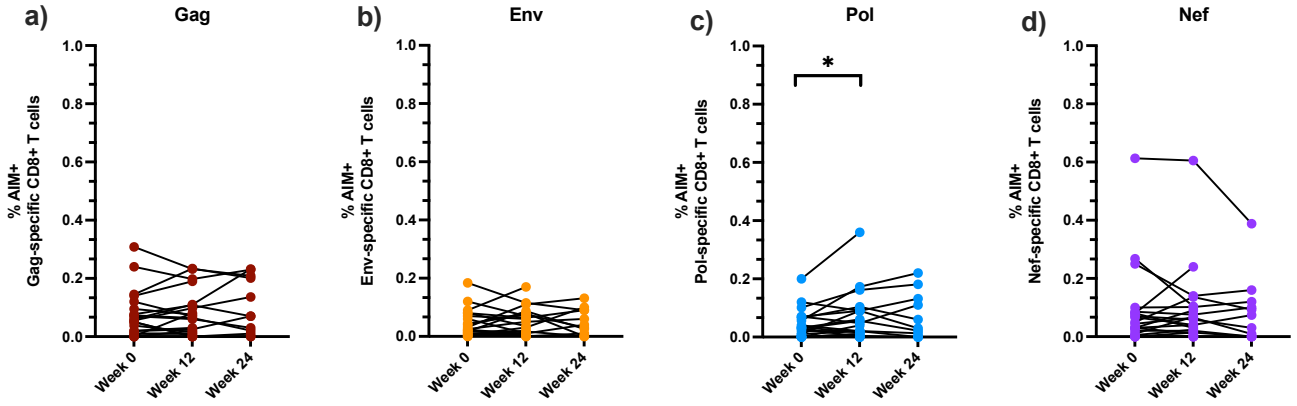
Figures 4.16a-d. Line graphs showing HIV-specific CD8+ cell proliferation to a) Gag, b) Env, c) Pol and d) Nef respectively between Weeks 0, 12 and 24 for Arm A (BNab-treated) participants that were virally suppressed and off ART (n=22). Each data point represents the percentage of background-subtracted proliferating CD8+ T cells as a percentage of all T cells. Repeated measures ANOVA were used to determine significance; P<0.05 indicates significance and are marked by asterisks (*).

In addition to assessing T-cell functionality via proliferation assays, we investigated whether combination bNAbs had any effect on HIV-specific T cell activation (via the upregulation of activation-induced markers such as OX-40, CD137 and CD69) in Arm A participants that were virally suppressed off ART. To achieve this, percentage of activated T cells were combined via a Boolean analysis as previously described in Chapter 2 to provide an overall percentage of AIM+ cells. In the CD4+ compartment, we observed

significant increases in Gag-specific AIM+ cells on a Wilcoxon matched-pairs signed rank test (p=0.0353), but not on a repeated measures ANOVA (p=0.0640) **(Figure 4.17a)**. In the CD8+ compartment, we observed significant increases in Pol-specific AIM+ cells between Weeks 0-12 (p=0.0402), although this was not significant after correction for multiple comparisons using a repeated measures ANOVA **(see Figures 4.17-18a-d).**



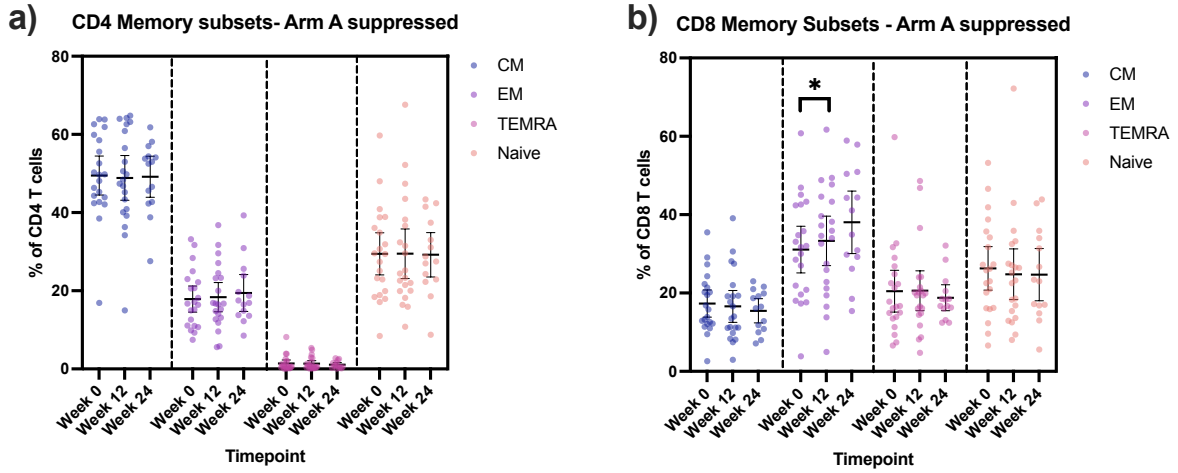
Figures 4.17a-d Line graphs showing the background-subtracted frequency of AIM+ CD4+ T cells towards a) Gag, b) Env, c) Pol and d) Nef in Arm A participants that are virally suppressed off ART between Weeks 0, 12 and 24 (n=22). Repeated measures ANOVA were used to determine significance; P<0.05 indicates significance and are marked by asterisks (*).



Figures 4.18a-d. Line graphs showing the background-subtracted frequency of AIM+ CD8+ T cells towards a) Gag, b) Env, c) Pol and d) Nef in Arm A participants that are virally suppressed off ART between Weeks 0, 12 and 24 (n=22). Repeated measures ANOVA were used to determine significance; P<0.05 indicates significance and are marked by asterisks (*).

Combination bNAbs are associated with skews toward Th1 helper cell subsets and a Tbet^{hi}EOMES^{lo} phenotype in Arm A "virally suppressed" individuals off ART

Given that interferon-gamma ELISpot assays are generally skewed towards detecting Th1-like responses, we next considered whether the increases in Gag-specific T cell immunity observed were accompanied by an expansion of memory T cells or T-helper cells over time (44). To achieve this, PBMCs were phenotyped via flow cytometry to isolate different memory T-cell and T-helper cell subsets from Arm A participants that remained virally suppressed off ART. Memory T cell subsets were gated on using the surface markers CCR7 and CD45RA. Significant increases were observed in CD8 effector memory subsets between Weeks 0 and 12 via a univariate Wilcoxon matched-pairs signed rank test (p=0.046), but not via a repeated measures ANOVA **(Figure 4.19b)**. There were also significant increases in Th1 helper subsets between Weeks 0-12 only (p=0.0135) **(Figure 4.19c)** (however this was not significant after adjustment for multiple comparisons).



Figures 4.19a-b. Scatter graphs showing a) CD4 and b) CD8 memory subsets in Arm A participants that are virally suppressed off ART between Weeks 0-24 (n=21). Coloured dots represent different memory subsets: central memory (CM), terminally differentiated (TEMRA) effector memory (EM), and naïve cells respectively. Error bars represent the mean with 95% confidence intervals. P values were calculated using a Wilcoxon matched pairs signed ranked test. P<0.05 indicates significance and are denoted by asterisks (*).

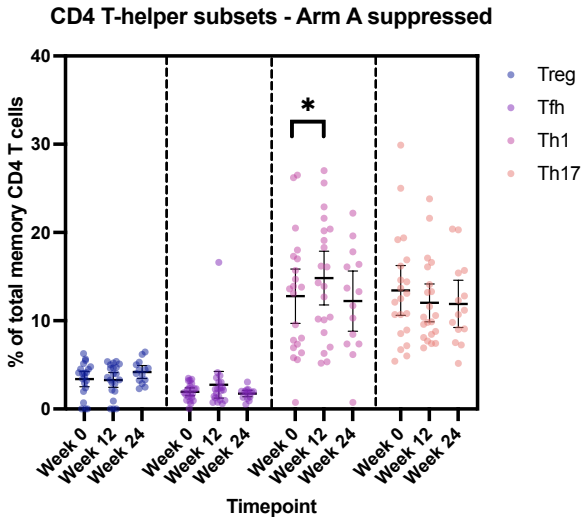
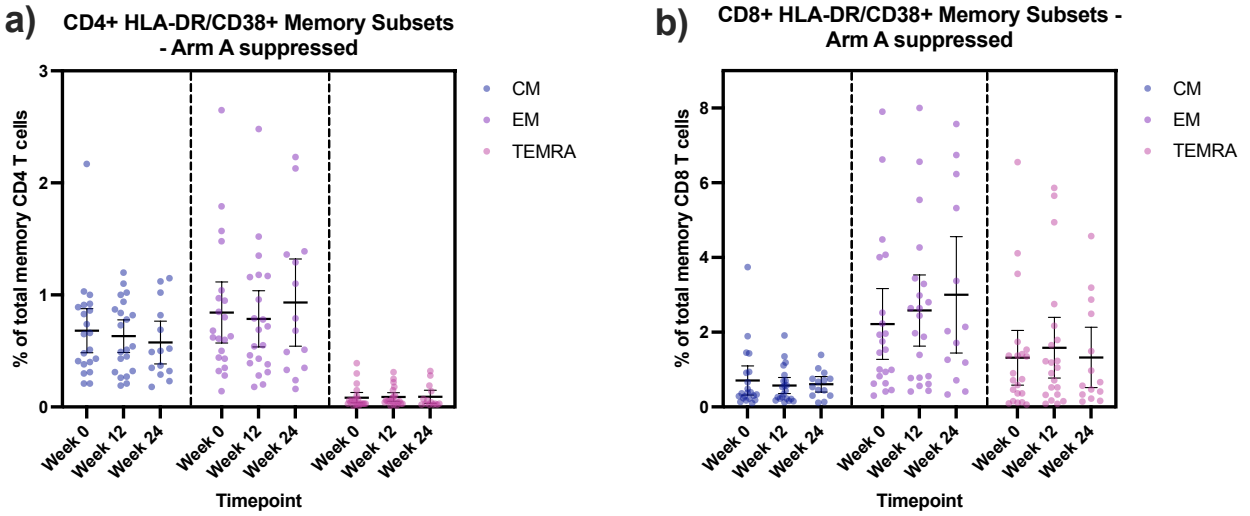


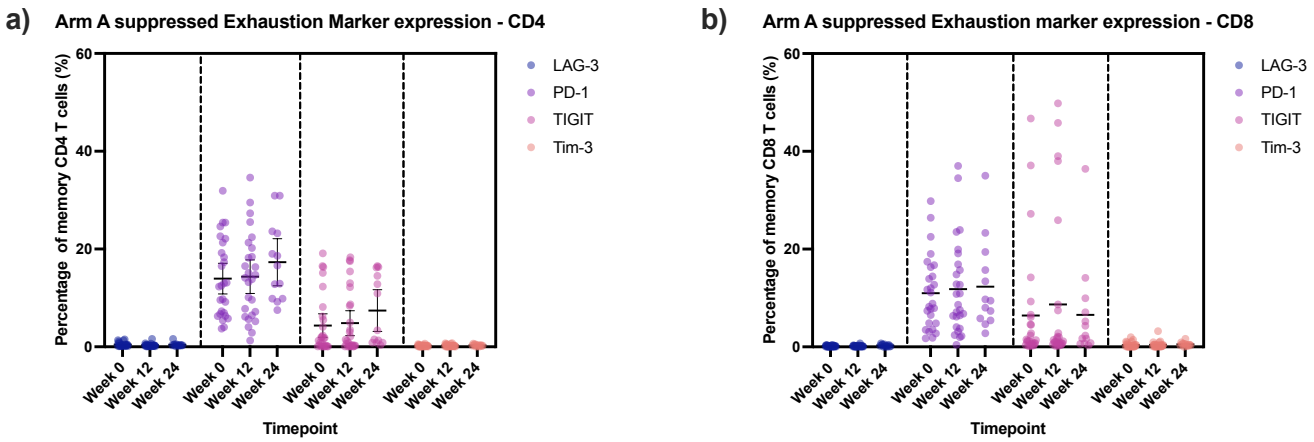
Figure 4.19c. Scatter graphs showing the CD4 T-helper subsets in Arm A participants that are virally suppressed off ART between Weeks 0-24 (n=21). Coloured dots represent T-regulatory (Treg), T-follicular helper (Tfh), T-helper1 (Th1) and T-helper 17 (Th17) cells respectively. Error bars represent the mean with 95% confidence intervals. P values were calculated using a repeated measures ANOVA.

For participants in Arm A, we also investigated changes in activation and exhaustion marker expression over time, as some evidence suggests that stimulation with a therapeutic vaccine or similar agent could potentially cause a reactivation of the latent reservoir in people living with HIV, as well as increases in T-cell activation which could negatively affect immune responses and induce immune dysregulation (45). To investigate whether this was the case in our cohorts, the double positive HLA-DR/CD38+ of CD4+ and CD8+ T cells were examined. There were no significant changes observed in CD4+ or CD8+ T cell compartments between Weeks 0-12 **(Figures 4.20a-b).**

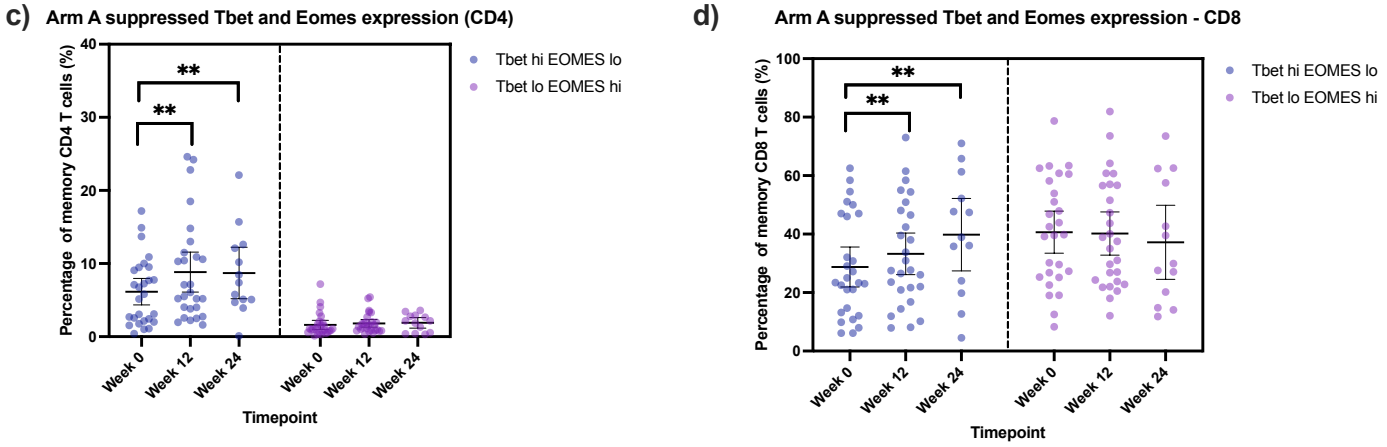


Figures 4.20a-b. Scatter graphs showing the double positive HLA-DR/CD38+ cells in both the a) CD4+ and b) CD8+ compartments between Weeks 0 and 12, in Arm A participants that are virally suppressed off ART (n=21). Coloured dots represent different memory subsets: central memory (CM), effector memory (EM), and terminally differentiated (TEMRA) cells respectively.

We then investigated if administering combination bNAbs to Arm A participants who remained virally suppressed off ART led to increased expression of exhaustion markers over time. Determining the level of exhaustion marker expression in these participants was important to ensure that administration of bNabs did not cause excessive immune activation and subsequent T-cell exhaustion. Notably, there was no significant increase in PD-1, LAG-3, Tim-3 or TIGIT expression between Weeks 0, 12 and 24 in either the CD4+ or CD8+ compartments **(Figures 4.21a-b).** Next, we investigated whether there were changes in the differential expression of the transcription factors Tbet and Eomes, as these have been differentially linked to specific phenotypes in HIV; for example, high expression of T-bet has been demonstrated in long term non progressors, and conversely the expression of a $Tbet^{lo}EOMES^{hi}$ phenotype in virus specific CD8 T cells has been associated with poor polyfunctionality (46). Thus, it would be clinically relevant to determine whether bNAbs are associated with a shift towards a favourable $Tbet^{hi}EOMES^{lo}$ phenotype. We observed significant increases in $Tbet^{hi}EOMES^{lo}$ T cells in both the CD4+ and CD8+ compartment between Weeks 0-12 (p=0.006 and p=0.0032 respectively), and Weeks 12-24 (p=0.006 and p=0.0048 respectively) **(Figures 4.12c-d).**



Figures 4.21a-b. Scatter graphs showing the expression of exhaustion markers LAG-3, PD-1, TIGIT and Tim-3 in both the a) CD4+ and b) CD8+ compartments between Weeks 0, 12 and 24. Arm A participants that are virally suppressed off ART (n=27).



Figures 4.21c-d. Expression of c) Tbet^{hi}EOMES^{lo} and d) Tbet^{lo}EOMES^{hi} populations in Arm A participants that are virally suppressed off ART (n=27). Repeated measures ANOVA was used to determine significance; $p<0.05$ indicates significance and are marked by asterisks (*).

4.4 Hypotheses 2: Arm B participants that receive dual long-acting bNabs during viraemia show evidence of increases in HIV-specific T cell immunity

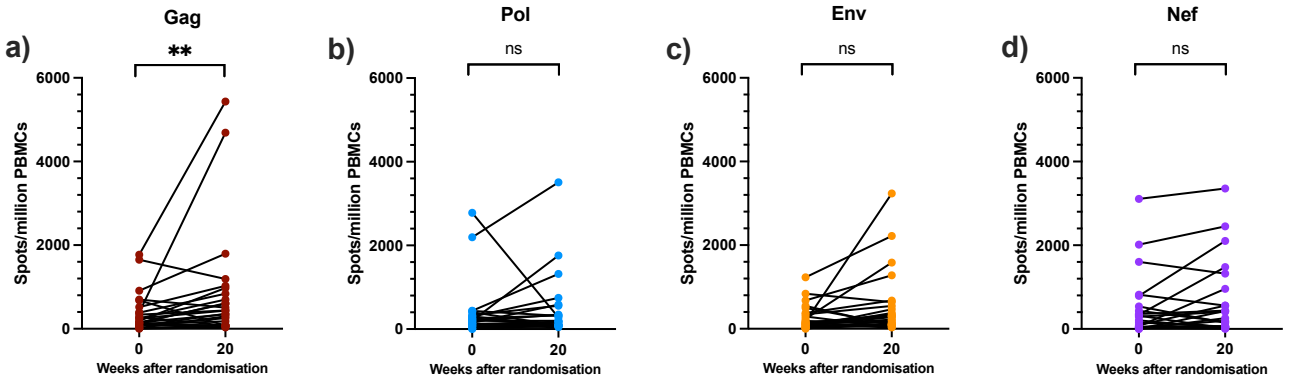
Next, we compared HIV-specific T cell immunity in Arm A participants, who remained virally suppressed off ART, to Arm B participants, who received bNAbs followed by 24 weeks of ART. In Arm B, participants received bNabs in the context of low level viraemia (compared to Arm A where participants received bNabs while on suppressive ART); we hypothesised that administering bNAbs during viraemia may allow more effective antigen-antibody immune complex formation and subsequent immune mediated enhancement. We predicted that administering bNAbs in Arm B might lead to similar or even stronger HIV-specific T cell responses compared to Arm A.

4.4.1 Methods

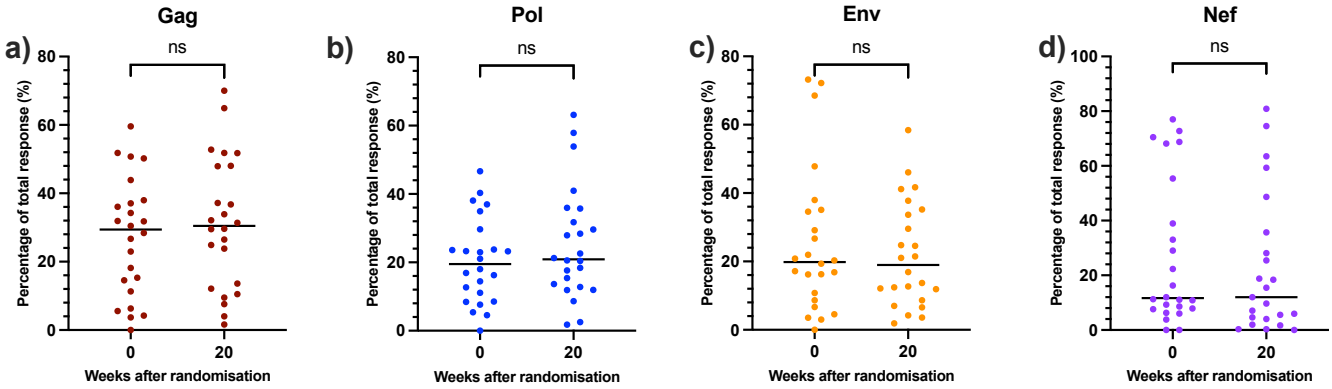
To determine whether similar effects in HIV-specific T-cell immunity were observed, all available Arm B participants that were officially unblinded were analysed at Week 0 and Week 20. A direct and fair comparison between Arm A and B is not possible as Arm B participants experience a period of viraemia/viral rebound prior to receiving bNabs and restarting ART; however, Week 20 was chosen because this timepoint was post-bNAbs and after participants had received a prolonged period of ART. Given the suppressive effects of ART, observing T-cell responses at this timepoint is scientifically relevant, as we expect that most of the circulating viral antigen would have been cleared; thus, any subsequent increase in response may be attributed to bNAbs. Thus, PBMCs of participants at Week 0 and 20 were analysed using the same T cell functional assays used for Arm A (see previous section) including interferon-gamma ELISpot with epitope analysis as previously outlined (see Chapter 2 Methods). Statistical analyses was performed using GraphPad prism Version 10. To compare responses at different timepoints, Wilcoxon matched-pairs signed rank tests were performed to determine significance.

4.4.2 Results- Arm B

First, we determined whether there were increases in HIV-specific interferon-gamma secretion in Arm B participants. Interestingly, Gag-specific interferon-gamma secreting T cells increased between Weeks 0 and 20 (p=0.0087) which is similar to the observations in Arm A **(Figure 4.22a).** However, there were no significant changes observed in other HIV proteins **(Figures 4.22b-d).** There were also no significant changes in percentage of response for any HIV protein **(Figures 4.23a-d).**

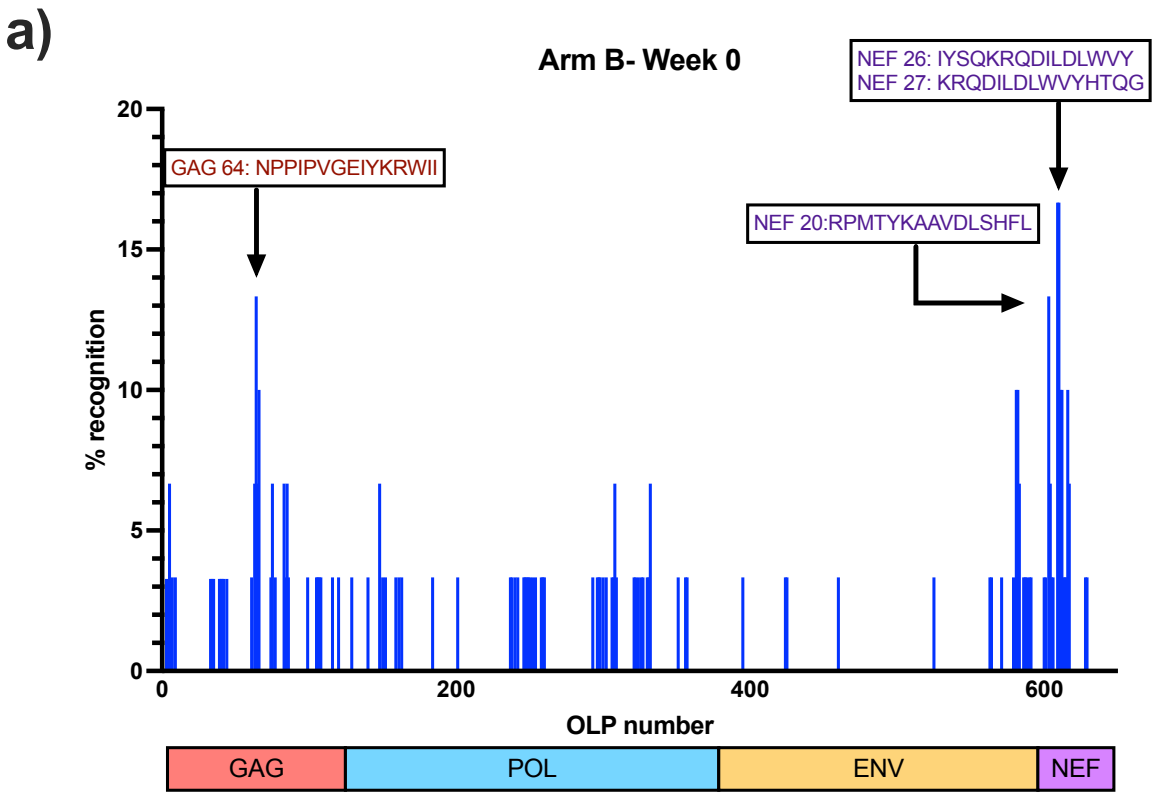


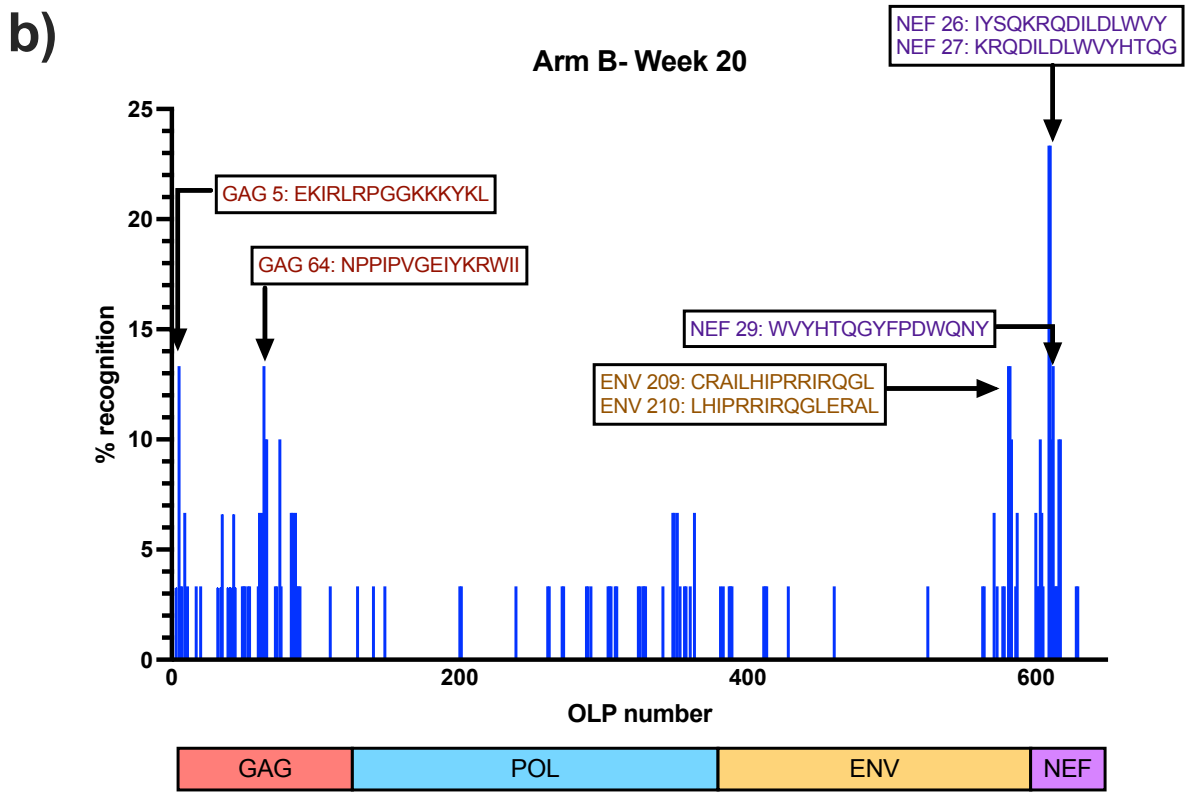
Figures 4.22a-d. Graphs showing the total a) Gag, b) Pol, c) Env and d) Nef "pooled" responses in Arm B participants post-bNAbs and on ART at Weeks 0 and 20 respectively (n=25). Each coloured dot represents a separate data point expressed in spots per million PBMCs. Statistical significance was calculated using a Wilcoxon matched-pairs signed rank test; significant results are shown as asterisks (*).



Figures 4.23a-d. Graphs showing the percentage change in the response of a) Gag, b) Pol, c) Env and d) Nef "pooled" responses in Arm B participants post-bNAbs and on ART at Weeks 0 and 20 respectively (n=25). Each coloured dot represents a separate data point expressed in spots per million PBMCs. Statistical significance was calculated using a Wilcoxon matched-pairs signed rank test; significant results are shown as asterisks (*).

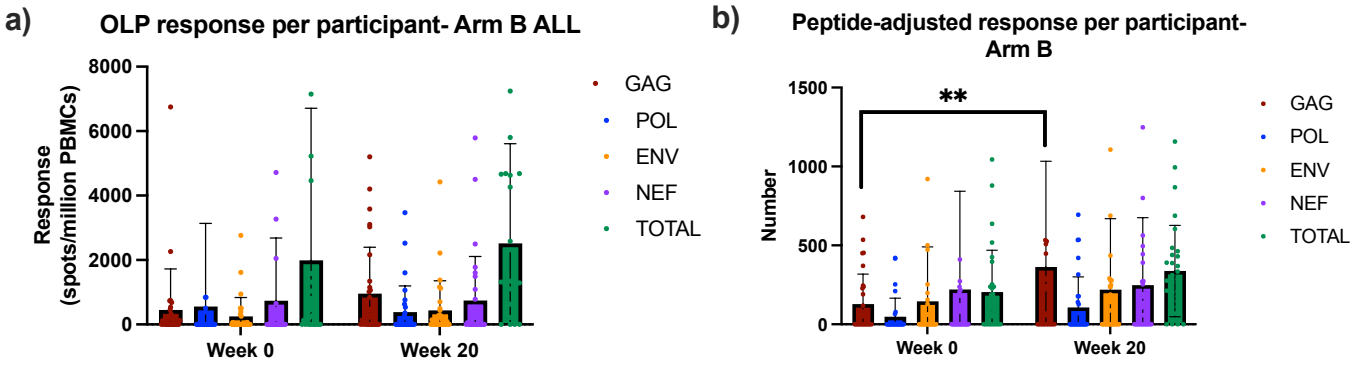
Next, we examined the frequency of recognised OLPs across the HIV genome at Week 0 and 20 in Arm B participants. At Week 0, dominant responses were observed to Gag (GAG 64: NPPIPVGEIYKRWII) and Nef OLPs (NEF 26: IYSQKRQDILDLWVY, NEF 27: KRQDILDLWVYHTQG and NEF 20: RPMTYKAAVDLSHFL) **(Figure 4.24a).** At Week 20, the highest responses were also directed towards NEF 26 and 27, as well as NEF 29 (WVYHTQGYFPDWQNY). Additional OLPs in Gag (EKIRLRPGGKKKYKL and NPPIPVGEIYKRWII) and Env (CRAILHIPRRIRQGL and LHIPRRIRQGLERAL) were also highly dominant **(Figure 4.24b)**. A full of list of recognised OLPs is summarised in **Appendix Table 4.2.**





Figures 4.24a-b. Bar graphs showing the frequency of recognised OLPs at Week 0 (a) and 20 (b) in Arm B participants. Each line indicates the percent recognition of each OLP. Dominant response across all HIV regions are labelled with arrows and their respective OLP sequence.

Next, we examined the OLP and peptide-adjusted responses for Arm B participants between Week 0 and 20. While we did not observe any increases in OLP responses between Week 0 and 20 **(Figure 4.25a)**, the Gag peptide adjusted response significantly increased between Week 0 and 20 (p=0.0011) **(Figure 4.25b)**. However, there were no significant changes in the percentage of OLP response or the number and percentage of OLPs targeted per participant **(Figures 4.26c-e)**.



Figures 4.25a-b. Bar graphs showing changes in absolute OLP and peptide adjusted responses for Arm B participants (n=30) between Weeks 0 and 20. Different colours represent responses to either Gag, Pol, Env, Nef or total proteins. P values were calculated using repeated measures ANOVA; p values <0.05 indicate significance.

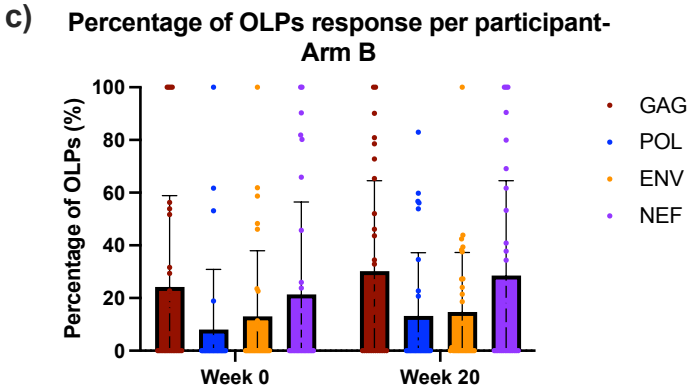


Figure 4.25c. Bar graphs showing change in the percentage of OLP responses for Arm B (n=30) between Weeks 0 and 20. Different colours represent responses to either Gag, Pol, Env, Nef or total proteins. P values were calculated using repeated measures ANOVA; p values <0.05 indicates significance.

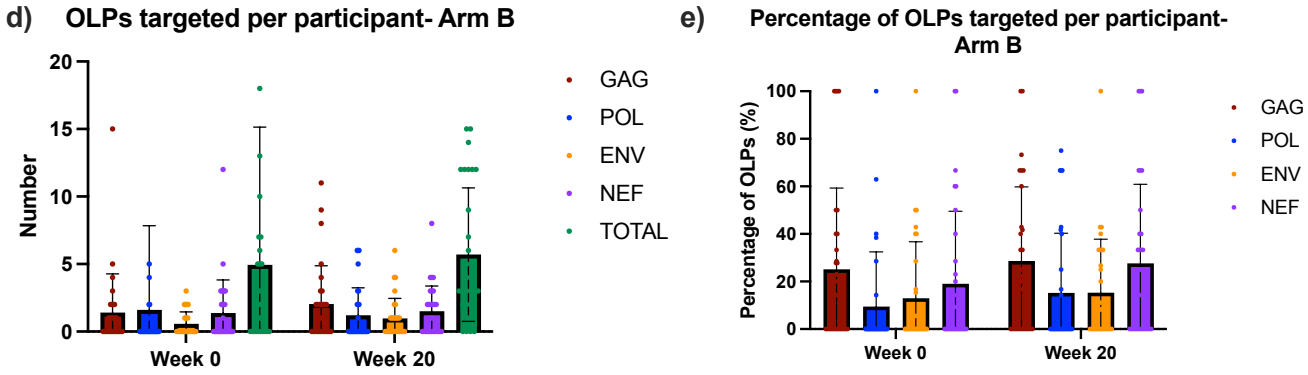
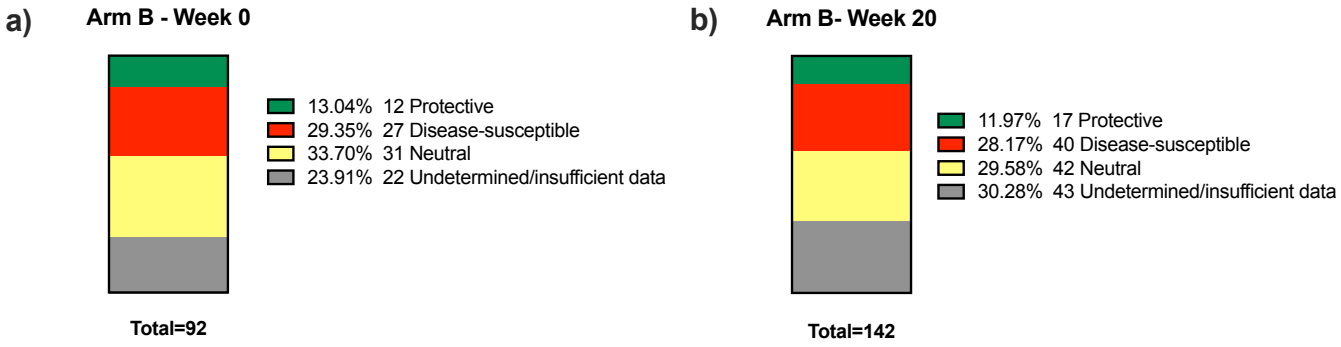
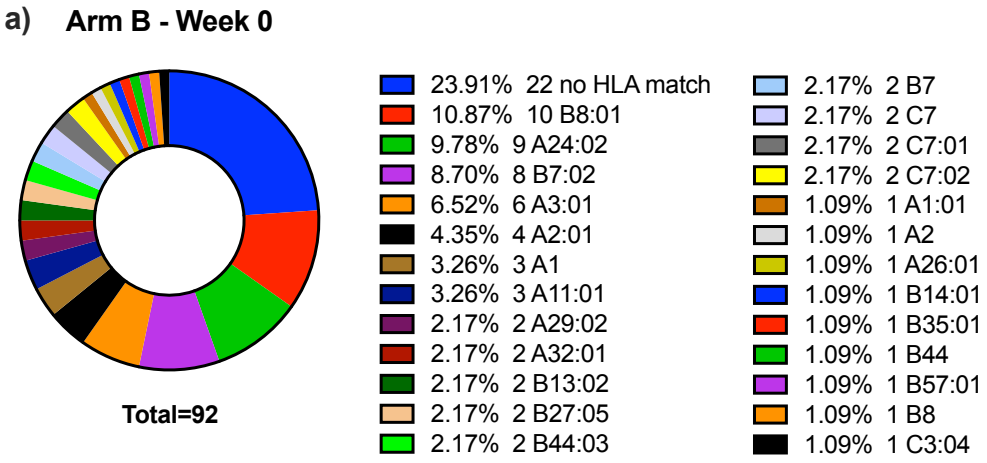


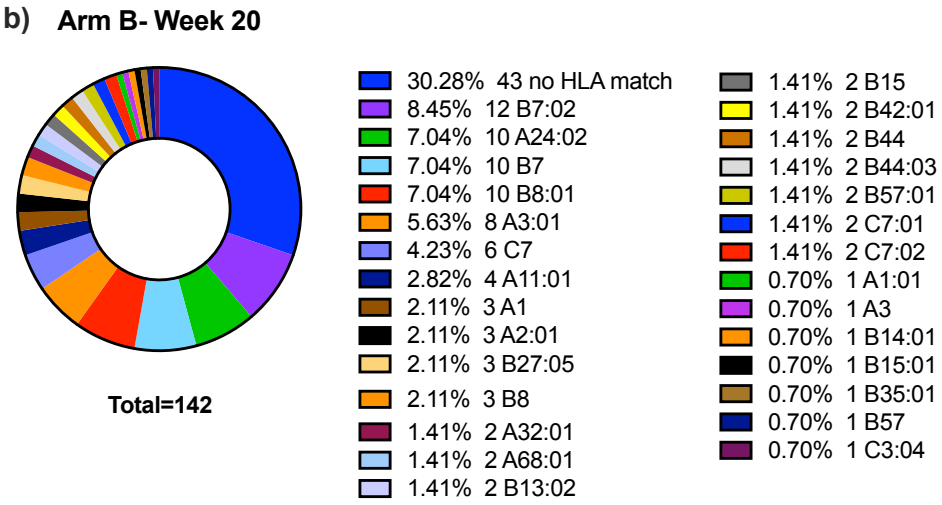
Figure 4.25d-e. Bar graphs showing change in the d) number and e) percentage of OLPs targeted per participant for Arm B participants between Weeks 0 and 20. Different colours represent responses to either Gag, Pol, Env, Nef or total proteins. P values were calculated using repeated measures ANOVA; p values <0.05 indicates significance.

Next, we examined whether there were changes in the HLA-class I restricted responses between Week 0 and 20; notably, there were no significant shifts towards either protective or disease-susceptible alleles between both timepoints **(Figures 4.27a-b)**. When we examined the distribution of HLA-class I restricted responses, the majority of responses were consistently restricted by the same HLA-class I alleles (e.g A24:02, B8:01 and B7:02). Interestingly, there was an increase in B7-restricted responses compared to baseline where these responses were negligible **(Figures 4.28a-b)**. When we examined the percentage of OLPs targeted by all Arm B participants, this increased slightly for Gag (28.38% -> 35.67%) and decreased for Pol (32.43-21.05%) **(Figures 4.29a-b)**.

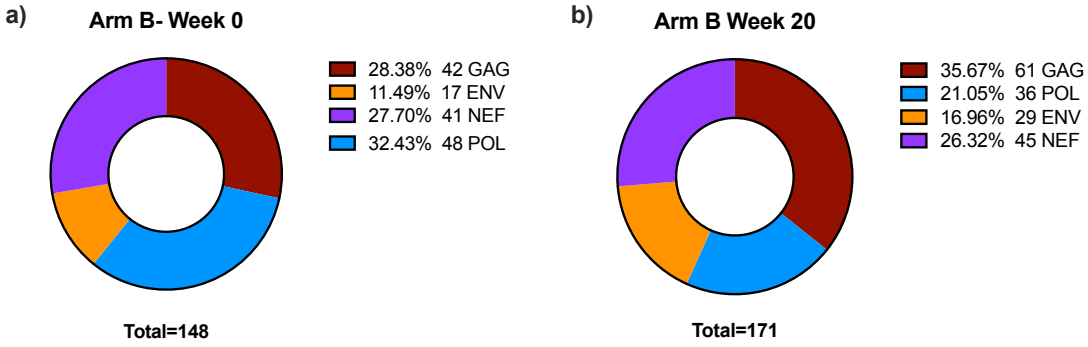


Figures 4.27a-b. Bar graphs showing the HLA-class I restricted responses at a) Week 0 and b) Week 20 in Arm B participants respectively. Each colour represents a different category of HLA-restricted response. If no match was found, this was written as "undetermined".





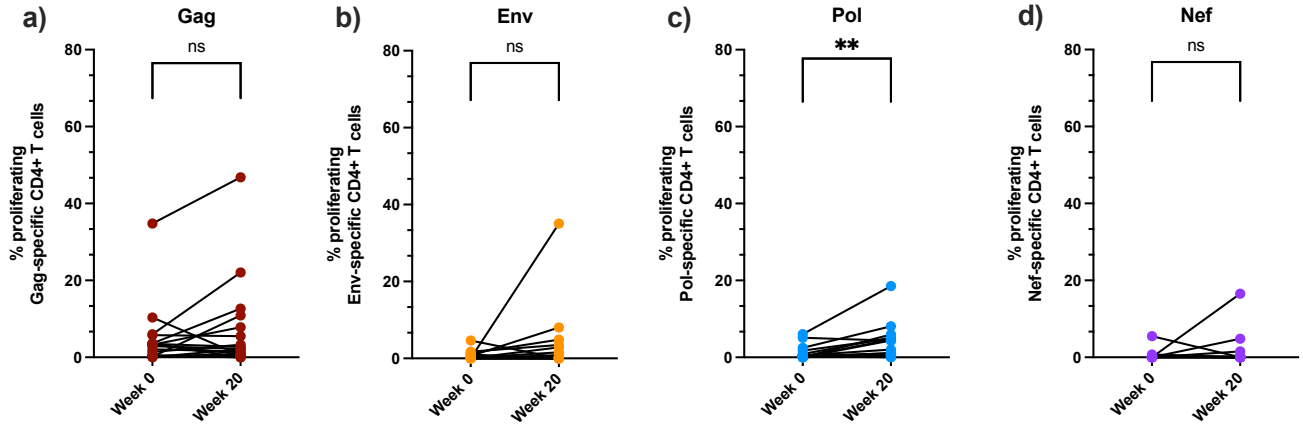
Figures 4.28a-b. Pie charts showing the HLA-class I restrictions of OLP responses at a) Week 0 and b) 20 in Arm B participants respectively. Each colour represents responses that are restricted to a particular HLA-class I allele. If no match was found, this was written as “no HLA match”.



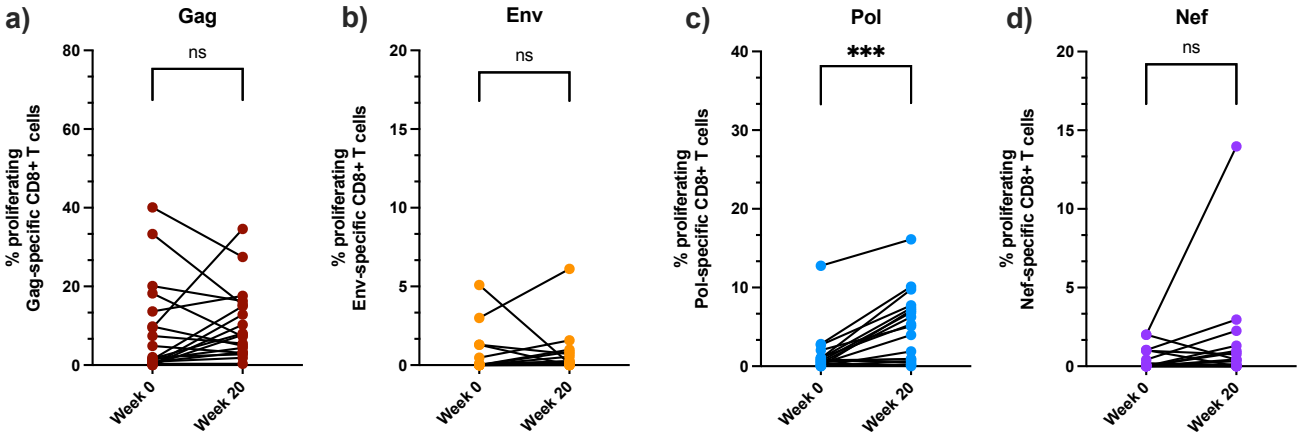
Figures 4.29a-b. Pie charts showing the percentage of responses towards Gag, Pol, Env and Nef in Arm B participants at a) Week 0 and b) Week 20 respectively. Each colour represents a different HIV protein.

Combination bNAbs are associated with increases in HIV-specific T cell proliferation in Arm B suppressed individuals post-bNabs and on ART

Next, T-cell proliferation was analysed to determine if combination bNAbs resulted in increases in HIV-specific T cell immunity in Arm B participants. We observed increases in Pol-specific CD4 (p=0.0012) and CD8 (p=0.0002) proliferation **(Figures 4.30c and Figure 4.31c);** however, in contrast to Arm A participants there were no increases in Gag-specific T-cell proliferation (p=0.356 and p=0.441 respectively) **(Figures 4.30a and 4.31a).**

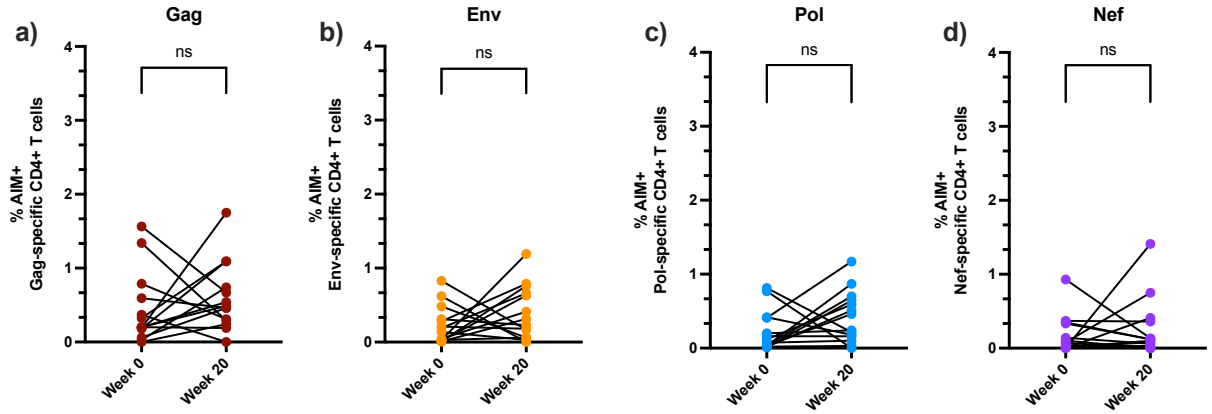


Figures 4.30a-d. Line graphs showing HIV-specific CD4+ cell proliferation to a) Gag, b) Env, c) Pol and d) Nef respectively between Weeks 0 and 20 for Arm B participants post-bNabs and on ART (n=19). Each data point represents the percentage of background-subtracted proliferating CD4+ T cells as a percentage of all T cells. Wilcoxon matched-pairs signed rank tests were performed to determine significance; significant results are marked with asterisks (*).

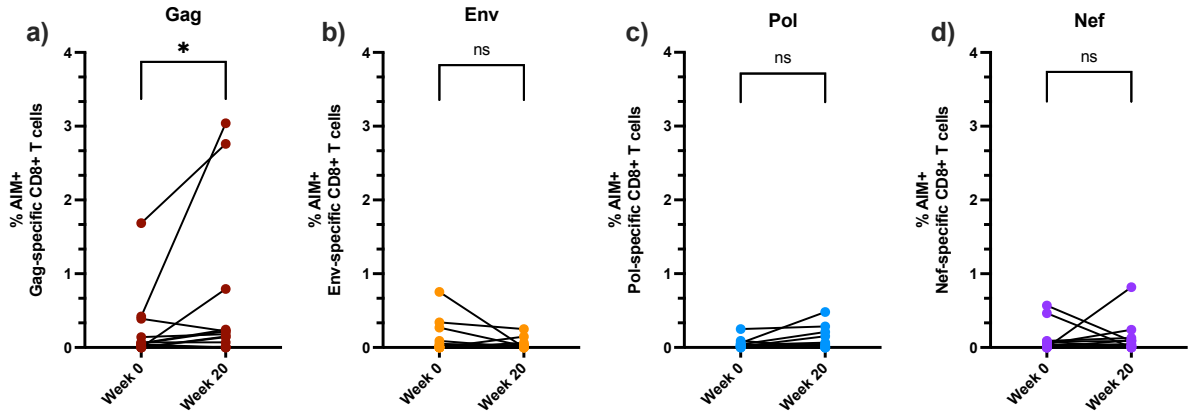


Figures 4.31a-d. Line graphs showing HIV-specific CD8+ cell proliferation to a) Gag, b) Env, c) Pol and d) Nef respectively between Weeks 0 and 20 for Arm B participants post-bNabs and on ART (n=19). Each data point represents the percentage of background-subtracted proliferating CD8+ T cells as a percentage of all T cells. Wilcoxon matched-pairs signed rank tests were performed to determine significance; significant results are marked with asterisks (*).

Next, AIM+ CD4 and CD8 responses were analysed to determine if bNAbs in Arm B participants resulted in increases in HIV-specific T cell activation between Week 0 and 20. In comparison to Arm A participants that were virally suppressed off ART, Arm B participants demonstrated marginally significant increases in Gag-specific CD8 AIM+ cells (p=0.0444) **(Figure 4.33a),** but not for CD4s (p=0.502). No other HIV proteins showed significance **(Figures 4.32b-d and Figures 4.32b-d).**

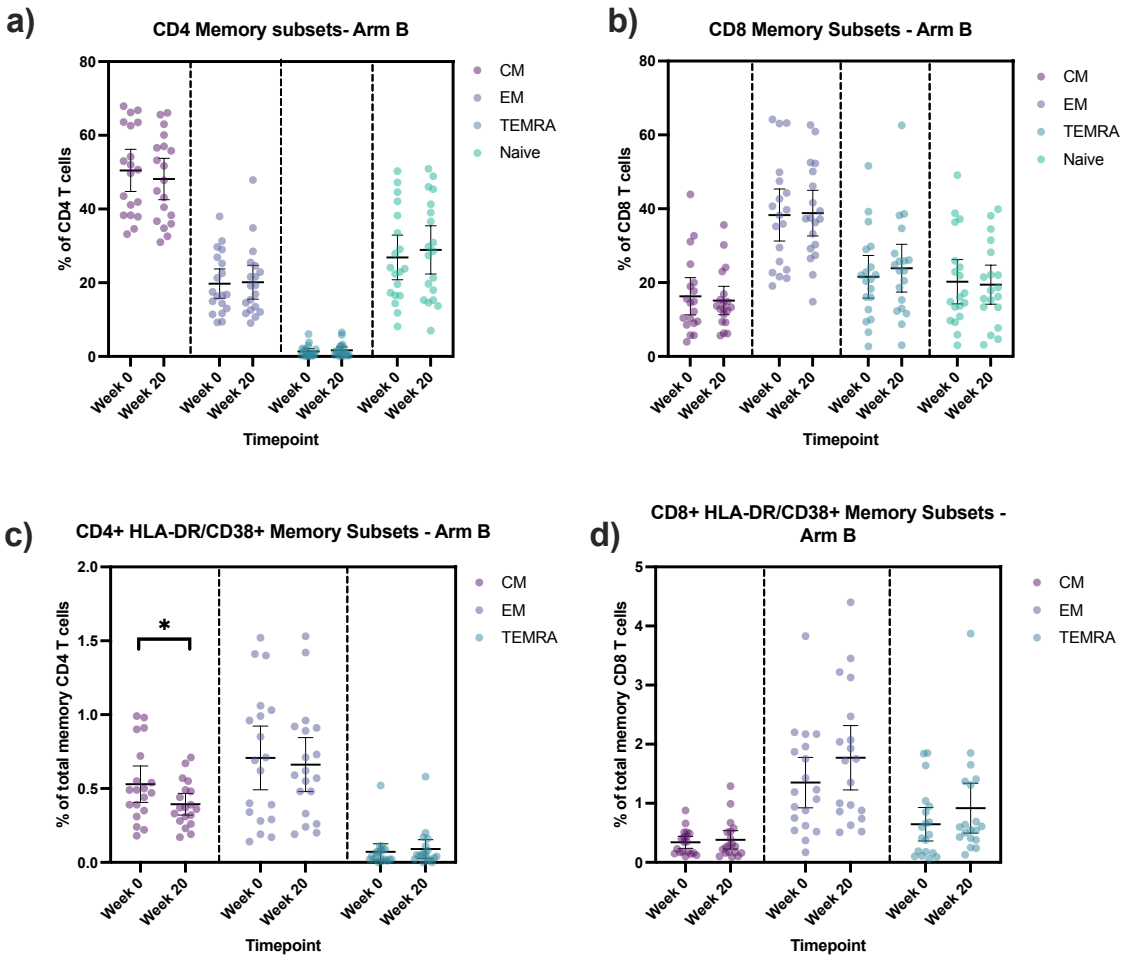


Figures 4.32a-d (see legend overleaf)

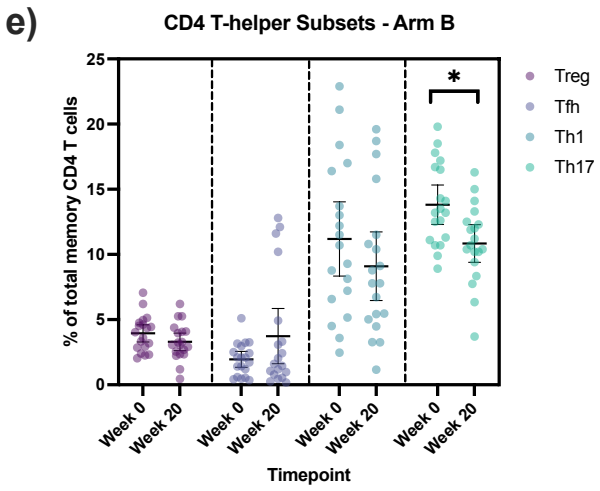


Figures 4.32a-d and 4.33a-d. Line graphs showing HIV-specific CD4+ and CD8+ AIM+ responses to a) Gag, b) Env, c) Pol and d) Nef respectively between Weeks 0 and 20 for Arm B participants post-bNabs and on ART (n=19). Each data point represents the percentage of background-subtracted CD4+ and CD8+ AIM+ cells as a percentage of all T cells. Wilcoxon matched-pairs signed rank tests were performed to determine significance; significant results are marked with asterisks (*).

Finally, the immune phenotype of Arm B participants were analysed to determine if there were similar increases in Th1 helper cell subsets and a Tbet^{hi}EOMES^{lo} phenotype as seen in Arm A. Notably, there were no significant changes in CD4 or CD8 T cell memory subsets between Weeks 0 and 20. In the CD4+ subsets, there was a slight decrease in central memory T cells expressing double positive HLA-DR and CD38 (p=0.0188) **(Figure 4.34c)**, as well as a decrease in Th17 cells between Weeks 0 and 20 (p=0.0126) **(Figure 4.34e)**.

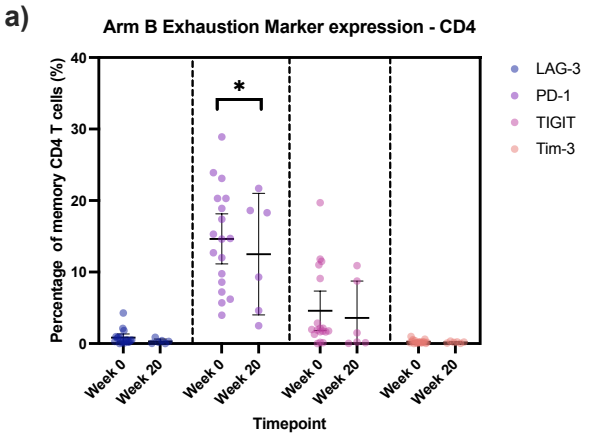


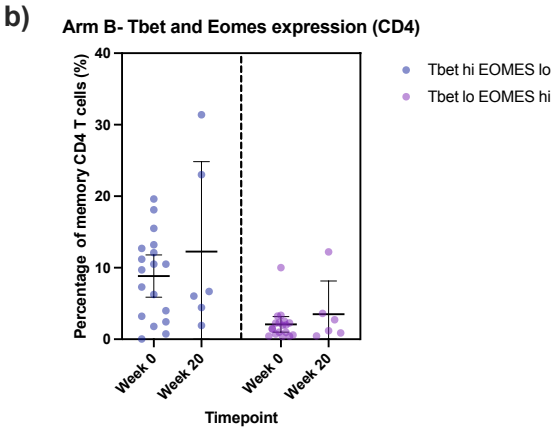
Figures 4.34a-d. Scatter graphs showing the a) CD4 and b) CD8 memory subsets, the HLA-DR/CD38 expressing c) and d) CD8 cells, as well as the e) CD4 T-helper subsets between Weeks 0 and 20 in Arm B participants (n=25). Coloured dots represent different memory subsets: central memory (CM), effector memory (EM), and terminally differentiated (TEMRA) cells respectively.



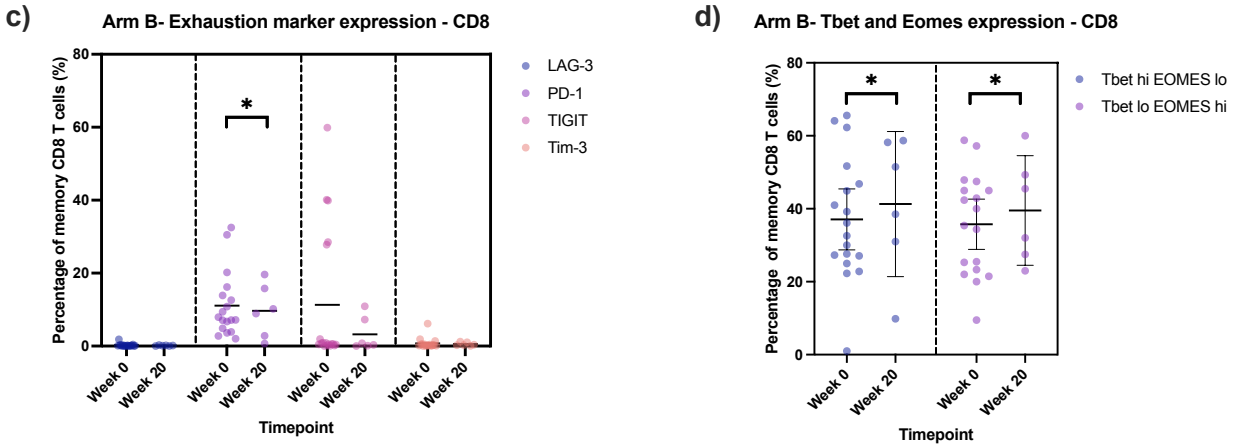
Figures 4.34e. Scatter graphs showing the CD4 T-helper subsets between Weeks 0 and 20 in Arm B participants (n=25). Coloured dots represent different memory subsets: central memory (CM), effector memory (EM), and terminally differentiated (TEMRA) cells respectively.

In a similar fashion to Arm A participants, we sought to determine whether there were any increases in exhaustion marker expression between Week 0 and Week 20 (post-bNAbs and after ART resumption). Notably, there were no significant increases in expression of the markers LAG-3, Tim-3 or TIGIT; however, CD4 and CD8 PD-1 expression decreased between Weeks 0 and 20 (p=0.05) **(Figures 4.35a and c).** When analysing the levels of Tbet and EOMES, Tbet^{hi}EOMES^{lo} and Tbet^{lo}EOMES^{hi} populations appeared to increase between Weeks 0 and 20 (p=0.0342 and p=0.0320 respectively), although the sample size in this analysis was too small to draw proper conclusions (n=8) **(Figures 4.35b and d).**





Figures 4.35a-b. Scatter graphs showing the expression of exhaustion markers LAG-3, PD-1, TIGIT and Tim-3 in both the a) CD4+ and b) CD8+ compartments between Weeks 0 and 20 in Arm B participants (n=18 at Week 0, n=8 at Week 20). Wilcoxon matched-pairs signed rank tests were performed to determine significance; significant results are marked with asterisks (*).



Figures 4.35c-d. Expression of c) Tbet^{hi}EOMES^{lo} and d) Tbet^{lo}EOMES^{hi} populations in Arm B participants (n=18 at Week 0, n=8 at Week 20). Due to the small sample size, only one sample t tests could be performed for tests of comparison; p values <0.05 are indicated by the asterisks (*).

4.5 **Hypotheses 3:** Significant inter-assay correlations are observed between T-cell assays that measure HIV-specific T cell immunity, and some of these are predictive immune correlates for viral rebound

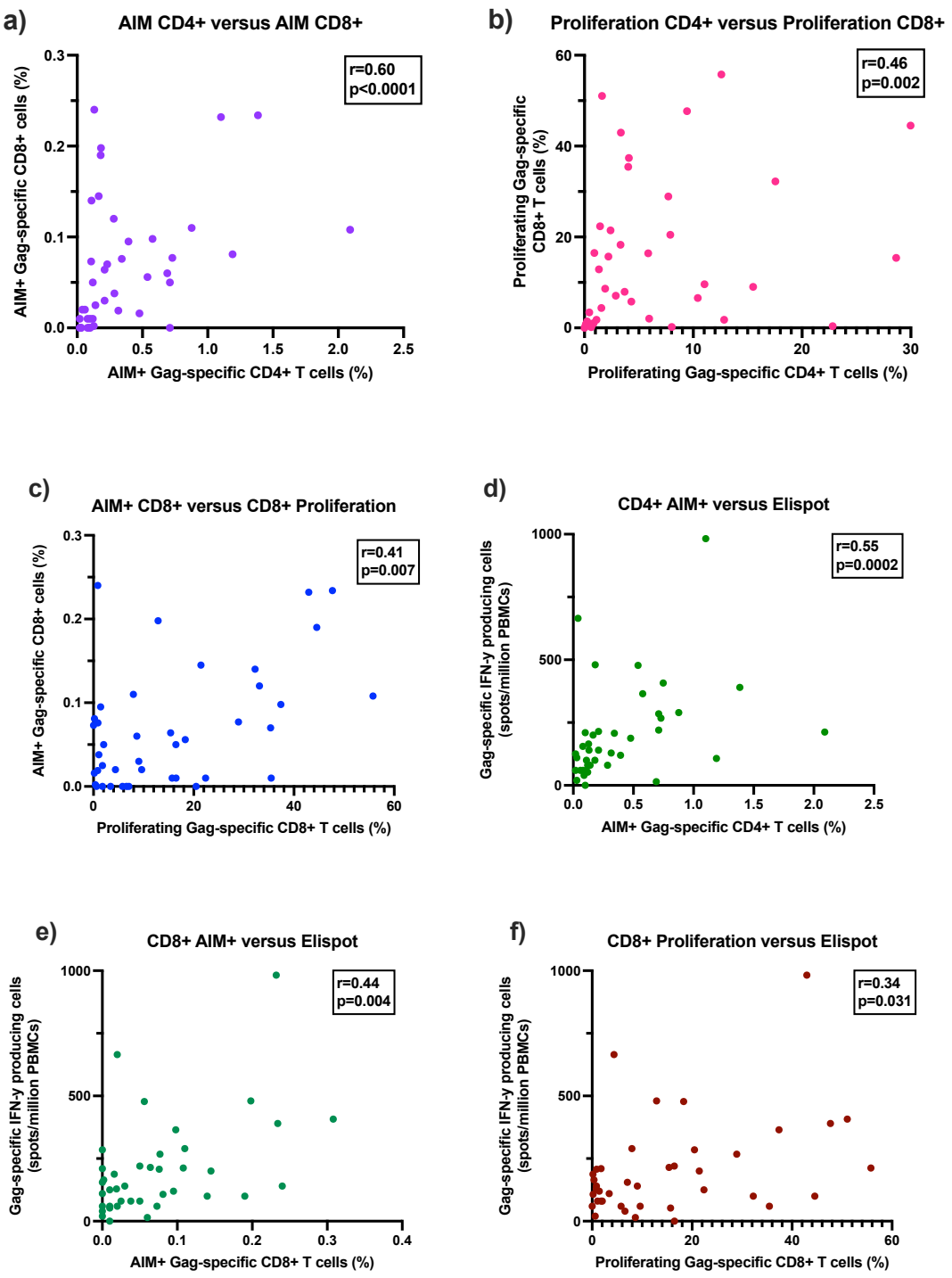
Finally, we aimed to determine whether HIV-specific T cell immunity in Arm A participants could serve as immune correlates for viral rebound. Previous data from the eCLEAR trial that used the single short-acting bNab 3BNC117 demonstrated that Gag-specific interferon-gamma secretion predicted the duration of ART-free or virological control (38). While data exists on the relationship between HIV-specific T cell responses (such as Gag) and viral control (47), limited data exists on how HIV-specific T cell immunity post-bNAbs could contribute to viral control and delayed viral rebound. The hypothesis is that Gag-specific T cell responses may significantly correlate with time to viral rebound, given the existing evidence that Gag-specific T cell responses have been significantly correlated with a reduction in viraemia (47). In addition,

we also examined relationships between different T-cell assay responses, which could inform the selection of optimal assays for future HIV research and cure trials.

4.5.1 Methods and Results- Part 1

HIV-specific functional T-cell responses demonstrate inter-assay correlations in Arm A participants that are virally suppressed off ART

First, we determined whether there were any inter-assay correlations between HIV-specific T cell responses observed in different functional T-cell assays (proliferation, AIM and ELISpot). For the purposes of this thesis, the analysis focused on determining correlations between Gag-specific responses of different T-cell assays, as these responses are most likely beneficial for viral control (47). To demonstrate this, all timepoints from Arm A participants between Weeks 0 and 24 that were virally suppressed during analytical treatment interruption were examined. As expected, there were strong positive correlations observed between AIM+ Gag CD4 and CD8 responses ($p<0.0001$), as well as between proliferative Gag CD4 and CD8s ($p=0.0036$) **(Figures 4.36a-b respectively).** When comparing AIM+ Gag responses with proliferative responses, there were weak positive correlations observed between AIM+ Gag CD8 responses and proliferative CD8 responses ($p=0.0074$), but not for CD4s ($p=0.386$) **(Figure 4.36c)**. When compared to ELISpot responses, both AIM+ Gag CD4 and CD8 responses correlated with Gag ELISpot response ($p=0.0003$ and $p=0.0042$, **Figures 4.36d-e** respectively). Finally, proliferative Gag CD8 (but not CD4) responses demonstrated a weak positive correlation with ELISpot Gag responses ($p=0.0312$, **Figure 4.36f**). A correlation summary table is shown below in **Table 4.2**.



Figures 4.36a-f. Scatter graphs showing correlations between different Gag-specific functional T-cell assays in Arm A participants that are virally suppressed off ART. X and Y axes are labelled with the T-cell assay response (proliferation, AIM, or ELISpot); each coloured dot represents one paired data point. Analyses was done via Spearman's rank correlation coefficients, $p<0.05$ indicates significance.

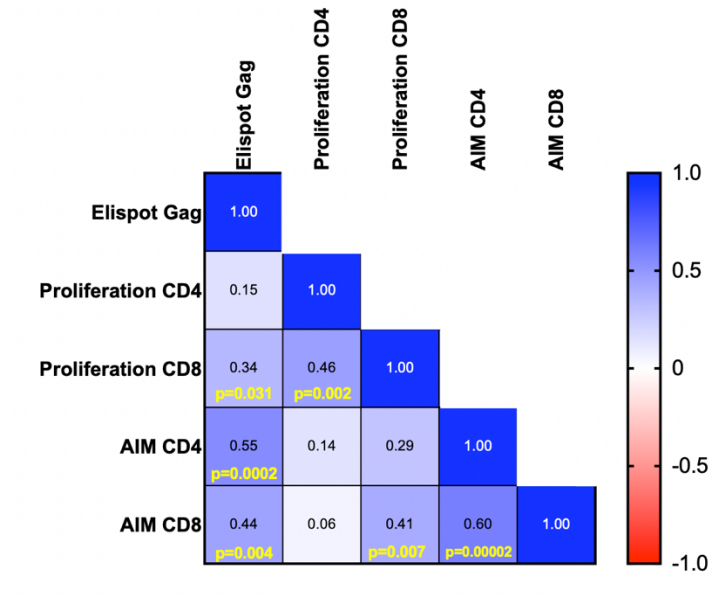
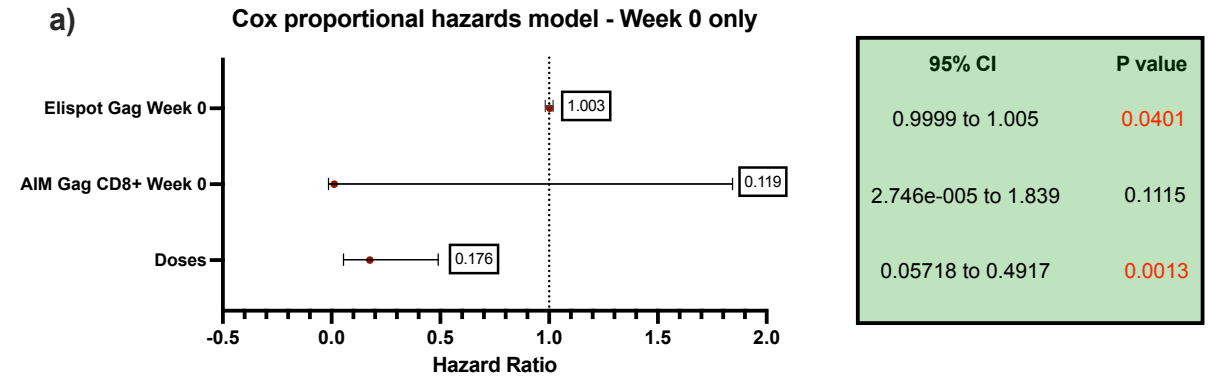


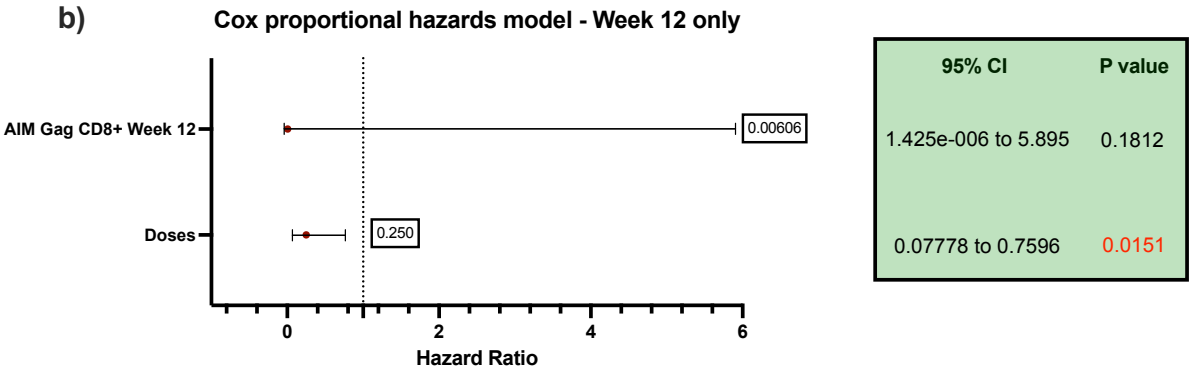
Table 4.2. Correlation table showing Gag-specific responses for different functional T-cell assays in Arm A participants that are virally suppressed off ART. Non-parametric Spearman correlations were calculated; R values are shown above p values in each box. P values <0.05 were considered significant. Only significant values are shown as yellow text.

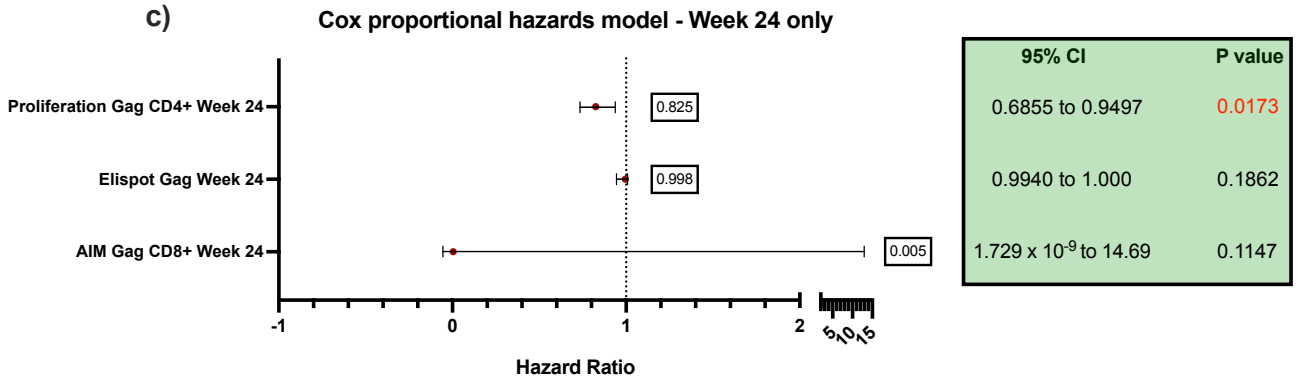
4.5.2 Methods and Results- Part 2

To determine the variables that influenced time to viral rebound, we performed a Cox proportional hazards model, where event was defined as time to viral rebound (in weeks) after the last dose of bNAbs. We focused on Arm A participants that remained virally suppressed off ART and produced three models, where each model only had responses from one timepoint (for example, Week 0, 12 or 24 responses only as separate variables). Due to the consistent link between Gag responses and viral control, we only included Gag responses from each T-cell assay as variables; we also included the number of bNAb doses received as an additional variable. For selection of relevant variables and to prevent data overfitting, we performed a lasso regression as a regularisation technique using R version 4.2.2 to determine the variables for inclusion in the Cox proportional hazards model (Results for lasso regressions are shown in **Appendix Table 4.3**).

In the Week 0 model, bNab dose was significantly associated with a lower likelihood of rebound (hazard ratio HR=0.176, p=0.0013), while ELISpot Gag responses were marginally associated with a higher likelihood of rebound (however the hazard ratio was equivocal in this case as the confidence intervals intersected zero) (**Figure 4.37a**). In the Week 12 model, bNAb doses were also associated with a lower likelihood of rebound although the hazard ratio was quantifiably lower than at Week 0 (**Figure 4.37b**). Notably, the Week 24 model showed that CD4+ proliferative responses to Gag were associated with a lower likelihood of rebound (HR = 0.825, p=0.0173) **(Figure 4.37c).**







Figures 4.37a-c. Cox proportional hazards models using bNAb doses plus either a) Week 0, b) Week 12 and c) Week 24. Numbers adjacent to the red data points indicate the hazard ratio; Black bars represent the 95% confidence intervals for each variable. P<0.05 indicates significance and significant values are shown in red text.

4.6 Discussion

This chapter investigated the impact of dual long-acting combination bNAb therapy (3BNC117-LS and 10-1074-LS) on HIV-specific T cell immunity in ART-treated participants with PHI. To examine this, we first used ELISpot assays to measure changes in interferon-gamma secretion in virally suppressed Arm A participants post-bNAbs. Interestingly, we observed increases in total Gag-specific interferon-gamma secreting T cells between Weeks 0 to 12, as well as increases in the percentage of Gag-specific responses between Weeks 0 and 24. This suggests that bNAbs may be stimulating increased Gag-specific T cell immunity as well as shifting dominant responses towards Gag epitopes over time. These findings concur with the landmark study by Niessl et al. which showed that ART-treated individuals post-bNAbs that made detectable interferon-gamma ELISpot responses showed broader targeting of Gag epitopes during the ATI period. They also observed significant increases in Gag-specific CD4 and CD8 interferon-gamma producing T cells in the first 6-7 weeks post-bNAbs (although this was measured via intracellular cytokine staining and not via ELISpot). Taken together, these findings provide evidence that combination bNAbs may be

associated with a shift in immune dominance towards (protective) Gag epitopes in the context of viral suppression off ART, at least in the short term. Notably, Niessl et al. used short acting versions of the bNAbs, compared to the modified long acting versions used in this trial; this could explain why we have observed shifts towards Gag responses up to Week 24 compared to only Week 12 in their study (37).

To further characterise the epitope specific responses associated with this change, we analysed the OLP responses by categorising Arm A participants into "early" and "late" rebounders using week 20 as the definition for viral rebound. Interestingly, we demonstrated that "late" rebounders made more OLP responses between Weeks 12 to 24; while the absolute number of Gag OLPs targeted per participant decreased, the percentage of Gag OLPs appeared to increase. This could suggest that "late" rebounders are making fewer, but more narrowly directed responses towards Gag between Weeks 12 to 24 which might explain the prolonged viral suppression observed in this group. In contrast, "early" rebounders showed decreases in total peptide-adjusted responses, as well as percentage of Gag OLPs targeted, between Weeks 12 and 24. This indicates that early rebounders tend to produce less Gag responses over time; however, we cannot make firm conclusions due to the small sample size in this sub-group (n=8).

We then examined the changes in OLPs targeted by all participants in each subgroup. While statistical analysis was not performed, we observed a persistent increase in Gag OLPs targeted in the "late" group, compared to a decrease in the "early" group. To determine whether these differences could be attributed to changes in immune dominance, we examined the recognition of OLPs across both groups; remarkably, we did not observe any striking differences between the main OLPs recognised in each group. Notably, a significant percentage of participants in both groups recognised Gag peptide IYKRWIILGLNKIVR at Week 0; at Week 24, responses to Gag peptides EKIRLRPGGKKKYKL and AGTTSTLQEQIGWMT, and one Nef

peptide WVYHTQGYFPDWQNY dominated. In this case, dominant responses towards these Gag and Nef regions are more likely attributed to an enrichment of common HLA-class I alleles.

Next, we looked at HIV-specific T cell proliferation and observed that Gag CD4+ and CD8+ proliferative responses increased between Weeks 0 and 12 in participants that remained virally suppressed and off ART; for CD8+ proliferation, this increased up to Week 24 post-bNabs. These findings prompt further investigation into the role of T-cell proliferation as a method of measuring HIV-specific T cell responses in future HIV cure trials involving bNAbs. Notably, these results also align with previous observations that emphasise the important relationship between protective Gag responses and viral control (48,49). We also observed increases in Pol-specific CD4+ proliferation between Week 0 and 12; although less described, some studies have linked Pol responses with control of viraemia (50–52). While we noticed increases in Nef-specific CD4 proliferation between Weeks 0 and 12, the relevance of Nef-specific responses to viral control is less likely as previous studies have not shown correlations between Nef-specific CD8 responses and viraemic suppression; in fact, the opposite is often true (47). Against this argument is a recent study by Adland et al. done in chronic clade C infection where they demonstrated that CD8 targeting of certain Nef epitopes could contribute to viral suppression; however, more investigation is needed to determine if this also applies to acute clade B infection (53). It is likely that the Nef responses observed in our study are merely "passenger" responses that exert weak pressure on the virus rather than responses that drive immune control (54).

With regards to activation markers, we used the AIM assay which has also been utilised by previous landmark studies to measure HIV-specific T cell activation (37,38). Our trial incorporated three activation markers for CD4+ T cells (OX-40, CD137 and CD69), two for CD8+ T cells (CD137 and CD69) as well as CD25 (see Chapter 2 for explanation of gating strategies); this panel has been optimised and used previously in

other vaccine trials (55). Interestingly, we observed significant increases in Gag and Pol-specific CD4+ and CD8+ AIM+ cells between Weeks 0 to 12 in Arm A participants that were virally suppressed off ART. This concurs with the increased Gag-specific responses observed in both ELISpot and proliferation. Previous studies that used short acting versions of 3BNC117 and 10-1074 in individuals with HIV off ART also showed increases in AIM+ Gag-specific CD8 responses up to 12 weeks post-bNAbs, but not for other HIV proteins (Pol, Nef and Env) (37).

The slight difference in observed results could be due to a number of factors; for instance, Niessl et al. incorporated PD-L1 as an AIM marker versus OX-40 in our panel (although both are clearly documented markers of T-cell activation) (37) and were limited by their small sample size (n=9). In another study by Rosas Umbert et al, Gag-specific AIM+ CD8 T cells were significantly higher at 3 and 12 months after initiating ART in participants receiving adjunctive 3BNC117 in addition to romidepsin, when compared to controls receiving ART only (38). In comparison, their study only used the short-acting bNab 3BNC117 prior to ART initiation, whereas Arm A participants received dual long-acting bNAbs on ART followed by an ATI. Like Niessl et al., they used PD-L1 instead of OX40 which may explain the difference in AIM+ cells. Another key difference is the gating strategy used; in the CD4 compartment, for example, both authors did a Boolean analysis using double positive cells to produce an overall percentage of total AIM+ cells (i.e PDL1+CD137+, PDL1+CD69+ and so forth). In contrast, we gated on CD4+ T cells that were double positive for CD25 and either OX-40, CD137 or CD69. Both gating strategies have been used by previous studies and may partially explain the divergence in results observed (37,38,55).

As an extension of T-cell functionality, we also performed immune phenotyping of PBMCs in Arm A participants. Previous studies have shown that therapeutic vaccination in early ART recipients can induce polyfunctional T cell responses similar to those in HIV controllers and expand the central memory pool

(56,57). Elite controllers often have higher frequencies of stem-like memory T cells and a broadly directed central memory phenotype capable of suppressing viral replication (58). In Arm A participants who were virally suppressed off ART, we did observe increases in CD8 effector memory T-cells between Weeks 0 and 12, but not towards Week 24. We also observed significant increases in Th1 CD4 cells between the same timepoints. This result concurs with existing knowledge on Th1 cells as key players in cell-mediated immunity via production of cytokines such as interferon-gamma. Studies have shown that HIV controllers maintain a highly effective Th1 effector T cell population against Gag in the setting of low antigenaemia (59). A disrupted Th1/Th2 ratio has been linked to disease progression (60), and strong Th1 proliferation is negatively correlated with lower HIV RNA and proviral DNA loads (61). Our ELISpot assays, which primarily measure Th1 responses, also showed increased interferon-gamma secretion towards Gag between Weeks 0 and 12. Thus, our findings suggest that combination bNAbs may be inducing expansion of Th1 helper cells and CD8 effector memory cells that are responsible for subsequent viral control.

In addition to the above findings, we also observed significantly higher percentages of the $Tbet^{hi}EOMES^{lo}$ phenotype in Arm A participants that were virally suppressed off ART at Weeks 12 and 24 post-bNAbs. This result is remarkable given the existing links between T-bet expression and a long term non-progressor phenotype; conversely, low levels of Tbet (i.e a $Tbet^{lo}$ $EOMES^{hi}$ phenotype) are typically associated with an exhausted, transitional memory phenotype with poorer CD8 functionality (46,62). Notably, combination bNAbs may be driving selection towards more favourable phenotypes over time, and these are occurring in conjunction with increases in Th1 expression and Gag-specific T cell immunity.

In comparison to virally suppressed Arm A participants, we also examined Arm B participants to determine if receiving bNAbs during low level viraemia followed by ART-induced viral suppression was associated with similar changes in HIV-specific T cell immunity. We compared responses between Week 0 and Week

20, a timepoint around 3 months post-bNabs and ART resumption where we would expect the circulating antigen from prior viraemia to have washed out. Contrary to our expectations, we did not observe any increase in Gag-specific CD4 or CD8 proliferation in Arm B participants, although Pol-specific CD4 and CD8 proliferation did increase between Weeks 0 and 20. A significant confounder in this analysis is that Arm B participants experienced a period of acute viraemia prior to receiving bNAbs and ART. Thus, it is uncertain whether any observed increases in response are protective or potentially due to the spillover effects of antigen circulation (although this effect has been controlled for using a 24-week washout period with ART). One might argue, however, that viraemia usually results in a global increase in HIV-specific T cell immunity across all HIV proteins; however, we have only observed specific increases in Pol-specific proliferation here. To further support this argument, previous studies have also reported similar increases in Pol-specific CD8+ T cells after 3 months in individuals with HIV that received 3BNC117 and initiated ART (although this was in comparison to controls receiving ART only (38); Pol-specific CD8+ T cells have also linked with viral suppression in some cases. Further analysis is required at later timepoints in this study to determine if the Pol-specific responses observed are protective or beneficial for viral control.

In our analysis of interferon-gamma secretion in Arm B participants, we also observed similar increases in Gag-specific responses from Week 0 to 20. Arm B participants also made significantly higher responses per Gag OLP between Weeks 0 to 20. In our epitope analysis, we observed dominant OLP responses to Gag (NPPIPVGEIYKRWII) and Nef (IYSQKRQDILDLWVY and KRQDILDLWVYHTQG) regions. At Week 20, these responses remained dominant with the addition of Gag peptide EKIRLRPGGKKKYKL. In this case, dominant responses to these regions likely reflect epitopes that are restricted by commonly enriched HLA-class I alleles present in our Arm B participants, such as B8:01 for NPPIPVGEIYKRWII. Furthermore, the frequent targeting of Nef region IYSQKRQDILDLWVYHTQG reflects the immunogenicity of this region, which is commonly recognised by numerous HLA-class I alleles (as shown in the Los Alamos HIV database).

Similarly, our inferred HLA-class I epitope mapping showed that predicted HLA-class I restricted responses did not significantly change between Weeks 0 and 20, nor did the percentage of responses towards each HIV protein.

Our ELISpot analysis was limited by the number of timepoints sampled and the epitope mapping performed was only inferred. Also, we measured interferon-gamma secretion responses using bulk PBMCs which means observed responses could be due to other interferon-gamma secreting immune cells such as natural killer cells (although T-cell driven interferon-gamma production is more likely given that they form the majority of PBMCs). Nevertheless, this assay still provides a robust functional readout of antiviral immunity. To strengthen the evidence for this hypothesis, future studies could measure interferon-gamma responses to T-cell specific fractions, such as via T-cell enrichment or sorting. Furthermore, other methods that detect the expression of multiple cytokines (such as the fluorospot) in addition to interferon-gamma may provide enhanced insights into the mechanisms and predictors of viral control post-bNabs. Overall, these findings suggest that combination bNAbs (given during viraemia) are associated with increases in the magnitude of Gag responses. Whether or not these responses correlate with subsequent control off ART is uncertain; in fact, previous studies suggest that the expression of interferon-gamma alone is unlikely to be sufficient for a protective immune response (63–68). It is likely that a combination of different T-cell assays are required to properly estimate the effect of T cells on viral control post-bNAbs. Overall, further investigation of HIV-specific T cell responses during the 2nd ATI in Arm B participants, coupled with definitive epitope mapping for HLA-class I restrictions, will determine whether these observed responses could confer protection.

With regards to other aspects of T-cell functionality, we also observed significant increases in Gag-specific CD8 AIM+ (versus CD4 in Arm A participants) cells in Arm B participants between Weeks 0 and 20, which

concurs with previous literature (38) (although the increases observed were fairly small). For T-cell phenotypes, we observed reductions in the number of double positive HLA-DR/CD38+ CD4+ T cells as well as PD1+ CD4 and CD8 T cells. These effects could be due to the initiation of suppressive ART between Week 0 and 20. We also noticed increases in both $Tbet^{hi}Eomes^{lo}$ and $Tbet^{lo}Eomes^{hi}$ CD4 and CD8 cells; however, we cannot make definitive conclusions due to the small sample size used (n=8). It is possible that the short period of viraemia experienced by Arm B participants may explain the findings observed, compared to our Arm A counterparts that did not rebound. Analysis of further timepoints will determine whether these changes persist off ART.

Finally, we determined if HIV-specific T cell responses in Arm A participants that remained virally suppressed could predict time to viral rebound. The cox proportional hazards models showed that the number of bNAb doses were associated with a significantly lower likelihood of viral rebound from the last bNAb dose, when including Week 0 and Week 12 responses as variables. Remarkably, we also observed that Gag CD4 proliferation at Week 24 significantly reduces the likelihood of rebound. One possible conclusion based on this data is that early responses produced in the first 12 weeks are a reactive response to low level viraemia during ATI (as we have observed in our previous analysis of Gag ELISpot and AIM data). However, participants that continue to produce responses beyond 12 weeks are more likely to rebound later, possibly because these responses are more durable and protective. This might explain why virally suppressed Arm A participants off ART tend to produce more Gag directed responses by 24 weeks (as seen in our ELISpot data). These findings have potentially important clinical implications for determining immune predictors of viral rebound and future studies should investigate whether these T-cell immune correlates are also observed in other interventional trials using bNAbs. In addition to these findings, we also observed significant positive correlations between AIM CD8 and Proliferation CD8 responses, as well as ELISpot versus CD4/CD8 AIM and CD8 proliferation. These strong inter-assay

correlations are crucial for designing future HIV cure trials that rely on T-cell assays as functional readouts. This analysis was limited by the relatively small sample size, as we only included datapoints from participants who remained virally suppressed for the first 24 weeks. This resulted in fewer timepoints available for analysis, especially at Week 24 where more participants have already rebounded in Arm A. Future studies should confirm these correlations.

In summary, this chapter describes how dual-long acting bNabs (3BNC117-LS and 10-1074-LS) can potentially boost HIV-specific T-cell immunity in participants that are virally suppressed off ART or after ART re-initiation. Responses observed tend to be skewed towards Gag and sometimes Pol, and a CD4 proliferative response towards Gag in virally suppressed Arm A participants was associated with a reduced likelihood of time to viral rebound. Additionally, combination bNabs are associated with Th1 skewing and an increased expression of favourable $Tbet^{hi}$ $EOMES^{lo}$ phenotypes, although more research is required to determine the durability and impact of these phenotypic changes. A summary of the findings from this chapter are shown in **Figure 4.38**. In conclusion, these findings shed light on how bNabs influence cell-mediated immunity with and without ART in the context of viral suppression, and how these responses may contribute to viral control in a therapeutic setting.

	Arm A (bNAbs during aviraemia)	Arm B (bNabs during viraemia)
T-cell functionality	**↑ Gag CD4/CD8 proliferation** **↑ Gag ELISpot**	**↑ Pol CD4/CD8 proliferation** **↑ Gag ELISpot** **↑ Gag CD8 AIM**
T-cell phenotypes	↑ Th1 skew ↑CD8 Effector Memory (Wk 0-12) ↑ $Tbet^{hi}EOMES^{lo}$	↑ $Tbet^{hi}EOMES^{lo}$

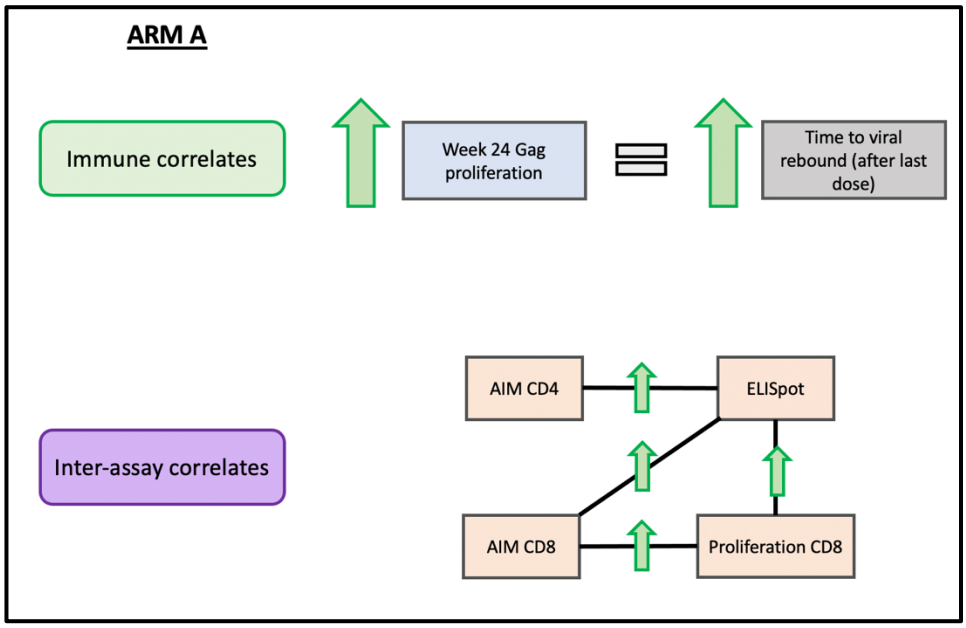


Figure 4.38. Summary of main findings from Chapter 4.

Chapter 4: References

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Chapter 5

Immune mechanisms of post-bNab "control" in the context of spontaneous viral suppression

Contributions to this chapter

I would like to express my thanks to all the participants and staff that made the RIO clinical trial possible; this includes the RIO pilot study research investigators, clinicians and research staff at the respective clinical recruitment sites, as well as the trial steering committee.

Sample collection and processing for the RIO clinical trial were performed by members of Prof. Graham Taylor's lab at Imperial College London, UK. Dr. Ane Ogbe and Dr. Ming Lee assisted with the RIO trial study design and initial setup as well with experimental expertise. The T-cell data presented here have been produced in collaboration with Dr. Mohammed Altaf and Carla Nel who helped with T-cell assay design, optimisation and results acquisition for the ELISpot, proliferation, immune e phenotyping and AIM assays. Penny Zacharopoulou provided data on bNAb sensitivity at baseline and helped with DNA library preparation and next generation sequencing data.

NB: Due to the ongoing blinded status of the study at the time of submission, we used a combination of real-time unblinding and available pharmacokinetic data to infer treatment arms of participants. Although the results presented here are preliminary and do not reflect the final formal report, we expect them to be highly comparable.

5.1 Introduction

HIV-specific T cells have frequently been implicated as a potential mechanism of achieving durable immune-mediated viral control. Some of the earlier studies looking at SHIV infection in rhesus macaques have demonstrated a clear association between CD8 T cells and viral suppression. For instance, one study showed that the administration of an anti-CD8 monoclonal antibody to SHIV infected rhesus macaques resulted in depletion of CD8 T cells and increased circulating viral loads directly after administration (1). These findings were also supported by another study by Jin et al; they administered an anti-CD8 monoclonal antibody to six SHIV-infected rhesus macaques and demonstrated significant rises in plasma viraemia following a reduction in CD8 T cells. Conversely, these effects were not observed in control macaques that were inoculated with a control antibody (2). More recent studies have confirmed the causality of these findings in ART-treated macaques and showed that CD8+ T cells contributed to viral suppression during ART and depletion of these resulted in plasma viraemia (3).

In human studies, HIV-specific T cell responses play crucial roles in controlling viral load during initial infection and determining the viral load set point (3). Early studies looking at individuals in PHI reported that participants with detectable CD8 responses towards Gag, Pol and Env were able to control viraemia versus participants with absent initial CTL responses, who showed evidence of persistent viraemia and declining CD4 T cell counts (4). Similarly, in symptomatic PHI patients, dominant CD8 responses towards the (Env) gp160 protein exhibited control of acute plasma viraemia, compared to patients with low or undetectable virus-specific CTL activity who showed poor control (5). In addition, another large comparative study done in 33 participants with primary HIV-1 infection demonstrated that early induction of Env-specific memory CTL responses was associated with control of viral replication and reduced declines in CD4+ T cell counts (6).

More recently, some studies have characterised the HIV-specific T cell responses at an epitope level; one early study by Addo et al looked at 57 participants at different stages in HIV infection and reported that responses to p24 Gag and Nef had the highest epitope densities and were the most frequently recognised HIV proteins (7); however, the breadth and magnitude of responses between individuals was very heterogenous and no significant correlations were found between responses and plasma viral load (7). Unsurprisingly, participants that made the strongest and broadest responses were those in untreated chronic HIV infection. In contrast, results from later study done in untreated chronic HIV infection found that increasing breadth of Gag-specific responses was associated with decreasing viraemia, while the opposite correlation was observed with increasing viraemia (8). These associations with CD8-specific T cell responses were also found to be independent of HLA type and unrelated to epitope sequence conservation (8). Although less well defined, a similarly designed study looking at individuals at different stages of HIV infection also demonstrated that Gag/Env ratios were a significant predictor of viral control, and that HIV-specific CD4 responses distinguishes HIV controllers from progressors (9). The contribution of HLA-class II restricted CD4 T cell responses has also been reported in another study by the same author, where they reported that a subgroup of HLA-DRB1 variants were linked to lower viraemia seen in HIV "controllers", and that these protective responses were primarily towards Gag and Nef (10). To investigate HLA-class I determinants of HIV control, another large study examined single nucleotide polymorphisms (SNPs) in over 974 controllers and 2648 progressors. They found that conformational properties on specific amino acid residues in the HLA-B peptide binding groove were significant determinants of viral load, depending on the amino acid expressed (11).

Several studies have also implicated the crucial role of HIV-specific T cell proliferation on viral control. During acute infection virus specific CD8+ T cells have been associated with declining viraemia and control of viral replication; however recent studies found that this usually becomes impaired after 1 year of

infection (12,13). It is likely that ongoing viral replication, in addition to the loss of HIV-specific T helper cells, which are often lost early on in infection and provide crucial help to HIV-specific CD8 T cells, are the reasons for this reduced proliferative capacity in chronic infection (14–16). Moreover, recent studies have demonstrated that the addition of autologous CD4+ T cells as well as vaccine-mediated induction of HIV-specific CD4 T helper responses was able to restore HIV-specific CD8 proliferation, suggesting that this defect is potentially reversible and poses important implications for immunotherapeutic interventions in HIV (13).

Further work has been done to characterise the HIV-specific CD8+ proliferative capacity of non-progressors in comparison to progressors. Long-term non progressors (LTNPs) have been shown to maintain the proliferative capability and cytolytic activity of CD8+ T cells, whereas it is impaired in individuals with HIV disease progression (17–19). More importantly, evidence shows that these responses were associated with enhanced perforin expression (17). Another study looking at HIV non-progressors reported that CD8+ T cells had higher polyfunctionality as measured via IFN-γ, MIP-1β, CD107a, TNF-α, and IL-2 (20). While this study focused on bulk T cell proliferative responses, other studies suggest that control of infection is primarily mediated by subsets HIV-specific proliferating T cells that are restricted to protective HLA-class I alleles. Moreover, CD8 T cells that are restricted by certain protective alleles can resist replicative defects, which makes them conducive to expansion and antiviral effector activities (12). Current evidence also suggests that central memory CD8 T cells (Tcm) are more effective in conferring protective immunity compared to effector (Te) and effector memory (Tem) CD8 T cells, because of their ability to proliferate in response to cognate antigen (21,22). Thus, the proliferative capacity of specific HLA-restricted CD8 T cells are likely to be key components of an effective T-cell response.

In line with previous literature on the role of HIV-specific CD8 T cells in viral control, the investigation of specific individuals that can control HIV in the absence of ART, including elite and post-treatment controllers, has garnered interest in recent years. The role of T cell immunity in post-treatment control has been identified in PTC participants from the "CHAMP" study as seen in Etemad et al., where PTCs were found to have more robust Gag-specific CD4+ T cell responses and lower levels of CD4+ T cell exhaustion than post-treatment non-controllers (23). Similarly in the VISCONTI study, post-treatment controllers had a skewed distribution of memory CD4 T cells towards transitional memory cells. However, PTCs have also been shown to possess several features that would make them unfavourable to long-term viral control; for instance, they have lower levels of CD4 and CD8 T cell activation, weaker CD8+ T cell responses and often lack the "protective" HLA-B alleles that are present in elite controllers (24)(ECs). In comparison, elite controllers are often in enriched with "protective" HLA-B alleles and have HIV-specific CD8 T cells with a superior proliferative and cytolytic capacities (17,25). With regards to post-bNab viral control, existing evidence has shown that the administration of short-acting combination bNabs 3BNC117 and 10-1074 has been associated with increased Gag-specific CD8 T cell immunity during ART interruption, as well as increased CD4 polyfunctionality (26). In another study where 3BNC117 was administered prior to ART initiation in conjunction with romidepsin, ART-naïve individuals demonstrated increases in Gag and Pol-specific responses post-ART initiation compared to receiving ART alone, as well as increased interferon-gamma release at 12 months (27).

Despite these findings, there is limited data on the immune mechanisms underpinning viral suppression in individuals with primary HIV infection that have received bNAbs followed by a treatment interruption. While current evidence suggests an association between bNAbs and enhanced HIV-specific T cell immunity, these results need to be confirmed using a placebo-controlled study to determine whether differences in T-cell immunity are observed pre- and post-bNabs. Moreover, the immunological changes

that occur during spontaneous viral suppression after receiving bNabs are also unclear. Additionally, are there any immunological differences to individuals that show rapid viral rebound, and how do T-cell responses influence and shape viral evolution? To answer these questions, this chapter will focus on selected Arm B participants in the RIO clinical trial that exhibited a degree of spontaneous viral suppression during the 2nd ATI period after receiving dual long-acting bNabs. These will be compared to Arm B participants that exhibited rapid viral rebound to determine if T-cell immunity can account for any differences in viral suppression. Finally, we examine a participant that was able to spontaneously achieve complete viral suppression post-bNAbs in the absence of ART and investigate whether this is driven by T-cell mediated mechanisms. The following hypotheses will be examined in this chapter:

5.2 Hypotheses:

1. Spontaneous viral control (in selected Arm B participants) is potentially T-cell mediated
2. Epitopic responses that are associated with spontaneous viral control are influencing viral evolution over time
3. Selected Arm B participants that experience rapid viral rebound post-bNabs are associated with poorer HIV-specific T cell responses
4. HIV-specific T cell immunity in one participant (003-001) is contributing to complete viral suppression post-bNAbs

5.3 **Hypothesis 1:** Spontaneous viral control (in selected Arm B participants) is potentially T-cell mediated

In Arm B, participants first underwent a period of ATI. After rebound, they received bNabs during low level viraemia followed by a 6-month washout period of suppressive ART. Subsequently, participants underwent a second period of ATI. Prior to commencing the second bNAb dose substudy, four participants received one bNAb dose prior to ART followed by a second period of ATI. Remarkably, all four participants rebounded to a lower viral set point with fluctuating viral loads during the second stage of ATI. The key question is whether this spontaneous maintenance of a lower viral set point is associated with any immunological changes during ATI, and whether this may be conferring a degree of partial viral control. While complete viral suppression was not achieved in three of the four participants, understanding how HIV-specific T cell immunity could be impacting this reduction in viraemia is important to determine factors affecting viral control.

5.3.1 Methods

To answer this question, these three Arm B participants in question were analysed for HIV-specific T cell functionality using the same assays previously described in Chapter 4. All four participants received ART regimens in accordance with NHS and British HIV Association best practice guidelines. Demographics of the participants are shown in **Table 5.1**. All four participants were sensitive to 3BNC117-LS and 10-1074-LS at baseline and had a viral load of less than 20 copies/ml at the time of randomisation. In accordance with protocol for Arm B participants, all four participants received a placebo infusion (0.9% NaCl infusion, normal saline) followed by an analytical treatment interruption (ATI) two days later. Upon viral rebound,

all participants restarted ART followed by combination bNAb infusion. After 24 weeks of ART, participants underwent a 2nd period of ATI. Viral load dynamics were analysed at each sampled timepoint.

PARTICIPANT	GENDER	AGE (YRS)	RACE	HIV CLADE	HLA CLASS I ALLELES	TIME FROM HIV DX TO ART (DAYS)	YEARS ON ART BEFORE TI (YRS)	ART MEDICATIONS	CD4 COUNT (WEEK 0)
001-002	M	26	White other	B/CRF02	A2:01/11:01 B7:05/06/15:01 C3:03/15:05	26	8	Abacavir/ Lamivudine/ Dolutegravir	930
001-003	M	35	White Other	AG	A3:01/11:01 B35:03/55:01 C3:03/04:01	35	5	Tenofovir DF/ Emtricitabine/ Dolutegravir	1440
002-001	M	52	White English	B	A3:01/24:02 B7:02/7:02 C7:02/7:02	33	3	Lamivudine/ Dolutegravir	648
003-001	M	44	Black British	B	A33:01/68:01 B35:01/42:01 C7:01/17:01/02	18	4	TAF/ Emtricitabine/ Elvitegravir/ cobicistat	938

Table 5.1. Participant demographics for selected Arm B participants that spontaneously achieved a lower viral load set point during the 2nd ATI.

5.3.2 Results

Combination bNAbs (given during viraemia) are associated with spontaneous viral control during ATI and maintenance of a lower viral set point (in four Arm B participants)

First, we examined the viral load dynamics in these participants. All four Arm B participants spontaneously controlled viral load in the 2nd ATI with the maintenance of a lower viral set point. The viral load dynamics are shown in **Figures 5.1a-d**. Each participant will be described individually.

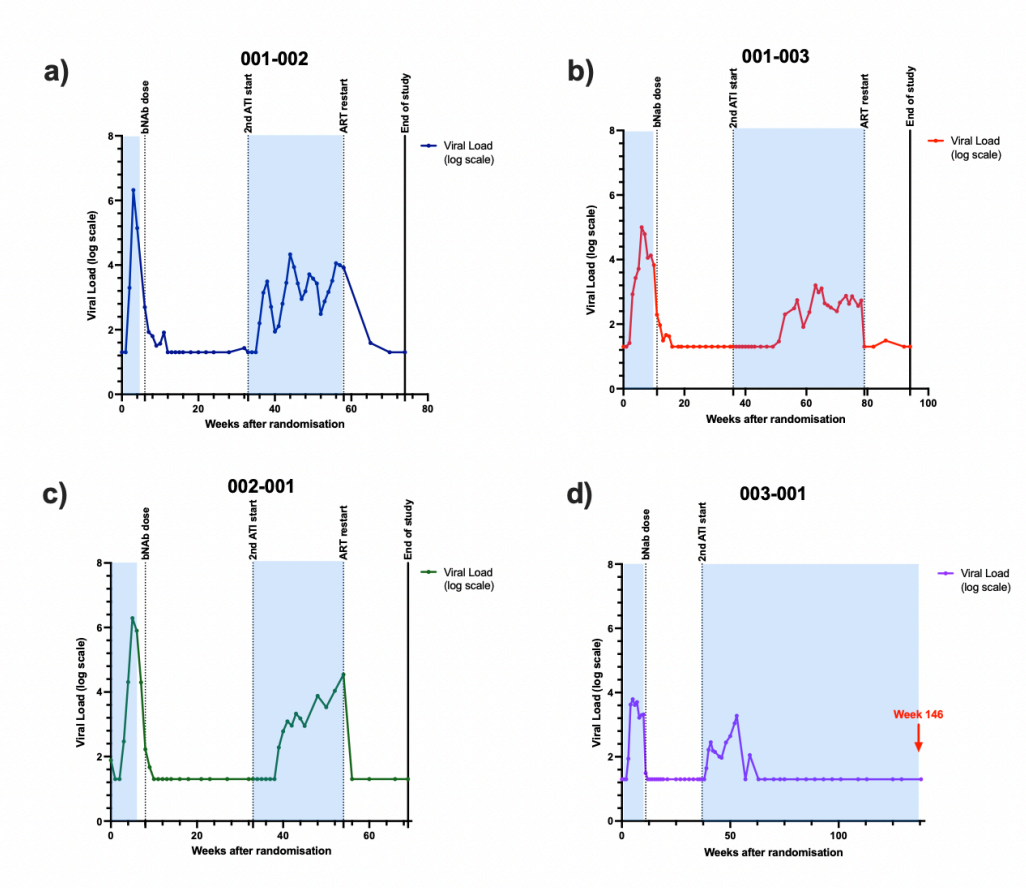
In participant 001-002, viral rebound occurred after a few weeks of treatment interruption (peak viral load 140000 copies/ml), and ART was restarted at Week 4 followed by open label bNAb dosing at Week 6. After 24 weeks of ART, the participant commenced the 2nd ATI and achieved a peak viral load of 21400 copies/ml, which was much lower than the initial peak viraemia in the 1st ATI. After remaining off ART for 25 weeks, the participant restarted ART at Week 58, achieved complete viral suppression below 20 copies/ml and proceeded until the end of study timepoint (**Figure 5.1a**).

Similarly, participant 001-003 experienced rapid viral rebound within 5 weeks of ATI (peak viral load 100000 copies/ml). ART was restarted at Week 10, followed by open label bNAb dosing at Week 11. During the 2nd ATI, the participant remained undetectable for another 15 weeks until viral rebound at a lower set point (peak viral load 1600 copies/ml). ART was restarted at Week 78; viral suppression below 20 copies/ml was achieved and the participant proceeded to the end of study timepoint (**Figure 5.1b**).

In participant 002-001, viral rebound occurred rapidly at Week 3 (peak viral load 1949844 copies/ml), followed by ART restart at Week 7 and open label bNAb dosing at Week 8. The participant started 2nd ATI at Week 33 and began rebounding after 6 weeks. A lower peak viral load was maintained compared to the 1st ATI (34674 copies/ml). ART was resumed at Week 54 and full viral suppression below 20 copies/ml was achieved. The participant was followed up until the end of study timepoint as per protocol (**Figure 5.1c**). However, it is important to note that participant 002-001 restarted ART prior to reaching a true "viral load set point", and thus we cannot comment whether a lower set point was actually maintained.

Participant 003-001 demonstrated remarkable viral load dynamics. Rebound occurred within the first 10 weeks of the 1st ATI (peak viral load 2094 copies/ml), followed by open label bNAb dosing at Week 11. After ART interruption during the 2nd ATI at Week 37, the participant experienced rapid viral rebound

(peak viral load 1893 copies/ml), which was lower than the initial rebound during the first ATI; however, viral load was then spontaneously suppressed to less than 20 copies/ml one week after peak viral rebound. Apart from one viral blip of 113 copies/ml after re-suppression, viral load remained at undetectable levels for more than one year. Currently, the participant remains undetectable (**Figure 5.1d)**.



Figures 5.1a-d. Viral load dynamics of Arm B participants a) 001-002, b) 001-003, c) 002-001 and d) 003-001 from the RIO clinical trial. Note: 003-001 has not yet reached the end of study timepoint and has had an undetectable viral load since viral rebound during the 2nd ATI (in the context of undetectable ART drug levels).

Lowered viral set points during the 2nd ATI is associated with enhanced HIV-specific T cell immunity in three out of four Arm B participants

Given that we observed lower viral load set points in four Arm B participants during the 2nd ATI post-bNabs, we next determined whether this was associated with increases in HIV-specific T cell immunity. For the purposes of this chapter, participant 003-001 will be discussed in a later section. To investigate this, T-cell

functional assays were performed to compare changes in HIV-specific T cell dynamics during the 1st and 2nd ATI, including HIV-specific T cell proliferation, activation, interferon-gamma secretion and phenotyping of bulk, unstimulated PBMCs from each participant (methods are described previously in Chapter 2).

First, HIV-specific interferon-gamma secretion was measured using a matrix-based screening ELISpot assay with overlapping HIV-1 consensus B peptides was used to "screen" for dominant responses across Gag, Pol, Env and Nef. Based on the positive responses seen at screening, a subsequent "confirmatory" ELISpot assay was performed using single HIV-1 consensus B overlapping peptides to "confirm" the positive responses (see Methods in Chapter 2). Out of the four participants screened, three participants showed enhanced dominant responses to Gag (and Nef in some cases) during the 2nd ATI after receiving bNabs. These enhanced T-cell responses were compared with the responses observed in HIV-specific T-cell proliferation and activation (Methods described previously). A summary of the T-cell responses from all assays are shown in **Table 5.2**.

Participant 001-002

In participant 001-002, high baseline responses to Pol peptides FNLPPVVAKEIVASC and MASDFNLPPVVAKEI were recorded at baseline (280 and 225 spots/million respectively). After rebound and bNAb dosing in the 2nd ATI, responses to both Pol peptides had declined (92.5 and 187.5 spots/million) while new responses to EVGFPVRPQVPLRPM increased (57.5 spots/million). However, none of the responses were enhanced significantly during the second ATI and responses towards Pol and Nef peptides remained lower at the end of study timepoint compared to baseline. In terms of HIV-specific proliferation, strong Gag CD4 and CD8 proliferative responses were detected prior to the 2nd ATI, however these responses decreased during viral rebound. After ART resumption, Gag-specific responses increased to

levels that were similar to baseline responses. In the AIM assay, Gag-specific CD4 T cell activation increased while on suppressive ART; on the other hand, Gag-specific CD8 responses were negligible. During the 2nd ATI, this response was maintained during viraemia and after ART resumption.

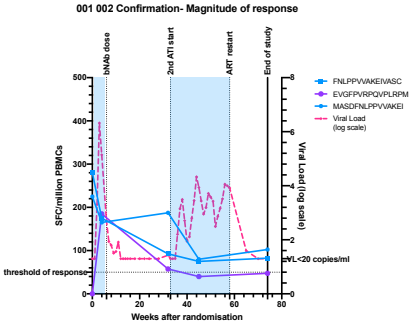
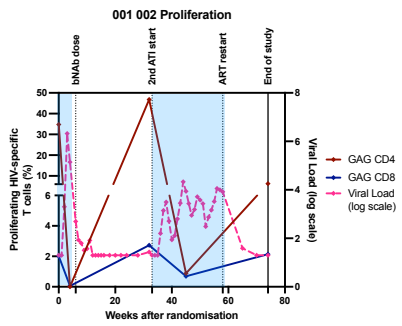
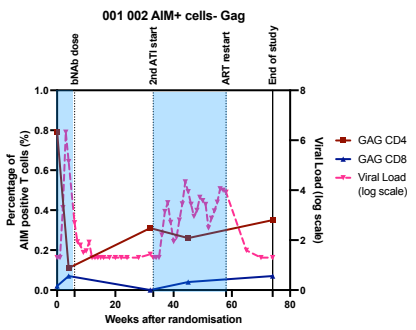
Participant 001-003

In participant 001-003, responses were made to two Gag peptides (EKIRLRPGGKKKYKL and EMMTACQGVGGPGHK) and one Env (SWSNKSLDEIWDNMT) peptide. Responses to both Gag overlapping peptides increased during viral rebound (535 -> 957.5 spots/million and 10 -> 127.5 spots/million respectively). During the 2nd ATI, responses to both EKIRLRPGGKKKYKL and EMMTACQGVGGPGHK increased during viraemia and remained elevated up to the point of ART restart (1035 spots for EKIRLRPGGKKKYKL and 97.5 spots for EMMTACQGVGGPGHK respectively). In contrast, responses to Env peptide SWSNKSLDEIWDNMT declined between the 2nd ATI and ART resumption (277.5 -> 62.5 spots/million). At the end of study timepoint, responses to Gag peptides declined to similar levels seen at baseline (535 -> 405 spots/million for EKIRLRPGGKKKYKL and 10 -> 35 spots/million for EMMTACQGVGGPGHK), while responses to Env peptide SWSNKSLDEIWDNMT were lower than seen at baseline (500 -> 5 spots/million).

With regards to HIV-specific proliferation, there were observed spikes in both Gag CD4 and CD8 responses during the 2nd ATI, although Gag CD8 responses dominated. After ART resumption, Gag-specific CD8 proliferation remained at higher levels compared to baseline. For HIV-specific T cell activation, Gag CD4 and CD8 responses increased during viraemia in the 1st ATI. During the 2nd ATI post-bNAbs, there were further increases in Gag CD4 and CD8 AIM+ responses, and these were maintained at higher levels after ART resumption compared to baseline.

Participant 002-001

In participant 002-001, many single-peptide responses were observed. To simplify the analysis, only the dominant responses were examined (full list of responses are shown in **Table 5.3**). High baseline responses were recorded for Nef peptides PGPGIRYPLTFGWCF and IRYPLTFGWCFKLVP (5032.5 and 4227.5 spots/million respectively). During viral rebound, dominant responses to Nef and Gag increased and subsequently declined during ART-induced viral suppression. Upon viral rebound in the second ATI, dominant responses to Gag and Nef increased and remained significantly higher than baseline (7275 for PGPGIRYPLTFGWCF and 6560 spots/million for IRYPLTFGWCFKLVP, and 4200 and 5025 spots/million for EKIRLRPGGKKKYKL and GKKKYKLKHIVWASR respectively). Similarly, both Gag and Nef-specific T-cell proliferation increased during the 2nd ATI. For Gag, this increase was seen more in the CD8-specific response and remained elevated compared to baseline. For Nef, HIV-specific CD8 responses increased steadily and was over 3-fold higher after ART resumption than at baseline. Additionally, we also observed clear increases in both Gag and Nef-specific CD8 AIM+ cells at viraemic timepoints in both the first and second ATI.

Participant	ELISpot (single peptides)	Proliferation assay	AIM assay
001-002			

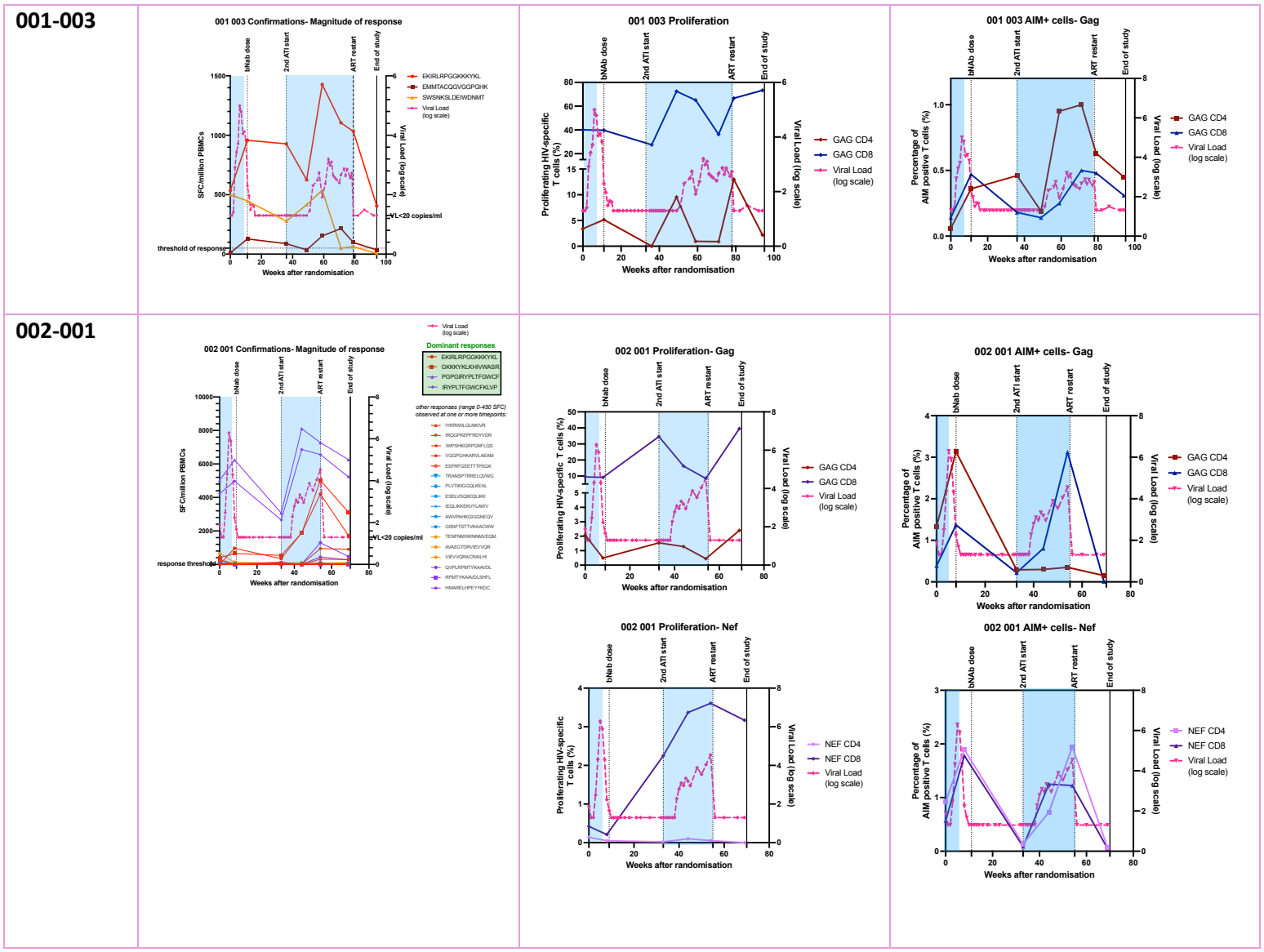


Table 5.2. HIV-specific T-cell responses seen in A) ELISpot, B) proliferation and C) AIM assays for participants 001-002, 001-003 and 002-001 in Arm B. Coloured lines represent responses to either HIV proteins or single overlapping peptides (OLPs); viral loads are plotted as dotted pink lines. Only dominant responses seen in A) ELISpot single peptide assays are shown for the proliferation and AIM assay.

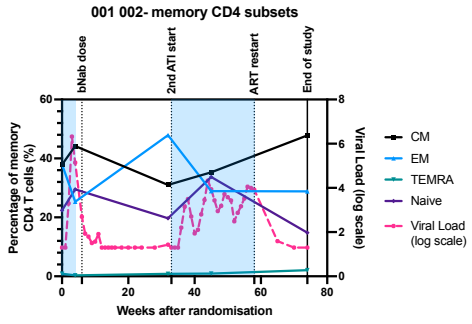
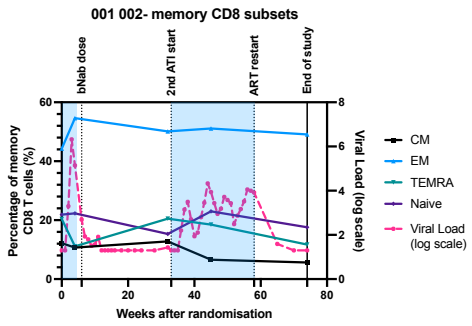
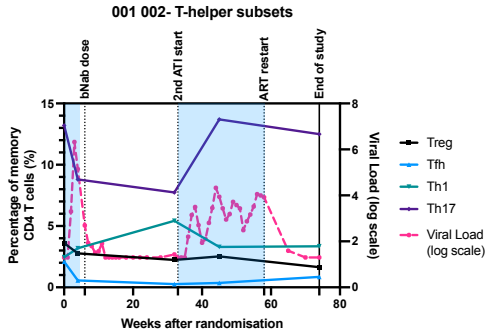
Participant	HIV protein	OLP	Response at viral rebound (1st ATI) (spots/million)	Response at viral rebound (2nd ATI) (spots/million)
001-002	Pol	MASDFNLPPVVAKEI	165	80
	Pol	FNLPPVVAKEIVASC	175	75
	Nef	EVGFPVRPQVPLRPM	185	40
001-003	Gag	*EKIRLRPGGKKKYKL	957.5	1032.5

	Gag	*EMMTACQGVGGPGHK	127.5	97.5
	Env	SWSNKSLDEIWDNMT	447.5	62.5
002-001	Gag	*EKIRLRPGGKKKYKL	967.5	4200
	Gag	*GKKKYKLKHIVWASR	652.5	5025
	Gag	IYKRWIILGLNKIVR	37.5	45
	Gag	IRQGPKEPFRDYVDR	7.5	60
	Gag	VGGPGHKARVLAEAM	0	950
	Gag	IWPSHKGRPGNFLQS	7.5	0
	Gag	ESFRFGEETTTPSQK	7.5	320
	Pol	TRANSPTRRELQVWG	7.5	55
	Pol	PLVTIKIGGQLKEAL	7.5	10
	Pol	ESELVSQIIEQLIKK	0	25
	Pol	IEQLIKKEKVYLAWV	0	15
	Pol	AWVPAHKGIGGNEQV	7.5	30
	Pol	GSNFTSTTVKAACWW	22.5	55
	Env	TENFNMWKNNMVEQM	137.5	95
	Env	AVAEGTDRVIEVVQR	0	0
	Env	VIEVVQRACRAILHI	77.5	20
	Nef	QVPLRPMTYKAAVDL	0	1295
	Nef	RPMTYKAAVDLSHFL	12.5	15
	Nef	*PGPGIRYPLTFGWCF	6242.5	7275
	Nef	*IRYPLTFGWCFKLVP	4977.5	6560
	Nef	HMARELHPEYYKDC	0	450
003-001	Gag	*ACQGVGGPGHKARVL	222.5	400
	Gag	*VGGPGHKARVLAEAM	297.5	295
	Nef	*IYSQKRQDILDLWVY	167.5	95
	Nef	*KRQDILDLWVYHTQG	147.5	30

Table 5.3. HIV-specific T cell responses to all single overlapping peptides (OLPs) in the ELISpot "confirmatory" assay for Arm B participants 001-002, 001-003, 002-001 and 003-001 during the first (pre-bNabs) and second (post-bNAbs) ATI. Peptides that are highlighted in red represent dominant peptide responses; these were further examined in a downstream ELISpot assay using HLA-restricted peptides.

Spontaneous viral control during the 2nd ATI is associated with changes in T-cell phenotype in Arm B participants

Next, we wanted to determine if spontaneous viral suppression observed during the 2nd ATI was associated with phenotypic changes in T-cell subsets in Arm B participants. To achieve this, immune phenotyping of PBMCs were performed for Arm B participants described previously. First, we examined the memory T-cell and T-helper subsets in each participant. A summary of each participant is shown in **Table 5.4**.

Participant	Memory T-cell subsets	T-helper cell subsets
001-002	 	

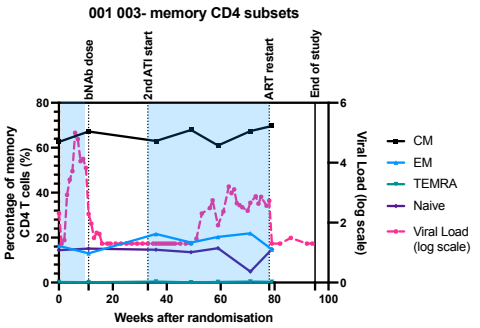
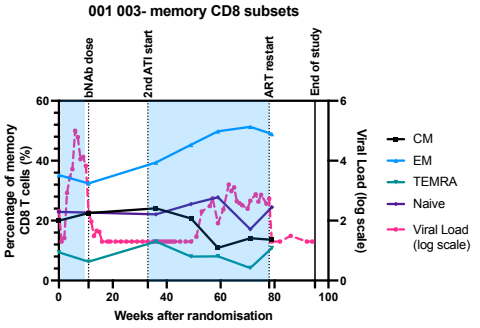
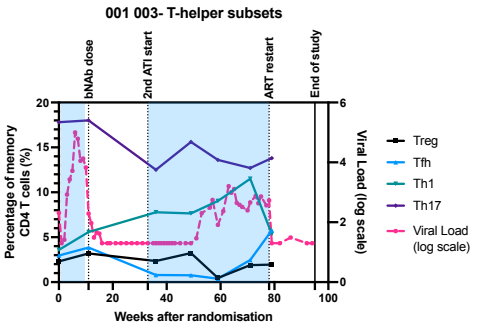
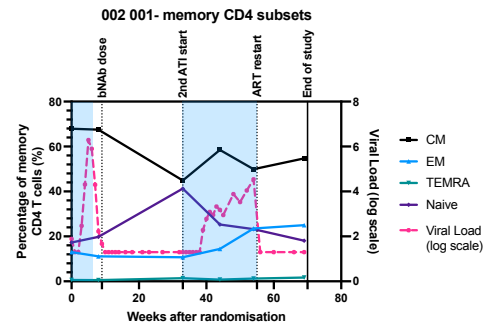
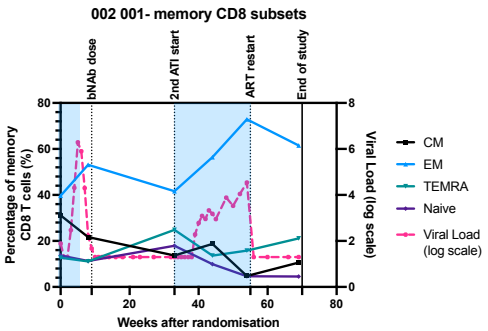
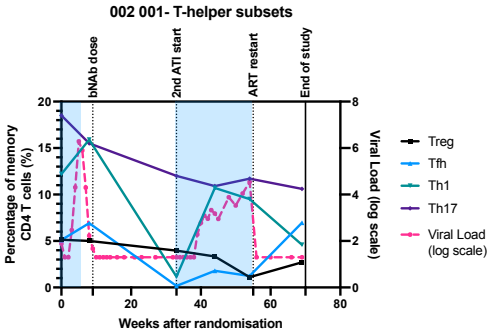
001-003	 	
002-001	 	

Table 5.4. Summary table of the memory T-cell subsets and T-helper subsets in Arm B participants 001-002, 001-003 and 002-001 respectively.

In participant 001-002, the memory CD8 subsets remained fairly static throughout the 1st and 2nd ATI. For the memory CD4 subsets, there was a slight increase in the effector memory T-cell population before the 2nd ATI; however, this decreased to levels that were similar to baseline after the 2nd ATI. Similarly, there were no dynamic changes in T-helper subsets between timepoints; there was a slight increase in Th17 cells during the 2nd ATI, however levels returned to baseline after the ATI period.

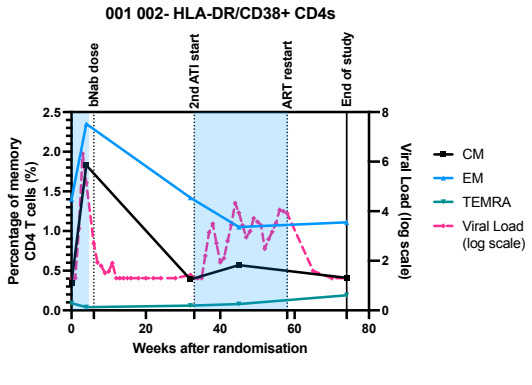
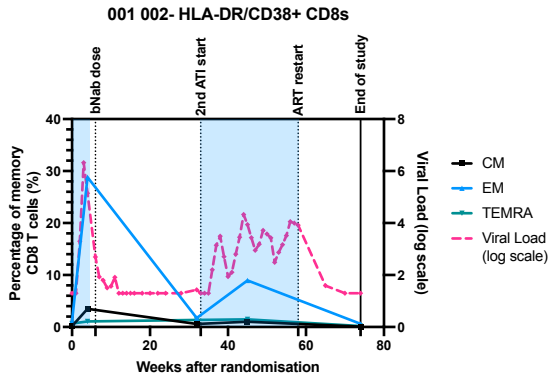
In participant 001-003, memory CD4 subsets remained similar to baseline levels throughout the 1st and 2nd ATI; however, in the memory CD8 subsets there was a small observed increase in effector memory T-cells during the 2nd ATI, and this remained higher than baseline levels at the point of ART resumption. Interestingly, this was accompanied by a steady increase in Th1 cells from baseline to the 2nd ATI as well; notably, levels dropped at the point of ART resumption but remained higher than baseline.

For participant 002-001, there were no remarkable changes in memory CD4 subsets over time. However, analysis of memory CD8 subsets showed increases in effector memory T-cells during the 1st and 2nd ATI. This increase was enhanced during the 2nd ATI compared to the 1st ATI. With regards to T-helper subsets, there was a gradual decline in Th17 cells from baseline to the 2nd ATI. In addition, there were sharp increases in Th1 cell populations during viraemic timepoints in the 1st and 2nd ATIs.

Next, we examined the activation of memory T cell subsets over time by gating on double-positive HLA-DR/CD38+ memory T cells. These are summarised in **Table 5.5**. In participant 001-002, there was an increase in activated CD4 effector memory T cells during the first ATI; this decreased during suppressive ART and did not increase during the 2nd ATI. In the memory CD8 population, there was an increase in HLA-DR/CD38+ effector memory T cells in both the 1st and 2nd ATI, although the increase was smaller in the 2nd ATI. Interestingly, in participant 001-003, we observed large increases in the activated effector memory

CD4 and CD8 T-cell population in both periods of ATI. Notably, the increase was larger during the 2nd ATI compared to the 1st ATI and these higher levels were maintained at the point of ART resumption compared to baseline.

In participant 002-001, we also observe similar increases in the population of activated effector memory T cells during viraemia in the 1st and 2nd ATIs. As expected, levels of activated effector memory T cells decreased after ART resumption and viral re-suppression. For participant 003-001, we observed fluctuating levels of activated central memory T cells, but more remarkably, there was a steady increase in activated effector memory T cells from baseline to the point of spontaneous viral suppression in the 2nd ATI. In the activated CD8 memory T cells, there was a large increase in activated effector memory T-cells during viral rebound in the 1st ATI, however this decreased during viral suppression on ART. During the 2nd ATI, there was a small increase in this population but not to the extent observed during the 1st ATI.

Participant	HLA-DR/CD38+ memory CD4 T cells	HLA-DR/CD38+ memory CD8 T cells
001-002		

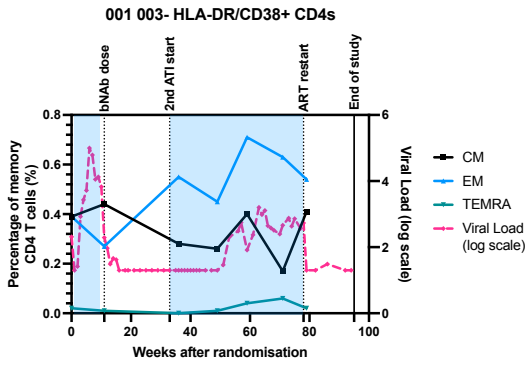
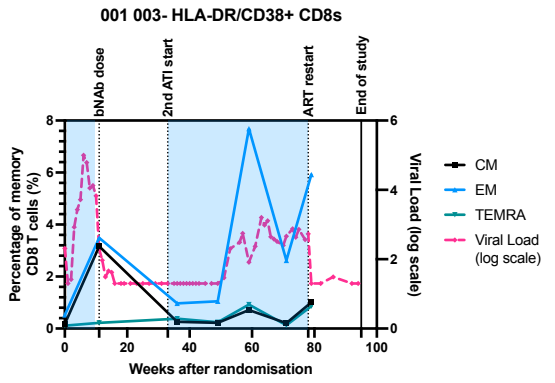
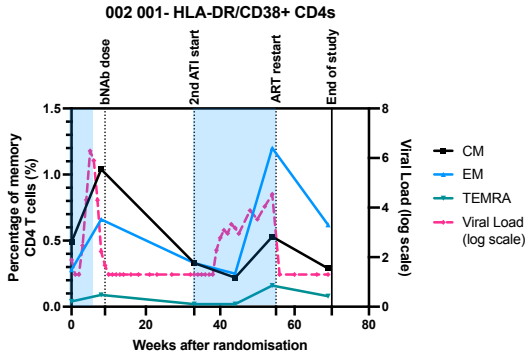
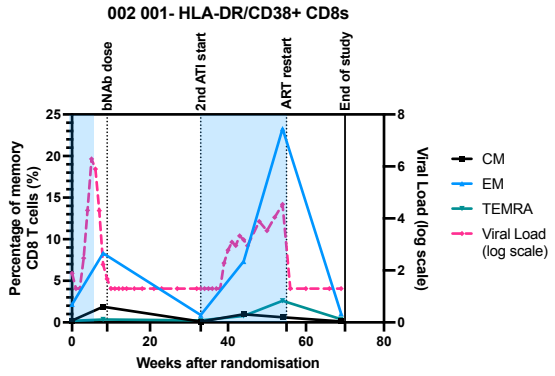
001-003		
002-001		

Table 5.5. Summary table showing the percentages of double positive HLA-DR/CD38+ CD4 and CD8 memory T cells in participants 001-002, 001-003 and 002-001 respectively.

Discussion

Although we only looked at a few participants, the findings presented in this section suggest that the association between spontaneous viral control and enhanced HIV-specific T cell immunity is heterogeneous; 2 of our 3 participants showed enhanced Gag-specific T cell immunity (via CD8 proliferation, AIM+ cells, and peptide-specific responses) during the period of control, however the same responses in participant 001-002 were not increased compared to baseline. Moreover, Pol and Nef-specific responses (as observed by ELISpot) did not increase in participant 001-002 during the period of control. Similarly, we observed increases in the CD8+ and HLA-DR/CD38+ CD8+ effector memory

compartment in 001-003 and 002-001, but not for 001-002. Due to the small sample size, more participants are needed to determine whether similar trends in response are observed in cases of spontaneous viral control. Since our phenotyping data was limited to unstimulated PBMCs, it would also be interesting to determine these increases are observed in the HIV-specific memory T cell fraction. To further characterise the enhanced HIV-specific T cell immunity and confirm the responses observed in 001-003 and 002-001, we subsequently deconvoluted the "optimal" peptide responses for these participants in the next section.

5.4 Hypothesis 2: Epitopic responses that are associated with spontaneous viral control are influencing viral evolution over time

In three out of four Arm B participants, viral rebound in the 2nd ATI, but at lower levels than observed in the 1st ATI. Additionally, viral load was spontaneously maintained at a lower set point and showed minor fluctuations between measurements. In addition to achieving partial viral control in the absence of ART, three Arm B participants produced dominant interferon-gamma responses to Gag (and sometimes Nef) overlapping peptides in ELISpot assays, and these were enhanced during the 2nd ATI compared to the 1st ATI. However, the ELISpot assays used HIV consensus B overlapping peptides and therefore may not be reflective of the mutational changes occurring in circulating plasma virus at any given timepoint.

Understanding the underlying changes in plasma HIV sequences at different timepoints in conjunction with the peptide-specific responses would help determine whether we are testing responses to the correct overlapping peptides. We may also infer how selective immune pressure is influencing viral evolution. Existing literature shows that targeting epitopes in Gag are associated with viral control because the Gag protein is more conserved and mutationally constrained, which means that mutations in

this region typically result in a significant loss of viral fitness. We may expect that the dominant responses observed (especially to Gag) may be associated with high fitness cost mutations that could explain the enhanced viral control observed during the 2nd ATI.

5.4.1 Methods (Part 1): Examining viral evolution

To compare the epitope-specific responses to their respective plasma viral sequences in each participant, we first determined the sequences of circulating plasma viruses at timepoints during the 1st and 2nd ATIs. Full methods are described previously in Chapter 2. Briefly, RNA was extracted from plasma samples of the four selected Arm B participants. Following RNA extraction, nested PCRs were performed to amplify Gag (and Nef) regions in the HIV-1 consensus B genome. Then, the purified PCR product was taken forward for library prep followed by next generation sequencing (NGS). Sequencing reads were then aligned with the HIV-1 consensus B genome to determine the relevant amino acid sequences. For some timepoints where plasma virus could not be amplified using nested PCRs, near full length sequences were retrieved from the trial collaborators at the Rockefeller University (New York, USA). Details of the plasma viral sequences at each timepoint are shown in **Table 5.6**.

5.4.2 Results (Part 1)

Immune escape is observed in two Arm B participants that coincides with enhanced Gag (and Nef)-specific T cell immunity during ATI

	001-003		002-001		003-001	
	GAG Sequence 1	GAG Sequence 2	GAG	NEF	GAG	NEF
UJ	EKIRLRPGGKKKYKL	EMMTACQGVGGPGHK	EKIRLRPGG KKKYKLKHIVWASR		ACQGVGGP**A**HKARVLAEAM	**V**YSQKRQDILDLWVYHTQG
BM	EKIRLRPGGKKKYKL	EMMTACQGVGGPGHK				
ZJ						
TK	EKIRLRPGGKKKYKL	EMMTACQGVGGPGHK	EKIRLRPGG KK**R**YKLKHI**M**WASR		ACQGVGGP**A**HKARVLAEAM	**V**YSQKRQDILDLWVYHTQG
AY			EKIRLRPGG KK**S**YKLKHI**M**WASR	PGPG**V**R**F**PL TFGWCFKLVP		
EZ	EKIRLRPGGKKKYKL	EMMTACQGVGGPGHK				
CJ	EKIRLRPGGKKKYKL	EMMTACQGVGGPGHK				

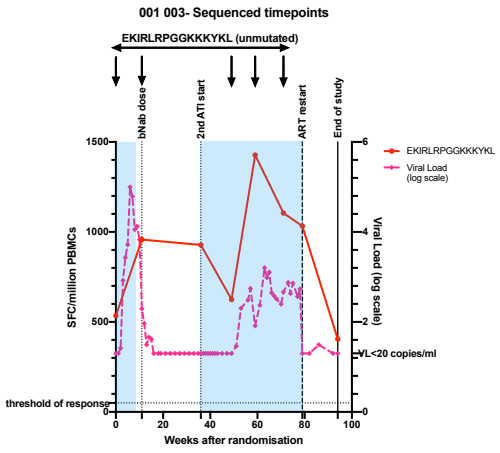
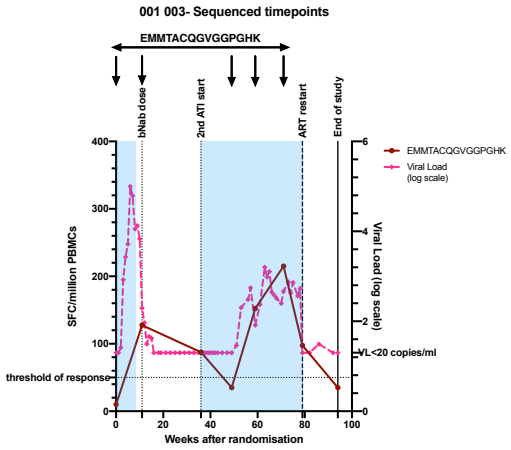
Table 5.6. HIV-1 consensus B amino acid sequences for RIO participants 001-003, 002-001 and 003-001. Timepoints are shown as two-letter codes, which correspond to different timepoints during treatment interruption. Only sequences that could be retrieved from next-generation sequencing are shown. Sequences were retrieved from a combination of in-house sequences and near-full length sequencing from Rockefeller university (28,29). Red bolded and underlined amino acids represent point mutations in sequences. Dark red sequences indicate sequences that are intact but defective/non-functional.

Key: UJ = baseline
BM= stage 1 viral rebound
ZJ=stage 2 start, on ART
TK= stage 2 viraemic
AY=stage 2 ART restart
EZ, CJ =stage 2 ATI

First, we examined whether participants showed evidence of mutations in plasma viral sequences over time to determine if the epitope-specific response observed were driving immune escape. A summary of the changes in plasma virus sequences is shown in **Table 5.7**. Responses will be described individually.

In participant 001-003, responses were made to Gag overlapping peptides EKIRLRPGGKKKYKL and EMMTACQGVGGPGHK. These regions were examined at multiple timepoints, including baseline and viral rebound during the 1st and 2nd ATIs. At all timepoints, no point mutations were observed in EKIRLRPGGKKKYKL and EMMTACQGVGGPGHK from baseline up to the 2nd ATI.

For participant 002-001, dominant responses were made towards EKIRLRPGGKKKYKLKHIVWASR in Gag, and to PGPGIRYPLTFGWCF in Nef. Both Gag and Nef sequences in this region were examined for mutations. In Gag, no mutations in the EKIRLRPGGKKKYKLKHIVWASR sequence were observed at baseline. However, at Week 48 during 2nd ATI where detectable viraemia was present, two point mutations were observed in the Gag sequence; at position 29, there was a mutation from lysine (K) to arginine (R), as well as another point mutation at position 37 from valine (V) to methionine (M). At Week 55 (timepoint AY), additional point mutations at position 29 from arginine (R) to serine (S). However, no further mutations were observed at position 37. For Nef, no sequences could be amplified successfully at baseline; however, the sequence retrieved during the 2nd ATI during viral rebound showed evidence of point mutations at positions 133 from isoleucine (I) to valine (V) and at position 135 from tyrosine (Y) to phenylalanine (F).

001-003		

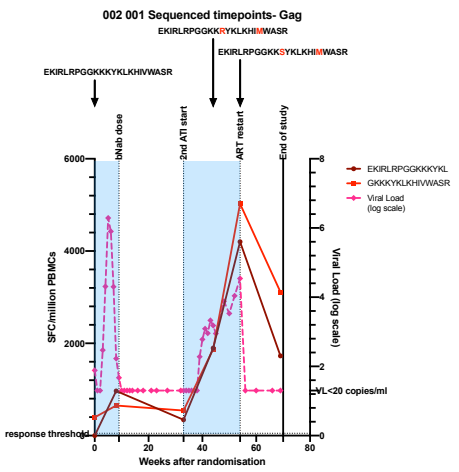
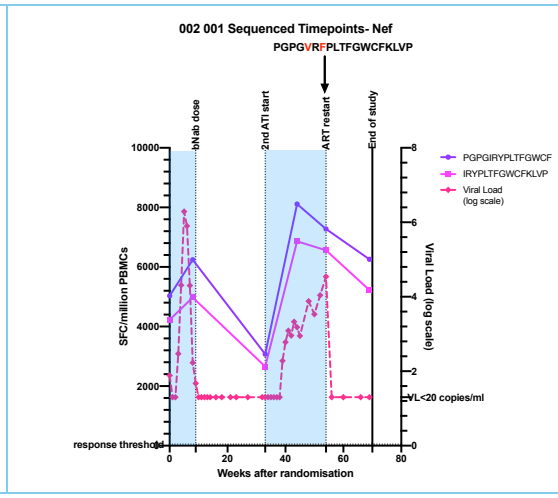
002-001		

Table 5.7. Plasma viral sequences for Arm B participants 001-003 and 002-001 at specific timepoints during the 1st and 2nd ATI (Gag and Nef for 002-001, and Gag for 001-003). Black downward arrows indicate timepoints where plasma viral sequences were amplified. Epitope-specific responses to Gag and Nef are shown in red and pink/purple lines respectively; viral loads are plotted in pink.

5.4.3 Methods (Part 2): Determining optimal epitopes

Based on available plasma virus sequences, we then sought to determine the "optimal" epitopes for each participant. The purpose of determining the optimal peptide responses was to provide a measure of functional avidity for each peptide-specific response; in other words, an indication of the binding affinity of a given peptide to the MHC-restricted peptide binding complex of a T cell. Another important reason for defining optimal epitopes is to examine viral evolution in participants across multiple ATIs and investigate whether these HIV-specific T cell responses are exerting a selective immune pressure that is driving viral escape.

To achieve this, a list of optimal peptides to test was generated using two parameters. Firstly, the HLA-class I alleles of each participant were typed using sequence specific amplification (SSP) (see Chapter 2). Then, these alleles were used to retrieve the most likely HLA-restricted peptide sequences for each participant, using a combination of existing evidence in the literature (HLA handbook), the Los Alamos

database epitope maps which provides HLA-restrictions of all documented peptides, and the Los Alamos database epitope location finder (ELF) tool which provides predicting binding affinities of different peptides according to their HLA-class I restriction and anchoring residues. This predicted binding affinity tool was based on the NetMHC 4.0 pan software which has been tested and validated, and is being updated regularly with new epitopes (30). Based on the above search criteria, the following list of predicted "optimal" peptides for each participant were generated (**Tables 5.8a-b**). The HLA-class I alleles for each participant are shown in **Table 5.8d**.

Table 5.8a

001-003 (n=10)	Sequence	Amino acid position	Most likely HLA-class I (A/B/C) allele restriction
Gag	KIRLRPGGK	18-27	A3:01
Gag	KIRLRPGGKK	18-28	B35
Gag	RLRPGGKKK	20-28	A3:01
Gag	RLRPGGKKKK	20-29	A3
Gag	RLRPGGKKKY	20-29	A3:01
Gag	IRLRPGGKKK	19-28	A3/A11
Gag	RLRPGGKK	20-27	A3:01 (predicted)
Gag	ACQGVGGPGHK	349-359	A11
Gag	CQGVGGPGHK	350-359	A11
Gag	GVGGPGHK	352-359	A11

Table 5.8b

002-001 (Gag = 11, Nef = 5)	Sequence	Amino acid position	Most likely HLA-class I allele restriction
Gag	KIRLRPGGK	18-26	A3:01
Gag	KIRLRPGGKK	18-27	B35
Gag	RLRPGGKK	20-27	A3:01 (predicted)
Gag	RLRPGGKKK	20-28	A3:01
Gag	RLRPGGKKKK	20-29	A3
Gag	RLRPGGKKKY	20-29	A3:01
Gag	IRLRPGGKKK	19-28	A3/A11
Gag	RPGGKKKYK	22-30	B7
Gag	KYKLKHIVW	29-37	A24:02
Gag (variant)	RLRPGGKK**R**Y	20-29	A3
Gag (variant)	RLRPGGKK**S**Y	20-29	unknown
Nef	RYPLTFGW	134-141	A24:02
Nef	YPLTFGWCF	135-143	B7
Nef	RYPLTFGWCF	134-143	A24
Nef (variant)	FPLTFGWCF	135-143	A24:02
Nef (variant)	RFPLTFGWCF	134-143	A24:02

Tables 5.8a-b. List of predicted optimal peptides (Gag for 001-003, and Gag and Nef for 002-001) for each Arm B participant. Each row contains the relevant HIV protein, predicted sequence (either consensus or variant), amino

acid position and the suspected HLA-class I allele restriction. Bolded red letters in sequences indicate point mutations in the participant plasma viral sequence; these sequences were included in the list.

RIO participant	**HLA-A**	**HLA-B**	**HLA-C**
001-003	03:01/11:01	35:03/55:01	03:03/04:01
002-001	03:01/24:02	07:02/07:02	07:02/07:02
003-001	33:01/68:01	35:01/42:01	07:01/17:01/02

Table 5.8d. HLA-class I alleles for Arm B participants that show spontaneous viral control during ATI.

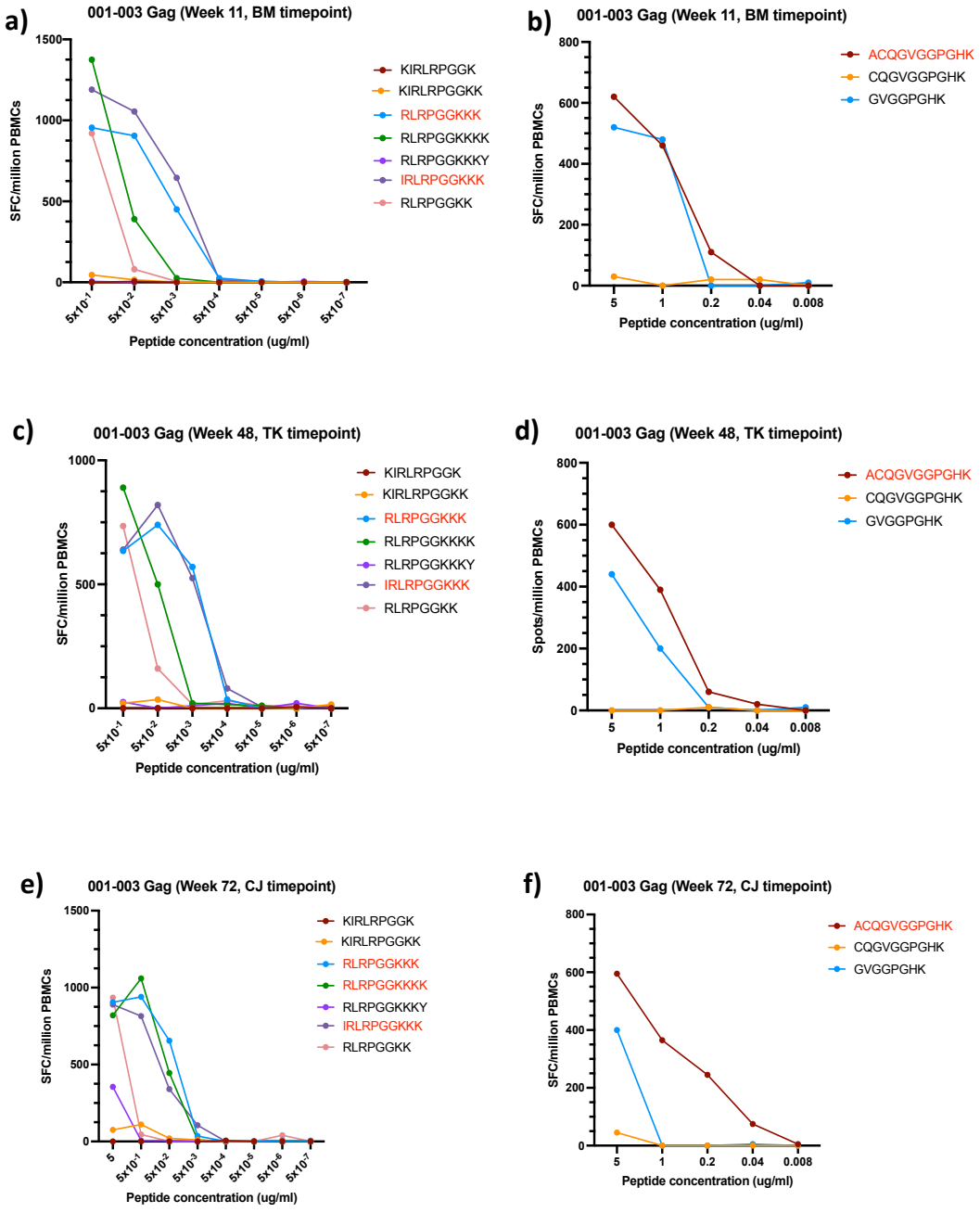
After a list of predicted optimal peptides were generated, ELISpot assays using these HLA-restricted peptides were performed. Detailed methods of ELISpots using HLA-restricted peptides are outlined in Chapter 2. Timepoints were selected during the first and second ATIs to examine any potential changes in viral evolution and immune escape. For a more accurate comparison of viral evolution and HIV-specific T cell responses, the timepoints that were used for viral sequencing were the same timepoints used for the ELISpot assays. Serial dilution curves were plotted and examined at each timepoint to determine which HLA-restricted peptide was producing the "optimal" response and showed less tapering at lower peptide concentrations.

5.4.4 Results

Dominant epitope-specific responses to Gag (and Nef) remain unchanged despite mutational changes in plasma virus over time

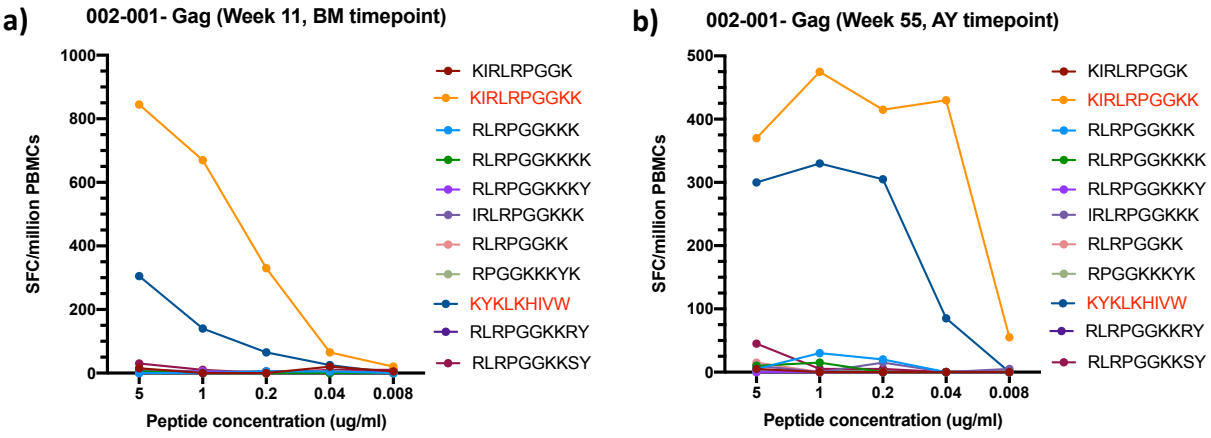
In participant 001-003, no evidence of mutational escape was observed at all analysed timepoints during the first and second ATIs for Gag regions EKIRLRPGGKKKYKL and EMMTACQGVGGPGHK. At viral rebound during the 1st ATI (Week 11, timepoint BM), the highest binding affinities were observed for RLRPGGKKK and IRLRPGGKKK (**Figures 5.2a-b**). During the second ATI at Week 48 (TK timepoint), the strongest

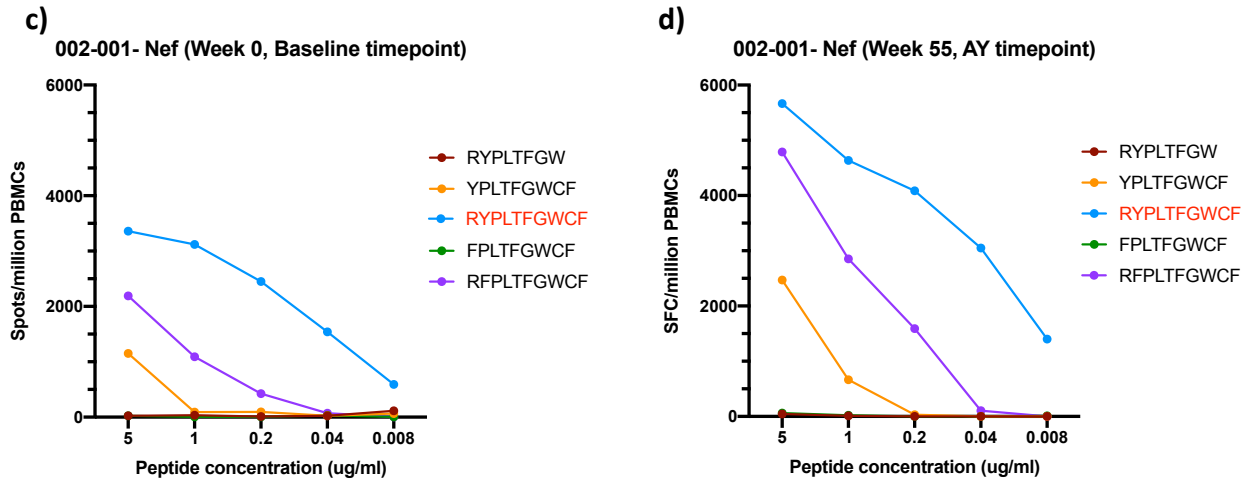
responses were observed towards the same HLA-restricted peptides RLRPGGKKK and IRLRPGGKKK as well. For the Gag region EMMTACQGVGGPGHK, ACQGVGGPGHK was the likely "optimal" at the first rebound (BM) timepoint, and this was also observed at the Week 48 (TK) timepoint during the second ATI (**Figures 5.2c-d**).



Figures 5.2a-f. Peptide-response curves using a list of predicted "optimal" peptides that were generated for participant 001-003. Coloured lines represent responses to different HLA-restricted peptides; the optimal peptide(s) are shown as red coloured text in the key on the right.

In participant 002-001, there were no mutations observed for the baseline Gag sequence EKIRLRPGGKKKYKLKHIVWASR; however, point mutations were subsequently observed at Weeks 44 (EKIRLRPGGKK**R**YKLKHI**M**WASR) and 55 (EKIRLRPGGKK**S**YKLKHI**M**WASR). With regards to HLA-restricted Gag responses at baseline, the strongest response was made towards KIRLRPGGKK and, to a lesser extent, KYKLKHIVW. Despite mutations in the Gag sequence, responses towards KIRLRPGGKK and KYKLKHIVW were preserved during the second ATI at the point of ART restart (Week 55) (**Figures 5.3a-b**). For Nef, no sequence could be retrieved at baseline, however there were point mutations observed at Week 55 (PGPG**V**R**F**PLTFGWCFKLVP). In terms of HLA-restricted Nef responses, the strongest responses were made to peptide RYPLTFGWCF at baseline, and this dominant response was subsequently maintained at Week 55 (as well as an increased response to RFPLTFGWCF) (**Figures 5.3c-d**).





Figures 5.3a-d. Peptide-response curves using a list of predicted "optimal" peptides that were generated for participant 002-001. Coloured lines represent responses to different HLA-restricted peptides; the optimal peptide(s) are shown as red coloured text in the key on the right.

Discussion

In this section, we observed that immune escape is fairly heterogeneous between the Arm B participants analysed. For 001-003, dominant responses to Gag epitopes remained highly similar between the 1st and 2nd ATIs with sequence conservation between the three analysed timepoints. It is uncertain whether these responses are responsible for the spontaneous viral control observed, although it is possible that mutations in those specific regions (EKIRLRPGGKKKYKL and EMMTACQGVGGPGHK) could be associated with high fitness costs which significantly hinders immune escape.

In comparison for 002-001, we observed immune escape in specific Gag regions as evidenced by multiple point mutations that occurred during the 2nd ATI (also see Discussion); concurrently, dominant epitope responses towards Gag and Nef epitopes at the same timepoints were identical to those observed in the 1st ATI. For 002-001, we were unable to obtain Nef sequences at baseline so cannot definitively comment on whether immune escape occurred during the 2nd ATI; however, Nef optimal epitope responses were

directed towards the unmutated epitope rather than the mutated variant, which could explain why this participant eventually lost control. Given existing evidence, it is more likely that the Gag mutations observed were responsible for the loss of control here. Our optimal epitope analysis also demonstrates the complexity of epitope binding and the significance of single amino acid changes in determining the binding affinity of epitopes. Despite analysing only two participants, the findings here support the hypothesis that in some cases, dominant responses to Gag are responsible for at least the initial period of spontaneous viral control. More investigation is required to characterise the potential impact of these mutations on viral fitness and subsequent viral control (also see Discussion).

5.5 Hypothesis 3: Selected Arm B participants that experience rapid viral rebound post-bNabs are associated with poorer HIV-specific T cell responses

In the previous section, we examined three participants that received bNabs during viraemia, remained on suppressive ART for 24 weeks, and subsequently exhibited a degree of spontaneous viral control during the 2nd ATI. We also demonstrated that this viral control was associated with enhanced HIV-specific T cell immunity, and dominant epitope responses to Gag (and Nef for 002-001) persisted despite mutational changes in the plasma virus of participants. However, it is uncertain whether similar changes in T-cell functionality and phenotype also occur in participants that show rapid viral rebound during treatment interruption. Thus, understanding the functional and phenotypic changes in participants that show poor control of viral replication off ART is equally important to decipher whether any factors are key determinants of viral control post-bNAbs and in the absence of ART.

5.5.1 Methods

To determine whether similar changes in T-cell functionality and phenotype were observed in participants that exhibited poor control, 4 individuals in Arm B that rebounded rapidly during the 2nd ATI were chosen as comparator participants. T-cell functional assays including ELISpot, proliferation and AIM were performed at multiple timepoints during ATI and on ART in a similar fashion as for Arm A. Due to the staggered design of the clinical trial, we were unable to obtain data for all timepoints for the proliferation and AIM assays, as well as for immune phenotyping. While we have included all available data, the limitations of our study prevented us from obtaining a more comprehensive dataset. Because of this, only complete datasets for the ELISpot assays are examined in this analysis. Future research will focus on addressing these gaps, including precise epitope mapping and a thorough analysis of viral evolution during ATI.

5.5.2 Results

Rapid viral rebound was observed during ATI in four Arm B participants that received bNAbs during viraemia and on suppressive ART

Participant demographics are shown in **Table 5.9a**. In terms of viral load dynamics, participant 001-017 rebounded within 3 weeks of the 1st ATI (peak viral load 21,900 copies/ml). The participant then received bNAbs during viraemia, went on ART, and received another bNAb dose at Week 30 (**Figure 5.4a**). Despite two doses of bNAbs, the participant rebounded within 3 weeks of the 2nd ATI with an even higher peak viral load than the 1st ATI (286,000 copies/ml). Complete viral re-suppression was achieved after ART resumption. In participant 001-019, viral rebound occurred after just 2 weeks in the 1st ATI (41,500 copies/ml) (**Figure 5.4b**). Similarly, participant 001-017 demonstrated rapid viral rebound 7 weeks after

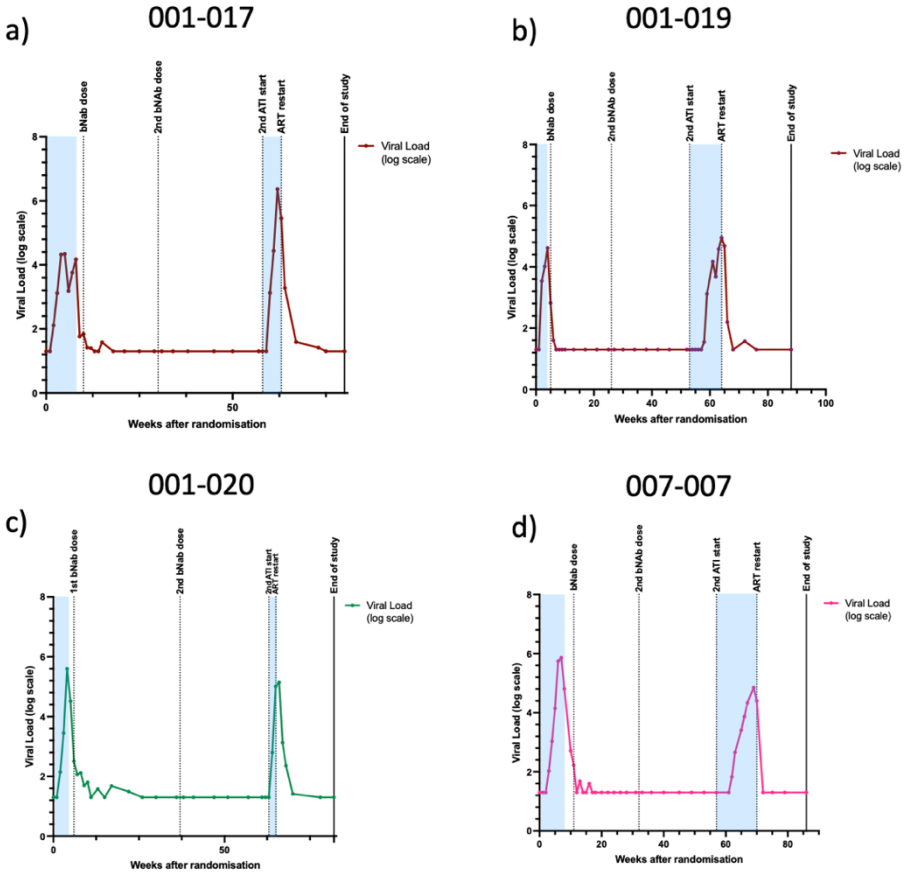
the start of the 2nd ATI (89,200 copies/ml), with a higher viral load at rebound compared to the previous ATI. In 001-020, rebound occurred 3 weeks after ATI (399,000 copies/ml). Despite receiving two bNAb doses plus ART, rebound occurred after 4 weeks in the 2nd ATI; however, peak viraemia was lower than previously observed (140,000 copies/ml) (**Figure 5.4c**). Finally, participant 007-007 also showed no difference in time to viral rebound between the 1st and 2nd ATIs (4 weeks in both cases) (**Figure 5.4d**). Peak viraemia was slightly lower in the 2nd ATI (71057 versus 728699 copies/ml). A summary of viral load dynamics is shown below in **Table 5.9b**.

PARTICIPANT	GENDER	AGE (YRS)	RACE	HIV CLADE	YEARS ON ART BEFORE TI (YRS)	ART MEDICATIONS	CD4 COUNT (WEEK 0)
001-017	M	32	Black	B	5	Abacavir/ Lamivudine/ Raltegravir	535
001-019	M	29	White Scottish	C	4	Tenofovir DF/ Emtricitabine/ Dolutegravir	927
001-020	M	46	White English	B	9	Tenofovir DF/ Emtricitabine/ Raltegravir	1084
007-007	M	49	White English	B	6	Rilpivirine/ Dolutegravir	554

Table 5.9a. Participant demographics for four Arm B participants that experienced rapid viral rebound.

	1st ATI		2nd ATI	
Participant	Time to rebound (Weeks)	Peak viral load (copies/ml)	Time to rebound (Weeks)	Peak viral load (copies/ml)
001-017	3	21,900	3	286,000
001-019	2	41,500	7	89,200
001-020	3	399,000	4	140,000
007-007	4	728,699	4	71,057

Table 5.9b. Time to rebound and peak viral load for Arm B participants 001-017, 001-019, 001-020 and 007-007 respectively during the 1st and 2nd ATIs.



Figures 5.4a-d. Viral load dynamics of Arm B participants a) 001-017, b) 001-019, c) 001-020 and d) 007-007. Coloured lines indicate viral loads, blue shaded areas indicate periods of treatment interruption.

Early aborted control during ATI post-bNAbs is associated with dominant responses towards Env and Nef in four Arm B participants

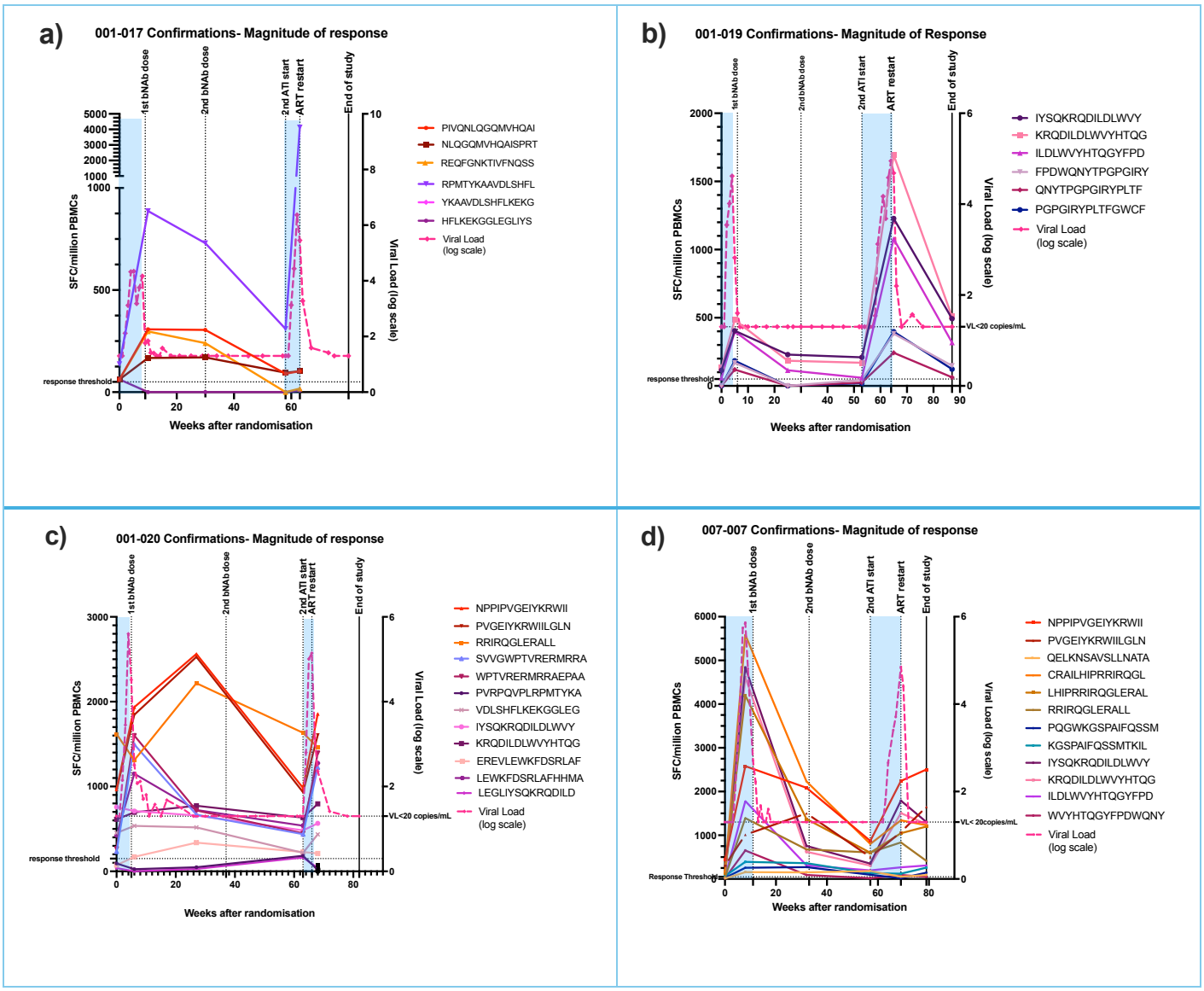
Next, we analysed the HIV-specific T cell responses for these four participants. We first investigated the interferon-gamma responses to single overlapping peptides and observed that all four participants made dominant responses to either Env or Nef peptides. We will examine these responses individually for each participant; responses to dominant peptides are summarised in **Table 5.10**.

For 001-017, the responses to Nef peptide RPMTYKAAVDLSHFL dominated at baseline (137.5 spots/million). This increased during viral rebound (887.5 spots/million) and fell after two bNAb infusions

and subsequent ART resumption. During the 2nd ATI, the response to RPMTYKAAVDLSHFL significantly increased (4132.5 spots/million). Due to issues with sample quality, the end of study timepoint could not be analysed. Smaller responses were also made to the Gag region PIVQNLQGQMVHQAISPRT (two adjacent Gag overlapping peptides) and Env peptide REQFGNKTIVFNQSS (**Figure 5.5a**). For 001-019, all dominant responses were mapped to the Nef regions IYSQKRQDILDLWVYHTQG and ILDLWVYHTQGYFPDWQNYTPGPIRYPLTFGWCF. Responses to these regions increased during the 1st ATI. During the 2nd ATI, responses increased significantly during viral rebound and fell after ART resumption (**Figure 5.5b**).

For participant 001-020, dominant responses were made to 2 overlapping Gag peptides (NPPIPVGEIYKRWII and PVGEIYKRWIILGLN), 1 Env peptide (RRIRQGLERALL) and 2 Nef peptides (SVVGWPTVRERMRRA and WPTVRERMRRAEPAA). During viral rebound in the 1st ATI, responses to these peptides increased and decreased post-bNAbs while on suppressive ART. During viral rebound in the 2nd ATI, responses to these peptides increased (as expected) (**Figure 5.5c**).

In participant 007-007, the strongest dominant responses were made towards Env peptides (CRAILHIPRRIRQGL and RRIRQGLERALL), and Nef peptides (IYSQKRQDILDLWVY and KRQDILDLWVYHTQG). During viral rebound in the 1st ATI, responses to CRAILHIPRRIRQGL and RRIRQGLERALL (5585 and 1390 spots/million respectively) increased, as well as for IYSQKRQDILDLWVY and KRQDILDLWVYHTQG (4840 and 4610 spots/million respectively). These responses decreased post-bNAbs and on suppressive ART; however, during viral rebound in the 2nd ATI responses to these Env and Nef peptides increased less than during the 1st ATI (**Figure 5.5d**).



Figures 5.5a-d. ELISpot assays using single overlapping peptides (OLPs) for Arm B participants a) 001-017, b) 001-019, c) 001-020 and d) 007-007. Each colour represents a response to a separate overlapping peptide. Blue shaded areas represent periods of treatment interruption. Viral loads are plotted in pink.

Participant	HIV protein	OLP	Response at viral rebound (1st ATI) (spots/million)	Response at viral rebound (2nd ATI) (spots/million)
001-017	Gag	PIVQNLQGQMVHQAI	307.5	102.5
	Gag	NLQGQMVHQAISPRT	167.5	102.5
	Env	REQFGNKTIVFNQSS	297.5	17.5
	Nef	RPMTYKAAVDLSHFL	887.5	4132.5
	Nef	YKAAVDLSHFLKEKG	932.5	3437.5
	Nef	HFLKEKGGLEGLIYS	0	7.5

001-019	**Nef**	IYSQKRQDILDLWVY	229	1227.5
	Nef	KRQDILDLWVYHTQG	184	1692.5
	Nef	ILDLWVYHTQGYFPD	114	1077.5
	Nef	FPDWQNYTPGPGIRY	0	382.5
	Nef	QNYTPGPGIRYPLTF	0	242.5
	Nef	PGPGIRYPLTFGWCF	0	397.5
001-020	**Gag**	NPPIPVGEIYKRWII	1935	1855
	Gag	PVGEIYKRWIILGLN	1845	1605
	Env	RRIRQGLERALL	1310	1460
	Nef	SVVGWPTVRERMRRA	1495	1225
	Nef	WPTVRERMRRAEPAA	1600	1395
	Nef	PVRPQVPLRPMTYKA	25	15
	Nef	VDLSHFLKEKGGLEG	535	435
	Nef	LEGLIYSQKRQDILD	0	40
	Nef	IYSQKRQDILDLWVY	710	565
	Nef	KRQDILDLWVYHTQG	695	795
	Nef	EREVLEWKFDSRLAF	170	210
	Nef	LEWKFDSRLAFHHMA	1150	1275
007-007	**Gag**	NPPIPVGEIYKRWII	2580	2240
	Gag	PVGEIYKRWIILGLN	1010	1070
	Env	QELKNSAVSLLNATA	155	50
	Env	CRAILHIPRRIRQGL	5585	1340
	Env	LHIPRRIRQGLERAL	4195	1045
	Env	RRIRQGLERALL	1390	835
	Pol	PQGWKGSPAIFQSSM	255	15
	Pol	KGSPAIFQSSMTKIL	390	120
	Nef	IYSQKRQDILDLWVY	4840	1790
	Nef	KRQDILDLWVYHTQG	4610	1505
	Nef	ILDLWVYHTQGYFPD	1780	265
	Nef	WVYHTQGYFPDWQNY	650	90

Table 5.10. HIV-specific T cell responses to all single overlapping peptides (OLPs) in the ELISpot "confirmatory" assay for Arm B participants 001-017, 001-019, 001-020 and 001-017 during the first (pre-bNabs) and second (post-bNAbs) ATI.

Although optimal epitopes were not defined for these participants, we examined the dominant responses made in each participant and used the amino acid sequences to predict the most likely epitope and HLA-class I restriction (see Chapter 2). The HLA class I alleles and predicted HLA-restricted peptides are shown

in **Tables 5.11 and 5.12** below. Notably, none of the participants had protective HLA class I alleles that been previously defined in the literature.

Participant	**HLA-A**	**HLA-B**	**HLA-C**
001-017	01:01/04N/33:03	13:02/53:01	04:01/08:04
001-019	02:01/03:01	35:01/47:01	06:02/06:02
001-020	01:01/04N	08:01/08:01	07:01/07:01
007-007	02:01/02:01	07:02/08:01	07:01/07:02

Table 5.11. HLA-class I alleles for Arm B participants 001-017, 001-019, 001-020 and 007-007.

Participant	**HIV protein**	**Amino acid sequence**	**Predicted optimal epitope**	**HLA class I allele restriction**
001-017	**Gag**	PIVQNLQGQMVHQAISPRT	MVHQAISPR	A*33:03
	Env	REQFGNKTIVFNQSS	EQFGNKTIV	B*13:02
	Nef	RPMTYKAAVDLSHFL	AAVDLSHFL	C*8:04
001-019	**Nef**	IYSQKRQDILDLWVYHTQGYFPD	TPGPGIRY	B*35:01
001-020	**Gag**	NPPIPVGEIYKRWIILGLN	EIYKRWIIL	B*8:01
	Env	RRIRQGLERALL	QGLERALL	B*8:01
	Nef	SVVGWPTVRERMRRAEPAA	WPTVRERM	B*8:01
	Nef	LEWKFDSRLAFHHMA	DSRLAFHHM	B*8:01
007-007	**Gag**	NPPIPVGEIYKRWII	PPIPVGEIY	B*35:01
	Env	CRAILHIPRRIRQGLERAL	IPRRIRQGL	B*7:02
	Nef	IYSQKRQDILDLWVYHTQG	RQDILDLWV	B*13:02

Table 5.12. Dominant regions in either Gag, Env or Nef that were targeted by Arm B participants. In cases where dominant responses to overlapping peptides were made, the whole region was used for epitope prediction. The predicted peptide binding affinity was used to select the most likely optimal epitope.

Discussion

In this section, we showed that certain Arm B participants that experienced rapid viral rebound during the 2nd ATI produced dominant responses mainly towards Env and Nef. Although 3 out of 4 participants made dominant Gag responses, we suspect that these targeted responses are in regions that are not particularly protective and are mostly restricted by disease-susceptible alleles. In comparison, the participants that achieved spontaneous control (section 1.5) produced dominant responses towards primarily Gag (and Nef in the case of 002-001), which may explain the divergence in viral control observed between the two groups of participants. However, the 4 participants described here were enriched in disease-susceptible alleles and the small sample size may confound the trends observed. Overall, we demonstrate that early aborted control is linked to a preponderance of Env and Nef-specific responses; additional analysis of T-cell functionality and phenotype for these participants are currently underway to determine whether these readouts differ from participants that exhibit spontaneous control.

5.6 Hypothesis 4: HIV-specific T cell immunity in one participant (003-001) is contributing to complete viral suppression post-bNAbs

In the previous Arm B participants that were examined in Hypotheses 1 and 2, spontaneous viral control occurred post-bNabs during the 2nd ATI, and the achievement of a lower viral set point coincided with enhanced T-cell immunity towards Gag (and Nef) epitopes in two out of three participants. In these cases, viral replication was only partially controlled, and all three participants eventually restarted ART. In comparison, one Arm B participant (003-001) demonstrated evidence of complete viral suppression after viral rebound in the 2nd ATI and has persistently undetectable viral loads to date with no evidence of viral rebound. The rare phenotype of this participant raises several important questions. Firstly, is the

spontaneous viral suppression observed in this participant associated with enhanced HIV-specific T cell immunity, and if so, what aspects of T-cell functionality could be contributing to this viral control? Secondly, how are these changes in T-cell immunity driving viral evolution (if any)? Finally, are there are phenotypic changes occurring during this period of spontaneous viral suppression? Answering these questions will be important for determining immune correlates and elucidating potential T-cell mediated mechanisms of viral control in the absence of ART. We hypothesise that this spontaneous viral suppression observed is being driven by enhanced Gag-specific T cell immunity, and that immune pressure on specific Gag epitopes is driving mutational changes in the plasma virus resulting in a high fitness cost and subsequent viral control.

5.6.1 Methods

To determine whether HIV-specific T cell immunity could be responsible for spontaneous viral suppression in this participant, we sampled timepoints during the 1st and 2nd ATIs, as well as timepoints beyond the point of viral suppression. HIV-specific T cell immunity was analysed using functional T-cell assays as previously described. Epitopic responses were also compared to plasma viral sequences at respective timepoints to analyse viral evolution during ATI.

5.6.2 Results

Spontaneous viral suppression (in 003-001) is associated with increases in HIV-specific T cell immunity over time

First, we investigated whether spontaneous viral suppression in participant 003-001 was associated with changes in T cell immunity via ELISpot, HIV-specific proliferation and also T-cell activation. Results are shown below (**Table 5.13**). In the single overlapping peptide ELISpot assays, 003-001 made responses to Gag overlapping peptides (ACQGVGGPGHKARVL and VGGPGHKARVLAEAM), and Nef peptides (IYSQKRQDILDLWVY and KRQDILDLWVYHTQG) that were not observed at baseline. During viral rebound in the 1st ATI, these responses increased significantly, presumably due to viraemia. During suppressive ART, both Gag and Nef responses decreased; however, in the 2nd ATI responses to Gag peptides increased significantly (12.5 -> 400 for ACQGVGGPGHKARVL and 22.5 -> 295 for VGGPGHKARVLAEAM) and coincided with the point where spontaneous viral suppression was achieved. In comparison, responses to Nef peptides IYSQKRQDILDLWVY and KRQDILDLWVYHTQG also increased during the 2nd ATI, but not to the extent observed during the 1st ATI.

Similarly, we observed increases in Gag-specific CD8 proliferation during the 1st and 2nd ATI; during the 2nd ATI, there was a spike in responses after spontaneous viral suppression followed by a gradual decline over time. There were also increases in Nef CD4 responses during viraemia, however these increased during viraemia and fell rapidly after viral suppression was achieved. With regards to HIV-specific T cell activation, there were increases in Gag and Nef-specific CD4 responses after viraemia in the 1st and 2nd ATIs, but these were not observed in the CD8 compartment.

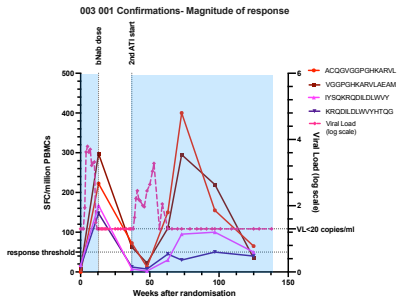
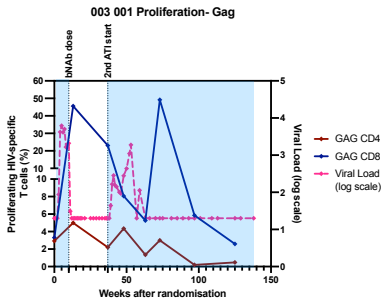
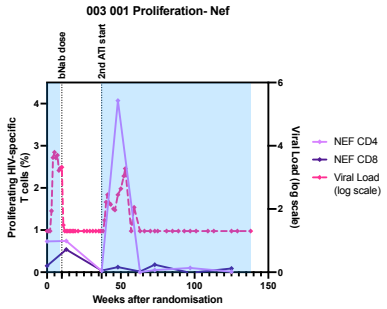
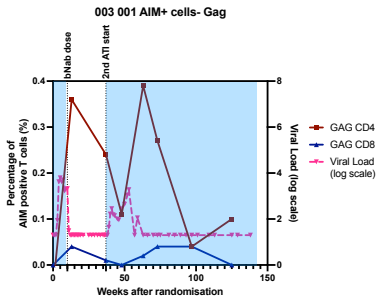
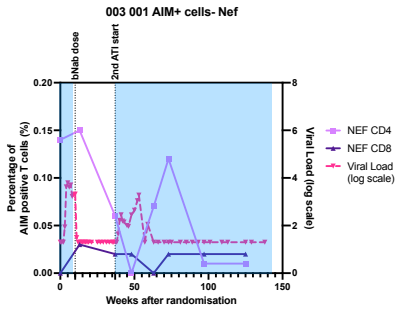
Participant	A) ELISpot (single peptides)	B) Proliferation assay	C) AIM assay
003-001		 	 

Table 5.13. HIV-specific T-cell responses seen in A) ELISpot, B) proliferation and C) AIM assays for participants 003-001. Coloured lines represent responses to either HIV proteins or single overlapping peptides (OLPs); viral loads are plotted as dotted pink lines. Only dominant responses (Gag and Nef) are shown for the proliferation and AIM assay.

Next, we examined whether there were any changes in T-cell phenotypes over time. Due to trial logistics, we only had limited datasets for timepoints during the 2nd ATI. Notably, we did not observe any changes in memory CD4 or CD8 populations during the 1st or 2nd ATIs. With regards to the T-helper subsets, we observed a decline in Th17 cells from baseline to the point of viral suppression in the 2nd ATI, as well as an increase in Tfh and Th1 subsets that increased significantly during the 2nd ATI. Then, we looked at the activated memory CD4 and CD8 T cells. In the CD4 compartment, we observed an increase in HLA-DR/CD38+ effector memory T cells during the 2nd ATI at the point of viral rebound. In the CD8 compartment, this increase was less prominent, and was significantly lower than the increase observed in the 1st ATI. A summary of T-cell phenotyping for 003-001 is shown below in **Table 5.14.**

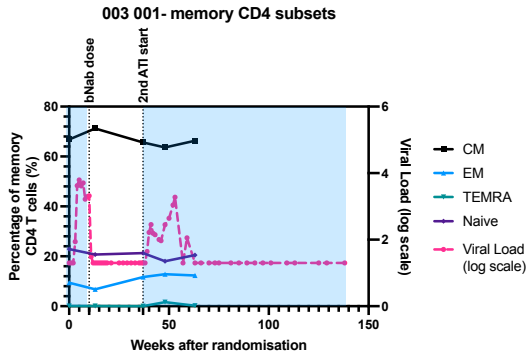
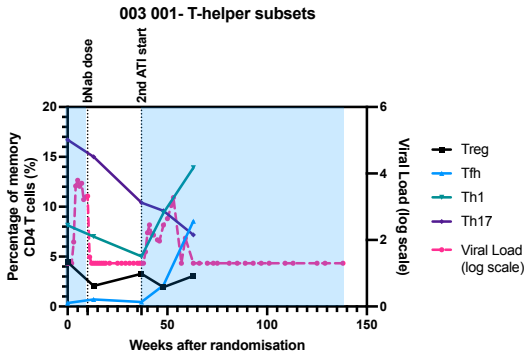
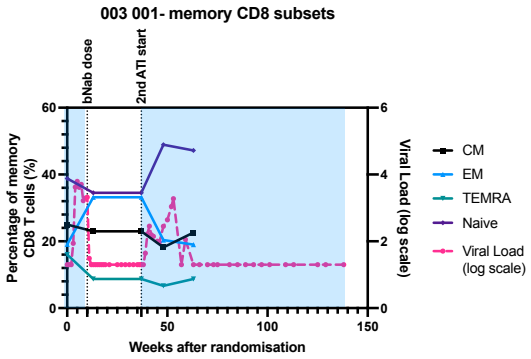
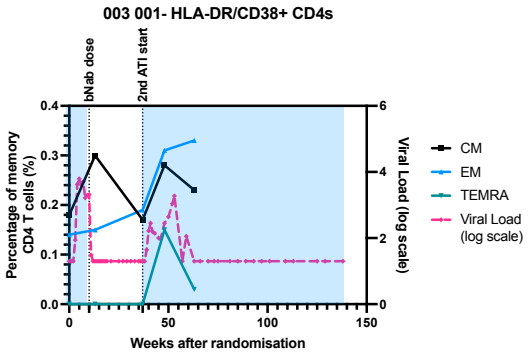
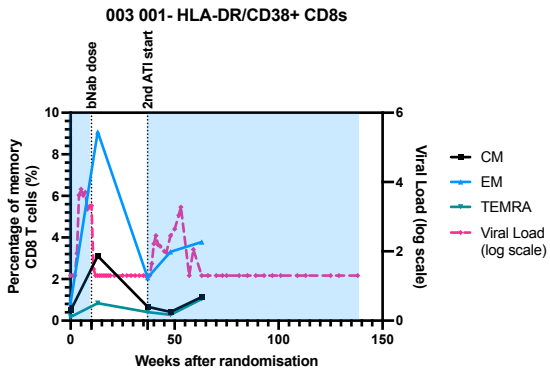
Participant	Memory CD4/CD8 subsets	T-helper subsets
003-001		
		
	HLA-DR/CD38+ double positive CD4s	HLA-DR/CD38+ double positive CD8s
		

Table 5.14. Summary table showing the memory CD4/CD8 populations, T-helper subsets and percentages of double positive HLA-DR/CD38+ CD4 and CD8 memory T cells in participant 003-001.

Optimal epitope responses demonstrate a gradual shift over time in response to plasma virus

Next, we examined the Gag and Nef plasma virus sequences of participant 003-001 in Gag and Nef at timepoints during the 1st and 2nd ATI. Regions were chosen based on the responses observed in the single overlapping peptide assays. We then proceeded to define the optimal epitopes for this participant using a list of predicted HLA-restricted peptides to test in a separate ELISpot assay (**Table 5.15**).

With regards to the plasma virus sequences of 003-001, there were point mutations observed in the baseline Gag sequence (ACQGVGGP**A**HKARVLAEAM) and the baseline Nef sequence. Notably, both the Gag and Nef sequences did not show evidence of further mutations during the 1st and 2nd ATIs up to the point of complete viral suppression.

When we examined the predicted HLA-restricted peptide responses, we observed strong responses towards both the original (GPGHKARVL) and variant (GP**A**HKARVL) peptide at the first rebound timepoint at Week 13. At Week 72, when the participant achieved spontaneous viral suppression during the 2nd ATI, a higher response was observed towards GP**A**HKARVL and this became more evident at lower peptide concentrations. With regards to Nef, point mutations were observed in the baseline sequence (**V**YSQKRQDILDLWVYHTQG) and remained unchanged at Week 72 after complete viral suppression in the 2nd ATI. In terms of peptide responses, only peptide KRQDILDLWVY made detectable responses at baseline and Week 72 (**Table 5.16**).

003-001 (Gag=2, Nef= 2)	Sequence	Amino acid position	Most likely HLA-class I allele restriction
Gag	**GPGHKARVL**	355-363	B42:01
Gag	**GPAHKARVL**	355-363	B42:01
Nef	**KRQDILDLWVY**	103-113	C7:01
Nef	**RQDILDLWVY**	104-113	C7:01

Table 5.15. List of predicted optimal peptides for participant 003-001. Each row contains the relevant HIV protein, predicted sequence (either consensus or variant), amino acid position and the suspected HLA-class I allele restriction. Bolded red letters in sequences indicate point mutations in the participant plasma viral sequence; these sequences were included in the list.

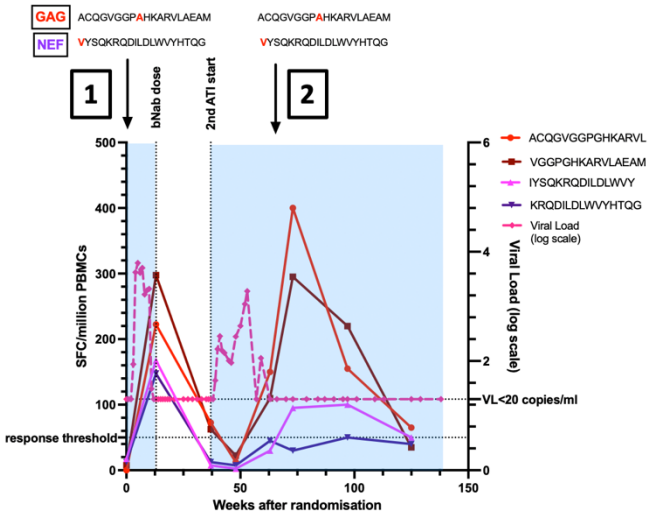
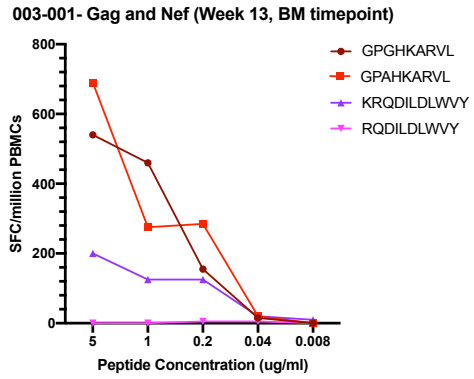
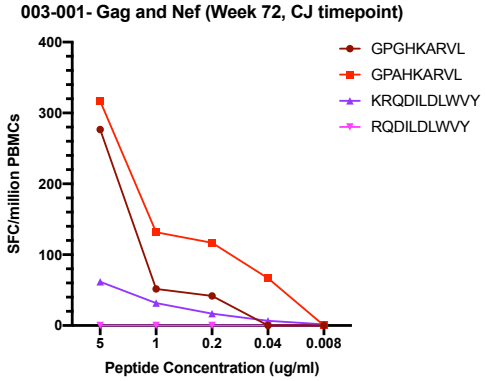
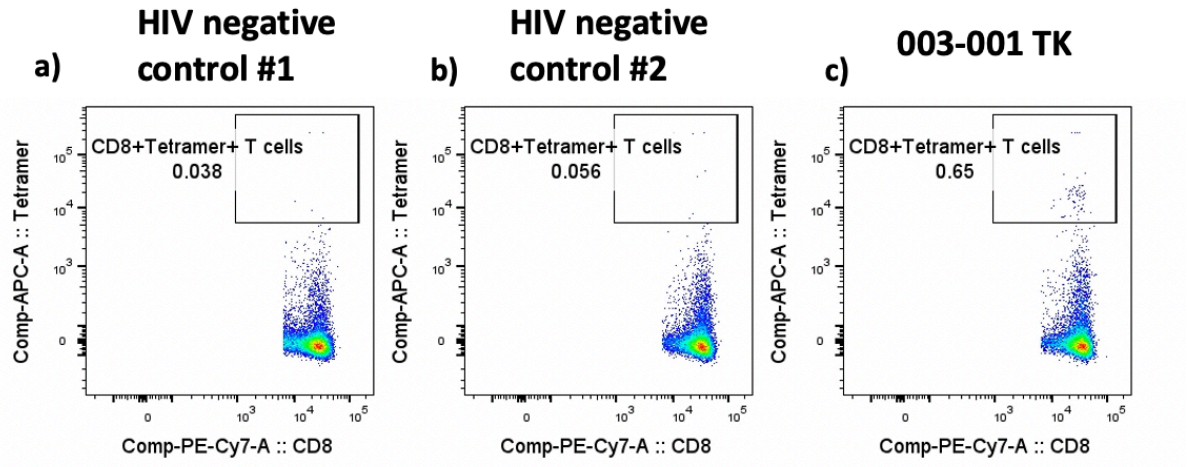
Plasma virus sequences (003-001)	**HLA-restricted peptide ELISpot assays (003-001)**
	 

Table 5.16. Plasma viral sequences for Arm B participants 003-001 during the 1st and 2nd ATI (Gag and Nef). Black downward arrows indicate timepoints where plasma viral sequences were amplified. Epitope-specific responses to Gag and Nef are shown in red and pink/purple lines respectively; viral loads are plotted in pink.

Next, we analysed the changes in antigen-specific T cells via tetramer staining (see Methods). At the time of writing this thesis, we were only able to do preliminary testing of the predicted optimal peptide GPGHKARVL (with a predicted HLA-class I allele B42:01). Our preliminary tetramer titration results at Week 49 (timepoint TK) during the 2nd ATI revealed that there were detectable CD8+tetramer+ cells (0.65%) that were not significantly detected in healthy negative controls (0.038% and 0.056% respectively)

(Figures 5.6a-c). Further investigation is currently underway to quantify tetramer-positive cells at multiple timepoints during ATI (pre and post-bNAbs).



Figures 5.6a-c. Preliminary tetramer stains on a-b) two HIV negative controls and c) participant 003-001 at Week 49 during the 2nd ATI, using the tetramer HLA-B*42:01-GPGHKARVL.

5.7 Discussion

HIV-specific T cell immunity has been frequently implicated in spontaneous viral control in HIV. As such, the study of elite controllers – individuals that can suppress viral replication in the absence of ART - has garnered significant interest. Studies report that elite controllers exhibit polyfunctional T-cell responses and proliferative capacities compared to progressors (17,25), and demonstrate an enhanced ability to suppress viral replication as demonstrated *in vitro*. Furthermore, elite controllers have been shown to possess higher frequencies of stem-like memory T cells (31,32), and have an abundant pool of expandable, Gag-specific central memory T cells that have a strong virus inhibitory capacity (32). Elite controllers have also been associated with favourable changes in host genetics and are enriched in certain HLA class I alleles such as HLA-B*57 and B*27 (33,34). On the contrary, a larger subset of individuals -known as post-treatment controllers – have been shown to have poorer CD8 T cell responses compared to elite controllers, and often possess risk-associated alleles (24). Despite extensive investigation, there are

numerous questions regarding the immune mechanisms that influence viral control, which is in part due to the scarcity of these individual subsets and the limited number of studies performed (23,35).

With regards to post-bNAb control in HIV, recent studies done in SHIV-infected rhesus macaques have demonstrated direct associations between CD8 T cell depletion and viral rebound (36,37). Moreover, short-acting combination bNAbs have been associated with enhanced Gag-specific T cell immunity, both during treatment interruption and on ART (26,27). While this evidence suggests that post-bNAb control could be T-cell mediated, there is limited data on the underlying immune mechanisms driving this control. More importantly, are there any specific immune signatures of spontaneous viral control, and how do these differ from individuals that show rapid loss of control? To answer these questions, we performed an in-depth analysis of HIV-specific T cell immunity in four Arm B participants that exhibited spontaneous viral control post-bNabs in the 2nd ATI, and compared these to four Arm B participants that experienced rapid viral rebound. Of the four post-bNAb controllers, we also did a deeper investigation into participant 003-001, who achieved an undetectable viral load post-bNAbs in the 2nd ATI and remains undetectable to date.

In the four Arm B participants that showed spontaneous viral control, three of the four participants (001-003, 002-001 and 003-001) demonstrated enhanced interferon-gamma responses to Gag and Nef (002-001 and 003-001) peptides which coincided with a lower viral set point in the 2nd ATI. On the contrary, participant 001-002 made responses to Pol and Nef peptides that decreased during the 2nd ATI, despite achieving a lower viral set point. There are a few reasons that could explain the divergence in interferon-gamma responses seen. Firstly, while interferon-gamma responses are a robust measure of virus-specific CD8 T cell responses, they are produced by the majority of individuals infected with HIV, including those that fail to control infection (3,38,39). Thus, an increased production of interferon-gamma does not

necessarily correlate with control of viral replication; in fact, interferon-gamma secreting HIV-specific CD8 T cells are often not the same as cells that target HIV as shown in one study (34). Rather, both the magnitude and specificity of the response must be taken into account. In the case of 001-002, the peptides recognised in Pol and Nef (FNLPPVVAKEIVASC, MASDFNLPPVVAKEI and EVGFPVRPQVPLRPM) are likely restricted to common HLA class I alleles that are not particularly protective or risk-associated, and thus are simply "passenger" responses that are produced as a result of ongoing viral replication. In comparison, both 001-003 and 002-001 showed recognition of the Gag region EKIRLRPGGKKKYKL, which has been extensively described as a region that is targeted by elite controllers and individuals with superior HIV control (40,41). Given the correlation between the magnitude of Gag-specific immune responses and viral control, it is reasonable to infer that at least some of these responses contribute to the suppression of viraemia.

With regards to the enhanced interferon-gamma responses observed, however, one caveat is that these responses were a result of viraemia that occurred during the 1st ATI, rather than being a direct "vaccine-like" effect of the combination bNAbs. The argument is that the viraemia induced during the 1st ATI may have triggered a surge in virus-specific T cell responses (which are not necessarily protective). To address this limitation, an additional Arm C of the RIO trial has been approved. This requires participants to undergo a period of treatment interruption-induced viraemia prior to Stage I of the study, where participants go through two rounds of ATI before receiving bNAbs and interrupting ART for a third time. Results from this Arm will determine whether the enhanced T cell responses observed in Arm B participants are being boosted by bNAbs or are an after-effect of viraemia. Another argument against this is that existing studies done with multiple structured treatment interruptions showed no association between the number of ATIs and response; in fact, there was an inverse correlation between viraemia during ATI and response in ELISpot (42). Thus, the theory that treatment interruptions could be producing

an “auto-vaccination” effect is controversial (although other factors prior to ATI have been shown to affect responses during ATI, as mentioned in Chapter 3).

In this chapter, we also deduced the optimal epitopes for Arm B participants that showed spontaneous viral control and compared these with plasma viral sequences at multiple timepoints during ATI, including post-bNAbs. For 001-003, we observed the highest binding affinity to Gag peptides IRLRPGGKKK, RLRPGGKKK, and ACQGVGGPGHK, and responses to these remain unchanged pre and post-bNAbs. Notably, peptide IRLRPGGKKK is an epitope that is also restricted by the protective allele HLA-B*27:05 (43). Thus, responses to this epitope may be responsible for the lower viral set point observed in this participant. Furthermore, A11-restricted epitope ACQGVGGPGHK has been recognised by elite controllers in HIV and was clinically linked to a lower viral set point during treatment interruption and slower progression (44,45).

For 002-001, strong responses were made to A24:02-restricted epitope KYKLKHIVW, which has been reported as a highly immunogenic, A-list epitope. Interestingly, one study found that mutations in this epitope were associated with a decrease in viral fitness, which may explain the role of this response in controlling viral replication (46). On examination of viral evolution, we also observed several point mutations in this Gag region at Week 44 and 55 during the 2nd ATI (EKIRLRPGGKK**R**YKLKHI**M**WASR and EKIRLRPGGKK**S**YKLKHI**M**WASR). The first point mutation observed (**R**YKLK) is a variant sequence that has previously been recognised, although its association with viral fitness is undetermined (47,48). In contrast, while the exact variant epitope RLRPGGKK**S**Y has not been previously reported, a mutation to KK**S**Y has been documented as an escape variant that results in a reduced viral replicative capacity (49). With regards to Nef, 002-001 made responses to A24-restricted epitope RYPLTFGWCF and a slightly lower response to variant epitope R**F**PLTFGWCF. Interestingly, epitope R**F**PLTFGWCF has been reported as a

non-susceptible escape variant (50), which means that individuals tend to produce a diminished response to this epitope. This may explain why our participant responded better to the unmutated epitope compared to the variant, although it is unclear in this case whether responses to these epitopes are detrimental to viral fitness. Overall, these results demonstrate that responses to Gag epitopes (seen in 001-003 and 002-001) persist during multiple rounds of ATI, and that in some cases these responses could be inducing fitness cost mutations. Further investigation is required to determine whether these boosted responses are due to bNabs or simply triggered by viraemia.

To further examine whether spontaneous viral control could be explained by immunological changes post-bNAbs, we investigated other aspects of T-cell functionality, including proliferation, activation and changes in memory subsets. We observed increases in both Gag CD8 proliferation and CD4 AIM+ responses during the 2nd ATI for both 001-003 and 002-001. We also observed increases in Th1 and CD8 effector memory T cells during the 2nd ATI after receiving bNabs and ART. There were also increases in HLA-DR/CD38+ CD4 and CD8 effector memory T cells, which might be expected given the ongoing viraemia occurring during ATI. While our sample size was small (n=2), we have also observed increased expression of Th1 cells in Arm A participants that remained virally suppressed off ART (see Chapter 4), which suggests that this response could be protective. However, whether or not this response is sufficient to maintain long-term viral control is uncertain. In comparison to these two individuals, participant 001-002 – who also spontaneously controlled viraemia in the 2nd ATI – did not show significant changes in memory or activated T cell subsets. In this case, mechanisms other than T cells may be conferring this control. While one could argue that this participant did not express any protective HLA-class I alleles, neither 001-003 or 002-001 were particularly enriched in these alleles either; in fact, 002-001 was homozygous for the frequently reported disease-susceptible allele B*7:02. Thus, host genetics alone cannot explain these differences, and this has been suggested by previous literature as well (34).

It is more likely that a combination of both innate and adaptive immunity is required to achieve spontaneous viral control. Overall, our assessment of T-cell functionality in these spontaneous controllers support the hypothesis that bNAbs may be inducing changes in HIV-specific T cell immunity that may drive control. Our analysis mainly focused on bulk PBMCs with peptide pool stimulation, followed by flow cytometric gating to determine CD4/CD8 specific responses. To extend the findings from this chapter, future studies should focus on delineating specific responses that could be driving spontaneous viral control by using, for example, HLA-restricted peptides to determine epitope-specific proliferation as well as sorting of T cells to better define antigen-specific CD4/CD8 cell populations.

The next hypothesis was to determine whether the changes in T-cell immunity observed were similar or different to other Arm B participants that rebounded rapidly. To achieve this, four Arm B participants who showed rapid loss of control in the 2nd ATI. In comparison to Arm B participants that spontaneously controlled with dominant Gag responses, all four participants made dominant responses to Env and Nef peptides. While these participants also made responses to Gag peptides, preliminary HLA-restricted epitope prediction showed that responses to these Gag regions (such as B*8:01-restricted EIYKRWII) have been previously associated with a loss of viraemic control in progressors (51), and have been shown to precede loss of control in viraemic controllers (52). One of the four participants (001-017) made a response to predicted epitope GQMVHQAI, which is restricted to the highly protective allele B*13:02 (53). However, the main dominant responses in this participant were to the Nef peptide RPMTYKAAVDLSHFL, which were almost 4-folds higher during the 1^{st} and 2^{nd} ATI compared to the Gag peptide PIVQNLQCQMVHQAI at the same timepoints; thus, it is possible that this protective Gag response was insufficiently high enough to achieve control. The predicted Nef epitope AAVDLSHFL has been previously reported, although not restricted to HLA-C*8:04 as seen in 001-017; this epitope has been frequently

associated with viral escape, and is thought to induce immunological footprint responses rather than limiting viral load (54).

There are some caveats with this analysis. Firstly, we only analysed four participants in Arm B, and coincidentally three of the four participants had disease-susceptible alleles (such as B*8:01, B35:01 and B*7:02). Thus, this comparison may be skewed, as individuals with these alleles are more likely to recognise peptide sequences that are associated with frequent immune escape. However, due to the staggered nature of data acquisition for the RIO trial, it was not logistically possible to sample more Arm B participants, so we instead performed an in depth analysis of a small number of participants that had available timepoints. Also, as previously mentioned, host genetics only partially influence viral control, and so other factors are likely contributing to this early loss of control post-bNAbs. In addition, due to logistical and experimental factors we were unable to define the optimal epitopes or perform tetramer staining for these participants. This would have provided additional data on how these epitopic responses influence viral evolution and whether this differs from other participants that spontaneously control. As such, predicted optimal epitope data is partly speculatory. However, the data presented provides useful insights into the divergence of responses seen in individuals that spontaneously control post-bNAbs versus individuals that rapidly lose control. Further studies with a larger sample size should confirm these findings and determine whether bNAbs could be mechanistically conferring this control.

The final hypothesis was to determine whether immune mechanisms post-bNAbs could be contributing to the complete viral suppression observed in participant 003-001. To summarise, participant 003-001 rebounded rapidly during the 2nd ATI post-bNAbs and ART; however, he spontaneously suppressed viral load and subsequently achieved undetectable viraemia to date (in the absence of ART). At the point of viraemia in the 2nd ATI, we observed an increase in Gag CD8 proliferation, which decreased gradually after

viral suppression was achieved. Similarly, there was a spike and subsequent fall in Nef-specific CD4 proliferation as well. On the contrary, Gag CD4 AIM+ responses appear to peak during viraemia and were maintained at high levels. Moreover, we observed an increase in Tfh and Th1 cell subsets after viral suppression as well. As later timepoints have not been sampled for this participant, it is difficult to determine the durability of these responses. The spontaneous viral suppression could arise from two possible mechanisms; one possibility is the removal of antigen due to enhanced HIV-specific T cell immunity which has resulted in a potential case of functional cure. On the contrary, another argument is that there is a loss of T-cell responses due to viral clearance by alternative mechanisms such as reservoir targeting.

We also observed similar trends in Gag-specific interferon gamma secretion. Interestingly, we observed an increase in Gag-specific response to optimal peptides GPGHKARVL and GPAHKARVL after viral rebound in the 2nd ATI, as well as an optimal response to Nef peptide KRQDILDLWVY. However, after complete viral suppression responses to both Gag and Nef decreased (as seen in the single peptide ELISpot data). As previously mentioned, this may be an indication of reduced circulating antigen and enhanced viral clearance, which results in a waning of response. Another potential possibility, although not yet proven, is that bNAbs have induced a functional cure in this participant. At the time of writing this thesis, ongoing reservoir measurements are being performed to determine whether there is presence of intact provirus at timepoints beyond Week 70 where complete viral suppression was achieved. Notably, while this participant has the disease-susceptible allele B*35:01, this allele has also been associated with post-treatment control in the VISCONTI cohort (24). Additionally, the response towards the Gag peptide (GPGHKARVL) is likely restricted by B*42:01; this has been associated with protection in Clade C infection, but less evidence exists for Clade B (55). Interestingly, our preliminary tetramer staining results showed detectable CD8+tetramer+ cells specific to HLA-B*42:01-GPGHKARVL at Week 49; further investigation is

underway to determine whether these antigen-specific T cells are expanded at later timepoints during the 2nd ATI and, more importantly, whether similar percentages of antigen-specific T cells are detected towards the variant form (GPAHKARVL) over time.

Our examination of Gag and Nef sequences in these targeted regions did not show evidence of mutation between baseline and the 2nd ATI timepoints. One theory is that the mutated variant epitope, GP**A**HKARVL, could be highly detrimental to viral fitness and thus more susceptible to immune targeting. This may explain why we observe a mild shift in immune dominance towards the variant Gag epitope at Week 72, compared to Week 13 where responses to the unmutated and variant epitopes were similar at each peptide concentration. Notably, little or no evidence has been reported regarding this variant epitope; thus, further assays should be done to assess viral fitness and infectivity and determine whether this epitope is associated with high fitness costs. Understanding this would help elucidate determinants of spontaneous viral control for this participant.

Overall, this chapter provides relevant insights into the immune mechanisms that could be contributing to spontaneous, post-bNAb viral control. We noted that spontaneous viral control often coincided with responses towards protective Gag epitopes in regions that have high associated fitness costs to the virus. Moreover, we showed increases in Gag-specific proliferation and activation post-bNAbs towards in these participants. While the comparison was partially incomplete, we were able to demonstrate that in terms of interferon-gamma secretion, participants that showed a rapid loss of control often made responses to Env and Nef as well as Gag regions that were frequently associated with immune escape and poor viral control. Finally, we performed an in-depth analysis of participant 003-001, who demonstrated complete viral suppression post-bNabs and showed that this was associated with transient increases in HIV-specific proliferation, activation and targeting of Gag (and to a lesser extent, Nef) epitopes. However, our analysis

may have been confounded by an over-representation of participants with disease-susceptible alleles, small sample size and insufficient timepoint sampling during the 2nd ATI and post-viral suppression. Further analysis at timepoints beyond Week 72 in participant 003-001 are in progress to determine the durability of HIV-specific T-cell immunity in the context of spontaneous viral suppression, and to determine its influence on viral evolution and immune escape. Current investigation is being done to establish whether this spontaneous viral suppression is attributed to innate immune mechanisms, such as NK-cell mediated immunity which has not been explored in this chapter. A summary of findings from this chapter are shown in **Figure 5.7.**

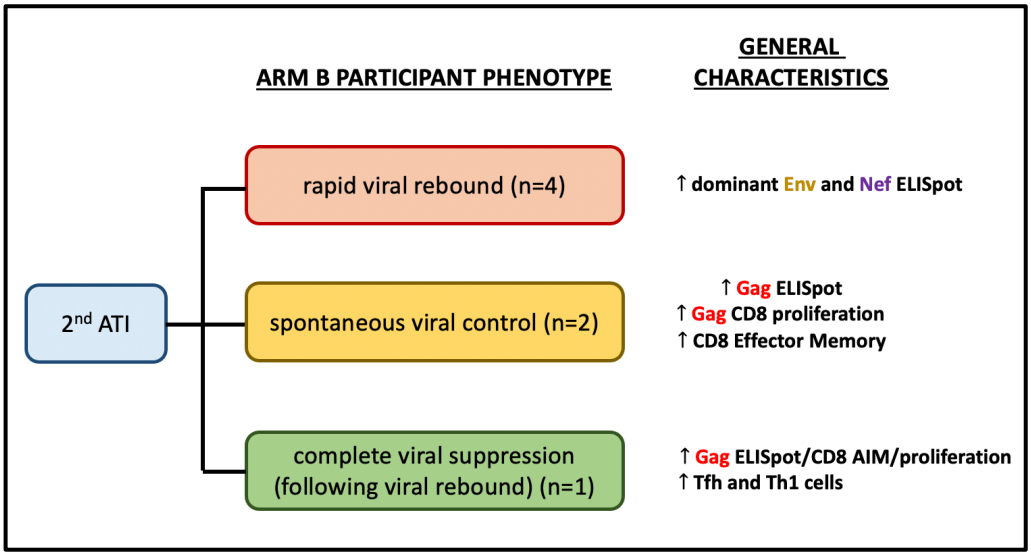


Figure 5.7. Summary of findings in Chapter 5.

Chapter 5: References

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Concluding Remarks

Despite recent advances in the treatment and management of HIV infection, ART is not a functional cure and the development and persistence of the viral reservoir is a significant barrier to eradication. Since the discovery of HIV in 1983, extensive investigation has been done to determine factors and therapeutic interventions that could achieve an ART-free remission. Studies in elite controllers of HIV have consistently implicated HIV-specific T cells as a key contributor to viral control off ART. Initial studies used treatment interruptions to determine immune correlates of viral rebound in the absence of ART. Some studies hypothesised that inducing viraemia could act as an "autovaccination" effect on T cells resulting in prolonged viral control, however evidence supporting this has been controversial. Recently, the passive transfer of broadly neutralising antibodies (bNAbs) in both animal and human studies has been shown to induce long-term viral suppression during treatment interruption and off ART, which is theorised to be attributable to a T-cell mediated "vaccinal" effect. However, there is limited data on the impact of treatment interruption on HIV-specific T cell responses and whether shifts in immunodominance are observed. Also, further investigation is required on how bNAbs could induce changes in HIV-specific T cell immunity during ART interruption, and how these may influence viral control and rebound.

To answer these questions, this thesis explored the changes in HIV-specific T cell immunity in individuals with primary HIV infection in several different contexts during ATI; this included the impact of ART duration (and the absence of ART) on T-cell immunodominance, the effects of bNabs given during viraemia or aviraemia on HIV-specific T cell immunity, and T-cell responses in the context of spontaneous viral control and how it shapes viral evolution. In Chapter 3, we explored the immunological impact of treatment interruption itself on HIV-specific T cell immunity in primary HIV infection, and how this was

influenced by the duration of ART: either two years of ART (PITCH cohort), or 48 weeks of ART versus no ART (SPARTAC cohort). This enabled us to understand the impact of early ART on HIV-specific T-cell responses during treatment interruption, which may inform therapeutic strategies to deliver an ART-free mediated viral suppression. In this chapter, we demonstrated that individuals receiving two or more years of ART exhibited shifts in immune dominance towards Gag and/or Pol that occurring during or post-ATI. In addition, one participant showed enhanced Gag-specific responses during viral suppression in ATI, which may have been contributing to viral control. In comparison, those receiving 48 weeks of ART demonstrated increased dominance towards Gag responses during ATI, compared to ART-naïve participants that less Gag-directed responses during ATI over time (1).

After establishing that treatment interruption induces shifts in immunodominance during ATI, the next question was to determine whether dual, long-acting combination bNAbs were associated with enhanced HIV-specific T cell immunity and whether there were immune correlates that predicted time to viral rebound. To test these hypotheses, we examined HIV-specific T cell immunity in two arms of the RIO randomised clinical trial: Arm A, where participants received combination bNAbs during aviraemia followed by ATI, and Arm B, where participants received combination bNabs during viraemia followed by a washout period of ART and a 2nd ATI. We demonstrated that in both cases, combination bNAbs were accompanied by increases in Gag-specific T cell immunity; in Arm A participants that remained virally suppressed off ART, increases in interferon-gamma secretion and proliferation were observed at least 12 weeks post-bNAbs, while in Arm B we observed increases in interferon-gamma secretion and T-cell activation for at least 20 weeks post-bNAbs. Interestingly, Arm B participants demonstrated increases in Pol-specific proliferative responses rather than to Gag as seen in Arm A. Additionally, this subset of Arm A participants were enriched for Th1 cells after 12 weeks and tended towards a Tbet^{hi}EOMES^{lo} phenotype which was observed up to 24 weeks post-bNAbs. Finally, we demonstrated positive correlations between

T cell responses observed in the proliferation, AIM and ELISpot assay, and showed that Gag proliferative responses at Week 24 in Arm A participants significantly predicted time to viral rebound. These findings add strength to existing data that supports the immune enhancing effects of bNabs during ATI/on ART (2,3), and sheds light on the possible T-cell mediated immune mechanisms that could be driving viral control.

As previous data has demonstrated clear links between HIV-specific T cells and spontaneous viral control in HIV, we then wanted to determine whether such associations could be observed in our RIO participants that displayed similar phenotypes. To do this, we examined four RIO participants in Arm B that achieved a lower viral load set point during the 2nd ATI, and compared these phenotypes to four other Arm B participants that showed evidence of rapid viral rebound. Our results showed that three of the four participants demonstrated enhanced Gag (and sometimes Nef)-specific T cell immunity as evidenced by interferon-gamma ELISpot, proliferation and T-cell activation that coincided with spontaneous viral suppression. In addition, optimal epitope analysis using interferon-gamma ELISpot revealed that the magnitude of these Gag responses was maintained despite viral evolution between both periods of treatment interruption, and that some epitopes were restricted by protective HLA alleles and associated in the literature with fitness cost mutations. While our data was incomplete, preliminary analysis showed that comparator participants that showed rapid viral rebound demonstrated dominant responses to predicted epitopes in Env and Nef that were not protective according to the literature, although these findings were partially confounded by risk-associated HLA class I alleles that were enriched in these participants.

Finally, we did an in-depth analysis of a participant who achieved complete viral suppression post-bNAbs to date, and found that spontaneous viral suppression was associated with coincidental increases in Gag-

specific T cell proliferation, activation and interferon-gamma secretion (although this waned over time). Although reasons for the decrease in the response over time remain unclear, current investigation is underway to determine the HIV-specific T cell responses at later timepoints during viral suppression. Viral reservoir sequencing is also underway to look for any evidence of intact provirus and determine whether this participant may have achieved a functional cure. In terms of optimal responses, we also demonstrated a gradual shift in dominance towards Gag epitope variants (i.e GP**A**HKARVL) over time; although the underlying mechanisms for this remain unclear, we hypothesise that this Gag variant epitope poses a high fitness cost on the virus, which may explain why this epitope was being increasingly targeted over time and the subsequent spontaneous viral control that occurred. Overall, this chapter demonstrated that spontaneous viral control is associated with enhanced T cell immunity towards Gag in some cases, with potential resultant effects on viral fitness. However, further work is required to determine if these findings can replicated in other studies involving bNAbs.

The cohorts described in this thesis enabled a thorough investigation into the shifts in HIV-specific T cell immunity that occur in the absence of ART; for instance, we were able to examine a group of ART-naive participants from a historical cohort (SPARTAC) when ART was not considered the standard of care, which would not be possible today. We demonstrated that the absence of ART erodes the production of Gag responses over time, compared to 48 weeks of ART where the potential to make new Gag responses increases. This confirms previous findings that have supported the protective, immune preserving effects of early ART (4,5). While Gag-specific responses have been consistently associated with control, we also observed increases in Pol-specific responses in two-year ART-treated participants in the PITCH cohort. Existing data regarding the association between Pol responses and viral control are available but less well explored than for Gag; further investigation should be done to determine the relevance of these responses to viral control off ART. Moreover, we were unable to examine other aspects of T-cell

functionality in the PITCH and SPARTAC; future studies would benefit from incorporating additional readouts such as T cell proliferation and activation to determine if they produce similar results. Where possible, treatment interruption studies should aim to include longer durations of ATI to allow a more comprehensive longitudinal analysis of HIV-specific T cell immunity, which was limited to some extent in our cohorts due to rapid viral rebound in most cases.

Given the current interest around combination bNab therapy and the "vaccinal" effect, examining data from the RIO clinical trial provided an opportunity to analyse this hypothesis in greater detail, and to compare the effects of bNAbs given during aviraemia (Arm A) and viraemia (Arm B). While we did observe increases in Gag-specific T cell immunity in both instances (which supports existing literature), we observed remarkable increases in Pol-specific proliferation in Arm B which were absent in Arm A. Whether or not this enhanced proliferation was due to viraemia during bNab administration is unclear, and whether this effect is observed beyond Week 20 which was the last analysed timepoint. Interpreting these results in the context of ATI rather than on ART may provide key insights into this trend. In addition, the viraemia induced by the first treatment interruption is a potential confounder here. Regardless, it remains to be determined whether these proliferative responses are associated with viral control off ART in Arm B; ongoing investigation is underway to see these responses persist during ATI. Nevertheless, we have shown that bNAbs are associated with increases in HIV-specific T cell immunity, and may at least partially contribute to viral control off ART.

With regards to achieving a T-cell mediated remission, a crucial key question is which T-cell responses could achieve this, and whether there are any predictors of viral rebound. Through the RIO cohort, we demonstrated that in Arm A participants that remained virally suppressed, Gag-specific proliferation at Week 24 significantly reduced the likelihood of viral rebound. In comparison, we did not observe any

significant correlations between Gag responses in interferon-gamma ELISpot or AIM responses with viral rebound. This raises the question of which responses best predict the loss of control. One possibility is that the timing of response is crucial for viral control; for example, observing an increase in responses in the first 12 weeks (as we have seen for Gag in interferon-gamma ELISpot) may represent an acute-phase response that is reactive rather than protective, and are purely attributed to the immune enhancing effects of bNAbs. Conversely, more durable memory responses that continue beyond, for example, Week 24, are more indicative of a vaccine-like response, that persist after bNAbs have fallen below subtherapeutic concentrations. In support of this argument, we have also observed shifts in immunodominance towards Gag in virally suppressed Arm A participants at Week 24. To confirm this hypothesis, later timepoints should be examined to see if the durability of these responses impact viral rebound.

In spite of the findings presented in this thesis, numerous questions still remain regarding the immune-enhancing effects of combination bNAbs. We have used a combination of bNAbs that has been extensively studied and evidenced in the literature; however, further efforts to test other combinations of bNAbs are required to determine if these influence HIV-specific T cell immunity in a similar fashion. In addition, there is still no consensus on the most appropriate T-cell assay to use to measure bNAb-induced T cell immunity, and which of these best predicts viral rebound. Given the fairly novel nature of bNAb-based interventional studies, future research should incorporate multiple assays (ELISpot, proliferation and AIM) as well as other cytokine based analyses to determine the best functional readouts, as this could significantly impact monitoring and management during ATI. While we have not explored this, further work should be done to determine how enhanced T-cell immunity during spontaneous viral suppression influences viral fitness, as this will provide key insights into epitope-specific responses that are driving viral control. Additional investigation into potential innate immune responses that contribute to this control are also warranted,

and are currently being explored in the trial. Finally, comprehensive pharmacokinetic analysis and modelling of bNAbs is of paramount importance to the assessment of bNAb-induced T-cell immunity, and future studies should investigate this in conjunction with T-cell readouts when interpreting immune correlates of viral rebound.

To conclude, this thesis explores the potential "vaccine-like" effects of T cells in primary HIV infection through multiple lenses; in individuals that have received two years of ART, 48 weeks of ART or no ART prior to ATI, as well in individuals that received combination bNAbs during viraemia or aviraemia. While our data shows trends towards Gag-specific T cell immunity during ATI with or without bNAbs, the key question that remains is whether these responses are sufficient for achieving a T-cell mediated remission in the long term. Further confirmation of these findings in other cohorts with primary HIV infection are also necessary. Ultimately, the data presented sheds light on how adaptive immunity may contribute to a T-cell-mediated suppression in HIV, and provides grounds on which to further elucidate the underlying mechanistic effects of bNAbs on T cells in a dynamic immune environment.

Concluding Remarks: References

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Appendix Chapter A

The RIO Clinical Trial

- a randomised phase IIb placebo-controlled trial of antiretroviral therapy plus dual long-acting HIV-specific broadly neutralising antibodies (bNAbs) in participants diagnosed with recent HIV infection

A.1 Background and rationale

ART has dramatically improved the survival of people living with HIV, and induces viral suppression as well as effectively preventing onward transmission. However, ART is not considered a functional cure due to persistence of the latent viral reservoir. Given the drawbacks of ART, including adherence issues, toxicities, resistance, and social stigma, there is a pressing need for alternative therapeutic approaches that can achieve a functional cure, eliminating the need for daily medication (1–6).

Recently, broadly neutralising antibodies (bNAbs) against the Env protein have been demonstrated as an emerging solution to address these challenges. Several clinical studies performed in non-human primates have shown that bNAbs are able to induce long term viral suppression and significantly delay viral rebound (7–9). BNAbs are uniquely able to penetrate the heavily glycosylated glycan shield on the surface of HIV-1, and subsequently recognise and target conserved epitopes on the Env glycoprotein (10). In addition to neutralising free floating virions, bNAbs also exhibit effector functions via their Fc domain which enables effector mediated cell killing via antibody-dependent cellular phagocytosis (ADCP), cytotoxicity (ADCC) and opsonisation (11,12). Most interestingly, it is hypothesised that bNabs form antigen-antibody immune complexes, which could stimulate increased dendritic cell uptake and antigen presentation and result in enhanced CD8 T cell responses known as the “vaccinal” effect (13–16). While neutralising antibodies are produced in 10-30% of individuals in chronic HIV infection, only 1% of these produce very potent broadly neutralising antibodies and initial attempts to produce these ex vivo had limited success and potency (17,18). However, recent advances in single cell cloning techniques and the development of high through-put neutralisation assays have resulted in the development of second generation bNabs, with a greater potency and breadth (19–24).

Initial attempts to use bNabs as passive immunotherapy in people living with HIV were of limited success; this was mainly due to the rapid emergence of viral escape and selection for drug-resistant strains, as well as autoreactivity in some cases (17,25–27). To address this problem, recent trials have used combinations of two or more bNAbs targeting non-overlapping epitopes; among different bNAb combinations, several studies have demonstrated that co-administering the CD4 binding site antibody, 3BNC117, and the V3 glycan antibody, 10-1074 are an effective combination for suppressing viraemia (28–31). For example, the landmark study by Mendoza et al. showed that combination bNabs 3BNC117 and 10-1074 induced prolonged viral suppression for between 15 to more than 30 weeks in 9 HIV-1 infected individuals, provided that they had antibody-sensitive reservoirs. Notably, none of the individuals developed resistance to either bNAb (31). In a similar study by Gaebler et al., prolonged viral suppression of more than 20 weeks was achieved in 13 out of 17 individuals that received the same combination of bNAbs as in seen in Mendoza et al (32). In an effort to reduce dosing frequency, bNAbs have been modified with -LS mutations - Met428Leu (M428L) and Asn434Ser (N434S) – which enhances binding affinities to the neonatal Fc receptor and significantly extends serum half-life (up to 3-4 fold in the case of VRC01-LS compared to wild-type VRC01, for example) (33–36). Currently, there are a number of trials investigating the effects of these modified bNabs **(Table A.1).**

While the clinical and pre-clinical observations in bNAb studies suggest they could potentially enhance immune mediated control of HIV, the mechanisms underlying this are still unclear. It is hypothesised that bNabs could exert a “vaccine-like” effect on T cells due to the formation of antigen-antibody immune complexes with subsequent priming of the adaptive immune response. Furthermore, while the combination of bNAbs 3BNC117 and 10-1074 have been tested in humans as single arm proof of principle studies with demonstrable effects on viral suppression in the absence of ART, these findings need to be extended using dual long-acting versions of these antibodies. Thus, the RIO randomised controlled trial is

a statistically powered trial designed to test the hypotheses of whether dual-long acting bNabs 3BNC117-LS and 10-1074-LS are effective in controlling HIV replication in adults living with HIV that initiated ART during acute HIV infection. In addition, this trial permits a detailed analysis into the changes in HIV-specific T cell immunity that occur during treatment interruption after receiving bNabs, and provides insights into virological and immune correlates of HIV remission while off ART.

Table A.1. Summary of clinical trials using combination therapy of >=2 broadly neutralising antibodies (Table adapted from Thavarajah et al. (1)).

Clinical Trial Study	Year	bNAb	Study Objective	Study Population	Ref.
NCT02825797 (Bar-on et al.)	2018	3BNC117 + 10-1074	Safety, pharmacokinetics, and antiviral activity	Viremic HIV-1-infected individuals with antibody sensitivity and have been on/off ART, n = 7	(29)
NCT028 (Mendoza et al.)	2018	3BNC117 + 10-1074	Antiviral activity incl. neutralization efficacy and latent reservoir	ART-treated and virally suppressed HIV-1-infected indivi-duals with antibody sensitivity undergoing ATI, n = 11	(31)
NCT02824536 (Cohen et al.)	2019	3BNC117 + 10-1074	Safety and pharmacokinetics	HIV-1-negative individuals, n = 24	(28)
NCT02825797 (Niessl et al.)	2020	3BNC117 + 10-1074	Antiviral activity, including Gag-specific CD8+ and CD4+ T-cell responses	Aviremic HIV-1-infected individuals undergoing ATI, n = 9	(13)
NCT03526848 (Gaebler et al.)	2022	3BNC117 + 10-1074 + ART	Antiviral activity incl. neutralization efficacy and reservoir size	HIV-1-infected individuals in the presence or absence of ART, n = 26	(32)
NCT03571204 (Sneller et al.)	2022	3BNC117 + 10-1074	Safety, pharmacokinetics, and antiviral activity including reservoir size	HIV-1-infected individuals, who initiated ART during early/acute infection, undergoing ATI (n = 14), ART-naïve HIV-1-infected individuals with viraemic control (n = 5), n = 19	(30)
PACTR201808919297244 (Mahomed et al.)	2022	VRC07-523LS + PGT121	Safety and pharmacokinetics	HIV-1-negative women, n = 45	(37)
PACTR202003767867253 (Mahomed et al.)	2023	VRC07-523LS + CAP256V2-LS	Safety, pharmacokinetics, and antiviral activity	HIV-1-negative women, n = 42	(38)
NCT03205917 (Julg et al.)	2022	VRC07-523LS + PGDM1400 + PGT121	Safety, pharmacokinetics, and antiviral activity	HIV-1-negative individuals (n = 24) and viremic, ART-naïve HIV-1-infected individuals (n = 5), n = 29	(39)
NCT03928821 (Sobieszczyk et al.)	2023	VRC07-523LS + PGDM1400 + PGT121 + 10-1074	Safety, pharmacokinetics, and antiviral activity	HIV-1-negative individuals who were randomly assigned to either dual- (n = 18) or triple-bNAb therapy (n = 9), n = 27	(40)

NCT03565315 (Awan et al.)	2024	VRC07-523LS + 10E8VLS	Safety and pharmacokinetics	HIV-1-negative individuals, n = 8	(41)

A.2 RIO Clinical Trial- Summary

A.2.1 Description of Cohort

The RIO trial is a randomised, placebo-controlled double-blind two-arm prospective phase II trial. Participants recruited to the RIO clinical trial (EUDRACT Number: 2019-002129-31, Clinicaltrials.gov number NCT04319367) are individuals who have started ART within 3 months of confirmed primary HIV infection (PHI), and have remained virally suppressed on ART for at least 12 months (<50 HIV RNA copies/ml). The full list of inclusion and exclusion criteria can be found in the **Appendix (Section 4.1-4.2)**. A majority of the participants will be recruited directly from the HEATHER cohort (Ethics Ref: NRES Committee West Midlands - South Birmingham 14/WM/1104; Current Date and Version No: 30/9/2015; Version 3.0) which consists of more than 350 individuals with treated PHI (previously described). Eligible consenting participants that are not enrolled into the HEATHER study but fulfil the same eligibility criteria may also have been enrolled into the trial. Informed consent was taken for all participants by Good Clinical Practice (GCP) trained members of the trial research team at respective clinical sites. Research study visits were undertaken at various clinical sites around the UK, in accordance with the trial study protocol (full list can be found in **Appendix Section 4.3**). Regarding interventional study visits, these were undertaken at the NIHR Imperial Clinical Research Facility (ICRF) at the Hammersmith Hospital (London, UK).

A key factor affecting eligibility for recruitment to RIO is sensitivity to the combination bNAb therapy administered (3BNC117-LS and 10-1074-LS), as numerous previous studies have shown that combination bNAbs were effective provided that the individuals had antibody-sensitive reservoirs (31). Thus, bNab sensitivity was assessed prior to recruitment using viral sequences from proviral HIV DNA extracted from participant peripheral blood mononuclear cells. HIV Env genes were subsequently amplified from provirus

via single genome amplification. Based on a minimum of 20 Env genes per candidate (sequenced using Illumina technology), a computational algorithm was then used to determine the sensitivity of these sequences. This was done by inspecting specific amino acid residues on the protein chain (42). Based on this analysis, participants with viral sequences that appear to be resistant to one or both bNAbs were not eligible to participate in RIO.

A.2.2 Trial Objectives

Primary objective: To determine the efficacy of dual bNAb infusion of 10-1074-LS and 3BNC117-LS at sustaining virological control within 20 weeks following initial ATI compared with placebo infusion.

Secondary objectives:

- To evaluate the safety of dual bNAb infusion of 10-1074-LS and 3BNC117-LS.
- To determine the role of viraemia at the time of bNAb administration in subsequent virological control.
- To determine the contribution of circulating bNAbs to virological control compared with a sustained impact following antibody elimination.
- To document the experiences and determine the attitudes of participants to the interventions and the treatment interruption.

Tertiary objective

- To determine the mechanisms by which bNAbs infusions can induce sustained virological control after stopping ART.

A.3 Study Design

The RIO trial is a randomised, double-blinded and placebo-controlled prospective phase II study that primarily aims to determine the efficacy of dual bNAb infusions of 10-1074-LS and 3BNC117-LS in inducing sustained virological control following an analytical treatment interruption (ATI), compared with a placebo infusion (EUDRACT Number: 2019-002129-31, ISRCTN Number / Clinical trials.gov Number: NCT04319367). Details of the bNAbs used are shown in **Appendix Section 4.4.** The primary endpoint of the study was time to viral rebound within 20 weeks of ATI. A total of 72 participants were recruited across multiple collaborating clinical centres in the UK, and randomised in a 1:1 ratio to either Arm A (n=36) or Arm B (n=36). The sample size was calculated to ensure sufficient statistical power; detailed statistical power considerations are described in **Appendix Section 4.5**. Recruited participants were either diagnosed with primary or early stage HIV infection, have been administered ART in accordance to NHS best practice and British HIV Association guidelines within 6 months of diagnosis, and have had an undetectable HIV viral load for >=12 months. At the time of randomisation, the designed ART regimen must include INSTI or bPI; participants on other regimens were switched to INSTI or bPI at least 4 weeks prior.

Arm A participants

A schematic diagram of the study design is shown below **(Figure A.1)**. In Arm A, participants are given an intravenous (IV) infusion of dual long-acting 3BNC117-LS and 10-1074-LS bNAbs, followed by an ATI two days later. ATI is continued until participants meet the ART restart criteria for viral rebound, by which they will subsequently restart ART and be followed-up for 16 weeks or to the end of study timepoint. The rationale of this arm is to test the hypothesis that administering dual long-acting bNAbs will result in sustained viral suppression during ATI, compared to the placebo group in individuals who have received ART during primary HIV infection without bNabs prior to ATI.

Arm B participants

In Arm B, participants received placebo infusions followed by an ATI two days later **(Figure A.1).** The 1st ATI represents Stage 1 of the study, which continues up until the point of viral rebound or the end of study. Upon meeting the criteria for viral rebound, participants will restart ART followed by an immediate unblinded IV infusion of dual long-acting 3BNC117-LS and 10-1074-LS bNAbs, followed by a washout period of ART for 24 weeks. The significance of infusing bNabs during viraemia is based on existing evidence from macaque SHIV models that viral suppression in bNab-infused rhesus macaques may be mediated by CD8 T cells. It is though that this process may be driven by immune complex formation in the context of low level viral replication, and depletion of CD8 T cells was associated with aborted viral control (43). The effects of bNAbs on CD8 T cell responses in humans are still largely unexplored; the concept is that viraemia induced by the initial ATI may enable more effective binding of these bNAbs and facilitate immune complex formation due to increased viral antigen production, and subsequently enhance protective CD8 responses. After bNAb administration, the 24 week washout period with ART is based on the assumption that the concentration of bNabs will have fallen to undetectable levels by this timepoint, which removes the potential therapeutic effects of bNAbs as a possible confounder for any viral suppression observed in the 2nd ATI. After 24 weeks of ART, unblinded Arm B participants will then undergo a 2nd ATI and begin Stage 2 of the trial, until viral rebound or the end of study timepoint is reached **(Figure A.1).** A full summary of the trial design is shown in the Appendix (**Figure A.1**).

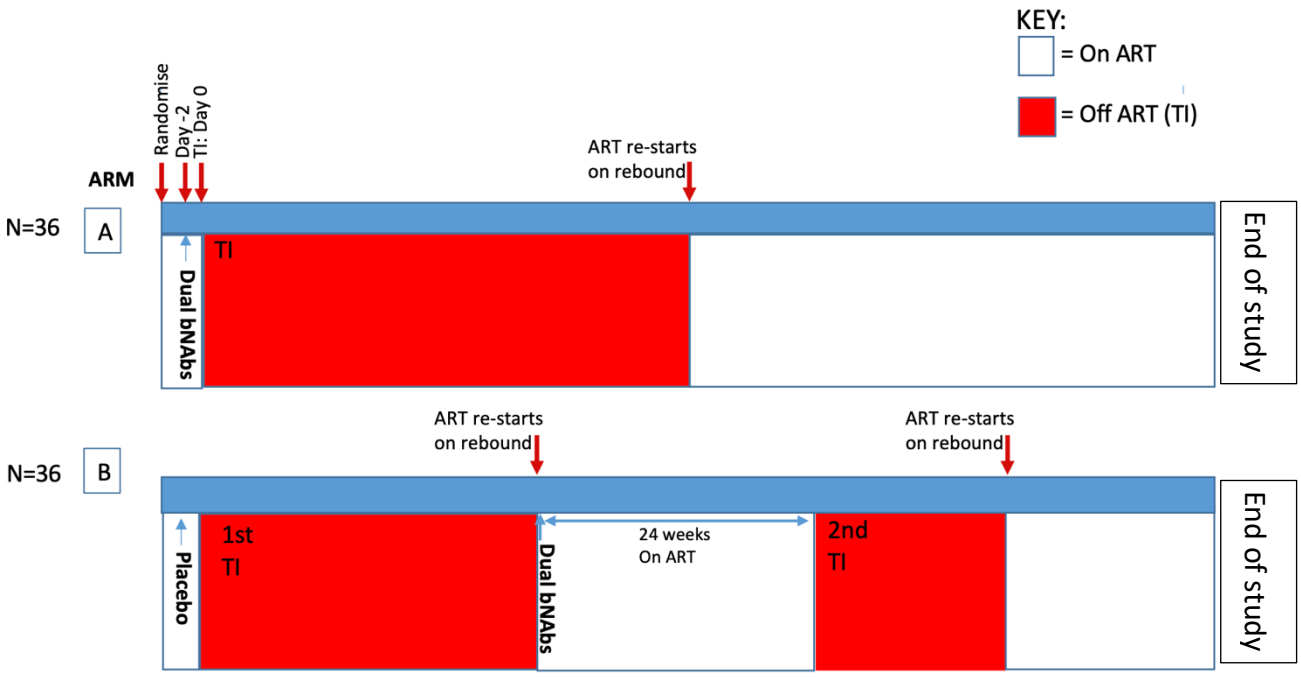


Figure A.1. Schematic diagram summary of the RIO clinical trial design.

A.3.1 Second dose sub-study

Participants that maintained viral suppression for 20 weeks or more (without fulfilling the criteria for viral rebound and ART restart) were permitted to have a second bNab infusion. Second dose infusions were only given to consenting RIO participants in Arm A or B, whilst maintain the initial blinding as determined during enrolment **(Figure A.2).** For participants randomised to Arm A, those that did not meet the criteria for viral rebound by Week 20 were offered a second dose of dual bNAbs. The reason for offering a second combination bNab infusion in this Arm was to determine whether a second bNAb infusion would provide a significantly longer period of viral control compared to a single infusion. Participants that did not consent to a second dose of bNAbs were followed up as per the main study schedule.

For participants randomised to Arm B, participants restarted ART after meeting the viral rebound criteria and received one bNAb infusion. 20 weeks after resumption, Arm B participants were then offered a second dose of open label dual bNAbs while on ART **(Figure A.3).** The rationale for offering a second bNAb infusion is that this would provide further insights into the effects of dual bNab therapy while on ART on viral control and measures of the HIV reservoir. More importantly, it would useful to observe whether a second bNAb infusion would prolong time to viral rebound. For participants that did not choose to have a second ATI but were still currently in the study, they were still offered a second dose beyond Week 20.

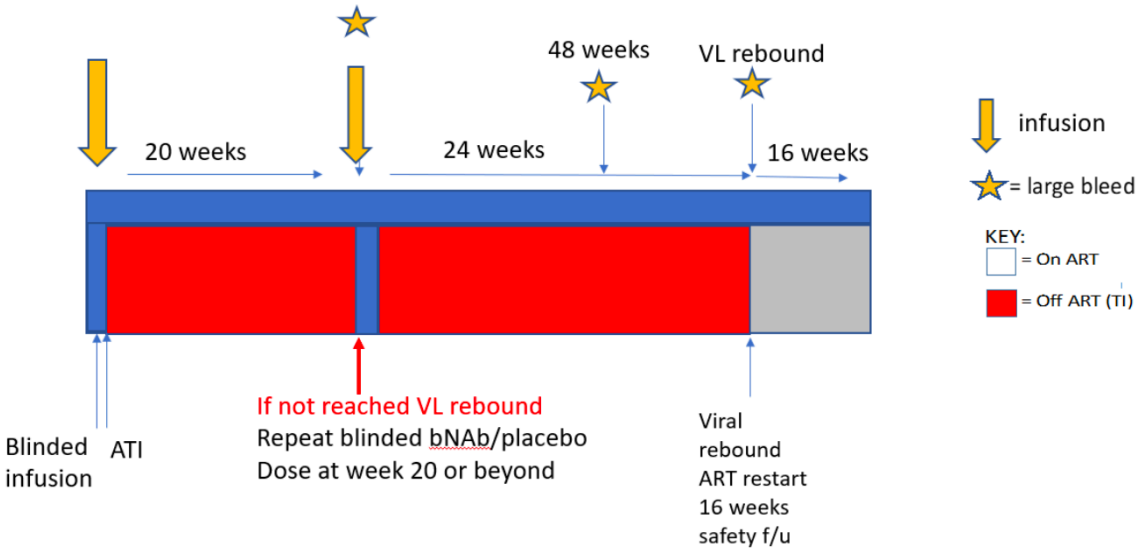


Figure A.2. Second dose sub-study assessment schedule for Arm A participants of the RIO clinical trial.

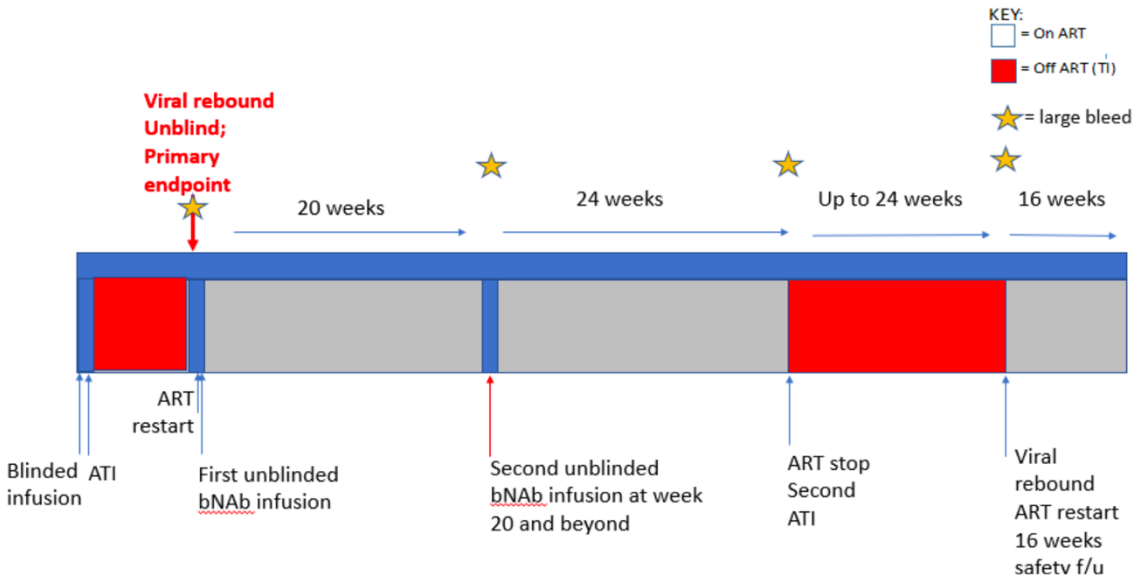


Figure A.3. Second dose sub-study assessment schedule for Arm B participants of the RIO clinical trial.

A.4 ATI monitoring and follow-up

All participants received compulsory monitoring of CD4 count and plasma HIV viral load using NHS validated measurements following ART interruption. Viral loads were measured at least weekly for the first 8 weeks during TI, followed by every two weeks afterwards until viral rebound or until ART was restarted according to the algorithm **(Figure A.4).** After restarting ART, participants were followed up at weeks 2, 4, 8 and 12 for viral load measurements or until their viral load became undetectable, and were followed up until the end of study timepoint. Blood were taken from participants for isolation of PBMCs for research assays at various timepoints throughout the follow-up schedule. Between 50 to 200mls of blood was taken depending on the timepoint. Full details of Arm A and B participant follow-up and trial assessment schedules are shown in the **Appendix (Tables 4.1a-c)**. The specific criteria for ART restart is shown below.

ART restart criteria (adapted from trial protocol by Lee et al (44)):

1. Viral rebound confirmed by HIV VL measurement from venous blood:

a. Sustained HIV viral load ≥1000 copies/ml for 6 Weeks +/− 7 days.

b. ≥100,000 copies/ml for two readings, 7 days apart +/− 7 days.

c. Instances outside of the above two categories but are close to these definitions and necessitate ART restart for clinical reasons. These instances will be individually reviewed by an independent panel (Endpoint Adjudication Committee) to confirm if they meet the definition for viral rebound.

2. CD4 count drops to less than 350 cells/μL.

3. Clinical symptoms attributable to ATI.

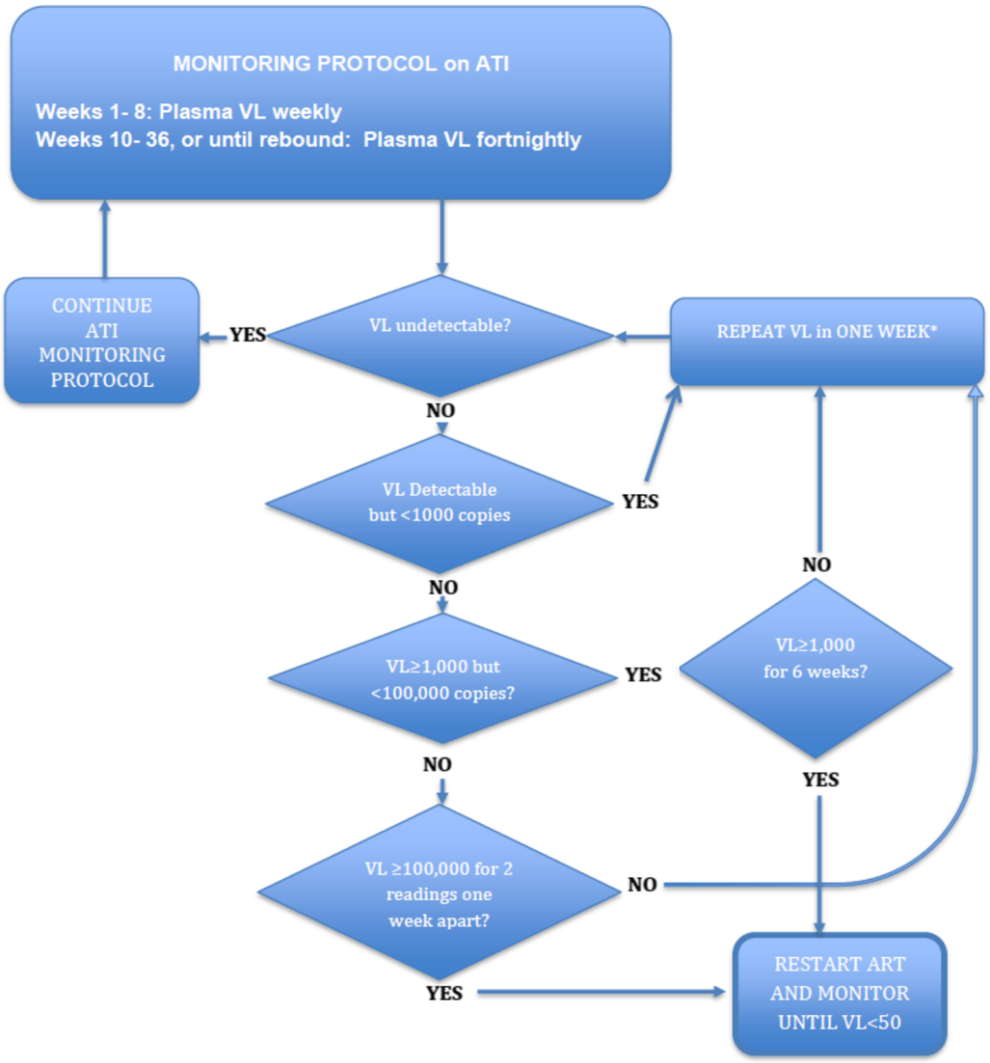
4. Participant preference.

5. Major concerns over risk mitigation for HIV transmission.

6. Positive for COVID-19 (SARS-CoV-2 PCR positive) during ATI.

A.4.1 Sampling of PBMCs and Plasma

Collection of PBMCs and plasma and viral load monitoring were done according to the official trial assessment schedule (see **Appendix Tables 4.1a-c** and **Appendix Figure A.1** respectively). In addition to these, extra bloods were taken as exploratory research samples in order to fulfil the tertiary objectives of the RIO trial (discussed previously). In participants whose plasma viral load remained undetectable, additional plasma (4 x 9ml ACD tubes) were taken for research purposes. A summary of the key research assays that were/will be performed are outlined in the **Appendix (Table 4.2).**



*Recurrent low grade viraemia (i.e <1000) or consecutive VL results that are inconsistently above and below 1,000 or 100,000 copies will need discussion with trial investigators.

Figure A.4. Monitoring protocol on analytical treatment interruption (ATI) for the RIO clinical trial.

Appendix Chapter A: References

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Appendices

Section B.1 Eligibility criteria for the RIO Clinical Trial

Inclusion criteria

- Aged ≥ 18 to ≤ 60 years old at screening.
- Able to give informed written consent including consent to long-term follow-up.
- Willing and able to comply with visit schedule and provide blood sampling.
- Started ART within a maximum of 6 months of estimated date of primary HIV infection, based on one of the following six criteria:
    1. Positive HIV-1 serology within a maximum of 24 weeks of a documented negative HIV-1 serology test result (can include point of care test (POCT) using blood for both tests). The estimated time of infection is taken as the midpoint between the dates of the negative HIV-1 serology or POCT test and positive HIV test at diagnosis, unless there was clear evidence for a transmission event, in which case that date will be taken.
    2. The date of a positive p24 antigen result and a negative HIV antibody test.
    3. The date of a negative HIV antibody test with either detectable HIV RNA or proviral DNA.
    4. Recent infection testing algorithm (RITA) reported as 'Incident' confirming the HIV-1 antibody avidity is consistent with recent infection (within the preceding 16 weeks). The estimated date of infection is assumed to be 2 months prior to the date of the incident test result.
    5. The date of a weakly reactive or equivocal 4th- generation HIV antibody antigen test.
    6. The date of an equivocal or reactive antibody test with less than 4 bands on western blot.
- Stable on ART with suppressed undetectable HIV viral load (VL) 'target not detected' using local assays for ≥1 year.
- No evidence of viral insensitivity to either 10-1074 or 3BNC117 antibodies based on proviral sequen- cing algorithm.
- Hepatitis B virus (HBV) surface antigen or HBV DNA, hepatitis C virus (HCV) Ag or HCV RNA negative or HCV anti-core antibody negative.
- No significant comorbidities.
- Nadir CD4 > 350 cells/μL.
- Current CD4 count > 500 cells/μL or CD4:CD8 ratio >1.
- On integrase inhibitor (INSTI) or boosted protease inhibitor (bPI)-based regimen at time of randomisation, if previously on non-nucleoside re- verse transcriptase inhibitor (NNRTI) has switched at least 4 weeks prior to randomisation.
- Adequate haemoglobin (≥120 g/L for males, ≥110 g/ L for females).
- Weight ≥ 50 kg.
  Females capable of becoming pregnant must agree to use hormonal contraception, intrauterine device, intrauterine hormone-releasing system, or to complete abstinence from at least 2 weeks before the first bNAb/placebo infusion and for 20 months after the last bNAb infusion.
- Participants must have been vaccinated against coronavirus (COVID-19), at least 4 weeks prior to enrolment.

B.2 Exclusion Criteria

- Previous ischaemic heart disease (ST or non-ST myocardial infarction, QRISK3 10-year cardiovascular risk > 20%, stable angina, unstable angina, stroke)
- Any current or past history of malignancy, excluding squamous cell skin cancers.
- Concurrent opportunistic infection or other comorbidity or comorbidity likely to occur during the trial, e.g. malabsorption syndromes, autoimmune disease.
- Any contraindication to receipt of British HIV Association (BHIVA) recommended combination antiretrovirals [14].
- HTLV-1 co-infection
- SARS-Cov-2 infection confirmed by SARS-Cov-2 RT-PCR positive result from nasopharyngeal swab up to 72 h prior to randomisation/dosing visit. Individuals at high risk from severe COVID-19 disease who may be defined in accordance with National Health Service England guidance [15] as vulnerable and shielded (as per the view of participant's physician)
- Current or planned systemic immunosuppressive therapy (inhaled or topical corticosteroids are allowed.)
- Participation in any other clinical trial of an experimental agent or any non-interventional study where additional blood draws are required; participation in an observational study is permitted.
- History of anaphylaxis or severe adverse reaction to antibody infusions, or hypersensitivity to 3BNC117- LS or 10-1074-LS or to or any constituent products or excipients
- Treatment with intravenous immunoglobulin or other monoclonal antibody treatments planned during the duration of the trial.
- Clinically significant abnormal blood test results at screening including:
  a. Moderate to severe hepatic impairment as defined by significant liver impairment with evidence of advanced fibrosis or cirrhosis with decompensation.

B.3 List of participating clinical sites in the RIO Clinical Trial

1. Imperial College Healthcare NHS Trust, London, UK
2. Guy's and St Thomas Hospital NHS Foundation Trust, London, UK
3. Central and North West London NHS Foundation Trust, London, UK
4. Royal Free London Hospital NHS Foundation Trust, London, UK
5. Chelsea and Westminster Hospital NHS Foundation Trust, London, UK
6. Barts Health NHS Trust, London, UK
7. Brighton and Sussex University Hospitals NHS Trust, Brighton, UK

B.4 Description of bNAbs used in the RIO clinical trial

- The RIO trial involves administration of two bNAbs: 10- 1074-LS and 3BNC117-LS, and placebo infusions (0.9% sodium chloride solution). 3BNC117-LS is a recombin- ant, fully human monoclonal antibody (mAb) of the IgG1κ isotype that specifically binds to the CD4 binding site (CD4bs) within HIV-1 envelope gp-120.
- 10-1074-LS is a recombinant, fully monoclonal antibody of the IgG1λ isotype that specifically binds to the base of the V3 loop within the HIV external membrane glycoprotein, gp120.
- The original antibodies 3BNC117 and 10-1074 were identified and cloned at the Rockefeller University from an HIV-positive individual. Both 3BNC117-LS and 10- 1074-LS differ from the original parental antibodies by two amino acid substitutions which were introduced for the purpose of extending their biological half-life.

- Placebo arm B will receive 0.9% sodium chloride (normal saline) infusion identical in appearance and volume to the bNAb infusions.
- Investigator brochures (IB) for 10-1074-LS and 3BNC117-LS IB will be used in the trial.
- Both bNAbs will be stored at 5 ± 3 °C, and each bNAb vial contains a nominal 150 mg/ml of bNAb protein in a 1.0-mL buffered solution.

bNAb dosage and administration

- 3BNC117-LS and 10-1074-LS will each be administered at a dose of 30 mg/kg and 10 mg/kg respectively. Prior to infusion, each IMP is diluted to a final volume of 100 mL in a single normal saline injection. Each IMP is then administered as a separate intravenous infusion over a period of approximately 30 min per bNAb. IMP infusion should be administered through a 0.2 or 0.22 micron in- line filter.

B.5 Sample Size and statistical power considerations

- Underlying viral rebound rates at 36-weeks of 90% and 55% in the control and intervention groups respectively, (i.e. 10% and 45% free from rebound by 36 weeks; hazard ratio 0.35), will be detected with 90% power using a two-sided log-rank-test at the 5% significance level provided there are 31 participants per arm and no loss to follow-up or return to ART treatment prior to 36-week primary outcome. We propose to recruit 72 participants in order to provide an allowance of approximately 10% return to ART treatment, and loss to follow- up, prior to the earlier of either 36-weeks or a primary event.

- For patients in arm B we will look at the within-person difference in 'length of time undetectable (in days)' between first and second period. Assuming a standard deviation of 14 days and a conservative correlation between the number of days in the first and second period of 0.7, 36 patients will allow detecting a difference of 6.6 days between the two periods with 90% power and two sided alpha 0.05, including 15% dropout rate.

- The primary endpoint of the previous main trial design compares the time to viral load rebound by study arm out to week 36 after randomisation. The change in second dual bNAb dosing at week 20 will not impact the overall study power comparing placebo with dual bNAb, even though the primary endpoint will now be reported at week 20. This is because we expect to see the majority of those in the placebo arm rebounding before 20 weeks, and therefore the shift from 36 to 20 weeks has no significant impact on the statistical analysis. Unpublished data from the PITCH study in which participants with treated early HIV infection (I.e., very similar to the placebo arm) stop therapy confirm that rebound almost always occurs within 8 weeks (Figure 9).

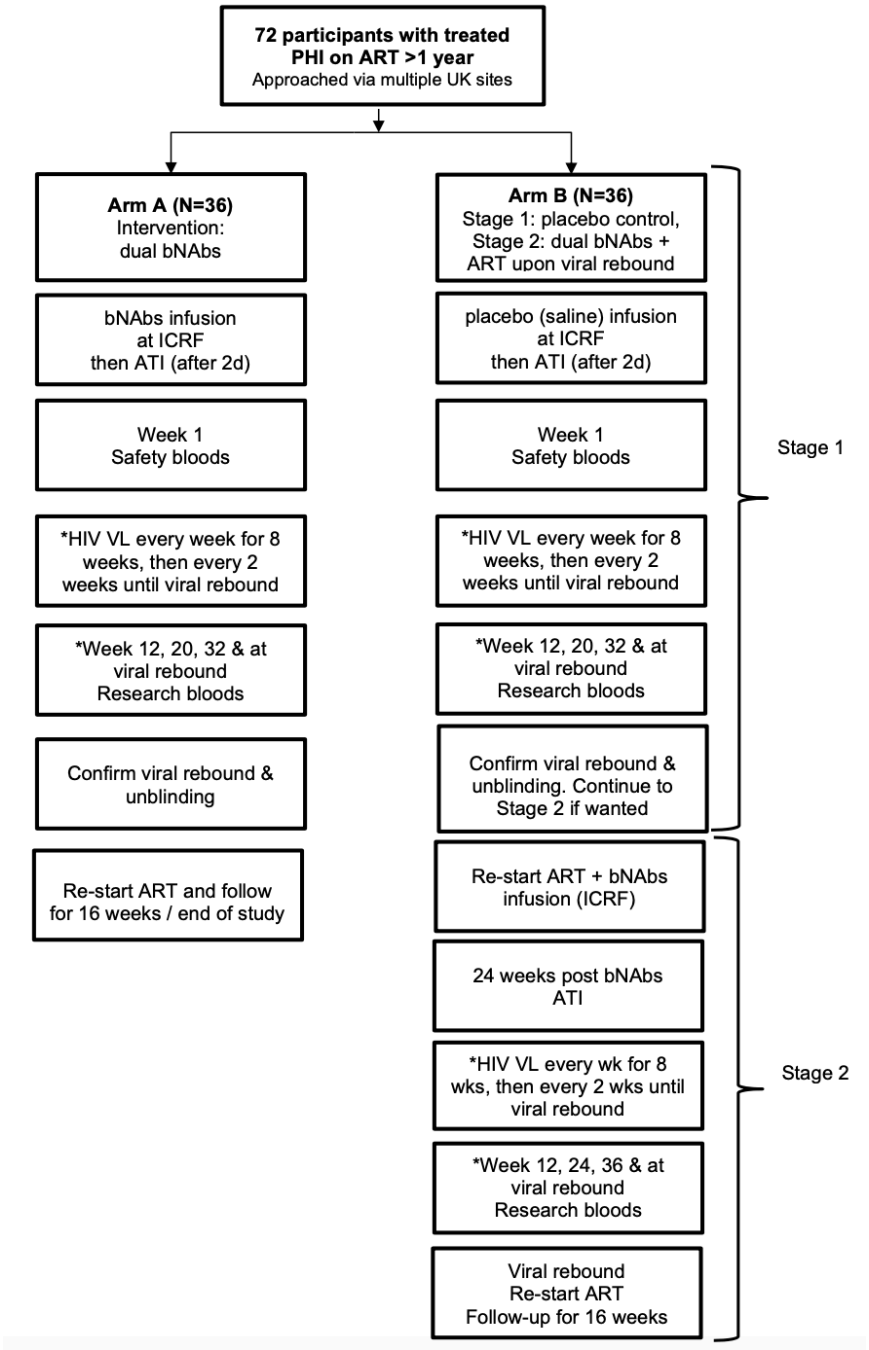


Figure B.1. Summary of trial design for Arm A and Arm B, including details on blood taking schedule, Stage 1 and Stage 2 descriptions and viral load measurement schedules.

Assessment	Blood volume (ml)	Screening[1]	Enrolment[1]	Randomise/ Dosing	ATI start[2]	Wk 1	Wk 2	Wk 3	Wk 4	Wkly (VL visit)	Wk 8	Wk 10	Wk 12	2-wkly to Wk 18 (VL visit)	Wk 20	4-wkly Wk 24 to 28 (VL visit)	Wk 32	4-wkly 36, 40+ (VL visit)	Wk 44[3]	Viral rebound & re-start ART visit[4,7]	2-wkly until und/able (VL visit)	4-wkly to week 16 (VL visit)	End of study / 16 wks on ART	Annual post study visit[5]	LINS[5] VL
Visit		*S1*	*S2*	*A1*	*A2*	*A3*	*A4*	*A5*	*A6*	*A7-A9*	*A10*	*A11*	*A12*	*A13-A15*	*A16*	*A17-A18*	*A19*	*A20-A21*	*A22+*	*AU1*	*AU2-X*	*AU3-Y*	*AU4*	*AU5*	*U1*
Timing		*-90d to -10d*	*-3d to -1d*	*-4-to-2d*	*0*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*R0*	*+/- 5 d*	*+/- 5 d*	*+/- 5 d*	*+/- 14d*	*N/A*
Consent		x																							
Eligibility		x	x																						
ART interruption																									
Re-start ART					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x					
Physical examination		x		x	x	x														x	x (wk 4 only)				
Vital signs (BP, HR, temperature, weight, height at screening only)		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
COVID-19 symptoms check		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Medical & medication History		x																							
Unblinding																				x					
QOL questionnaire		x						x															x		
ECG		x																							
Adverse Event tracking		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
bNAb/placebo infusion				x																					
Blood tests (local labs)																									
HBVsAg/DNA	6	x																							
HCV Ag/RNA		x																							
HTLV-1		x																							
Rapid Plasma Reagin (RPR)		x																							
Clotting (INR)	4	x																							
Haematology (WCC&differential, Hb, platelets, FBC)	4	x		x		x		x		x		x		x		x		x			x (wk 12)				
LFTs (ALT, Bili, ALP, Albu, AST)	4	x		x		x		x		x		x		x		x		x			x (wk 12)				
Biochemistry		x		x		x		x		x		x		x		x		x			x (wk 12)				
CD4 count, CD8 count, CD4:CD8 ratio	4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
HIV viral load	7	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
SARS-Cov-2 antibodies[6]	7	x																							
Urine test / dipstick (protein/blood)		x		x		x		x		x		x													
Pregnancy test (if applicable)		x		x																			x		
uPCR		x																							
[6]SARS-Cov-2 Swab (PCR)			x																						
Research bloods																									
bNAb sensitivity[8]		20																							
Viral genotype (resistance)	7																			7					
Immunology, sequencing, gene expression	45-200			200						45		45		200	45 (wk 16)	200	45	200	200*	200			200	45	
Plasma for ART drug levels and serum for bNAb levels & ADA	16			16			16		16		16		16	16 (wk 16)	16	16	16	16	16	16			16		
HIV reservoir (Q4PCR assay)	45			45									45		45		45		45*	45			45		
Bleed Volume RESEARCH		*20*		*261*			*16*		*61*		*61*		*261*	*61 (wk16)*	*261*	*61*	*261*	*61*	*261*	*268*			*261*	*45*	
Bleed Volume CLINICAL		*36*	*11*	*19*	*11*	*19*	*19*	*11*	*19*	*11*	*19*	*11*	*15*	*36*	*19*	*11*	*19*	*11*	*19*	*11*	*11*	*19*	*11*		*11*
Sub-total research	2159																								
Sub-total clinical	379																								
Total	**2538**																								

Appendix Table B.1a. Trial assessment schedule for Arms A and B for Stage 1 of the RIO clinical trial.

Assessment	Blood volume (ml)	Viral rebound & re-start ART visit[4]	bNAb DOSE (one week later)[7]	Wk 1	Wk 2	Wk 3	Wk 4	Wkly (VL visit)	Wk 8	2-wkly (VL visit) until 24 wks on ART[10]	SECOND ATI -	Wk 1 after ATI	Wk 2 after ATI	Wk 3 after ATI	Wk 4 after ATI	Wkly (VL visit)	Wk 8 after ATI	Wk 10 after ATI	Wk 12 after ATI	2-wkly Wk 14 to22 (VL visit)	Wk 24 after ATI	4-wkly Wk 28 & 32 (VL visit)	Wk 36 after ATI	4-wkly 40, 44+[3] (VL visit)	Viral rebound & re-start ART visit	2-wkly until und/able (VL visit)	4-wkly once suppressed (VL visit)	End of study / 16 wks on ART	Annual post study visit[5]	LINS[5] VL
Visit		*B1*	*B2*	*B3*	*B4*	*B5*	*B6*	*B7-9*	*B10*	*B11 - B17*	*B18*	*B19*	*B20*	*B21*	*B22*	*B23-B26*	*B27*	*B28*	*B29*	*B30-B34*	*B35*	*B36-B37*	*B38*	*B39-B40+*	*C1*	*C1-X*	*C1-Y*	*C4*	*C5*	*U1*
Timing		*R0*	*+/- 10d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 5d*	*24 wks +/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*R0*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 14d*	*N/A*
ART interruption																														
[9]On ART		x	x	x	x	x	x	x	x																					
Re-start ART		x									x	x	x	x	x	x	x	x	x	x	x	x	x	x						
Physical examination		x	x	x																					x	x (wk 4 only)				
Vital signs (BP, HR, temperature, weight, height at screening only)		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
COVID-19 symptoms check		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Unblinding		x																												
QOL questionnaire																											x			
Adverse Event tracking		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
bNAb infusion			x																											
Blood tests (local labs)																														
Haematology (WCC&differential, Hb, platelets, FBC)	4	x		x	x		x		x			x	x		x		x		x		x		x			x (wk 12)			
LFTs (ALT, Bili, ALP, Albu, AST)	4	x	x	x	x		x		x			x	x		x		x		x		x		x			x (wk 12)			
Biochemistry		x		x	x		x		x			x	x		x		x		x		x		x			x (wk 12)			
CD4 count, CD8 count, CD4:CD8 ratio	4	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
HIV viral load	7	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
Urine dipstick (protein, blood)		x	x				x		x			x			x		x													
Pregnancy test (if applicable)		x	x																								x			
uPCR		x																												
[6]SARS-Cov-2 Swab (PCR)																														
Research bloods																														
Viral genotype (resistance)	7	7																							x					
Immunology, sequencing, gene expression	45-200		200		45		45		45		200			45		45		200	45 (wk 16&20)	200	45	200	45	200			200	45	
Plasma for ART drug levels and serum for bNAb levels & ADA	16		16				16		16		16		16			16		16	16 (wk 16&20)	16	16	16	16	16			16		
HIV reservoir (Q4PCR assay)	45		45								45					45		45		45		45		45			45		
Bleed Volume RESEARCH		*7*	*261*		*45*		*61*		*61*		*261*		*16*		*61*		*61*		*261*	*61 (wk 16&20)*	*261*	*61*	*261*	*61*	*261*			*261*	*45*	
Bleed Volume CLINICAL		*19*	*15*	*19*	*19*	*11*	*19*	*11*	*19*	*11*	*11*	*19*	*19*	*11*	*19*	*11*	*19*	*11*	*19*	*11*	*19*	*11*	*19*	*11*	*19*	*11*	*11 [19/wk12]*	*11*		*11*
Sub-total research	2306																													
Sub-total clinical	405																													
Total	**2711**																													

Appendix Table B.1b. Trial assessment schedule for Arms A and B for Stage 2 of the RIO clinical trial.

Assessment	Blood volume (ml)	Screening[1]	Enrolment[1]	Randomise/ Dosing	ATI start[2]	Wk 1	Wk 2	Wk 3	Wk 4	Wkly (VL visit)	Wk 8	Wk 10	Wk 12	2-wkly to Wk 18 (VL visit)	Week 20+	2nd dose	Week 1 after 2nd dose	Wk 4 after 2nd dose	2-wkly to wk 12 (wks 6-10 after 2nd dose)	Wk 12 after 2nd dose	4-wkly to wk20 (wks 16 - 20 after 2nd dose)	Week 24 after 2nd dose	4-wkly (wks 28 - 32+ after 2nd dose)	Week 36 after 2nd dose	4 wkly (wks 40, 44+)[3]	E15-16+	Viral rebound & re-start ART visit[4,7]	2-wkly until und/able (VL visit)	4-wkly to week 16 (VL visit)	End of study / 16 wks on ART	Annual post study visit[5]	LINS[5] VL
Visit		*S1*	*S2*	*A1*	*A2*	*A3*	*A4*	*A5*	*A6*	*A7-A9*	*A10*	*A11*	*A12*	*A13-A15*	*E1*	*E2*	*E3*	*E4*	*E5-E7*	*E8*	*E9-10*	*E11*	*E12-E13*	*E14*	*E15-16+*		*AU1*	*AU2-X*	*AU3-Y*	*AU4*	*AU5*	*U1*
Timing		*-90d to -10d*	*-3d to -1d*	*-4-to-2d*	*0*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 2d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+4wks*	*+/- 2d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*	*+/- 5d*		*R0*	*+/- 5 d*	*+/- 5 d*	*+/- 5 d*	*+/- 14d*	*N/A*
Consent		x													x**																	
Eligibility		x	x																													
ART interruption																																
Re-start ART					x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x						
Physical examination		x		x	x	x										x	x									x	x(wk 4 only)				
Vital signs (BP, HR, temperature, weight, height at screening only)		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
COVID-19 symptoms check		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			x
Medical & medication History		x																														
Unblinding																										x						
QOL questionnaire		x					x																						x			
ECG		x																														
Adverse Event tracking		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
bNAb/placebo infusion				x												x																
Blood tests (local labs)																																
HBVsAg/DNA	6	x																														
HCV Ag/RNA		x																														
HTLV-1		x																														
Rapid Plasma Reagin (RPR)		x																														
Clotting (INR)	4	x																														
Haematology (WCC&differential, Hb, platelets, FBC)	4	x		x		x	x		x		x		x		x	x	x		x		x	x	x	x				x (wk 12)			
LFTs (ALT, Bili, ALP, Albu, AST)	4	x		x		x	x		x		x		x		x	x												x (wk 12)			
Biochemistry		x		x		x	x		x		x		x		x	x												x (wk 12)			
CD4 count, CD8 count, CD4:CD8 ratio	4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x			x
HIV viral load	7	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x			x
SARS-Cov-2 antibodies[6]	7	x																														
Urine test / dipstick (protein/blood)		x		x		x		x		x		x				x																
Pregnancy test (if applicable)		x		x																									x			
uPCR		x																														
[6]SARS-Cov-2 Swab (PCR)			x																													
Research bloods																																
bNAb sensitivity[8]		20																														
Viral genotype (resistance)	7																									7						
Immunology, sequencing, gene expression	45-200			200					45		45		200	45 (wk 16)	200			45	45 (wk 8)	200	45	200	45	200	45		200			200	45	
Plasma for ART drug levels and serum for bNAb levels & ADA	16			16			16		16		16		16	16 (wk 16)	16			16	16 (wk 8)	16	16	16	16	16	16		16			16		
HIV reservoir (Q4PCR assay)	45			45							45				45					45		45		45			45			45		
Bleed Volume RESEARCH		*20*		*261*			*16*		*61*		*261*		*261*	*61 (wk16)*	*261*			*61*	*61 (wk8)*	*261*	*61*	*261*	*61*	*261*			*268*			*261*	*45*	
Bleed Volume CLINICAL		*36*	*11*	*19*	*11*	*19*	*19*	*11*	*19*	*11*	*19*	*11*	*15*	*36*	*19*	*19*	*19*	*15*	*11*	*15*	*11*	*15*	*15*	*15*	*15*		*11*	*11*	*19*	*11*		*11*
Sub-total research	2543																															
Sub-total clinical	**469**																															
Total	**3011**																															

Appendix Table B.1c. Trial assessment schedule for Arms A and B for the second dose sub-study of the RIO clinical trial.

Table B.2. Summary of key research assays performed as part of the tertiary/exploratory endpoints in the RIO clinical trial.

Assay	Sample	Purpose	Time-point
T cell immunology			• Pre-ART where available • On ART • Pre-bNAb • Fortnightly during ATI • Following viral suppression
HIV-specific T cell intracellular cytokine staining for CD4 and CD8 phenotypic and functional responses	PBMC	Screen CD4 and CD8 T cell responses to HIV in response to bNAbs	
HIV ELISpots: γ-interferon responses with peptides targeted according to ICS responses	PBMC	Higher resolution analyses of specific responses to peptide level and how they are impacted by intervention	
CD8 tetramer responses targeted to specific peptides	PBMC	Further in-depth resolution of CD8 T cell functionality at cellular level	
HLA Class I and II typing (4-digit)	PBMC	Profiling of HLA alleles to shape immune responses and confer advantage/disadvantages	
T cell killing assays	PBMC	Functional killing assay to augment intracellular cytokine staining data	
Natural Killer (NK) cell responses			
Flow cytometry for NK cell phenotype and function	PBMC	Characterisation of NK function and profile in response to bNAbs	
ADCC and NK cell killing	PBMC	Functional assessment of bNAbs using characterised killing protocols	
NK-like population analyses (flow cytometry)	PBMC	High resolution characterisation of cells that fall between the innate and adaptive responses	
Virology			
Next-generation sequencing (NGS) single genome amplification (SGA) of HIV ENV: bNAb sensitivity	PBMC	Env sequencing to screen for bNAb sensitivity	Screening
Full length HIV sequencing	Plasma CD4 T cells	Full length haplotype analysis of viral and proviral populations pre and post intervention to determine correlations with response	• Pre-ART (virus) where available (HEATHER cohort) • On ART, and pre-ATI (provirus) • Rebound (virus) • Post resuppression (provirus)
HIV Integration site analysis	CD4 T cells	Understanding of clonality of viral reservoir and relationship of integration sites to reactivation potential and susceptibility to inhibition	Pre-ATI
HIV DNA and cell-associated RNA quantitation	CD4 T cells	Standard molecular analyses of proviral and HIV transcript quantitation, both surrogate markers of persistent infection and biomarkers of remission and rebound	• Pre-ART where available (HEATHER cohort) • On ART • Pre-bNAb • Pre-ATI • Post resuppression
Host genomics			
Transcriptome profiling* *Initially bulk, but with single-cell 10× resolution dependent on results	CD4 and CD8 T cells NK cells	Full host genomic analyses at bulk and single cell (likely tetramer driven for CD8 cells) to understand host determinants of outcomes	• Pre-ART where available (HEATHER cohort) • On ART • Pre- ATI
Pharmacokinetics and anti-drug antibodies			
bNAb pharmacokinetics Quantitation of 'anti-drug antibodies' (ADA)	Plasma	To determine bNAb plasma concentrations To determine development of inhibitory antibodies against bNAbs	• Post bNAb infusion

Chapter 4

Arm A "Early"- Week 0				Arm A "Early"- Week 24			
HIV protein	OLP number	Peptide sequence	Percentage recognition (%)	HIV protein	OLP number	Peptide sequence	Percentage recognition (%)
GAG	5	EKIRLRPGGKKKYKL	12.5	GAG	5	EKIRLRPGGKKKYKL	12.5
GAG	9	HIVWASRELERFAVN	12.5	GAG	60	AGTTSTLQEQIGWMT	12.5
GAG	66	IYKRWIILGLNKIVR	12.5	POL	108	IKVKQLCKLLRGTKA	12.5
GAG	73	PKEPFRDYVDRFYKT	12.5	POL	111	TKALTEVIPLTEEAE	12.5
GAG	84	ILKALGPAATLEEMM	12.5	POL	112	TEVIPLTEEAELELA	12.5
POL	243	NSDIKVVPRRKAKII	12.5	POL	156	VVSLTDTTNQKTELQ	12.5
ENV	3	NYQHLWRWGTMLLGM	12.5	POL	159	ELQAIHLALQDSGLE	12.5
ENV	167	LWNWFDITNWLWYIK	12.5	POL	160	IHLALQDSGLEVNIV	12.5
ENV	168	FDITNWLWYIKIFIM	12.5	ENV	3	NYQHLWRWGTMLLGM	12.5
ENV	173	IGLRIVFAVLSIVNR	12.5	NEF	20	RPMTYKAAVDLSHFL	12.5
ENV	199	WNLLQYWSQELKNSA	12.5	NEF	22	VDLSHFLKEKGGLEG	12.5
ENV	209	CRAILHIPRRIRQGL	12.5	NEF	29	WVYHTQGYFPDWQNY	12.5
ENV	210	LHIPRRIRQGLERAL	12.5				
NEF	39	VEEANEGENNSLLHP	12.5				
NEF	49	HMARELHPEYYKDC	12.5				

Arm A "Late"- Week 0				Arm A "Late"- Week 24			
HIV protein	OLP number	Peptide sequence	Percentage recognition (%)	HIV protein	OLP number	Peptide sequence	Percentage recognition (%)
GAG	60	AGTTSTLQEQIGWMT	9.1	GAG	5	EKIRLRPGGKKKYKL	9.1
GAG	66	IYKRWIILGLNKIVR	9.1	GAG	60	AGTTSTLQEQIGWMT	9.1
GAG	89	VGGPGHKARVLAEAM	9.1	GAG	66	IYKRWIILGLNKIVR	9.1
POL	70	KDFRKYTAFTIPSIN	9.1	NEF	19	QVPLRPMTYKAAVDL	9.1
NEF	21	YKAAVDLSHFLKEKG	9.1	NEF	21	YKAAVDLSHFLKEKG	9.1
NEF	32	QNYTPGPGIRYPLTF	9.1	NEF	29	WVYHTQGYFPDWQNY	9.1
NEF	33	PGPGIRYPLTFGWCF	9.1	NEF	32	QNYTPGPGIRYPLTF	9.1
				NEF	33	PGPGIRYPLTFGWCF	9.1
				NEF	35	LTFGWCFKLVPVEPE	9.1

Table 4.1. OLP recognition at Weeks 0 and 24 in both the “early” and “late” rebounders”. OLPs are written across rows with their respective HIV protein, number, and peptide sequence. To examine for immune dominance, we set an arbitrary recognition threshold of 5% of greater. Only the highest recognised peptides are shown here.

Arm B - Week 0				Arm B - Week 20			
HIV protein	OLP number	Peptide sequence	Percentage recognition (%)	HIV protein	OLP number	Peptide sequence	Percentage recognition (%)
NEF	26	IYSQKRQDILDLWVY	16.7	NEF	26	IYSQKRQDILDLWVY	23.3
NEF	27	KRQDILDLWVYHTQG	16.7	NEF	27	KRQDILDLWVYHTQG	23.3
GAG	64	NPPIPVGEIYKRWII	13.3	GAG	5	EKIRLRPGGKKKYKL	13.3
NEF	20	RPMTYKAAVDLSHFL	13.3	GAG	64	NPPIPVGEIYKRWII	13.3
GAG	66	IYKRWIILGLNKIVR	10	ENV	209	CRAILHIPRRIRQGL	13.3
ENV	209	CRAILHIPRRIRQGL	10	ENV	210	LHIPRRIRQGLERAL	13.3
ENV	210	LHIPRRIRQGLERAL	10	NEF	29	WVYHTQGYFPDWQNY	13.3
NEF	28	ILDLWVYHTQGYFPD	10	GAG	65	PVGEIYKRWIILGLN	10
NEF	29	WVYHTQGYFPDWQNY	10	GAG	66	IYKRWIILGLNKIVR	10
NEF	33	PGPGIRYPLTFGWCF	10	GAG	75	VDRFYKTLRAEQASQ	10
GAG	5	EKIRLRPGGKKKYKL	6.7	ENV	211	RRIRQGLERALL	10
GAG	63	WMTNNPPIPVGEIYK	6.7	NEF	20	RPMTYKAAVDLSHFL	10
GAG	65	PVGEIYKRWIILGLN	6.7	NEF	28	ILDLWVYHTQGYFPD	10
GAG	75	VDRFYKTLRAEQASQ	6.7	NEF	33	PGPGIRYPLTFGWCF	10
GAG	83	DCKTILKALGPAATL	6.7	NEF	34	IRYPLTFGWCFKLVP	10
GAG	85	LGPAATLEEMMTACQ	6.7	GAG	9	HIVWASRELERFAVN	6.7
POL	25	RWKPKMIGGIGGFIK	6.7	GAG	35	NLQGQMVHQAISPRT	6.7
POL	185	MASDFNLPPVVAKEI	6.7	GAG	43	IPMFSALSEGATPQD	6.7
POL	209	GSNFTSTTVKAACWW	6.7	GAG	61	STLQEQIGWMTNNPP	6.7
ENV	211	RRIRQGLERALL	6.7	GAG	63	WMTNNPPIPVGEIYK	6.7
NEF	21	YKAAVDLSHFLKEKG	6.7	GAG	83	DCKTILKALGPAATL	6.7
NEF	34	IRYPLTFGWCFKLVP	6.7	GAG	84	ILKALGPAATLEEMM	6.7
				GAG	86	ATLEEMMTACQGVGG	6.7
				POL	225	IHNFKRKGGIGGYSA	6.7
				POL	226	KRKGGIGGYSAGERI	6.7
				POL	228	YSAGERIVDIIATDI	6.7
				POL	240	LWKGEGAVVIQDNSD	6.7
				ENV	199	WNLLQYWSQELKNSA	6.7
				NEF	4	WPTVRERMRRAEPAA	6.7
				NEF	17	EVGFPVRPQVPLRPM	6.7
				NEF	21	YKAAVDLSHFLKEKG	6.7

Table 4.2. OLP recognition at Weeks 0 and 24 in both the “early” and “late” rebounders”. OLPs are written across rows with their respective HIV protein, number, and peptide sequence. To examine for immune dominance, we set an arbitrary recognition threshold of 5% of greater. Only the highest recognised peptides are shown here.

Lasso Regression – Dose + Week 0 responses only	
Variable	**Coefficient**
Lambda	0.141
Dose	-1.14
AIM Gag CD4+ Week 0	-
AIM Gag CD8+ Week 0	-1.92
Proliferation Gag CD4+ Week 0	-
Proliferation Gag CD8+ Week 0	-
ELISpot Gag Week 0	0.00114
Lasso Regression – Dose + Week 12 responses only	
Variable	**Coefficient**
Lambda	0.093
Dose	-1.00
AIM Gag CD4+ Week 12	-
AIM Gag CD8+ Week 12	-2.95
Proliferation Gag CD4+ Week 12	-
Proliferation Gag CD8+ Week 12	-
ELISpot Gag Week 12	-
Lasso Regression – Dose + Week 24 responses only	
Variable	**Coefficient**
Lambda	0.18
Dose	-
AIM Gag CD4+ Week 24	-
AIM Gag CD8+ Week 24	-2.79
Proliferation Gag CD4+ Week 24	-0.094
Proliferation Gag CD8+ Week 24	-
ELISpot Gag Week 24	-0.0014

Table 4.3. Lasso regressions performed using T-cell responses at Weeks 0, 12 and 24 as variables, along with bNab doses received. Numbers indicate the coefficient size of each variable respectively; larger coefficients indicate that the variable is a more significant predictor of time to viral rebound (from last bNAb dose). Dashes represent variables with a coefficient of "zero" (i.e are not significant predictors of the outcome). Lambda values represent the fitting of the model and controls the level of regularisation. Optimal lambda values were calculated in R software for calculation of coefficients. Variables with significantly non-zero coefficients were taken forward and run in a Cox proportional hazards model.