CHARACTERISATION OF HIV RESERVOIRS IN BLOOD AND GUT ASSOCIATED LYMPHOID TISSUE

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of Imperial College London

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Declaration

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John Thornhill

July 2018
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Acknowledgements

I would like to thank all the volunteers who participated in this research, your altruism, and enthusiasm is admirable, and without it, this work would not have been possible. I am indebted to the HEATHER study team, the St. Mary’s Hospital Endoscopy Unit, the Translational Gastroenterology Unit in Oxford, as well as the Jefferiss Wing and Clinical Trials Centre staff at St Mary’s Hospital, London. In particular, Jonathan Hoare, Simon Peake, Rob Goldin, Heather Lewis, Kristin Kuldanek, Kanta Mahay, Rebecca Hall, Julie Fox and Sarah Fidler for their help in running the HEATHER gut study. I am grateful to all the staff and students at the Peter Medawar Building for Pathogen Research in Oxford who have been a wonderful source of friendship, support, advice and encouragement. In particular, to Cathy DL, Chan P, Chloe T, Chris W, Emily A, Emily H, Genevieve M, Helen B, Kate S, Kate W, Jodi G, Lian L, Masa M, Matt J, Mattie P, Morgane G, Natalia O, Nicola R, Prabh P, Tianqi & Tony B who have been great lab buddies in some shape or form! I would also like to thank Carolina Herrera, Graham Taylor, Jonathan Underwood and Bora Mora Peris at Imperial College for their expert advice and support in navigating the PhD experience. I would also like to acknowledge the Medical Research Council (MRC) and the British HIV Association for their funding of the HEATHER gut study, and the MRC for their support through a Clinical Research Training Fellowship.

I am extremely grateful to my supervisors Prof Sarah Fidler & Prof John Frater whose intellect, wise counsel and support made my PhD a thoroughly fruitful, positive and enjoyable experience.

To my family, I am eternally grateful for a lifetime of love & encouragement.

Lastly, Tiago, you are always my source of inspiration, humour, wisdom and love. Thank you for your unwavering support. Amo-te para sempre.
**Abstract**

Antiretroviral therapy (ART) has dramatically improved survival for people living with HIV (PLWH). However, a stable pool of latently infected cells precludes a cure. Initiation of ART during primary infection (PHI) has been used as a strategy to limit seeding of the HIV reservoir and enhance immune recovery. Gut-associated lymphoid tissue (GALT) is targeted early in HIV infection with significant immune damage and remains one of the principal sites of latent HIV infection on ART. However, data characterising immune recovery & the HIV reservoir in the GALT of these early treated individuals are lacking.

Using concurrent peripheral blood (PBMC) and biopsy samples from the terminal ileum and rectum, I characterised the HIV reservoir across anatomical sites in treated PHI. I tested the hypothesis that ART initiated in PHI results in normalisation of immune parameters across anatomical sites. Furthermore, I characterised cellular populations known to support HIV persistence and tested for associations between HIV DNA and gut markers of inflammation and immune dysfunction.

This thesis provides evidence of compartmentalised immune recovery in treated PHI with enhanced recovery in the PBMC compartment compared to GALT. Furthermore, the burden of latent HIV infection was highest in the terminal ileum compared to all other sites. Markers of inflammation, bacterial translocation and TFH lineage associated with HIV DNA in GALT while markers of T cell exhaustion did not.

Even when started in PHI, ART did not normalise markers of immune function in GALT, and HIV DNA persisted at high levels in GALT despite years of suppressive ART. Bacterial translocation and the B cell - T cell interaction may contribute to HIV persistence in GALT. A better understanding of the drivers of viral persistence and immune dysfunction in GALT can inform the design of future HIV cure approaches.
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Abbreviations

AIDS  Acquired immunodeficiency syndrome
ADCC  Antibody-dependent cellular cytotoxicity
APOBEC  Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ART  Antiretroviral therapy
Ascl-2  Achaete-scute complex homolog-2
CD  Cluster of differentiation
cGAS  Cyclic GMP-AMP synthase
CHI  Chronic HIV infection
CPM  Copies per million
ddPCR  Digital droplet polymerase chain reaction
ddPCR  Digital droplet polymerase chain reaction
ELISA  Enzyme-linked immunosorbent assay
FBC  Full blood count
fCD8  Follicular cytotoxic CD8 T cell
G6PD  Glucose-6-phosphate dehydrogenase deficiency
GALT  Gut-associated lymphoid tissue
GFR  Glomerular filtration rate
GI  Gastrointestinal
HDAC  Histone deacetylase
HIV  Human immunodeficiency virus
HLA  Human leukocyte antigen
IBS  Irritable bowel syndrome
ICOS  Inducible T cell co-stimulator
ICRs  Immune checkpoint receptors
IFN-γ  Interferon-γ
IL  interleukin
INR  International normalised ratio
IP-10  Interferon gamma-induced protein 10
Lag-3  Lymphocyte-activation gene 3
LRAs  Latency reversing agents
LTR  Long terminal repeat
mAb  Monoclonal antibody
MAdCAM-1  Mucosal vascular addressin cell adhesion molecule-1
MCP-1  Monocyte chemoattractant protein 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MHC</td>
<td>Major-histocompatibility complex</td>
</tr>
<tr>
<td>MIP-1 α</td>
<td>Macrophage inflammatory protein-1α</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>Macrophage inflammatory protein-1β</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal mononuclear cells</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
</tr>
<tr>
<td>msRNA</td>
<td>Multiply spliced RNA</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human primates</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-Nucleoside Reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside/Nucleotide Reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive sleep apnea</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral mononuclear blood cells</td>
</tr>
<tr>
<td>PC</td>
<td>Principal Component</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death-1</td>
</tr>
<tr>
<td>pDCs</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PLWH</td>
<td>People living with HIV</td>
</tr>
<tr>
<td>PLWH</td>
<td>People living with HIV</td>
</tr>
<tr>
<td>PR</td>
<td>Per rectum</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>PTC</td>
<td>Post-treatment control</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>QVOA</td>
<td>Quantitative viral outgrowth assay</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>sCD14</td>
<td>Soluble CD14</td>
</tr>
<tr>
<td>sCD163</td>
<td>Soluble CD163</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>T ileum</td>
<td>Terminal ileum</td>
</tr>
<tr>
<td>TCM</td>
<td>Central memory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>T EM</td>
<td>Effector memory T cells</td>
</tr>
<tr>
<td>TFH</td>
<td>T Follicular helper cells</td>
</tr>
<tr>
<td>TIGIT</td>
<td>T cell immunoreceptor with Ig and ITIM domains</td>
</tr>
<tr>
<td>TILDA</td>
<td>Tat/rev Induced Limiting Dilution Assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tim-3</td>
<td>T-cell immunoglobulin domain and mucin domain-3</td>
</tr>
<tr>
<td>T&lt;sub&gt;N&lt;/sub&gt;</td>
<td>Naïve T cells</td>
</tr>
<tr>
<td>T&lt;sub&gt;REG&lt;/sub&gt;</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>T&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>Tissue-resident T cells</td>
</tr>
<tr>
<td>T&lt;sub&gt;TM&lt;/sub&gt;</td>
<td>Transitional memory T cells</td>
</tr>
<tr>
<td>uPCR</td>
<td>Urine protein creatinine ratio</td>
</tr>
<tr>
<td>usRNA</td>
<td>Unspliced RNA</td>
</tr>
<tr>
<td>VL</td>
<td>Viral load</td>
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Chapter 1

Introduction

1.1. HIV epidemiology

The Human Immunodeficiency Virus (HIV) has been one of the most significant infectious challenges of the late 20th and early 21st centuries. The first case reports of pneumocystis pneumonia in gay men, which would subsequently be recognised as an opportunistic infection of gay-related immune deficiency (GRID), now known as the acquired immune deficiency syndrome (AIDS), were published in the MMWR reports in 1981. By 1983, Barre-Sinoussi et al. had identified a T lymphotrophic retrovirus as the likely causative organism of AIDS. During the early 1980s treatment options were limited and the prognosis for people living with HIV/AIDS was poor and primarily focused on the treatment of opportunistic infections and palliative care. The initial medical and scientific challenges focused on case identification, understanding the natural history, delineating the mode of transmission and development of a diagnostic antibody test. Since its initial discovery, our understanding of the HIV virus has increased exponentially; this has led to significant developments in HIV therapeutics and clinical care, with a dramatic improvement in the prognosis for people living with HIV. However, there is no cure for HIV and the number of individuals living with the disease today is the greatest since the epidemic began.

There are currently an estimated 37.6 million individuals globally living with HIV, and 35 million people have died from HIV related illness since the beginning of the epidemic. The highest burden of HIV infection is in low and middle-income countries in Sub-Saharan Africa and South East Asia, which together account for almost 80% of people living with HIV globally. A modest decrease in new infections has been observed since 2010, this is predominately due to decreased new infections in Southern Africa. However, a considerable (approximately 60%) increase in new infections has been observed in Central Asia and Eastern Europe. Key at-
risk groups of individuals continue to account for a significant proportion (44% in 2015) of new infections globally, these include men who have sex with men (MSM), people who inject drugs, people in prisons, sex workers and their clients, young women particularly in sub-Saharan Africa and transgender people. Two distinct HIV viruses, namely HIV-1 and HIV-2, are responsible for disease in humans. Both HIV-1 and HIV-2 were the result of the zoonotic transfer of viruses affecting chimpanzees and sooty mangabeys respectively. Globally, HIV-1 is responsible for the vast majority of disease in humans and is the focus of this thesis; any future reference to HIV relates to HIV-1 unless otherwise specified. Phylogenetically, HIV-1 can be classified into four groups M, N, O, and P. Each of these groups reflects the introduction of a different simian immunodeficiency virus (SIV) from naturally infected primates into humans. HIV group M is the only group to have undergone pandemic spread in humans, group O accounts for less than 1% of global infections, while groups N and P account for a handful of cases each. Rapid viral turnover and highly error-prone replication has resulted in the enormous genetic variation seen in HIV group M. There are currently nine subtypes or clades of HIV group M (A-D, F-H, J, K). In addition, hybrids of these clades exist and are called circulating recombinant forms (CRFs). HIV clades and CRFs have distinct geographical spread, and have evolved in response to different host and viral factors; while some studies have suggested that different clades have different impacts on disease progression.
1.2. The HIV Life Cycle

HIV is a single-stranded enveloped RNA retrovirus. The HIV-1 genome consists of two identical 9.2 kb single-stranded RNA molecules within the virion, while the persistent form of HIV is proviral double-stranded DNA within infected cells. There are four main regions in the HIV genome. Firstly, the Long Terminal Repeat (LTR), which are identical sequences which flank each end of the genome. The LTR is a regulatory region and is responsible for driving HIV gene expression. Secondly, the gag-pol gene encodes two polyprotein precursors. These include the proteins of the nucleocapsid, other structural proteins and three viral enzymes: protease, reverse transcriptase and integrase. Thirdly, the env gene encodes the exterior gp120 protein and the transmembrane protein - gp41. Finally, the fourth region includes the HIV accessory genes: tat, rev, nef, vif, vpr and vpu.

HIV utilises cell surface proteins, primarily CD4, for entry into human cells. The initial entry step involves an interaction between the HIV envelope glycoprotein gp120 and the CD4 molecule on the cell surface. This, in turn, allows gp120 to interact with one of the chemokine receptors CCR5 or CXCR4 inducing a conformational change in envelope glycoproteins, ultimately allowing for the dissociation of gp120 from gp41. The gp41 protein is responsible for the fusion of viral and cellular membranes and as a result the release of the viral core in the cell cytoplasm. Single-stranded HIV RNA in the cytoplasm is then available for reverse transcription into double-stranded complementary DNA c(DNA). Retroviruses require integration into the host genome to facilitate their replication; the cDNA pre-integration complex is transported to the cell nucleus where it is integrated into host chromatin by viral integrase. The integrated viral DNA also referred to as the provirus, is then transcribed to produce viral RNA. Initially, only Tat, Rev and Nef genes are expressed with the production of multiply-spliced mRNAs. As the infection progresses, secondary to higher levels of Rev, there is the production of unspliced mRNA which encodes the structural and accessory proteins Gag, Pol, Env, Vif, Vpr, and Vpu. The unspliced RNA products are then transported out of the nucleus where they are translated in the cytoplasm. After translation, Env and Gag-
Pol proteins migrate to the plasma membrane where they start to assemble into progeny virions. Viral budding through the cytoplasm is accompanied by Nef-induced downregulation of CD4 on the cell membrane. HIV protease is then required for the production of infectious virions; it cleaves a number of proteins from the Gag-Pol polyprotein precursor, including itself, reverse transcriptase and integrase encoded in the pol gene, and the matrix, capsid, and nucleocapsid proteins encoded in the gag gene.
1.3. Natural History of HIV infection

1.3.1. HIV transmission

Sexual transmission is the most common route of transmission for HIV, with initial infection events occurring predominately although not exclusively through physical breaches in the mucosal tissue of the genital tract and ano-rectum. The risk of infection per sex act is low; reports suggest a transmission rate of approximately 0.1% for unprotected receptive vaginal intercourse and 1.4% for unprotected receptive anal intercourse. The risk of transmission is also impacted by a number of host and viral factors, the most significant being the level of the HIV viral load. Additional factors influencing transmission are concurrent STIs, contraception and pregnancy and male circumcision status. After breaching the epithelial and mucus barriers, HIV can interact with local immune cells in the genital and rectal mucosa where a host of potential targets cells are present; these include dendritic cells, macrophages and activated CCR5+ CD4 T cells. In addition, dendritic cells have surface dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) which binds HIV, virus bound to dendritic cells is then transported to local lymphoid tissue with subsequent infection of CD4 T lymphocytes. Studies in macaques have demonstrated that seeding of the local lymphoid tissue occurs rapidly, usually within one to three days after initial infection.

Despite the identification of a mixture of HIV quasi-species in semen and cervicovaginal secretions from an HIV+ partner, a single founder virus is often successful at achieving infection; in approximately 80% of heterosexual transmissions and 60% in men who have sex with men (MSM). Regardless of the route of exposure, lymphoid tissue in the gut is the focus of viral replication during acute HIV infection, where there is an abundance of preferential target cells including CCR5+CD4+, αβ7 CD4+ and T helper 17 (Th17) cells. During this initial stage of infection, viral replication is confined to mucosal and lymphoid tissue and not detectable in peripheral blood for a period of approximately ten days; this is referred to as the eclipse phase.
1.3.2. Primary HIV infection

It is important to distinguish primary from acute HIV infection; primary HIV infection (PHI) includes the acute phase of infection, which usually refers to the period from initial infection up to the symptomatic period around the time of seroconversion, while PHI is often defined as the first six months post-infection. Seroconversion occurs with the development of HIV antibodies, and a viral load set point is achieved. Clinically, acute HIV is often associated with a febrile illness and may be misdiagnosed as an infectious mononucleosis-like syndrome. The clinical symptoms of acute HIV infection are non-specific and are reported in approximately 43% of individuals. Symptoms include fevers, sweats, malaise, lethargy, anorexia, nausea, myalgia, arthralgia, headaches, sore throat, diarrhoea, lymphadenopathy and a maculopapular rash. Given the non-specific symptomatology, HIV testing is indicated in all sexually active individuals. It is essential to consider the time lag for the development of antibody responses and p24 antigen or viral RNA detection when acute HIV infection is suspected.

Certain diagnostic tests may miss very early infection; figure 1.3.1 shows the differential development of viral proteins and HIV RNA, HIV DNA and HIV-specific antibodies. Fourth generation combination tests detect HIV antibody and p24 antigen and have a window period of approximately 14 days. Staging systems of acute HIV infection have been developed based on the expected time of viral detection, antibody and p24 antigen detection and western blot evolution; Fiebig staging is one of the most frequently referenced and is summarised in Figure 1.3.1. In the U.K a test measuring the avidity of HIV antibodies is available through Public Health England, it has been used in combination with clinical data to determine recent HIV infection; is known as recent HIV test algorithm (RITA).
### 1.3.3. Untreated HIV infection

In untreated HIV infection, there is a clinically asymptomatic stage of infection with a continual loss of circulating CD4 T cells; on average between 50–70 cells/year. The time between infection and development of AIDS varies greatly between individuals with certain viral and host factors being predictive of disease progression, such as viral load set-point and HLA type. One study from San Francisco in the 1980s estimated median time of 9.8 years from infection to the onset of AIDS. The mechanism by which HIV causes immune dysfunction is likely multifaceted and not only due to CD4 T cell depletion secondary to cytolytic viral infection. HIV induces a host of immunological abnormalities, which contribute to bystander cell death and other sequelae of chronic immune activation, ultimately leading to progressive immune dysfunction and AIDS.
1.3.4. Elite Controllers

As discussed, the majority of individuals living with HIV and not on ART exhibit high HIV viral loads in plasma and progressive CD4 T cell loss, on average 61 cells/µL/year\textsuperscript{48}. However, a small proportion of HIV infected individuals demonstrate a degree of viral control without ART; such individuals are referred to as “elite controllers” or “HIV controllers” (HICs). Various definitions of elite controllers have been proposed; the CASCADE and ANRS CO18 cohorts define elite controllers as individuals who have had “at least five consecutive viral load measurements below 400 or 500 copies/ml, the last one being measured at least 5 years after the date of seroconversion, while never having received ART”\textsuperscript{49, 50}. These individuals are rare, with reports suggesting that approximately 1% of people living with HIV (PLWH) are elite controllers\textsuperscript{50, 51}. These individuals are of particular interest in HIV cure research as they may offer insights into mechanisms of spontaneous HIV control. Approximately half of subsequent elite controllers have an initial period of viraemia prior to control; however, at seroconversion, these elite controllers have lower viral loads and higher CD4 T cell counts than non-controllers\textsuperscript{50}. Host genetics including the HLA alleles HLA-B27, HLA-B51, HLA-B*5701, HLA-B*5703, and HLA-B*5801 are associated with control of HIV and slower progression to AIDS\textsuperscript{52}. Cytotoxic CD8 T lymphocyte (CTL) responses are thought to play an important role in the viral control seen in elite controllers; Sáez-Cirión et al. have demonstrated that CTLs from elite controllers have greater capacity to control HIV infection ex vivo compared to non-controllers\textsuperscript{53}, which may be perforin-mediated\textsuperscript{54}. Interestingly, a study of elite controllers demonstrated stronger polyfunctional mucosal CD4 and CD8 T-cell responses compared to non-controllers, but these responses were not necessarily seen in peripheral blood\textsuperscript{55}, suggesting that efficient mucosal immune responses may be necessary for elite control. The innate immune systems, in particular, plasmacytoid dendritic cells, may also be important for viral control in elite controllers\textsuperscript{56}. Measures of HIV reservoir are lower in controllers even in tissues sites with lower HIV DNA and RNA in GALT compared to non-controllers\textsuperscript{57}. In addition, HIV controllers had lower measures of bacterial translocation than progressors\textsuperscript{58}. Taken
together, these data suggest that elite control is a consequence of superior immune surveillance of HIV resulting in decreased measures of HIV persistence\textsuperscript{57}, immune activation\textsuperscript{59} and exhaustion; the ability to manipulate the mechanisms responsible may be an opportunity to induce viral control in progressors. In addition to the elite controllers described here, another form of viral control is recognised in individuals who have initiated ART during PHI, termed post-treatment control which is discussed in greater detail below.
1.4. Antiretroviral Therapy

The advent of effective antiretroviral therapy (ART) has dramatically improved the prognosis for individuals living with HIV\(^8\). ART drug development has focused primarily on interference with various stages of the HIV life cycle, see figure 1.4.1. The first class of ART drugs licensed for clinical use was the dideoxynucleoside analogues, also known as nucleoside reverse transcriptase inhibitors (NRTIs); these nucleoside analogues inhibited the action of reverse transcriptase preventing the reverse transcription of HIV into viral DNA\(^60\). In 1987, the initial clinical studies were commenced with Zidovudine (AZT) monotherapy and demonstrated benefit over no treatment, but limited benefit in terms of long-term survival\(^61, 62\). Didanosine (ddI) and other nucleoside agents were subsequently developed and had a marginal benefit over Zidovudine monotherapy\(^63-65\). However, these agents were associated with significant toxicities and the emergence of HIV drug resistance\(^66-72\). The drug resistance which emerged following treatment with single agents developed the notion that combination ART may be required in HIV therapeutics\(^73\). Subsequent clinical trials of dual therapy with nucleoside ART agents such as AZT and ddI or zalcitabine (ddC) showed a benefit in terms of overall survival and prevention of disease progression\(^74, 75\). In the early 1990’s there was development of new classes of ART drugs such as Protease inhibitors (PI), these agents blocked the action of the HIV protease enzyme, preventing cleavage of \textit{gag} and \textit{gag-pol} protein precursors, inhibiting the production of new virions and new rounds of infection\(^76, 77\). The development of these newer agents meant that combination triple agent therapies could attack HIV at different stages of the viral replication cycle.

Studies of dual and triple therapy with NRTIs and a protease inhibitor conferred substantial benefit in survival in advanced disease and heralded in the era of highly active antiretroviral therapy \(^78-80\). The initial enthusiasm for protease inhibitor-containing HAART regimens was dampened somewhat by the high pill burden, interactions with food, interactions with drugs and adverse events associated with the first-generation protease inhibitors\(^81-85\).
The approval of a number of new agents in the late 1990s included novel agents in the non-nucleoside reverse transcriptase inhibitors class (NNRTI); these non-competitive inhibitors of reverse transcriptase worked by inhibiting the movement of proteins that are essential to reverse transcriptase’s function in carrying out DNA synthesis. The arrival of these newer agents, such as nevirapine and efavirenz, meant that a multitude of new treatment options now existed for combination therapy. Also, the NNRTI class had potentially more favourable side effect and dosing profiles, as well as equal efficacy in controlling HIV replication when compared to the earlier protease inhibitors.

The introducing of HAART from 1995, as the standard of care for HIV, resulted in a dramatic decline in the morbidity and mortality among individuals living with HIV. The expectation of treatment with the newer combinations was suppression of viral replication. Subsequently, the focus of ART clinical research shifted towards the development of more tolerable and efficacious regimens and agents with a higher barrier to HIV drug resistance. The development of single tablet regimen (STR) formulations, which contain a combination of three active ART agents, has allowed for significant treatment simplifications for people living with HIV (PLWH). Such STRs mean one tablet once a day is the mainstay of ART for a large proportion of individuals living with HIV. However, controversy exists surrounding the contribution that these STRs make to ART adherence and if the increased cost of these regimens are justifiable in an era where generic ART is available.

Development of newer ART agents has continued throughout the past decade with the introduction of the integrase strand inhibitor (INSTI) class which target and inhibit the strand transfer reaction of HIV integrase. Drugs in this class include raltegravir, dolutegravir and bictegravir, all of which are highly efficacious, with relatively few adverse effects and no significant CYP3A4 drug interactions. In randomised studies comparing INSTI regimens to NNRTI and PIs containing regimens, the INSTI was better tolerated with fewer treatment discontinuations. INSTI containing regimens are now the recommended initial regimen in US guidelines.
Figure 1.4.1 HIV life cycle and mechanism of action of ART\textsuperscript{7}

The HIV life cycle is outlined in the below. The drugs which target each step in the life cycle are indicated in \textit{italics}. (1) HIV enters the target cell through an interaction between the HIV envelope glycoprotein, and the target cells surface molecules, CD4 (Attachment inhibitors) and a co-receptor, either CXCR4 or CCR5 (CCRS antagonists). (2) Fusion of viral and cellular membranes allows for the release of the viral core in the cell cytoplasm where viral RNA is reverse transcribed into viral DNA (NRTIs, NNRTIs). (3) The resulting pre-integration complex is transported to the cell nucleus, where the HIV viral DNA is integrated into the host genome (INSTIs). (4) HIV DNA is then transcribed into viral mRNA using the host transcriptional enzymes. (5) Translation of new viral proteins occurs in the cytoplasm (6) with subsequent formation of mature virions (Maturation Inhibitors).


While the benefits of HAART quickly became apparent, concurrent randomised data was not available to guide the optimal timing of initiation of ART for adult HIV infection. Observational studies demonstrated poorer outcomes with respect to mortality and disease progression in those who delayed ART until CD4 T cells counts were less than 200 per cubic millimeter\textsuperscript{94-97}. Accordingly, treatment guidelines in the United States and the UK initially advised treatment initiation between 200 and 350 CD4 cells per cubic millimeter\textsuperscript{98,99}. Strategies of episodic ART, guided by CD4 T cell counts, were employed to overcome problems associated with long-term ART use, such as difficulty with adherence, adverse events and cost\textsuperscript{100}. However, this strategy was abandoned based on the results of the SMART randomised control trial which demonstrated that CD4 count-guided episodic use of ART was associated increased risk of
opportunistic infections and death \textsuperscript{101}. The NA-ACCORD study provided further evidence, using cohort data, that initiation at higher CD4 T cell counts may be beneficial\textsuperscript{102}. The HTPN052 study supported the use of ART for all individuals living with HIV as a strategy to prevent onward transmission of HIV\textsuperscript{22}. Ultimately; it was the findings of the START and TEMPRANO randomised control trials that supported initiation of ART for all individuals living with HIV regardless of CD4 T cell count\textsuperscript{103, 104}. The START investigators found a decrease in both AIDS and non-AIDS events in the immediate ART initiation arm and importantly no increased rates of ART-related side effects were observed in those initiating earlier ART, irrespective of CD4 count\textsuperscript{104}. The findings of the START and TEMPRANO trials led to the immediate changes in the WHO and National and International ART guidelines for adults living with HIV, recommending ART initiation irrespective of CD4 count\textsuperscript{105, 106}. However, these guidelines made no special case for those presenting with PHI, and while the recommendation was for initiation of ART for all PLWH, removing the previous CD4 count threshold, there were no time preferences placed on ART initiation in PHI.

As previously discussed; timely initiation of ART during primary HIV infection (PHI) may offer benefits to the individual with respect to immune recovery, limiting of HIV reservoir seeding and prevention of onward transmission\textsuperscript{22, 107-109}. The SPARTAC, randomised control trial, demonstrated that 48 weeks of ART in PHI delayed time to disease progression compared with deferred ART initiation, but this overall delay was not significantly longer than the duration of treatment\textsuperscript{110}. Also, it showed that, unlike the SMART trial which enrolled individuals with chronic stage HIV infection, short-term treatment was a safe intervention in PHI, this may have particular relevance in future HIV eradication studies involving a treatment interruption in individuals who have commenced ART during PHI.

Following the recommendation for all PLWH to initiate and remain on ART, UNAIDS has set the ambitious targets of 90:90:90 \textsuperscript{111}. These targets aim for the diagnosis of 90% of those living with HIV, ensuring that 90% of PLWH are in HIV care and on ART, and achieving viral suppression in 90% of those on ART, by 2020\textsuperscript{111}. This admirable goal faces considerable
challenges; global access to ART is limited by educational, gender, healthcare and economic inequalities\textsuperscript{111, 112}. Furthermore, the cost of ART poses a significant burden to the economies of those countries most significantly affected by HIV and while access to generic formations has gone some way to address this issue, they are not universally available \textsuperscript{113}. The global burden of HIV is highest in resource-limited countries where the challenges to provide adequate HIV care go far beyond the provision of ART. Trained healthcare providers, access to laboratory diagnostics, monitoring and resistance testing, are some of the other critical facets in the cascade of care that are necessary to provide effective long-term treatment for HIV in these settings.

Drug resistance resulting in treatment failure is now uncommon in healthcare settings with unrestricted access to ART. Newer INSTI regimen such as Dolutegravir have a high genetic barrier to resistance. Furthermore, availability of drug classes with different mechanisms of action including fusion inhibitors, CCR5 antagonists\textsuperscript{114} and newer agents such as maturation and attachment inhibitors\textsuperscript{115} ensure that novel ART regimens can be constructed to achieve viral suppression despite resistance in treatment-experienced individuals. However, HIV drug resistance in many low and middle-income countries presents difference therapeutic challenges due to limited access to genotypic resistance testing and second or third line ART. These challenges have been highlighted by recent reports of high rates of pre-treatment drug resistance in low and middle-income countries in Africa and Latin America, where the reported prevalence estimates of NNRTI resistance is now approximately 10\%\textsuperscript{116}. In a separate study, increased virological failure rates of up to 38\% have been reported in African cohorts, and while these findings are not necessarily linked, they do highlight the need for HIV resistance surveillance in such settings\textsuperscript{117}.

In conclusion, despite the undeniable success of ART, challenges remain and remind us that options for HIV treatment beyond ART should be considered in the management of HIV infection.
1.5. HIV reservoir

1.5.1. Definition of HIV reservoir

Following transmission of HIV, a reservoir of latently infected cells is established rapidly\textsuperscript{118}, focused within CD4\textsuperscript{+} bearing cells, especially within the lymphoid tissue of the gut, lymph nodes and genital tract\textsuperscript{119-121}. Data from Non-Human Primates (NHP) has shown the reservoir is seeded very early and only if ART is initiated immediately (within <3 days from viral challenge), then reservoir development may be attenuated\textsuperscript{29}. A recent study in humans has demonstrated that even individuals initiated on ART during Fiebig I had viral rebound on cessation of therapy\textsuperscript{122}. Finzi et al. estimated the mean half-life of the latent reservoir to be 43.9 months, implying that once established, this pool of latently infected cells can persist for the lifetime of the host\textsuperscript{123}. It is the presence of this reservoir that precludes HIV cure with ART alone. Defining the functional HIV reservoir is important, as many measures of HIV reservoir include replication incompetent virus rather than virus capable of recrudescence. For this discussion, the HIV reservoir can be considered as a pool of “long-lived cells containing non-defective provirus in a non-expressing but inducible state”\textsuperscript{124}.

1.5.2. Characterisation of HIV reservoirs

The HIV reservoir consists predominately of infected memory CD4\textsuperscript{+} T cells; naive CD4\textsuperscript{+} T cells are infected at a lower frequency\textsuperscript{125}. In the absence of HIV infection, the majority of CD4\textsuperscript{+} cells are present in a resting state, such as central memory (T\textsubscript{CM}) and naïve (T\textsubscript{N}) cells. Resting memory CD4 T cells have a long lifespan with a half-life of 40–44 months\textsuperscript{126}. Therefore, more than 70 years of ART would be required for elimination of this cellular reservoir. Furthermore, naïve cells have an even longer half-life than memory T cells\textsuperscript{127}. In response to infection, there is the transformation of these resting cells into more short-lived activated effector T cells (T\textsubscript{EM}); HIV preferentially replicates in activated CD4\textsuperscript{+} T cells\textsuperscript{128}. In general, these activated effector T cells are depleted by ongoing infection or apoptosis. However, some survive and
revert to a resting state resulting in long-lived latently infected memory T cells. The model of activated CD4 T cells reverting back to a resting state is the generally accepted mechanism of HIV latency. However, recent evidence suggests that latency may also result from the direct infection of resting CD4 T cells, particularly in lymphoid tissue. Accordingly, the HIV reservoir in peripheral blood mononuclear cells (PBMCs) consists predominately of latently infected $T_{CM}$ or Transitional ($T_{TM}$) CD4 T Cells. The distribution of HIV-DNA in CD4 subsets may also have implications for persistence; in the VISCONTI study – a cohort of post-treatment controllers, the $T_{TM}$ subset (which are shorter-lived compared to $T_{CM}$) was the main contributor to the HIV-1 reservoir. The T cell subset population which constitutes the latent reservoir may be different in non-PBMCs, for example in the gut, effector memory T ($T_{EM}$) cells have been shown to account for the greatest proportion of the cellular pool. New cellular reservoirs continue to be described, such as CD4$^+$ T cells with stem cell-like properties; this population although rare are significant as they have cellular mechanisms to resist apoptosis and persist for extremely long periods of time. A greater understanding of how the subtypes of memory T cells contribute to viral latency and persistence and the effect of treatment strategies on the latent T cell subset populations will be needed to inform strategies to eliminate these long-lived reservoirs. In addition to memory T cells subsets, some CD4 T cells with specialised functions are enriched for HIV DNA such as T Follicular Helper Cells and Th17 cells, these are discussed in greater detail in subsequent sections.

Monocytes and macrophages may also act as cellular reservoirs for HIV, particularly in tissue sites such as the gut, lung and brain. HIV DNA has been detected in astrocytes and macrophages in brain tissue from HIV infected individuals. However, true latency in these cells has not been clearly demonstrated; while circulating monocytes may harbour HIV, these cells are transcriptionally active and are likely recently infected. Work in macaques and African green monkeys found that myeloid cells containing HIV DNA in tissue had evidence of T cell phagocytosis rather than true latent infection.
1.5.3. Quantification of the HIV Reservoir

The methods used to quantify the latent HIV reservoir vary and no single optimal assay has been established. Figure 1.5.1 outlines the various measures that are used to quantify the latent reservoir. Precisely what is being measured by the different technologies and how relevant this is clinically, remains unknown. Traditionally, the quantitative viral outgrowth assay (QVOA) has been considered the gold standard for reservoir quantification. However, limitations of this assay exist; QVOA requires high cellular input, is expensive, labour intensive and time-consuming, furthermore, repeat stimulation of cells has been shown to induce additional viral outgrowth that may not reflect in vivo mechanisms.

Measurement of HIV nucleic acids is relatively inexpensive and amenable to high throughput; total cell-associated, integrated and 2-LTR circles, are all DNA forms of HIV which can be measured by quantitative polymerase chain reaction (qPCR) or digital droplet polymerase chain reaction (ddPCR). Total and integrated HIV DNA overestimate the size of the replication competent reservoir as they include measurement of defunct virus DNA which is incapable of replication. This is particularly relevant when we consider recent data which suggests the size of the replication competent reservoir may be 60-fold greater than previously estimated when measured using sequencing techniques to quantify the replication-competent proviral reservoir. Interestingly, the only measure which has been shown to predict time to viral rebound after stopping ART to date is peripheral blood CD4 ‘Total HIV DNA.’

Measurement of cell-associated viral RNA species such as us-RNA and ms-RNA have been used to assess levels of viral transcription; us-RNA is found at higher levels towards the end of the HIV life cycle with higher us-RNA/ms-RNA ratios associated with T cell activation and disease progression.

Assays to characterise and quantify the viral reservoir are in need and continue to evolve; assays which measure HIV transcription post activation such as the Tat-Rev Limited Dilution Assay (TILDA) is less expensive, less time consuming than QVOA and is highly correlated
with existing measures of reservoir\textsuperscript{150}. Recently, a novel reporter cell-based assay which measures tat-induced activation has been developed to measure replication-competent virus\textsuperscript{151}.

When evaluating the clinical significance of the measured residual HIV reservoir, it may not be enough to quantify the proviral reservoir; sequence data\textsuperscript{147,152}, epigenetic factors\textsuperscript{153} and proviral integration sites\textsuperscript{154,155} also affect latency and need to be considered. Furthermore, the current techniques used to measure HIV reservoir are often restricted by the input requirement of a large number of viable cells, this is particularly true when measuring HIV reservoir in tissue sites. Imaging techniques have been employed for quantification of total body reservoir in macaques using copper labelled SIV gp120-specific antibodies measured by antibody targeted positron emission tomography (PET)\textsuperscript{156}, but this technology has not yet been translated to humans. Given the limitations in quantifying the HIV reservoir in tissue sites, it is reasonable to evaluate surrogate, non-viral measures of the reservoir. Such measures may include immunological or inflammatory markers in tissue, blood or plasma which reflect the HIV tissue reservoir; these measures will be essential to guide future HIV eradication intervention studies\textsuperscript{157}.

1.5.4. Cellular correlates of HIV reservoir

Markers of immune activation and inflammation have been associated with HIV disease progression\textsuperscript{158-163}. CD4 T cell count, in particular, has guided clinical care since the beginning of the HIV epidemic\textsuperscript{164-166}. However, its utility in identifying individuals at risk of non-AIDS morbidity and mortality in the era of ART is less clear and was recently highlighted by the START study\textsuperscript{104}. Other markers of immune function, such as the CD4/CD8 ratio may be superior in those with robust CD4 counts. Also, low CD4/CD8 ratio predicts increased levels of immune activation and higher HIV DNA in chronic HIV infection\textsuperscript{167-172}. Higher expression of negative inhibitory receptors, such as PD-1, TIGIT, and Tim-3 is evident in HIV infected individuals; these receptors are collectively referred to as immune checkpoint receptors (ICRs)
Figure 1.5.1 Measures of HIV reservoir

This figure illustrates the estimated number of cells infected with HIV that persist on ART according to the methodology used to quantify HIV reservoir. There are approximately 100–1,000 cells with HIV genomes (total HIV DNA) per million CD4 T cells, but only a fraction of these cells makes CA HIV RNA. A smaller proportion have detectable HIV proteins or release virions into the supernatant. A smaller fraction of cells generates replication-competent HIV in vitro after stimulation by TCR ligation (∼1/10^6 cells). However, sequencing of viral genomes indicates that the true size of the reservoir (intact genomes; ∼60/10^6 cells) might be much larger than this.  


or exhaustion markers. Increased expression of ICRs is associated with HIV disease progression. In addition to their utility in predicting disease progression, expression of ICRs and activation markers are also closely associated with measures of the HIV reservoir. T cell activation and CD4 expression of the ICRs, PD-1, TIGIT, Tim-3, and Lag-3 have all been shown to relate to reservoir size in PBMCs. Specific ICR pathways such as PD-1-PDL1 have also been implicated in maintaining HIV latency by inhibiting T cell activation.

A novel and reportedly highly specific marker of HIV reservoir in resting CD4 T cells, CD32, was recently proposed by Descours et al. However, these findings have not been reproduced in work by other groups, including our own. Another potential marker, CD30
has been suggested by Henrich et al., who demonstrated that CD30 expression on CD4 T cells identified transcriptionally active CD4 T cells in GALT and PBMCs. Much of the work discussed above has examined the expression of immune markers in PBMCs, but the expression of these markers in blood does not necessarily reflect their expression in tissue. Also, measurement of these markers in tissue may provide insight into mucosal immune function and prove a suitable surrogate for reservoir quantification and assessment in tissue sites. As previously mentioned, specific CD4 T Cell types, such as TFH and Th17, are enriched for latent HIV. These cell types are predominately found in tissue sites, understanding the relevance of ICR and activation marker expression on these cells will be necessary to further delineate the HIV reservoir in tissue sites. Finally, therapeutic antibodies which target ICRs are available or are in clinical development. Therefore, a complete characterisation of their expression in tissue is required before use of these agents in clinical studies.

1.5.5. Mechanisms of HIV reservoir persistence on ART

Many strategies for HIV eradication focus on accelerating the decay or purging the HIV reservoir. Therefore, it is critical that we understand the mechanisms which support and maintain the HIV reservoir on ART. Evidence exists for ongoing viral replication in sanctuary sites, which are poorly accessed by ART and have limited immune surveillance. However, this assertion is not universally accepted. The ability to demonstrate the evolution of viral sequences on ART would support the view that ongoing viral replication occurs on ART treatment. Brodin et al. reported that the proviral DNA sequences on ART are similar to those in replicating virus before ART initiation, with no evidence of ongoing viral evolution on treatment. Other studies have also shown a lack of viral evolution in sanctuary sites such as gut and lymph node. Contrary to this, Lorenzo-Redondo et al. published evidence that viral evolution and trafficking between tissue compartments continues in individuals on ART. However, other groups have contested their findings and methodology.
intensification studies have also shown an increase in HIV viral proteins, including us-RNA and 2-LTR circles, with the addition of raltegravir to standard three-drug ART \(^{191, 192}\); these findings suggest that treatment intensification with an integrase inhibitor drug blocks new rounds of ongoing productive infection. Poor tissue penetration of certain GALT and lymphoid tissue provide a mechanism for replication at these sites \(^{184}\). Next generation in situ hybridisation techniques used by Estes et al. have also shown an abundance of HIV RNA\(^+\) cells in GALT of ART-treated macaques and humans, in association with low tissue drug concentration of ART \(^{185}\).

Different mechanisms of cellular transmission of HIV may also contribute to productive HIV infection on ART. The high density of cells in tissue sites may favour cell to cell transmission of HIV\(^{193}\). Cell to cell transmission, as opposed to cell-free transmission, is associated with multiple infecting virions, increasing the likelihood of infection and reduced ART efficacy\(^{194, 195}\).

Homeostatic proliferation of the latently infected cells provides a mechanism for expansion of the latent reservoir on ART. Chomont et al. identified that homeostatic proliferation as a key mechanism of HIV persistence in the T\(^{\text{TM}}\) CD4 T cell subset \(^{130}\). The proviral integration site into the human genome also supports reservoir proliferation; work by Wagner and Maldarelli has shown preferential viral integration at sites associated with oncogenes and the cell cycle, respectively \(^{155, 196}\). Importantly, these clonally expanded populations are capable of producing infectious virus \(^{146, 197}\).
1.6. The Immune system and HIV

1.6.1. Basic Immunology

The immune system is an organised collection of cells and molecules which functions to protect the host against infection. The cells of the immune system are derived from pluripotent haematopoietic stems cells in the fetal liver and in bone marrow, which can differentiate into either myeloid or lymphoid progenitor cells. To establish an infection, pathogens such as HIV must first penetrate the surface barriers such as the skin and mucosa of the body cavities. Broadly, the immune system can be divided into innate and adaptive arms, both of which are impacted by HIV infection.

The innate immune response is rapid, not antigen-specific and not dependent on immune memory, which requires previous exposure to a pathogen. The principal cellular components of the innate immune system are phagocytic cells (neutrophils, monocytes and macrophages), inflammatory mediators (eosinophils and basophils), dendritic cells and natural killer cells. Innate cells can discriminate between ‘self’ and ‘foreign’ molecules using so-called pattern recognition receptors (PRRs), for example, macrophages possess mannose receptors not normally expressed on the cells of vertebrates. Once a pathogen is recognised as foreign, macrophages and neutrophils can engulf the microorganism by phagocytosis. Interestingly, mannose receptors have been implicated in CD4 independent infection of astrocytes by HIV. Toll-like receptors (TLRs) present on macrophages and dendritic cells are important PRRs and are responsible for the recognition of a range of Pathogen–Associated Molecular Patterns (PAMPs). TLRs mediate rapid activation of innate immunity, by triggering the production of pro-inflammatory cytokines and through the upregulation of co-stimulatory molecules. In fact, TLRs are recognised as mediators of the persistent immune activation seen in chronic HIV infection.

Innate cells also play an important role in the presentation of antigen to the cells of the adaptive immune system, classic antigen presentation cells (APCs) include dendritic cells,
macrophages and B cells. APCs migrate to draining lymph nodes where they present antigen to T cells using the major-histocompatibility complex molecules (MHC) on their cell surface. The interaction between APCs and T cells is restricted by the class of MHC molecules on the APC, namely class I (HLA-A, B, and C) or class II (HLA-DP, DQ and DR). CD4 binds to an invariant part of the MHC class II molecule, whereas CD8 binds to an invariant part of the MHC class I molecule. MHC class I molecules are expressed on all nucleated cells while MHC class II molecules that signal CD4 helper T cells to secrete cytokines are found only on APCs such as dendritic cells, B cells and activated macrophages.

Natural killer (NK) cells, as the name suggests can identify and destroy infected cells using pro-apoptotic cytoplasmic granules and via Fas/FasL-dependent or TRAIL-dependent apoptotic signals. They recognise their targets in one of two ways. One mechanism called antibody-dependent cellular cytotoxicity (ADCC), involves the activation of NK cells by the binding of the FcγRIIIA receptor (CD16), present on a proportion of NK cells, to the Fc domain of IgG, which in turn links to the viral antigen on the surface of the infected target cell. The second mechanism for identification of infected targets by NK cells involves balancing of signals to the killer activating receptors and killer-inhibitory receptors (KIRs) on NK cells. The killer activating receptors recognise molecules expressed on the surface of all nucleated cells, while KIRs recognise MHC class I molecules. However, when both receptors are engaged, the inhibitory signal prevails. Certain infections, including HIV, can interfere with MHC I expression and its downregulation makes cells a target for elimination by NK cells. However, HIV has developed a mechanism to evade NK cell killing, whereby the viral protein Nef downregulates only HLA-A and -B but not -C or -E from the cell surface of infected cells.

The innate immune system serves as the first line of defence against foreign pathogens, early sensing by PRRs results in rapid immune activation with the production of pro-inflammatory cytokines and chemokines, such as type I and type III interferon. In acute HIV infection, viral-associated PAMPs initiates the innate response thereby recruiting dendritic cells,
macrophages and NK cells the site of infection and local lymphoid tissue, controlling viral spread and activating and supporting adaptive immune responses.\textsuperscript{205}

The adaptive immune system depends on B lymphocyte and T lymphocyte responses; the latter depends on the presentation of antigen by APCs to recognise antigen. In addition to the generation of effector lymphocytes, the adaptive immune response generates memory T and B cells which can mount superior immune response on exposure to a cognate antigen. In contrast to the PRRs on innate immune cells, lymphocytes possess highly specific antigen surface receptors, BCR (Immunoglobulins) on B lymphocytes, and the T-cell receptors (TCRs) on T lymphocytes. The human genome is composed of approximately 45\% of transposable elements, and it is the variable recombination of those elements which encode the TCR and BCR genes; this allows for the generation of a vast repertoire of antigen receptors.\textsuperscript{206, 207}

T lymphocytes are derived from stems cells in the bone marrow, and undergo development in the thymus, through a process of positive and negative selection, referred to as ‘thymic education’.\textsuperscript{202, 208} This process involves the positive selection of T cells with TCRs that can recognise self MHC molecule and negative selection of TCRs which have a high affinity for self-peptides.\textsuperscript{209} Also, at this stage of T cell development, the immature CD4 and CD8 double positive T lymphocytes downregulate one of these molecules and commits to either a CD4 or CD8 T cell lineage. The TCR is a transmembrane molecule which consists of $\alpha/\beta$ or $\gamma/\delta$ heterodimers. The process of thymic selection described above is usual for those with the $\alpha/\beta$ heterodimer while a proportion of T cells possess the $\gamma/\delta$ heterodimer; these cells leave the thymus early and are thought to play a role in mucosal defences, such as in the epithelium of the gastrointestinal (GI) tract.\textsuperscript{210}

The antigen-TCR interaction alone is not sufficient for T cell activation and proliferation; an additional requirement is the engagement of co-stimulatory molecules such as CD28 (the ligand for the B7 receptor on dendritic cells)\textsuperscript{211} and CD40 ligand (which binds to CD40 on B cells).\textsuperscript{212} Following Activation of T and B cells, there is clonal selection and expansion of
receptors with relevant specificities. CD4 T cells have been characterised into different T-helper cells (Th) subsets based on their function and cytokine production, including Th1 (Interferon-$\gamma$, IL-2), Th2 (IL-4, IL-5, IL-6 & IL-10), Th17 (IL-17, IL-22) and TFH (IL-21). Th1 and Th2 orchestrate the helper T cells response to intracellular and extracellular pathogens respectively while Th17 are particularly important in the immune response at mucosal surfaces.

Cytotoxic CD8 cells are important for elimination of virally infected cells. Naïve CD8+ T cells are activated by APC engagement with TCR in association with MHC class I molecules, and similar to CD4 T cells they also require the engagement of co-stimulatory molecules. Once activated an effective CD8 response requires clonal expansion and the differentiation of naïve cells into primary effector cells; these cells are capable of recognising peptides from virally infected target cells leading to the direct killing of antigen-bearing cells through perforins, granzymes, and Fas/FasL interaction\textsuperscript{213}.

B cells act as APCs; however, their primary function is the production of antibodies. Antibodies are complex structures composed of two identical light chains and two identical heavy chains; the amino acid sequence of the latter determining the antibody class (IgM, IgG, IgA, IgD or IgE)\textsuperscript{214}. On B cells these antibodies act as the BCR, and when secreted by plasma cells they can be directly protective by preventing microorganism binding to a cellular target. However, more often they function by working in tandem with other components of the immune system such as complement or through the process of antibody-dependent cellular cytotoxicity. Class switching and production of high-affinity antibodies requires B cell engagement with specialised CD4 helper cells - T follicular helper cells, which occurs in germinal centres of B cell follicles.
1.6.2. The Immune response to HIV

HIV infection induces a vigorous immune response to acute HIV infection. However, viral and host factors mean that this response is unable to eliminate and prevent chronic viral infection, resulting in persistent immune activation and dysfunction.

**Innate immune responses to HIV**

The early innate response to HIV is induced by sensing of viral particles or infected cells by PRRs. TLRs on plasmacytoid dendritic cells and conventional myeloid dendritic cells, induce a range of pro-inflammatory cytokines including type 1 interferon, IP-10, TNF-α and MCP-1. These result in the recruitment of a host of immune cells such as dendritic cells, macrophages and CD4 T cells to the site of infection (most often mucosal sites) and local lymphoid tissue. Importantly, type I interferon production has been shown to upregulate HIV restriction factors included APOBECs and TRIM-5, although not until day ten post-infection. HIV also acts to counter these measures; work in macaques, by Barouch et al., has shown that in very early infection the HIV virus triggers host responses, such as the NLRX1 and TGF-β pathways, that suppress early innate and adaptive immunity. In addition, intracellular cytoplasmic innate sensors which detect foreign DNA and RNA can induce an interferon response; such innate sensors include cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING).

**Adaptive T-cell responses to HIV**

Dendritic cells from mucosal tissue sites migrate to secondary lymphoid tissue where they present antigen to CD4 T cells; this facilitates infection of CD4 T cells by dendritic cell-specific C-type lectin (DC-SIGN) which binds HIV Env gp120 and brings virus in close proximity to CD4 T cells. HIV’s tropism for CD4 ensures that these cells are rapidly infected and depleted.

Acute HIV infection results in extensive activation and expansion of cytotoxic CD8 T cells, with the first CD8 responses evident at the end of the eclipse phase of infection. The majority
of early CD8 responses are HIV-specific\textsuperscript{224, 225}, and contribute to viral control and the establishment of viral equipoise, where the rate of viral elimination and virus production are balanced, termed the viral set point\textsuperscript{224, 226}. T cell responses are initially directed at Env and Nef, while responses to the more conserved Gag p24 and Pol proteins tend to arise later\textsuperscript{227-229}. After peak viraemia, selective pressure, driven by the error-prone step of reverse transcription, results in rapid viral evolution and the emergence of CD8 driven escape mutants; with viral epitopes no longer recognised by the pool of HIV specific CD8 T cells\textsuperscript{230}. Founder virus sequences are replaced rapidly with those of escape mutants, within ten days in one study\textsuperscript{227}. Following primary infection, the avidity of CD8 T cell clones tends to wane\textsuperscript{231}. However, individuals exhibiting the elite controller phenotype maintain CD8 T cells with higher TCR avidity than non-controllers and polyfunctional responses which target conserved viral epitopes\textsuperscript{232-234}.

Memory CD4 T cells are the target of HIV infection and are rapidly depleted in acute HIV infection\textsuperscript{2, 235}. Despite this, CD4 T cell responses are important in the control of HIV; both CD8 and B cells responses are dependent on CD4 T cell help\textsuperscript{236, 237}. Immune exhaustion of HIV specific CD4 T cells has been associated with disease progression\textsuperscript{175, 238}, while studies of elite controllers have shown that individuals with the propensity to control HIV have intact CD4 function and lower measures of CD4 T cell exhaustion and senescence\textsuperscript{239, 240}. Furthermore, HIV-specific CD4 T cells with cytotoxic potential have been associated with viral control\textsuperscript{241}.

**Antibody responses to HIV:**

B cells generate early IgM responses to HIV during acute HIV infection, but these are non-neutralising\textsuperscript{242}. Antibody class switching and development of high-affinity antibodies requires B cell-T cell interaction and somatic hypermutation\textsuperscript{223, 243}. Neutralising antibodies develop in most individuals in the first few months after infection but these quickly select for viral resistance mutations, usually in HIV Env\textsuperscript{244, 245}. In rare individuals, after years of uncontrolled infection, there is the development of broadly neutralising antibodies which are likely driven by exposure to antigen\textsuperscript{246}. The determinants of who will develop BNAbs are unclear; however,
HIV superinfection with a new strain of virus may be one of the driving forces\textsuperscript{247}. Passive administration of the BNAbs – VRC01 has been shown to prevent HIV infection in animal models\textsuperscript{248, 249}. The role of BNAbs in chronic HIV infection is less clear; slower HIV disease progression has been described in long-term non-progressors with BNAbs targeting \textit{Env} and \textit{Gag}\textsuperscript{250, 251}. However, the presence of BNAbs in the absence of ART does not confer any benefit regarding disease progression\textsuperscript{252}.

### 1.6.3. Immune activation and T cell exhaustion in HIV Infection

Elevated markers of inflammation and immune activation are a feature of untreated HIV infection\textsuperscript{253}. Uncontrolled viral replication and the associated increased expression of immune activation markers, in particular, CD38 on CD8 T cells, was identified early in the epidemic as a marker of disease progression\textsuperscript{254, 255}. Elevated markers of immune activation have been described on NK cells\textsuperscript{256}, B lymphocytes\textsuperscript{257} monocytes\textsuperscript{258}, neutrophils\textsuperscript{259} and plasmacytoid dendritic cells\textsuperscript{260}. This immune activation results in elevated levels of a host of proinflammatory cytokines in plasma and tissue including IL-1\(\beta\), IL-6, IL-8, tumour necrosis factor (TNF), sCD14, sCD163, MIP-1\(\alpha\), MIP-1\(\beta\), RANTES, and IFN-\(\gamma\)-inducible protein (IP-10)\textsuperscript{253}. Increased levels of these inflammatory markers in HIV infected individuals have been associated with disease progression and non-AIDS morbidity\textsuperscript{253}. For example, higher levels of the monocyte activation marker IP-10 in PHI has been associated with disease progression\textsuperscript{261} and HIV DNA, with the source of highest IP-10 production thought to be small intestine\textsuperscript{262}. sCD14 is shed from monocytes in response to lipopolysaccharide stimulation and has been suggested as a marker of bacterial translocation to the gut\textsuperscript{263}. Microbial translocation occurs when disruption to the integrity of the gut mucosal barrier allows transfer of bacterial products into the circulation resulting in persistent inflammation and immune dysfunction\textsuperscript{58, 264}. The mechanisms leading to mucosal barrier dysfunction in HIV in part relates to loss of gut Th17 cells during early HIV infection\textsuperscript{265-267}, while more recent evidence suggests that dysbiosis may
also play a role\textsuperscript{268, 269}. Markers of gut epithelial barrier dysfunction including sCD14 have been associated with mortality in treated HIV infection\textsuperscript{270}.

While a robust immune response is essential for clearance of viral infections, persistent antigenic stimulation and T cell activation in the context of chronic viral infections can result in an exhausted T cell phenotype where cells have decreased effector function and limited antiviral capacity ultimately contributing to viral persistence\textsuperscript{271}. T cell exhaustion is characterised by reduced proliferative capacity of T cells and a progressive loss in the ability to produce effector cytokines such as IL-2, TNF-\(\alpha\), IFN-\(\gamma\) and \(\beta\)-chemokines\textsuperscript{272}. Higher HIV viral load has been associated with a decline in polyfunctional anti-viral CD8 T cells capable of producing multiple cytokines\textsuperscript{273} and with increased expression of inhibitory receptors such as PD-1\textsuperscript{193, 274}, TIGIT\textsuperscript{174} and Tim-3\textsuperscript{275}. Expression of T cell exhaustion markers on CD4 T cells has also been shown to identify cell populations enriched for latent HIV infection\textsuperscript{177}. In addition to the loss of effector functions, exhausted virus-specific CD4 T cells primed during persistent infections express CXCR5 and other markers of TFH cells though to be dependent on chronic IFN-I signaling\textsuperscript{276}. In PHI, expression of exhaustion markers on CD8 T cells has been associated with diseased progression\textsuperscript{163} while their expression on both CD4 and CD8 cells in PHI has been associated with time to virological rebound on treatment cessation\textsuperscript{277}. 
1.7. HIV and the Gut

1.7.1. Gut Immunology

The gastrointestinal-associated lymphoid tissue (GALT) is the largest and one of the most complex facets of the immune system. In fact, it has been estimated that T lymphocytes, plasma cells, and macrophages in the gut account for 85% of total body lymphoid tissue\textsuperscript{278}.

The epithelial cells of the gut mucosa act as the first line of defence against potential pathogens and form the crypts and villi characteristic of the GI tract. This layer also includes goblet cells (more common in the colon) and paneth cells (small intestine only) responsible for the production of mucus and anti-microbial peptides, respectively. The immune cells of the GI mucosa encounter more antigen than any other body compartment and must have the ability to recognise harmful pathogen and tolerate harmless antigens such as food proteins\textsuperscript{279}. Microfold (M) cells are specialised enterocytes, which lack surface microvilli and the usual thick layer of mucus\textsuperscript{279} M cells endocytose antigen from the intestinal lumen, see figure 1.7.1. However, M cells do not express MHC class II molecules and, therefore, pass on antigen to professional antigen presenting cells (APCs) – usually dendritic cells. These APCs move to T-cell areas and B cell follicles, where they can interact with naïve lymphocytes\textsuperscript{279,280}.

Functionally the Gut-associated lymphoid tissue can be broadly divided into immune inductive and effector sites. The inductive sites include mesenteric lymph nodes (MLN), Peyer’s patches in the small intestine, and follicular aggregates in the large intestine. These collections of secondary organised lymphoid tissues are the primary sites of intestinal immune priming. Maturation of the naïve T-cells in the organised lymphoid tissue in GALT results in the expression of specific GI homing markers (including $a_4b_7$ and CCR9), through retinoic acid signalling\textsuperscript{281}, which facilitate their homing back to the mucosal effector compartments from the peripheral circulation\textsuperscript{282}. Inductive sites also support IgA class switching and clonal expansion of B-cells which occurs in response to antigen-specific TFH activation\textsuperscript{283}.
The effector immune sites in GALT are diffusely distributed throughout the gut mucosa and include the lymphocytes in the lamina propria and intraepithelial lymphocytes. The lamina propria comprises diverse immune cells including dendritic cells, macrophages, B cells and T cells while the epithelium is composed mainly of T cells. Intraepithelial lymphocytes (IELs) are those found in the epithelial layer, the majority of these are CD8-positive α/β T cells. However, approximately 15 per cent are γ/δ T cells. CD4 T cells predominate in the lamina propria but occasionally are also found in the epithelial layer.

There are structural and functional differences between the small and large intestine. Anatomically the small intestine spans from the pylorus to the ileocecal valve, while the large intestine begins in the caecum and ends in the anus. The small intestine is covered in characteristic small villi or microvilli which project into the lumen of the GI tract. The early part of the small intestine (duodenum and jejunum) is particularly important in digestion and absorption of nutrients which it does through the action of intestinal enzymes and epithelial cells respectively, while the ileum contains the highest frequency of Peyer’s patches. In contrast, the large intestine lacks villi, and its primary function is the absorption of water and elimination of undigested waste. It is also colonised with trillions of commensal bacteria which are essential for normal GI function.

The immunological milieu differs between the gut and peripheral blood and also along the GI tract itself, see figure 1.7.2. The relative proportions of CD4 memory subsets which populate the gut mucosa differ when compared to the peripheral blood; central memory (T_{CM}) and naïve (T_{N}) T cell subsets predominate in the peripheral blood while transitional memory (T_{TM}) and effector memory (T_{EM}) are the most common subsets in gut mucosa. In addition, the frequency of CD8 T cells and intraepithelial lymphocytes differ along the GI tract. Furthermore, CD4 T subsets with specialised function including Th17 and T_{Reg} are distributed at different frequencies along the GI tract; Th17 cells preferentially located in the lamina propria of the small bowel but are also found at lower number in the colon, while the opposite pattern is seen for T_{Reg} cells, with highest frequency in the colon. In contrast, Th1 and Th2 cells are
Figure 1.7.1 The gut mucosal immune system

This figure illustrates from of the main cell types involved in the immune response in the gut mucosa, including M cells which endocytose antigen from the mucosal surface, dendritic cells which play a role in antigen presentation, and the cells B and T lymphocytes of the adaptive immune system. These are found in organised lymphoid structures such as Peyer’s patches and also throughout the lamina propria. Plasma cells produce IgA, an important antibody on mucosal surfaces.


found at relatively similar frequencies along the GI tract. B cell populations are also found throughout the GI tract, in particular, IgA producing plasma cells. IgA2 producing cells being most abundant in colonic tissues and IgA1 in the small intestine. Innate lymphoid cells (ILCs), effector cells of the innate immune system, are also found throughout the GI tract, with ILC3s found at highest frequency in the small intestine.

Other gut resident cells include invariant natural killer T cells and mucosal-associated invariant T (MAIT) cells. MAIT cells are CD161+ CD8 T cells, are activated following viral infection and have been shown to play a role in mucosal immunity. Furthermore, the frequency of MAIT cells is depleted in early HIV infection and fails to recover.
Macrophages have a critical function in the gut immune environment; in addition to phagocytosis and antigen presentation, they are also involved in maintaining gut homeostasis\textsuperscript{284}. The colon possesses a higher frequency of macrophages when compared to the small intestine\textsuperscript{294} These constitutive differences in the immune environment between the peripheral blood and gut and also along the GI tract itself are important considerations when evaluating the mechanisms of HIV-associated immune dysfunction and HIV persistence.

1.7.2. HIV infection of GALT

The gut is a key site of infection and CD4 T cell depletion during primary HIV infection\textsuperscript{2}, which is in part due to specific characteristics of CD4 cells in the gut, which allow for rapid and sustained infection of gut CD4 T cells. Also, a significant proportion of uninfected CD4 T cells die by apoptosis and proptosis\textsuperscript{295, 296}.

There are some notable differences between T-cell populations in peripheral blood and GALT that are of particular importance with respect to predisposing T cells in GALT to HIV infection and HIV persistence. CCR5 is the primary co-receptor for HIV. Therefore, HIV preferentially infects and replicates in CCR5\textsuperscript{+} CD4 T cells\textsuperscript{297}. CCR5 is expressed on the majority of CD4\textsuperscript{+} T cells in GALT, and as such, these cells are massively depleted in acute HIV infection\textsuperscript{2, 298, 299}.

CD4 T cell depletion during PHI in the gut has been shown to be greater in effector sites compared to inductive sites\textsuperscript{2, 32} and the recovery of CD4 T cells in GALT is often limited even with antiretroviral therapy, with absolute numbers below that of healthy controls\textsuperscript{2, 32, 298, 300}. Furthermore, CD4 recovery in GALT may lag behind that in PBMCs\textsuperscript{301}, suggesting alternative mechanisms of immune recovery; collagen deposition and fibrosis of lymphoid tissue may contribute to the poor immune reconstitution in tissue sites\textsuperscript{302, 303}. 
Figure 1.7.2 The immune apparatus and constituent immune cells vary along the intestinal tract.

The figure demonstrates regional specialisation in the mouse intestine, indicating how antigenic content (red graphs), gut-associated lymphoid tissue (GALT; green graphs) and various immune cells (blue and orange graphs) change in frequency along the length of the intestinal tract.

Figure reproduced with permission. Mowat et al. Nature Reviews Immunology 14, 667–685 (2014) doi:10.1038/nri3738
The activation status of T cells in GALT differs from that in blood. In health, T cells in the gut are highly activated due to ongoing exposure of antigen from the GI tract. This baseline activated phenotype, in tandem with the fact that they are mainly (>98%) of the memory (CD45RO+) phenotype, provides a pool of ongoing targets for productive HIV infection in the gut. Furthermore, loss of mucosal integrity a consequence of microbial translocation and direct viral toxicity to enterocytes causes local inflammation, which again promotes T cell activation and de novo infection.

As previously discussed, trafficking of CD4 T cells to gut tissue is facilitated by their expression of α4β7 integrin. MAdCAM-1 is the ligand of α4β7 is expressed on the vasculature of all GI tissues. α4β7 integrin binds to the HIV envelope protein gp120 facilitating infection. Consequently, α4β7 expressing CD4 T cells are highly susceptible to productive HIV infection. In addition, the high concentration of susceptible gut-homing activated α4β7+ CD4+ T cells in mucosal surfaces likely contribute to development of an optimal environment for cell-to-cell spread. In the SIV model, preferential infection of α4β7 expressing CD4 T cells in peripheral blood has been shown during acute infection. Furthermore, higher α4β7 expression on CD4 T cells has been associated with increased risk of HIV acquisition and disease progression.

Th17 cells are highly permissive to productive HIV infection and are preferentially depleted in the gut. The loss of these IL-17 and IL-22 producing mucosal CD4 T cells may play a role in driving systemic immune activation, as these cells regulate epithelial homeostasis and are essential in maintaining mucosal epithelial integrity. Studies in humans and macaques have shown that Th17 recovery is poor despite ART initiation and that higher frequency of Th17 cells is associated with lower bacterial translocation. The barrier function of the gut mucosa is maintained by intact tight junctions between epithelial cells and the cooperation of other immune cells within the gut mucosa. As previously discussed the early infection and depletion of Th17 cells and other IL-17 producing cells contributes to the damage of the epithelial barrier. Also, the balance of mucosal Th17 and Treg cells is affected by
HIV; an expansion of T_{reg} cells is seen in the rectal and duodenal mucosa during chronic HIV infection\textsuperscript{321, 322}. This skewed Th17/T_{reg} ratio is, in part, mediated by Indoleamine 2,3 dioxygenase-1 (IDO-1) production by dendritic cells and monocytes. IDO-1 converts tryptophan into kynurenine, which skews CD4\textsuperscript{+} T-cell differentiation towards a T_{reg} phenotype instead of Th17 phenotype supporting bacterial translocation and immune activation\textsuperscript{266}. Ultimately this loss of integrity of the epithelial barrier seen in HIV/SIV infection allows for translocation of bacterial pathogens\textsuperscript{264} which in turn drive chronic immune activation\textsuperscript{58}.

As previously discussed, this chronic immune activation and associated increased measures of bacterial translocation, such as intestinal fatty acid binding protein (I-FABP), Zonulin-1 and sCD14 have been associated with HIV disease progression and mortality\textsuperscript{270}. Early ART, at least in the SIV model, has been shown to limit bacterial translocation, immune activation and HIV disease progression\textsuperscript{323}. While in humans, a modest reduction in T cell activation in rectal and duodenal GALT was observed with ART initiated in chronic infection\textsuperscript{324}.

### 1.7.3. Markers of immune function in GALT

The relative expression of many immune markers differs between blood and GALT. Studies have used the CD4/CD8 ratios in GALT as a surrogate of immune dysfunction and immune recovery\textsuperscript{2, 32, 325}. In health, lower CD4 percentage has been reported in GALT compared to blood\textsuperscript{2}. Shacklett et al. observed a mean CD4/CD8 ratio of 2.2 in rectal GALT from healthy controls and a ratio of 0.4 in HIV-infected individuals, of note only two of the ten HIV-infected individuals were on ART at the time of their biopsy\textsuperscript{325}. Mowat et al. also report a CD4/CD8 ratio of approximately 2.0 in the lamina propria of HIV uninfected individuals\textsuperscript{284}. Mehandru et al. using immunohistochemistry, measured the CD4/CD8 ratio in inductive sites in GALT; for HIV-uninfected controls a ratio of 2.4 (range 1.6-3.1) was observed while in those with untreated early/acute HIV infection the ratio was 1.3 (range 0.9-2.0), with lower ratios driven by higher CD8 counts rather than CD4 depletion\textsuperscript{32}. In health, discordance in the expression of other immune markers has also been described between blood and gut\textsuperscript{300}. This finding
highlights the importance of the inclusion of healthy controls in studies examining gut immune function. For example, in health, consistent elevation of the majority of markers of immune activation has been described in gut T lymphocytes\textsuperscript{300}, suggesting a more activated immune environment in the gut. Recent work has also reported lower perforin and granzyme expression on CD8 T cells in GALT compared to peripheral blood\textsuperscript{326, 327}. These studies demonstrate the importance of tissue sampling to understand the immune milieu in disease states and reinforces the assertion that measurement of peripheral markers of immune function does not necessarily reflect the tissue immune environment.

### 1.7.4. HIV reservoir and persistence in GALT

The majority of research into the HIV reservoir is carried out on CD4 T cells from the peripheral blood. One of the important considerations, when we interpret this research, is that less than two per cent of total body lymphocytes reside in the peripheral circulation; the vast majority are found in the lymphoid tissue of lymph nodes and the GI tract\textsuperscript{328}. Lymphoid tissue and not PBMCs has been shown to be the primary reservoir for HIV infection for people virally suppressed on ART\textsuperscript{329}. As mentioned above; there is a preferential infection of gut-associated lymphoid tissue (GALT) in acute HIV infection with concurrent seeding of HIV reservoir\textsuperscript{2, 330}. Persistence of HIV DNA has been demonstrated in GALT after years of suppressive ART, with levels of HIV DNA in GALT that are up to 5-fold higher than those measured from peripheral blood CD4\textsuperscript{+} cells\textsuperscript{331}. A recent publication used radio-labelled anti-gp120 to characterise the whole body HIV reservoir in macaques; this study identified GALT as well as nasal-associated lymphoid tissue (NALT) as two of the key compartments of viral reservoirs\textsuperscript{156}. Estes et al. used next generation \textit{in situ} hybridisation (ISH) technology to identify HIV DNA\textsuperscript{+} cells in tissue sites, thereby determining the anatomical locations of the HIV reservoir pre- and post-ART. On ART, HIV DNA declined in lymph node but not in rectal tissue, while 98% of HIV RNA\textsuperscript{+} cells were found in GALT on ART, suggesting that GALT may be particularly important in supporting HIV persistence through ongoing HIV replication or viral production\textsuperscript{185}. However, data are mixed on the compartmentalisation of viral replication on ART HIV in
sanctuary sites. Van Marle et al. sequenced HIV Nef from multiple gut sites in viremic individuals and reported evidence of viral diversity between anatomically distinct areas of the gut, suggesting compartmentalised viral replication. In contrast to this, Evering et al. performed single genome sequencing of HIV Env, on GALT and PBMC samples from ART-treated individuals and found no evidence of virus evolution. Imanichi et al. also found no evidence of compartmentalisation between ileum and colon. These findings have been corroborated more recently by Josefsson et al., who reported stable viral genetic sequences in GALT and PBMCs from individuals on ART for more than four years. However, Lorenzo-Redondo et al., using next-generation sequencing and a Bayesian evolution model make a strong case for evidence of viral evolution within lymph nodes with subsequent trafficking of productive virus to blood. This assertion is supported by the finding that specific antiretrovirals have poor penetration into lymphoid and gut tissue sites, thereby allowing ongoing viral replication. The potential for ongoing replication in some ART-treated individuals is evident; Estes et al. demonstrated CD4 RNA+ cells by ISH in ART-treated individuals. In support of this, treatment intensification studies with raltegravir have resulted in a decrease in us CA-RNA in the terminal ileum supporting the hypothesis that there is ongoing replication in this site. However, these findings may reflect virus production from latent cells rather than ongoing replication.

The majority of studies measuring HIV reservoir in the gut have reported a higher level of HIV DNA in GALT compared to PBMCs. However, an earlier study found no difference. While most studies involved sampling from one gut site, some have measured HIV DNA in multiple sites in the GI tract. One such study highlighted different burdens of HIV DNA along the GI tract; biopsies were taken from the duodenum, terminal ileum, ascending colon and rectum from eight HIV infected individuals on ART. Significantly higher DNA levels were found in all GALT sites compared to PBMCs with the highest median HIV DNA level measured in the rectum; however, there was no significant difference between HIV DNA levels in the gut sites in this study. Using the HIV DNA levels measured from this
study the author estimated that gut accounts for 83-95% of all HIV infected cells in the body. \(^{339}\) They also reported higher RNA / DNA ratios in the ileum suggesting a more transcriptionally active reservoir in that site. \(^{335}\) As already mentioned, treatment intensification studies with raltegravir have also identified the terminal ileum as an important site for HIV persistence with decreased HIV CA-RNA levels post-treatment intensification.\(^{191}\)

The CD4 T cell subsets present in gut sites may also contribute to HIV persistence; while \(T_{CM}\) and naïve CD4 T cells predominate in blood, \(T_{CM}\) and \(T_{EM}\) are the subsets most frequently found in GALT. \(^{328}\) Yukl et al. measured HIV DNA and HIV RNA in sorted CD4 T cell subsets in GALT, using tissue obtained from the terminal ileum and rectum in virally suppressed HIV+ individuals on ART; they found highest DNA levels in \(T_{EM}\) cells in gut sites compared to \(T_{CM}\) in peripheral blood. \(^{132}\)

There are abundant CD4 T cells subsets with specialised function in the gut that are known to be preferentially infected and enriched for HIV DNA; T follicular helper (TFH) cells are found in GALT lymphoid tissue and are a well-established cellular HIV reservoir.\(^{136, 340-345}\) TFH cells are found within the germinal centres of lymphoid follicles where they provide B cell help promoting high-affinity antibody responses.\(^{346, 347}\) Impaired TFH function has been described in HIV infected individuals\(^{236, 348, 349}\) with the destruction of up to 50% of germinal centres in the terminal ileum during the first 80 days of acute HIV infection.\(^{350}\) Also, TFH cells are preferentially infected\(^{342}\) and enriched for latent HIV\(^{135, 136, 351}\). Their location in the germinal centres which predominately exclude CD8 T cells may serve as a barrier to their elimination.\(^{186}\) In fact, in elite controlling macaques with otherwise suppressed viral replication off ART, germinal centres have been shown to be the only site of ongoing viral replication.\(^{186}\) This important CD4 subset is discussed in greater detail in chapter 5. As discussed previously, Th17 cells in the gut play an essential role in maintaining gut mucosal integrity.\(^{352}\) Th17 cells, characterised phenotypically by CCR6\(^+\) expression, have also been shown to harbour replication-competent HIV DNA during ART in the colon\(^ {353}\) and blood.\(^ {137}\) Th17 cells are long lived\(^ {354}\) and as such may support HIV persistence on ART.\(^ {355}\) The recently described tissue-
resident memory CD4 T (T_{RM}) cells are found in lung, skin and mucosal surfaces and do not traffic through the peripheral circulation \(^{356}\). Phenotypically they are distinguished from other T_{EM} populations by their expression of CD69 \(^{357}\). In adipose tissue CD69\(^+\) T cell have been found to harbour HIV DNA\(^{358}\) and highlight the potential role of these cells in the HIV tissue reservoir\(^{359}\). Other non-conventional T cells found in the gut such as gamma-delta (\(\gamma\delta T\)) cells \(^{360}\), NKT cells\(^{361}\) and MAIT \(^{293}\) cells are negatively impacted by HIV infected, but their contribution to the HIV reservoir is unknown.

The Yukl group have reported that non-CD4 T cells in gut harbour HIV DNA and have suggested these cells may be of monocyte/macrophage lineage \(^{362}\). The role of macrophages as true reservoirs of HIV is controversial\(^{363}\). HIV DNA and HIV RNA have been detected within macrophages with some groups suggesting true latent infection \(^{140,364}\). However, others suggest that this may reflect phagocytosis of infected cells rather than actual latent infection of macrophages \(^{365,366}\). In the humanised mouse model, HIV persistence has been demonstrated in macrophages, and these cells may be a potentially important cellular reservoir for HIV in tissue\(^{367}\).

Treatment factors may also contribute to HIV persistence in GALT. A recent paper demonstrated higher tissue penetration of raltegravir compared to dolutegravir. However, no significant differences in GALT HIV RNA, DNA, or immunologic markers were observed in that study\(^{368}\). Studies have also demonstrated lower levels of other ART in lymphoid tissue sites. Fletcher et al., using lymph node and GALT samples, measured the drug concentrations of ART in tissue and found lower levels in some locations, occasionally below the level of quantification \(^{184}\). In support of this, Lorenzo-Redondo et al. using viral evolution data, suggest ongoing replication in lymphoid tissue relates to poor penetration of ART in these sites \(^{183}\).
1.8. Approaches to and HIV eradication and cure

Antiretroviral Therapy (ART) confers a dramatic survival benefit, but treatment is lifelong, expensive and has problems of long-term adherence to therapy, drug toxicity, and risks the development of drug resistance. Consequently, there is patient, scientific, and public health support for management options beyond life-long medication.\(^\text{369}\)

1.8.1. Definition of HIV cure

Only one case of true HIV eradication has been reported.\(^\text{370}\) While such a sterilising HIV cure is highly desirable; a more achievable goal may be viral “remission” whereby the absence of on-going viral replication off therapy limits the risk of viral transmission, immune dysfunction and avoids life-long drug exposure and associated toxicities. Such a cure has been coined a functional cure.

1.8.2. Strategic use of ART in primary HIV infection

ART initiated during PHI has been used as a strategy to limit the size of the HIV-1 reservoir and augment immune recovery. The recent identification of a cohort of individuals who exhibit viral control after ART in PHI, known as post-treatment controllers (PTC) has re-invigorated the use of ART in PHI.\(^\text{131, 373}\) The original cohort of PTC individuals is from a French cohort referred to as the VISCONTI cohort. The PTC seen in these individuals adds to the substantial evidence in support of early ART as a strategy to enhanced recovery of immune markers associated with disease progression, such as CD4 T cells count\(^\text{109}\) and markers of immune activation.\(^\text{371}\)

As previously discussed amongst elite controllers, HIV-specific CD8\(^+\) immune responses are associated with viral control.\(^\text{53}\) The development of CTL viral escape mutants allows HIV to evade the cytotoxic T cell response early in untreated infection.\(^\text{230}\) However, treating with ART during acute infection may prevent the development of such variability in proviral \textit{gag} DNA epitopes; work by Deng et al. has shown that unless ART is started within 3 months of
infection, the vast majority (>98%) of latent viruses carry CTL escape mutations \textsuperscript{374}. Therefore, the initiation of early ART may alter the natural history of HIV disease, limiting viral diversity and allowing the development of immune response without mutational escape.

HIV related damage to the innate immune system in PHI, and more specifically to mucosal NK cell function, has been described \textsuperscript{375}. Recent data from the VISCONTI group suggests that NK cells in PTC patients retain the high capacity to control HIV infection, \textit{in vitro} \textsuperscript{376}. These data indicate that preventing irreversible damage to this arm of the immune system by use of early ART may be important to achieve PTC.

ART initiated during PHI seems to affect the distribution of HIV-1 DNA amongst CD4 T cell subsets limiting the reservoir in longer-lived central memory T cell (T\textsuperscript{CM}) subsets \textsuperscript{121}. Not all CD4 T cells subsets are equal with respect to longevity, and therefore the distribution of HIV-DNA in CD4 subsets may have implications for persistence; in the VISCONTI cohort, the T\textsuperscript{TM} subset (which are shorter lived) was the main contributor to the HIV-1 reservoir in those with PTC \textsuperscript{131}.

**Clinical trial evidence supporting immediate ART initiation at HIV transmission**

ART commenced in PHI reduces immune activation \textsuperscript{371} and enhances recovery of other immune indices such as CD4 T cell count \textsuperscript{109} and CD4/CD8 ratio \textsuperscript{377}. Those individuals exhibiting PTC in VISCONTI had low levels of immune activation \textsuperscript{131}, limiting the number of activated CD4 cells available as preferential targets for de novo HIV infection \textsuperscript{378}. Immune exhaustion markers such as PD-1 have been associated with HIV disease progression \textsuperscript{379}, recent data from the SPARTAC group highlights the importance of such markers in predicting HIV viral rebound with T cell exhaustion markers PD-1, Tim-3 and Lag-3 measured before ART predicting time to the return of viraemia \textsuperscript{380}.

SIV models demonstrate the very early establishment of an HIV reservoir, with evidence of seeding even when ART was initiated three days after viral infection \textsuperscript{29}. Findings from the macaque model, while informative, cannot be directly extrapolated to humans as the
characteristics of the virus and host significantly differ. In humans, the HIV reservoir is also established early; observational cohort studies have identified that even when ART is initiated within ten days from symptoms of HIV seroconversion quantifiable levels of HIV reservoir can be detected. However, evidence suggests that immediate ART commenced within weeks to months after HIV seroconversion can markedly reduce the subsequent size of the reservoir, as measured by HIV DNA, integrated HIV DNA, and viral outgrowth assays. Very early ART is associated with the lowest measures of HIV reservoir; Ananworanich et al. have shown that HIV DNA levels are lower in those starting ART in Fiebig stage I compared to Fiebig II or III. However, for sexual transmission of HIV, outside of clinical trials, it is exceptionally rare for individuals to present and initiate ART within such short timelines. Furthermore, initiation of very early ART alone does not induce viral control. A recent study by Colby et al. undertook a treatment interruption in individuals who had initiated ART during Fiebig stage I and had received a median of 2.8 years of ART. All eight individuals in this study experienced rapid viral rebound on cessation of ART. Amongst seroconverting adults within the SPARTAC trial, the closer to transmission ART was initiated, as well as the longer duration of therapy before treatment interruption, the longer the period of PTC was observed.

In a well-described cohort of young Thai men, HIV viral reservoir was already detectable within up to ten days post-infection and seeding of this reservoir was identified in gut mucosal biopsies. The impact of early ART on the gut HIV reservoir in rectal tissue has also been studied; undetectable HIV DNA levels were noted in three of seven individuals treated during acute HIV infection, while in another study treatment with ART during acute infection compared to ART initiated in chronic infection.

Extrapolation of this concept to perinatally infected infants has also been the subject of much interest. For the majority of infant cohorts, viral rebound is observed almost universally amongst the majority of infants interrupting therapy. In HIV-1-infected newborns, initiation of ART within 72 hours of birth leads to a reduction in viral reservoir size. The Mississippi
baby case reported that ART initiated within 36 hours of delivery, followed by 18 months of viral suppression with subsequent treatment interruption provided several years of PTC before viral recrudescence was observed. In the case of an infant in Milan who was commenced on ART within 12 hours of birth until three years of age, viral rebound was seen within two weeks of ART cessation. In these cases, assessment of HIV reservoirs in blood using HIV-1 DNA, and replication-competent viral reservoirs assays, before treatment interruption, did not predict viral rebound. However, in the case of the Milan infant, increased immune dysfunction (immune activation and HIV specific CTL responses) consistent with ongoing HIV replication before treatment interruption was noted. These findings suggest that the assays employed in these cases did not accurately measure viral reservoir that correlated with the inducible virus and suggests that unmeasured viral sanctuary sites might have been the source of viral recurrence.

**Duration of ART**

If the strategy of immediate ART in either adults with PHI or immediate initiation of ART amongst perinatally infected infants is adopted, the next question is what is the optimal duration of ART before planned treatment interruption that might best confer post-treatment control (PTC)? Data from the SPARTAC clinical trial demonstrates a direct association between ART duration when (commenced in PHI) and a longer time to viral rebound after treatment interruption. However, in the SPARTAC trial, a much lower proportion of individuals experienced viral control at two years (n=4) when compared with VISCONTI, suggesting either that 48 weeks of ART is probably an insufficient duration of therapy to confer PTC. Alternatively, it may be that ART was not initiated soon enough after PHI (in this trial eligible participants had a maximum of 6 months window from a previous negative test, with median time from estimated date of infection and ART initiation being 12 weeks). The post-hoc analysis of SPARTAC identified a greater impact of therapy if started less than 12 weeks from seroconversion and although the numbers were too small to confirm this, it is possible that PTC is more common if ART is started sooner; as seen in the Thai and
VISCONTI cohorts\textsuperscript{121,131}. The optimal or minimum duration of ART to achieve PTC remains unclear; in VISCONTI the median time on ART was 36.5 months (range 12-92) – ultimately a prospective study designed to study PTC will be needed to answer this question.

1.8.3. Post-treatment control (PTC)

We have previously discussed the rare individuals who spontaneously control HIV in the absence of any antiretroviral therapy (ART) – termed “elite controllers”\textsuperscript{391}. This phenotype is associated with host protective human leukocyte antigen (HLA) types\textsuperscript{392} and chemokine receptor genotypes\textsuperscript{393}, strong HIV specific CD8 responses,\textsuperscript{394} and a slower disease progression and lower morbidity\textsuperscript{391}. However, the absence of detectable viral replication in plasma does not normalise overall morbidity compared with age-matched uninfected controls\textsuperscript{395-397}, and trials of ART are currently underway to test whether treatment is still beneficial over deferral for elite controllers\textsuperscript{398,399}.

For the majority of people living with HIV, immunological decline is inevitable, and initiation of ART is necessary to confer viral control and immune recovery. Despite years of normalisation of markers of immune function (CD4 total count, CD4 \% and CD4/CD8 ratio)\textsuperscript{400,401}, for most people, stopping ART is accompanied by rapid viral rebound (within 2-4 weeks after TI) and has been shown to confer an increase in all-cause mortality\textsuperscript{101} and hence is not recommended. However, recent findings, from several cohorts, describe a phenomenon of post-treatment viral control (PTC) on interruption of ART\textsuperscript{131,402,403}. In such situations, interruption of ART is not universally accompanied by viral recrudescence, but instead confers significant periods of time (> 2 years) off therapy with a sustained undetectable ( < 50 copies HIV RNA/ml) viral load\textsuperscript{131,376} How common this phenotype is remains uncertain; from large observational cohorts it appears to be between 5-10\% of the population. PTC has now been well described in various European cohorts\textsuperscript{131,403,404}, with the incidence of PTC reported between 15.6\% in the VISCONTI cohort\textsuperscript{131} and 5.5\% in CASCADE\textsuperscript{403}. These cohorts are summarised in table 1.8.1. These findings are tantalising; it appears that a period on ART if
commenced in PHI has the capacity to alter the natural history of disease progression for certain individuals. Importantly, these individuals differ in phenotype from spontaneous *elite controllers*; they are not enriched for the previously described protective HLA alleles and do not demonstrate strong CD8+ HIV-specific responses. However common to both groups are low levels of measures of peripheral blood HIV-1 viral reservoirs.

There appears to be a critical, albeit uncertain, viral reservoir threshold below which post-treatment control maybe feasibly predicted. Data from the VISCONTI cohort indicates the quantity of HIV DNA-1 (median 1.71 log copies/10⁶ PBMC) is much lower in PTC than non-controllers and unexpectedly, these levels continued to decrease off ART. Furthermore, long-lived CD4 T cell subsets (i.e. naïve CD4 T cells) contributed less to the reservoir in viral controllers. These data suggest low reservoir levels may be a prerequisite for PTC. This is supported by data from the SPARTAC randomised control trial which examined intermittent ART (for a duration of 12 weeks versus 48 weeks) versus – standard of Care (no ART until CD4 guided ART initiation) in PHI on viral set point and disease progression. In this study, four individuals controlled plasma viraemia (<400 copies/ml) for at least two years after stopping therapy. From this cohort, total HIV-1 DNA level (but not integrated HIV DNA) at the time of treatment interruption in the 48-week arm was associated with time to viral rebound. Nonetheless, low measures of HIV reservoir using quantification of total HIV DNA /CD4+ T-cell assay does not accurately predict or guarantee PTC, and the optimal measure of the reservoir to do this is yet to be defined.
# Table 1.8-1 Summary of Post-Treatment Control cohorts

<table>
<thead>
<tr>
<th>Trial/Study</th>
<th>Cohort Characteristics</th>
<th>Number of cases of PTC Rates (% of overall cohort)</th>
<th>Time to ART Initiation (IQR)†</th>
<th>Duration of ART before Interruption / Median (IQR)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VISCONTI</td>
<td>PTC definition: HIV RNA levels &lt;400 copies/mL for at least 24 months after ART interruption. Original case series included 14 cases of PTC – a recent update includes 20 cases. The median duration of remission was 9.3 years, with the longest period of control of more than 12 years</td>
<td>N=20</td>
<td>&lt; 10 weeks</td>
<td>3.04 years (N/A)</td>
</tr>
<tr>
<td>CASCADE</td>
<td>Total cohort 25629, of whom 259 interrupted ART initiated in PHI. PTC definition: 2 consecutive measurements showing HIV RNA level &lt;50 copies/mL or re-initiation of ART after TI</td>
<td>N=11 (5.5%) [95% CI, 3.1-9.0]</td>
<td>≤ 3 months</td>
<td>1.3 years (0.8-2.0)</td>
</tr>
<tr>
<td>SWISS</td>
<td>Total cohort = 32 patients who ART initiated in PHI. PTC definition: plasma HIV RNA &lt;50 copies/ml during follow-up period (range 0.8-2.5 years).</td>
<td>N=3 (9%)</td>
<td>≤ 4 months</td>
<td>1.5 years (1.3-1.6)</td>
</tr>
<tr>
<td>Primo-SHM RCT</td>
<td>53 patients randomised to ART treatment arms (24 or 60 weeks). PTC Definition: VL below 100 copies/ml at 36 weeks</td>
<td>N=4 (7.5%)</td>
<td></td>
<td>24 weeks or 60 weeks</td>
</tr>
<tr>
<td>SPARTAC RCT</td>
<td>243 individuals randomised to short course ART (12 or 48 weeks). PTC definition: HIV RNA &lt;400 copies/ml for at least 24 months</td>
<td>N=4 (1.6%)</td>
<td>Median 12 weeks (IQR 9-15)</td>
<td>12 weeks or 48 weeks</td>
</tr>
<tr>
<td>Seattle Cohort</td>
<td>Total cohort of 22 individuals initiating ART in PHI. PTC definition; VL&lt;500 copies/ml ≥24 months</td>
<td>N=1 (4.5%)</td>
<td>≤ 3 months</td>
<td>&gt;0.9 years</td>
</tr>
<tr>
<td>OPTIPRIM RCT – ANRS 147</td>
<td>RCT of 24 months of 5 vs 3 drug ART in PHI. Total cohort of 90, 75 interrupted ART. PTC definition HIV RNA &lt;50 copies/ml 24-month post TI</td>
<td>N=1</td>
<td>36 days</td>
<td>2 years</td>
</tr>
</tbody>
</table>

† IQR stated only where available. Abbreviations: IQR, Interquartile range; PTC, Post-treatment control; N/A, Not available; ART, Antiretroviral therapy; TI, Treatment interruption; VL, Viral load; PHI, Primary HIV Infection; RCT, Randomised control trial
1.8.4. Latency Reversing Agents

Histone deacetylase inhibitors (HDACi) are epigenetic modifiers and have been investigated to purge the latent reservoir in early pilot studies. Vorinostat has been shown to disrupt HIV latency \(^408\), and a multi-dose trial resulted in increased expression of HIV RNA after 14 days of treatment\(^409\). However, there was no evidence for a reduction in the size of the HIV reservoir as measured by total DNA \(^410\). Data from a primary T cell model suggests that post-transcriptional blocks also contribute to latency \(^411\), this was based on work using the reactivating agent's vorinostat and disulfiram which increased viral transcription but failed to enhance viral translation effectively. In contrast to this, the more potent HDAC inhibitor romidepsin has shown promise with the safe induction of HIV-1 transcription resulting in plasma viremia \(^412\). The potential adverse effects of new therapeutics must also be considered as, for example, the suggestion that HDAC inhibitors suppress the ability of CTL to kill HIV-infected cells \(^413\). Individual patient safety with HDAC inhibitors must also be considered; vorinostat caused non-specific gene expression 84 days following administration \(^414\), and more potent HDAC inhibitors may have worse and potentially more long-lasting safety profiles. These safety concerns are particularly pertinent when we consider the evidence that people living with HIV are already at increased risk of non-AIDS morbidities such as cancer \(^415\). These results illustrate the importance of understanding the cellular factors and pathways involved in viral transcription and productive viral replication and how such an understanding is needed for the development of appropriate drugs to safely target HIV latency. Although it is likely the HDAC inhibitors in addition to ART alone are not the answer to an HIV cure, if they can be used to establish proof-of-principle that ‘latent’ provirus can be activated and reawakened, it will provide a springboard for further mechanistic studies and drug development. Accordingly, they remain an interesting drug class.

Activating the latent reservoir is unlikely to be a sufficient alone to eradicate or allow control of HIV infection off ART for the majority of individuals, and likely augmentation or restoration of immune function will be needed in tandem. In vitro models have shown that demonstrate
that stimulating HIV-1-specific CTLs prior to reactivating latent HIV-1 may be essential for successful eradication efforts. Such approaches targeting the HIV reservoir have been coined ‘Kick and kill’ where the immune system is first primed followed by reactivation of the latent HIV reservoir. A number of studies are ongoing or have recently reported on the use of HDAC inhibitors in combination with therapeutic vaccination. The BCN-02 study demonstrated viral control in individuals who had received romidepsin in combination with a prime-boost vaccination strategy. The RIVER study used the same vaccine in combination vorinostat and results from this study contribute data to chapter 7 of this thesis.

1.8.5. Other HIV Eradication Approaches

The use of other immune modulators in combination with viral activators is also being assessed as an approach to control HIV. PD-1 is a T cell exhaustion marker expressed on cells that have lost much of their effector functions; blocking this receptor seems to restore some immune efficacy. In patients on effective ART, there is a correlation between HLA-DR and PD-1 expression and the frequency of cell-associated HIV RNA or DNA. Agents that interact with PD-1 have been used with some success in malignant melanoma, and PD-1 is currently under investigation as a potential therapeutic target for latent HIV.

ART Intensification strategies have yielded limited success in suppressing low-level viral replication by ultrasensitive assays. However, intensification does lead to decreased levels of immune activation and inflammatory markers. Such strategies may form part of a combination approach with the aim of a functional cure for HIV.

Gene Therapy approaches have been employed to render CD4 T cells resistant HIV infection, in an attempt to replicate the success of the only case of HIV cure – the Berlin patient, who received a bone marrow transplant from a donor with a homozygous 32-bp deletion (Δ32/Δ32) in CCR5 conferring resistance to HIV infection by R5 tropic virus. Zinc finger nucleases have been used to engineer autologous CD4 T cells with this CCR5 deletion ex vivo inducing a reduction in HIV viral on reinfusion of the CCR5 deleted CD4 T cells.
Broadly Neutralising Antibodies (BNAbs) have been used with limited success. As previously discussed naturally arising BNAbs are found in a subset of HIV-1-infected patients after years on therapy. This has led to the isolation developed of monoclonal antibodies with broad and potent activity against HIV for therapeutic use. In the macaque model, BNAbs have been successful in preventing infection with SIV. Studies examining the role of BNAbs as a strategy to control viral rebound on cessation of ART have had less success; two single-arm studies using the BNAb VRC01 in individuals with chronic HIV infection failed to show a durable benefit of VRC01, all participants rebounded after treatment interruption and rebound virus demonstrated resistance to the BNAb tested.

A large number of HIV therapeutic vaccines are in development or currently in early phase clinical trials. A range of vaccination strategies are being tested included DNA, RNA, peptide, viral vector and dendritic cell vaccines. Early data from one study has been promising; the BCN02 study used a prime-boost vaccination protocol in addition to the HDAC inhibitor romidepsin. The vaccine antigens were designed to elicit T cell responses on highly conserved parts of HIV, including elements from the Gag, Pol, Env and Vif proteins and an early analysis has demonstrated viral control in some individuals six months after treatment interruption.

Non-human primate studies using a monoclonal antibody targeting α4β7 integrin has had promising results in macaques. SIVmac239 infected monkeys received 90 days of ART initiated five weeks post infection and then underwent infusions of the α4β7 integrin antibody every three weeks from week 9-32. Following treatment interruption in this study, all eight animals who had received the α4β7 antibody treatment controlled viral replication for over nine months. Phase I clinical studies with this antibody are now underway in HIV infected individuals.

A summary of some of the broad range of therapeutics currently being tested or in clinical development with the aim of HIV control or eradication are outlined in table 1.8.2 below.
<table>
<thead>
<tr>
<th>Trial (Clinicaltrials.gov identifier)</th>
<th>Location</th>
<th>Phase</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic HIV Infection</td>
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<tr>
<td>* Also has an Acute infection arm</td>
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<tr>
<td>Towards HIV Functional Cure - ULTRA-STOP (NCT01876962)</td>
<td>France</td>
<td>NA</td>
<td>A Pilot Study Evaluating the Maintenance of Viral Suppression After 24 Weeks of Therapeutic Interruption in Chronic HIV-1 Infected Patients with a Low Circulating HIV-DNA Reservoir</td>
</tr>
<tr>
<td>ACTG A5308 (NCT01777997)</td>
<td>USA</td>
<td>IV</td>
<td>Single-Arm, Open-Label Study to Evaluate the Effect of Fixed-Dose TDF/FTC/RPV on T-Cell Activation, Absolute CD4+ Count, Inflammatory Biomarkers and Viral Reservoir in Treatment-Naive HIV-1 Controllers</td>
</tr>
<tr>
<td>*New Era Study: Treatment with Multi-Drug Class (MDC) HAART in HIV Infected Patients</td>
<td>Germany</td>
<td>III</td>
<td>A Multicenter, Open-label, Non-randomized Trial to Evaluate Treatment with MDC HAART and Its Impact on the Decay of Latently Infected CD4+ T Cells</td>
</tr>
<tr>
<td>Effect of A CCR5 Co-receptor Antagonist On the Latency And Reservoir Of HIV-1 (NCT01950325)</td>
<td>Spain</td>
<td>II</td>
<td>Pilot Study Of The Effect Of Maraviroc On The Latency And Reservoir Of HIV-1 In Patients Taking Highly Active Antiretroviral Therapy</td>
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<tr>
<td>Acute HIV</td>
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<tr>
<td>Antiretroviral Therapy for Acute HIV Infection (NCT0079626)</td>
<td>Thailand</td>
<td>III</td>
<td>Subjects with acute HIV infection are randomised to receive standard HAART (TDF/FTC/EFV) or mega-HAART (TDF/FTC/EFV + MVC +RAL)</td>
</tr>
<tr>
<td>Early cART and cART in Combination with Autologous HIV-1 Specific CTL Infusion in The Treatment of Acute HIV-1 (NCT02231281)</td>
<td>China</td>
<td>III</td>
<td>The purpose of this study is to assess the ability of the early initiation of cART or cART in combination with autologous HIV-1 specific cytotoxic T lymphocyte (CTL) infusion achieve a post-treatment control among treatment-naïve acute HIV-1 infection arm</td>
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<tr>
<td>Those with * below also have an acute infection arm</td>
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<tr>
<td>Antibody Trials</td>
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<tr>
<td>* Also has an Acute infection arm</td>
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<tr>
<td>3BNC117 - Broadly Neutralizing Monoclonal Antibody (NCT02018510)</td>
<td>USA</td>
<td>I</td>
<td>A Phase 1, Open-Label, Dose-escalation Study of the Safety, Pharmacokinetics and Antiretroviral Activity of 3BNC117 (broadly neutralising) Monoclonal Antibody in HIV-infected and HIV-uninfected Volunteers</td>
</tr>
<tr>
<td>BMS-936559 – Anti PD L1 (NCT02028403)</td>
<td>USA</td>
<td>I</td>
<td>This study will evaluate the safety, PK data, and immune response to BMS-936559, (administered by IV infusion), in HIV-infected people receiving cART VL&lt;50</td>
</tr>
<tr>
<td>VRC 601 - Broadly Neutralizing Monoclonal Antibody (NCT01950325)</td>
<td>USA</td>
<td>I</td>
<td>This is the first clinical trial of the VRC-HIVMAB060-00-AB (VRC01) monoclonal antibody. This is a dose-escalation study to examine safety, tolerability, dose and pharmacokinetics of VRC01</td>
</tr>
<tr>
<td>*CHERUB 001 - Intravenous immunoglobulin</td>
<td>UK</td>
<td>I</td>
<td>A proof of concept study examining the effect of high dosage IVIG in ART-treated acute infection on the CD4+ T cell HIV reservoir</td>
</tr>
<tr>
<td>Latency Reserving Agents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vorinostat: HDACi (NCT01365065)</td>
<td>Australia</td>
<td>II</td>
<td>To assess safety and effect on HIV transcription of Vorinostat in Patients Receiving Suppressive cART</td>
</tr>
<tr>
<td>Vorinostat: HDACi (NCT01319383)</td>
<td>USA</td>
<td>I/II</td>
<td>A study of the Effect of Vorinostat on HIV RNA expression in resting CD4+ T Cells of HIV-infected Patients Receiving Stable Antiretroviral Therapy</td>
</tr>
<tr>
<td>Disulfiram (NCT01944371)</td>
<td>USA</td>
<td>I/II</td>
<td>To determine the safety, pharmacology and bioactivity of disulfiram in treated HIV-infected adults. The primary hypothesis is that three days of disulfiram will result in an increase in HIV transcription in CD4+ T-cells</td>
</tr>
<tr>
<td>Poly-IICLC: TLR-3 agonist (NCT02071095)</td>
<td>USA</td>
<td>I/II</td>
<td>Investigating the adjuvant, Poly-IICLC to establish if it is safe and well tolerated in HIV-1-infected subjects on combination antiretroviral therapy</td>
</tr>
<tr>
<td>ALT-803: recombinant human superagonist interleukin-15 complex (NCT02191098)</td>
<td>USA</td>
<td>I</td>
<td>Proof of Principle Study of Pulse Dosing of IL-15 to Deplete the Reservoir in HIV Infected People on Optimized ART with Undetectable Plasma HIV RNA</td>
</tr>
<tr>
<td>Romidepsin: HDACi (NCT01933594)</td>
<td>USA</td>
<td>I/II</td>
<td>A Study of Single Dose Romidepsin in HIV-Infected Adults with Suppressed Viremia on cART to Assess Safety, Tolerability, and Activation of HIV-1 Expression</td>
</tr>
</tbody>
</table>
### Table 1.8-2 continued

<table>
<thead>
<tr>
<th>Gene Therapy</th>
<th>Number of Sites</th>
<th>Design</th>
<th>USA</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal-1: Safety Study of a Dual Anti-HIV Gene Transfer Construct Cal-1 to Treat HIV-1 Infection (NCT01734850)</td>
<td>1</td>
<td>I/II</td>
<td>A study looking at whether an experimental gene transfer agent, LVsh5/C46 (also known as Cal-1), can inhibit HIV infection by removing CCR5 from bone marrow &amp; WBCs and producing a protein named C46.</td>
<td></td>
</tr>
<tr>
<td>VXR496: Tolerability and Therapeutic Effects of Repeated Doses of Autologous T Cells with VXR496 in HIV (NCT020295477)</td>
<td>1</td>
<td>I/II</td>
<td>Open-label, Study to Evaluate the Tolerability, Trafficking and Therapeutic Effects of Repeated Doses of Autologous T Cells Transduced with VXR496 in HIV Infected Subjects</td>
<td></td>
</tr>
<tr>
<td>MazF-T: Redirected MazF-C4D4 Autologous T Cells for HIV Gene Therapy (NCT01787994)</td>
<td>1</td>
<td>I</td>
<td>Evaluate the Safety &amp; Immunogenicity of Autologous CD4 T Cells Modified with a Retroviral Vector Expressing the MazF Endoribonuclease Gene in HIV+ patients</td>
<td></td>
</tr>
<tr>
<td>SB-728-T: Dose Escalation Study of Autologous T-cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases (NCT01044654)</td>
<td>1</td>
<td>I</td>
<td>Dose Escalation Study of Autologous T-Cells Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases SB-278 in HIV-Infected Patients Who Have Exhibited Suboptimal CD4+ T-Cell Gains During Long-Term cART</td>
<td></td>
</tr>
</tbody>
</table>

**Novel Combination Therapies**

* Also has an Acute infection arm

- **RIVER**: Research In Viral Eradication of HIV Reservoirs. Safety and Efficacy of the HDACi Vorinostat & Vaccine when combined with a CD4 Cell Count > 600/mm³
- **REDUC**: Safety and Efficacy of the HDACi Romidepsin & Vaccine Vacc-4x for Reduction of Latent HIV Reservoir (NCT02229665)
- **Repeat Doses of SB-728mR-T (Zinc finger nucleases) After Cyclophosphamide Conditioning (NCT02222665)**
- **Vacc-4x + Lenalidomide vs. Vacc-4x +Placebo in HIV-1-infected ART (NCT01704781)**
- **CD4-ZETA Gene-Modified T Cells With and Without Exogenous IL-2 In HIV Patients (NCT01044654)**

**Therapeutic Vaccines**

- **DermaVir**: topically applied DNA vaccine (NCT00711230)
- **MAG-pDNA + rSVIN HIV-1 Gag: DNA + viral vector vaccines (NCT01859325)**
- **GTU-multiHIV + LIPO-5: DNA + lipopeptide vaccines (NCT01489285)**
- **VAC-3S: peptide-based vaccine (NCT02041247)**
- **AGS-004: Dendritic cell vaccine (NCT02022428) IGHID 1309 Study**
- **A Pilot Study of a Dendritic Cell Vaccine in HIV-1 Infected Subjects (NCT00833781)**

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**1.8.6. Biomarkers in HIV eradication studies**

Interventional studies aimed at HIV eradication will need reproducible, measurable endpoints. The ultimate clinically relevant test of HIV remission or eradication currently requires a treatment interruption with subsequent monitoring for viral rebound. We know that the vast majority of individuals who stop ART will experience measurable viral rebound within two weeks. Accordingly, a biomarker to accurately predict individuals most likely to be able to safely interrupt therapy and have the best chance of PTC are needed. Such a biomarker is likely to be multi-dimensional, not merely quantifying the reservoir but also qualifying its replication competency using sequence data and potentially integration site analysis, in addition to clinical parameters. Assessment of the immunological factors associated with HIV control will also be required. It seems unlikely that a single biomarker alone will predict those who can control but instead a combination of marker incorporated into a predictive algorithm.

Multiple laboratory assays have been developed to identify and quantify the size of the HIV reservoir, but to date, none have accurately been able to predict viral rebound after treatment interruption. Previous work from our group has shown that the best predictor of time to viral rebound after interrupting ART is total HIV DNA from peripheral blood CD4+ T-cells. These findings were from the SPARTAC trial, a randomised controlled study comparing...
short course ART in primary infection with deferred therapy. This study demonstrated that after 48 weeks of ART individuals with high levels of Total HIV-DNA experienced plasma viraemia quicker than those with lower levels of HIV DNA. These findings suggest that a viral reservoir threshold may exist below which post-treatment viral control may be a feasible outcome. Note that whilst total HIV DNA measured from blood predicted time to viral rebound it did not conversely predict PTC. Amongst case reports of individuals in apparent viral remission off therapy, measurements of total HIV DNA from blood whilst correlating with time to viral rebound, failed to predict viral rebound. Such a prospective study has yet to be undertaken. These observations, suggest that current measurement of viral reservoirs from blood samples are insufficient, to accurately predict potential and sampling from alternative important HIV reservoir sites such as the gut is required.

1.8.7. Ethical Considerations in HIV eradication research

Research into curing HIV-1 infection engenders important ethical considerations. Life expectancy for people living with HIV and receiving ART approaches that of the general population and, accordingly, virological measures, morbidity and quality of life outcomes rather than mortality now predominate as primary endpoints in clinical trials of new interventions. Researchers must balance the potential, and still unknown, lifelong ART-related toxicities with the impact of long-term increased inflammation and immune activation observed even for people on successful suppressive ART with the benefits of being cured of HIV or virally suppressed off therapy. Any large-scale clinical trials of potentially toxic interventions assessing HIV cure must have strict safety and acceptability safeguards in place. Furthermore, full and transparent engagement with the HIV community and industry partners as well as policymakers in the design of such trials will be paramount, in particular concerning the potential risk of viral transmission for those when virus rebound may occur.
1.9. Summary

In summary, ART initiated in PHI offers an opportunity to limit the impact of HIV infection and may provide early treated individuals with the best opportunity for success in HIV eradication interventions. The gut immune system is a principal target site during early HIV infection and important in disease persistence during treated infection. Understanding the interaction between HIV and the gut immune system in this key population of treated PHI individuals is critical to developing effective HIV cure interventions. Identification of appropriate markers of gut HIV reservoir and immune function will be needed to understand the biology of the reservoir in this anatomical site and to guide future HIV cure interventions studies.

1.9.1. Thesis Aims

The aims of this thesis are to:

i. Characterise the HIV reservoir and markers of immune function and inflammation in blood and gut-associated lymphoid tissue of individuals treated during PHI

ii. Assess the impact of ART initiated during PHI on HIV reservoir and markers of immune function across anatomical sites including the peripheral blood, terminal ileum and rectum.

iii. Identify biomarkers associated with HIV reservoir and immune function in GALT which can be used in future studies aimed at HIV eradication
Chapter 2

Methods

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Chapter 2.

Methods

2.1. Clinical Cohorts

2.1.1. The UK register of HIV Seroconverters (UKHSC)

The UK HIV Seroconverters Cohort (UKHSC) is an observational cohort of routine clinic data from individuals with defined HIV seroconversion dates collected between 1994-2014\textsuperscript{438}. Patients were eligible for this study if they were: 16 years or older at the time of their first HIV antibody positive test, had confirmed PHI according to the criteria outlined in table 2.2.1 below and were willing to give written informed consent. The data collected by the register included: sex, ethnic group, probable route of HIV transmission, annual CD4 counts, details of therapy and prophylaxis prescribed, AIDS-defining events and vital status\textsuperscript{438}, CD8 counts were also available for a subset of individuals. Follow up information was collected annually. This study was funded by the Medical Research Council (MRC). Clinical data from this cohort were used in chapter 3.

2.1.2. The SPARTAC trial

The SPARTAC (Short Pulse Anti-Retroviral Therapy At Sero-Conversion) trial was a randomised control trial of short course ART in primary HIV infection. It was designed to test whether a short period of treatment with ART in the early stages of HIV infection can delay the need for ART in the longer term. It enrolled adults with primary infection who were within six months of seroconversion who were then randomised to receive ART for 48 weeks, 12 weeks, or no therapy (standard of care, SOC). The primary endpoint was the time from randomisation to either CD4 $<$350 cells/mm\textsuperscript{3} or initiation of continuous ART. It completed participant follow up in 2010. A total of 366 participants were randomised from 35 sites in Australia, Brazil, Europe and Africa; 40% were from the UK and 35% South Africa. Of these, 60% were men (90% MSM) and 40% African women. The results were published in 2013\textsuperscript{110}. PBMCs and
clinical data from this study were used in chapter 3, and chapter 5 and the details of the samples used are outlined in the respective chapters.

2.1.3. The HEATHER cohort

The HEATHER study (HIV Reservoir targeting with Early Antiretroviral Therapy) is an observational cohort of individuals with documented Primary HIV infection (PHI) - within three months of HIV acquisition; who have commenced on immediate Antiretroviral Therapy (ART). It enrolled over 250 individuals across three clinical sites in London, UK. The recruiting sites were St. Mary’s Hospital, Guys and St Thomas’s Hospital and 56 Dean St, all in London. Primary HIV infection was confirmed according to the criteria outlined in table 2.2.1. Inclusion criteria for the study included confirmed laboratory evidence of PHI and initiation of ART within three months of the date of HIV diagnosis with uninterrupted ART. Individuals with hepatitis B or C co-infection at screening were not eligible. Clinical data from this study was used in all results chapters. All participants gave informed consent before screening. Recruitment for studies within the HEATHER cohort was approved by the West Midlands—South Birmingham Research Ethics Committee (reference 14/WM/1104).

2.1.4. The HEATHER gut study

The HEATHER gut study was a sub-study of the main HEATHER study. Individuals who consented to HEATHER at all sites were potentially eligible for this sub-study. Inclusion criteria were as for the main study. Addition exclusion criteria for the HEATHER gut study included a diagnosis of bleeding disorders or diagnosis of Inflammatory bowel disease and Individuals on immunosuppressive therapy. The gut sub-study had two study visits, the first was a screening visit and the second involved a colonoscopy procedure with biopsies taken from the terminal ileum and rectum in addition to concurrent blood sampling. There was an optional third and fourth study visit which consisted of a repeat colonoscopy and gut biopsy approximately one year after the first procedure, with a preceding re-screening visit. The colonoscopy procedure was at St Mary’s Hospital (Imperial College Healthcare NHS Trust).
HIV viral load and lymphocyte subset counts were taken around the time of the colonoscopy procedure. Participants were reimbursed £100 for each colonoscopy. The study visits and procedures at each visit are outlined in table 2.1.1. Following completion of the HEATHER gut study participants were followed up as part of the main HEATHER study. The HEATHER gut study was approved by the West Midlands—South Birmingham Research Ethics Committee (reference 14/WM/1104) as an amendment to the main study program.

Table 2.1-1 HEATHER gut study visits and procedures

<table>
<thead>
<tr>
<th>Study Visit</th>
<th>Visit 1 Screening</th>
<th>Visit 2 1st Colonoscopy</th>
<th>Visit 3 Safety visit</th>
<th>Visit 4 2nd Colonoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timeline</td>
<td>Day -42 to -1</td>
<td>Day 0</td>
<td>Within 14 days prior to visit 4</td>
<td>Day 365 +/- 28</td>
</tr>
<tr>
<td>PIS given to eligible individuals</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Informed consent</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colonoscopy consent</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Review eligibility</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Medical/ARThistory review</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Physical exam</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 T cell count</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma HIV VL</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full blood count</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clotting (INR)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC for HIV DNA</td>
<td>X</td>
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<td></td>
</tr>
<tr>
<td>PBMC for flow cytometry</td>
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<td></td>
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<tr>
<td>Colonoscopy</td>
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</tr>
<tr>
<td>Rectal Biopsy</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileum Biopsy</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“X” indicates a procedure was carried out at that study visit
Abbreviations: PIS, patient information sheet; VL, viral load; INR international normalised ratio; PBMC, peripheral blood mononuclear cells
2.1.5. The RIVER study

The RIVER (Research In Viral Eradication of HIV Reservoirs) trial was a proof of concept-randomised study. It investigated the impact of immediate ART initiation at the time of HIV acquisition comparing the impact of additional HIV vaccination and latency-reversing agents, vorinostat, a histone deacetylase inhibitor (HDACi) with ART alone (see figure 3). The primary endpoint of this study was total HIV DNA in PBMCs. The study recruited n= 62 participants at five sites in London and the South East of England, namely Imperial College NHS Healthcare Trust, Chelsea and Westminster NHS Foundation Trust, Guy's and St Thomas’ NHS Foundation Trust and Brighton and Sussex University Hospitals NHS Trust. All study participants completed the trial protocol by December 2017. The main study primary outcome compared measures of total HIV DNA in blood CD4+ T cells between the two arms. The RIVER study contributed data to chapter 7. The RIVER study was approved by the South Oxford Research Ethics Committee (reference 14/SC/1372).

2.1.6. The RIVER gut study

Individuals who had completed the RIVER study were followed up as part of the HEATHER study protocol. These individuals were offered the opportunity to participate in the HEATHER gut study. As such, a number of individuals who had completed the RIVER study underwent colonoscopy and gut biopsy as part of the HEATHER gut study. Therefore, the ethical approval for colonoscopy and biopsies in this cohort was as for the HEATHER gut study (reference 14/WM/1104). Data on this cohort of individuals is presented in chapter 7.
2.2. Criteria for primary HIV infection

In all studies primary HIV infection was confirmed by any one of the criteria outlined in table 2.1.2. Additional criteria unique to the SPARTAC trial and the UKHSC are detailed in chapter 3. The estimated date of HIV seroconversion (EDI) was calculated according to the method of PHI confirmation as shown in table 2.1.1. If individuals had confirmed PHI by more than one of the criteria listed, the following order of preference was used to calculate EDI: B (HIV-1 antibody negative with positive PCR or positive p24 Ag), D (equivocal HIV-1 antibody test supported by a repeat test within 2 weeks showing a rising optical density), E (clinical seroconversion syndrome with antigen positivity), A (a negative HIV antibody test within 6 months of a positive test), and C (RITA consistent with recent infection).

Table 2.2-1 Criteria for diagnosis of PHI

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Calculating estimated date of seroconversion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> HIV-1 positive antibody test within six months of an HIV-1 negative antibody test</td>
<td>Midpoint of most recent negative test and positive test</td>
</tr>
<tr>
<td><strong>B</strong> HIV-1 antibody negative with positive PCR (or positive p24 Ag or viral load detectable)</td>
<td>Date of test</td>
</tr>
<tr>
<td><strong>C</strong> RITA (recent incident assay test algorithm) assay result consistent with recent infection</td>
<td>Date of test -120 days</td>
</tr>
<tr>
<td><strong>D</strong> Equivocal HIV-1 antibody test supported by a repeat test within two weeks showing a rising optical density</td>
<td>Date of equivocal test</td>
</tr>
<tr>
<td><strong>E</strong> Having clinical manifestations of symptomatic HIV seroconversion illness supported by antigen positivity</td>
<td>Date of test</td>
</tr>
</tbody>
</table>
2.3. HEATHER Study Procedures

**Informed Consent**

A medical or nursing professional who was part of the HEATHER research team counselled patients and obtained informed consent for both the HEATHER and HEATHER gut sub-study, a minimum of 24 hours was given to the individual to read and consider the study Patient Information Leaflet (PIL) prior to consent. Additional written informed consent was obtained for the colonoscopy and biopsy procedure in keeping with routine NHS clinical procedures. The endoscopist took this on the day of the procedure.

**Colonoscopy and gut biopsies**

To undergo the colonoscopy procedure participants were asked to lie on their left side on a trolley with knees slightly bent. The endoscopist administered midazolam as a sedative (optional) through an intravenous cannula and supplemental oxygen was given by nasal prongs. Blood pressure, heart rate and pulse oximetry were monitored throughout the procedure. The colonoscope was inserted through the anus and into the large bowel. Air was introduced to expand the bowel. Biopsy specimens were collected with a standard biopsy forceps. Up to 12 biopsies were taken from the terminal ileum and rectum for research purposes. 6-8 biopsies from each site were placed immediately in complete media (as described below) and put on ice and processed within 3 hours. Up to four biopsies from each site were put directly from biopsy into RNA later and stored overnight at 4°C and then at -80°C for subsequent DNA extraction. 2 biopsies from each site were put immediately on complete media, the tissue explants were then cultured for 21 days as described below.
2.4. Laboratory Procedures

2.4.1. Isolation of mucosal mononuclear (MMC) cells from gut biopsy samples

Rectal and terminal ileum biopsies (up to 8 from each site) were collected at endoscopy and immediately placed in complete media; RPMI-1640 media with 5% heat-inactivated fetal bovine serum (FBS), 0.04 mg/ml gentamicin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2mM L-glutamine. Biopsies were transported on wet ice and processed within 3 hours of sampling. On arrival in the lab, samples were transferred to a gentleMACS C tube (Miltenyi Biotec) and washed in 1mM dithiothreitol (DTT) solution and then put on a shaker at room temperature for 15 minutes. The biopsies were then washed three times in PGA solution (penicillin/streptomycin, gentamicin and amphotericin solution. PGA solution contained Hanks' Balance Salt Solution without Ca²⁺ and Mg²⁺, with 0.04 mg/ml gentamicin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 250 µL/ml amphotericin B. Biopsy samples subsequently underwent collagenase and mechanical digestion using a Collagenase D (Roche) solution (1 mg/ml) for 30 minutes and a gentleMACS dissociator (Miltenyi Biotec), respectively. Collagenase D solution contained 15ml of complete media with Collagenase D (1mg/ml) and DNAase (0.01 mg/ml). The gentleMACS “shake” programme settings were “Brain01” prior to the 30-minute incubation and “B01” at the end of the 30-minute incubation. The resulting cell suspension was then strained using a 70 µM filter and washed with PGA. The washed cells were then counted, and viability was assessed using a Muse™ Cell Analyzer (Millipore). Cells were then used immediately for staining. In developing and optimising the protocol for DNA extraction an aliquot of collagenase digested MMCs was stored in Trizol for later DNA extraction for some samples.

2.4.2. Isolation of cells from tonsil tissue

Tonsillar tissue was dissected and mechanically digested, prior to cryopreservation of the cellular suspension.
2.4.3. HIV DNA quantification

HIV DNA was quantified using a quantitative PCR, this method was previously optimised and published by Dr James Williams and Matt Jones. Kathleen Gaertner developed the ‘total’ HIV-1 primers and probe.

**PBMC CD4 enrichment**

PBMC samples were CD4 enriched by negative selection (Dynabeads, Invitrogen or EasySep Human CD4 Enrichment Kit, StemCell). CD4+ T cell DNA was extracted (Qiagen AllPrep DNA/RNA Mini Kit) and used as input HIV DNA for PCR.

**Albumin and total HIV DNA qPCR**

Cell copy number was quantified in triplicate at a 1:25 dilution using an albumin qPCR assay. The master mix contained 2x Lightcycler 480 Probes Master Mix (Roche), 200nM Probe (FAM- CCT GTC ATG CCC ACA CAA ATC TCT CC – BHQ-1) and 250nM of each primer (Forward: GCT GTC ATC TCT TGT GGG CTG T, Reverse: AAA CTC ATG GGA GCT GCT GGT T) with 10µl DNA sample in a total volume of 25µl.

Following cellular quantification using the albumin qPCR, 25,000 input cells per 10µl were assayed in triplicate for the ‘total’ HIV DNA assay. The master mix for the total assay contained 500nM Probe (FAM – AGT RGT GTG TGC CCG TCT GTT G – BHQ-1), 500nM LTR_OS primer (GRA ACC CAC TGC TTA ASS CTC AA) and 500nM LTR_AS primer (TGT TCG GGC GCC ACT GCT AGA GA) (MWG) and 2x Roche LightCycler 480 probes master mix, in a total volume of 25ul. Both qPCR amplifications were performed using the following program: one cycle of 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The data were analysed using the Roche LightCycler software.
2.4.4. DNA and RNA extraction from gut tissue

**DNA and RNA extraction from gut biopsy tissue (RNA later samples)**

HIV DNA was quantified in genomic DNA extracted from gut biopsy tissue which had been stored in RNA later. This tissue included all cell types within the biopsy specimen and was not CD4 enriched. Gut biopsy tissue samples were placed in 2-ml screw-capped tubes, prefilled with 1.4-mm (diameter) ceramic beads and which contained 600ul Qiagen RLT Plus buffer and 6µl of β-mercaptoethanol. The tube contents were then homogenised using a MagNA Lyser (Roche) set at 6000rpm for 45 seconds. RNA and DNA were then extracted using a Qiagen AllPrep DNA/RNA Mini Kit as per the manufacturer’s protocol. An adaptation was made to the RNA extraction protocol whereby the RNA columns were treated with DNAase for 30 minutes between washes to reduce DNA contamination.

**Trizol Extraction of DNA & RNA from gut tissue (MMC samples)**

This method was initially tested for DNA and RNA extraction from gut tissue. However, it was superseded by the method described above. Cells were pelleted by centrifugation (1500xg for 10 minutes). The supernatant was then carefully removed and the cell pellet was re-suspended in 50µl PBS. TRlzol (950µl) reagent (Life Technologies) was added, and the cells were lysed by pipetting up and down several times. The homogenised sample was then incubated for 5 minutes at room temperature. At this stage, the homogenised sample was stored or at -80°C.

The DNA and RNA extraction procedures were as follows: 200µl of chloroform was added to the TRIZol solution, the tube was capped tightly and shaken vigorously by hand for 15 seconds. The solution was then incubated for 2-3 minutes on ice and then centrifuged at 12,000xg for 15 minutes. After centrifugation, the mixture separated into 3 phases; a lower red phenol-chloroform phase containing proteins and DNA, the interphase containing proteins and DNA and a colourless upper aqueous phase containing RNA.
For RNA extraction the aqueous phase was carefully removed by pipetting and placed in a new tube. 2µl of glycogen (20mg/mL) was then added to the aqueous phase sample followed by 500µl of 100% isopropanol; the sample was mixed by pipetting. The sample was then incubated for >1 hour at -20°C. The sample was subsequently centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was then carefully removed, and 1mL ice-cold 75% ethanol was added to wash. This wash step was repeated twice. After the final wash, the RNA pellet was allowed to air-dry for 2-5 minutes. The pellet was re-suspended the in 30µl DNase/RNase free distilled H$_2$O.

For DNA extraction 500µl back extraction buffer (BEB) was added to the interphase and organic phase, mixed by hand and incubated for 10 minutes at room temperature. The BEB contained 4M Guanidine Thiocyanate, 50mM Sodium Citrate, 1M Tris (free base) all dissolved in distilled H$_2$O. The solution was then centrifuged at 12,000 x g for 15 minutes. The aqueous phase (containing DNA) was then transferred to a clean tube. 2µl glycogen (20µg/ml) followed by 400µl isopropanol were then added. The samples were mixed by shaking and incubated at 5 minutes at room temperature. This tube containing precipitated DNA was then centrifuged at 12,000 x g for 5 minutes. 1ml 75% ethanol to wash the pellet followed by repeat centrifugation. The wash step was repeated three times. After the final wash step, the DNA pellet was allowed to air dry for 3-5 minutes. The pellet was then Re-suspended in 8mM NaOH. 1M HEPES was added to adjust the pH of the sample (~pH 7), followed by 50mM of EDTA to a final concentration of 1mM (1:50 dilution). The DNA concentration was measured by nano-drop, and the sample was stored at -80°C.

**2.4.5. Total HIV DNA quantification in gut tissue samples**

**Total HIV DNA quantification in gut tissue**

Cell copy number and total HIV DNA was quantified on the DNA extracted from gut tissue samples as described for PBMCs above. Results were then reported as “copies per million gut cells”, as the tissue sample was not CD4 enriched.
Calculation of copies of HIV DNA per million CD4 gut cells

The frequency of CD4 T cells within biopsy specimens was estimated using data from flow cytometry plots. The percentage of Live, Epcam negative, singlets that were CD4+ was used to calculate the level of HIV DNA per million CD4 T cells. Of note, this method assumed that all HIV DNA measured was within CD4 T cells.

2.4.6. Flow Cytometry

PBMC and mucosal mononuclear cell Staining Protocol

Cryopreserved PBMCs were thawed in RPMI-1640 medium supplemented with 10% FBS (R10), L-glutamine, penicillin and streptomycin containing 2.7 Kunitz units/mL of DNase (Qiagen). Mucosal mononuclear cells were stained immediately upon isolation (without freezing). For all flow cytometry panels' cells were stained in BD Horizon Brilliant Stain Buffer (BD) containing all extracellular antibodies and Live/Dead Near IR at 1 in 300 dilutions (Life Technologies) at room temperature for 20 minutes. This was followed by fixation and permeabilisation using the Human FoxP3 Buffer Set (BD Pharmingen) as per manufacturer's protocol with intracellular staining. The antibody panels used are shown in table 2.4.1 below. All samples were acquired on a LSR II (BD). The same machine was used for all experiments with daily calibration with Rainbow Calibration Particles (Biolegend) to maximise comparability between days. Data were analysed using FlowJo Version 10.4.1 (Treestar).

Table 2.4-1 Antibody Panels for Flow Cytometry

† indicates intracellular antibody *indicates marker was omitted for some samples

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### Panel 2 – Gut exhaustion /CD32 Panel Antibodies

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### Panel 4 – b7/CCR9 Panel Antibodies

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<td>Miltenyi</td>
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2.4.7. Gut biopsy/explant culture protocol

Biopsies were collected in 10mls of complete media at endoscopy. In preparation, Gelfoam (Pfizer) pieces were cut and soaked in complete media. The contents of each collection tube were poured into a Petri plate. A 96 well U-bottom plate was prepared with 200 µl of complete media in two wells (one for terminal ileum & one for rectum); and a 24 well plate with 300 µl of complete media in two wells (as above) and a piece of pre-soaked Gelfoam in each well. One biopsy was transferred each well.

For the 96 well plate: the supernatant was harvested at 24 hours and then stored at -80°C. The remaining tissue was transferred to a tube with 500 µl of RNAlater, which was stored overnight at 4°C and then at -80°C.

-For the 24 well plate: the supernatant was harvested at days 3, 7, 11 and 15. During every harvest 220 µl of the supernatant was removed and replaced with fresh complete media. The harvested supernatant was stored in tubes at -80°C. At day 21 in addition to harvesting all the supernatant, the remaining tissue was transferred a tube with 500 µl of RNAlater kept overnight at 4°C and then stored at -80°C.

2.4.8. Human Immunoglobulin Isotyping

This protocol describes the procedure for Human Immunoglobulin Isotyping for IgA, IgM, IgG1, IgG2 IgG3 & IG4 which is presented in chapter 5. The Milliplex® map human immunoglobulin isotyping magnetic bead kit was used according to the manufacturing instructions. Briefly, 50 µL of each standard or control was added to the appropriate wells. 50 µL of diluted sample was then added into the appropriate wells. 25 µL of the mixed beads were added to each well. The 96 well plate was then sealed and incubated with agitation on a plate shaker for 1 hour at room temperature. The plate was then washed two times. 25 µL of anti-human κ and λ light chain detection antibody was then added. The plate was then resealed and incubated with
agitation on a plate shaker for 30 minutes at room temperature. 25 μL of diluted streptavidin-phycoerythrin was then added to each well containing the 25 μL of detection antibody, followed by incubation on a plate shaker for 30 minutes at room temperature. The well contents were then removed, allowing the plate to soak on the magnet for 60 seconds before aspiration. The contents were then resuspended in 150 μL of sheath fluid to all wells. The plate was then run and data acquired on a Bio-RAD analyser.

2.4.9. Human IL-6

The IL-6 data presented in chapter 3 was measured in plasma using a standard quantitative sandwich enzyme immunoassay technique, using the Quantikine® Human IL-6 Immunoassay HS ELISA. The catalogue number of this kit was HS600B. The procedure was carried out according to the manufacturer’s instructions.

2.4.10. D-Dimer

This assay was applied to samples from the “pTFH clinical cohort” (Chapter 5). Human D-Dimer was measured in plasma by ELISA using the RayBio® Human D-Dimer ELISA Kit according to the manufacturers instructions.

2.4.11. Indirect ELISA to detect anti-IgA and anti-IgG, gp120 and p24 in human plasma samples

A quantitative ELISA assay was used to measure IgA and IgG antibody responses to gp120 and p24 in plasma samples from the pTFH cohort in chapter 5. The protocol is summarised below.

Medium-binding ELISA plates were coated with 50μl per well of α-human κ and α-human λ (1:1 ratio diluted 1:500) capture antibodies, and the capture antigen (1μg/ml) either Conserved clade B SOSIP trimer (Soluble HIV-1 Env Trimer, BG505 SOSIP.664 gp140, provided by Prof Rogier Sanders) or p24 (recombinant p24 - amino acids 1-189 - HIV-1 HxB2
produced in E. coli from Fitzgerald Industries International) and incubated for 1 hour at 37°C. The ELISA plates were then washed with PBS-0.05% Tween 20 (PBST) and blocked by adding 200μl of assay buffer to each well and incubated for another 1 hr at 37°C. Assay buffer was prepared by adding 5g Bovine Serum Albumin (BSA) + 250μl Tween® 20 to 500ml of 1X DPBS (Gibco). The Standard Curve dilutions were prepared by serial dilutions of the known standard in assay buffer. 50μl of sample/standard/control was then added in triplicate wells to the plates and incubated for 1 hour at 37°C. The plate was washed with PBST after incubation. Goat α-Human IgA-HRP or Goat α-Human IgG-HRP detection antibody was diluted in assay buffer and added at 50μl per well. After incubation for 1 hour at 37°C, plates were washed with PBST. 50μl of TMB substrate (Kirkegaard & Perry Labs - KPL, SureBlue TMB 1-Component Peroxidase Substrate) was then added to each well and incubated for 10 minutes at room temperature in the dark. The reaction was stopped by the addition of 50μl per well of Stop Solution (KPL, Cat No 50-85-06). Absorbencies were read at 450nm using a Synergy HT Multi-Detection microplate reader (BioTek Instruments, Inc., Burlington, VT).

2.4.12. Enzyme immunoassay (EIA) for the qualitative detection of p24 core antigens of the HIV-1

In chapter 7, HIV p24 antigen in biopsy culture supernatant was measured using the INNOTEST HIV Antigen mAb (96T) EIA kit 444. The procedure is outlined below.

Briefly, 100 μl of conjugate working solution one was added to each test well. Then 100 μl of the specimen was added to each test well. The strips were then covered with an adhesive plate sealer and incubated for 60 minutes at 37°C. After incubation, each well was washed five times with the wash solution supplied. 200 μl of conjugate working solution 2 (biotinylated murine anti-p24 monoclonal antibodies, containing 0.049% Proclin 300 as preservative, bovine albumin as stabiliser and heat-inactivated mouse serum) was then added to each well. The strips were again covered with an adhesive sealer and incubated for 30 minutes at 37°C. Each well was washed a further five times with wash solution. 200 μl of peroxidase substrate and chromogen solution was then added to each test well. This was then incubated for 30
minutes at room temperature (15-30°C). The reaction was stopped by adding 50 μl sulfuric acid (1-2 mol/l) to each well. The absorbance of the solutions in the wells was read within 10 minutes at 450 nm with a microplate reader.

2.4.13. Human Magnetic Luminex Assays

A Human magnetic Luminex multiplex assay was used to measure the biomarkers listed in Table 2.3.2, in plasma and explant culture supernatant samples. These results are presented in chapters 4, 5 & 7. The assay procedure was carried out according to the manufacturer’s instructions and is summarised below.

Table 2.4-2 Biomarkers measured using Human Magnetic Luminex assay

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50μl of the sample (diluted if necessary) and the standard was added to each well. 50μl of the diluted microparticle cocktail was then added to each well. The plate was then sealed and
incubated for 2 hours on a horizontal orbital microplate shaker (800rpm) and then washed. The plate was secured to a magnetic device for each wash step to prevent loss of microparticles. 50μl of the diluted biotin-antibody cocktail was then added to each well, incubated for 1 hour and then washed. 50μl of diluted streptavidin-PE was then added to each well, incubated for 30 minutes and then washed. The microparticles were then resuspended in 100μl of wash buffer and put on the shaker for 2 minutes. The plate was then run and data acquired on a Bio-RAD analyser.

2.4.14. Immunohistochemistry

Immunohistochemistry for staining for Bcl-6 and CD8 was performed by Dr Naomi Guppy at UCL advanced diagnostics. A stained tonsil section is shown in chapter 5.

2.5. Statistical Analysis

The specific statistical tests used for each analysis are outlined in each chapter. Unless otherwise stated non-parametric tests were used to compare continuous variables throughout this thesis. Where three groups were compared, a Kruskal-Wallis test (unpaired data) was used with corrections made by multiple comparisons using Dunn’s test. Correlative analyses were performed using Spearman’s rank correlation. For all tests, p values <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism (GraphPad Software) version 7.0b, SPSS (IBM SPSS Statistics version 24), R version 3.4.0 (2017-04-21) and R studio Version 1.0.143 (© 2009-2016 RStudio, Inc.). The details of the regression models used are outlined in each chapter.
2.6. Ethical Approval

2.6.1. Healthy control PBMC

In chapter 6, recruitment of healthy individuals (used as a comparison group to primary HIV infection cohort and for fresh blood phenotyping was approved by the Sheffield Research Ethics Committee (reference 16/YH/0247).

In all chapters, PBMCs isolated from leukocyte cones obtained through an anonymous donation to NHS Blood and Transplant (UK) was approved by NHS Blood and Transplant and the National Research Ethics Service Oxfordshire Research Ethics Committee.

2.6.2. HIV infected – treated during chronic infection PBMCs

In Chapter 3, the HIV infected cohort who were treated with ART during the chronic stage of HIV infection donated PBMC samples under the Imperial College Communicable Disease Group Biobank (REC reference is 09/H0606/106)

2.6.3. Tonsillar Tissue

In chapter 5 and 6, Tonsil tissue was obtained from one HIV+ individual undergoing routine tonsillectomy, two months after acquiring HIV, through the Imperial College Communicable Disease Group Biobank (REC reference is 09/H0606/106)

2.6.4. The SPARTAC Trial

The SPARTAC trial was approved by the following authorities: The Medicines and Healthcare products Regulatory Agency (UK), the Ministry of Health (Brazil), the Irish Medicines Board (Ireland), the Medicines Control Council (South Africa) and the Uganda National Council for Science and Technology (Uganda). It was also approved by the following ethics committees in the participating countries: the Central London Research Ethics Committee (UK), Hospital Universitário Clementino Fraga Filho Ethics in Research Committee (Brazil), the Clinical
Research and Ethics Committee of Hospital Clinic in the province of Barcelona (Spain), the Adelaide and Meath Hospital Research Ethics Committee (Ireland), the University of Witwatersrand Human Research Ethics Committee, the University of Kwazulu-Natal Research Ethics Committee and the University of Cape Town Research Ethics Committee (South Africa), Uganda Virus Research Institute Science and ethics committee (Uganda), the Prince Charles Hospital Human Research Ethics Committee and St Vincent’s Hospital Human Research Ethics Committee (Australia) and the National Institute for Infectious Diseases Lazzaro Spallanzani, Institute Hospital and the Medical Research Ethics Committee, and the ethical committee of the Central Foundation of San Raffaele, MonteTabor (Italy).

2.6.5. The HEATHER study

Recruitment for studies within the HEATHER cohort was approved by the West Midlands—South Birmingham Research Ethics Committee (reference 14/WM/1104).

2.6.6. The RIVER study

The RIVER study was approved by the South Oxford Research Ethics Committee (reference 14/SC/1372).
Chapter 3

CD4/CD8 ratio as a marker of immune recovery and HIV-1 reservoir in treated Primary HIV

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Chapter 3.

CD4/CD8 ratio as a marker of immune recovery and HIV-1 reservoir in treated Primary HIV infection

3.1. Introduction

Initiation of ART as soon as possible after acquiring HIV infection has been suggested as a strategy to enhance immune recovery and limit HIV-1 reservoir seeding. The potential benefits of such early ART have been highlighted by the VISCONTI cohort who have observed an unusually high frequency and a long period of sustained virological control after interruption of ART which was initiated during PHI. There may be an immunological and virological threshold below which PTC might be more feasible. Therefore, the immunological and HIV reservoir parameters of individuals treated during PHI warrant further investigation and may offer insight into the propensity for HIV control in this population.

Given the greater frequency of HIV viral control in treated PHI cohorts compared to those treated during chronic infection, these early treated individuals are often deemed to have the greatest chance of success with interventions aimed at HIV eradication. Biomarkers which can predict HIV viral rebound on treatment cessation are lacking, and the identification of such biomarkers remains a challenge for the field. Hence, in the absence of a clinically relevant measure, of virological “cure”, the only current tool to explore viral remission involves stopping ART. In most cases, this is usually accompanied by a rapid rebound in viraemia with the potential associated risks of immune damage and onward viral transmission.

CD4 T cell count and CD8 T cell count are routinely measured in the monitoring of individuals living with HIV-1 infection. The total CD4 T-cell count is a validated surrogate marker of disease progression for HIV/AIDS and early ART has been shown to enhance its recovery. Also, CD8 T cell activation has also been shown to predict disease progression in untreated HIV disease and in PHI.
The disruption of T-cell homeostasis by HIV induces CD4 T cell depletion and CD8 T-cell expansion resulting in an inverted CD4/CD8 ratio, which persists in chronic HIV infection despite many years of ART. While recovery of total CD4+ T-cell counts is the expectation of treatment with ART, the CD4/CD8 ratio often fails to normalise when ART is initiated in chronic infection with reported normalisation frequency of CD4/CD8 ratio ranging from 6-26.3%. Low CD4/CD8 ratio in individuals on suppressive ART has been shown to predict disease progression in chronic HIV infection, in particular in relation to non-AIDS morbidity and mortality, including cancer. Also, it has been independently associated with persistently elevated markers of T-cell activation and immune senescence and measures of HIV viral reservoirs.

The CD4/CD8 T-cell ratio may, therefore, better reflect immune function than total CD4 count alone and may also reflect the size of the viral reservoir. While the risk of AIDS-related illness and opportunistic disease is significantly reduced once CD4 counts have recovered to levels >350 cells/mm³, the risk of non-AIDS morbidity persists.

This chapter has been divided into three sections firstly I examine the impact of ART in primary HIV infection on the recovery of the CD4/CD8 ratio by testing the hypothesis that CD4/CD8 ratio recovery would be enhanced following immediate ART initiation initiated in PHI compared to chronic infection. Secondly, I test the association between CD4/CD8 ratio and time to viral rebound and disease progression and thirdly, I test for associations between the CD4/CD8 ratio and markers of immune function and HIV reservoir.
**Aims**

The overarching aims of this chapter are to explore the impact of early ART on the recovery of the CD4/CD8 ratio, examine its utility in predicting disease progression and time to viral load rebound and to investigate the associations of the CD4/CD8 ratio with the HIV reservoir and markers of immune function in treated PHI.

**Hypothesis**

The following Hypotheses are tested in this chapter are:

1. *Earlier initiation of ART is associated with shorter time to CD4/CD8 ratio > 1.0*

2. *Higher CD4/CD8 ratio is associated with a longer time to disease progression*

3. *Higher CD4/CD8 ratio at treatment interruption (TI) is associated with longer time to viral load rebound on TI*

4. *There is association between CD4/CD8 and HIV DNA in treated primary HIV infection*

**Objectives**

The objectives of this chapter are:

1. *Examine the effect of time to initiation of ART on CD4/CD8 recovery in individuals with known estimated dates of HIV infection*

2. *Identify the biomarkers, including CD4/CD8 ratio, associated with HIV disease progression and time to viral load rebound after ART interruption amongst treated PHI*

3. *Determine the association (if any) between CD4/CD8, HIV reservoir and markers of immune function in treated primary and treated chronic HIV infection.*
3.2. Methods

3.2.1. Cohort description

**SPARTAC (Short Pulse Anti-Retroviral Therapy At Sero-Conversion) Trial**

The SPARTAC trial was a randomised control trial of short course ART in primary HIV infection\(^{110}\) compared with no immediate ART as was the standard of care at the time the study was conducted. It was designed to test whether a short period (12 or 48 weeks) of treatment with ART in the early stages of HIV infection can delay the need for ART in the longer term compared with no immediate ART. It enrolled adults with primary infection who were within six months of seroconversion who were then randomised to receive ART for 48 weeks, 12 weeks, or no therapy (standard of care, SOC). The primary endpoint was the time from randomisation to either CD4 <350 cells/mm\(^3\) or initiation of continuous ART. It completed participant follow up in 2010. A total of 366 participants were randomised from 35 sites in Australia, Brazil, Europe and Africa; 40% were from the UK and 35% South Africa. Of these, 60% were men (90% MSM) and 40% African women. The results were published in 2013\(^ {110}\)

A subset of the SPARTAC cohort with CD4 and CD8 data available, were included in the analyses in this chapter. The clinical and study characteristics of those included are outlined in the corresponding results section.

**The UK register of HIV Seroconverters (UKHSC)**

The UK HIV Seroconverters Cohort (UKHSC) is an observational study which enrolled a cohort of consenting individuals who agreed to the capture of routine clinic data from individuals with defined HIV seroconversion dates collected between 1994-2014. The data collected by the register included: sex, ethnic group, probable route of HIV transmission, annual CD4 counts, details of therapy and prophylaxis prescribed, AIDS-defining events and vital status\(^ {438}\). Follow up information was collected annually. For purposes of the analysis presented here data were restricted to two clinical centres able to provide CD8 T cell data as these were not routinely collected within UKHSC.
The HEATHER cohort

HEATHER is an observational study of treated PHI. It includes only individuals who have commenced on ART within three months of confirmed PHI. It is described in greater detail in chapter 4.

Study definitions

A normal CD4/CD8 T-cell ratio was defined as ≥ 1.0⁴⁶²; a sensitivity analysis was carried out at a CD4/CD8 ratio of 1.2.

The definition of Primary HIV infection was based on those used in the UKHSC ⁴³⁸ and SPARTAC ¹¹⁰ protocols. One or more of the following criteria were required for classification as primary infection (i) a positive HIV-antibody test within 6 months after a negative test (ii) a negative HIV-antibody test with a positive HIV RNA viral load, (iii) a low level of HIV antibodies (optical density units [OD], <0.6) according to a serologic testing algorithm for recent infection, (iv) an equivocal HIV-antibody test with a repeat test within 2 weeks showing an increase in the level of HIV antibodies or (v) clinical manifestations of symptomatic HIV seroconversion illness supported by antigen positivity and less than 4 positive bands on Western blot analysis.

Fiebig staging was not undertaken for the majority of individuals included in these analyses. For the individuals from the SPARTAC trial, nine participants were at Fiebig stage I infection while the remainder were at stages V or VI. No staging was undertaken for UK Register participants, though I anticipate that the majority were at Fiebig stages V and VI.

Estimated date of seroconversion (EDSC) was calculated for individuals with PHI. Similarly, EDSC was calculated according to the SPARTAC study protocol, it was estimated as the midpoint between the most recent negative or equivocal test and the first positive test for patients who met criterion (i) or (iv) as the date of the test for patients who met criterion (ii) (iii), or (v).
Viral load rebound was defined as HIV VL > 400 copies/ml in this analysis while disease progression as outlined the SPARTAC trial has been defined a composite of a CD4 T cell count of <350 cells/mm³ or initiation of long-term ART\textsuperscript{110}

**Statistical Analysis**

Cox proportional hazard models and time-to-event methods were used to test the effect of the interval between PHI and ART initiation on time from ART initiation to normalisation of the CD4/CD8 ratio. The multivariate analyses were adjusted for sex, the time between ART initiation and the baseline reading, year of ART initiation, age at ART initiation, risk group, ethnicity and enrolment from an African site (as a proxy for ethnicity), baseline CD4 count and HIV-1 viral load. Follow-up was censored at date last recorded result. The factors adjusted for in the multivariate analysis should account for differences between the randomised SPARTAC cohort and the observational UKHSC cohort.

Cox proportional hazard models and time-to-event methods were also used to test the effect of the CD4/CD8 ratio at PHI (baseline) on time to disease progression endpoint and time to HIV viral load rebound. These Cox models were censored at the earlier of a break in ART treatment (>7 days) or last visit before 24th April 2014, whichever was earlier. Multivariate analyses were adjusted for sex, risk group, ethnicity, enrolment from an African site, year of seroconversion, the interval between baseline ratio and ART initiation, and both CD4+ count, HIV-1 viral load and age at ART initiation.
3.3. Results

To test the hypotheses outlined at the beginning of this chapter I used data from three different subsets of individuals from the SPARTAC trial, the UKHSC and the HEATHER study according to the question which was being asked. Therefore, I have split the results section into three parts to illustrate which dataset contributed to the analysis.

3.3.1. CD4/CD8 ratio as a clinical biomarker in PHI

573 individuals from the SPARTAC trial and UK Register of HIV seroconverters had confirmed PHI and subsequently commenced on ART and as such were eligible for this inclusion in this analysis, see figure 3.3.1. CD4/CD8 ratio at the time of seroconversion (baseline) and before initiation of ART was abnormal (<1.0) in 482/573 (84%) individuals, with a median (IQR) CD4/CD8 ratio of 0.45 (0.27, 0.71). The clinical characteristics of those with an abnormal CD4/CD8 ratio during PHI is outlined in table 3.3.1, briefly the majority of individuals were male (77.6%), MSM (73%), of white (47.9%) or unknown (47.9%) ethnicity and a median (IQR) age of 34 (27,41) years.

Figure 3.3.1 Flowchart of individuals from the SPARTAC trial & UK register of HIV seroconverters included in the time to normalisation of CD4/CD8 ratio analysis
Table 3.3-1 Baseline characteristics of HIV seroconverters included in time to CD4/CD8 normalisation analysis

<table>
<thead>
<tr>
<th></th>
<th>Initiated ART &lt;6 months after PHI diagnosis N=309</th>
<th>Initiated ART ≥6 months after PHI diagnosis N=159</th>
<th>Total N=468</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>219 (70.9%)</td>
<td>144 (90.6%)</td>
<td>363 (77.6%)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>123 (39.8%)</td>
<td>101 (63.5%)</td>
<td>224 (47.9%)</td>
</tr>
<tr>
<td>Black</td>
<td>13 (4.0 %)</td>
<td>7 (4.4%)</td>
<td>20 (4.3%)</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>173 (56.0%)</td>
<td>51 (32.1%)</td>
<td>224 (47.9%)</td>
</tr>
<tr>
<td><strong>Risk group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSM</td>
<td>203 (65.9%)</td>
<td>138 (86.8%)</td>
<td>341 (73.0%)</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>103 (33.4%)</td>
<td>19 (12.0%)</td>
<td>122 (26.1%)</td>
</tr>
<tr>
<td>IDU</td>
<td>1 (0.3%)</td>
<td>0 (0.0%)</td>
<td>1 (0.2%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.3%)</td>
<td>2 (1.3%)</td>
<td>3 (0.6%)</td>
</tr>
<tr>
<td><strong>Initial drug regimen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI based regimen</td>
<td>251 (81.2%)</td>
<td>33 (20.8%)</td>
<td>284 (60.7%)</td>
</tr>
<tr>
<td>NNRTI based regimen</td>
<td>49 (15.9%)</td>
<td>111 (69.8%)</td>
<td>160 (34.2%)</td>
</tr>
<tr>
<td>NNRTIs only</td>
<td>1 (0.3%)</td>
<td>11 (6.9%)</td>
<td>12 (2.6%)</td>
</tr>
<tr>
<td>Other</td>
<td>8 (2.6%)</td>
<td>4 (2.5%)</td>
<td>12 (2.6%)</td>
</tr>
<tr>
<td><strong>Time in months from SC to ART Initiation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>2.48 (1.33, 3.35)</td>
<td>29.07 (15.2, 51.1)</td>
<td>3.37 (1.93, 16.07)</td>
</tr>
<tr>
<td><strong>Age (years) at ART initiation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>33 (27, 41)</td>
<td>38 (30, 42)</td>
<td>34 (27, 41)</td>
</tr>
<tr>
<td><strong>CD4 count/mm³ at ART initiation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>547 (408, 687.0)</td>
<td>290 (220, 400)</td>
<td>465 (307, 622)</td>
</tr>
<tr>
<td><strong>CD8 count/mm³ at ART initiation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>980 (711, 1370)</td>
<td>930 (700, 1309)</td>
<td>965.5 (710, 1350)</td>
</tr>
<tr>
<td><strong>CD4:CD8 ratio at ART initiation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>0.55 (0.36, 0.82)</td>
<td>0.32 (0.19, 0.45)</td>
<td>0.45 (0.27, 0.71)</td>
</tr>
</tbody>
</table>

Abbreviations MSM, men who have sex with men; IDU, injecting drug use; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; IQR, interquartile range; ART antiretroviral therapy
To understand the relationship between CD4 count and CD4/CD8 ratio during PHI, I next examined the proportion of individuals who had abnormal CD4/CD8 according to CD4 T cell count categories. These CD4 T cell count categories were based on the historical treatment guideline levels at which treatment with ART was indicated, namely <350, 350-500 and >500 cells/mm$^3$. Of 84, 155 and 334 individuals presenting with CD4 T cell counts <350, 350-500, and >500 cells/mm$^3$, 95% (n=80), 91% (n=141) and 78% (n=261) had abnormal CD4/CD8 ratios, respectively. The median CD4/CD8 ratio (IQR) at baseline in PHI was 0.30 (0.21, 0.42) for those with CD4 count <350 cells/mm$^3$, 0.46 (0.31, 0.66) for those with CD4 350-500 cells/mm$^3$ and 0.55 (0.35, 0.80) if CD4 was >500 cell/mm$^3$. These data suggest that majority of individuals in PHI have an abnormal CD4/CD8 ratio at the time of PHI regardless of CD4 T cell count and even in those with CD4 counts of >500 cells/mm$^3$.

3.3.2. Enhanced normalisation of CD4/CD8 ratio with earlier ART in PHI

I next investigated the impact of the timing of ART on the recovery of the CD4/CD8 ratio in treated PHI. Specifically, I examined the effect of the interval between diagnosis of PHI and ART initiation on time from ART initiation to normalisation of CD4/CD8 (>1.0). This was tested using time-to-event methods and Cox proportional hazards models. All models were censored at either a break in ART treatment (>7 days) or last visit before 24th April 2014, whichever was earlier. The factors adjusted for in multivariate analyses were sex, risk group, ethnicity, enrolment from an African site, year of seroconversion, the interval between baseline ratio and ART initiation, and both CD4 T cell count, HIV-1 viral load and age at ART initiation. For illustration purposes, I used an arbitrary cut-off of 6 months to examine the effect of a dichotomised duration of HIV infection at the initiation of ART; ART started <6 months and ART initiated ≥6 months of the estimated date of seroconversion. The cut off at which the CD4/CD8 ratio has normalised is generally accepted to be 1.0. However, a ratio of 1.2 has been used by some groups and I performed a sensitivity analysis using this value. A further sensitivity analysis was performed using a cut-off of 0.5.
I found strong evidence that those with longer time between PHI and ART initiation were less likely to achieve normal CD4/CD8 ratio (aHR [95%CI] =0.98 per month increase [0.97, 0.99], p<0.001) after adjusting for confounding variables (including baseline HIV-1 viral load and CD4 count). The only other significant covariate in the model was baseline CD4 T cell count, with normalisation more likely as count increased per 50 cell/mm$^3$ increase, (HR=1.12 [95%CI 1.09, 1.15] p<0.001), see table 3.2 2. Results from the sensitivity analysis (normal CD4/CD8 ratio >1.2) were qualitatively unchanged.

Table 3.3-2 Multivariate analysis of factors associated with time to CD4/CD8 ratio normalisation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Adjusted Hazard Ratio</th>
<th>95% Confidence Interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Months from SC</td>
<td>0.98</td>
<td>(0.97, 0.99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD4 count at ART initiation</td>
<td>1.12</td>
<td>(1.09, 1.15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(per 50 cells/mm$^3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of ART initiation</td>
<td>0.99</td>
<td>(0.95, 1.04)</td>
<td>0.775</td>
</tr>
<tr>
<td>Age at ART initiation</td>
<td>0.99</td>
<td>(0.98, 1.01)</td>
<td>0.440</td>
</tr>
<tr>
<td>Risk group (compared with MSM)</td>
<td></td>
<td></td>
<td>0.052</td>
</tr>
<tr>
<td>MSW</td>
<td>0.83</td>
<td>(0.40, 1.72)</td>
<td></td>
</tr>
<tr>
<td>IDU</td>
<td>51.2</td>
<td>(6.44, 406.5)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity (compared to White)</td>
<td></td>
<td></td>
<td>0.375</td>
</tr>
<tr>
<td>Black</td>
<td>1.94</td>
<td>(0.86, 4.34)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1.31</td>
<td>(0.70, 2.49)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1.23</td>
<td>(0.85, 1.79)</td>
<td></td>
</tr>
<tr>
<td>Enrolment from an African site</td>
<td>1.31</td>
<td>(0.50, 3.25)</td>
<td>0.617</td>
</tr>
</tbody>
</table>

I next used an arbitrary cut-off of 6 months to examine the effect of a dichotomised duration of HIV infection at initiation of ART; ART started <6 months and ART initiated ≥6 months of estimated date of seroconversion. Of the 468 individuals with abnormal CD4/CD8 ratio initiating ART, 309 commenced ART within 6 months, and 159 initiated ≥6 months from seroconversion, see figure 3.3 1. The baseline characteristics of <6month and ≥6 months groups are shown in table 3.1 1. After a period of one year from the date of starting ART; 129/275 (46.9%) individuals in the <6-month group normalised the CD4/CD8 ratio, compared with 16/127 (12.6%) in the ≥6-month group. Overall median [95%CI] time to normalisation of
CD4/CD8 ratio was 715 [408, 988] days; 172 [117, 280] days for those in the <6 months group, and 1344 [1080, 1630] days in the ≥6 months group. Those initiating ART within 6 months from seroconversion were significantly more likely to achieve a CD4/CD8 ratio above 1.0 (HR [95%CI] =2.47 [1.67, 3.66], p<0.001), as illustrated in the Kaplan-Meier plot in figure 3.3.2.

**Figure 3.3.2 Kaplan–Meier plots of the probability of time to normalisation of the CD4/CD8 ratio by ART<6-months or ART≥6-months group**

Lines indicate those on antiretroviral therapy (ART) within six months of estimated date of seroconversion (dashed line) and those who deferred ART to more than six months (solid line).

Additionally, I explored the relative contribution of CD4 and CD8 T cell count recovery to the higher rates of CD4/CD8 normalisation in the ART <6-months group (early ART). The graphs of the CD4 and CD8 T cells counts are shown in Figures 3.3.3 and 3.3.4 respectively. Of note, fewer observations were available beyond 24 months. In the <6 months (early ART) group a significant decline in the CD8 count is noted on the first six months, suggesting that the contraction of the CD8 compartment may contribute greatest to the rapid normalisation of the CD4/CD8 ratio in this group. However, to investigate the driving factor behind the normalisation of the CD4/CD8 ratio over 48 months I fitted linear mixed models of CD4 and CD8 count, adjusting for early ART (<6-months group). The early ART variable was more
significant for the CD4 count than CD8, suggesting that lack of normalisation is driven mainly by poor CD4 recovery.

Figure 3.3.3 Mean CD4 T cell count in ART<6-months and ART≥6-months according to time from ART initiation

Figure 3.3.4 Mean CD8 T cell count in ART<6-months and ART≥6 months group according to time from ART initiation
3.3.3. CD4/CD8 ratio in PHI predicts HIV disease progression

The SPARTAC trial began recruitment in August 2003 at which time the CD4 T cell count threshold for treatment initiation in the UK was 350 cells/mm$^3$\textsuperscript{[453]}\textsuperscript{[463]}. The disease progression endpoint of SPARTAC was a composite of a CD4 T cell count of <350 cells/mm$^3$ or initiation of long-term ART. Therefore, I next investigated if the baseline CD4/CD8 ratio was predictive of disease progression in untreated HIV infection in those whom treatment was not yet indicated. This analysis included only individuals from SPARTAC and the UK register of HIV seroconverters who had not received ART (i.e. both treatment arms of SPARTAC were excluded) and who had a CD4 T cell count of >350 cells/mm$^3$. 286 ART-naïve individuals with baseline CD4 counts > 350 cells/mm$^3$ contributed to this analysis, the median time to the disease progression endpoint was 1.51 [95%CI 1.32, 2.37] years. In the first instance, I included baseline CD4/CD8 ratio as a continuous variable adjusting in the multivariate analysis for sex, age at PHI, risk group, ethnicity and enrolment from an African site, baseline CD4 count and HIV-1 viral load. Also, I assessed for collinearity to identify any correlation between baseline CD4 T cell count and CD4/CD8 ratio in the model, a variance inflation factor (VIF) of $\sim$1.5 suggests there was no significant collinearity between these variables. Higher CD4/CD8 ratio at seroconversion was independently associated with lower risk of the primary endpoint (aHR [95% CI] =0.32 [0.20, 0.51], p<0.001). I then tested the CD4/CD8 ratio as a categorical variable using the arbitrary categories of <0.5, 0.5 – 1.0, and >1.0. Compared to individuals with CD4/CD8 ratio >1.0 at PHI, those with lower ratios had greater risk of disease progression (ratio <0.5: HR [95%CI] = 2.89 [1.74, 4.79], p<0.001, ratio >0.5–≤1.0: HR [95%CI] = 1.97 [1.17, 3.30], p=0.010), figure 3.3 4. These data suggest that CD4/CD8 ratio may be a useful independent predictor of disease progression in those with CD4 counts above traditional treatment indication thresholds.
Figure 3.3.5 Kaplan Meier plots of the probability of initiating ART or CD4<350 by CD4/CD8 ratio at time of seroconversion

Lines indicate CD4 CD8 ratio (a) <0.5, (b) ≥0.5 & ≤1.0 or (c) >1.0. This analysis used data (n=286) those from the UK Register of Seroconverters and SPARTAC (including only those who were not randomized to starting treatment.

Legend

- (a) Abnormal Ratio <0.5
- (b) Abnormal Ratio ≥0.5, ≤1.0
- (c) Normalized ratio >1.0
3.3.4. CD4/CD8 ratio as a predictor of time to viral rebound on cessation of ART

For the vast majority of individuals interrupting ART, viral rebound is observed and occurs relatively quickly (within weeks). However, there is an absence of validated biomarkers which can predict those individuals who will have rapid viral rebound. It has been previously shown in that CD4/CD8 ratio is related to total HIV-1 DNA, which was predictive of time to viral load rebound in one study. Accordingly, I next examined CD4/CD8, as a surrogate for total HIV-1 DNA, to see how it performed as a biomarker for viral load rebound on treatment cessation. I investigated the association of CD4/CD8 with time to viral rebound amongst a subset of the two cohorts previously described (SPARTAC & UKSHC). Viral load rebound was defined as HIV VL > 400 copies/ml in this analysis. For inclusion in this analysis, individuals were required to have initiated transient short-term ART during PHI. 207 individuals contributed data; the flow diagram for inclusion in this analysis is shown in figure 3.3.6

Figure 3.3.6 Flow diagram of individuals included time to HIV viral rebound after ART interruption in PHI analysis

This flow chart outlines the individuals who were included in the time to HIV viral rebound analysis. Participants from both the SPARTAC trial and UKHSC contributed data. Only those individuals had initiated ART, achieved an undetectable HIV VL, had CD4 and CD8 data available and who then interrupted therapy were included. The number of individuals fulfilling each of these criteria is indicated by “n” below.
3.3.6. The baseline characteristics of individuals included in this analysis are outlined in Table 3.3-3. In summary, 142 (69%) were male, median age and time from seroconversion to ART initiation was 37 years and 84 (IQR 57-106) days respectively; while median ART duration was 3.37 months. 203 individuals reached the endpoint (VL> 400 copies/ml) at a median time of 31 days, 4/207 did not experience virological rebound after a maximum of 3.1 years.

<table>
<thead>
<tr>
<th>Table 3.3-3 Baseline Characteristics of Individuals included in the analysis of factors associated with time to viral rebound after ART interruption in PHI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N (IQR)</strong></td>
</tr>
<tr>
<td>Total included in the analysis</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Median age / years</td>
</tr>
<tr>
<td>Median days from EDSC to ART initiation</td>
</tr>
<tr>
<td>Median time on ART / months</td>
</tr>
<tr>
<td>Median CD4/CD8 ratio:</td>
</tr>
<tr>
<td>at HIV seroconversion</td>
</tr>
<tr>
<td>at the date of ART initiation</td>
</tr>
<tr>
<td>at time of ART stop</td>
</tr>
<tr>
<td>ART initiation HIV VL / log copies per ml</td>
</tr>
<tr>
<td>Median time to viral load rebound / days</td>
</tr>
<tr>
<td>Median year of seroconversion</td>
</tr>
</tbody>
</table>

**Univariable analysis**

In the unadjusted analysis, the factors that were significantly associated with shorter time to viral load rebound were HIV viral load at seroconversion (p=0.003) and ART initiation (p=0.001), while higher CD4/CD8 ratio at HIV seroconversion (p=0.02) and ART initiation (p=0.01) and longer duration of ART (0.03) were associated with longer time to viral rebound. The complete results of the unadjusted analyses are shown in table 3.3.4.

**Multivariable analysis**

I next used Cox proportional hazard models to investigate the role of the CD4/CD8 ratio as a predictor of time to viral load rebound. This model was adjusted for sex, age, route of HIV
exposure, enrolment from an African site (as a proxy for ethnicity), time from seroconversion to ART initiation, HIV viral load (log copies /ml), duration on ART at time of interruption and time from both viral load & CD4/CD8 reading to ART Initiation. Different models were developed using the CD4/CD8 ratio as a continuous and categorical variable. CD4/CD8 as a continuous variable at ART initiation (p=0.39) and all other time-points was no longer significant in the multivariable analysis. However, a borderline association remained when the ratio at the time of ART start was ≥ 1.2 (p=0.049), as compared with <1.2, see table 3.4.5.

The other factors which remained significant in the multivariable analysis were higher HIV viral load at the time of starting ART (p=0.01) which was associated with shorter time to viral rebound and more prolonged duration on ART (p=0.045) which was associated with longer time to rebound.

Table 3.3-4 Unadjusted analysis of factors associated with time to viral load rebound after treatment interruption in PHI

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio [95% Confidence Interval]</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.76 [0.56-1.04]</td>
<td>0.085</td>
</tr>
<tr>
<td>Age Category</td>
<td>0.96 [0.86-1.09]</td>
<td>0.539</td>
</tr>
<tr>
<td>Exposure Category</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSM</td>
<td>2.77 [0.68-11.28]</td>
<td>0.155</td>
</tr>
<tr>
<td>MSW</td>
<td>2.17 [0.53-8.90]</td>
<td>0.283</td>
</tr>
<tr>
<td>Seroconversion HIV VL (Log copies / ml)</td>
<td>1.23 [1.07-1.42]</td>
<td>0.003</td>
</tr>
<tr>
<td>ART Initiation HIV VL (Log copies / ml)</td>
<td>1.27 [1.11-1.46]</td>
<td>0.001</td>
</tr>
<tr>
<td>Time from seroconversion to ART Initiation / days</td>
<td>0.99 [0.998-1.00]</td>
<td>0.189</td>
</tr>
<tr>
<td>CD4/CD8 ratio:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>at HIV Seroconversion</td>
<td>0.67 [0.47-0.94]</td>
<td>0.021</td>
</tr>
<tr>
<td>at ART initiation</td>
<td>0.64 [0.44-0.91]</td>
<td>0.015</td>
</tr>
<tr>
<td>at time of ART interruption</td>
<td>0.76 [0.58-1.01]</td>
<td>0.058</td>
</tr>
<tr>
<td>Duration on ART (months)</td>
<td>0.97 [0.933-0.997]</td>
<td>0.032</td>
</tr>
<tr>
<td>CD4/CD8 ratio categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ART initiation CD4/CD8 ≥ 1.0 versus &lt;1.0</td>
<td>0.59 [0.40-0.85]</td>
<td>0.005</td>
</tr>
<tr>
<td>ART initiation CD4/CD8 ≥ 1.2 versus &lt;1.2</td>
<td>0.43 [0.27-0.70]</td>
<td>0.001</td>
</tr>
<tr>
<td>CD4/CD8 at ART interruption ≥ 1.2 compared to &lt;1.2</td>
<td>0.72 [0.53-0.98]</td>
<td>0.034</td>
</tr>
<tr>
<td>Year of HIV Seroconversion</td>
<td>0.97 [0.90-1.04]</td>
<td>0.347</td>
</tr>
<tr>
<td>CD4 &gt; or &lt; 900 cell/mm³ at ART interruption</td>
<td>1.14 [0.83-1.57]</td>
<td>0.415</td>
</tr>
</tbody>
</table>
Table 3.3-5 Multivariable analysis of factor associated with time to viral load rebound during ART interruption in PHI

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio [95% Confidence Interval]</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.72 [0.27-1.96]</td>
<td>0.522</td>
</tr>
<tr>
<td>Age Category</td>
<td>-</td>
<td>0.796</td>
</tr>
<tr>
<td>Exposure category</td>
<td>-</td>
<td>0.282</td>
</tr>
<tr>
<td>Enrolment from an African site</td>
<td>1.63 [0.80-3.29]</td>
<td>0.178</td>
</tr>
<tr>
<td>Time from seroconversion to ART Initiation</td>
<td>1.00 [0.999-1.001]</td>
<td>0.601</td>
</tr>
<tr>
<td>ART Initiation HIV Viral Load (Log copies / ml)</td>
<td>1.22 [1.04-1.44]</td>
<td>0.017</td>
</tr>
<tr>
<td>Duration on ART (months)</td>
<td>0.96 [0.93-0.99]</td>
<td>0.045</td>
</tr>
<tr>
<td>ART initiation CD4/CD8 ≥ 1.2 compared to &lt;1.2*</td>
<td>0.59 [0.345-0.997]</td>
<td>0.049</td>
</tr>
</tbody>
</table>

*Model for CD4/CD8 ratio as a continuous variable was not significant and a poorer model fit (as measured by AIC), and is not shown

3.3.5. CD4/CD8 ratio correlates with markers of immune function & HIV reservoir

I have shown in the previous sections than early ART enhanced the normalisation of the CD4/CD8 ratio and that this ratio can predict time to viral load rebound and disease progression. I next wanted to test associations of the CD4/CD8 ratio with markers of immune function and HIV reservoir. The biomarkers of interest for this analysis included markers of HIV reservoir (total HIV DNA), immune activation (HLA-DR) and immune checkpoint receptors (PD-1, TIGIT & Tim-3). PBMCs were available for HIV infected individuals (n=121) at five different stages of HIV infection: (i) Group A; baseline PBMC samples (from around the time of seroconversion), (ii) Group B; PBMC samples (longitudinal) from individuals treated with ART at 1-year, (iii) Group C; PBMC samples (longitudinal) from ART naïve individuals at 1-year, (iv) Group D; PBMCs from individuals treated since PHI for >1-year and (v) Group E; PBMCs from individuals treated since chronic HIV infection for >1-year. A flow diagram of the individuals included in each group is shown in figure 3.3 7. The clinical characteristics of each group are summarised in table 3.3 6.
Figure 3.3.7 Flowchart of clinical cohorts used to test the association of HIV reservoir and markers of immune function with CD4/CD8 ratio

This flowchart illustrates the five groups compared in the analysis examining the association between the CD4/CD8 ratio and markers of immune function. Group A (n=49) is the measurements around the time of seroconversion, before initiating ART in those with confirmed PHI. Group B (n=29) is the subset of group A who have received 48 weeks of ART. Groups C (n=17) is the subset of group A who have remained ART-naïve at 48 weeks. Group D (n=17) is measurements from individuals treated with ART in PHI but who have had varying durations of ART (at least one year). Group E (n=9) is measurements from individual who have initiated ART during chronic stage HIV infection and who have varying durations of ART (but at least one year).

Abbreviations: PHI, primary HIV infection; ART; antiretroviral therapy; CHI, chronic HIV infection, PBMC, peripheral blood mononuclear cells;

In summary, the study groups and subsequent analysis are stratified by group based on the timing of initiation of ART and the duration of ART. Group A includes individuals identified at the time of PHI prior to ART initiation. Groups B and C are composed of the individuals from Group A after 48 weeks of follow-up, who have received either 48 weeks of ART or are ART naïve, respectively. Group D also includes individuals identified at PHI, but this group has had a longer duration of ART (>1-year) while Group E includes individuals who commenced on ART in chronic HIV infection and who have had at least >1-year of ART. Of note in the two
groups without a defined period of ART, the median duration of ART was significantly longer in Group E (106 months) compared to Group D (26 months).

Table 3.3-6 Baseline characteristics of cohorts contributing data to the analysis testing the association of CD4/CD8 ratio with HIV reservoir and biomarkers of immune function

<table>
<thead>
<tr>
<th></th>
<th>Group A Baseline PHI Pre-ART</th>
<th>Group B ART-treated PHI 48 Weeks</th>
<th>Group C ART-naive PHI 48 Weeks</th>
<th>Group D ART-treated PHI &gt;1 Year</th>
<th>Group E ART-treated Chronic HIV &gt;1 Year</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>49 (100%)</td>
<td>29 (100%)</td>
<td>17 (100%)</td>
<td>16 (94%)</td>
<td>9 (100%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td>33 (27.5-41.0)</td>
<td>34 (27.5-42.5)</td>
<td>31.5 (27-40.5)</td>
<td>45 (29.5-46.5)</td>
</tr>
<tr>
<td><strong>Baseline CD4 T cell count (cells/mm³)</strong></td>
<td>574 (464-758)</td>
<td>570 (516-732)</td>
<td>567 (518-704)</td>
<td>470 (421-567)</td>
<td>264 (178-338)</td>
</tr>
<tr>
<td><strong>Baseline HIV RNA (log₁₀ copies/ml)</strong></td>
<td>4.8 (4.2-5.8)</td>
<td>4.95 (4.19-5.87)</td>
<td>4.68 (4.15-5.2)</td>
<td>5.7 (5.20-5.79)</td>
<td>4.95 (4.63-5.69)</td>
</tr>
<tr>
<td><strong>Months between PHI diagnosis &amp; PBMC sampling</strong></td>
<td>0.5 (0.25-0.75)</td>
<td>13.5 (12-15)</td>
<td>13 (13-14)</td>
<td>26 (19.8-32.8)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Months on ART at time of PBMC sampling</strong></td>
<td>0 (10-11)</td>
<td>11 (10-11)</td>
<td>0 (10-11)</td>
<td>26 (19.8-32.3)</td>
<td>106.4 (41.5-186.4)</td>
</tr>
<tr>
<td><strong>Current CD4/CD8 ratio-F†</strong></td>
<td>0.72 (0.48-1.14)</td>
<td>1.3 (0.94-1.72)</td>
<td>0.75 (0.49-1.22)</td>
<td>1.13 (0.79-1.71)</td>
<td>1.35 (1.12-1.78)</td>
</tr>
<tr>
<td><strong>Current CD4/CD8 ratio-A</strong>*</td>
<td>0.59 (0.35-0.88)</td>
<td>0.89 (0.67-1.39)</td>
<td>0.55 (0.36-0.82)</td>
<td>1.10 (0.87-1.4)</td>
<td>0.85 (0.7-0.9)</td>
</tr>
<tr>
<td><strong>Current HIV-1 DNA (log₁₀ copies per million CD4 T cells)</strong></td>
<td>4.04 (3.45-4.31)</td>
<td>3.25 (3.01-3.39)</td>
<td>NA (2.96-3.23)</td>
<td>3.14 (3.39-3.78)</td>
<td>3.49 (3.39-3.78)</td>
</tr>
</tbody>
</table>

Demographic and clinical characteristics of participants included in analysis Values given represent n (%) for categorical variables and median (interquartile range) for continuous variables.

*Baseline" refers to measurement around the time of seroconversion for those with PHI while for CHI it refers to the measurement at the time of HIV diagnosis

† CD4/CD8 ratio calculated using T-cell percentages from flow cytometry plots

* CD4/CD8 ratio calculated using absolute T-cell counts

EDI; estimated date of infection NA=Not available
Two methods for measuring CD4/CD8 ratio were used in this analysis, firstly the CD4/CD8 ratio was calculated using absolute CD4 and CD8 T cells count as are reported during routine clinical HIV care measurements, and secondly using CD4 and CD8 T cell percentage data obtained from flow cytometry plots. These have been denoted throughout this chapter by the suffix ratio-A and ratio-F respectively. CD4/CD8 ratios measured by flow cytometry plots were consistently higher than those measured using the absolute T cell count method for all groups, although it was only statistically significantly higher in group B. However, both measurements of CD4/CD8 ratio remained highly correlated with each other, $r=0.73$, $p<0.0001$ (figure 3.3 8d). Both measures have been used in the correlation analysis described, while the absolute count ratio alone was used for the regression analyses. The CD4/CD8 ratio for each group (by both measurement method is illustrated in figure 3.3 8a. The CD4/CD8 ratio-A method (figure 3.3 8c) demonstrated greater differences between groups and is discussed below. As expected the CD4/CD8 ratio was lowest in the ART naïve groups (Group A & C) with a median (IQR) ratio of 0.55 (0.35-0.88) and 0.50 (0.41-0.69) respectively; CD4/CD8 ratio recovery is seen in the ART-treated group at week-48 (Group B) with a significantly higher median ratio of 0.89 (0.68-0.89) in this group compared to both baseline PHI (Group A, $P=0.0019$) and the ART naïve group at 48 weeks (group C, $P=0.043$), $P$ values were calculated using Kruskal-Wallis test & corrected for multiple comparisons. CD4/CD8 ratios in individuals treated with ART in PHI and continued beyond one year (group D) were also significantly higher than ART-naïve individuals at 48-weeks (group C, $P=0.007$) and baseline PHI (group A, $P=0.0002$). However, while pairwise comparison of group D and group E (duration of ART >1 year in both groups) was significant ($P=0.04$, by Mann-Whitney test) with a higher median ratio of 1.1 (0.84-1.5) in the PHI group versus 0.85 (0.7-0.9) in the CHI group, it was no longer statistically significant when corrected for multiple comparisons; this may reflect the small sample size in group E.
Figure 3.3.8 CD4/CD8 ratio measured by absolute T cell count (A) and Flow Cytometry (F) across study groups

The Tukey box and whiskers plots in (a-c) below illustrate the CD4/CD8 ratios measured using flow cytometry plots (F) in blue and absolute (A) T cell count in red. The central box represents the values from the lower to upper quartiles (25 to 75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value (1.5 times the IQR) excluding outliers which are represented by an individual symbol. Figure (a) compares CD4/CD8 ratio across study groups using both methods of ratio calculation. P values (Mann-Whitney test) compare the methods for each group. Figure (b) and figure (c) illustrate the CD4/CD8 ratio across study groups calculated by Flow Cytometry (F) and Absolute T Cell count respectively. The scatter-plot in figure (d) demonstrates the correlation between measured values of CD4/CD8 ratio-F and CD4/CD8 ratio-A. *P<0.01, **P<0.001, ***P<0.0001
HIV reservoir measurements across study groups

Total HIV DNA was measured across the study groups to determine the HIV reservoir burden in each group (data was not available for group C). This is illustrated in the jitter plot in figure 3.3 9. The reciprocal pattern of that seen for CD4/CD8 ratio was observed; highest total HIV DNA level was seen at baseline (group A) with a median (IQR) HIV DNA of 4.04 (3.45-4.31) log_{10} copies/10^6 CD4 T cells. This was significantly higher when compared with HIV DNA levels measured for both later time-points of those treated in PHI; group B [3.3 (2.9-3.4) log_{10} copies/10^6, P=0.0003] and group D [3.14 (2.9-3.2) log_{10} copies/10^6, P<0.0001], after correction for multiple comparisons. Pairwise comparison of group D and group E highlighted significantly (P<0.001) higher total HIV DNA levels in the chronic ART-treated (E) group [3.5 (3.4-3.8) log_{10} copies/10^6] compared to the PHI-ART treated (D) group, but as for CD4/CD8 this was no longer significant when corrected for multiple comparisons. This data is consistent lower HIV DNA levels in individuals treated at the time of primary HIV infection compared to chronic stage disease^372.

Figure 3.3.9 Total HIV DNA measured across study groups

Total HIV DNA levels for each study group are shown in the jitterplots below. Each symbol represents the HIV DNA level for a single individual in Log_{10} copies per 10^6 CD4 T cells. The overall P value was calculated using a Kruskal-Wallis test, P values which remained statistically significant after correction for multiple comparisons using Dunn’s test are represented by *** (P<0.001) or ****(P<0.0001)
Activation and Immune Checkpoint Receptor (ICR) marker expression on T cells across study groups

The expression of the activation marker HLA-DR and ICRs (PD-1, TIGIT & TIM-3) was assessed across study groups. A different flow cytometry antibody panel was used to assess the expression of ICRs and HLA-DR for Groups D & E and did not include TIGIT. Therefore, the subsequent results only include comparisons of groups which were assessed using the same antibody panels, i.e. Groups A, B & C are compared with each other (using Kruskal-Wallis test, corrected for multiple comparisons) and group D & group E were compared.

Figure 3.3.10 Expression of ICRs and HLA-DR on CD4 T cells across study groups

These Jitter Plots show (a) PD-1, (b) TIGIT, (c) Tim-3 & (d) HLA-DR expression on CD4 T cells across study groups. The letters on the x-axis denotes the study group. A=Baseline PHI, B=Week 48 ART-treated, C=Week 48 ART Naïve, D=ART treated in PHI>1 year & E=ART treated in CHI>1 year. Two separate statistical comparisons were made; Groups A,B & C were compared with each other using a Kruskal-Wallis test and corrected for multiple comparisons, Groups D & E were compared using a Mann-Whitney test. *P<0.05, **=P<0.01 & ***P<0.001
Overall a pattern of decreased expression of ICRs and HLA-DR on both CD4 and CD8 T cells was observed following ART initiation was observed, see figures 3.3 10 & 3.3 11. In the CD4 T cell compartment, there was significantly lower PD-1, Tim-3 and HLA-DR expression in the ART-treated group (B) at 48 weeks compared to baseline. No significant difference was seen between the long-term treated groups (D & E) for any of the markers assessed, however this likely relates to small sample size. On CD8 T cells significantly lower expression of PD-1 and HLA-DR was observed in the ART-treated group at 48 weeks (group B) compared to baseline PHI (Group A) and the ART-naïve group at 48 weeks (group C). In the long-term treated

Figure 3.3.11 Expression of Immune Checkpoint Receptors and HLA-DR on CD8 T cells across study groups

These Jitter Plots represent (a) PD-1, (b) TIGIT, (c) Tim-3 & (d) HLA-DR expression on CD8 T cells across study groups. As in the previous figure the letters on the x-axis denote the study group. A=Baseline PHI, B=Week 48 ART-treated, C=Week 48 ART Naïve, D=ART treated in PHI>1 year & E= ART treated in CHI>1 year. Again, two separate statistical comparisons were made; Groups A,B & C were compared with each other using a Kruskal-Wallis test and corrected for multiple comparisons, Groups D & E were compared using a Mann-Whitney test. *P<0.05, **=P<0.01 & ****P<0.0001
groups (D & E) only HLA-DR expression on CD8 was significantly lower in the PHI ART-treated group (D) compared to the CHI ART-treated group (E). These data suggest a decrease in ICR and HLA-DR following ART in both T cell compartments, with the exception of TIGIT which was expressed at similar levels across all groups with available data

**Correlations of Baseline CD4/CD8 ratio with HIV reservoir and markers of immune function**

To understand if the CD4/CD8 ratio is a useful biomarker in PHI, I next wanted to determine the relationship (if any) between CD4/CD8 ratio measured around the time of seroconversion and HIV DNA and markers of immune function. The results, ordered by hierarchical clustering are illustrated in the correlation matrix in figure 3.9.12. The variables in the analysis include all baseline measures of CD/CD4 ratio, baseline ICR and HLA-DR expression on CD4 and CD8 T cells, baseline and week 48 total HIV DNA measurements, baseline CD4 and HIV VL. Hierarchical clustering identifies “clusters” of variables which are highly correlated with each other, of note the measures of CD4/CD8 ratio and baseline CD4 T cell count were strongly correlated with each other. Similarly, the majority of immune checkpoint receptors have a positive correlation with each other and with HIV measures of HIV DNA. Interesting the baseline CD4/CD8 ratio-A (and CD4/CD8 ratio-F) has a significant inverse correlation with both baseline and week 48 total HIV DNA. Higher CD4/CD8 ratio was also associated with lower expression of Tim-3, PD1 & HLA-DR on CD4 T cells and lower baseline HIV-1 viral load. Given the high level of correlation between variables, I fitted a stepwise linear regression model with CD4/CD8 as the dependent variable to identify the factors most predictive of baseline CD4/CD8 ratio. The model ($R^2=0.43$) identified baseline CD4 HLA-DR expression ($\beta=-0.292$, $P=0.02$) and HIV VL ($\beta=-0.52$, $p<0.0001$) as the variables most predictive of baseline CD4/CD8 ratio. I next investigated the utility of CD4/CD8 ratio as an independent predictor of baseline HIV-DNA, adjusting for baseline CD4 and HIV VL, however, only baseline viral load remained predictive of total HIV DNA at the time of seroconversion in this model.
Figure 3.3.12 Correlation Matrix illustrating the association between variables around the time of seroconversion.

The correlogram below is a graph of the correlation coefficients between the listed variables around the time of seroconversion. The colour and size of each circle represents the value of the correlation coefficient, with a scale (-1 to +1) on right side of the graph. Blue represents a positive correlation coefficient, while red represents a negative correlation. Hierarchical clustering was used to identify correlation patterns (in bold squares) and the variables are listed accordingly. Significance levels are not shown.

Legend:

Wk 48 CD4CD8_A = CD4/CD8 ratio (by absolute T cell count) at week 48
SC CD4 = CD4 count around the time of seroconversion
Base CD4CD8 ratio_F = CD4/CD8 (by flow plot) closest to seroconversion
Base CD4CD8 ratio_A = CD4/CD8 (by absolute T cell count) closest to seroconversion
Wk 48 HIV DNA = total HIV DNA measured at week 48
Baseline HIV DNA = total HIV DNA measured closest to seroconversion
SC HIV RNA = HIV viral load closest to seroconversion
Correlations of Week 48 CD4/CD8 ratio with HIV reservoir and markers of immune function

I next tested the associations of the CD4/CD8 ratio after 48 weeks of ART (group B) to examine relationship in ART treated individuals, these are illustrated in the correlation matrix in figure 3.3.13. Using the same methods described in the previous section, I again found that current measures of CD4/CD8 ratio and baseline CD4/CD8 ratio were positively correlated, as was the expression of HLA-DR & ICRs on CD4 and CD8 T cells at week 48. Higher CD4/CD8 ratio-A at week 48 was associated with lower baseline HIV-1 DNA (P<0.05) and with lower week 48 HIV DNA, however, this did not achieve statistical significance. Also, higher week-48 CD4/CD8 ratio-A was associated with significantly lower CD4 HLA-DR, CD4 PD-1, CD4 TIGIT and CD8 PD-1 expression.

Figure 3.3.13 Correlation Matrix illustrating the association between variables in the ART treated group at 48 weeks

The correlogram below is a graph of the correlation coefficients between the listed variables at week 48 in the ART-treated group (B). The colour and size of each circle represents the value of the correlation coefficient, with a scale (-1 to +1) on right side of the graph. Blue represents a positive correlation coefficient, while red represents a negative correlation. Hierarchical clustering was used to identify correlation patterns (in bold squares) and the variables are listed accordingly. Significant levels are not shown. Legend as for Figure 3.3.12
I then used linear regression analyses to determine if CD4/CD8 ratio measured at week 48 was independently associated HIV-1 DNA at that time-point. The final model (R²=0.44) was adjusted for baseline CD4 count and baseline viral load, again only baseline viral load (β=0.69, P=0.002) was predictive of total HIV-1 DNA.

**Correlations of CD4/CD8 ratio with HIV reservoir and markers of immune function beyond one year of ART.**

Our final analyses tested the associations of CD4/CD8 ratio in individuals who had received more than one year of ART. Data from both PHI (group D) and CHI (group E) contributed to this analysis. The correlation matrix is shown in figure 13.3 14. Baseline and current CD4/CD8 ratio are still positively correlated with each other although this association is no longer statistically significant. Similarly, higher current CD4/CD8 ratio is associated with lower CD4 PD1 expression, lower HLA-DR expression on CD4 and CD8 T cells, and shorter duration between HIV diagnosis and ART initiation, however these are not statistically significant. The strongest associations were seen between current CD4/CD8 ratio-A and current HIV DNA (r=-0.59, P=0.003) and between current HIV DNA and duration from HIV diagnosis to ART initiation (r=0.71, p<0.001).

I next developed a linear regression model to examine the predictors of HIV-1 DNA in this ART treated cohort. The model considered the differences between group d and e (i.e. timing of ART initiation and variable duration of treatment) and were adjusted for duration of time from HIV diagnosis to time of ART initiation and duration of ART. In the univariable analysis, time in months from HIV diagnosis (β=0.47 P=0.02) and current CD4/CD8 ratio-A (β=-0.66, P=0.001) were predictive, however, baseline HIV RNA was no longer predictive (β=-.01, P=0.96). The multivariable model included baseline CD4 count, baseline HIV RNA, current CD4/CD8 ratio-A, duration on ART and time from HIV diagnosis to ART initiation, only current CD4/CD8 ratio-A (β=-0.56 P=0.04) and duration from HIV diagnosis to ART initiation (β=0.57 P=0.02) remained significant in the adjusted model. The linear regression analysis results are summarized in table 1.3.15.
Figure 3.3.14 Correlation matrix of immune function and HIV reservoir CD4/CD8 ratio measurements after at least one year of ART

Individuals included in this analysis initiated ART in both chronic HIV infection (n=9) and PHI (n=17) and had varying durations of ART. The correlogram below shows the correlation coefficients between the listed variables from the long-term ART treated groups (D & E). The colour and size of each circle represents the value of the correlation coefficient, with a scale (-1 to +1) on right side of the graph. Blue represents a positive correlation coefficient, while red represents a negative correlation. Hierarchical clustering was used to identify correlation patterns (in bold squares) and the variables are listed accordingly. Significance levels are not shown.

Legend:
- **Current_CD4CD8 ratio_F** = CD4/CD8 ratio (by flow plot) at time of PBMC sampling
- **Current HIV DNA** = Total HIV DNA measured at time of PBMC sampling
- **Months Diagnosis to ART** = The no. of months between diagnosis of HIV and ART initiation
- **Baseline CD4** = CD4 count around the time of HIV diagnosis
- **Base CD4CD8 ratio_A** = CD4/CD8 (by absolute count) closest to seroconversion
- **Baseline HIV RNA** = HIV viral load closest to HIV diagnosis
Table 3.3-7 Results of univariate and multivariable linear regression models examining predictors of total HIV DNA in long-term ART treated individuals

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate Analysis</th>
<th>Multivariable Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$</td>
<td>$P$ value</td>
</tr>
<tr>
<td>Baseline HIV RNA ($\log_{10}$ copies/ml)</td>
<td>-0.01</td>
<td>0.96</td>
</tr>
<tr>
<td>Baseline CD4 T cell count (cells/mm$^3$)</td>
<td>-0.28</td>
<td>0.21</td>
</tr>
<tr>
<td>Current CD4/CD8 ratio-A</td>
<td>-0.66</td>
<td>0.001</td>
</tr>
<tr>
<td>Duration from HIV diagnosis to ART initiation (months)</td>
<td>0.47</td>
<td>0.02</td>
</tr>
<tr>
<td>Duration on ART (months)</td>
<td>0.40</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Variables in the multivariable model include Baseline HIV RNA and CD4 T cell count, current CD4/CD8 ratio, months on ART and time from HIV diagnosis to ART initiation

Model fit: adjusted $R^2 = 0.431$
3.4. Conclusion

3.4.1. Summary of Chapter 3

The first section of this chapter addresses hypothesis 1; the data clearly demonstrates that the earlier ART is initiated from EDI there is an observed enhanced normalisation of CD4/CD8. Individuals in this study were 2% less likely to normalize the CD4/C8 ratio for each month delay in ART initiation from EDI.

In the second section, I address the 2\textsuperscript{nd} and 3\textsuperscript{rd} hypothesis; I found that higher CD4/CD8 ratio at seroconversion was associated with a longer time to disease progression and in those who were treated with ART in PHI and then underwent a treatment interruption higher CD4/CD8 ratio was independently associated with a longer time to virological rebound.

Finally, in the third section I tested the 4\textsuperscript{th} hypothesis and examined the associations between CD4/CD8 ratio and HIV DNA and markers of immune function in HIV infected individuals with different treatment characteristic. I found that higher CD4/CD8 ratios were associated with lower T cell expression of HLA-DR & ICRs and lower total HIV DNA in treated PHI. Seroconversion HIV viral load was most predictive of HIV DNA at baseline and still after one year of ART. However, in individuals treated for a number of years with ART, CD4/CD8 ratio was predictive.

3.4.2. Discussion

This data supports the concept of using early ART as a strategy to enhance immune recovery, and is in keeping with findings from other cohorts of early treated individuals \cite{170}. Furthermore, I found an increased probability of CD4/CD8 ratio normalisation for each month closer to seroconversion that ART was initiated. Previous work by Le et al. had demonstrated enhanced CD4 T cell count recovery with ART commenced within four months of primary HIV infection, however this work did not examine the effect of early ART on CD4/CD8 ratio\cite{109}. The reported normalisation rates of CD4/CD8 ratio in treated chronic HIV infection are low and comparable.
to those initiating ART ≥ 6 months in our study\textsuperscript{401, 451, 452}. Higher rates of CD4/CD8 ratio normalisation have been reported in two studies of treated chronic infection, but only after a median of 10 years on ART\textsuperscript{449, 451}. Accordingly, the additional benefits of commencing ART in PHI are now discussed in the UK national HIV treatment guidelines which have cited our work on CD4/CD8 ratio\textsuperscript{106}. In addition, I found smaller HIV reservoir size in individuals commenced on ART during PHI confirming the published findings of Jain et al. and Hocqueloux et al.\textsuperscript{371, 372}.

HIV treatment guidelines now recommend ART treatment for all individuals regardless of stage of HIV infection or CD4 T cell count. These recommendations are based on the findings of the START randomised control trial, which examined the benefit (of all-cause mortality) of starting ART at higher CD4 T cell counts (>500 cells/mm\textsuperscript{3}). The START investigators reported that ART initiated at CD4 counts of greater than 500 cell/mm\textsuperscript{3} was associated with lower incidence of AIDS-related event, non–AIDS-related event, or death from any cause\textsuperscript{465} Perhaps surprisingly the majority of events (68%) occurred in patients with seemingly normal CD4 T cell counts (> 500 cells/mm\textsuperscript{3}), suggesting that a robust CD4 count may not be indicative of other immune perturbations. Recent work by the same group demonstrated that those over 50 years of age, and with CD4/CD8 ratios of less than 0·5, had the greatest benefit by starting immediate ART\textsuperscript{167} Our work illustrates the role of the CD4/CD8 ratio as a biomarker which is predictive of disease progression in untreated HIV infection and associated with markers of immune dysfunction in treated primary infection. Serrano Villar et al. have previously shown an inverse correlation between CD4/CD8 ratio and markers of immune activation\textsuperscript{169}, while our data additionally demonstrates a relationship with immune checkpoint receptors.

While an inverse correlation was observed between CD4/CD8 ratio and HIV DNA in all groups, it was only independently associated with HIV DNA in individuals who had received ART for a number of years. The main driver of HIV DNA during the first year of PHI was baseline viral load. The later association of CD4/CD8 with HIV DNA may suggest higher HIV DNA is associated with poorer immune recovery. In support of this higher CD4/CD8 ratios (particularly
after 1 year of ART) were associated with lower measures of immune dysfunction such as PD-1 and HLA-DR expression on both CD4 and CD8 T cells. Overall this data suggests that the factors which determine HIV reservoir size are primarily related to virological and immunological factors around the time of seroconversion rather than on ART with a clear beneficial effect of early ART initiation.

CD4/CD8 ratio may have additional potential uses as a biomarker in HIV cure research. Whilst there remains a lack of consensus as to the optimal measurement of viral reservoir\textsuperscript{147}, quantification of reservoir size using total HIV-1 DNA remains the best clinical biomarker to predict both disease progression and virological rebound amongst individuals interrupting ART\textsuperscript{148}. I have demonstrated that higher CD4/CD8 ratio, a biomarker routinely measured as party of clinical care, associates with lower HIV reservoir size and can predict time to viral rebound in those interrupting therapy. Chun et al. has previously shown a relationship between CD4/CD8 ratio in treated chronic infection\textsuperscript{456}, while our data confirms this in treated primary HIV infection and additionally demonstrates its potential utility in predicting time to viral rebound. I propose that CD4/CD8 ratio would be a useful biomarker in HIV cure studies as part of an algorithm to identify optimal candidates for cure interventions or treatment interruption.
Chapter 4

Characterisation of the HIV reservoir in gut-associated lymphoid tissue during treated primary HIV infection

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Chapter 4.

Characterisation of the HIV reservoir in gut-associated lymphoid tissue during treated primary HIV infection

4.1. Introduction

Gut-Associated Lymphoid Tissue (GALT) is the largest reservoir of HIV in the body, at levels often five times higher than in blood\(^{185,331}\). However, the majority of clinical studies assessing the HIV reservoir in humans have involved sampling from peripheral blood mononuclear cells (PBMCs). Consequently, the HIV reservoir in human GALT is less well described. The HIV reservoir in GALT is seeded very early in the course of HIV infection\(^{29}\). CD4 T cells in GALT are preferentially infected and depleted during acute HIV infection, in part due to the increased activation levels of CD4 T cells in GALT and the high expression of CCR5, a crucial co-receptor for HIV entry into CD4 T cells\(^{2}\). The ensuing damage to the integrity of the mucosal barrier leads to microbial translocation and potential long-term immune activation\(^{58}\).

Furthermore, evidence of immune dysfunction is evident in GALT despite many years of ART\(^{331}\). Early ART during primary HIV presents an opportunity to limit reservoir seeding and facilitate immune recovery. However, data on the impact of ART in PHI on GALT immune function are limited and mixed. Very early ART during Fiebig stages I and II have has been shown to preserve mucosal Th17 cells and limit immune activation\(^{466}\), while ART later in primary infection was not associated with immune recovery\(^{318}\).

GALT may also play a unique role in supporting HIV persistence. There is a dichotomy of opinion on the presence of ongoing HIV replication in tissue sites during ART. Recent work by Estes et al. has demonstrated HIV RNA\(^+\) cells in GALT of individuals on ART; however, this may reflect viral production from the latent reservoir rather than ongoing replication. The gut is host to a highly specialised immunological environment, containing CD4 and CD8 T cell subsets found less frequently in the peripheral circulation. Some of these subsets, such as TFH and CCR6\(^+\) CD4 T cells have been shown to support latent HIV infection. Such
differences in the constituent immune cells of the gut immune and niche functions along the gastrointestinal tract may be important factors for HIV persistence\textsuperscript{284}.

While studies of the HIV reservoir in PBMCs offer valuable insights into the landscape of latent HIV infection, they ignore critical cellular niches which may sustain the HIV reservoir. In addition, blood measures of latent HIV infection have failed to accurately predict HIV rebound on treatment cessation in cases of apparent functional HIV cure\textsuperscript{388, 429}. If HIV replication or immune recovery are compartmentalised, the sampling of gut tissue or a suitable surrogate plasma marker will be required. In this chapter, I characterise the HIV reservoir and markers of immune function in GALT during treated primary HIV infection. To assess immune recovery, I compare the immune milieu in GALT along different anatomical sites of the gastrointestinal tract of early-treated HIV infected individuals with that of healthy controls. I also assessed for associations between markers of immune function and inflammation and the HIV reservoir to delineate the potential mechanisms supporting HIV persistence in GALT.
**Aims**

In this chapter, I aimed to characterise the proportion of CD4\(^+\) T cells in the terminal ileum, rectum and PBMCs that are latently infected with HIV and to assess the impact of early ART on immune recovery within these anatomical sites. I assessed immune recovery in GALT and PBMCs by comparing the expression of immune activation and exhaustion markers of early-treated HIV infected individuals with healthy controls. My overarching aim was to determine if measures of HIV persistence and immune dysfunction were more evident in one particular anatomical site. Finally, I assessed for associations between peripheral markers of inflammation and HIV reservoir to identify a surrogate marker of gut HIV reservoir and potentially delineate mechanisms which support HIV persistence in GALT.

**Hypothesis**

The following Hypotheses are tested in this chapter are:

1. **Measures of Total HIV DNA are higher in both terminal ileum and rectum compared to PBMCs.**

2. **ART in PHI normalises markers of immune dysfunction in PBMCs but not in GALT sites.**

3. **Expression of markers of immune dysfunction and inflammation in GALT associate with levels of HIV DNA in GALT**

**Objectives**

The objectives of this chapter are:

1. **Characterise HIV reservoir along the GI tract and PBMCs during treated PHI**

2. **Characterise the expression of ICRs and activation markers along the GI tract and in PHI during treated PHI and compare their expression with healthy controls**

3. **Test for associations between markers of immune dysfunction and inflammation & the HIV reservoir**
4.2. Methods

4.2.1. Clinical Cohorts

Two clinical cohorts contributed to the analysis presented in this chapter. HIV infected individuals were enrolled from the HEATHER study (outlined in the methods chapter) into the HEATHER Gut study (REC reference 14/WM/1104), while healthy control gut tissue was obtained from individuals recruited at routine endoscopy through the Translational Gastroenterology Unit (TGU) in Oxford (REC reference: 11/YH/0020). Recruitment of additional healthy control PBMCs was approved by the Sheffield Research Ethics Committee (REC reference: 16/YH/0247).

4.2.2. Heather Gut Study Assessments

All Study assessments were performed at St Mary’s Hospital Imperial College NHS Trust. Each participant attended for between two and four study visits; those in the longitudinal group had two colonoscopy procedures and four study visits, while those in the cross-sectional group underwent a single colonoscopy and two study visits. Having given written informed consent, participants underwent a screening assessment to determine their eligibility according to the inclusion/exclusion criteria. The inclusion & exclusion criteria are outlined below in table 4.2.1. The timing and procedures carried out at each study visit are outlined in table 4.2.2 below.

Table 4.2-1 Table 4.2-2 Heather gut study inclusion & exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion criteria:</th>
<th>Exclusion criteria:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 positive males or females.</td>
<td>Unable or unwilling to give informed consent</td>
</tr>
<tr>
<td>Aged 18 years or above.</td>
<td>Pregnant or lactating: at enrolment, or during sub-study</td>
</tr>
<tr>
<td>Signed Informed Consent</td>
<td>Documented bleeding disorders</td>
</tr>
<tr>
<td>Patient aware of and understands their HIV status</td>
<td>Diagnosis of Inflammatory bowel disease</td>
</tr>
<tr>
<td></td>
<td>Individuals on immunosuppressive therapy</td>
</tr>
<tr>
<td></td>
<td>Persistent anaemia - haemoglobin &lt;10g/dL</td>
</tr>
<tr>
<td></td>
<td>Hepatitis B and/or C co-infection at time of enrolment</td>
</tr>
</tbody>
</table>
# Table 4.2-3 Study Visits for the HEATHER gut study

<table>
<thead>
<tr>
<th>Study Visit</th>
<th>Procedures &amp; assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Screening</strong></td>
<td></td>
</tr>
<tr>
<td>This was the initial study visit.</td>
<td>Informed consent</td>
</tr>
<tr>
<td></td>
<td>History and physical examination</td>
</tr>
<tr>
<td></td>
<td>Pregnancy test - serum (for all women with childbearing potential)</td>
</tr>
<tr>
<td></td>
<td>Blood tests for clotting (PT/INR) and full blood count (FBC), CD4 &amp; CD8 count, HIV viral load</td>
</tr>
<tr>
<td></td>
<td>A discussion explaining bowel preparation for the colonoscopy procedure</td>
</tr>
<tr>
<td><strong>Baseline – 1st Gut Biopsy</strong></td>
<td></td>
</tr>
<tr>
<td>This visit occurred at least one day but not more than 90 days after the screening visit. Baseline visit should occur when all results of screening evaluations are available to confirm eligibility.</td>
<td>Review of inclusion &amp; exclusion criteria</td>
</tr>
<tr>
<td></td>
<td>Vital signs; blood pressure &amp; pulse</td>
</tr>
<tr>
<td></td>
<td>Review of medical history and medications</td>
</tr>
<tr>
<td></td>
<td>Pregnancy test (for all women with childbearing potential)</td>
</tr>
<tr>
<td></td>
<td>Colonoscopy informed consent, procedure &amp; gut biopsy</td>
</tr>
<tr>
<td><strong>Visit 3 (optional)</strong></td>
<td></td>
</tr>
<tr>
<td>This visit is only relevant to individuals who underwent a second colonoscopy. It was a safety visit before the second colonoscopy. It occurred within 42 days preceding the final visit.</td>
<td>Blood tests; FBC, clotting, CD4 &amp; CD8 count &amp; HIV viral load</td>
</tr>
<tr>
<td></td>
<td>Confirmation that participants are happy to continue in the study</td>
</tr>
<tr>
<td></td>
<td>Review of medical history and inclusion/exclusion criteria</td>
</tr>
<tr>
<td><strong>Final – 2nd Gut Biopsy (optional)</strong></td>
<td></td>
</tr>
<tr>
<td>This was the final study visit. It involved a second colonoscopy which occurred 365 days (+/- 90 days) from the baseline visit.</td>
<td>Review of medical history &amp; medications</td>
</tr>
<tr>
<td></td>
<td>Vital signs; blood pressure &amp; pulse</td>
</tr>
<tr>
<td></td>
<td>Pregnancy test</td>
</tr>
<tr>
<td></td>
<td>Colonoscopy informed consent, procedure &amp; gut biopsy</td>
</tr>
</tbody>
</table>

**Abbreviations:** 
- FBC, full blood count
- PT, prothrombin time
- INR, international normalised ratio

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4.2.3. Heather Gut Study Procedures

Informed Consent

A medical or nursing professional who was part of the HEATHER research team counselled patients and obtained informed consent for the HEATHER Gut-study, a minimum of 24 hours was given to the potential participant to allow time to read and consider the study participant information sheet before consent. Additional written informed consent was obtained for the colonoscopy and biopsy procedure in keeping with routine NHS clinical procedures. The endoscopist took this on the day of the procedure.

Bowel Preparation

Standard bowel preparation was prescribed, this laxative preparation was given to clean the bowel and allow for appropriate visualisation of the gut mucosa at endoscopy; two sachets of the laxative Moviprep were taken on the day before the colonoscopy at 2 pm and 6 pm procedure. Study participants were also advised to fast from lunchtime on the day prior to the procedure.

Colonoscopy & Gut Biopsies

The colonoscopies were carried out at St Mary’s Hospital, Imperial College NHS Trust. The endoscopist outlined the procedure, any treatment, possible risks, expectations and side effects prior to obtaining written consent. To undergo the colonoscopy procedure participants were asked to lie on their left side on a trolley with knees slightly bent. The option of receiving a sedative and a painkiller through an intravenous cannula in the hand or arm with the aim of making the procedure more comfortable was offered to all participants. Blood pressure, heart rate and pulse oximetry were monitored throughout the procedure. The colonoscopy procedure involved passing the colonoscope through the anus and into the large bowel with subsequent incubation of the terminal ileum. Biopsy specimens were collected with standard forceps. Biopsies were firstly taken from the terminal ileum and then the rectum.
4.2.4. Healthy control GALT biopsies

Healthy Control gut tissue was obtained through from donors to the Translational Gastroenterology Unit (TGU) tissue biobank, Oxford; biopsy samples were taken from terminal ileum and rectum (8-10 at each site) with 30mls of blood taken concurrently. Laboratory processing, analysis and storage of samples was conducted at the Peter Medawar Building for Pathogen Research, University of Oxford, as per the HEATHER gut study. The ethical approval for this aspect of the project was obtained through the research tissue bank ethics (REC Ref: 11/YH/0020) and IBD Cohort (REC Ref: 09/H1204/30).

4.2.5. Isolation of mucosal mononuclear cells from gut biopsies

This procedure is described in detail in the chapter 2. To summarise, rectal and terminal ileum biopsies (up to 12 from each site) were collected at endoscopy and immediately placed in complete media; RPMI-1640 media with 5% heat-inactivated fetal bovine serum (FBS), 0.04 mg/ml gentamicin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2mM L-glutamine. Biopsies were processed within 3 hours of sampling. Samples were washed in 1mM dithiothreitol (DTT) solution and then with a penicillin/streptomycin, gentamicin and amphotericin (PGA) solution (Hanks’ Balance Salt Solution without Ca^{2+} and Mg^{2+}, with 0.04 mg/ml gentamicin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 250 µL/ml amphotericin B). Biopsy samples subsequently underwent collagenase and mechanical digestion using Collagenase D (1 mg/ml) for 30 minutes and a gentleMACS dissociator (Miltenyi Biotec), respectively. The resulting cell suspension was then strained using a 70 µM filter, washed with PGA and the washed cells were then used for staining. Up to four biopsies from the terminal ileum and rectum were put directly from biopsy into RNA later and stored at -80°C for subsequent DNA extraction.
4.3. Results

4.3.1. Heather Gut Study Cohort

The Heather Gut Study enrolled individuals from the main HEATHER study which is described in chapter 2. Briefly, HEATHER is an observational study of treated primary HIV infection. It has enrolled over 350 individuals with confirmed PHI based on the criteria outlined in the methods chapter. Individuals in the HEATHER study were offered to take part in the HEATHER gut study, an optional study which involved a colonoscopy and biopsy sampling from the terminal ileum and rectum in addition to blood sampling, as outlined above. A subset of individuals consented to longitudinal sampling with a second colonoscopy and biopsy taken approximately one year after the initial biopsy. Twenty-nine individuals were initially enrolled in the HEATHER study; eight were excluded for reasons outlined below. Ten individuals underwent longitudinal sampling. The clinical characteristics of the twenty-one individuals included in the analysis in the chapter are summarised in table 4.3.1. The reasons for exclusion were as follows; individuals who had received vorinostat before study entry (n=4), ART initiated in chronic HIV infection (n=2) and diagnosis of inflammatory bowel disease (n=2). All participants in the HEATHER gut study were male, and their risk for HIV exposure was MSM. Primary HIV infection was confirmed by positive p24 antigen and negative HIV antibody test in 3/21 (14%) of individuals; by negative to positive test interval in 14/21 (67%) and incident RITA test in 4/21 (19%). The time from HIV diagnosis to ART initiation in this cohort of treated HIV seroconverters was relatively short with a median of 21(12-31) days from HIV diagnosis to initiation of ART. The median HIV viral load (VL) at time of diagnosis was 5.6 (4.2-6.0) log copies per ml (CPM). The median absolute CD4 T cell count at diagnosis was 515 (433-768) cells/mm$^3$, while the median CD8 T cell count was 900 (712-1208) cells/mm$^3$. An inverted CD4/CD8 ratio was observed in the majority of individuals at the time of diagnosis with a median (IQR) ratio of 0.5 (0.4—0.8). This had normalised in a large proportion by the time of the first gut biopsy when the median (IQR) CD4/CD8 ratio was 1.2 (0.9-1.7).
The median (IQR) time on ART at the time of first gut biopsy was 24 (17-36) months, at which time the median CD4 T cells count was 768 (638-957) cells/mm$^3$. All individuals had an undetectable HIV viral load at the time of colonoscopy. The majority of study participants (76%) had received between 12 and 36 months of ART at the time of their first gut biopsy. Four individuals had received 48 months or more at the time of biopsy, the longest being on ART for 96 months at biopsy. In those individuals who underwent a second colonoscopy the median (IQR) time on ART at the second biopsy was 35 (26-46) months. One individual was diagnosed with Hepatitis C between his first and second biopsy; however, he was treated with directly acting antivirals (DAAs) in the interval between biopsies and was hepatitis C RNA negative in plasma at the time of his second gut biopsy. There were no serious adverse events, related to the endoscopy procedure or otherwise during the study. Successful endoscopy and intubation of the terminal ileum were performed in all study participants in the HIV positive group.

4.3.2. Healthy Control Cohort

A cohort of 19 individuals was recruited as “healthy controls” through the translational gastroenterology unit (TGU) in Oxford. These individuals underwent colonoscopy as part of their routine medical care and consented for additional biopsies to be taken for purposes of research. Failure to intubate biopsy the terminal ileum occurred in two individuals. The clinical characteristics of this cohort are summarised in table 4.3.2. There are some important differences to highlight between the HEATHER gut study cohort and the healthy control cohort. The healthy control cohort were significantly older (P<0.0001) than the HEATHER gut cohort with a median (IQR) age of 60 (55-71) years and 35 (27-43) respectively. In addition, a proportion of healthy controls (42%) were female while all the HEATHER study participants were male. One individual was diagnosed with active cancer and their biopsy samples were excluded from the analysis, individuals with a history of cancer but no active disease at the time of biopsy were included unless otherwise stated.
<table>
<thead>
<tr>
<th>Study ID</th>
<th>Age</th>
<th>CD4+ T cell count (cells/mm$^3$)</th>
<th>CD8+ T cell count (cells/mm$^3$)</th>
<th>CD4/CD8 ratio</th>
<th>HIV VL (Log$_{10}$ CPM)</th>
<th>Time from PHI* to ART (days)</th>
<th>Time VL &lt;50 (days)</th>
<th>Time on ART at 1st Biopsy (months)</th>
<th>HIV viral load (Log$_{10}$ CPM)</th>
<th>Time on ART at 2nd Biopsy (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut 1</td>
<td>55</td>
<td>705</td>
<td>2628</td>
<td>0.27</td>
<td>5.53</td>
<td>21</td>
<td>339</td>
<td>19</td>
<td>0.5</td>
<td>839</td>
</tr>
<tr>
<td>Gut 2</td>
<td>35</td>
<td>278</td>
<td>NA</td>
<td>NA</td>
<td>7.13</td>
<td>28</td>
<td>92</td>
<td>21</td>
<td>1.0</td>
<td>707</td>
</tr>
<tr>
<td>Gut 3</td>
<td>56</td>
<td>188</td>
<td>NA</td>
<td>NA</td>
<td>5.70</td>
<td>17</td>
<td>60</td>
<td>81</td>
<td>2.6</td>
<td>957</td>
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<tr>
<td>Gut 4</td>
<td>34</td>
<td>752</td>
<td>912</td>
<td>0.82</td>
<td>5.18</td>
<td>54</td>
<td>22</td>
<td>23</td>
<td>1.2</td>
<td>1208</td>
</tr>
<tr>
<td>Gut 5</td>
<td>37</td>
<td>968</td>
<td>NA</td>
<td>NA</td>
<td>4.20</td>
<td>26</td>
<td>115</td>
<td>33</td>
<td>2.4</td>
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<td>49</td>
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<td>20</td>
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<td>3</td>
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<td>Gut 11</td>
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<td>443</td>
<td>443</td>
<td>1.00</td>
<td>3.23</td>
<td>28</td>
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<td>14</td>
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<td>Gut 13</td>
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<td>398</td>
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<td>0.36</td>
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<td>35</td>
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<td>860</td>
<td>1220</td>
<td>0.70</td>
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<td>23</td>
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<td>Gut 15</td>
<td>44</td>
<td>515</td>
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<td>4.39</td>
<td>10</td>
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<td>48</td>
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<td>382</td>
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<tr>
<td>Gut 16</td>
<td>30</td>
<td>423</td>
<td>888</td>
<td>0.48</td>
<td>4.85</td>
<td>13</td>
<td>530</td>
<td>15</td>
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<td>Gut 17</td>
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<td>449</td>
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<td>18</td>
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<td>17</td>
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<tr>
<td>Gut 18</td>
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<td>789</td>
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<td>NA</td>
<td>5.59</td>
<td>46</td>
<td>209</td>
<td>33</td>
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<td>Gut 19</td>
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<td>548</td>
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<td>Gut 20</td>
<td>24</td>
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<td>0.54</td>
<td>6.70</td>
<td>33</td>
<td>1682</td>
<td>96</td>
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<td>0.40</td>
<td>5.84</td>
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<tr>
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<td>543</td>
<td>869</td>
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<td>7</td>
<td>487</td>
<td>16</td>
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<td>NA</td>
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<td>9</td>
<td>0</td>
<td>22</td>
<td>1.9</td>
<td>1158</td>
</tr>
</tbody>
</table>

**Abbreviations:** CPM, copies per million; PHI, primary HIV infection; ART, antiretroviral therapy; VL, viral load; NA, not available. *refers to time from date of HIV diagnosis to starting ART. All participants were male.
### Table 4.3-2 Health Control Patient Characteristics

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Age</th>
<th>Gender</th>
<th>Year of endoscopy</th>
<th>Indication for Colonoscopy</th>
<th>Medical History</th>
<th>Active Cancer*</th>
<th>Cancer details</th>
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<tr>
<td>GI 1657</td>
<td>56</td>
<td>M</td>
<td>2016</td>
<td>Polyp of colon</td>
<td>Sickle cell, asthma, G6PD, OSA</td>
<td>No</td>
<td>-</td>
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<tr>
<td>GI 1658</td>
<td>74</td>
<td>M</td>
<td>2016</td>
<td>Cancer screening</td>
<td>None</td>
<td>No</td>
<td>Prostate cancer, diagnosed July 2017</td>
</tr>
<tr>
<td>GI 1932</td>
<td>59</td>
<td>F</td>
<td>2016</td>
<td>Diverticular disease</td>
<td>None</td>
<td>No</td>
<td>-</td>
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<tr>
<td>GI 1933</td>
<td>43</td>
<td>F</td>
<td>2016</td>
<td>Benign neoplasm – sigmoid</td>
<td>Acute gastritis</td>
<td>No</td>
<td>Low-grade mucinous tumour appendix removed 2014</td>
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<tr>
<td>GI 1931</td>
<td>66</td>
<td>M</td>
<td>2016</td>
<td>Rectal polyp</td>
<td>None</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>GI 1937</td>
<td>57</td>
<td>F</td>
<td>2016</td>
<td>Cancer Screening</td>
<td>Rectal cancer</td>
<td>No</td>
<td>T3, N0, M0, L0, V0, R0 Rectal resected 2014</td>
</tr>
<tr>
<td>GI 1938</td>
<td>69</td>
<td>M</td>
<td>2016</td>
<td>Coeliac Disease</td>
<td>Coeliac disease - diagnosed 2016</td>
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<td>-</td>
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<tr>
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<td>71</td>
<td>M</td>
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<td>T cell Lymphoma</td>
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<td>-</td>
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<td>36</td>
<td>F</td>
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<td>IBS, Hidradenitis suppurativa</td>
<td>No</td>
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<td>GI 2382</td>
<td>73</td>
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<td>-</td>
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<td>2017</td>
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<td>-</td>
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<tr>
<td>GI 670</td>
<td>77</td>
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<td>-</td>
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<td>GI 2542</td>
<td>71</td>
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<td>F</td>
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<td>Hypertension</td>
<td>No</td>
<td>-</td>
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<tr>
<td>GI 3035</td>
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<td>Haemorrhoids</td>
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<td>-</td>
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<tr>
<td>GI 3037</td>
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<td>M</td>
<td>2018</td>
<td>Polyps</td>
<td>Rheumatoid arthritis</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; G6PD, glucose-6-phosphate dehydrogenase deficiency; OSA, obstructive sleep apnea; IBS, irritable bowel syndrome; PR, per rectum

*refers to active cancers at the time of endoscopy
4.3.3. HIV DNA levels in GALT

HIV DNA Levels in Terminal Ileum & Rectum in treated during PHI

HIV DNA levels were measured in the terminal ileum and rectal biopsies from individuals enrolled in the HEATHER gut study. Measurements were expressed as copies per million guts cells and as copies per million CD4 T cells. Participants (n=20) who had received at least three months of ART were included; individuals who had received any investigational agent in as part of another study were excluded. The median (IQR) time on ART at the time of gut biopsy was 34 (19.5-46) months. Median HIV DNA levels expressed as \( \log_{10} \) copies per million CD4 T cells, were 3.49 (3.36-4.0) and 3.44 (3.09-3.66) in the terminal ileum and rectum respectively, with significantly higher levels in the terminal ileum (\( p=0.008 \), using Wilcoxon paired rank test). When total HIV DNA was expressed as \( \log_{10} \) copies per million gut cells, higher levels were again observed in the terminal ileum, with a median (IQR) of 2.76 (2.59-3.09) in the terminal ileum compared to 2.71 (2.50-2.96) in the rectum, although this was only borderline statistically significant (\( p=0.06 \)). Rectal HIV DNA levels were positively correlated with those in the terminal ileum when measured as CPM CD4 T cells (\( r=0.55 \ p=0.01 \)) and CPM gut cells (\( r=0.66 \ p=0.002 \)), see figure 4.3.1.

Figure 4.3.1 Total HIV DNA Levels in GALT

Total HIV DNA levels in terminal ileum and rectum from the HEATHER gut cohort are shown in (a) expressed as copies per million CD4 T cells and (b) expressed as copies per million gut cells. P values were calculated using a Wilcoxon test. Abbreviations; CPM, copies per million; T Ileum, terminal ileum.

(a) HIV DNA CPM CD4 T cells  
(b) HIV DNA CPM gut cells
The HIV reservoir in GALT did not decay over time

Ten individuals enrolled in the HEATHER gut study had longitudinal sampling from GALT sites, with HIV DNA measured at two time points. The median (IQR) duration of ART at the time of biopsy one was almost two years, at 22 (16-31) months, while at the time of biopsy two it was 35 (28-44) months. The median (IQR) interval between biopsies was 13 (12-13) months. The median (IQR) HIV-1 DNA in terminal ileum at the time of the first and second gut biopsies (figure 4.3.2a), expressed as $\log_{10}$ HIV DNA CPM gut cells, was 2.68 (2.50-2.96) and 2.67 (2.62-3.08) respectively; these were not significantly different ($p=0.23$). In rectal issue (figure 4.3.2c), the median (IQR) HIV DNA, expressed as $\log_{10}$ CPM gut cells, was 2.42 (2.21-2.92) at first biopsy and 2.68 (2.47-2.99) at second biopsy; again, these were not significantly different ($p=0.16$). The changes in HIV DNA measured for each individual, from biopsy one to biopsy two, in each gut site is shown in figure 4.3.2b (terminal ileum) and figure 4.3.2c (rectum); in general, the direction of change was the same in each GALT site (i.e. an increase in HIV DNA in the terminal ileum was associated with an increase in rectum within an individual).

As the majority of latent HIV is thought to reside in CD4 T cells and the relative frequency of CD4 T cells can differ between biopsy samples, I also calculated HIV DNA in $\log_{10}$ CPM CD4 T cells. This allowed me to make direct comparisons with measures of HIV DNA in matched PBMC samples; as these were enriched for CD4 T cells prior to HIV DNA quantification and therefore HIV DNA was measured as CPM CD4 T cells. In the terminal ileum (figure 4.3.3a), there was no significant difference in median (IQR) HIV DNA measured as $\log_{10}$ CPM CD4 T cells, which was 3.47 (3.30-3.88) at biopsy one and 3.52 (3.34-3.52) at biopsy two ($p=0.77$). In rectal tissue (figure 4.3.3c), the median (IQR) HIV DNA at biopsy one was 3.12 (2.94-3.66) $\log_{10}$ CPM CD4 T cells and 3.44 (3.05-3.67) $\log_{10}$ CPM CD4 T cells; again, these were not significantly different ($p=0.32$). I also looked at
the change in HIV DNA levels in the PBMC compartment over time (figure 4.3.3e); the median (IQR) HIV DNA PBMCs, expressed as log_{10} CPM CD4 T cells, at biopsy one was 3.04 (2.79-3.36) and 2.99 (2.66-3.22) at biopsy two, these were not statistically significant (p=0.20). However, HIV DNA at biopsy two was almost universally lower or equivalent when compared to HIV DNA at biopsy one (figure 4.3.3f).

Overall these data suggest that there is heterogeneity in terms of HIV DNA decay in GALT over time in ART-treated individuals, with some experiencing an increase in HIV DNA on ART, while others had decreased. However, overall there was no overall statistically significant change over time. In PBMCs, HIV remained stable or tended to decrease over time, but again this was not statistically significant.

**Figure 4.3.2 Longitudinal total HIV DNA (log_{10} CPM gut cells) in GALT**

Longitudinal HIV DNA measures, expressed as CPM gut cells, in (a) terminal ileum and (c) rectum for individuals in the longitudinal cohort of the HEATHER gut study. (b) shows the change in HIV DNA in terminal ileum from biopsy 1 to biopsy 2 for each individual, while (d) shows the change in HIV DNA in rectum from biopsy 1 to biopsy 2 for each individual.
Figure 4.3.3 Longitudinal total HIV DNA (log_{10} CPM CD4 T cells) in GALT and PBMC

Longitudinal HIV DNA in CPM CD4 T cells are shown for (a) terminal ileum, (c) rectum & (e) PBMC. The change in HIV DNA from biopsy 1 to biopsy 2 for each individual is shown for (b) terminal ileum, (d) rectum & (f) PBMC. P values were calculated using Wilcoxon’s test.

HIV DNA is higher in GALT sites compared to peripheral blood

I next examined the distribution of HIV DNA across GALT and PBMC anatomical sites (figure 4.3.4a). Matched (all three anatomical sites) GALT and PBMC data were available for 19 individuals. Two-time points were included in the analysis for eight individuals,
giving a total of 28 observations. The median (IQR) HIV DNA was significantly higher in terminal ileum at 3.47 (3.32-3.95) compared to 3.43 (3.06-3.66) \( \log_{10} \) CPM CD4 T cells in the rectum \( (p=0.049) \). The terminal Ileum also had significantly higher reservoir when compared to PBMC; where the median (IQR) HIV DNA was 3.01 (2.88-3.21) \( \log_{10} \) CPM CD4 T cells \( (p<0.001) \). Rectal HIV DNA was also significantly higher than PBMC DNA \( (p=0.008) \). There was a significant association between PBMC measures of HIV DNA and those in the terminal ileum \( (r=0.59 \ p=0.001) \) and rectum \( (r=0.48 \ p=0.009) \), see figure 4.3.4b & 4.3.4c.

**Figure 4.3.4 HIV DNA measurements across all anatomical compartments**

The jitter plot in (a) shows HIV DNA levels in the terminal ileum, rectum and PBMCs; the line indicates the median. The correlation between HIV DNA measured in PBMCs and (b) terminal ileum and (c) rectum is also shown. Group comparisons in (a) were made using Friedman’s test. Non-parametric tests (Spearman’s) was used to test associations. Abbreviations: CPM, copies per million; T. Ileum, terminal ileum.

(a) Total HIV DNA measured across anatomical sites

(b) Correlation between PBMC & terminal ileum HIV DNA

(c) Correlation between PBMC & rectal HIV DNA
4.3.4. Clinical Correlates of HIV DNA in the GALT

Duration of ART

I next examined the relationship between duration of ART at the time of gut biopsy and HIV DNA; no significant relationship between ART duration and any measures of HIV reservoir in GALT or PBMCs was observed. The correlations between duration of ART and measures of HIV reservoir in rectal tissue were as follows: CPM CD4 T cells, \( r=0.21 \) \( P=0.28 \); CPM gut cells, \( r=0.17 \) \( P=0.40 \). Similarly, in the terminal ileum, no significant relationship was observed between duration of ART and HIV reservoir: CPM CD4 T cells, \( r=-0.06 \) \( P=0.75 \). Also, in PBMCs, no correlation was observed; \( r=-0.11 \) \( P=0.56 \). I was also interested in testing if the duration of ART at the time of biopsy affected the decay dynamics of HIV DNA (i.e. does DNA decrease during the first year of ART but not in subsequent years on ART). Using the longitudinal cohort only I plotted the time on ART for those who had HIV DNA measured at two time points (figure 4.3.5). No pattern was identified to suggest that the duration of ART impacted on the decay dynamics of HIV DNA in any site.

Figure 4.3.5 Duration of ART and HIV DNA measurements

These plots illustrate paired (n=10) HIV DNA measures (indicated by symbols and connecting line) and duration on ART at the time of gut biopsy. The data presented only includes study participants who had biopsies at two time points. Data is shown for (a) terminal ileum (b) rectum and (c) PBMC. The gut study ID is also shown.
Baseline HIV Viral Load

I have shown in chapter 3 that baseline viral load was predictive of total HIV DNA after one year of ART. Therefore, I examined the relationship between baseline HIV viral load and total HIV DNA in PBMCs and GALT in the HEATHER gut cohort. For individuals who had longitudinal sampling, two HIV DNA measurements were available, the one closest to an ART duration of one year was used; the median (IQR) time on ART for this analysis was 25 (17-39) months. Baseline HIV viral load was predictive of HIV DNA in PBMCs on ART ($r=0.49 \ p=0.03$) but not in either of the GALT tissue sites, using both measures of HIV DNA, see figure 4.3.6.

Figure 4.3.6 Baseline HIV viral load and measures of HIV DNA in GALT and PBMCs

These scatter plots show the relationship between baseline HIV viral load measured in plasma and GALT HIV DNA, measured in $\log_{10}$ (a) CPM CD4, (b) CPM gut cells and in (c) PBMCs, CPM CD4 T cells. Correlations tested by Spearman’s r. Abbreviations: VL, viral load; CPM, copies per million.

(a) Baseline HIV VL & GALT HIV DNA CPM CD4 cells

(b) Baseline HIV VL & GALT HIV DNA CPM gut cells

(c) Baseline HIV VL & PBMC HIV DNA CPM CD4 cells
CD4/CD8 ratio

In chapter 3, I have previously shown that CD4/CD8 ratio is predictive of HIV-1 DNA in PBMCs after several years on ART. Therefore, I next wanted to test for associations between CD4/CD8 ratio and HIV DNA in GALT. Baseline CD4/CD8 ratio in blood was significantly associated with all measures of HIV reservoir on ART in all anatomical sites. These are illustrated in figure 4.3.7; the correlation coefficients for baseline CD4/CD8 ratio and each measure of HIV DNA across anatomical sites were as follows: terminal ileum (HIV DNA CPM CD4 T cells, r=-0.45 P=0.05; HIV DNA CPM gut cells, r=-0.51 P=0.02), rectum (HIV DNA CPM CD4 T cells, r=-0.59, P=0.006; HIV DNA CPM gut cells, r=-0.60 P=0.005) and PBMC (HIV DNA CPM CD4 T cells, r=-0.48 P=0.03).

Figure 4.3.7 Baseline CD4/CD8 ratio and measures of HIV DNA in GALT and PBMCs

These scatter plots show the relationship between baseline CD4/CD8 ratio measured in blood, near the time of seroconversion, and HIV DNA measured in GALT expressed as (a) Log_{10} CPM CD4 T cells, (b) Log_{10} CPM gut cells and (c) in PBMCs expressed as Log_{10} CPM CD4 T cells. r= Spearman’s correlations. Abbreviations; CPM, copies per million; T. Ileum, terminal ileum.

(a) Baseline CD4/CD8 ratio & GALT HIV DNA CPM CD4 cells

(b) Baseline CD4/CD8 ratio & GALT HIV DNA CPM gut cells

(c) Baseline CD4/CD8 ratio & PBMC HIV DNA CPM CD4 cells
I next explored the relationship between CD4/CD8 measured at the time of gut biopsy (concurrent CD4/CD8 ratio) and HIV DNA in GALT and PBMC sites. Initial analysis failed to demonstrate a significant relationship in any site (figure 5.3.8 a-c). The lack of association was driven by two observations, which were from the same individual, measured one year apart, namely GUT 3. Of note this participant had received a substantially longer duration on ART; 94 months compared to a median of 33 months for the overall cohort. Therefore, I performed an outlier analysis based on the duration of ART data and identified three observations (GUT 3 both time points and GUT 20) as outliers. When I excluded these (figure 5.3.8 d-f), I observed a significant correlation between concurrent CD4/CD8 ratio and HIV DNA in all sites except in rectum when quantified as CPM gut cells ($r$=-0.35 $P$=0.1). The statistically significant correlations with concurrent CD4/CD8 ratio were as follows; terminal ileum (CPM CD4 T cells, $r$=-0.62 $P$=0.002; CPM gut cells, $r$=-0.54 $P$=0.008), rectum (CPM CD4 T cells, $r$=-0.57 $P$=0.005) and PBMC (CPM CD4 T cells, $r$=-0.55 $P$=0.006).
Figure 4.3.8 Concurrent CD4/CD8 ratio and HIV reservoir in GALT & PBMCs

The scatterplots below illustrate the relationship between concurrent CD4/CD8 ratio measured in PBMCs and GALT HIV reservoir measured as (a) HIV DNA CPM CD4 T cells, (c) HIV DNA CPM gut cells and in (e) PBMCs as HIV DNA CPM CD4 T cells; (a), (c) & (e) includes ‘outliers’ in terms of duration of ART treatment which are shown as unfilled symbols. (b), (d) & (f) show the same data with outliers excluded. $r$ = Spearman’s correlation. Abbreviations: GALT, gut-associated lymphoid tissue; CPM, copies per million; PBMC, peripheral blood mononuclear cells; T. Ileum, terminal ileum.
4.3.5. Immune recovery in GALT and PBMCs during treated PHI

In order to determine the impact of ART in PHI on markers of immune dysfunction on T cells in GALT and PBMCs, I measured the CD4/CD8 ratio, the expression of the ICRs, PD-1, TIGIT and Tim-3 and the expression of the activation markers HLA-DR and CD38 on CD4 and CD8 T cells across anatomical sites in treated PHI individuals and made comparisons with healthy controls.

GALT CD4/CD8 ratio

In health, lower CD4/CD8 ratios are evident in gut sites compared to PBMCs, as shown in figure 4.3.9 (a). In treated PHI, there were differences observed in the CD4/CD8 ratio in the terminal ileum (P=0.009) and PBMC (P=0.001) compartments between individuals treated during PHI and healthy controls, figure 4.3.9 (b). No significant difference in the ratio was observed between HIV+ individuals and healthy controls in the rectum (P=0.70). These data suggest that recovery of CD4/CD8 differs within gut sites, with normalisation in the rectum but not in the terminal ileum. Differences were also observed in the PBMC compartment; however, the median in PBMCs was greater than 1.0 in HIV+ individuals.

Figure 4.3.9 CD4/CD8 across anatomical sites in healthy controls and HIV infected individuals

The CD4/CD8 across anatomical sites is shown in (a) healthy controls (unfilled symbols) only while (b) includes both healthy controls and HIV infected individuals (filled symbols). Terminal ileum is shown in red triangles, rectum in blue circles and PBMCs in yellow squares. P values were calculated using a Mann-Whitney test. Abbreviations; T Ileum, terminal ileum; HC, healthy controls.

(a) CD4/CD8 ratio: healthy controls

(b) CD4/CD8 ratio: HIV+ and Controls
**Expression of ICR in healthy control GALT**

In the first instance, I wanted to describe the pattern of ICR expression in GALT in the absence of HIV infection. Therefore, I examined the pattern of ICR expression in healthy controls. The expression, on CD4 T cells, of all the ICRs measured, namely PD-1, TIGIT and Tim-3 was significantly higher in both the terminal ileum and rectum when compared to PBMCs. On CD8 T cells the rectum but not the terminal ileum had higher expression of PD-1 and Tim-3 compared to PBMCs. Also, CD8 PD-1 expression was also higher in the rectum compared to the terminal ileum, see figure 4.3.10.

I postulated that the higher expression of PD-1 and TIGIT in the CD4 T cell compartment in GALT might relate to the higher frequency of T follicular helper cells (TFH) in GALT which characteristically express these markers. Therefore, I examined the relative expression of ICRs on CD4 T cells on CXCR5+ and CXCR5- CD4 T cells in GALT and compared this with their expression on bulk CD4 cell from PBMCs, see figure 4.3.11. TIGIT and PD-1 expression were highest in the CXCR5+ CD4 subset in terminal ileum and rectum. To understand if the increased expression of ICRs in GALT was driven by their presence on CXCR5+ cells I next compared their expression on CXCR5- and bulk PBMCs. Interestingly, median TIGIT expression was not significantly different between CXCR5- subsets and bulk CD4 T cells in terminal ileum (16.2% and 8.9% respectively, P=0.07) and rectum (12.4% and 8.87% respectively, P=0.70), suggesting that higher TIGIT expression on CD4 T cells in GALT relates in part to increased frequency of TFH cells. Median PD-1 expression was also highest on the CXCR5+ CD4 T cell subsets (terminal ileum 72.9%, rectum 71.2%), with an intermediate level of expression on CXCR5- CD4 T cells (terminal ileum 39.2%, rectum 48.5%) and the lowest expression on bulk CD4 PBMCs (14.95%). No significant differences were seen in Tim-3 expression across subsets, except in the rectum where higher expression of Tim-3 was noted on the CXCR5-...
CD4 T cell subset (median 7.0%) compared to the expression on bulk CD4 T cells (median 2.0%), P=0.03.

**Figure 4.3.10 Expression of ICRs in GALT and PBMCs of healthy controls**

These jitter plots show the expression of the PD-1, TIGIT and Tim-3 on (a) CD4 T cells and (b) CD8 T cells in the terminal ileum (red triangles), rectum (blue circles) and PBMCs (yellow squares) of healthy controls. P values were calculated using a Kruskal Wallis test, corrected for multiple comparisons. Statistical significance levels are indicated * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001. Abbreviations: TI, terminal ileum; R, rectum, PBMC, peripheral blood mononuclear cells; ns, not statistically significant.
Figure 4.3.11 ICR expression on CXCR5⁺ and CXCR5⁻ CD4 T cell subsets in GALT and bulk CD4 PBMCs in healthy controls

These jitter plots show the expression of ICRs on CXCR5⁺ and CXCR5⁻ subsets in terminal ileum cells and (b) CD4 T cells in the terminal ileum (red triangles), rectum (blue circles) and PBMCs (yellow squares) of healthy controls. P values were calculated using a Kruskal Wallis test, corrected for multiple comparisons. Statistical significance levels are indicated * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001. Abbreviations: TI, terminal ileum; R, rectum, PBMC, peripheral blood mononuclear cells; ns, not statistically significant.
ICR expression on CD4 T cells in GALT in HIV+ individuals and healthy controls.

I next looked at ICR expression on bulk CD4 T cells from HIV infected GALT and made comparisons with ICR expression in healthy control GALT. Significantly higher expression of all ICRs was observed on bulk CD4 T cells of HIV infected individuals compared to controls in the rectum, but not in the terminal ileum or PBMC compartment. In the rectum, median PD-1 expression on CD4 T cells in HIV infected individuals and healthy controls was 66.1% and 52.1% respectively (P=0.02); median TIGIT expression was 41.0% and 20.1% respectively (P=0.0004), while median Tim-3 expression on bulk CD4 cells was 10.1% and 6.5% respectively (P=0.03).

Based on my earlier finding that a proportion of PD-1 and TIGIT expression on bulk CD4 T cells could be attributed to functional expression of these markers on CXCR5+ CD4 T cells in GALT. I next wanted to assess for differences in ICR expression on CXCR5+ CD4 T cell subsets between HIV infected individuals and healthy controls, as higher ICR expression on this population was more likely to represent true T cell exhaustion. These data are summarised in figure 4.3.12. There was a trend towards higher median PD-1 expression on CXCR5+ CD4 T cells in HIV infected individuals compared to healthy controls in the terminal ileum (48.2% and 39.2% respectively, p=0.06). There was also significantly higher PD-1 expression in the rectum of HIV infected individual compared to controls (61.3% and 48.5% respectively, p=0.03). There was no significant difference in the median PD-1 expression on PBMCs between individuals with HIV and controls (12.4% and 10.3% respectively, p=0.64). A similar pattern was observed for TIGIT expression on CXCR5+ CD4 T cells between HIV infected individuals and healthy controls; median TIGIT in terminal ileum was 22.8% and 16.2% respectively p=0.01; in the rectum median TIGIT expression was 25.9% and 12.1% respectively p<0.0001, while there was no significant difference between the two groups in PBMCs (8.7% and 10.1% respectively, p=0.46). Tim-
3 expression on CXCR5 CD4 T cells was only significantly higher in the rectum of HIV infected individuals compared to controls (11.1 and 6% respectively p=0.02), although a trend towards higher Tim-3 expression was also observed in HIV+ terminal ileum compared to healthy controls (6.5% and 5.2% respectively p=0.09), again no significant differences were seen in the PBMC compartment (2.1% and 1.4% respectively p=0.26). In contrast to the differential expression of PD-1 and TIGIT in CD4 cells observed in the CXCR5- subsets between HIV+ individuals and healthy controls, no significant difference in the expression of these markers on CXCR5+ CD4 T cells subset was seen in any anatomical site. These data suggest that increased PD-1 and TIGIT expression related to HIV and potential T cell exhaustion in GALT may only be evident on the CXCR5-CD4 T cells subsets and expression of these markers on the CXCR5+ subset are related to the phenotype of these cells. Therefore, alternative markers such as Tim-3 may be more useful at evaluating T cell exhaustion GALT and particularly in the CXCR5+ CD4 T cell subset.

**ICR expression on CD8 T cells in GALT in HIV+ individuals and healthy controls.**

In the CD8 T cell compartment, no differences were observed in the expression of any of the ICRs in the terminal ileum between HIV+ GALT and healthy controls, see figure 4.3.13. In rectal tissue, significantly higher TIGIT expression was observed in HIV+ GALT when compared to healthy control GALT (P=0.005), but no differences were seen in terminal ileum and PBMCs. There was a trend towards higher Tim-3 expression on CD8 T cells in HIV+ individuals compared to healthy controls in all anatomical sites, these data are summarised in figure 4.3.13.
Figure 4.3.12 ICR expression on CXCR5+ CD4 T and CXCR5+ CD4 T cells in HIV infected individuals compared to healthy controls across anatomical sites.

These jitter plots illustrate (a) PD-1, (c) TIGIT and (e) Tim-3 expression on CXCR5+ CD4 T cells (left column) comparing HIV infected individuals (closed symbols) and healthy controls (open symbols) in terminal ileum (red), rectum (blue) and PBMCs (yellow). Expression of (b) PD-1, (d) TIGIT and (f) Tim-3 on CXCR5+ CD4 T cells (right column) is also shown. All P values are shown and were calculated using a Mann-Whitney test. Abbreviations: T Ileum, terminal ileum; PBMC, peripheral blood mononuclear cells; HC, healthy controls.
Figure 4.3.13 ICR expression on CD8 T cells in HIV infected individuals across anatomical sites

The jitter plots show (a) TIGIT, (b) PD-1 & (c) Tim-3 expression on CD8 T cells across anatomical sites in terminal ileum (red), rectum (blue) and PBMC (yellow). P values are calculated using a Mann Whitney test. Abbreviations; T Ileum, terminal ileum; HC, healthy controls; HIV+, HIV positive

Expression of activation markers across anatomical sites in healthy controls

The expression of activation markers varies throughout the gut in healthy controls. In this dataset, a lower median HLA-DR expression on CD4 T cells was observed in the terminal ileum (1.1%) compared to the rectum (1.9%) and PBMCs (2.1%), although these differences were not statistically significant. Similarly, in the CD8 compartment, no significant differences were seen in median HLA-DR expression when the terminal ileum (0.9%) rectum (1.2%) and PBMC (1.3%) compartments were compared. In contrast, the median CD38 expression on CD4 T cells was significantly higher in the terminal ileum (73.0%) when compared to both rectum (42.6%) and PBMC (45.5%) compartments, both P<0.01 after correction for multiple comparisons. The CD38 expression on CD8 T cells in healthy GALT was significantly higher in the terminal ileum (76.6%) and rectum (85.5%) when compared with PBMCs (45.9%), both P=0.003 after correction for multiple comparisons.

CD4 T cell activation marker expression in GALT in HIV+ individuals compared to controls

To examine the impact of early ART on T cell activation across tissue sites, I compared the expression of HLA-DR and CD38, as well as the co-expression of HLA-DR and CD38
and the co-expression of HLA-DR and PD-1 between HIV infected individuals and healthy controls. Figure 4.3.14 shows the expression of these markers on CD4 T cells in GALT and PBMCs. The terminal ileum was the compartment with the most consistent elevation of all measures of CD4 T cell immune activation when compared with healthy controls. Median terminal ileum CD4 HLA-DR expression was 1.7% and 0.8% in HIV infected and healthy controls respectively, P=0.005; median terminal ileum CD4 CD38 expression was 82.3% and 73% respectively, p=0.06; median terminal ileum co-expression of HLA-DR and CD38 was 1.7% and 0.9% respectively, p=0.04; while median terminal ileum co-expression of HLA-DR and PD-1 was 1.4% and 0.45% respectively, P<0.0001. In the rectum, immune activation on CD4 cells was higher in HIV infected individuals compared to healthy controls by all measures except for CD4 HLA-DR (p=0.13); median CD4 CD38 in the rectum was 69.4% and 42.5% respectively, P<0.001; median rectal co-expression of HLA-DR and CD38 on CD4 T cells was 2.2% and 1.1% respectively, P=0.007; while median rectal CD4 HLA-DR and PD-1 co-expression was 1.1% and 1.1% respectively, P=0.05. In the PBMC compartment, there were no significant differences in any of the measures of CD4 T cell activation between HIV infected individuals and healthy controls. However, there was a trend towards higher CD38 expression on CD4 cells in HIV+ PBMCs compared to healthy controls with a median expression of 55.2% and 45.7% respectively, P=0.08.

**CD8 T cell activation marker expression in GALT in HIV+ individuals compared to controls**

I next examined the expression of activation markers on CD8 T cells across anatomical sites. The pattern of expression of activation markers on CD8 T cells was less consistent than on CD4 T cells. In health, I have shown that the terminal ileum and rectum have much higher CD38 expression on CD8 T cells compared to PBMCs while HLA-DR expression was comparable across anatomical sites. In the terminal ileum, only HLA-DR and PD-1
co-expression on CD8 T cells were significantly higher in HIV+ individuals compared to healthy controls, with a median expression of 1.0% and 0.2% respectively, P=0.0002.

**Figure 4.3.14 Expression of activation markers on CD4 T cells in HIV positive individuals and healthy controls across anatomical sites**

The Jitter plots below show the expression of (a) HLA-DR (b) CD38 (c) HLA-DR and CD38 co-expression and (d) HLA-DR and PD-1 co-expression on CD4 T cells, in HIV+ (filled symbols) and healthy controls (open symbols), from the terminal ileum (red), rectum (blue) and PBMC (yellow). P values were calculated using a Mann-Whitney test. Abbreviations; T Ileum, terminal ileum; PBMC, peripheral blood mononuclear cells; HIV+, HIV infected individuals; HC, healthy controls.

In the rectum, median CD8 HLA-DR (3.4% and 1.2%, P=0.005) and median HLA-DR & CD38 co-expression (3.1% and 0.9% P=0.002) on CD8 T cells was significantly higher in HIV infected individuals compared to healthy controls. There was also a trend towards higher HLA-DR & PD-1 co-expression on CD8 cells in rectal tissue (P=0.08). Unlike in the CD4 compartment, higher expression of activation markers on CD8 T cells was seen
in the PBMC compartment of HIV infected individuals compared to healthy controls; median HLA-DR on CD8 T cells was 3.1% and 1.3% respectively P=0.02; median CD38 expression on CD8 T cells was 53.2% and 45.9% respectively P =0.01; while median co-expression of HLA-DR and CD38 on HIV infected CD8 T cells compared to controls was 1.7% and 0.9% respectively P=0.01. These data are summarised in figure 4.3.15.

**Figure 4.3.15 Expression of activation markers on CD8 T cells in HIV positive individuals and healthy controls across anatomical sites**

The Jitter plots below show the expression of (a) HLA-DR (b) CD38 (c) HLA-DR and CD38 co-expression and (d) HLA-DR and PD-1 co-expression on CD8 T cells in HIV+ (filled symbols) and healthy controls (open symbols), from the terminal ileum (red), rectum (blue) and PBMC (yellow). P values were calculated using a Mann-Whitney test. Abbreviations; T Ileum, terminal ileum; PBMC, peripheral blood mononuclear cells; HIV+, HIV infected individuals; HC, healthy controls.
The relationship between HIV DNA and immune marker expression in GALT

In chapter 3 I have shown that ICR expression is associated with latent HIV infection in PBMCs. Given the higher expression of ICRs and activation markers in GALT, I next tested for a correlation between the expression of these markers and HIV DNA in the gut. Also, I tested for associations between ICR expression on the non-follicular CD4 T cell subset (CXCR5^− CD4^+) to account for the higher expression of these markers in on follicular (CXCR5^+CD4^+) T cells in GALT.

In the first instance, I tested for associations in terminal ileum GALT. Earlier, I have demonstrated a significantly higher level of CD4 T cell activation and a trend towards higher ICR expression on the CXCR5 CD4 T cell subset in HIV+ terminal ileum compared to controls. However, despite higher expression of these markers on CD4 T cells in the terminal ileum, no biologically plausible significant associations between HIV DNA and ICRs or activation marker expression were observed. Of note, a higher terminal ileum CD4/CD8 ratio was associated with lower HIV DNA expressed as CPM CD4 T cells (r=-4.7 P=0.02). Furthermore, the expression of ICRs and activation markers were positively correlated with each other. The correlation matrix in figure 4.3.16 summarises the terminal ileum correlations.

In rectal tissue, higher levels of both activation and ICR marker expression was observed in HIV+ GALT. However, as was the case in the terminal ileum, no significant correlations were observed between measures of HIV DNA and either ICR or activation marker expression on CD4 and CD8 T cells in the rectum. There was a borderline significant positive correlation between total rectal DNA (CPM CD4 T cells) and the Tim-3 expression on rectal CD8 T cells (r=0.35 P=0.08). CD8 Tim-3 expression in turn was significantly associated with the rectal CD4/CD8 (r=-0.6 P=0.001). Of note, PD-1 expression on CD4 and CD8 T cells were most highly correlated with and clustered with markers of immune
activation, however, PD-1 expression was also positively associated with TIGIT and Tim-3 expression. The correlation matrix in figure 4.3.17 summarises the correlations in the rectum.

**Figure 4.3.16 Correlogram summarising the relationship between ICR and activation marker expression and HIV DNA in the terminal ileum**

Positive and negative associations are shown in shades of blue and red respectively. The narrower the ellipse, the stronger the association. P values are shown within the ellipse, an absent or P value of ”0” indicates a P value of <0.01. Variables are ordered by hierarchical clustering. Abbreviations; Non-Fcd4, Non-follicular CD4 T cells; T Ileum, terminal ileum; cpm, copies per million. CD4 CD8 ratio refers to terminal ileum CD4/CD8 ratio.
**Figure 4.3.17** Correlogram summarising the relationship between ICR and activation marker expression and HIV DNA in the rectum

Positive and negative associations are shown in shades of blue and red respectively. The narrower the ellipse, the stronger the association. P values are shown within the ellipse, an absent or P value of "0" indicates a P value of <0.01. Variables are ordered by hierarchical clustering. Rectangles indicate those variables which cluster together. Abbreviations; Non-Fcd4, Non-follicular CD4 T cells; T Ileum, terminal ileum; cpm, copies per million.
4.3.6. Soluble Markers of Inflammation and Microbial Translocation in treated PHI

Chronic immune activation has been linked to morbidity and mortality in HIV and has been linked to microbial translocation. The early damage to the integrity of the gut epithelium by HIV supports ongoing immune activation and may support gut HIV persistence. I have shown above that measures of T cell immune activation and exhaustion in GALT are elevated in people living with HIV despite early ART. Therefore, I next investigated if higher measures of inflammatory cytokines, chemokines and markers of bacterial translocation would be present in the plasma of HIV infected individuals compared to controls. Using a Luminex® multiplex assay, I measured the cytokines IL-4, IL-5, IL-6, IL10, IL12 p70 and IL-15, the chemokines MCP-1, MIP-1α, MIP-1β and IP-10 as well as sCD163, MAdCAM-1, L selectin, CD40 and CD40 ligand in the plasma of HIV infected individuals and healthy controls. After correction for multiple comparisons I found significantly higher plasma levels of IL-4 (p=0.004), IL-10 (P<0.0001), IL-15 (P=0.0001), MIP-1α, (P<0.0001), MCP-1 (P<0.001), IP-10 (P=0.04), sCD40 (P=0.0001) and MAdCAM-1 (p=0.03) in HIV+ individuals compared to controls. Figure 4.3.18 summarises these data.

I also measured inflammatory cytokines and chemokines in the terminal ileum and rectum from HIV infected individuals only. Gut biopsies (one from each site) were placed in culture within one hour of the biopsy procedure and the supernatant, which was harvested at 24 hours, was used to measure the level of the analytes listed above (sCD163 and sCD14 were not measured in tissue supernatants). After adjusting for multiple comparisons sCD40 (P=0.003), IL-12 p70 (p=0.0001), MIP-1β (P=0.0001), IL-15 (0.008) and IL-5 (P=0.05) were all found at higher levels in the terminal ileum culture supernatants compared to rectal culture supernatants suggesting greater immune activation in this site.
**Figure 4.3.18 Plasma soluble markers of inflammation in HIV infected individuals and healthy controls**

The bars graphs in this figure show the mean concentration of the corresponding analyte in HIV infected (n=30) and healthy controls. Whiskers indicate the standard deviation. Data was Log$_{10}$ transformed, and multiple T-tests applied to test for group differences. Statistical significance was determined using the Bonferroni-Dunn method, with alpha = 0.05. Adjusted P values are indicated by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001. Only significant P values are shown.

However, it is worth noting the analytes were measured in the supernatant of unstimulated tissue, and therefore the levels of some of the analytes were relatively low. Interestingly higher MAdCAM-1 and IL-10 levels were also measured in the terminal ileum; however, this was no longer significant after adjusting for multiple comparisons. IL-6 and MCP-1 levels were higher in rectal tissue but again were not significant after adjusting for multiple comparisons. These data are summarised in figure 4.3.19.

**Figure 4.3.19 Soluble markers of inflammation measured in terminal ileum and rectal tissue culture supernatants**

The bars graphs in this figure show the mean concentration of the corresponding analyte in HIV infected and healthy controls. Whiskers indicate the standard deviation. Data was Log$_{10}$ transformed, and multiple T-tests applied to test for group differences. Statistical significance was determined using the Bonferroni-Dunn method, with alpha = 0.05. Adjusted P values are indicated by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001, only significant P values are shown.
Associations of Plasma markers of inflammation and bacterial translocation with HIV DNA in gut sites

I have demonstrated increased levels of immune activation in GALT during treated PHI. Furthermore, markers of inflammation and gut microbial translocation are elevated in HIV infected individuals compared to controls. Therefore, I explored the relationship between these soluble plasma markers and measures of HIV reservoir in gut tissue sites. The correlogram in figure 4.3.20 outlines the associations between total HIV DNA in GALT and plasma markers of bacterial translocation and inflammation. As previously discussed, the measures of HIV DNA in the gut correlated strongly with each other. Interestingly all measures of gut DNA are associated with soluble CD14. Markers of systemic inflammation IL-6 and IP-10 also clustered with HIV DNA and sCD14. In addition, many of the inflammatory cytokines (IL-4, IL-5 and IL-10) were positively correlated with each other, as were markers of lymphocyte migration to the gut (MAdCAM-1 and L Selectin) and CD40-CD40L.

To further understand the relationship between these soluble inflammatory markers and to overcome collinearity, I used a LASSO regression analysis to perform variable selection. I developed an independent model for each total HIV DNA measure, i.e. total HIV expressed as copies per million gut and copies per million CD4 T cells in both the terminal ileum and rectum. The LASSO model applies a penalty to the sum of the absolute values of the regression coefficients; this forces some of the variable coefficients to zero thereby removing unimportant variables resulting in a final model of predictive variables. The results of the four LASSO models are summarised in table 4.3.3 below. Lambda ($\lambda$) is the penalty coefficient applied, and the optimal Lambda is calculated using cross-validation of the dataset. The cross-validation plot for model 1 is shown in figure 4.3.21. The optimal value for lambda is the one which has the lowest mean square error and is indicated by the vertical dotted line on the right of the graph.
Figure 4.3.20 Associations between soluble inflammatory markers in plasma and HIV DNA in GALT

This correlation matrix includes observations from n=32, and the variables are ordered by hierarchical clustering. Positive correlations are indicated in blue while negative correlations are in red. The narrower the ellipse, the higher the correlation coefficient. P values are shown in the ellipse and were calculated using Spearman’s test. The rectangles illustrate those variables which cluster together. Absent P values indicate a significance level of <0.01. Abbreviations; cpm, HIV DNA copies per million.
Figure 4.3.21 Cross Validation Plot of Lasso Model 1

This plot shows the mean-square error (MSE) of the model (red points) plus/minus the standard error (whiskers) for each value of lambda. The minimum $\lambda$ is indicated by the horizontal dotted line on the right while the dotted line on the left is the largest $\lambda$ at which the MSE is within one standard error of the minimal MSE. The numbers on the horizontal above the graph indicate the number of variables included for a given value of $\lambda$.

Figure 4.3.22 Model 1 - change in the $\beta$-coefficients of variables over a range of $\lambda$ values

The plot shows the $\beta$-coefficients of the variables (coloured lines and corresponding coloured labels for selected variables are on the right y-axis) over a range of values of $\lambda$. The model used the $\lambda$ value at which the MSE was within one standard error of the minimal MSE, as indicated by the dotted vertical line on the right.
The model also allows us to visualise the shrinkage of the $\beta$ coefficients for each variable over a range of lambda values. This is how the model excludes less significant variables and is illustrated in figure 5.3.22 for model 1.

It is also possible to understand the proportion of variance that the model accounts for based on the addition or omission of variables. This is summarised in figure 5.3.23

**Figure 4.3.23 Model 1: fractional deviance explained**

The variance explained according to the inclusion or exclusion of variables is shown in the plot below.

The variable most consistently associated with HIV DNA in GALT across all models was soluble CD40; in fact, CD40 positively correlated with all measures of HIV DNA in both the terminal ileum and rectum. sCD14 was also strongly associated with HIV DNA in all sites except in model 2 (terminal ileum HIV DNA CPM CD4 T cells). Other predictors of HIV DNA in GALT included the pro-inflammatory cytokine IL-6 and IL-4. Higher RANTES predicted lower HIV DNA levels in both gut sites and higher plasma IL-17A was predictive of lower HIV DNA in the rectum. MAdCAM-1 was strongly predictive of HIV DNA in terminal ileum but not in the rectum, although a non-significant inverse association was
also evident in the rectum. The relationship between HIV DNA and selected significant variables from the Lasso models are shown for the rectum and the terminal ileum in figures 4.3.24 and figures 4.3.24, respectively.

Table 4.3-3 Lasso regression models of gut total HIV DNA and plasma inflammatory markers

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*Only variables with β coefficients >0 which were included in the final models are shown.
The prefix "s" indicates soluble. Abbreviations; CPM, copies per million.
Figure 4.3.24 Correlations between HIV DNA in the rectum and the plasma inflammatory markers which were predictive in the LASSO model

The scatter plot shows the correlation between HIV DNA in the rectum, expressed as CPM gut cells, and plasma (a) sCD40, (b) sCD14, (c) RANTES and (d) MAdCAM-1. P values were calculated using a non-parametric test and Spearman’s coefficient, r is shown. The prefix s- denotes soluble. Abbreviations; CPM, copies per million; Log, Log10.
Figure 4.3.25 Correlations between HIV DNA in the terminal ileum and the plasma inflammatory markers which were predictive in the LASSO model

The scatter plot shows the correlation between HIV DNA in the terminal ileum, expressed as CPM gut cells, and plasma (a) sCD40, (b) sCD14, (c) RANTES, (d) MAdCAM-1, (e) IL-4 and (f) IL-6. P values were calculated using a non-parametric test and Spearman’s coefficient, r is shown. The prefix s- denotes soluble. Abbreviations; CPM, copies per million; Log, Log_{10}.
4.4. Conclusion

4.4.1. Discussion

The gut contains the largest number of lymphocytes in the human body\textsuperscript{278,328} with only 2\% of CD4 T cells found in peripheral circulation\textsuperscript{467}. In this chapter, I have quantified and characterised the reservoir in two anatomically and immunologically distinct regions of the GI tract. In keeping with published data, I found higher levels of HIV DNA in the gut tissue compared to PBMCs\textsuperscript{331,335}. Chun et al. have previously estimated the frequency of HIV DNA in CD4 T cell in GALT to be five times greater than in peripheral blood\textsuperscript{331}. I also observed an enrichment of HIV DNA in GALT compared to PBMCs, albeit at a lower level with approximately 3.5-fold higher HIV DNA levels in GALT compared to PBMC. This lower level of enrichment may relate to the treatment characteristics of the two cohorts, the HEATHER cohort all initiated ART in primary infection while the Chun et al. cohort did not. Also, the cohort in the Chun et al. study had received a much longer duration of ART, ranging from 4.8 to 9.9 years. While the half-life of the latent HIV reservoir in blood is estimated to be between 6–44 months\textsuperscript{123,468,469}, the rate of decay in GALT is unknown. Ramratnam et al. have previously reported slower rates of decay in individuals with intermittent plasma viremia\textsuperscript{469}. Therefore, if there is ongoing HIV viral replication in gut sites on ART, slower decay in these sites would account for relatively higher GALT HIV DNA levels with longer duration of ART. I did not observe a relationship between duration of ART and HIV DNA in GALT in our study, this is not unsurprising when I consider the slow rate of reservoir decay; while not statistically significant, some individuals in our study had an increase in HIV reservoir in gut sites, while the trend in the PBMC compartment was towards a decrease. Previous studies examining HIV reservoir in PBMCs have shown a rapid decay in HIV DNA levels in the first year of ART with slow decay thereafter\textsuperscript{372}. Viard et al. studied the decay of the HIV reservoir in PBMCs of chronically
treated individuals and reported a $0.5 \log_{10}$ drop in HIV DNA in the first year of ART, a $0.18 \log_{10}$ drop in the second year with no significant decrease afterwards. Initial gut sampling in our cohort was performed at a median of 25 months post-ART initiation. Furthermore, as previously mentioned I did not see any significant changes in HIV DNA measures in those with longitudinal sampling; while acknowledging the limitations of our sample size to detect small differences, my data suggest that the pattern of decay in the gut reflects that in blood with a suggestion of increasing GALT reservoir in some individuals.

I observed a broad range of GALT HIV DNA levels in the HEATHER cohort despite relatively similar time to initiation and duration of ART. Work in blood suggests that virological and host immune factors around the time of seroconversion predict time to disease progression and the HIV reservoir on ART. Earlier work by the SPARTAC collaborators demonstrated that HIV viral load at seroconversion predicted HIV-1 DNA levels in PBMCs on ART. Yerly et al. examined the relationship between HIV RNA and HIV DNA in blood and lymph node and also found a relationship between baseline HIV RNA and DNA levels. Similarly, baseline plasma viral load in our cohort was predictive of on ART HIV DNA levels in PBMCs. However, baseline viral load did not correlate with GALT HIV DNA levels. The baseline factor most predictive of on ART GALT DNA was the plasma CD4/CD8 ratio, as was concurrent CD4/CD8 ratio suggesting the host immune response is important in determining and maintaining the size of the gut HIV reservoir.

Early ART has been shown to enhance immune recovery in blood and GALT. Work from the RV254/SEARCH 010 cohort, which diagnoses and treats very early HIV infection (mostly Fiebig I-III) in Thailand showed an increase in CCR5+ CD4 T cells in the sigmoid colon of early-treated patients. Further work by the same group demonstrated a
protective effect of ART started in Fiebig I/II, preventing the depletion on Th17 cells in the sigmoid colon. However, persistent immune dysfunction evident by the poor recovery of CD4 T cells is seen even in these very early treated individuals. I hypothesised that the differences observed in HIV DNA levels along the GI tract in our cohort related to the compartmentalisation of immune recovery in treated PHI. Firstly, I observed a discrepancy in the recovery of the CD4/CD8 ratio between the terminal ileum and rectum, suggesting a more limited immune recovery in the former. I also observed anatomical variation in the expression of immune activation and exhaustion markers. Overall, T cells in the GALT expressed significantly higher levels of almost all of the markers of immune dysfunction assessed when compared to PBMCs. In general, CD4 T cells in the terminal ileum of HIV infected individuals had a more activated immune phenotype while CD4 T cells in the rectum had higher expression of T cell exhaustion markers. The higher expression of PD-1 and TIGIT in the GALT of healthy controls also suggest a physiological function of these markers in GALT. It has been suggested that the high amount of PD-1 expressed by germinal centre TFH cells contributes to limiting TFH cell proliferation in germinal centres through dampening of TCR signalling. As such, Tim-3 may be a more appropriate marker of T cell exhaustion in the gut. However, given the limited cell number and poor viability of mucosal mononuclear cell I was unable to assess for functional T cell exhaustion. Interestingly Tim-3 expression on CD8 T cells had the strongest correlation with HIV DNA in rectal tissue, albeit non-statistically significant. I did not see a positive correlation between markers of immune activation in the gut and HIV DNA. In fact, I observed a negative association with the expression of CD38 on CD4 T cells in the terminal ileum. Such findings have previously been reported by Yukl et al., who showed a negative correlation between HIV DNA and HLA-DR and CD38 co-expression in rectal tissue. Their findings and ours support the hypothesis that the activation of HIV specific cells may enhance viral clearance and induce apoptosis in non-specific T-cells in GALT.
thus explaining the negative correlation seen in the gut\textsuperscript{335}. While I did not observe a significant positive correlation between ICR or activation marker expression in GALT, other groups have; a recent report by Khoury et al. demonstrated an association between integrated HIV DNA and PD-1 in rectal tissue but not in other sites\textsuperscript{137}, of note total HIV DNA PD-1 was not measured in that study.

One of the important findings from this thesis is the different levels of HIV DNA observed along the GI tract. HIV DNA levels were significantly higher in the terminal ileum compared to the rectum. Published reports suggest that terminal ileum may be a site of ongoing replication on ART; a treatment intensification study found a decrease in CA-RNA in the terminal ileum of those whose treatment was intensified with the integrase inhibitor raltegravir\textsuperscript{191}, such findings may indicate that raltegravir inhibited new rounds of ongoing replication. A potential mechanism for higher HIV DNA levels in this site is the poor penetration of some ART into tissues. This hypothesis is supported in work by Fletcher et al. which has demonstrated sub-therapeutic levels of ART in lymphoid tissue of ART-treated individuals with evidence of ongoing viral replication\textsuperscript{184}. More recent work by Estes et al. demonstrated HIV RNA\textsuperscript{+} cells in the GALT of individuals on ART supporting the case for ongoing HIV replication in GALT tissue sites. In addition to higher DNA levels in the terminal ileum, I also observed a high a frequency of CD4 T cell activation and higher PD-1 and TIGIT expression on CXCR5\textsuperscript{-} CD4 T cells in this site. Coupled with this I found higher levels of IL-5, IL-12 p70, IL-15 and MIP-1\textbeta in explant supernatants from the terminal ileum compared to the rectum suggesting greater inflammation in this site on ART\textsuperscript{473-475}. While some evidence suggests an absence of viral evolution in GALT in individuals with treated PHI\textsuperscript{189}, ongoing low-level HIV viral replication or even virus production from latently infected cells in GALT sites offer a plausible explanation for the observed increased immune activation in these sites. Guadalupe et al. have previously
demonstrated ongoing viral replication in GALT sites, even in PHI-treated individuals on ART \(^\text{476}\), while Mehandru et al., consistent with our findings report increased levels of immune activation in GALT but not in PBMCs of individuals treated during PHI\(^\text{338}\). The studies by Guadalupe and Mehandru involved sampling from one gut site only, namely the jejunum and colon respectively, and therefore, unlike the HEATHER study was unable to describe differences in immune recovery along the GI tract within ART-treated individuals\(^\text{338, 476}\).

I also observed a relationship between gut HIV DNA levels and plasma markers of microbial translocation and systemic inflammation. In the lasso model, sCD14 strongly associated with HIV DNA in the gut. This finding highlights a potential mechanism whereby ongoing microbial translocation, a consequence of ongoing immune dysfunction in the gut either supports maintenance of the HIV reservoir or alternatively the observed immune dysfunction is induced by viral production from the HIV reservoir itself. Furthermore, plasma sCD40 was consistently associated with the gut HIV reservoir, the CD40 molecule is found on the surface of B-cells, macrophages, and dendritic cells and shedding of CD40 is a consequence of the B cell – T cell interaction through CD40/CD154 signalling and soluble CD40 can inhibit the interaction through negative feedback\(^\text{477, 478}\). Elevated CD40 levels have described in association with liver injury in hepatitis B \(^\text{478}\) and hepatitis C infection\(^\text{479}\). The association of a marker of the B cell – T cell interaction with HIV DNA in tissue sites in our study is interesting and warrants further investigation. I also observed a consistent inverse correlation between RANTES and HIV DNA in tissues, highlighting the role of this antiviral chemokine in control of HIV reservoir in tissue. Previous work has shown an antiviral effect of RANTES in alveolar macrophages supporting its role as an antiviral factor in tissues\(^\text{480}\). Interestingly, Chun et al. have previously shown that \(\beta\)-chemokines play a role in the inhibition of viral replication in the
latent HIV reservoir in some LTNPs and patients receiving HAART, but not in chronically infected patients on ART \textsuperscript{481}. A surprising finding in my data was the inverse association between MAdCAM-1 and HIV DNA levels in the terminal ileum. MAdCAM-1 has been suggested as a biomarker of HIV induced inflammation\textsuperscript{482}. Elevated MAdCAM-1 in plasma was associated with lower HIV DNA levels in the terminal ileum; while no clear mechanism for this is apparent, it is worth highlighting that I measured soluble MAdCAM-1 in plasma which may not be directly related to the MAdCAM-1 expression on the vascular endothelium. A possible explanation for this finding is that this soluble form of MAdCAM-1 may compete with HIV gp-120 in its binding to $\alpha 4\beta 7$ thereby limiting infection.

In summary, I have demonstrated higher levels of HIV DNA in terminal ileum GALT compared to peripheral blood and rectal tissue and shown that the HIV reservoir in GALT is stable over time. My data also suggest clear compartmentalisation of immune recovery between the PBMC and GALT compartments, with differences also evident along the GI tract itself. While the expression of markers of immune function was not associated with HIV DNA, markers of bacterial translocation and systemic inflammation were, suggesting a relationship between incomplete immune recovery and the resulting mucosal barrier dysfunction and HIV DNA levels in GALT.
Chapter 5

The impact of ART in primary HIV infection on germinal centre T cells

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Chapter 5.

The impact of ART in primary HIV infection on germinal centre T cells

5.1. Introduction

T Follicular helper cells (TFH) correspond to a subset of memory CD4+ T cells expressing high levels of CXCR5 and PD-1 on their cell surfaces and are vital components of the adaptive immune system; they reside in germinal centres (GC) within B cell follicles in secondary lymphoid tissue. They are relevant to HIV persistence, especially in tissue sites as they are preferentially infected and may act as a cellular sanctuary for HIV. TFH cells were first described by Kim et al. who identified a subset of the germinal centre resident, CXCR5 expressing CD4 T cells, with enhanced ability to induce antibody production from B cells. TFH cells support the humoral immune response and allow for efficient antibody production, they interact with B cells, through the cytokine IL-21. This interaction promotes B cell proliferation and differentiation into plasma cells and is necessary for antibody class switching, ultimately allowing for the production of high-affinity targeted antibodies. The discovery of the transcription factor which drives TFH differentiation and function, B-cell lymphoma 6 (Bcl-6), lead to the classification of TFH as an independent lineage of CD4 T cells.

Transcriptional Profile of T Follicular Helper Cells

CXCR5 promotes T cell and B cell migration into lymphoid tissue B cell follicles in response to its interactions with CXCL13. Gene-expression profiling of TFH cells have identified other molecules which play an essential role in the development, function and
migration of TFH, these include; BCL6, PD-1, IL21, ASCL2 and ICOS. Furthermore, when compared to other CD4 T cell subsets TFH cells have a unique gene expression profile, they do not express T-bet, RORyt, GATA3 or Foxp3 and have limited ability to produce Th1/Th2/Th17 related cytokines\textsuperscript{488, 489}.

The differentiation of naïve CD4 T cells towards a TFH lineage is initiated in the T cell zone of lymph nodes by antigen-presenting dendritic cells (DCs). This T cell priming by dendritic cells involves an interaction between the T cell receptor (TCR) and the costimulatory molecules CD28 and ICOS\textsuperscript{490, 491}. Upon activation of antigen-specific CD4 T cells, there is upregulation of the transcription factor aschaete-scute homolog 2 (Ascl2); this results in downregulation of CCR7 and upregulation of CXCR5, allowing migration of the so-called pre-TFH cell to lymphoid tissue B cell follicles\textsuperscript{492}. DCs also secrete IL-6 and IL-12, which in turn induces Bcl-6 expression in pre-TFH cells, in a STAT3-dependent manner\textsuperscript{493, 494}. These pre-TFH cells also require B cell presentation for optimal TFH development and GC formation\textsuperscript{495, 496}. A second signal is required from cognate antigen-primed B cells resulting in stable Bcl-6 expression in the pre-TFH\textsuperscript{497}. Bcl-6, in tandem with other transcription factors such as IRF4, BATF and c-maf then drives the expression of TFH cell signature genes critical for T cell-B cell interaction, including; CXCR5, ICOS, Sh2d1a Pdcd1 and CD40L\textsuperscript{485}. IL-27 can induce c-maf expression in association with ICOS\textsuperscript{498, 499}. C-maf, in turn, induces IL-21 production in CD4 TFH cells. IL-21 then acts as an autocrine cytokine, promoting pre-TFH cell differentiation and homeostatic maintenance of TFH cells. IL-21 also plays a role in the differentiation of germinal centre B cells into memory and plasma cells. It is this optimal interaction between B cells and TFH which determines the magnitude of the germinal centre reaction which in turn controls affinity maturation of B cells and therefore the breadth of the antibody response\textsuperscript{500-502}. 
Peripheral T Follicular Helper Cells

The relationship and function of circulating peripheral T-follicular helper cells (pTFH) to the bona fide TFH resident in the germinal centre are much debated. CXCR5+ CD4 T cells with TFH-like phenotype, from now on referred to as pTFH, can be found in peripheral blood. There are phenotypic and functional differences when pTFH are compared to germinal centre TFH. Although no universally agreed phenotype exists, pTFH have been described as those CD4 cells which express CXCR5 alone\(^{503}\) to those that express CXCR5\(^*\), CCR7\(^{\text{low}}\), PD-1\(^*\) and ICOS\(^*\)\(^{504}\). Others have reported pTFH to co-express CCR7 and are therefore included in the central memory CD4 T cell subset\(^{484, 505}\). However, the lack of a standardised phenotypic definition for pTFH may confuse some of the reported findings in studies of pTFH and HIV. One of the clear phenotypic differences between pTFH and their germinal centre counterparts is the lower PD-1 and Bcl-6 expression seen on pTFH\(^{506}\), see figure 4.3.1. Expression of CXCR5 and PD-1 on pTFH cells is stable and not upregulated upon non-specific antigen or cytokine stimulation. Also, pTFH expressing these markers have been shown to support B cell differentiation into plasmablasts. However secondary signals from B-cells, such as CD40L or ICOS interactions, as well as IL-21 secretion are also required\(^{503, 507, 508}\). One of the primary aims of this chapter is to describe the phenotypic and functional differences between pTFH and GC TFH in treated primary HIV infection and to understand the association of this subset with HIV DNA in blood and tissue.

HIV and T Follicular Helper Cell Dysfunction

In health, TFH cells support B cells to proliferate and differentiate into plasma cells, leading to efficient antibody production. However, the role of TFH in the setting of chronic viral infections is less clear. Consequently, this chapter also aims to explore the relationship between TFH cells, humoral immunity and mechanisms for HIV viral persistence.
role of TFH cells has been described in the context of other chronic viral infections. TFH have been associated Lymphocytic Choriomeningitis Virus (LCMV) clearance in mice, the development of HCV specific antibodies and an active Hepatitis B phenotype.509-511.

The interaction between HIV infection and TFH is complex; evidence suggests that HIV induces dysregulation of TFH. Chronic untreated HIV infection results in the expansion of TFH cells in lymph nodes.512 However, the expanded TFH cells are dysfunctional and unable to provide adequate B cell help.513,236,512 This expansion of HIV-specific TFH cells is associated with HIV viraemia, interestingly the majority of the expanded TFH cells are specific for Gag- as opposed to Env.512 This observed expansion of TFH is in contrast to the expected depletion of activated total CD4 T cells seen in untreated HIV infection and is thought to be driven by persistent antigenic stimulation, mediated in part by IL-6 signalling.514. It is this abnormal expansion of TFH cells, caused by the failure of viral antigen clearance, which drives skewed B cell differentiation and impaired antibody production.512 It has also been proposed the PD-1 triggering on TFH cells by PD-L1 on germinal centre B cells may be a mechanism for TFH dysfunction.236. In fact, PD-1 blockade has been shown to enhance HIV-specific immunoglobulin production in vitro and triggering of PD-1 on TFH cells lead to a decrease in IL-21 production.236. Loss of regulatory control by T Follicular Regulatory (TFR) cells may also play a role in the inefficient germinal centre responses seen in untreated HIV infection.515,516.

pTFH have the potential to provide B cell help.503,517,518. However, Boswell et al. showed that pTFH cells have different cytokine production (such as IL-21, IL-2 and IL-17) when compared with germinal TFH (IL-4, IL-10 and IL-21, but compromised production of IL-2 and IL-17).517. Locci et al. have found that pTFH with the phenotype PD-1+CXCR3−CXCR5+ are the CD4 T cells subset with the greatest potential to provide B cell help.507. In chronic ART-treated virally suppressed individuals, pTFH are functionally
impaired, with reduced ability to provide B cell help, when compared with those pTFH from elite controllers. Conversely, vaccine-induced antibody responses to influenza in HIV infected individuals are associated with preservation of pTFH function; measured by the secretion of CXCL13 and IL-21. A recent study by Schultz et al. used an IL-21 capture assay to identify circulating IL-21+CD4+ T cells; these have been shown to be transcriptionally and phenotypically pTFH cells. IL-21–producing blood CD4+ T cells have been described in acute and chronic HIV infection, and elevated frequencies of HIV-specific IL-21–producing CD4+ T cells have been associated with viral control. The ALVAC+AIDSVAX HIV vaccine study found an expansion of HIV-specific IL-21+ pTFH cells, supporting the idea that induction of TFH cells is required for superior humoral responses against HIV.

Taken together these data suggest that for some individuals, HIV infection depletes pTFH numbers and impairs their function. In contrast, those better able to maintain robust TFH functionality have a higher propensity for viral control; data from elite controllers suggest that these individuals have more intact TFH function and humoral responses with a lower frequency of latent infection of TFH cells than non-controllers. One of the clinical challenges will be understanding how we can prevent or modulate TFH dysfunction. ART may impact the function of TFH cells; a degree of reversibility of TFH dysfunction has been demonstrated with ART in chronic HIV infection, while a recent study investigated the impact of ART in acute HIV infection on TFH function suggests that very early ART may preserve pTFH function and B-cell memory. However, this required very early initiation of ART (Fiebig stage I/II) and may not be feasible outside of a clinical study. In addition to the provision of B cell help, there is growing evidence for the role of pTFH and IL-21 signalling in support of the antiviral function of CD8 and NK cells. This is particularly
interesting when we consider follicular CD8 (fCD8) T cells and the role they may have in controlling viral replication, and this is discussed in the fCD8 section below\textsuperscript{527, 528}.

**T Follicular Helper Cells, HIV persistence and the HIV Reservoir**

TFH cells in both the germinal centre and the peripheral circulation have been shown to be preferentially infected with HIV and enriched for HIV-1 DNA\textsuperscript{134-136, 340, 343}. The absence of HIV viraemia in peripheral blood does not necessarily reflect viral suppression in lymphoid tissue\textsuperscript{184}. Although controversial, lymphoid tissue is thought to be the primary site of ongoing HIV replication during chronic ART-treated HIV infection \textsuperscript{342, 343}. Indeed, B cell follicles have been suggested as a sanctuary site for HIV where ongoing replication may occur in elite controllers \textsuperscript{186}. Germinal centre TFH and pTFH cells are implicated in HIV persistence as they support viral replication during treated infection and serve as an important cellular reservoir of HIV-1 DNA\textsuperscript{134, 135, 351}.

Germinal centre TFH cells are highly permissive to SIV and HIV infection\textsuperscript{514} and the TFH and CXCR5\textsuperscript{+} PD-1\textsuperscript{+} populations in lymph node have been shown to be most effective in supporting HIV replication\textsuperscript{136}. A study looking at the role of pTFH defined phenotypically as CD45RA\textsuperscript{−} CCR7\textsuperscript{+} CXCR5\textsuperscript{+}, found that pTFH and in particular, PD1\textsuperscript{+} pTFH cells to be more permissive to HIV infection than non-pTFH cells. They also demonstrated greater HIV production (measured by p24 expression after anti-CD3/anti-CD28 stimulation) and higher frequencies of 2-LTR circles in the pTFH cells compared to non-pTFH cells, confirming the idea that TFH cells support HIV persistence during ART-treated HIV infection \textsuperscript{340}.

Non-human primate studies have demonstrated that “elite controlling monkeys” who had HIV-specific CD8\textsuperscript{+} mediated viral control in extrafollicular sites, had evidence of ongoing viral replication in germinal centres. They showed that HIV-specific CD8\textsuperscript{+} T cells are
excluded from germinal centres in these otherwise elite controlling animals. Boritz et al. have published data supporting this mechanism of persistence in humans; using samples from elite controllers, they detected viruses in lymph nodes which had genetic and transcriptional markers of active replication, most abundantly within PD1+, TFH-enriched cell populations.

**Follicular Cytotoxic T cells (fCD8)**

During Primary HIV infection, naive antigen-specific cytotoxic CD8+ T cells differentiate into effector cells with the potential to control viraemia. However, viral escape ensures rapidly. Traditionally it is thought that such cytotoxic CD8 T cells are excluded from B cell follicles, as such, these follicles act as immune privileged sites in HIV chronic HIV infection. While it is clear that CD4+ T cells utilise CXCR5 to localise to B cell follicles, there is little known about the ability of cytotoxic CD8 cells to enter B cell follicles and control infection at this site. Recent work by Le et al. and Leong et al. have demonstrated that antigen-specific CXCR5+ CD8+ T cells which they have coined ‘follicular cytotoxic CD8 T cells’ migrated to B cell follicles, where they have the potential to eradicate virus-infected TFH cells and B cells. Studies of SIV infection in rhesus macaques have demonstrated an expansion of SIV specific fCD8 cells is associated with greater viral control. In human studies, Petrovas et al. have found that fCD8 cells in lymph nodes from HIV infected individual have greater cytolytic potential than non-fCD8. However, this finding is still debated, with some evidence that fCD8 may be functionally exhausted. fCD8 have also been described in peripheral blood, at low frequency. Nonetheless, their frequency inversely correlated with HIV viral load.
**Aims**

In this chapter, I aim to characterise the phenotype of pTFH and germinal centre TFH in PHI, and to examine the impact of ART commenced in PHI on the frequency, phenotype and function of these cells. Additionally, I characterise fCD8 T cells in PHI. Finally, I explore the relationship between both CD4 and CD8 follicular T cells and the HIV reservoir in treated PHI.

**Hypothesis**

The following Hypotheses are tested in this chapter are:

1. ART commenced in PHI preserves the frequency and function of peripheral and germinal centre TFH

   ii. In PHI, higher TFH cells are associated with lower measures of HIV reservoir

   iii. In PHI, higher fCD8 cells are associated with lower measures of HIV reservoir

**Objectives**

The objectives of this chapter are:

1. Characterise the phenotype of pTFH and germinal centre TFH in PHI

2. Characterise the phenotype of fCD8 cells in PHI

3. Assess the impact of ART in PHI on measures of TFH

4. Test for associations between both TFH markers & fCD8 and HIV reservoir at seroconversion and during treated PHI
5.2. Methods

5.2.1. Description of clinical cohorts

Part 1 – pTFH Clinical Cohort

Individuals with confirmed PHI from the HEATHER and SPARTAC studies are included in the analysis presented in the Results - Part 1 section of this chapter. These cohorts are described in greater detail in chapter 2 – section 2.1.2 and 2.1.3. Additional inclusion criteria for the pTFH clinical cohort included, known estimated date of infection (EDI), a follow-up period of at least one year, and PBMC sample availability.

For purposes of analysis, the pTFH clinical cohort was divided into ART-treated and ART-naïve individuals. ART-treated individuals were defined as those who had commenced ART within 90 days of EDI and who remained on ART for at least one year. This group included individuals from the SPARTAC (ART-48 week) arm and HEATHER. ART-naïve individuals included those with known EDI who did not initiate ART within the first year of the EDI. This group was composed of individuals from SPARTAC (standard of care arm).

The PBMC analysis was performed at two time-points (i) baseline; the initial sample that was taken pre-ART and closest to EDI and at (ii) one year; the PBMC sample taken approximately one year after EDI.

Part 2 – GALT TFH Clinical Cohort

The second part of this chapter uses data from the HEATHER gut study to characterise the tissue TFH cells. The cohort used for this analysis are described in chapter 2 – section 2.1.4 and the demographic details are outlined in chapter 4 -section 4.3.1 (HIV infected) and 4.3.2 (healthy controls).
5.2.2. Laboratory Methods

Flow Cytometry

For the pTFH cohort, expression of CD3, CD19, CD4, CD8, CXCR5, PD-1, TIGIT, Tim-3, HLA-DR, Bcl-6 and IL-21 on resting PBMC was measured using flow cytometry; the antibodies used are shown in table 4.3.1. IL-21 was measured only in individuals from the SPARTAC study. Surface and intracellular staining and acquisition of data were performed as described in chapter 2.

IL 6, D-Dimer & CXCL13 Measurement

In part 1, IL-6, D-Dimer and CLCX13 were measured in plasma by ELISA as previously outlined in chapter 2 – section 2.4.9 and section 2.4.10.

In Part 2, all soluble biomarkers (including IL-6 and CXCL13) were measured using the Magnetic Luminex assay as outlined in chapter 2 – section 2.4.13.

Immunoglobulin Measurement

Total IgM, IgA, IgG1, IgG2, IgG3 and IgG3 types were using the Milliplex human immunoglobulin isotyping magnetic bead panel as outlined in chapter 2 – section 2.4.8.

HIV, Total, specific anti-consensus gp120 HIV-1 clade B and specific anti-p24 IgG and IgA responses were measured by ELISA, as previously described in chapter 2 – section 2.4.11.

Total & Integrated HIV-1 DNA measurement

Total HIV DNA was measured by quantitative qPCR and is described in chapter 2 – sections 2.4.3 and 2.4.5. CD4 T cell enrichment was performed prior to HIV DNA quantification in PBMCs but not on MMCs from the gut-associated lymphoid tissue. Reporting of total HIV DNA measurements from gut samples was therefore reported as
copies per million “gut” cell. For comparison with PBMC measures of total HIV DNA, the total HIV-1 DNA level expressed as copies per million CD4 T cell in the gut was also calculated using the CD4 T cell count derived from each participant flow cytometry plot. Integrated HIV-1 DNA was measured on a subset of individuals at baseline using the Alu-gag qPCR according to the method devised by Liszewski $^{535}$.

**Statistical Analysis**

Continuous variables were compared between groups using non-parametric tests throughout. Where three groups were compared, a Kruskal-Wallis test was used; pairwise comparisons were performed on all combinations of groups only if the overall test p-value was <0.05. Correlative analyses were performed using Spearman’s rank correlation. The ‘corrplot’ package in R was used to construct the correlograms shown.

Linear mixed models were used to assess the impact of ART on pTFH.

Principal Component Analysis (PCA) was used to perform dimension reduction of the original datasets, at the time-points baseline and at one-year. Variables in the original dataset with more than ten per cent missing data were excluded from the PCA. For the remaining variables, missing data were imputed using the “missMDA” package in R; this method uses a regularised iterative PCA function for imputation. The `prcomp` function in R was used to perform PCA. The contribution of each principal component to the overall variance dataset is presented in the scree plots shown. Only principal components (PC) with eigenvalues of greater than one was included in subsequent correlation and regression analyses.

The PCA plots and variable correlation plots generated may be interpreted as follows; the correlation plots summarise the relationship between variables and their contribution to
the first two principal components. The angle between the variable is an approximation of the correlation between the variables; a small angle indicates that variables are positively correlated, vectors at right angles have no correlation, while vectors in opposite quadrants (i.e. approximately 180°) are negatively correlated. The PCA Bi-plot illustrates the contribution variables and individuals to the principal components. The labelled coloured arrows in the bi-plot represent the contribution of each variable to PC1 and PC2 while the points/circles describe the contribution of each individual to PC1 and PC2. Individuals who are on the same side of a given variable have a high value for this variable while individuals who are on the opposite side of a given variable have a low value for this variable.

Analyses were performed using GraphPad Prism version 6.0f (GraphPad Software, La Jolla, California, USA), IBM SPSS Statistics for MAC, version 24 (IBM Corp., Armonk, N.Y., USA) or R version 3.2.2.

**Absolute TFH and CXCR5⁺ CD4 T cell counts**

The absolute number of TFH and CXCR5⁺ CD4 T cells at baseline and one year was estimated using the absolute total CD4 T cell count, measured using as part of routine clinical care and concurrent flow cytometer data (e.g. absolute CXCR5⁺ CD4 T cell frequency = Total CD4 T cells count multiplies by percentage CXCR5 expression on CD4 T cells).
5.3. Results – Part 1 (PBMC)

This results section has been divided into two parts, the first characterises and examined the impact of ART started in PHI on peripheral T follicular helper (pTFH) cells while the second section focuses on germinal centre TFH cells in Gut-associated Lymphoid Tissue (GALT).

5.3.1. The phenotype of peripheral circulating TFH cells in primary HIV infection

Firstly, to demonstrate the phenotypic differences between pTFH and GC TFH, I show a flow cytometry plot representative of GC TFH in figure 5.3.1 (a). This shows CXCR5 and PD-1 expression on CD4 T cells from HIV+ tonsil; a distinctive TFH population is identified with high expression of both CXCR5 and PD-1. This phenotype holds true for GC TFH cells in GALT, but peripheral-circulating TFH cells have a less clearly defined double positive population due to lower levels of CXCR5 and PD-1; figure 5.3.1 (b) shows the CXCR5 and PD-1 expression from a representative PBMC HIV+ sample; a considerable proportion of CD4+ T cells express only CXCR5 with lower PD-1 expression and lack of a clear TFH population. B-cell lymphoma 6 (Bcl-6) is the transcription factor which drives TFH cell differentiation and function and phenotypically identifies TFH. Figures 5.3.1 (b & d) illustrate the expression of Bcl-6 across the CD4 T cells according to CXCR5 and PD-1 expression in tonsil and PBMC CD4 T cells on a representative sample from the HEATHER cohort. The Bcl-6 expression is highest in the “bona fide” TFH population in tonsil tissue. Accordingly, I will examine the pTFH and germinal centre TFH separately in this results chapter. The results section will focus initially on peripheral-circulating TFH only, while the second section will focus on germinal centre TFH from GALT samples from individuals in the HEATHER study, who also had PBMC samples available for comparisons with germinal centre TFH.
Figure 5.3.1 Flow plots of T follicular helper cells in tonsil tissue and PBMC

The expression of CXCR5 & PD-1 (gated on CD4^+ cells) identifies TFH, Pre-TFH and Non-TFH cells in (a) tonsil and (c) PBMCs. Bcl-6 expression, according to CXCR5 and PD-1 expression, in tonsil and PBMC (b) and (d), respectively is shown.

5.3.2. Peripheral Circulating T Follicular Helper cell (pTFH) clinical cohort

A total of 56 individuals from the SPARTAC randomised control trial (n=37) and the HEATHER study (n=19) contributed data for this analysis; these are referred to as the pTFH cohort. The pTFH cohort was subdivided into ART-treated (n=31) and ART-naïve individuals (n=25). This nomenclature was based on whether or not they had received ART during the first 48 weeks of PHI. Table 5.3.1 summarises the clinical characteristics of the pTFH clinical cohort at baseline (i.e. both ART-treated and ART-naïve groups) and the ART-treated and ART-naïve groups, separately. In the ART-treated group, the median (IQR) time to ART initiation from HIV diagnosis was 34 (13-53) days. At baseline, there were no significant differences between ART-treated and ART naïve individuals with respect to baseline CD4 (p=0.3) CD4/CD8 ratio (p=0.1), HIV viral load (p=0.7) or age
A larger proportion of individuals in the ART-treated group were diagnosed with PHI by a p24 antigen positive / HIV antibody negative compared with the ART-naïve group (23% and 8%, respectively). Conversely, a larger proportion of the ART-naïve group, compared to the ART-treated group were diagnosed with PHI by western blot (20% and 6%, respectively). PHI diagnosis using either of these methods, p24+/HIV antibody negative or evolving western blot indicates a stage II to IV using Fiebig classification and correspond to very early infection\textsuperscript{38}. All other methods of PHI classification were similar across groups.

Table 5.3-1 Clinical Characteristics of the pTFH Cohort

<table>
<thead>
<tr>
<th>Study enrolled in:</th>
<th>Baseline(\dagger) (n=56)</th>
<th>ART-Treated (n=31)</th>
<th>ART-Naive (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPARTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEATHER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at EDI</td>
<td>33 (27-41)</td>
<td>34 (27-42)</td>
<td>31 (28-40)</td>
</tr>
<tr>
<td>Median baseline (IQR):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 T cell count (cell/mm(^3))</td>
<td>573 (485-722)</td>
<td>608 (520-739)</td>
<td>560 (383-693)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.58 (0.33-0.78)</td>
<td>0.63 (0.37-0.90)</td>
<td>0.42 (0.32-0.69)</td>
</tr>
<tr>
<td>HIV Viral Load (log(_{10}) CPM)</td>
<td>4.9 (4.3-5.7)</td>
<td>5.0 (4.3-5.8)</td>
<td>4.8 (4.3-5.7)</td>
</tr>
<tr>
<td>Method of PHI diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV Ab negative/HIV PCR(^*) or p24(^+)</td>
<td>9 (16%)</td>
<td>7 (23%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Negative to Positive Test</td>
<td>27 (48%)</td>
<td>15 (48%)</td>
<td>12 (48%)</td>
</tr>
<tr>
<td>Evolving Western blot</td>
<td>7 (13%)</td>
<td>2 (6%)</td>
<td>5 (20%)</td>
</tr>
<tr>
<td>RITA</td>
<td>13 (23%)</td>
<td>7 (23%)</td>
<td>6 (24%)</td>
</tr>
<tr>
<td>Time from EDI to ART initiation(^*) (days)</td>
<td>NA</td>
<td>72 (24-118)</td>
<td>NA</td>
</tr>
<tr>
<td>Days from HIV diagnosis to ART initiation(^*)</td>
<td>NA</td>
<td>34 (13-53)</td>
<td>NA</td>
</tr>
<tr>
<td>Initial ART regimen(^*) contains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease Inhibitor</td>
<td>12 (39%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NNRTI</td>
<td>NA</td>
<td>2 (7%)</td>
<td>NA</td>
</tr>
<tr>
<td>Integrase Inhibitor</td>
<td>3 (10%)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Missing Data</td>
<td>14 (42%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values given represent n (%) for categorical variables and median (interquartile range) for continuous variables. \(\dagger\) Includes both ART-treated and ART-naïve individuals at baseline. *Data are shown for ART-treated individuals only. Abbreviations: EDI, estimated date of infection; IQR, interquartile range; CPM, copies per million.
5.3.3. Peripheral circulating T Follicular Helper cells in PHI

Using samples from the SPARTAC and HEATHER cohorts, I examined the effect of ART started in PHI on the frequency, phenotype and function of peripheral TFH (pTFH) at baseline pre-ART initiation and week 48 after EDI for both ART-treated and ART-naive groups.

TFH-associated maker expression at seroconversion

In this analysis, high or low expression of the cell surface markers CXCR5 and PD-1, the transcription factor Bcl-6 and the cytokine IL-21 were used to identify pTFH. pTFH were defined as CD4 T cells which co-express CXCR5 and PD-1. I began by assessing the expression of Bcl-6 and IL-21 across four circulating peripheral CD4 T cell populations defined according to their expression of CXCR5 and PD-1, these are illustrated in figure 5.3.2 (a). Q1, TFH, Q3 and Q4 refer to CXCR5LowPD1High, CXCR5HighPD1High, CXCR5HighPD1Low, and the CXCR5LowPD1Low expression on CD4 T cells, respectively. This characterisation was performed on resting CD4+ T cells in all individuals (n=56) at baseline (i.e. closest sample to seroconversion and pre-ART).

pTFH accounted for a median (IQR) of 2.5% (1.8-3.6%) of all circulating CD4 T cells at baseline. Higher Bcl-6 and IL-21 expression were observed in the TFH population (CXCR5HighPD1High) and Q2 (CXCR5HighPD1Low) compared to all other groups [Q1 (CXCR5LowPD1High) & Q4 CXCR5LowPD1Low]). However, no difference was seen between the CXCR5 expressing groups, i.e. TFH and Q3 CXCR5HighPD1Low groups. CXCR5+ CD4 T cells (TFH plus Q3) accounted for a median (IQR) of 9.0% (6.2-12.6%) of all circulating CD4 T cells. Given the similar expression of Bcl-6 and IL-21 on all CXCR5+ (TFH plus Q3) and the low frequency of TFH in peripheral circulating CD4 T cells, all further analysis was carried out on this group in addition to TFH.
Figure 5.3.2 Baseline expression of follicular markers, ICR and HLA-DR according to CXCR5 and PD1 expression

The flow plot in figure (a) illustrates CXCR5 and PD-1 expression on CD4 T cells in PBMCs. The colour coded quadrants highlight the populations referred to in the subsequent jitter plots. Quadrant 1 (Q1), in red, includes CXCR5\(^{+}\)PD1\(^{+}\) CD4 cells. Quadrant 2 (Q2) in yellow includes TFH cells, i.e. CXCR5\(^{+}\)PD1\(^{+}\) CD4 cells. Quadrant 3 (Q3) in green includes CXCR5\(^{+}\)PD1\(^{-}\) CD4 cells and quadrant 4 (Q4) in blue includes the double negative, CXCR5\(^{-}\)PD1\(^{-}\) population. Figure (b-f) shows Bcl-6, IL-21, TIGIT, Tim-3 and HLA-DR expression across the four CD4 T cell populations respectively. The symbols ****, ***, and ** represent the P value of <0.0001, <0.001 and <0.01 respectively. Differences between groups were calculated using a Friedman test and corrected for multiple comparisons using Dunn’s test.

(a) Quadrants

(b) Bcl-6

(c) IL-21

(d) TIGIT

(e) Tim-3

(f) HLA-DR

Interestingly, expression of TIGIT expression appeared to be discriminatory at identifying the TFH population, with a significantly higher TIGIT expression on TFH cells compared to all other groups (all p<0.0001), figure 5.3.2d. Greater T cell activation, as measured by HLA-DR expression was seen in both PD1\(^{\text{High}}\) quadrants with significantly higher HLA-DR expression in Q1 and TFH compared to all other quadrants, see figure 5.3.2f.
5.3.4. Impact of ART in PHI on the frequency of pTFH

To address my first hypothesis, I examined the frequency of TFH and CXCR5 expression on CD4 T cells, at baseline and after one year, in ART-treated and ART-naïve individuals, see figures 5.3.3 and figure 5.3.4. At year one, the median CXCR5 expression on CD4 T cells was significantly lower (p=0.04) in the ART-naïve individuals (8.4%) compared to the ART-treated individuals (10.5%). However, no there was no significant difference in the median co-expression of CXCR5 and PD-1 (TFH) between groups at one year (ART-naïve 2.3%; ART-treated 2.9%)

In addition, I measured the absolute number of TFH and CXCR5+ CD4 T cells at baseline and one year in the ART-treated and ART-naïve groups. There were significantly higher absolute numbers of both TFH and CXCR5+ CD4 T cells in the ART-treated group compared to the ART-naïve group at 1 year (TFH, 79 cells/mm³ versus 32 cells/mm³; CXCR5+CD4 T cells, 18.2 cells/mm³ versus 8.4 cells/mm³), these findings are illustrated in figure 5.3.4.

Figure 5.3.3 Impact of ART on the expression of CXCR5 and PD1 on CD4 T cells

These jitter plots illustrate the percentage expression of (a) CXCR5 and (b) co-expression of CXCR5 & PD1 on CD4 T cells at the time of seroconversion (baseline) and after one year in ART-treated (ART 1-year, in blue) and ART-naïve (Naïve 1-year, in green) individuals. P value was calculated using a Kruskal Wallis test, corrected for multiple comparisons.

(a) CXCR5 expression

(b) CXCR5 & PD-1 co-expression
I next used a linear mixed effects model to test the impact of ART on the frequency of TFH and CXCR5+ CD4 T cells over time; a significant interaction for time and absolute cell numbers between treatment groups (i.e. ART-naïve vs ART-treated) was noted for both TFH, $F(1,41) = 6.159, p=0.017$, and CXCR5+ CD4 T cells, $F(1,41) = 8.63, p=0.005$, see figure 5.3.5. Of note, the number of CXCR5+ CD4 and TFH cells was lower in the ART-naïve group at baseline. However, the interaction of ART remained significant. No such significant interaction was seen when a linear model was applied to the relative expression CXCR5 or co-expression of CXCR5 and PD-1 on CD4 T cells. However, an effect was seen when tested using group comparisons with significantly higher CXCR5 expression in the ART-treated group compared to the naïve group at year one ($p=0.04$), but not in CXCR5 and PD-1 co-expression.

These results indicate that ART preserves both absolute numbers of pTFH and CXCR5+ CD4 T cells when commenced in PHI, with some evidence to suggest that CXCR5 expression may also increase in ART-treated individuals.
Figure 5.3.5 Linear Mixed effect models of the impact of ART on CXCR5+ CD4 T cells and TFH cells

(a) A significant interaction between time point and absolute CXCR5+ CD4 T cell number is observed across treatment groups [ART-treated (blue line) and ART-naïve (green line). Error Bars represent the 95% confidence interval (CI). $F (1,41) = 8.63$, $p=0.005$.

(b) A significant interaction between time point and absolute TFH number is observed across treatment groups [ART-treated (blue line) and ART-naïve (green line). Error Bars represent the 95% confidence interval (CI). $F (1,41) = 6.159$, $p=0.017$. 
5.3.5. Impact of ART on Immune checkpoint and activation marker expression on pTFH in PHI

Expression of ICR has been associated with T cell exhaustion and dysfunction, while T cell activation has been associated with HIV disease progression. Therefore, I measured the expression of these markers, firstly on bulk CD4 and CD8 T cells and then CXCR5+ compared to CXCR5- CD4 T cells. Figure 5.3.6 outlines the relative expression of HLA-DR, PD-1, TIGIT and Tim-3 on CD4 T cells at baseline and after one year in ART-treated and ART-naïve individuals. CD4 T cell activation, as measured by HLA-DR, was significantly lower in ART-treated individuals at one year, when compared to baseline HLA-DR expression (p=0.0003) and HLA-DR expression in ART-naïve individuals at one year (p=0.0004). A trend towards lower PD-1 expression on CD4 T cells was also observed in the ART-treated group compared to baseline (p=0.056) but was not when compared to ART-naïve at one year (p=0.2)

Figure 5.3.6 ICR and Activation marker expression on CD4 T cells in PHI

The jitter plots in (a-d) illustrate relative HLA-DR, PD-1, TIGIT and Tim-3 expression on CD4 T cells, respectively. Grey circles represent Baseline measurements; Blue circles are measurements from ART-treated individuals after one year; Green circles are measurements from ART-naïve individuals at 1-year P values were calculated using Kruskal-Wallis test, corrected for multiple comparisons.
No significant effect of ART was observed in TIGIT or Tim-3 expression on CD4 T cells, in fact, Tim-3 expression was lower in the ART-naïve group when compared to ART-treated individuals at one year (P=0.01).

The jitter plots in Figure 5.3.7 illustrate the expression of HLA-DR, PD-1, TIGIT on Tim-3 on CD8 T cells at baseline and 1-year in ART-treated and ART-naïve groups. Expression of all markers, except Tim-3, were lower on CD8 T cells at one year in the ART-treated group when compared to baseline (HLA-DR P=0.0001; PD-1 P<0.0001; TIGIT P=0.0009; Tim-3 P=0.62) and when compared to ART-naïve individuals at 1-year (HLA-DR P=0.0002; PD=1 P<0.0001; TIGIT P<0.0001, Tim-3 P=0.076). Taken together these data suggest that ART commenced in PHI significantly reduces immune activation in the CD4 and CD8 compartments, compared to baseline and compared with individuals randomised to no immediate ART at one year.

**Figure 5.3.7 ICR and Activation marker expression on CD8 T cells in PHI**

The jitter plots in (a-d) illustrate relative HLA-DR, PD-1, TIGIT and Tim-3 expression on CD8 T cells, respectively. Grey circles represent Baseline measurements; Blue circles are measurements from ART-treated individuals after one year; Green circles are measurements from ART-naïve individuals at one-year P values were calculated using Kruskal-Wallis test, corrected for multiple comparisons.
ART also significantly reduced expression of the ICRs - PD-1 and TIGIT in the CD8 compartment, while PD-1 expression on CD4 T cells in ART-treated individuals was lower than untreated (although this was not statistically significant). Tim-3 expression in both CD4 and CD8 T cell compartments was similar between groups at year-1. In fact, Tim-3 expression was higher (but not significantly so) in ART-treated individuals at year-1.

Given the potential functional role of ICR expression on TFH cells, specifically PD-1 and TIGIT on TFH cells I next measured the expression of ICR and activation markers on CXCR5⁺ (follicular) and CXCR5⁻ (non-follicular) CD4 T cells. The expression of HLA-DR, PD-1, TIGIT and Tim-3 measured at baseline, and after one year, in ART-treated and ART naïve groups is illustrated in Figure 5.3.8. No differences were noted in the expression of any of the immune checkpoint receptors (PD-1, TIGIT or Tim-3) on follicular CD4 T cells between groups or at either time point; this suggests that commencement of ART does not affect the expression of these markers on follicular CD4 T cells. However, significantly lower PD-1 (P=0.03) and Tim-3 (P=0.01) expression were observed on non-follicular CD4 T cells in the ART-treated group at year-1 compared to baseline and lower TIGIT (P=0.04) expression in the ART-treated group compared to the ART-naïve group at year-1.

The expression of HLA-DR on CXCR5⁺ CD4 cells was lower in ART-treated individuals when compared to either baseline (P<0.0001) or ART-naïve individuals at year-1 (P=0.02). The same pattern was seen for CXCR5⁻ CD4 T cells with lower HLA-DR expression in ART-treated individuals when compared to baseline (P=0.0007) and ART-Naïve individuals at year-1 (P=0.0007). Taken together, these results show a decrease in immune activation with ART in PHI on both CXCR5⁺ and CXCR5⁻ CD4 T-cells, and while ART confers lower ICR expression on CXCR5⁻ CD4 T cells, no such effect is seen on CXCR5⁺ CD4 T cells.
Figure 5.3.8 Expression of (a) HLA-DR, (b) PD-1, (c) TIGIT and (d) Tim-3 on CXCR5+ (left column) and CXCR5- (right column) CD4 T cells.

These jitter plots illustrate relative HLA-DR, PD-1, TIGIT and Tim-3 expression on CXCR5+ and CXCR5- CD4 T cells populations around the time of seroconversion (grey circles) and after one year, in the ART-treated group (blue circles) and ART-naive group (green circles). CXCR5+ and CXCR5- CD4 T cells are shown on the left and right of the dashed line respectively. P values were calculated using Kruskal-Wallis test, corrected for multiple comparisons. Only significant P values are shown. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05
5.3.6. The Impact of ART initiated in PHI on measures of TFH function

IL-21, CXCL13 & Bcl-6 production

CXCL13 through its interactions with CXCR5 plays an important role in promoting T cell and B cell migration into B cell follicles, while IL-21 acts as an autocrine cytokine to promote pre-TFH cell differentiation and homeostatic maintenance of TFH cells\textsuperscript{487, 502}. Therefore, I measured IL-21 production on unstimulated CD4 T cells and CXCL13 from plasma at baseline and year one in both treatment groups to assess the impact of ART in these markers of pTFH function.

No significant differences in IL-21 production were observed on bulk CD4 T cells. However, a trend towards higher IL-21 was observed at baseline and in the ART-naïve group at year one compared to the ART-treated group at year one (P=0.09 and P=0.15, respectively). Significantly higher IL-21 was measured on follicular CD4 T cells at baseline and in the ART naïve group at year one compared to the ART-treated group at one year (P=0.01 and P=0.02, respectively). These data suggest that higher IL-21 expression is observed in the groups with persistent exposure to HIV antigen. These data are summarised in figure 5.3.9.

CXCL13 measured in plasma has been shown to correlate with germinal centre TFH function and associated with the production of broadly neutralising antibodies in untreated HIV infection\textsuperscript{536}. I examined the impact of early treatment on CXCL13 levels in a subset of individuals with available plasma (n=26) across our treatment groups and found a trend towards lower levels in the ART-treated individuals compared to baseline (P=0.06) and ART-naïve individuals at 1-year (P=0.15). However, no difference was observed in the frequency of B cells (CD19+ lymphocytes) between groups. These data are summarised in figure 5.3.10.
Higher Bcl-6 expression was seen in the ART-treated group at one year when compared to the ART-naïve group, but this was not statistically significant when corrected for multiple comparisons.

**Figure 5.3.9 IL-21 expression at baseline and one year in ART-treated and ART-naïve individuals on bulk CD4 T cells and CXCR5+ CD4 T cells.**

The jitter plots below illustrate IL-21 expression on (a) bulk CD4 and (b) CXCR5+ CD4 T cells at baseline (grey symbols) and 1-year in ART-treated (blue symbols) and ART-naïve individuals (green symbols). Overall P values were calculated using a Kruskal-Wallis test and corrected for multiple comparisons using Dunn’s test. Corrected P values are shown for between-group comparisons.

(a) IL-21 on CD4 T cells

(b) IL-21 on CXCR5+ CD4 T cells

**Figure 5.3.10 Plasma CXCL13 levels and B-cell frequency at baseline and one year in ART-treated and ART Naïve individuals**

These jitter plots show (a) CXCL13 levels in plasma and (b) CD19 expression on lymphocytes across study groups. Overall P values are calculated using a Kruskal-Wallis test; P-values corrected for multiple comparisons are shown above the groups being compared.

(a) Plasma CXCL13

(b) B cells / CD19 expression
**Immunoglobulin levels in primary HIV infection**

The interaction between TFH and B cells determines the magnitude of the germinal centre reaction leading to the affinity maturation of B cells and subsequently the breadth of antibody response. Therefore, to assess the potential impact of ART in PHI on antibody production I next looked at total and IgG subtype levels in plasma samples across the study groups. Limited plasma samples were available for this analysis (baseline; n=22, ART-treated 1 year; n=8, ART-naïve 1 year; n=12). Perhaps not unsurprisingly given the small sample size I did not observe any statistically significant difference in the frequency of total IgA, IgM, IgG1, IgG2, IgG3 or IgG4 across study groups. These data are summarised in figure 5.3.11. A trend towards lower median IgG3 (overall P=0.07) was seen in both the ART-naïve (78.9ng/ml) and ART-treated (92.5 ng/ml) groups at year-1 compared to baseline (157.6ng/ml), with the lowest levels observed in the ART-naïve group. IgG3 has previously been shown to be more effective at neutralising HIV-1 than IgG1 and is important in antibody-dependent cell cytotoxicity against HIV-1[^537]. Interestingly the lowest HIV-1 DNA level at baseline was observed in the individual (an outlier) with the highest measured IgG3 response.

**HIV specific antibody responses**

I next examined the frequency of IgG and IgA HIV-specific antibodies targeting the HIV gp120 envelope and HIV p24 gag proteins. In addition, I calculated the specific activity of these antibodies, which is the specific antibody titre as a proportion of the total antibody titre [e.g. Specific IgG gp120 activity = specific IgG gp120 antibodies (ug/ml) / Total IgG antibodies (ug/ml)]. Plasma samples were available for HIV specific antibody measurements for n=22 individuals at baseline, n=7 individuals in the ART-treated group and n=15 in the ART naïve group, at one year. Higher anti-gp120 IgG (P=0.02) and anti-gp120 IgG specific activity (P=0.02) was measured in the ART-naïve group at one year.
when compared to baseline, with a trend towards higher anti-gp120 IgG specific activity in the ART-naïve group compared to the ART-treated group (P=0.07) at one-year. No differences in anti-p24 IgG levels or specific activity was noted between groups. These data are summarised in figure 5.3.12. Similarly, the HIV specific IgA responses and activity to gp120 and p24 were not different between groups and these are shown in figure 5.3.13.

I next examined the IgG/IgA ratios across study groups. No difference in total IgG/IgA ratio was noted across study groups (Overall P=0.3, by Kruskal-Wallis, corrected for multiple comparisons by Dunn’s test). However, higher anti-gp120 HIV specific IgG/IgA ratio was observed in the ART-naive group (P=0.02) at one year but not in the ART-treated group (P=0.22) compared to baseline. The opposite trend (overall P=0.1, by Kruskal-Wallis, corrected for multiple comparisons by Dunn’s test) was seen for anti-p24 HIV specific IgG/IgA ratio with the highest ratio in the ART-treated group (P=0.09) compared to baseline. These data are summarised in figure 5.3.14. Finally, no correlation was seen between TFH and the development of HIV specific antibody responses.

**Broadly neutralising antibody responses (BNAbs)**

The breadth of neutralisation of HIV antibodies was measured by a collaborator for eight individuals included in this study. All of these individuals were ART-naïve as early ART would likely preclude the development of BNAbs, five had low breadth of neutralisation scores (range; 0-2). The other three individuals had medium to high neutralisation scores (range; 4-7). The median (IQR) time since PHI at time of measurement was 163 (113-220) weeks. The breadth of neutralising demonstrated a positive association with Bcl-6 MFI measured at baseline (r=0.76 p=0.03), and IL21 MFI at one year (r=0.90 p=0.2). No other significant associations were observed; in particular, the breadth of neutralisation did not correlate with TFH measures or HIV specific antibody responses presented previously.
Figure 5.3.11 Total (a) IgA, (b) IgM, (c) IgG1, (d) IgG2, (e) IgG3 and (f) IgG4 antibody levels across study groups

These jitter plots (a-f) illustrate total IgA, IgM, IgG1, IgG2, IgG3 and IgG4 levels at baseline (grey symbols) and year-1 in the ART-treated (blue symbols) and ART-naive individuals (green symbols). Overall P values were calculated using a Kruskal-Wallis test and corrected for multiple comparisons.

(a) IgA

Overall P=0.6

(b) IgM

Overall P=0.5

(c) IgG1

Overall P=0.7

(d) IgG2

Overall P=0.4

(e) IgG3

Overall P=0.07

(f) IgG4

Overall P=0.8
Figure 5.3.12 HIV specific IgG antibody responses

The Box plots below show the (a) Anti-gp120 specific IgG response, (b) the Anti-gp120 IgG specific activity, (c) Anti-p24 specific IgG response and (d) the Anti-p24 IgG specific activity. The grey, blue and green boxplots represent baseline measurements (n=22), ART-treated individuals (n=7) and ART naïve individuals (n=15) at 1-year, respectively. P-values were calculated using a Kruskal-Wallis test and corrected for multiple comparisons using Dunn’s test. Selected P values are shown.

(a) Anti-gp120 specific IgG

(b) Specific Activity anti-gp120 IgG

(c) Anti-p24 specific IgG

(d) Specific Activity anti-p24 IgG
Figure 5.3.13 HIV-specific IgA antibody responses

The Box plots below show the (a) Anti-gp120 specific IgA response, (b) the Anti-gp120 IgA specific activity, (c) Anti-p24 specific IgA response and (d) the Anti-p24 IgA specific activity. The grey, blue and green boxplots represent baseline measurements (n=22), ART-treated individuals (n=7) and ART naïve individuals (n=15) at 1-year, respectively. P-values were calculated using a Kruskal-Wallis test and corrected for multiple comparisons using Dunn’s test. Selected P values are shown.

Figure 5.3.14 Anti-gp120 and Anti-p24 IgG/IgA ratios

The box plots illustrate (a) anti-gp120 IgG/IgA ratio and (b) anti-p24 IgG/IgA ratio at baseline (grey), ART-treated individuals (blue) and ART-naïve individuals (green) at 1-year. Data are shown in Tukey Boxplot format, i.e. median, IQR and outliers. Group comparisons are made using a Kruskal-Wallis test and corrected for multiple comparisons. Selected P values are shown.
5.3.7. The relationship between the HIV reservoir & markers of immune function around the time of seroconversion

Immunological biomarkers measured pre-ART have been shown to associate with HIV reservoir and to predict time to viral load rebound on subsequent treatment cessation\textsuperscript{277}. Therefore, I was interested in exploring the relationship between T follicular markers and other markers of immune function, namely ICR and activation markers, around the time of seroconversion (baseline). I also tested for associations between these baseline variables and measures of HIV reservoir. Furthermore, I then used PCA to examine which groups of variables most closely associated with total HIV-DNA.

Correlates of T Follicular markers at seroconversion

Many surface markers have been used to define the phenotype of pTFH across different studies\textsuperscript{503, 507, 517, 518, 538, 539}. I defined pTFH as CD4 T cells which co-express the surface molecules CXCR5 and PD-1. In the first instance, I explored the expression of TFH-associated molecules on CD4 cells, such as CXCR5 and TIGIT, with absolute frequency of TFH cells. The absolute number of TFH cells at seroconversion was strongly associated with the percentage expression of CXCR5+ (r=0.68 p<0.0001) and CXCR5+PD1+(r=0.75 p<0.0001) on CD4 T cells, and also with the absolute number of CXCR5+ CD4 T cells (r=0.83 p<0.0001); perhaps not unsurprisingly, these markers were also highly correlated with each other. The transcription factor Bcl-6 was associated with CXCR5 expression on CD4 cells (r=0.35 p=0.008) and IL21 MFI on CD4 cells (r=0.64 P<0.001). Of note, CD4 Bcl-6 expression did not correlate with other pTFH markers; however, it was associated with higher CXCR5 expression on CD8 T cells (r=0.28 p=0.04), as was CD4 IL21 (r=0.42 p=0.01), CD19 (r=0.32 p=0.02) and co-expression of the TFH markers CXCR5 and PD-1 (r=0.32 p=0.02). Also, greater levels of CD8 T cell activation, measured by HLA-DR were associated with lower CXCR5 expressing CD8 cells (r=-0.27
These data suggest that pTFH have a role in supporting the expression of CXCR5 on CD8 T cells during seroconversion.

Interestingly, TIGIT expression on CD4 T cells was also highly correlated with the TFH phenotype (CXCR5 and PD-1 co-expression) \( (r=0.72 \, P<0.0001) \), as well as the majority of other T follicular markers. Therefore, I investigated if TIGIT expression on CD4+ and CXCR5+CD4+ T cells may be more discriminatory at identifying pTFH. I measured Bcl-6 expression across three CD4 T cell populations; CXCR5+PD1+, CXCR5+TIGIT+ and TIGIT+, see figure 5.3.15. However, Bcl-6 expression was significantly higher on CXCR5+PD1+ compared to CXCR5+TIGIT+ T cells \( (P=0.0002) \) suggesting that the former surface markers are the optimal ones to identify pTFH rather than TIGIT expression.

Higher levels of CD4 T cell activation, as measured by HLA-DR expression, was associated with lower expression of CXCR5 on CD4 T cells \( (r=-0.27 \, p=0.05) \) and lower absolute number of CXCR5+ CD4 T cells \( (r= -0.46, \, P=0.0005) \). In general, higher CD4/CD8 ratio was indicative of more favourable immune parameters; higher ratio was predictive of higher absolute pTFH \( (r=0.3 \, p=0.03) \), higher CD8 CXCR5 expression \( (r=0.42 \)
p=0.002), higher CD19 expression (r=0.53 p<0.0001), lower T cell activation (HLA-DR) on CD4 (r=-0.63 p<0.0001) and CD8 (r=-0.36 p=0.0003) T cells, lower CD4 PD-1 (r=-0.44 p=0.0008) & CD8 PD-1 (r=-0.47 p=0.0003) expression and lower TIGIT expression CD8 T cells (r=-0.32 p=0.02).

**Correlates of HIV reservoir at seroconversion**

Baseline HIV RNA (r=0.77, P<0.0001) and the CD4/CD8 ratio at seroconversion (r=-0.52 p=0.0002), as has been shown in earlier chapters, were again the parameter measured that most strongly associated with total HIV DNA. A similar pattern was seen for integrated HIV DNA; these associations are illustrated in figure 5.3.16.

Higher expression of CXCR5 on CD8 T cells (r=-0.45 p=0.002) and CD19 expression (r=-0.53 p=0.001) was associated with lower total HIV DNA, while increased frequency of absolute number of TFH (r=-0.47 p=0.03) and CXCR5+ CD4 T cells (r=-0.5 p=0.02) at seroconversion was associated with lower measures of integrated HIV DNA but not total HIV DNA, this relationship is further illustrated in the scatterplots in figure 5.3.17.

ICR and activation marker expression on both CD4 and CD8 T cells were significantly positively correlated with markers of HIV reservoir; total HIV DNA was significantly associated with PD-1 (r=0.29, P=0.052) and Tim-3 (r=0.47, P=0.001) expression on CD4 T cells and PD-1 (r=0.51, p=0.0003), Tim-3 (r=0.46, P=0.001) and TIGIT (r=0.38, p=0.091) expression on CD8 T cells. Integrated HIV-1 DNA was also correlated with ICR expression around the time of seroconversion; PD-1 (r=0.50 p=0.02) and Tim-3 (r=0.52 p=0.01) on CD8 T cells.

Also, higher levels of CD4 (r=0.34, P=0.021) and CD8 (r=0.67, p<0.001) immune activation, as measured by HLA-DR expression predicted higher baseline total HIV DNA activation marker expression, similar associations with T cell activation were observed for integrated HIV-1 DNA.
Figure 5.3.16 Scatter Plots of measures of HIV reservoir versus CD4/CD8 ratio and B-cells at seroconversion

The first column of scatter plots shows the relationship between Total HIV DNA and (a) absolute CD4/CD8 ratio, (c) CD4/CD8 measured by flow cytometry and (e) CD19 expression. The second column shows the relationship between integrated HIV DNA and (b) absolute CD4/CD8 ratio, (d) CD4/CD8 measured by flow cytometry and (f) CD19 expression. R= Spearman’s correlation
**Figure 5.3.17 Associations of Follicular T cells and measures of HIV reservoir at seroconversion**

The first column of scatter plots shows the relationship between Total HIV DNA and (a) absolute numbers of TFH, (c) absolute CXCR5+ CD4 T cells and (e) CXCR5 expression on CD8 T cells. The second column shows the relationship between integrated HIV DNA and (b) absolute numbers of TFH, (d) absolute CXCR5+ CD4 T cells and (f) CXCR5 expression on CD T cells. R= Spearman’s correlation.
Principal component analysis of seroconversion immune markers and their association with HIV DNA

Given the collinearity between baseline measurements and to further understand the relationship between baseline variables, I performed a principal component analysis (PCA). This PCA included all variables except integrated and total HIV-1 DNA. I excluded variables with more than 10% missing data from this analysis (i.e. CXCL13, Immunoglobulins, D-Dimer & IL-6). The scree plot in figure 5.3.18 (a) illustrates the contribution of each principal component (PC) to the total variance in the dataset, PC1, PC2, PC3 & PC4 account for approximately 24%, 18%, 9% and 9% of the variance in the dataset respectively. The PCA correlation plot in figure 5.3.19 summarises the relationship between variables and their contribution to the first two PCs.

Figure 5.3.18 Scree Plot illustrating contribution of each principal component to the overall variance at seroconversion.
The angle between the variable is an approximation of the correlation between the variables; a small angle indicates that variables are positively correlated, vectors at right angles have no correlation, while vectors in opposite quadrants (i.e. approximately 180°) are negatively correlated. Again, all CD4 T Follicular markers are positively correlated with each other and also with the expression of CXCR5 on CD8 cells. Expression of activation and immune check-point receptors on both CD4 and CD8 T cells are positively correlated with each other.

Figure 5.3.19 Principal Component Analysis (PCA) variable correlation plot at seroconversion

The plot below is a variable correlation plot. It shows the relationships between all variables. Variables that are positively correlated are grouped together. Negatively correlated variables are on opposite sides of the plot origin (opposed quadrants). The distance between variables and the origin measures the quality of the variables on the factor map. A high “contrib” indicates a good representation of the variable on the principal component. PC1 = Dim1, PC2 = Dim2
A higher CD4/CD8 ratio was associated with higher frequency of TFH cells and higher CXCR5+ CD8 cells, while a lower ratio was associated with higher expression of ICR and activation markers. Table 5.3.2 shows the variables which contribute to the first two principal components. The first principal component contributes 24% of the variance to the dataset and is composed (in order of contribution) of CD4/CD8 ratio, HIV viral load, CD4 HLA-DR expression, baseline CD4 count, PD-1 expression on CD4 and CD8 cells, CD4 HLA-DR, the absolute number of CXCR5+ CD4 T cells and Tim-3 expression on CD4 T cells, see table 5.3.2. The second principal component is composed primarily of follicular markers including CXCR5 and TFH (CXCR5 & PD-1) expression on CD4 T cells, absolute numbers of TFH and CXCR5+ CD4 T cells, and TIGIT expression on CD4 T cells. The main contributor to the 3rd principal component is Tim-3 expression on CD8 cells but also includes follicular markers such as CD4 Bcl-6 MFI, CD4 IL21-MFI and CXCR5 expression on CD8 T cells, while the 4th principal component includes a mix of ICR, activation and TFH marker expression, as well as CD19 and time from estimated date of infection (EDI) to ART start.

I next used the PCs to explore the relationship between baseline measures of immune function and total HIV DNA. PC1 was significantly associated with total baseline HIV DNA, (r=-0.76 p<0.0001) while no association was observed for PC2 (r=-0.13 p=0.38) or any other PC, see figure 5.3.20. These data suggest that the variables contributing to PC1, including concurrent CD4/CD8, baseline HIV RNA, and expression PD-1 and HLA-DR on both CD4 and CD8 T cells of are most predictive of total HIV DNA at seroconversion. pTFH markers were not predictive in this analysis. In summary, these data suggest that HIV reservoir around the time of seroconversion most greatly associated with traditional markers of HIV disease progression such as HIV RNA, CD4 count and CD4/CD8.
Expression of activation and ICR markers on T cells and CXCR5 on CD8 T cells also associated with HIV reservoir.

Table 5.3-2 Variables at seroconversion contributing to Principal Component 1 & 2

<table>
<thead>
<tr>
<th>Order of importance</th>
<th>Principal Component 1</th>
<th>Principal Component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baseline CD4/CD8 ratio (F)</td>
<td>CD4 CXCR5</td>
</tr>
<tr>
<td>2</td>
<td>Baseline HIV RNA</td>
<td>TFH</td>
</tr>
<tr>
<td>3</td>
<td>Baseline CD4/CD8 ratio (A)</td>
<td>Absolute TFH</td>
</tr>
<tr>
<td>4</td>
<td>CD4 HLA-DR</td>
<td>Absolute CXCR5</td>
</tr>
<tr>
<td>5</td>
<td>Baseline CD4 count</td>
<td>CD4 TIGIT</td>
</tr>
<tr>
<td>6</td>
<td>CD4 PD-1</td>
<td>CD8 TIGIT</td>
</tr>
<tr>
<td>7</td>
<td>CD8 PD-1</td>
<td>CD8 Tim-3*</td>
</tr>
<tr>
<td>8</td>
<td>CD8 HLA-DR</td>
<td>CD8 PD-1*</td>
</tr>
<tr>
<td>9</td>
<td>Absolute CXCR5</td>
<td>CD8 CXCR5*</td>
</tr>
<tr>
<td>10</td>
<td>CD4 Tim-3</td>
<td>CD8 HLA-DR*</td>
</tr>
</tbody>
</table>

* contributes less than 7% to PC

Figure 5.3.20 Correlation between HIV DNA and (a) PC1 and (b) PC2 at Seroconversion
5.3.8. T Follicular and ICR expression at 1-year from PHI in ART-treated and ART-naïve individuals

To understand the impact of ART commenced in PHI on the expression of T follicular and ICR marker I analysed the expression of these markers at one year in the individuals enrolled into the SPARTAC study who were randomly allocated with either receive ART (ART-treated) and those who had not (ART-naïve). My first question was to see if the pattern of expression of immune markers could differentiate the ART-treated and ART-naïve groups. Given the large number of variables I again used PCA to allow for dimension reduction. Total HIV DNA was not measured in the ART-naïve group at one year, and this variable was excluded from the PCA. Similarly, to the baseline PCA, measured variables with more than 10% missing values (CXLC13, D-Dimer, IL-6 and immunoglobulins) were excluded. PC1 was predominately made up of variables associated with immune activation and exhaustion, including PD1, TIGIT & HLA-DR expression on CD8 T cells and PD-1 TIGIT and HLA-DR expression on CD4 T cells.

Table 5.3-3 Variables at Year-1 (ART-treated & ART-naïve) contributing to principal component 1 & 2
This table lists the variables contributing to the 1st and 2nd principal components on order of importance. The variables included in this analysis are those measured in the ART-treated and the ART-naïve cohorts at year-1.

<table>
<thead>
<tr>
<th>Order of importance</th>
<th>Principal Component 1</th>
<th>Principal Component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD8 PD-1</td>
<td>CD4 CXCR5</td>
</tr>
<tr>
<td>2</td>
<td>CD4 HLA-DR</td>
<td>TFH</td>
</tr>
<tr>
<td>3</td>
<td>CD8 TIGIT</td>
<td>CD8 CXCR5 (fCD8)</td>
</tr>
<tr>
<td>4</td>
<td>CD8 HLA-DR</td>
<td>CD4 TIGIT</td>
</tr>
<tr>
<td>5</td>
<td>CD4 PD-1</td>
<td>CD8 Tim-3</td>
</tr>
<tr>
<td>6</td>
<td>CD4 count at Year-1</td>
<td>CD4 PD-1</td>
</tr>
<tr>
<td>7</td>
<td>CD4 TIGIT</td>
<td>CD4 Tim-3*</td>
</tr>
<tr>
<td>8</td>
<td>CD4/CD8 (F) at Year-1</td>
<td>CD4/CD8 (F) at Year-1*</td>
</tr>
<tr>
<td>9</td>
<td>CD8 CXCR5*</td>
<td>CD19*</td>
</tr>
<tr>
<td>10</td>
<td>CD4 Tim-3*</td>
<td>CD4 HLA-DR*</td>
</tr>
</tbody>
</table>

* contributes less than 7% to PC
Similar to baseline the PC2 was composed mainly of TFH associated markers, namely CXCR5+CD4, TFH, CXCR5+CD8 and TIGIT+CD4. Table 5.3.3 summarises the variables contributing to PC1 and PC2, respectively.

To explore the contribution of each individual (coloured coded as blue for ART-treated or yellow for ART-naïve) in the study to each variable measured I used a PCA Bi-plot as shown in figure 5.3.21. This Bi-plot illustrates the contribution of each variable to PC1 and PC2 (shown by the labelled coloured arrows). It also shows the contribution of each individual (blue and yellow circles) to PC1 and PC2. Individuals who are on the same side of the plot as a given variable have a high value for this variable while individuals who are on the opposite side of a given variable have a low value for this variable. PC1 and PC2 account for 31.6% and 19.6% of the variability in the data, respectively. Although some overlap exists, ART-treated (blue ellipse) and ART-naïve individuals (yellow ellipse) cluster separately on the biplot. At the year-one time-point, ART-naïve individuals tended to have higher expression of ICRs and activation markers (PC1), while ART-treated individuals have higher expression of follicular markers (PC2) and CD4/CD8 ratio. These data illustrate the role of ART in preserving T follicular markers and reducing immune activation and ICR expression.

**Correlation of Follicular T cell markers at year-1 in ART-treated individuals**

Our lack of understanding as to how the HIV reservoir is maintained on ART is a key barrier to HIV cure. Furthermore, it has been demonstrated that pTFH are a key cell type-enriched for HIV DNA on ART. Therefore, I next examined the associations between TFH markers and HIV DNA in ART-treated individuals at one year. Again, limited complete data was available for the variables IL-6, D-Dimer, CXCL13 and Immunoglobulins and these are excluded from the analysis presented here. In addition, integrated HIV DNA was not measured at one year due to limited sample availability.
Figure 5.3.21 PCA Bi-plot of Activation and ICR measurements from both ART-treated and ART naïve individuals at Year-1

PCA Bi-plot showing the contribution of each individual (plotted blue and yellow points) and each variable (coloured arrows) to the PC1 (Dim1) and PC2 (Dim2) after one year from PHI in ART-treated (blue) and ART-naïve (yellow) individuals.

The correlogram in figure 5.3.22 summarises the relationship between variables in ART-treated individuals after 1-year on ART. Similar to our findings at baseline, TFH measures; absolute frequency of CXCR5 & TFH, percentage expression of CXCR5 & TFH markers (i.e. co-expression of CXCR5 and PD-1) on CD4 T cells are highly correlated with each other. TIGIT expression on CD4 T cells at 1-year is again significantly correlated with absolute TFH and the percentage expression of TFH markers on CD4 T cells (r=0.76 p<0.0001 & r=0.73 p=0.0001, respectively). Bcl-6 expression and IL-21 MFI on CD4 T cells, similar to baseline, were highly correlated with each other (r=0.87 p=0.0005) but were not associated with expression of TFH markers on CD4 cells.

Longer time from HIV diagnosis to starting ART was associated with; a lower number of absolute CXCR5+CD4+ T cells (r=-0.67 p=0.002) and TFH (r=0.51 p=0.03). This
suggests that not only ART but early ART preserves pTFH, most likely by preventing overall CD4 T cells loss, although CD4 count at year-1 was not associated with time from HIV diagnosis to ART start (p=0.4).

Higher expression of CXCR5 (r=0.45 p=0.01) and CXCR5 & PD-1 co-expression (r=0.58 p=0.001) on CD4 T cells, as well as higher CD4/CD8 ratio, were again associated with a higher level of CXCR5 expression on CD8 T cells at year one. CD8 CXCR5 expression remained significantly associated with baseline total HIV DNA (r=-0.47 p=0.02), but the association was not statistically significant with total HIV DNA at one year (r -0.25 p=0.3). The associations with total HIV DNA at year one included; baseline total HIV DNA & HIV viral load (r=0.57 p=0.008 & 0.39 p=0.07, respectively) and baseline CD4/CD8 ratio (r=-0.60 p=0.003). In addition, concurrent expression of TIGIT on CD8 T cells and the absolute number of CXCR5⁺ CD4 T cells both had a positive association with HIV DNA at 1 year (r=0.54 p=0.0.001 and r=0.59 p=0.01, respectively).

Figure 5.3.22 Correlogram of statistically significant (P<0.05) associations between variables in ART-treated individuals at 1-year.
Factor reduction was performed using PCA; Table 5.3.4 summaries the contribution of the variables to PC1 and PC2, which accounted for approximately 20% and 19% of the variance respectively.

PC1 was composed mainly of TFH associated markers (in order of contribution); TFH, CD4 TIGIT, CD4 CXCR5, the absolute frequency of TFH and CXCR5+ CD4 T cells and the expression of CXCR5 on CD8 T cells. Also, PD-1 expression on CD4 and CD8 T cells, as well as TIGIT expression on CD8 T cells contributed to PC1. There was no significant correlation between PC1 and total HIV DNA measured at year one (r=0.33 p=0.13) see figure 5.3.23 (a). The variables contributing to PC2 at baseline (in order) were; baseline HIV RNA, baseline CD4 T cell count, baseline HIV DNA, baseline CD4/CD8 ratio, CXCR5 expression on CD8 T cells, CD4 T cell count at one year, and CXCR5+CD4 T cell count, CD4 Tim-3, CD4 HLA-DR and CD8 HLA-DR. PC2 was significantly associated with total HIV DNA measured at year one (r=0.47 p=0.03), see figure 5.3.23 (b). No other PC was significantly associated with HIV DNA at one year.

**Table 5.3-4 Variables at Year-1 (ART-treated only) contributing to principal component 1 & 2**

This table lists the variables contributing to the 1st and 2nd principal components on order of importance. The variables included in this analysis are those measured in the ART-treated and the ART-naïve cohorts at year-1.

<table>
<thead>
<tr>
<th>Order of importance</th>
<th>Principal Component 1</th>
<th>Principal Component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TFH</td>
<td>Baseline HIV RNA</td>
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<tr>
<td>2</td>
<td>CD4 TIGIT</td>
<td>Baseline CD4 Count</td>
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<td>3</td>
<td>CD4 CXCR5</td>
<td>Baseline HIV DNA</td>
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<td>4</td>
<td>Absolute TFH</td>
<td>Baseline CD4/CD8 ratio</td>
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<tr>
<td>5</td>
<td>Absolute CD4 CXCR5</td>
<td>CD8 CXCR5 (fCD8)</td>
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<td>CD8 CXCR5 (fCD8)</td>
<td>Year-1 CD4 Count</td>
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<td>CD8 PD-1</td>
<td>CD4 Tim-3</td>
</tr>
<tr>
<td>9</td>
<td>CD8 TIGIT</td>
<td>CD4 HLA-DR</td>
</tr>
<tr>
<td>10</td>
<td>CD8 Tim-3</td>
<td>CD8 HLA-DR</td>
</tr>
</tbody>
</table>
Figure 5.3.23 Correlation between HIV DNA and PC at 1-year in ART-treated individuals

The scatter plots show the association between HIV DNA and (a) PC1 and (b) PC2 at 1-year in ART-treated individuals. Correlations calculated using Spearman's test. Abbreviations: PC1, principal component 1; PC2 principal component 2.
5.3.9. Summary - Part 1

The first part of this chapter examined the phenotype of pTFH during primary HIV infection. Peripheral blood CXCR5 expressing CD4 T cells were phenotypically similar to pTFH cells (i.e. CXCR5\textsuperscript{High}PD\textsuperscript{High} CD4 T cells) and had comparable expression of Bcl-6 and IL-21. My data suggest that ART initiated in PHI preserved the absolute number of both CXCR5\textsuperscript{+} CD4 T cells and pTFH in blood compared to untreated PHI, and that the relative expression of CXCR5 on CD4 cells was also higher in ART-treated individuals after one year.

In addition, ART-treated individuals had decreased expression of activation and exhaustion markers compared to ART-naïve individuals at one year, in both CD4 and CD8 T cell compartments, although this was confined to the CXCR5\textsuperscript{+} CD4 T cells for ICR expression.

Finally, Absolute CXCR5\textsuperscript{+} CD4 T cell and TFH number, HLA-DR and PD-1 expression on CD4 and CD8 T cells, HIV viral load, CD4/CD8 ratio, and CXCR5\textsuperscript{+} CD8 T were all significantly associated with HIV reservoir in PBMCs at time of seroconversion. However, the PCA suggested that HIV viral load and the expression of activation markers and PD-1 more closely associated with HIV reservoir compared to TFH markers. Similarly, in ART-treated individuals at one year, the PC most strongly associated with HIV reservoir again was made up mainly by baseline HIV RNA, CD4/CD8 ratio and ICRs (Tim-3 on CD4 and CD8 T cells), but also included CXCR5 expression on CD4 and CD8 T cells.
5.4. Results – Part 2 (GALT)

The second section of the chapter characterises germinal centre (GC) TFH in GALT of individuals treated during PHI. The individuals contributing data to this section are those from the HEATHER gut study and the cohort characteristics have been outlined in section 4.3.1.

5.4.1. Collagenase use in flow cytometry to characterise TFH in GALT

As outlined in chapter 2, gut biopsies are digested using collagenase to prepare a suspension of mucosal mononuclear cells suitable for flow cytometry. Collagenase is produced by two separate and distinct genes in Clostridium histolyticum and is prepared from Clostridium histolyticum cultures\textsuperscript{540}. Several collagenase preparations are commercially available with varying levels of collagenase, caseinase, clostripain, and tryptic activities\textsuperscript{541}. This is an important consideration when staining for chemokines such as CXCR5 in tissue as the enzymatic content can impact upon the epitope of interest.

Figure 5.4.1 below shows the impact of using a collagenase with a normal to high tryptic activity on CXCR5 expression. Accordingly, collagenase D which has normal collagenase activity and very low tryptic activity (usually $<0.1$ U/mg) was used in this work.

**Figure 5.4.1 Effect of Collagenase with high tryptic activity (e.g. Collagenase A) on CXCR5 expression in tonsil tissue.**

The flow plot in shows (a) CXCR5 expression in Collagenase A treated & (b) mechanically digested tonsil tissue. Flow plots are shown are gated on CD45RA-CD4+ T cells.
5.4.2. TFH in GALT during treated PHI

The phenotype of TFH in a representative sample from HIV+ rectal tissue is shown in figure 5.4.2 (a). PD-1<sup>high</sup> and CXCR5<sup>high</sup> expression define the TFH population in GALT. In HIV+ terminal ileum, ICOS expression was significantly higher on TFH cells compared to bulk CD4 T cells, with a median expression of 30.3% and 4.4% respectively, P<0.0001. Similarly, Bcl-6 expression was higher on terminal ileum TFH cells compared to bulk CD4 T cells with a median expression of 14.0% and 3.5% respectively, P<0.0001; see figure 5.4.2 (b). The same pattern of expression was observed in HIV+ rectal tissue with a median ICOS expression of 47.9% and 16.1% on TFH cells and bulk CD4 T cells respectively, P<0.0001. Median Bcl-6 expression in rectum was also significantly higher on TFH compared to bulk CD4 T cells at 9.7% and 2.8% respectively, P<0.0001.

To understand the impact of treated PHI on TFH in cells in GALT, I next compared the frequency of TFH cells between HIV infected individuals and healthy controls in GALT, see figure 5.4.2 (c). No significant difference in the frequency of TFH cells was noted between HIV+ individuals and healthy controls in the terminal ileum (P=0.5). However, significantly higher TFH were noted in the rectal tissue of HIV+ individuals compared to healthy controls (P=0.004). A similar pattern was seen regarding Bcl-6 expression on CD4 T cells with no difference between groups in the terminal ileum (P=0.76), and higher Bcl-6 expression in HIV infected rectal tissue compared to healthy controls (P=0.02), see figure 5.4.2 (d).

I next investigated if the frequency of TFH cells in GALT changed over time. Figures 5.4.2 (e) and (f) show paired samples from individuals in the HEATHER gut study who had longitudinal sampling of the terminal ileum and rectum respectively. There was no significant difference in the frequency of TFH cells in either the terminal ileum (P=0.28) or
the rectum (P=0.19) between the first and second gut biopsy. Given the nature of gut biopsy sampling, variation in flow cytometry data may relate to the taking of biopsies from random mucosal sites. However, the longitudinal data presented here suggests consistency in the sampling method used.

Figure 5.4.2 TFH in GALT
The flow plot in (a) shows the TFH population in a representative sample from rectal MMCs based on CXCR5 and PD-1 expression on CD4 T cells. (b) shows the Bcl-6 expression on TFH (green) and Non-TFH (blue) cells in the same sample. The jitter plot in (c) illustrates the frequency of TFH cells in HIV+ (filled symbols) and healthy control (open symbols) in the terminal ileum (red triangles) and rectal (blue circle) GALT from the HEATHER gut cohort while (d) shows Bcl-6 expression. The frequency of TFH in those individuals with paired biopsy samples is summarised in (e) and (f) showing the terminal ileum and rectum respectively. P values were calculated using non-parametric tests.
5.4.3. p24 expression on TFH in GALT

I initially intended to perform cell sorting experiments on mucosal mononuclear cells (MMCs), sorting based on CXCR5 and PD-1 expression of CD4 T cells with subsequent quantification of HIV DNA on sorted subsets. However, the limited cell yield from gut biopsy tissue meant that the number of TFH cells after sorting was too low to quantify HIV DNA accurately. Alternatively, I measured p24 expression on unstimulated CD4 T cells to test for evidence of viral production from TFH cells in individuals on ART. Figure 5.4.3 shows representative flow plots of p24 staining and the expression of p24 on TFH and bulk CD4 T cells in the rectum. While no significant difference was observed between p24 expression on TFH and bulk CD4 T cells in the terminal ileum (P=0.16) and rectum (P=0.23), higher p24 expression was noted in the TFH population in some individuals, see figure 5.4.3 (c). However, in general, a significant amount of non-specific background staining was observed with the p24 antibody used; positive p24 staining was also observed in some HIV negative individuals. Therefore, these results should be interpreted with caution. A potential method to overcome this non-specific staining in future may be to use an additional p24 antibody, taking the double positive population as p24 positive.

Figure 5.4.3 p24 expression in HIV+ rectum

The flow dot plots in show representative staining in rectal GALT MMCs; (a) shows the p24 Fluorescence minus one (FMO) stain, while (b) show the fully stained sample. Using data from the HEATHER gut study (c) shows the p24 expression on TFH and bulk CD4 T cells from rectal MMCs. Data not shown for terminal ileum MMCs.
5.4.4. Markers of immune function on TFH in GALT during treated PHI

To assess immune dysfunction (or the absence of it) of TFH in GALT during treated PHI, I assessed the expression of Tim-3, TIGIT, HLA-DR/CD38 and ICOS on TFH between HIV infected individuals and healthy controls. Similar expression of TIGIT and Tim-3 was noted in both the terminal ileum and rectum when HIV+ was compared with healthy controls. Higher expression of the activation marker HLA-DR/CD38 was measured on TFH cells from HIV+ individuals compared to controls in both the terminal ileum (median 1.9% 1.0% respectively P=0.03) and rectum (median 2.2% 1.4% respectively, P=0.03). The TFH activation marker ICOS was also significantly higher on rectal TFH in HIV+ GALT compared to controls (median 37.4% & 16.7% respectively, P=0.002) with a trend towards higher ICOS expression in the terminal ileum of the HIV+ individuals (31.4 & 21.8% respectively, P=0.058)

**Figure 5.4.4 Markers of immune function on TFH in GALT**

The plots below compare the expression of (a) TIGIT, (b) Tim-3, (c) HLA-DR/CD38 & (d) ICOS expression on TFH between HIV+ (closed symbols) and healthy controls (open symbols) in terminal ileum (red triangles) and rectum (blue circles). P values were calculated using a Mann-Whitney test.
5.4.5. Follicular CD8 T cells in GALT during treated PHI

I next characterised the follicular CD8 (fCD8) T cell subset in GALT during treated PHI. For purposes of flow cytometry, fCD8 T cells were defined as CD3+CD8+CXCR5+ lymphocytes. Representative flow plots of the fCD8 populations in terminal ileum and rectum are shown in figure 5.4.6 (a) and (b) respectively. The frequency of fCD8 cells was significantly higher in both gut sites compared to PBMCs (both P<0.0001), see figure 5.4.6 (c); the median frequency of fCD8 was 4.3%, 7.2% and 1.1% in the terminal ileum, rectum & PBMCs respectively. To assess the impact of ART-treated PHI on fCD8 frequency in GALT, I compared the frequency of fCD8 T cells between HIV+ individuals and healthy controls in the terminal ileum and rectum. No difference in fCD8 frequency was noted in the terminal ileum between HIV+ & healthy controls (P=0.97), while significantly higher fCD8 were observed in the rectal tissue of HIV+ individuals (P=0.002). Using paired longitudinal samples from 10 individuals enrolled in the HEATHER gut study, I examined the frequency of fCD8 in GALT tissue over time; no significant changes were noted in the terminal ileum (P=0.43) or rectum (P=0.49) between the first and second gut biopsy.

I next examined the phenotype of fCD8 and non-follicular CD8 T cells (CXCR5−CD8+) in HIV+ GALT. Bcl-6 expression was significantly higher on fCD8 compared to non-follicular CD8 T cells in terminal ileum (median 11.9% and 0.7% respectively, P<0.0001) and rectum (median 7.9% and 0.4% respectively, P<0.0001). Higher expression of the cytolytic proteins, perforin and granzyme B was also noted on fCD8 compared to non-follicular CD8 T cells; the median perforin expression in terminal ileum was 14% and 0.5% respectively, P<0.0001 and in rectum, it was 10.1% and 1.4% respectively. Granzyme B expression was also higher on fCD8 cells compared to non-follicular CD8 cells in the terminal ileum (38.1% and 11.7% respectively, P<0.0001) and rectum (32.8% and 24.9% respectively, P=0.02). Of note, a trend towards higher perforin expression on fCD8 cells
was observed in the terminal ileum compared to the rectum (P=0.051). These data are summarised in figure 5.4.7 (a) and (b).

Figure 5.4.5 Immunohistochemistry staining of fCD8 in healthy control tonsil
Staining of control tonsil, Bcl-6 (brown) identifies the germinal centre (GC) while CD8 (red) cells are not usually in GCs, we confirm their presence, as indicated by the arrow below. (a) X10 (b) X20 magnification.
Figure 5.4.6 fCD8 T cells in GALT

The flow plots in (a) & (b) show the fCD8 T cell populations in a representative sample from terminal ileum & rectal MMCs. The jitter plot in (c) illustrates the frequency of fCD8 in HIV+ (filled symbols) and healthy control (open symbols) in terminal ileum (red triangles), rectum (blue circle) & PBMCs (yellow squares), while (d) compared fCD8 expression in GALT between HIV infected and healthy controls. The frequency of fCD8 in those individuals with paired biopsy samples is summarised in (e) and (f) showing the terminal ileum and rectum respectively. P values were calculated using non-parametric tests and corrections were made for multiple comparisons.
The immune checkpoint markers PD-1 and TIGIT were both expressed at significantly higher levels on fCD8 compared to non-follicular CD8 T cells in both GALT sites; median PD-1 expression in terminal ileum was 23.8% and 4.12% respectively (P<0.0001), and in rectum it was 40.6% and 15.3% respectively. Median TIGIT expression on fCD8 and non-follicular CD8 cells in terminal ileum was 41% and 24.7% respectively (P<0.0001) and in rectum it was 54.1% and 37.2% respectively, P<0.0001. These data are summarised in figure 5.4.7 (c) and (d). Tim-3 was the exception, with no difference in Tim-3 expression noted between fCD8 cells and non-follicular CD8 cells in the terminal ileum and higher expression of Tim-3 on non-follicular CD8 T cells in rectal tissue (P=0.005). Immune activation, as measured by HLA-DR and CD38 co-expression was also highest on fCD8 cells compared to the non-follicular CD8 T cells in both the terminal ileum (median 5.0% and 0.8% respectively, P<0.0001) and the rectum (median 4.9% and 3.3% respectively P=0.007), see figure 5.4.7 (f).

I also compared the expression of PD-1, TIGIT, Tim-3, HLA-DR/CD38, Granzyme B and Perforin on fCD8 cells between HIV infected individuals and healthy controls. TIGIT expression on fCD8 was higher in HIV+ individuals in the rectum (P=0.001) with a trend towards higher expression in the terminal ileum (P=0.058). No difference in PD-1 or Tim-3 expression on fCD8 cells was noted between HIV+ and healthy controls. Perforin expression on fCD8 cells was also similar between HIV+ individuals and healthy controls. However, Granzyme B expression was lower in the rectum of HIV+ individuals when compared to controls (P=0.03). Finally, fCD8 cells in the terminal ileum of HIV+ individuals were more activated compared to controls (P=0.01), as measured by HLA-DR & CD38 co-expression on CD8 T cells; no differences in activation were noted in the rectum between HIV+ & controls.

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In summary, fCD8 T cells are found at high frequency in GALT, particularly in rectal tissue during PHI. They exhibit an activated phenotype, have high expression of Bcl-6, PD-1 and TIGIT and greater cytolytic potential compared non-fCD8. When compared to healthy controls, fCD8 in HIV+ terminal ileum are more activated and maintain similar levels of cytolytic proteins compared to controls while in the rectum they have higher TIGIT & lower perforin expression.

**Figure 5.4.7 Phenotype of fCD8 T cells compared to non-follicular CD8 T cells in GALT**

The Jitter plots below compare the expression of (a) Perforin, (b) Granzyme B, (c) PD-1, (d) TIGIT, (e) Tim-3 and HLA-DR & CD38 co-expression between fCD8 T cells (filled symbols) and non-follicular CD8 T (unfilled symbols) cells in the terminal ileum (red triangles) and rectum (blue circles) of the HEATHER gut study cohort. This data includes HIV+ individual only. P values were calculated using a Wilcoxon pairs signed rank test. Abbreviations: fCD8, follicular CD8; Non-fCD8, non-follicular CD8 cells.
5.4.6. Correlations of Follicular Makers in GALT

The correlation matrices in figure 5.4.8 and figure 5.4.9 summarise the associations between TFH, fCD8 and measures of HIV DNA in the terminal ileum and rectum of HIV+ GALT. The frequency of TFH cells correlated with the frequency of fCD8 T cells in both the terminal ileum (r=0.49, P=0.01) and rectum (r=0.61, P=0.0005). TFH also correlated with CD19 expression in the terminal ileum (r=0.49 P=0.02) and in the rectum (r=0.73 P=0.0002), while CD19 expression was associated with fCD8 frequency in rectal tissue only (r=0.61, P=0.003), supporting the idea that these germinal centre cells support each other’s differentiation or respond to a common pathway in treated PHI. Also, higher ICOS expression on TFH cells in both the terminal ileum and (r= 0.45 P=0.04) the rectum (r=0.46, P=0.03) was associated with higher IL-21 and higher IL-10 (r=0.44, P=0.04) measured in PBMCs, in keeping with the role of ICOS in sustaining TFH activation & function. Also, IL-21 demonstrated a strong positive association with sCD40 (r=0.55 P=0.002), a marker we have previously shown to be predictive of HIV DNA in GALT.

The frequency of TFH inversely correlated with HIV DNA (CPM CD4 T cells) in the terminal ileum (r=-0.44 P=0.02). However, HLA-DR expression on TFH was positively correlated with both measures of HIV DNA in both the terminal ileum (CPM gut cells, r=0.44 p=0.02; CPM CD4 cells, r=0.40 p=0.05) and rectum (CPM gut cells, r=0.41 p=0.04; CPM CD4 cells, r=0.35 p=0.08); in the rectum ICOS (r=0.45 P=0.03) and TIGIT (r=0.40 P=0.04) expression on TFH were also associated with higher levels of HIV DNA, taken together these findings suggests that greater TFH activation may support latent infection GALT.

As discussed, fCD8 T cells associated with TFH in both the terminal ileum and rectum. In addition, higher Bcl-6 expression on TFH was correlated with higher fCD8 in both the terminal ileum (r=0.41 p=0.05) and rectum (r=0.40 P=0.04). Furthermore, in the terminal
ileum, fCD8 cells were associated with IL4 (r=0.42 P=0.04) and IL-15 (r=0.50 P=0.02) measured in TI explant supernatants. Interestingly, higher frequency of fCD8 T cells in the terminal ileum was associated with lower levels of HIV DNA in that site (r=-0.49, P=0.01) and suggests that higher frequency of these cytotoxic T cells with access to the germinal centre may limit HIV DNA.

In summary, measures of TFH, fCD8 and B cells associated with each other in GALT suggesting coordination of their function during treated PHI. Activation of TFH cells was associated with increased measures of HIV DNA in both gut sites, while higher frequency of fCD8 in the terminal ileum associated with lower HIV DNA in that site.
Figure 5.4.8 Correlations of follicular markers measured in the terminal ileum of HIV+ GALT

The correlation matrix shows the associations between follicular markers measured in terminal ileum and plasma. All markers refer to terminal ileum unless otherwise indicated, PBMC indicates the marker was measured in blood or plasma. Blue indicates a positive association while red indicates a negative association. P-values were calculated using Spearman’s test and are shown in each ellipse.

Abbreviations: fCD8, follicular CD8; TFH, T follicular helper cell; TI.DNA.gut, terminal ileum HIV DNA in copies per million gut cells; TI.DNA.cd4, terminal ileum HIV DNA in copies per million CD4 T cells.
**Figure 5.4.9 Correlations of follicular markers measured in the rectum of HIV+ GALT**

The correlation matrix shows the associations between follicular markers measured in rectum and plasma. All markers refer to rectum unless otherwise indicated, PBMC indicates the marker was measured in blood or plasma. Blue indicates a positive association while red indicates a negative association. P-values were calculated using Spearman’s test and are shown in each ellipse.

Abbreviations: fCD8, follicular CD8; TFH, T follicular helper cell; Rectum.cpm.gut, Rectum HIV DNA in copies per million gut cells; Log.Rect.cpm.cd4, Rectum HIV DNA in copies per million CD4 T cells.
5.5. Conclusion

These data characterise both peripheral and germinal centre TFH around the time of seroconversion. In addition, I have been able to examine the impact of ART initiated in PHI on the expression of TFH makers and measures of their function by comparing ART-treated with ART-naïve individuals in PBMCs, and ART-treated individuals with healthy controls in GALT.

My analysis of the pTFH cohort demonstrates dynamic changes in the pTFH compartment in PHI, and I found evidence that ART preserved the absolute number of pTFH cells. This finding is consistent with work by Boswell et al., who report lower pTFH in ART-naïve individuals, with a degree of pTFH recovery after ART initiation\(^{517}\). However, while their work focused on chronic infection, I demonstrate pTFH recovery with ART in PHI. Work by Lindqvist and others has demonstrated the expansion of GC TFH in lymph nodes during chronic HIV infection\(^{512}\). Interestingly, I observed evidence of GC TFH expansion in rectal GALT but not in the terminal ileum, supporting the hypothesis of compartmentalised immune recovery and immune responses to HIV.

While my data support the hypothesis that ART preserves the absolute number of total pTFH when initiated in PHI, which are otherwise lost without immediate/early therapy, it is possible that this finding may reflect preservation of the total number of CD4 T cells rather than selective preservation of pTFH. Pallikkuth et al. have shown that pTFH are more permissive to HIV infection than non-pTFH and in particular PD-1\(^+\) pTFH cells\(^ {351} \). In keeping with this finding, I found lower relative CXCR5 expression on CD4 cells in ART-naïve individuals after one year. However, there was no difference in CXCR5 and PD-1 co-expression on CD4 cells when comparing ART-naïve and ART-treated individuals at one year, this may reflect a reduction in PD-1 expression on CXCR5\(^+\)CD4 cells in ART-
treated individuals, due to decreased immune activation, rather than an actual decrease in the pTFH phenotype. HLA-DR expression was highest in the PD-1<sup>High</sup> populations, indicating that PD-1 expression may reflect activated CD4 T cells populations rather than selecting for pTFH. In contrast to the higher expression of Bcl-6 in GC TFH cells, PD-1<sup>-</sup>CXCR5<sup>+</sup> and PD-1<sup>-</sup>CXCR5<sup>-</sup> CD4 cells have similar levels of Bcl-6 expression, suggesting that CXCR5 expression alone may capture circulating CD4 T cells with a pTFH phenotype. These findings are in agreement with findings from HIV uninfected individuals and may have implications for future functional studies on the pTFH population as the low frequency of pTFH, as defined by CXCR5 and PD-1 co-expression, currently limits certain functional and cell sorting experiments.

I found that a higher frequency of pTFH cells at seroconversion was associated with lower measures of HIV reservoir. Also, higher GC TFH in terminal ileum GALT on ART associated with lower terminal ileum HIV DNA. Contrary to this, after one year of ART, the frequency of CXCR5<sup>+</sup> CD4 cells in PBMCs positively correlated with HIV DNA. This latter finding is in keeping with published work suggesting that pTFH are the major PBMC HIV reservoir within central memory CD4 cells in chronically infected individuals. An explanation for the negative correlation found between pTFH and HIV reservoir at seroconversion may relate to the hypothesis that higher pTFH reflect an efficient germinal centre immune response to HIV during acute HIV infection which limits HIV reservoir. On ART, the reduction of HIV antigen may change these dynamics; increased markers of GC TFH activation on ART correlated with higher GALT HIV DNA, such increased activation of GC TFH may be indicative of ongoing viral replication within germinal centres. These findings highlight the complex interaction between HIV and TFH, whereby TFH cells are preferential targets and cellular reservoirs for infection but are also important components of the immune response to HIV. My data supports the notion that GC and pTFH may play
different roles in supporting HIV persistence on ART. In support of this, an important recent paper by Boritz et al. suggested that in HIV controllers three interrelated mechanisms support HIV persistence namely ongoing infection of cells in lymphoid tissue, the survival and recirculation of some of these cells, and in peripheral blood the long-term persistence of proviruses in clonally expanded cells\textsuperscript{344}.

My data demonstrate a beneficial interaction between TFH and fCD8; pTFH cells (at the time of seroconversion) and GC TFH (in terminal ileum & rectum) associated with follicular CD8 T (fCD8) cells in PBMC and GALT. Also, higher expression of Bcl-6 on GC TFH associated with increased numbers of fCD8, while in PBMCs fCD8 cells were associated with higher IL21 MFI & Bcl-6 expression on pTFH at seroconversion. Work by Leong et al. has shown that Bcl-6 is required for fCD8 differentiation\textsuperscript{527}. These findings are also supported by recent work in rhesus macaques, which found evidence that higher frequency of IL-21\textsuperscript{+} SIV specific TFH cells was associated with the greater provision of T cell help to fCD8 and lower viral loads\textsuperscript{543}. fCD8 T cells expansion was noted in the rectum of HIV+ individuals compared to healthy controls, but there was no correlation with HIV DNA in this site. In contrast, I found that fCD8s were inversely correlated with total HIV DNA and HIV RNA in PBMC at seroconversion and with HIV DNA in the terminal ileum on ART. This is particularly interesting in the context of recent reports from murine models which suggest a role for fCD8s in controlling chronic viral LCMV infection\textsuperscript{527, 528}. While some investigators have reported decreased cytolytic function\textsuperscript{527, 544} of fCD8 others have demonstrated increased cytotoxic potential\textsuperscript{528, 545}; I clearly show in HIV+ GALT that fCD8 cells have a significantly higher expression of Granzyme B and Perforin. However, a functional assessment of these cells would be required to show that this increased expression confers enhanced killing. Petrovas et al. showed that fCD8s cells could be redirected to kill latently infected cells via bi-specific antibodies, highlighting their role in
targeting the latent reservoir in tissue sites\textsuperscript{545}. Another strategy which has been successful in enhancing the access of fCD8 T cells to B cell follicle has involved the use of IL-15 to redirect circulating CD8 cells to B cell follicles\textsuperscript{546, 547}. In support of this, I found a positive correlation between IL-15 measured in terminal ileum tissue explants supernatants and the frequency of fCD8.

T cell exhaustion has been proposed as a mechanism of T cell dysfunction in chronic viral infections\textsuperscript{274, 548-550}. My data suggests that treatment with ART had a differential impact on the expression of exhaustion & activation markers between CXCR5\textsuperscript{+} and CXCR5\textsuperscript{-} CD4\textsuperscript{+} T cell subsets in PBMCs. Persistent elevation of ICRs was seen on the CXCR5\textsuperscript{+} CD4 cells despite ART; the expression of TIGIT and PD-1 on CXCR5\textsuperscript{+} cells may not signify immune exhaustion but rather that their expression on CXCR5\textsuperscript{+} cells is a functional mechanism used to prevent inappropriate TFH proliferation and allow for the selective pressure of B cell responses. While on CXCR5\textsuperscript{-} CD4 T cells in PBMCs, ICR expression decreased when viral control was achieved with ART. Tim-3 expression decreased in both CXCR5\textsuperscript{+} and CXCR5\textsuperscript{-} CD4\textsuperscript{+} T cell subset and may be a better marker of T cell exhaustion on pTFH in the setting of PHI. Concerning GC TFH, similar levels of T cell exhaustion marker expression were seen between ART-treated individuals and controls. However, elevated TFH activation marker expression was noted in HIV\textsuperscript{+} GALT, compared to controls suggesting HIV induced TFH activation persists despite ART.

I measured surrogate markers of pTFH function in our study, the optimal experiment to assess pTFH function would involve a co-culture experiment of pTFH with B cells and include a measure of the ability of pTFH to provide B cell help. Due to limited cell numbers, I was unable to perform this experiment. Instead, I measured Bcl-6, IL-21 and CXCL13 as a surrogate marker for TFH function and activity. Apart from IL-21, I saw no differences in these markers between ART-naïve and ART-treated individuals. Surprisingly, IL-21 was
lowest in the ART-treated groups compared to all other groups. However, as IL-21 was measured on unstimulated CD4 T cells, the increased IL-21 observed in the ART-naive group does not necessarily represent preserved functional potential of pTFH in this group but may reflect continued exposure to HIV antigen in the ART-naïve group.

Previous work by Lindqvist et al. demonstrated a significant correlation between the Bcl-6 expression in TFH cells, measured in lymph node, and the total IgG antibody concentration in HIV-infected, untreated and treated individuals. I failed to see such an association in our cohort. However, in the pTFH cohort, I measured Bcl-6 expression in peripheral blood and not in the lymph node. Interestingly, I did observe an association between Bcl-6 expression on CD4 T cells and the breadth of broadly neutralising antibody response, supporting the hypothesis that the magnitude of TFH response is associated with the development of broadly neutralising antibodies. No significant relationship between CXCR5 expression and antibody responses was observed; this may be a consequence of the omission of CXCR3 from the TFH flow cytometry panel which in retrospective may have been important to include. Locci et al. have previously shown that PD-1+CXCR3−CXCR5+CD4+ T cells are the pTFH population most able to provide B cell help and have been associated with the development of broadly neutralising antibodies in HIV infected individuals.

IgG1 and IgG3 have been shown to be the IgG subclasses most important in anti-HIV antibody-dependent cellular cytotoxicity (ADCC). The results of the RV144 HIV vaccine trial suggested that vaccine-induced plasma IgA might inhibit ADCC by binding to IgG and thereby interfering with its effector function, in this study elevated IgG/IgA ratio was associated with increased protection against HIV acquisition in vaccine recipients. More recently Ruiz et al. have demonstrated that lower HIV-specific gp120 IgG/IgA ratios are associated with decreased in ADCC in untreated HIV-infection. I observed higher
HIV specific gp120 IgG in the ART-naïve group suggesting that a period of exposure to viraemia is required to mount an antibody response to env, this was not the case for responses to gag. Previous work by Binley et al. has shown that ART commenced in PHI inhibited the development of anti-gp120 antibodies while ART initiation in chronic infection had no such effect. Furthermore, higher gp120 IgG/IgA ratio was observed in the ART-naïve group, while HIV specific p24 IgG/IgA was elevated in the ART-treated group, suggesting that early ART has a differential impact on gag and env responses. However, antibody responses did not relate to TFH in our study; this may be explained by our measurement of bulk pTFH rather than HIV-specific pTFH.

In summary, these data show that ART initiated in PHI supports the preservation of TFH and reduces markers of TFH immune dysfunction. The relationship between TFH and HIV DNA was complex with different associations observed between ART-treated and ART-naïve individuals and between pTFH and GC TFH cells. At seroconversion higher pTFH was associated with lower HIV DNA. However, on ART traditional baseline variables such as viral load remained more predictive of HIV DNA than measures of pTFH. In GC GALT activation of TFH cells was associated with higher HIV DNA. Finally, fCD8 cells are present in HIV+ GALT and PBMCs, have increased cytotoxic potential and were associated with lower HIV DNA.
Chapter 6

CD32 as a marker of HIV reservoir

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Chapter 6.
CD32 as a marker of HIV reservoir

6.1. Introduction

One of the key challenges in eradicating HIV infection is the identification of cells which are latently infected\(^{468}\). However, identification of latently infected CD4\(^+\) T cells in peripheral blood and particularly in tissue sites has been challenging due to the lack of a specific marker on CD4\(^+\) T cells for latent HIV infection. If identified, such marker could be used as a target for HIV cure interventions. Expression of the immune checkpoint inhibitors PD-1, TIGIT and Lag-3 on CD4 T cells in peripheral blood have demonstrated enrichment for HIV-1 DNA\(^{177}\), while in rectal tissue PD-1 expression on CD4-T cells may represent a marker of HIV persistence\(^{555}\). However, while the ICRs identify cells enriched for HIV DNA, they are not definitive markers of HIV reservoir. Recent work by Descours et al. has suggested that CD32a (FcyRIIa) - a low-affinity IgG receptor expressed on myeloid cells and granulocytes (but which is not generally considered to be expressed on T cells\(^{556}\)) as a specific surface marker for latent HIV infection\(^{179}\). Whilst other groups have shown that in the periphery these cells can contain HIV DNA, the consistency of magnitude of enrichment has not been replicated\(^{180,181}\). It has also been suggested that CD32 is predominately expressed on a subset of activated CD4\(^+\) T cells containing transcriptionally active HIV after long-term ART\(^{181}\). Importantly, the significance of CD32 expression and the HIV reservoir in GALT has not been previously studied but is critical to our understanding of how this marker relates to the HIV reservoir. In this chapter, I examine the expression of CD32 and the association with HIV DNA in GALT of individuals who initiated treatment during PHI. The work presented here was a collaborative effort within our research group. The laboratory work completed by me includes the processing, HIV-1 DNA quantification, antibody staining, flow cytometry and analysis of all tonsil and
gut tissue samples presented. In addition, I completed the antibody staining, flow cytometry and analysis of the longitudinal PBMCs for the activation and T follicular markers presented. Dr Genevieve Martin completed the CD4 T cell lineage work presented. Dr Matthew Pace completed the CD4 depletion kit analysis and B cell and T cell phenotypic marker experiments. Dr Chan Phetsouphanh performed the cells sorting experiments.

**Aims**

The aim of this chapter was to characterise the expression of CD32 on CD4 T cells in terminal ileum, rectum and PBMCs and to test for associations between CD32 & HIV DNA

**Hypothesis**

i. **CD32 expression on CD4 T cells will correlate with HIV DNA in GALT**

**Objectives**

The objectives of this chapter are to:

i. **Compare the expression of CD32^+ CD4 T cells between ART-treated HIV positive individuals and healthy controls**

ii. **Describe the frequency of CD32^+ expression on CD4 T-cells in peripheral blood, terminal ileum and rectum during treat PHI**

iii. **Characterise the immune phenotype of CD32^+ expressing CD4 T cells across anatomical sites.**

iv. **Examine the relationship between HIV-1 DNA and CD32 expression in PBMCs and GALT**
6.2. Methods

The HEATHER Cohort

The HEATHER cohort is described in detail in chapter 2. For inclusion in the cohort, participants with identified PHI commenced ART within 3 months of diagnosis and did not have co-infection with Hepatitis B or C. Date of seroconversion was estimated as the midpoint of the dates of the most recent negative or equivocal test and positive test (criteria a and d above), the date of the test (b and e) or 120 days prior to the test date (c, the recency period of this assay). Terminal ileum and rectal samples were obtained from a subset of participants in HEATHER as part of the HEATHER gut study.

Terminal ileum, rectal and PBMC samples from healthy controls were obtained from individuals undergoing routine endoscopy from the Translational Gastroenterology Oxford Tissue Biobank. This cohort was previously described in chapter 2 and the clinical characteristics are shown in table 4.3.2.

Processing of tissue samples.

Gut tissue digestion was performed as described in chapter 2. Briefly, rectal and terminal ileum biopsies (up to 12 from each site) were collected at endoscopy and immediately placed in RPMI-1640 media with 5% heat-inactivated fetal bovine serum (FBS), 0.04 mg/mL gentamicin, 100 IU/mL penicillin, 0.1 mg/mL streptomycin and 2mM L-glutamine. Biopsies were processed within 3 hours of sampling. Samples were washed in 1mM dithiothreitol (DTT) solution and then with PGA solution (Hanks’ Balance Salt Solution with 0.04 mg/mL gentamicin, 100 IU/ml penicillin and 0.1 mg/mL streptomycin). Biopsy samples subsequently underwent collagenase and mechanical digestion using Collagenase D (1 mg/mL) for 30 minutes and a gentleMACS dissociator (Miltenyi Biotec), respectively. The
resulting cell suspension was then strained using a 70 µM filter, washed with PGA and used for staining.

**Measurement of HIV-1 DNA**

Quantification of HIV-1 DNA was carried out as described in chapter 2. Briefly this involved two qPCR reactions. The first round quantified the number of cells/µL, using primers targeting the Albumin gene, against known copy standards. A known cell number was then inputed into the second reaction (up to 25,000 cells per 10/µL) and total HIV-1 DNA was quantified using primers targeting the LTR region. For PBMC the starting DNA was from a CD4 T cell enriched sample while the GALT DNA was not CD4 enriched. GALT DNA was expressed as copies per million gut cells and copies per million gut CD4 T cells (based on frequencies from flow cytometry plots).

**Flow cytometry and cell sorting**

Mucosal cells were stained with LiveDead Near IR, anti-CD3 BV570, anti-CD4 BV605, anti-CD8 BV650 and anti-TIGIT PE-eFluor710. The following antibodies were also included: PD-1 PE-Cy7 (EH12.1), Tim-3 PE-CF594 (7D3), HLA-DR AlexaFluor700 (MAB)[BD] and CD32 PE (FUN-2)[BioLegend]. EPCAM APC-Vio770 (HEA-125) [Miltenyi Biotec] was also used to allow for the exclusion of epithelial cells. Staining was performed for 20 minutes at room temperature, followed by fixation and permeabilisation using the Human FoxP3 Buffer Set (BD Pharmingen) as per manufacturers protocol.

Cryopreserved PBMCs were thawed in RPMI-1640 medium supplemented with 10% FBS, L-glutamine, penicillin and streptomycin as above (R10) containing 2.7 Kunitz units/mL of DNase (Qiagen). Cells were stained in BD Horizon Brilliant Stain Buffer (BD) containing all antibodies and Live/Dead Near IR at 1 in 300 dilutions (Life Technologies) at 4°C for
30 minutes. Isotype controls for CD32 were prepared using an irrelevant IgG2bκ antibody (MPC-11)[BioLegend].

All samples were acquired on an LSR II (BD). The same machine was used for all experiments with daily calibration with Rainbow Calibration Particles (Biolegend) to maximise comparability between days. Data were analysed using FlowJo Version 10.8.0r1 (Treestar).

For sorting experiments, CD4 T cells were enriched from cells thawed as above by negative magnetic selection using EasySep Human CD4+ T cell Enrichment kit (StemCell Technologies). CD4 T cells were stained in R10 at 4°C using Live/Dead Near IR, anti-CD32 PE-Cy7, anti-CD3 FITC, anti-CD4 eFluor450. Sorting of CD32+ and CD32- CD4 T cells was performed using a Mo-Flo XDP.

**Statistical Analysis**

Continuous variables were compared between groups using non-parametric tests throughout. Comparisons between CD32+ and CD32- populations were performed using the Wilcoxon matched-pairs signed rank test. Where three groups were compared, a Kruskal-Wallis test (unpaired data); pairwise comparisons were performed on predetermined combinations of groups only if the overall test p-value was <0.05. Correlative analyses were performed using Spearman’s rank correlation. For all tests, p values <0.05 were considered statistically significant. Analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA) version 6.0f or IBM SPSS Statistics version 24.
6.3. Results

6.3.1. Clinical characteristics

The individuals included in this study included participants (n=18) from the HEATHER gut study and healthy controls (n=5) recruited through the Oxford TGU. The HIV+ cohort presented here is a subset of the HEATHER gut study presented in chapter 4 who had data available for CD32 staining. The clinical characteristics of those included in this analysis are summarised in table 6.3.1. There were some notable differences between the groups. The majority (3/5) of healthy controls were female while all the HIV+ group were male. In addition, the median age of the healthy controls was significantly older then HIV+ participants.

Table 6.3.1 Participant Characteristics

<table>
<thead>
<tr>
<th>Participant Category</th>
<th>Healthy Controls (n=5)</th>
<th>ART-Treated since PHI (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Age at biopsy, years</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71 (56-71)</td>
<td>38 (31-46)</td>
</tr>
<tr>
<td>Indication for colonoscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Research only</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>Colonic polyps</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Altered bowel habit</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Plasma HIV VL at biopsy, RNA copies per ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>NA</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Time on ART at biopsy, months</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>(19.3-44.3)</td>
</tr>
<tr>
<td>Time from EDI to ART initiation, days</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(53-88)</td>
</tr>
<tr>
<td>Plasma HIV RNA at HIV diagnosis, log10 RNA copies per ml</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.6-6.1)</td>
</tr>
</tbody>
</table>

Abbreviations: IQR (interquartile range) NA, not applicable; PHI, primary HIV infection; ART, antiretroviral therapy; EDI, estimated date of infection; VL, viral load.
6.3.2. HIV DNA levels in CD32 cohort

Firstly, I measured HIV DNA in unstimulated CD4^+ T cells from peripheral blood mononuclear cells (PBMCs), rectal GALT and terminal ileum GALT from those ART-treated individuals from the HEATHER cohort who commenced ART during PHI and were included in this analysis (n=18). The median (IQR) duration of ART at the time of HIV DNA measurement was 33.5 (19.3-44.3) months. Consistent with data presented in chapter 4, I observed significantly higher levels of HIV DNA in both rectal (P=0.004) and terminal ileum (P<0.0001) mucosa compared to matched PBMCs, see figure 6.3.1; the median (IQR) HIV DNA levels in the terminal ileum, rectum and PBMC were 3.49 (3.34-3.87), 3.44 (3.1-3.65) and 3.07 (2.89-3.22) log_{10} copies per million (CPM) CD4^+ T cells, respectively. Also, higher HIV DNA levels were observed in terminal ileum compared to the rectum, although not statistically significant after adjusting for multiple comparisons (P=0.7).

Figure 6.3.1 HIV DNA levels in GALT and PBMCs in HIV infected individuals

This jitter plot shows levels of HIV DNA (CPM CD4^+ T cells) in the terminal ileum, rectum and peripheral blood of HIV infected individuals. Each symbol represents one individual; the horizontal line through the symbols indicates the median. Comparisons between groups were done using a Kruskal-Wallis test and corrected for multiple comparisons using Dunn’s test. Abbreviations: CPM, copies per million T Ileum, terminal ileum; PBMC, peripheral blood mononuclear cells.
6.3.3. Characteristics of CD32 expressing CD4 T cells across different tissue compartments

I next compared CD32 expression on CD4+ T cells between the HIV infected individuals (n=18) and healthy controls (n=5). The median (IQR) CD32 expression in HIV infected individuals compared to healthy controls was 5.2% (4.2-7.8%) vs. 7.6% (4.7-9.4%) in terminal ileum, 5.8% (3.8-6.5%) vs. 7.3% (3.4-12.2%) in rectum and 4.7% (2.7-6.4%) vs. 3.7% (2.9-6.2%) in PBMC, respectively; no statistically significant differences were observed between HIV+ individuals and healthy controls in any anatomical site. The CD32 expression on CD4+ T cells from the HIV infected tonsil sample (n=1) was 4.6%.

Given the greater HIV DNA levels observed in the gut mucosal sites of the HIV infected cohort compared with peripheral blood, it follows that if CD32 is a marker of the HIV reservoir, then higher CD32 expression would be seen in those sites; I hypothesised that higher HIV levels in gut sites might correlate with greater CD32 expression. Accordingly, I examined the frequency of CD32 expression on CD4+ T cells in terminal ileum, rectal and peripheral blood mononuclear cells to determine if there is an association with HIV DNA. The gating strategy is shown in Figure 6.3.2 (a); in PBMCs and mucosal mononuclear cells (MMC) a tight lymphocyte gate was used to avoid monocyte contamination as these are known to express CD32, doublets were also excluded, in GALT tissue epithelial cells were excluded using a dump gate which included EPCAM and dead cells. When comparisons were made across anatomical sites, no significant differences were observed in median (IQR) CD32 expression on CD4+ T cells in terminal ileum, rectum and peripheral blood samples in HIV infected individuals; 5.2% (4.2-7.9), 5.8% (3.9-6.7) and 4.7% (2.7-6.4), respectively. These data are summarised in figure 6.3.2 (b) below.
Figure 6.3.2 CD32 expression in different anatomical compartments

(a) Representative CD32 gating strategy; data shown are gated on mucosal mononuclear cells from terminal ileum GALT. (b) Mucosal mononuclear cells from terminal ileum and rectal mucosa and peripheral blood mononuclear cells were used to evaluate the CD32 expression on CD4+ T cells across anatomical sites from HIV infected individuals. Each symbol represents an individual. Data are shown for HIV infected individuals only. No statistical difference in the frequency of CD32 expression on CD4+ T cells was observed between anatomical sites (rectum, terminal ileum & peripheral blood), P=0.7 by Kruskal-Wallis test. Abbreviations: TIleum, terminal ileum; PBMC, peripheral blood mononuclear cells.
6.3.4. CD32^+CD4^+ T cells exhibit an activated phenotype

Significantly higher activation as determined by HLA-DR expression was found on CD32^+ CD4^+ T cells compared to CD32^- CD4^+ T cells (figure 6.3.3a) in all anatomical compartments. Median HLA-DR expression on CD32^+ compared to CD32^- was as follows; terminal ileum 11.2% vs 0.7%; rectum 18.2% vs 1.5%; PBMC 5.2% vs 1.6%, all P<0.001; Wilcoxon test. No differences were observed in HLA-DR expression on CD32^- CD4^+ T cells in GALT sites compared to PBMC. However, higher HLA-DR expression was noted on CD32^- CD4^+ T cells in rectal tissue and terminal ileum tissue when compared with PBMCs (P<0.0001 and P=0.06 respectively. Kruskal-Wallis test, Dunn’s test correction for multiple comparisons), suggesting greater activation of the CD32^- CD4^+ T cells in gut compared to PBMCs, but not in the CD32^- CD4^+ T cell subset.

6.3.5. Expression of TFH markers on CD32 cells in tissue and PBMC

I next examined the expression of immunological markers known to associate with measures of HIV reservoir on CD32^+ CD4^+ T cell populations in HIV infected individuals. As discussed in chapter 5, Bona fide T follicular helper (TFH) cells are found predominately in germinal centres within lymphoid tissue and are enriched for HIV DNA. In this chapter, I again defined TFH based on the expression of the surface markers CXCR5, ICOS and PD-1, and the TFH transcription factor Bcl-6. I examined the expression of these markers on CD32^+ CD4^+ T cells compared with CD32^-CD4^+ T cells across tissue compartments (Figure 6.3.3 b-d). As expected, higher expression of all TFH markers (CXCR5, ICOS & Bcl-6) was observed in GALT CD4^+ T cells compared to peripheral blood mononuclear cells. The median expression of CXCR5 on CD32^+ CD4^+ T cells was significantly higher compared to CD32^- CD4^+ T cells in all compartments (terminal ileum 28.6% vs 14.2%, P<0.0001; rectum 38.7.0% vs 29.1%, P=0.0007; PBMC...
14.6% vs. 11.2%, P=0.001). Similarly, median ICOS expression was significantly higher on CD32+ compared to CD32- CD4+ T cells across all compartments in HIV infected individuals (terminal ileum 14.4% vs 4.5%; rectum 19.9% vs 8.4%; PBMC 2.7% vs 0.9%)

Figure 6.3.3 Expression of activation and TFH markers on CD32+ and CD32- CD4+ T cells

(a) HLA-DR expression on CD32+CD4+ T cells compared to CD32-CD4+ T cells in terminal ileal (△), rectal (○) and peripheral blood (□) mononuclear cells from HIV infected participants. Expression of T follicular markers (b) CXCR5 (c) ICOS and (d) Bcl-6 on CD32+ CD4+ T cells compared to CD32- CD4+ T cells in terminal ileal (△), rectal (○) and peripheral blood (□) mononuclear cells from HIV infected participants. Each symbol represents an individual; open symbols indicate CD32- populations and filled symbols indicate CD32+ populations. Bars indicate the median. Comparisons were made using a Wilcoxon test. Abbreviations: T Ileum, terminal ileum; PBMC, peripheral blood mononuclear cells.
all P values <0.0001). Bcl-6, the master transcription factor for TFH differentiation, was also expressed at higher levels on CD32^+ CD4^+ T cells in terminal ileum (6.5% vs 3.2%; P<0.0001), rectum (8.1% vs. 2.6%; P<0.003) and PBMC (1.0% vs. 0.1%; P<0.0001).

I then examined CD32 expression on the *bona fide* TFH population found in tissue, defined as CD4^+CXCR5^+PD1^{high} (Figure 6.3.4a). I confirmed a TFH phenotype based on higher Bcl-6 expression in the CD4^+CXCR5^+PD1^{high} population compared to non-TFH cells, i.e. CD4^+CXCR5^+PD1^{low} and CD4^+CXCR5^- CD4^+ T cells (Figure 6.3.4b); the median Bcl-6 expression in the TFH populations was 30.0% compared to 4.3% in the non-TFH population (P<0.0001). In this GALT tissue TFH population, we observed significantly higher CD32 expression on TFH compared to non-TFH populations; 9.5% and 5.2% respectively, P<0.0001 (Figure 6.3.4c). The CD32 expression on TFH cells and non-TFH cells in tonsil was 8.8% and 4.9% respectively.

**Figure 6.3.4 CD32 expression on TFH in GALT**

The flow plot in (a) is a representative plot of mucosal mononuclear cells from the terminal ileum, illustrating the GALT TFH population based on CXCR5^+ and PD-1^{high} expression. The jitter plot in (b) illustrates the mean Bcl-6 expression from GALT mucosal mononuclear cells (combined terminal ileum and rectum; n=38), on TFH and non-TFH CD4^+ T cells. The jitter plot in (c) illustrates the mean CD32 expression from GALT mucosal mononuclear cells (terminal ileum and rectum; n=38) on TFH and non-TFH CD4^+ T cells. Lines represent the median. P values calculated using a Wilcoxon test. Abbreviations; TFH, T follicular helper cells.
6.3.6. Immune checkpoint marker expression on CD32+ CD4+ T cells

The ICRs PD-1 and TIGIT have been shown to be preferentially expressed on the surface of latently infected CD4+ T cells in peripheral blood during treated HIV infection\textsuperscript{130, 177}. In rectal GALT, PD-1 expression on CD4+ T cells has been shown to have a positive correlation with integrated HIV DNA\textsuperscript{555}. Therefore, I hypothesised that expression of ICRs would be higher in CD32+ CD4+ T cells across anatomical sites. The expression of all ICRs on CD4+ T cells was higher in gut tissue compared to peripheral blood for all ICRs as previously shown in chapter 4. PD-1 and TIGIT expression was significantly higher on the CD32+ CD4+ T cell subsets compared to CD32- CD4+ T cell subsets across all anatomical sites (P<0.002 for all, by Wilcoxon test); the median PD-1 expression on CD32+ CD4+ T cells compared to CD32- CD4+ T cells were 84% vs. 27.7% in terminal ileum, 86.5% vs. 59.6% in the rectum and 26.8% vs. 16.4% in PBMC, respectively. TIGIT expression was also significantly higher on CD32+ CD4 T cells when compared to CD32- CD4+ T cells with median expression of 56.3% vs. 28.1% in terminal ileum, 56.6% vs. 41% in rectum and 15.4% vs. 12.5% in PBMC, respectively. These data are summarised in figure 6.3.5.

Tim-3 expression was significantly higher on CD32+ CD4+ T cells compared to CD32- CD4+ T cells in PBMC (median expression 3.7% vs. 1.9%, respectively) and the rectum (18.2% vs. 9.5%), P<0.001 for both. However, no significant difference in Tim-3 expression was noted between CD32+ and CD32- CD4+ T cells in the terminal ileum (median expression of 6.9% vs. 5.3%, P=0.4).
Figure 6.3.5 Expression of on ICRs on CD32+CD4+ T cells compared to CD32-CD4+ T cells across anatomical sites.

These jitter plots show (a) PD-1 (b) TIGIT and (c) Tim-3 expression on CD32+ and CD32- CD4+ T cells in terminal ileum (△), rectum (○) and peripheral blood (□) mononuclear cells from HIV infected participants. Each symbol represents an individual; open symbols indicate CD32- populations and filled symbols indicate CD32+ populations. Comparisons were made using a Wilcoxon test. The horizontal line indicates the median. Abbreviations: T Ileum, terminal ileum; PBMC, peripheral blood mononuclear cells.

6.3.7. CD32\textsuperscript{high} and CD32\textsuperscript{low} CD4 T cells in PBMCs and tissue

Published work completed by myself and other members of our research group has shown that CD32+ CD4+ T cells in peripheral blood exhibit two phenotypic populations, with differential expression of immune checkpoint and activation markers\textsuperscript{180}. The gating for CD32\textsuperscript{low} and CD32\textsuperscript{high} populations is based on CD32 expression on CD3 negative lymphocyte populations, which include B cells, and express CD32 at high levels (Figure 6.3.6a). The significance of these CD4+ CD32+ populations is unclear. Accordingly, I
examined the phenotype and frequency of CD32^{low} and CD32^{high} expression on CD4^{+} T cells in GALT and peripheral blood. No difference in the frequency of CD32^{low} events was noted across anatomical sites; however, CD32^{high} cells were more common in both GALT sites when compared to PBMC (Figure 6.3.6b); P=0.02, Kruskal-Wallis adjusted for multiple comparisons). The median (IQR) frequency of CD32^{high} cells in terminal ileum, rectum and PBMC was 0.069% (0.026-0.133%), 0.068 (0.024-0.125%) and 0.016% (0.011-0.057%). The frequency of CD32^{high} cells in tonsil tissue was 0.18% After adjusting for multiple comparisons there were significantly higher CD32^{high} CD4^{+} cells in terminal ileum compared to PBMC (P=0.03) and a trend towards higher CD32^{high} CD4^{+} T cells in rectum compared to PBMC (P=0.06), no significant differences were noted between GALT sites (P=0.99). Our earlier work in PBMCs has shown that the CD32^{high} population have a unique phenotype expressing both T cell and B cells markers (CD19, CD20 & TCRαβ), high levels of HLA-DR and lower expression of immune checkpoint markers than the CD32^{low} population. To further characterise this population which is more frequent in GALT I examined activation, immune checkpoint and TFH markers on CD32^{high} CD4^{+} T cells in GALT. PD-1 and TIGIT expression were greatest on the CD32^{low} population in both gut sites; PD-1 expression on the CD32^{low} subset was significantly higher than the CD32^{-} (P<0.0001; Figure 6.3.7) and CD32^{high} population in terminal ileum, a similar pattern was seen in PBMCs. In the rectum, it was also highest in the CD32^{low} subset but only significantly so when compared to CD32^{-} CD4^{+} T cells (P=0.0005; Figure 6.3.7) after adjusting for multiple comparisons. TIGIT expression followed a similar pattern with highest expression on CD32^{low} cells in both terminal ileum and rectum when compared to CD32^{-} CD4^{+} T cells (P<0.0001 and P=0.02, respectively; Figure 6.3.7) and compared to CD32^{high} cells (P=0.002 and P=0.11, respectively), no significant differences between TIGIT expression
Figure 6.3.6 Gating and frequency of CD32high CD4+ T cells across anatomical sites

(a) The flow plot on the left shows three distinct CD32 populations in CD3 negative cells; the gating on CD4+ T cells is based on these populations. Representative gating of the CD32low and CD32high gating on CD4+ T cells is shown on the right. (b) The jitter plot shows the frequency of CD32high T cells in gut sites and PBMCs in HIV infected individuals. Comparisons are made using a Kruskal-Wallis test, corrected for multiple comparisons. Lines indicate the median.
on PBMC CD32 subpopulations after adjusting for multiple comparisons. HLA-DR was expressed at very high levels on the CD32^{high} population in all anatomical sites in keeping with previous findings in the periphery (all P<0.01; Figure 6.3.7).

**Figure 6.3.7 Expression of activation and ICR markers on CD32 expressing CD4^+ T cell populations across anatomical sites**

These jitter plots show (a) HLA-DR (b) PD-1 (c) TIGIT & (d) Tim-3 expression on CD32^{neg}, CD32^{low} & CD32^{high} CD4^+ T cells populations in terminal ileum, rectum and PBMC. Overall P values were calculated using a Kruskal-Wallis test; lines indicate the median. Abbreviations; Neg, negative.

Interestingly the expression of all TFH associated markers was higher on the CD32^{high} subset across all anatomical sites, except for ICOS in the terminal ileum (Figure 6.3.8). CXCR5 expression was significantly higher on CD32^{high} cells, compared to CD32^- cells (P=0.0003 and P<0.0001) and CD32^{low} (P=0.01 & P=0.0007) in the terminal ileum and PBMC respectively; in the rectum, it was again highest in CD32^{high} cells but only achieved statistical significance compared to CD32^- cells (P=0.01). Bcl-6 expression was also
significantly higher on CD32\textsuperscript{high} cells compared to CD32\textsuperscript{−} and CD32\textsuperscript{low} subsets, in all anatomical sites (all P<0.05, after adjusting for multiple comparisons).

**Figure 6.3.8 Expression on TFH markers on CD32 expressing CD4\(^+\) T cell populations across anatomical sites**

The jitter plots show the (a) CXCR5 (b) ICOS & (c) Bcl-6 expression on CD32\textsuperscript{−}, CD32\textsuperscript{low} & CD32\textsuperscript{high} CD4\(^+\) T cell populations in terminal ileum, rectum and PBMC. Overall P values were calculated using a Kruskal-Wallis test; lines indicate the median. Abbreviations; Neg, negative
6.3.8. Relationship of CD32 expression with HIV reservoir

The greatest enrichment of HIV DNA in CD32* cells has been reported in the CD32\textsuperscript{high} population. Therefore, I next investigated whether there was a correlation between HIV DNA and CD32\textsuperscript{high} expressing cells across anatomical sites (Figure 6.3.9). No relationship was observed between HIV DNA and CD32\textsuperscript{high} expressing cells in any site. Similarly, no statistically significant relationship was observed between HIV DNA (log\textsubscript{10} copies per million CD4\textsuperscript{+} T cells) and the frequency of either CD32\textsuperscript{low} expression in PBMCs (r=-0.13 p=64) or in either GALT site (rectum r=0.37, p=0.13 and terminal ileum r=0.46, p=0.12).

While not statistically significant CD32\textsuperscript{low} expression was positively associated with HIV DNA in both gut site, this was not the case in PBMC.

We also tested for associations between HIV DNA and the expression of TFH markers and ICRs. No significant associations were observed in the terminal ileum. Interestingly, in rectal tissue the strongest association observed was between CD32 expression on TFH cells and HIV DNA in the rectum (r=0.66 p=0.003); CXCR5\textsuperscript{+} CD4\textsuperscript{+} T cells and HIV DNA (r=0.46 p=0.055) also showed a borderline line significant association.

Cell sorting experiments

Cell sorting experiments were only performed on PBMCs and not on gut samples due to limited cell numbers. Non-CD4 enriched PBMCs were sorted into the following populations: CD32\textsuperscript{-}, CD32\textsuperscript{low}, CD32\textsuperscript{*CD14\textsuperscript{+}}, and CD32\textsuperscript{high}. DNA was extracted from the sorted cells and total HIV-1 DNA quantified by qPCR. Higher levels of total HIV-1 DNA were noted in the CD32\textsuperscript{low} CD4 T cells populations for most individuals although the level of enrichment was low. Detectable total HIV-1 DNA was present in the CD32\textsuperscript{high} population in 7 out of 9 individuals and enriched in 4 of these. HIV-1 DNA was also detectable in CD32\textsuperscript{*CD14\textsuperscript{-}} sorted cells, see figure 6.3.10
Figure 6.3.9 Relationship between HIV DNA & CD32 expression on CD4\(^+\) cells

Correlations of HIV DNA (Log\(_{10}\) copies per 10\(^6\) CD4\(^+\) T cells) and CD32\(^{low}\) expression on CD4\(^+\) T cells in (a) terminal ileum, (c) rectum & (e) PBMCs. Correlations of HIV DNA (Log copies per 10\(^6\) CD4\(^+\) T cells) and CD32\(^{high}\) expression on CD4\(^+\) T cells in (b) terminal ileum, (d) rectum & (f) PBMCs. Correlations were tested using Spearman’s correlation. Abbreviations: CPM, copies per million; PBMC, peripheral blood mononuclear cell.

(a) Terminal ileum CD32\(^{low}\)

(b) Terminal ileum CD32\(^{high}\)

(c) Rectum CD32\(^{low}\)

(d) Rectum CD32\(^{high}\)

(e) PBMC CD32\(^{low}\)

(f) PBMC CD32\(^{high}\)
Figure 6.3.10 Total HIV-1 DNA levels as measured in sorted CD4 T cell population in PBMCs

The populations were sorted based on CD32 expression (CD32\(^{-}\), CD32\(^{\text{low}}\) and CD32\(^{\text{high}}\)) and CD32\(^{+}\)CD14\(^{+}\) expression as shown in (a). The plot in (b) shows the frequency of HIV DNA per cell in sorted CD4 T cells populations.
6.4. Conclusion

In studies of HIV reservoir in gut tissue, higher HIV DNA levels have been consistently detected in GALT when compared to PBMCs \(^{339, 557}\). Work investigating the role of CD32 as a marker HIV reservoir to date has focused on peripheral blood sampling where one group has reported it as a specific marker for latent HIV infection \(^{558}\), and more recently the lymph node where it was associated with transcriptionally active HIV \(^{181}\). Here, I have explored the phenotype of CD32 expressing CD4\(^+\) T cells and their relationship to the HIV reservoir in GALT from individuals treated in PHI. My data suggest that CD32 expression on GALT CD4\(^+\) T cells is not restricted to or upregulated by HIV infection, but rather that CD32 is highly co-expressed with other markers known to associate with HIV reservoir. My results do not support the role of CD32 as a highly specific marker for latent HIV infection and were unable to demonstrate a clear relationship between CD32 expression on CD4\(^+\) T cells and HIV DNA in either peripheral blood or gut tissue. However, although not achieving statistical significance the direction of the correlation between GALT HIV DNA and CD32 expression was positive, and our relatively small sample size may not have captured a weak association. The positive correlation was observed in both gut sites (but not in peripheral blood) suggesting CD32 expression in tissue sites may be more closely associated with HIV reservoir.

The role of CD32 expression on CD4\(^+\) T cells has not been fully elucidated, but evidence suggests that its expression is an activation-induced response \(^{559, 560}\). Consistent with this, CD32 expressing CD4\(^+\) expressing T cells in both blood and gut tissue sites in this study exhibited high levels of HLA-DR expression. This is in keeping with recent work by Abdel-Mohsen et al. and Wittner et al. which demonstrated high levels of CD69, HLA-DR and CD38 expression on CD32 expressing CD4\(^+\) T cells in lymph node \(^ {181, 561}\). In addition,
activated CD4+ T cells are preferentially infected with HIV and this offers a potential mechanism for enhanced infection of CD32 expressing CD4+ T cells.

Descours et al. reported the greatest enrichment of latent HIV in the CD32\textsuperscript{high} population\textsuperscript{179}. I observed a higher frequency of CD32\textsuperscript{high} cells in GALT sites compared to blood. In addition, this population had the highest expression of the follicular markers CXCR5, ICOS and Bcl-6, consistent with a TFH phenotype. However, no association between CD32\textsuperscript{high} expression and HIV DNA was seen. Due to low cell numbers, particularly from GALT tissue samples, a low frequency of CD32\textsuperscript{high} events was recorded in our study which limits the interpretation of this data.

I have shown that CD32+ CD4+ T cells express significantly higher CXCR5, ICOS, PD-1 and the transcription factor Bcl-6 suggesting that CD32+ CD4 T cells possess a T-follicular like phenotype. Also, TFH cells from both gut sites had significantly higher CD32 expression than non-follicular T cells. This finding may explain the positive, albeit non-significant, correlation seen between HIV DNA and CD32 expression in tissue sites as TFH cells are known to be preferentially infected by HIV and enriched for HIV DNA during treated HIV infection\textsuperscript{136, 345}. In support of this, we observed a significant association between HIV DNA and CD32 expression on TFH cells and between HIV DNA and CXCR5 expression on CD4+ T cells, both in rectal tissue. Our finding of a predominantly TFH phenotype for CD32 expressing CD4+ T cells contrasts with that of Abdel-Mohsen et al. who characterised these cells in the periphery and tonsil and suggested these had a T\textsubscript{H}2 (and to a lesser extent T\textsubscript{H}17) phenotype\textsuperscript{181}. Importantly, I examined the phenotype of bulk, rather than only memory, CD4+ T cells which could explain this discrepancy. It is also possible that these differences reflect biological differences between the periphery, tonsil and GALT – where differences in the size and composition of the HIV reservoir are also known\textsuperscript{132}. 269
Further work is needed to characterise the role of CD32 on these GALT TFH cells. Interestingly, recent work in SLE suggests that activated T-follicular helper cells express Fc receptors which may have a role in controlling their expansion. The results of this study suggest that expression of Fc receptors on TFH cells are involved in facilitating cellular interactions with B cells, an observation which may account for the association seen between total HIV DNA and CD32 expression in tissue sites. In addition to potentially facilitating interactions with B cells, it is also possible that CD32 is acquired on the cell surface from B cells through membrane transfer as a result of the cell-cell interaction (for example, by trogocytosis). This would be an explanation for the TFH phenotype I have described here in GALT, as these are CD4+ T cells which would be commonly interacting with B cells in tissues. It would also be consistent with our previous findings in the periphery that CD32 expressing T cells express both B and T cell markers. Due to sample limitations, I have not been able to directly test this hypothesis here.

In summary, my findings demonstrate that CD32 expression in the GALT is not a highly specific marker for overall HIV reservoir size, but instead identifies a population of activated CD4+ T cells with a T follicular like phenotype and is highly co-expressed with markers known identify latently infected CD4+ T cells.
Chapter 7

Interventions to reduce the HIV reservoir in gut-associated lymphoid tissue

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Chapter 7.

Interventions to reduce the HIV reservoir in gut-associated lymphoid tissue

7.1. Introduction

The results presented in this chapter describe measures of immune function and the HIV reservoir amongst HIV+ study participants receiving immediate ART in PHI with additional interventions. The study presented examined the impact of ART initiated in PHI with a latency reversal agent and a therapeutic HIV vaccine, compared with ART alone on measures of HIV DNA and immune function in a sub-study of participants enrolled into the RIVER trial.

As previously discussed, the HIV viral reservoir is established early in blood and GALT, very soon after HIV acquisition. ART very effectively controls viral replication in the blood CD4+ T-cells below the threshold of detection, usually within 8-12 weeks after starting treatment. Latently infected cells harbour integrated HIV DNA within their genome and form the HIV reservoir. The presence of a reservoir of latently HIV-infected cells means that ART alone is unable to cure HIV infection and interruption of therapy is invariably accompanied by a rebound in plasma viraemia. Pharmacological strategies to reactivate latent HIV have been employed, with the aim of depleting the HIV reservoir to a level below which HIV viral rebound will not occur. One class of agents which have been studied are the Histone Deacetylase (HDAC) inhibitors; as the names suggest they act by inhibiting histone deacetylase enzymes, blocking the removal of acetyl groups from histones, resulting in a relaxed chromatin state, thereby promoting reactivation of HIV. A number of HDAC agents such as vorinostat, panobinostat and romidepsin
have been studied in phase I and II clinical studies in humans. The combination of HDAC inhibitors with HIV therapeutic vaccines has been favoured as a strategy to prime the immune system prior to virus reactivation. This has been coined “kick and kill”, where the kick is the action of the LRA, while immune priming by a therapeutic vaccine provides the kill. Studies using vorinostat as a single agent in HIV infected individuals have demonstrated increases in CA-HIV but have not shown a reduction in the size of the latent HIV reservoir in blood. Elliot et al. also measured HIV CA-RNA and HIV DNA in the rectal tissue of participants receiving vorinostat, reporting a trend towards higher CA-RNA but no change in HIV DNA in the rectum; of note, this was a single arm study. Similarly, panobinostat as a single agent increased levels of unspliced CA HIV RNA but did not impact HIV DNA. Another single arm study used romidepsin in combination with a p24 gag peptide vaccine, with recombinant human granulocyte macrophage colony-stimulating factor (rhuGM-CSF) as local adjuvant, this study showed a reduction of HIV DNA in blood between screening and six weeks post treatment.

The RIVER gut study presented in this chapter studied the use of vorinostat in combination with a prime-boost vaccine in HIV infected individuals. The vaccine used was a combined ChAdV-HIVconsv prime (chimpanzee adenovirus 63-HIVconsv chimeric gene, nonreplicating vector) and MVA-HIVconsv (modified vaccine ankara-HIVconsv gene, nonreplicating vector) boost. This vaccine was designed to elicit broad cytotoxic responses towards fourteen of the most conserved regions of the HIV viral proteome. This vaccine has previously been studied in the phase I trial HIV-CORE-002 in HIV uninfected individuals where it induced broad and durable CD4 and CD8 T cells responses. This vaccine has also be used in studies of HIV infected individuals; the BCN-02 study presented preliminary findings from their single arm study; participants who were treated with ART during PHI received the ChAdV-HIVconsv prime followed by three doses of the...
Figure 7.1.1 Mechanism of Action of HDAC inhibitors

In the absence of HDAC inhibitors, expression of HIV RNA is inhibited by the action of HDAC which catalyses deacetylation of histone tails and keeps chromatin in a compacted state. Inhibition of HDACs by HDACi promotes histone acetylation by HATs leading to relaxation of the chromatin and initiation of transcription. Abbreviations: HDACs, histone deacetylases; HDACi, histone deacetylase inhibitors; HATs, histone acetyltransferases; LTR, long-terminal repeat.


MVA-HIVconsv. In addition, three weekly infusions of rompidespin were given between the 2nd and 3rd MVA-HIVconsv boost. Participants subsequently underwent a treatment interruption. Interestingly, five out of thirteen recipients of the intervention experienced viral control for several months\textsuperscript{418}. However, similar to many of the other HDAC inhibitor studies discussed, there was no control arm in this study. Inclusion of a control arm is particularly important when we consider that spontaneous periods of HIV control have been described in almost 8% of untreated HIV seroconverters\textsuperscript{405}.
There have been no long-term toxicities described in participants who had received 14 days of vorinostat after two years of follow up\textsuperscript{576}. However, the action of HDAC inhibitors is not specific for HIV and potentially off target reactivation of human endogenous retroviruses (HERVs) can occur\textsuperscript{577}. Furthermore HDAC inhibitors induce T cell activation\textsuperscript{567, 578} and vorinostat has been associated with persistent changes in host gene expression up to 84 days post administration\textsuperscript{570}. Therefore, in addition to examining the impact of vorinostat plus a prime-boost vaccine on GALT HIV reservoir, we also compare the activation and immune checkpoint marker expression in GALT between the intervention and the control arm.
Aims
My aim in this chapter was to compare gut measures of HIV reservoir namely; HIV-1 DNA from gut-associated lymphoid tissue and markers of immune function in GALT between study arms in a subset of individuals from the RIVER study. Of note, this was a proof of concept sub study which aimed to enrol five individuals from each study arm.

Hypothesis
The following hypotheses are tested in this chapter are:

i. HIV DNA in GALT will be lower in individuals treated with a HDAC inhibitor and vaccine compared to those treated with ART alone in the RIVER gut study

ii. Immune activation and ICR expression in GALT are similar in the intervention arm and the control arm of the RIVER gut study

Objectives
The objectives of this chapter are:

i. Compare measures of total HIV DNA between gut biopsy samples from terminal ileum, rectum and blood amongst participants randomised to the ART-alone ARM and the ART-plus-intervention of the RIVER trial

ii. Compare markers of immune function between gut biopsy samples from terminal ileum, rectum and blood amongst participants in both arms of the RIVER trial.

iii. Measure p24 expression in gut biopsy culture supernatants 7 days and 21 days post stimulation with PHA and IL-2
7.2. Methods

7.2.1. The RIVER Study

The RIVER trial is a proof of concept-randomised study investigating the impact of immediate ART initiation at the time of HIV acquisition comparing the impact of additional HIV vaccination and latency-reversing agents, vorinostat, a histone deacetylase inhibitor (HDACi) with ART alone (see figure 7.2.1). The study recruited n = 62 participants at five sites in London and the South East of England, namely Imperial College NHS Healthcare Trust, Chelsea and Westminster NHS Foundation Trust, Guy's and St Thomas' NHS Foundation Trust and Brighton and Sussex University Hospitals NHS Trust. All study participants completed the trial protocol by December 2017. The main study primary outcome compared measures of total HIV DNA in blood CD4+T cells between the two arms.

The design of RIVER trial is outlined in figure 7.2.1. ART was commenced in all individuals at enrolment. Randomisation occurred at week 24, eligibility criteria for randomisation included an undetectable viral load, negative HCV RNA, normal renal (eGFR >90mls/min/1.73m² and uPCR <30mg/mmol) and liver function (ALT less than 5 times the upper limit of normal) and QTc interval <440ms. Participants were randomised (1:1) to receive either ART-only or ART plus intervention. For all study participants, four drug ART was given and included a NRTI backbone, a boosted protease inhibitor and raltegravir. The intervention involved administration of a therapeutic vaccination followed by 10 doses of vorinostat. Vaccination involved intramuscular injection (into the deltoid muscle) of a ChAdV-HIVconsv prime (chimpanzee adenovirus 63-HIVconsv chimeric gene, nonreplicating vector) followed eight weeks later by an MVA-HIVconsv (modified vaccine ankara-HIVconsv gene, nonreplicating vector) boost. The first dose of vorinostat was
administered two days after the MVA-HIVconsv boost. The dosing schedule of vorinostat was 400mg orally every third day for a total of ten doses. The final study visit was 18 weeks post randomisation. The RIVER study treatment schedule is summarised in figure 7.2.2. After completion of the main study protocol, participants were invited to enrol in the HEATHER study for long-term follow up. RIVER participants who subsequently enrolled in HEATHER were then invited to take part in the gut study.

**Figure 7.2.1 RIVER Trial Design**

The schema below outlines the design of the RIVER trial. Individuals with confirmed PHI were enrolled and commenced on immediate ART. After 24 weeks of ART, participants were randomised to Arm A: in which individuals continued on ART alone or Arm B; where participants received a prime-boost vaccine and then 21 days of vorinostat. The primary outcome of the study was HIV DNA levels in CD4+ T cells. HIV DNA levels in GALT was the primary outcome of the RIVER gut study.
This figure was adapted with permission from the RIVER study protocol version 4.0

**Figure 7.2.2 RIVER study treatment schedule**

The treatment schedule for the ART-only arm (A) and the ART-plus-intervention arm (B) is shown in the schema below.

7.2.2. The RIVER gut study

Individuals who had completed the RIVER study protocol and who had consented and enrolled into the HEATHER study for long-term follow up were invited to have a gut biopsy as part of the HEATHER gut study protocol. Consenting individuals then underwent a colonoscopy, with terminal ileum and rectal biopsies taken as outlined in the HEATHER gut study procedures in chapter 4. Where possible and to ensure comparable treatment characteristics, the timing of the gut biopsy was within approximately one year of completion of the RIVER study protocol. A second colonoscopy and biopsy were not routinely offered to the RIVER gut study participants. However, two individuals had been enrolled in HEATHER prior to their participation in the RIVER study and who were subsequently randomised to the ART-plus-intervention arm; as such they had pre-vorinostat and vaccine biopsies which were included in the analysis.
7.2.3. Statistical Analysis

The main RIVER study primary endpoint was powered to determine a difference in measures of total HIV DNA from peripheral blood between the two arms with 90% power. However, the RIVER gut study was not powered to determine an impact between study arms and is an opportunistic and exploratory analysis of the impact of HDAC inhibitors on the GALT tissue reservoir. Comparisons were made between the study arms using non-parametric tests. Data from the healthy control cohort described in chapter 4 were included for illustrative purposes.

7.2.4. Measurement of p24 antigen in tissue explant supernatants

These procedures are outlined in detail in the methods chapter. To summarise, two biopsies from the terminal ileum and two biopsies from the rectum were cultured in separate wells (i.e. one biopsy per well). Individual biopsies were placed on gelfoam in a volume of 300 µl of complete media in a 24 well plate (four wells used in total). Two culture conditions for used; one explant from each anatomical site was treated with 5µg/ml of PHA and 100U/ml of IL-2 for 72 hours, during culture complete media was supplemented with 100U/ml of IL-2. The other explants were cultured in complete media only. Culture supernatant was harvested at days 3, 7, 11, 15 and 21. During every harvest, 220 µl of supernatant was removed and replaced with fresh complete media. Harvested supernatant in cryotubes at -80°C. At day 21, in addition to harvesting all the supernatant the remaining explant was transferred to a tube with 500 µl of RNALater, kept at 4°C overnight and then stored at -80°C.
7.2.5. Cytokine analysis on gut explant supernatants

One biopsy from the terminal ileum and rectum was placed on gelfoam in separate wells of 96 well U-bottom plate with 200 µl of complete media each well. The supernatant was harvested at 24 hours.

7.2.6. Total HIV DNA quantification

Total HIV DNA was quantified as outlined in the methods chapter and chapter 4.

7.2.7. ICR and activation marker expression on T cells

Surface expression if ICRs and activation markers and intracellular staining for transcription factors measured by flow cytometry as described in the chapter 2.
7.3. Results

7.3.1. RIVER Gut Study Clinical Characteristics

Eleven individuals were enrolled in the RIVER gut study, five in the ART-only arm and six in the ART-plus-intervention arm (receiving vorinostat and vaccine), all were male. The arms were well matched with no significant differences between characteristics. However, a trend towards higher CD4 count at biopsy was observed in the ART-only arm (P=0.06). Importantly, time from ART start to biopsy and time from finishing the RIVER protocol were not significantly different. The baseline characteristics are outlined in table 7.2.1

Table 7.3-1 Clinical Characteristics of the RIVER gut study

<table>
<thead>
<tr>
<th></th>
<th>ART-plus-intervention Arm (n=6)</th>
<th>ART-only Arm (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Age</td>
<td>39 (30-45)</td>
<td>35 (31-35)</td>
</tr>
<tr>
<td>PHI diagnosis criteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p24 Ag+/HIV Ab-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Negative to Positive test</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>RITA</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Results at Baseline*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV viral load, Log_{10} CPM</td>
<td>4.1 (3.9-5.3)</td>
<td>4.9 (4.6-5.6)</td>
</tr>
<tr>
<td>CD4 count cells/mm³</td>
<td>492 (435-561)</td>
<td>543 (449-548)</td>
</tr>
<tr>
<td>CD8 count cells/mm³</td>
<td>966 (810-1203)</td>
<td>879 (860-905)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.55 (0.35-0.90)</td>
<td>0.54 (0.49-0.58)</td>
</tr>
<tr>
<td>Time from EDI to ART start, days</td>
<td>75 (35-79)</td>
<td>65 (33-82)</td>
</tr>
<tr>
<td>Time from a positive HIV test to ART, days</td>
<td>23 (15-28)</td>
<td>13 (7-18)</td>
</tr>
<tr>
<td>Completed RIVER protocol to biopsy, months</td>
<td>9 (6-13)</td>
<td>6 (6-14)</td>
</tr>
<tr>
<td>Time from ART start to biopsy</td>
<td>22 (21-24)</td>
<td>17 (16-22)</td>
</tr>
<tr>
<td>Results at time of Biopsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 count cells/mm³</td>
<td>669 (626-904)</td>
<td>1058 (908-1167)</td>
</tr>
<tr>
<td>CD8 count cells/mm³</td>
<td>539 (480-571)</td>
<td>623 (572-668)</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.40 (1.18-1.5)</td>
<td>1.60 (1.28-2.13)</td>
</tr>
<tr>
<td>HIV viral load</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*Values given represent n (%) categorical variables and median (interquartile range) for continuous variables. Baseline refers to measure closest to serconversion. Abbreviations: EDI, estimated date of infection; RITA, recent infection testing algorithm
7.3.2. Total HIV DNA level in the GALT in the RIVER gut study

I first compared HIV DNA levels between study arms in GALT. Using combined data from the terminal ileum and the rectum, I observed higher HIV DNA, expressed as CPM gut cells in the ART-only arm compared to the ART-plus-intervention arm (P=0.04). However, when I compared the arms in the individual GALT sites, this was no longer statistically significant. The median (IQR) total HIV DNA in the terminal ileum was 2.8 and 3.1 in log$_{10}$ CPM gut cells in the ART-plus-intervention and ART-only arms respectively (P=0.25), while in the rectum it was 2.8 and 3.0 in log$_{10}$ CPM gut cells respectively (P=0.14). There was no difference between study arms in PBMC HIV DNA when measured in non-CD4 enriched cells (P=0.40). These data are summarised in figure 7.3.1.

**Figure 7.3.1 HIV DNA (copies per million cells) in the RIVER gut study**

The jitter plots below show the HIV DNA level expressed in (a) GALT expressed as copies per million gut cells and (b) PBMCs, expressed as copies per million PBMCs in the ART-plus-intervention arm (blue) and the ART-only arm (green) of the RIVER gut study. The horizontal line indicates the median. P-values were calculated using a Mann-Whitney test.

(a) Terminal Ileum and Rectal HIV DNA  (a) PBMC HIV DNA

<table>
<thead>
<tr>
<th>HIV DNA CPM gut cells</th>
<th>P=0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART plus intervention ARM</td>
<td>ART Only ARM</td>
</tr>
<tr>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIV DNA CPM PBMC</th>
<th>P=0.40</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART plus intervention ARM</td>
<td>ART Only ARM</td>
</tr>
<tr>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>3.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

I next tested for differences between the study arms when HIV DNA was measured in CPM CD4 T cells. Combined GALT data was not significantly different between study arms. In the terminal ileum, the median HIV DNA was 3.5 and 3.7 log$_{10}$ CPM CD4 T cells.
in the ART-plus-intervention and ART-only arms respectively (P=0.61), while in the rectum it was 3.4 and 3.7 log\(_{10}\) CPM CD4 T cells respectively (P=0.31). Similarly, in PBMCs no difference was seen between arms (P=0.13). These data are summarised in figure 7.3.2.

**Figure 7.3.2 HIV DNA (copies per million CD4 T cells) in the RIVER gut study**

The jitter plots below show the HIV DNA, expressed as copies per million CD4 T cells in (a) terminal ileum and rectal GALT and (b) PBMCs in the ART-plus-intervention (blue) and ART-only (green) arms of the RIVER gut study. Horizontal lines indicate the median. P-values are calculated using a Mann-Whitney test.

(a) Terminal Ileum and Rectal HIV DNA

(b) PBMC HIV DNA

Higher median HIV DNA was observed in the ART only arm for all measures of HIV DNA in all sites, although this was not statistically significant. Therefore, given the small sample size in each arm, I compared the RIVER dataset with another cohort of treated PHI, the HEATHER cohort. This allowed interpretation of the results in the context of a larger dataset. The levels of HIV DNA (both CPM cell and CPM CD4 cells) were comparable between the HEATHER cohort and both arms of the RIVER gut study suggesting that the differences observed are accounted for by the expected spread of the gut HIV DNA data. The comparisons of HIV DNA levels (CPM CD4 T cells) between the HEATHER and RIVER study arm in both the terminal ileum (overall P=0.69) and rectum (overall P=0.47) is shown in figure 7.3.3 (a) and (b) respectively.
7.3.3. p24 measured in tissue explant supernatant

Terminal ileum and rectal tissue explants were cultured for 21 days under stimulated (with PHA and IL-2) and unstimulated conditions. Data was available on four participants from the ART-plus-intervention arm and three from the ART-only arm. The supernatant harvested at days 7 and 21 was used to measure p24 gag expression. Overall levels of p24 were low with a range of 2.5-4.2 pg/ml. Activation of the explants (combined terminal ileum and rectum) resulted in higher p24 expression at day 7 (P=0.003) but not at day 21 (P=0.64), see figure 7.3.4 (a). The increase in p24 at day 7 was significant in the rectum (P=0.05) with a trend toward significance in the terminal ileum (P=0.06). I next tested for differences in p24 expression between study arms. While median p24 levels were consistently higher in samples from the ART-plus-intervention arm in both resting and activated samples, none of these differences were statistically significant. The p24 expression at day 7 and day 21, in resting and activated tissue explants from the terminal ileum and rectum, across study arms are shown in see figures 7.3.4 (b) and 7.3.4 (c)
Figure 7.3.4 p24 expression in tissue explants

The jitter plots below compare p24 expression (a) under resting and activated conditions at days 7 and 21, in (b) resting and (c) activated tissue explants across the vorinostat (blue) and ART-only (green) arms and (d) between the rectum and terminal ileum. (e) shows the correlation between HIV DNA and p24 expression at D7 in activated explants. P values were calculated using a Wilcoxon test for paired samples and a Mann-Whitney test for unpaired samples. Lines represent the median. The horizontal dotted line in (a) represents the negative wells. Abbreviations: TI, terminal ileum; D7, day 7; D21, day 21. VOR, vorinostat arm; ART, ART only arm.

(a) p24 Pre- and Post-Activation

(b) p24 in Resting Explants

(c) p24 in Activated Explants

(d) p24 in Rectum and Terminal Ileum

(e) p24 and HIV DNA: Day 7 Activated

Legend

△ Terminal Ileum
○ Rectum
△ Vorinostat Arm
○ ART Only Arm

P=0.003
P=0.64

P=0.63
P=0.40
P=0.50
P=0.54

P=0.29
P=0.54
P=0.80
P=0.68

P=0.93
P=0.13
P=0.03
P=0.14

P=-0.2
P=0.5
Using combined data from both study arms, I assessed for a difference in p24 expression between the rectum and the terminal ileum, figure 7.3.4 (d). At day 7, no differences were seen between groups in resting (P=0.93) or activated (P=0.13) tissue explants. At day 21, p24 was significantly higher in resting rectal tissue explants (P=0.03) and remained higher in the activated rectal explant tissue, but this was no longer statistically significant (P=0.14).

Finally, I assessed for any correlation between p24 measured in culture supernatants and HIV DNA at all time points and culture conditions, no statistically significant associations were observed. A representative plot is shown for activated tissue explants day 7 in figure 7.3.4 (e).

7.3.4. ICR and Activation marker expression

Previous studies using HDAC inhibitors have shown increased expression of markers of immune activation and increased gene expression associated with their use\textsuperscript{414, 569, 570}. Therefore, I compared the expression of ICRs and activation markers in the study arms of the RIVER gut study, to determine any differences in the expression of these markers in the terminal ileum and rectal tissue post vorinostat and therapeutic vaccination. Overall no significant differences in the expression of ICRs in GALT were observed between study arms. The expression of ICRs on CD4 and CD8 T cells extracted from both terminal ileum and rectal tissue samples is shown in figure 7.3.5. In the CD4 T cell compartment expression of PD-1 in terminal ileum (58.9% and 48.9% P=0.55), rectum (71.5% and 69.4%, P=0.54) and PBMC (29.5% and 16.7%, P=0.31) was similar between the ART-plus-intervention and ART-only arms. Similarly, no significant differences were observed for TIGIT and Tim-3 expression on CD4 T cells in the terminal ileum (P=0.55 and P=0.84 respectively), rectum (P=0.99 and P=0.93 respectively) and PBMCs (P=0.15 and P=0.60 respectively).
respectively). In the CD8 compartment, median PD-1 expression was higher in the ART-plus-intervention arm compared to the ART-only arm in GALT. However, these differences were not significantly different (terminal ileum 13.8% and 6.32%, P=0.31; rectum 45.4% and 35%, P=0.18). Similarly, TIGIT expression on CD8 T cells was higher in the ART-plus-intervention arm compared to the ART-only arm in GALT sites but not significantly so (terminal ileum 32.4% and 28.3%, P=0.84; rectum 49.9% and 37.9%, P=0.33). Tim-3 expression on CD8 T cells was similar between study arms in all sites (terminal ileum 13.1% and 9.6%, P=0.55; rectum 21.1% and 22.2%, P=0.66; PBMC 7.5% and 8.4%, P=0.42).

Activation marker expression was also similar across study arms. Median co-expression of HLA-DR and CD38 on CD4 T cells in the ART-plus-intervention arm compared to the ART-only arm was 1.6% and 2.0%, P=0.42, respectively in the terminal ileum, and 2.97% and 4.8%, P=0.93 in the rectum. In PBMCs, there was a trend towards higher HLA-DR & CD38 co-expression in the ART-plus-intervention arm compared to the ART-only arm, 2.2% and 0.9%, P=0.06. In the CD8 compartment, there were similar levels of HLA-DR and CD38 co-expression in the ART-plus-intervention and ART-only arms; in the terminal ileum (0.8% and 0.7%, P=0.99), rectum (3.38% and 5.86%, P=0.66) and PBMCs (3.6 and 1.7%, P=0.55). These results are summarised in figure 7.3.6.
Figure 7.3.5 Exhaustion marker expression in the RIVER gut study

The jitter plots below compare the expression of (a) PD-1, (b) TIGIT & (c) Tim-3 on CD4 T cells and (d) PD-1, (e) TIGIT & (f) Tim-3 on CD8 T cells between the ART-plus-intervention (blue) and ART only (green) arm of the RIVER gut study, across anatomical sites. *P*-values were calculated using a Mann-Whitney test. Abbreviations: T Ileum, terminal ileum.
Figure 7.3.6 Activation marker expression in the RIVER gut study

The jitter plots below compare the expression of (a) HLA-DR, (b) CD38 & (c) HLA-DR & CD38 co-expression on CD4 T cells and (d) HLA-DR, (e) CD38 & (f) HLA-DR & CD38 co-expression on CD8 T cells between the ART-plus-intervention arm (blue) and the ART only (green) arm of the RIVER gut study, across anatomical sites. P-values were calculated using a Mann-Whitney test.

Legend

△ Terminal ileum

〇 Rectum

□ PBMC
7.3.5. Longitudinal HIV DNA sampling

Two individuals enrolled in the RIVER gut study had gut biopsies prior to enrolment into the RIVER trial and were subsequently randomly allocated to the ART-plus-intervention study arm. These participants designated RIV10 and RIV12 had their first gut biopsies taken eight and six months prior to vorinostat and vaccination administration. The HIV DNA levels in GALT for these individuals were very similar pre- and post- the study intervention.

When measured as HIV DNA CPM gut cells, the HIV DNA level in the terminal ileum for participant RIV10 was 2.63 log CPM prior to the randomisation and 2.64 log CPM post the intervention; in the rectum, it was 2.46 and 2.49 log CPM respectively. For participant RIV12, the level in the terminal ileum was 2.65 log CPM pre- and 2.72 log CPM post-intervention, while in the rectum it was 2.27 log CPM and 2.41 log CPM. These differences were within the range of variability of the assay.

When measured as HIV DNA CPM CD4 T cells, the pre-randomisation and post-intervention levels for participant RIV10 were 3.41 and 3.34 log CPM CD4 T cells respectively in the terminal ileum and 3.05 and 3.11 log CPM CD4 T cells respectively in the rectum. For participant RIV12 the pre- and post- intervention levels were 3.85 and 3.52 log CPM CD4 T cells respectively in the terminal ileum and 2.97 and 2.88 log CPM CD4 T cells respectively in the rectum. Figure 7.3.7 shows the HIV DNA levels in CPM gut cells and CPM CD4 T cells, at the time of the first and second gut biopsy for participants RIV10 and RIV12.
**Figure 7.3.7 HIV DNA levels pre- and post vorinostat and vaccination**

HIV DNA levels in (a) CPM gut cells and (b) in CPM CD4 T cells pre- and post vorinostat and therapeutic vaccination in two participants of the RIVER gut study. Participant ID is shown on the right. Abbreviations: CPM, copies per million; VOR, vorinostat and therapeutic vaccination.

### 7.3.6. Markers of inflammation and microbial translocation

Reactivation of latent HIV may have unintended detrimental consequence in GALT including increased microbial translocation and resulting systemic inflammation. To test for this, I compared plasma level of sCD14, MAdCAM-1, sCD163, IL-6, IP-10 and sCD40 in the ART-plus-intervention arm and ART-only arm of the RIVER gut study. sCD163 levels were significantly higher in the ART-plus-intervention arm compared to the ART-only arm (P=0.03). No other significant differences in the plasma levels of any other of these markers were observed between the study arms. These results are summarised in figure 7.2.10 below. Finally, we measured IL-17A in the terminal ileum and rectal explant supernatants which were harvested after 24 hours of culture. No significant differences in IL-17A were measured between study groups in either the terminal ileum (P=0.48) or rectum (P=0.61).
Figure 7.3.8 Markers of Inflammation and microbial translocation in the RIVER gut study

The levels of (a) sCD14, (b) MAdCAM-1, (c) sCD163, (d) IL-6, (e) IP-10 and (f) sCD40 in plasma samples of the ART-plus-intervention (blue) and ART only (green) arms of the RIVER gut study are shown. The horizontal line indicates the median. P-values were calculated using a Mann-Whitney test. Abbreviations: s, soluble.
7.4. Conclusion

7.4.1. Discussion

These data suggest that vorinostat in combination with a therapeutic vaccine did not reduce the HIV DNA reservoir in the gut-associated lymphoid tissue of ART-treated individuals during PHI compared with ART alone. Median HIV DNA levels in GALT were lower in the ART-plus-intervention group in our study but did not achieve statistical significance for individual GALT sites. The small sample size likely accounts for the observed difference between groups as the overall range of HIV DNA levels in the RIVER gut study was similar to the range seen in the HEATHER gut study. In addition, in the two individuals with longitudinal HIV DNA sampling, pre and post-vorinostat and therapeutic vaccination HIV DNA levels were similar. Our findings are in keeping with other studies which have used HDAC inhibitors alone or in combination with other vaccination strategies\textsuperscript{409, 571, 579}. Only one study, which used the HDAC inhibitor romidepsin in combination with a p24 gag peptide vaccine, has demonstrated a reduction in HIV DNA in blood\textsuperscript{572}. Ultimately our gut sub-study of RIVER and those published to date have not been powered to detect a difference in HIV DNA levels in GALT or lack a control arm \textsuperscript{570, 574} and a larger randomised study with longitudinal biopsies in gut sites will be required to answer this question definitively.

The CLEAR study examined the use of panobinostat alone in fifteen HIV infected individuals and showed no durable reduction in HIV DNA levels in blood \textsuperscript{571}; sigmoid colonic biopsies were taken in nine individuals pre- and post- panobinostat with no difference seen in HIV DNA levels pre- and post- panobinostat\textsuperscript{571, 579}. A separate analysis from this study suggested that panobinostat was associated with decreased CD69 expression and increased IL-17A expression in colonic intestinal epithelium\textsuperscript{579}. We did
not see any differences IL-17A levels between study arms in either the terminal ileum or the rectum. However, unlike the CLEAR study where biopsies were taken soon after panobinostat treatment, gut biopsies in the RIVER gut study were taken approximately ten months after the intervention, suggesting that any increases seen in the CLEAR study may not be sustained. There is only one other study to date which has examined the impact of ART plus vorinostat on HIV DNA levels in GALT; Elliott et al. in a single arm study took longitudinal rectal tissue biopsy samples pre and post a two-week course of vorinostat\textsuperscript{569}. While they observed a transient increase in HIV CA-RNA in plasma, no differences were seen in with CA-RNA or HIV DNA levels in rectum tissue pre- and post-vorinostat treatment\textsuperscript{570}. I attempted to measure CA-RNA in DNAase treated RNA samples from terminal ileum and rectum but was unable to consistently detect a meaningful signal with our current assay. In those with detectable CA-RNA, the background signal (i.e. the signal from the non-reverse transcribed sample) was in the range of the reverse-transcribed samples suggesting DNA contamination. Therefore, CA-RNA was not measured in our samples.

Quantitative viral outgrowth assays (QVOA) are considered the gold standard in measuring the latent HIV reservoir in stimulated PBMCs\textsuperscript{580}. Based on this principle I attempted to measure the viral outgrowth potential of tissue explants by measuring p24 in tissue supernatant in stimulated explants at day 7 and 21. Explant stimulation with PHA and IL-2 resulted in an increase in p24 at day 7 of culture but not at day 21. This may relate to the poorer viability of the explant at day 21 compared to day 7. The traditional QVOA requires 14-21 days due to the relative insensitivity of the p24 ELISA\textsuperscript{580}; however, this may not be feasible given the poorer viability of tissue explants in more extended culture. Newer ultrasensitive p24 assays have been developed and may allow for p24 quantification at an earlier time-point\textsuperscript{581}. Another strategy to overcome the poor sensitivity
may be to use RT-qPCR for detection of HIV RNA in culture supernatants. I did not demonstrate any differences in p24 expression between study arms. However, I did detect higher p24 in rectal tissue compared to terminal ileum at day 21, suggesting greater potential for reactivation in this site. With further development, this novel approach may be a useful tool to study and characterise the HIV reservoir in tissue sites.

The long-term safety of HDAC inhibitors in PLWH is an important focus of HIV cure research, given the safety and efficacy of current ART regimens. Work by Mota et al. reported on virologic and immunological outcomes two years post ART plus vorinostat therapy, they found no significant changes in HIV CA-RNA, HIV DNA, plasma RNA, CD4 or CD8 T cell count. The RIVER gut study investigated the impact of ART-plus-intervention on immune activation and exhaustion markers in tissue sites; considering the hypothesis that reactivation of HIV in tissue may induce T cell activation and exhaustion. There were no significant increases in ICR or activation marker expression in GALT. However, a trend toward higher HLA-DR & CD38 co-expression on CD4 T cells was noted in PBMCs compared with ART alone. Recent evidence also suggests that HDAC inhibitor use may result in off-target reactivation with reports of transcriptional modulation of HERVs following vorinostat treatment. Contrary to this, previous studies with panobinostat have suggested a role for HDAC inhibitors in reducing inflammation in HIV; Hogh et al., reported lower plasma levels of interleukin-6, matrix metalloproteinase 9, E-selectin and soluble CD40 ligand with ART plus panobinostat treatment. While there was no elevation of the majority of markers of inflammation or microbial translocation in the RIVER gut study, higher sCD163 levels were observed in the ART-plus-intervention arm raising the possibility that HDAC inhibitor in combination with therapeutic vaccine use may contribute to microbial translocation and thus this finding warrants further investigation.
In Summary, vorinostat with therapeutic vaccination compared with ART-alone did not impact gut HIV reservoir in the RIVER study. However, this pilot study was limited by the small sample size. Also, HIV DNA may not be the optimal measure of the latent reservoir in tissue to assess in kick and kill intervention studies; as such we have trialled a novel assay which may have utility in measuring tissue viral outgrowth in future studies. Importantly there was no durable impact of ART-plus-intervention over ART-alone on activation or exhaustion marker expression in GALT. However, measures of microbial translocation were higher in the ART-plus-intervention group compared with ART alone, and these should be included in any future studies using HDAC inhibitors and/or therapeutic vaccines to monitor unintended negative consequences of their use.
Chapter 8
Discussion
Chapter 8.

Discussion

Latent HIV infection presents the main obstacle to HIV cure. The overarching aim of this thesis was to characterise the HIV reservoir and associated markers of immune dysfunction during treated PHI, in the largest anatomical collection of cells contributing to HIV persistence - those within GALT.

The main findings from this thesis are:

1. ART initiated in PHI enhances CD4/CD8 ratio normalisation
2. CD4/CD8 ratio correlates with HIV reservoir as measured by total HIV DNA in blood and gut sites
3. HIV DNA levels differ between the terminal ileum, rectum and PBMCs, with the highest levels observed in the terminal ileum
4. GALT measures of immune function are persistently abnormal despite ART initiated in PHI and years of suppressed viral replication, with evidence of compartmentalised immune recovery.
5. HIV DNA in GALT correlates with bacterial translocation and systemic inflammatory markers
6. Markers of TFH activation are associated with higher HIV DNA while higher fCD8 cells are associated with lower HIV DNA
7. CD32 is not a specific marker for HIV DNA in GALT but instead identifies CD4 T cells with an activated TFH phenotype
8. Vorinostat in combination with therapeutic vaccination in treated PHI did not impact upon the gut HIV reservoir.
I have identified the CD4/CD8 ratio as a biomarker of both immune function and HIV DNA in blood and PBMCs during treated PHI. In the era of ART, the traditional markers of HIV disease progression such as the total CD4 T cell count and the HIV viral load, continue to play a role identifying those individuals at risk of AIDS-related morbidity and disease control by ART respectively\textsuperscript{461}. However, these measures of HIV disease do not capture those with subtler immunological dysfunction which contributes to non-AIDS morbidity and mortality. The dramatic improvement in treatment strategies for PLWH since the discovery of the virus more than thirty years ago means that the expectation of therapy for the majority of PLWH is suppression of virus with substantial improvements in life expectancy\textsuperscript{8, 433, 460}. The findings from the START trial demonstrated that ART is advantageous for all PLWH regardless of CD4 count\textsuperscript{104}. Also, this study highlighted that a proportion of non-AIDS events are not predicted by CD4 T cell count alone. Studies have demonstrated the utility of CD4/CD8 ratio as an alternative predictive surrogate marker indicative of immune activation and immune exhaustion\textsuperscript{169, 171}, and a lower CD4/CD8 ratio has been associated with an increased risk of adverse clinical outcomes such as cardiovascular events and cancer\textsuperscript{169, 584}.

The data presented on CD4/CD8 ratio addresses all three of my aims outlined in the introduction. In chapter 4, I characterised the CD4/CD8 ratio across anatomical sites in treated PHI and have shown anatomical variation in normalisation of this biomarker. In chapter 3, I confirm the association between markers of immune dysfunction and CD4/CD8 ratio in PBMCs, illustrating a relationship between CD4/CD8 ratio and markers of T cell activation and exhaustion. Importantly, I show this in a PHI-treated population, a cohort where greater immune recovery is expected compared to chronically treated individuals and in whom the range of CD4/CD8 ratio may not have been sufficient to detect subtle immunological abnormalities. This was not the case, and I have shown that
CD4/CD8 ratio is an important marker of immune dysfunction in treated PHI. This is in keeping with findings from other groups who have shown an association between CD4/CD8 ratio and immune activation, exhaustion and clinical outcomes\textsuperscript{170, 448, 453, 585, 586}.

In chapter 4, I contribute novel insights into the role of the CD4/CD8 ratio as a marker of tissue reservoir, showing that CD4/CD8 ratio measured in blood was associated with the size of the HIV reservoir in GALT, in both the terminal ileum and rectum. While CD4/CD8 ratio has previously been shown to associate with HIV DNA in PBMCs \textsuperscript{172}, this has not been previously reported in GALT.

Findings from the VISCONTI, SPARTAC and CASCADE cohorts provide evidence that early ART in PHI may contribute to post-treatment viral control\textsuperscript{131, 405, 587}. As such, this thesis has focused on a similar cohort of individuals initiated on ART during PHI. While other investigators have demonstrated the benefit of early ART on CD4\textsuperscript{109} count and markers of immune activation\textsuperscript{371}, the data in chapter 3, is the first to demonstrate the enhanced normalisation of CD4/CD8 ratio in early treated individuals\textsuperscript{377}.

Identification of suitable markers to inform studies investigating HIV eradication or viral control was the third aim of this work and is a priority for the field of HIV cure research. It is particularly important to validate these markers in treated-PHI individuals as a proportion of these individuals may control the HIV virus on treatment cessation. In Chapter 3, I have shown that CD4/CD8 ratio is associated with the time to disease progression and time to viral rebound on treatment cessation; concluding that CD4/CD8 may be used as a component of an algorithm to predict sustained viral control after treatment interruption.

The CD4/CD8 ratio is measured routinely as part of HIV clinical care; this is particularly relevant when we consider HIV cure in a global context where the aim is to cure all individuals living with HIV, including those in resource-limited settings. The HIV epidemic
remains concentrated in sub-Saharan Africa and other emerging economies. Monitoring of treatment in these locations most heavily affected by HIV is often limited to lymphocyte subset counts, and even viral load testing is beyond the capacity of some healthcare systems. Therefore, if interventions aimed at HIV cure prove successful; it is vital to have affordable, discriminatory immune markers with the ability to predict those with the greatest chance of success; CD4/CD8 ratio may be such a marker.

The gut HIV reservoir has garnered much interest as an obstacle to HIV cure \(^{185, 472}\) and is the primary focus of this thesis. Several important publications have recently provided evidence that this tissue reservoir may support ongoing viral replication and persistence on ART\(^{183, 185}\). Characterising the HIV reservoir and immune function in GALT amongst individuals starting ART in PHI enhances our understanding of the biology of the reservoir and immunological differences between this compartment and blood, where such differences may be important in facilitating HIV persistence. In addition, the extent of immune recovery in each anatomical site may be relevant in predicting those who might control viral replication on ART cessation. While I have not shown direct evidence of ongoing viral replication in gut tissue sites, I have shown higher expression of markers associated with HIV persistence, such as activation and exhaustion markers, in gut sites with different patterns of immune recovery identified between blood and gut, and within individual gut sites. There was no significant correlation observed in the gut between these markers and the HIV reservoir in our study; this lack of correlation may relate to the measurement of these markers on bulk CD4 T cells rather than on memory T cell subsets, the latter being more relevant for latent HIV infection. Other groups have found a relationship between integrated HIV DNA and PD-1 expression on CD4 T cells in rectal tissue in the gut\(^{318, 555}\), but this metric of the reservoir was not measured the HEATHER gut study.
The data in chapter 4 highlights differences in the expression of markers of T cell activation and exhaustion and inflammatory cytokines in gut sites suggesting compartmentalised immune recovery in GALT with treated PHI, these differences in the immune milieu in GALT may be significant in supporting HIV persistence along the GI tract\textsuperscript{588}. In support of this idea, the HEATHER gut study is the first to demonstrate a significantly higher level of HIV DNA in one gut site compared to another. Yukl \textsuperscript{132}, Chun \textsuperscript{589} and others have demonstrated higher HIV reservoir in gut compartments compared to PBMCs but did not observe a statistically significant difference between individual gut sites\textsuperscript{132}. The finding of higher HIV DNA in the terminal ileum warrants further study; work by other groups has identified the terminal ileum as a potentially important site for HIV persistence \textsuperscript{191}, while Estes et al. identified the gut as the site with highest HIV RNA production on ART\textsuperscript{185}. Raltegravir intensification of standard ART resulted in a decrease in unspliced CA HIV RNA in the terminal ileum suggesting ongoing productive infection in this site\textsuperscript{191}. I was unable to measure CA HIV RNA in gut sites accurately but found increased expression of CD4 T cell immune activation markers and cytokines in the terminal ileum in the HEATHER gut study, supporting the assertion that this may be a site of productive infection. Cell to cell transmission of HIV provides a potential mechanism for HIV transmission in lymphoid tissue\textsuperscript{194}; such transmission of HIV would still efficiently trigger the pyroptotic death of CD4 T cells\textsuperscript{590} allowing progressive depletion of CD4 cells and ongoing inflammation despite suppressive plasma viremia. While we did not recruit a significant number of chronically treated individuals to the HEATHER gut study, it is also possible that the timing of ART initiation contributed to the difference in the burden of the reservoir along the GI tract, as such differences were not observed in studies of chronically treated individuals \textsuperscript{300, 335}. It is reassuring that the measures of the HIV reservoir in
independent anatomical sites correlated with each other. Therefore, in clinical studies measurement of the reservoir in one gut site may be sufficient predicting overall HIV reservoir burden. However, studies exploring the mechanisms of persistence and compartmentalised immune recovery would be advised to include sampling from more than one tissue site.

Given the nature of tissue sampling from GALT, it is important to highlight some limitations of my work. Random biopsies are taken from multiple sites to attain representative samples; however lymphoid tissue may not be obtained at each biopsy. For flow cytometry work six to eight pooled biopsy samples were combined to increase the probability of representative sampling. In addition, the limited samples that could be safely taken at biopsy meant that a limited yield of RNA and DNA could be extracted from biopsy samples. Therefore, despite initial intentions to measure HIV reservoir with more than one method, I decided to use one robust method of reservoir quantification in which quantification would be possible even with limited cellular input. I developed and optimised the technique for DNA extraction and quantification of HIV reservoir from gut biopsies. Several methodologies were tested to improve DNA yield from biopsy samples, including Trizol and Qiagen column extraction from collagenase digested mononuclear cells. Ultimately, extraction of DNA from tissue stored in RNA later yielded the greatest quantities of HIV. Yukl et al. previously tested HIV DNA quantification using samples which had been collected by snap freezing or processed immediately by collagenase digestion and reported similar results with both methods. The RNA later method described in this thesis has advantages over those previously described methods in that no specialised equipment or processing is required on the day of collection, making it particularly suitable for clinical settings and large-scale clinical trials. In future studies, use of this method could allow larger yields of DNA and allow measurement of the reservoir in GALT using more
than one method, allowing further characterisation of the gut HIV reservoir. However, it is important to note that this method does not allow for retrieval of a single cell suspension of mucosal mononuclear cells which could subsequently undergo cell sorting.

I have also identified a potential link between HIV gut reservoir and ongoing bacterial translocation as measured by sCD14. HIV reservoir levels in both the terminal ileum and rectum were associated with sCD14. A potential explanation for this finding is that higher viral production from those with larger gut reservoirs, contributes to greater viral production and immune damage, reducing local gut epithelial integrity and resulting in higher markers of bacterial translocation in plasma. Alternatively, poor immune recovery in the gut may sustain ongoing infection due to the availability of preferential targets for ongoing infection.

The link between HIV DNA and microbial translocation has been made in one previous study looking at chronically treated HIV infected individuals, in that study LPS levels correlated with HIV DNA levels in rectosigmoid biopsies\textsuperscript{334}. In addition, the HEATHER gut study data showed increased levels of inflammatory cytokines in the plasma and GALT of individuals with treated PHI compared to controls. In the lasso regression models in chapter 4, a number of proinflammatory cytokines were associated with increased gut reservoir supporting the hypothesis than ongoing bacterial translocation and inflammation associated with gut HIV DNA, with a higher level of most inflammatory cytokines observed in the terminal ileum compared to the rectum. My data also supports the investigational use of therapeutics which aim to reduce bacterial translocation, not only to limit immune activation but also to limit HIV DNA. Previous studies using rifaximin have shown a marginal benefit with respect to measures of bacterial translocation in immune non-responders\textsuperscript{591}. Interestingly, Dillion et al. have recently shown a compartmentalised type I interferon response during chronic untreated HIV infection, with IFN-β being more predominant in the gut. This may be relevant for strategies aiming to reduce bacterial
translocation; Dyavar et al. have shown in the macaque model that PD-1 blockade reduced IFN signalling which in turn was associated with increased expression of genes involved in the repair of tight junctions in the gut epithelium and decreased LPS levels\textsuperscript{592}. Use of these agents in PHI in addition to ART may have a role in limiting microbial translocation and gut HIV reservoir.

In this thesis I also characterised one of the most important cellular reservoirs of latent HIV in the gut tissue - TFH cells, these are an established niche for latency, and many groups have previously shown that TFH are preferential targets of HIV infection with increased frequency of proviral DNA in this cell type\textsuperscript{134, 136, 343, 351, 378, 593}. In addition, these cells support the antibody response to HIV meaning their dysfunction would limit the immune response to HIV\textsuperscript{236, 349, 594}. In chapter 5, I demonstrated that pTFH are preserved with ART initiated in PHI but did not show an association between HIV DNA and pTFH on ART with traditional viral and immune factors being more predictive of HIV DNA. In contrast to this, I did observe an association between the reservoir and TFH activation in gut sites. I also found an independent association between sCD40, a marker of the B cell – T cell interaction and HIV DNA in GALT. CD32 has also been cited as a marker of HIV reservoir\textsuperscript{179} and while I did not observe a correlation between this marker and HIV DNA in the gut, CD32 did identify cells with an activated TFH phenotype. Taken together, these data suggest an important role of the TFH – B cell interaction in supporting HIV persistence in gut tissue.

Flow cytometry experiments from chapter 5 demonstrated the presence of fCD8 T cells in GALT tissue of HIV infected individuals. This cell type has recently implicated in viral control within germinal centres in the LCMV model\textsuperscript{527}. In the HEATHER gut study fCD8 T cells were present at highest frequency in rectal tissue, also the gut site with lowest measures of HIV DNA. However, while the frequency of fCD8 inversely correlated with
HIV DNA in the terminal ileum, this was not the case in rectal tissue. Furthermore, while bulk CD8 T cells in the gut had lower expression of the cytotoxic granules perforin and granzyme B compared to those in PBMCs, fCD8 T cells in gut had higher expression of these molecules compared to their non-fCD8 counterparts, suggesting a greater cytotoxic potential of this subset. Additionally, the frequency of TFH correlated with fCD8. The pathway supporting fCD8 differentiation is yet to be fully elucidated, but the data in chapter 5 supports the idea that TFH may support fCD8 differentiation. This finding of lower HIV DNA in gut in association with fCD8 is important, as strategies to improve access of fCD8 to the germinal centre may limit HIV persistence in this important sanctuary site. Recent in vitro data demonstrated that fCD8 T cells could kill latently infected cells via bispecific antibodies \(^{546}\) and highlights how these cells may be harnessed to eliminate HIV within germinal centres.

The final chapter of my thesis explored the impact of vorinostat and a therapeutic vaccine to reduce the HIV gut reservoir and enhance immunity. This pilot study did not show any impact on the HIV GALT reservoir in those receiving the study intervention, compared with ART alone, these findings were consistent with work by other groups \(^{570}\). Importantly there was no signal to suggest increased levels of immune activation or exhaustion in individuals who had received the intervention compared with ART alone. Sample size limited this pilot study, and therefore any findings or indeed the absence of significant findings must be interpreted with this in mind. Higher levels of sCD163 were noted in the intervention group compared with ART alone group, suggesting bacterial translocation may be implicated in the mechanism of viral persistence in the gut. In contrast to our finding a previous study found a decrease in the expression of the early activation marker CD69 and increased IL-17A mRNA expression in colonic biopsies of individuals receiving panobinostat \(^{579}\). Finally, while the findings of this work are primarily relevant to the HIV field, they may also
be of interest to those working with immunotherapy agents in the cancer field. Data on ICR expression in gut CD4 and CD8 T cells in healthy controls may aid the understanding of the reported gastrointestinal side effects such as colitis which is common and often severe in recipients of novel agents targeting ICRs such as PD-1 inhibitors.  

8.1.1. Conclusions and Future Work

In this thesis, I have demonstrated that ART started in PHI can enhance the normalisation of markers of immune dysfunction, particularly in the PBMC compartments. I have also provided evidence of compartmentalised immune recovery, with elevated activation and ICR expression within the gut. While the markers of immune function did not correlate with HIV DNA in the gut, markers of microbial translocation and systemic inflammation did, supporting a link between gut HIV DNA, microbial translocation and persistent inflammation even after years of viral suppression amongst individuals treated during PHI. I have also characterised the HIV reservoir in blood, terminal ileum and rectum in treated PHI and shown that both CD4 and CD8 germinal centre T cells may be important for HIV persistence in the gut.

Assessment of immune function and HIV reservoir in tissue sites presents technical challenges related to limited cell number, poor cell viability and the ability to obtain representative samples. In my future work, I aim to incorporate recently developed technologies including next generation in situ hybridisation platforms such as RNAscope and tissue cytometry platforms to further explore the role of fCD8 T cells in HIV persistence. Such platforms, using HIV specific DNA and RNA probes in tandem with CD8 follicular and tissue-resident T cell markers would allow for the role of these cells in HIV persistence to be further elucidated. In addition, it would allow assessment of other potentially important cellular reservoirs in tissue such as macrophages. I also aim to
develop functional assays of GALT using the tissue explant model used in this thesis which will be essential to fully understand how HIV persists in GALT.

This thesis raises some interesting links between bacterial translocation and HIV persistence. Limiting bacterial translocation or the causes of it, which may include ongoing viral replication or production and subsequent immune damage in GALT tissue sites, may have a role to play in targeting the HIV reservoir in GALT. Interventions to reduce bacterial translocation should be tested, and I would be keen to explore the impact of such interventions on HIV tissue reservoir and immune function in future clinical studies.

In conclusion, this thesis confirms the role of HIV DNA in GALT as a barrier to HIV cure even amongst those treated early in PHI. Furthermore, this work identifies biomarkers and potential mechanisms related to HIV persistence in GALT and highlights the importance of GALT sampling in future HIV cure intervention studies.
List of Communications

Data contained in or relevant to this thesis published during my studies are listed below:

Publications


Conference Presentation


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Appendix A.

The HEATHER study

The HEATHER study is conducted as part of the CHERUB (Collaborative HIV Eradication of Reservoirs: UK BRC) collaboration.

CHERUB Steering Committee: Andrew Lever (University of Cambridge), Mark Wills (University of Cambridge), Jonathan Weber (Imperial College, London), Sarah Fidler (Imperial College, London), John Frater (University of Oxford), Lucy Dorrell (University of Oxford), Mike Malim (King’s College, London), Julie Fox (King’s College London), Ravi Gupta (University College London), Clare Jolly (University College London).

Participating Sites & Staff: St Marys Hospital, London (S Fidler, J Thornhill, K Mahay*, K Kuldanek*, H Lewis, R Hall ), Chelsea and Westminster Hospital, London (N Nwokolo, G Whitlock) Guy’s & St Thomas Hospital London (J Fox, T Solano, A Sharp, J Lwanga)


(* Left the study team before the trial ended.)
Appendix B

The SPARTAC Trial

The SPARTAC Trial Investigators:


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*Clinical Endpoint Review Committee:* N Paton, S Fidler.

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Appendix C

Flow Cytometry Gating

(i) General GALT gating strategy

Representative flow cytometry plots showing gating strategy for CD4 and CD8 T cells - sample show is gated on rectal mucosal mononuclear cells:
(ii) Immune checkpoint receptors expression on CD4 T cells

Representative gating of PD-1, TIGIT and Tim-3 from terminal ileum GALT (FMO is shown on the left and stained sample on the right)
(iii) Immune checkpoint receptors expression on CD8 T cells

Representative gating of PD-1, TIGIT and Tim-3 from terminal ileum GALT (FMO is shown on the left and stained sample on the right)
(iv) TFH marker expression on CD4 T cells

Representative gating of TFH, Bcl-6 & CXCR5 from GALT mucosal mononuclear cells (FMO is shown on the left and stained sample on the right)
Appendix D

Memory CD4 (CD45RA⁻) T Cells in GALT

Figure (a) below shows the representative staining of CD45RA on CD4 T cells in rectal GALT. Figure (b) shows the frequency of CD45RA⁻ cells in the terminal ileum, rectum and PBMCs. Figure (c) shows the HIV DNA levels across the terminal ileum, rectum and PBMC expressed as copies per million (cpm) CD45RA⁻ CD4⁺ T cells.

(a) Representative CD45RA staining in rectal GALT

(b) % of CD4 T cells that are CD45RA⁻

(c) Log HIV DNA cpm CD45RA⁻ CD4⁺ cells