T follicular helper cell dynamics during acute HIV-1 subtype C infection and relevance for T-helper cell-dependent responses

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PREFACE

The experimental work described in this dissertation was carried out at the HIV Pathogenesis Programme, School of Laboratory Medicine and Medical Sciences, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, from April 2014 to June 2018, under the supervision of Dr. Zaza Ndhlovu and Prof. Thumbi Ndung'u

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

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DECLARATION 1: PLAGIARISM

I, Omolara Baiyegunhi, declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Signed: …… Date: ……..

DECLARATION 2: MANUSCRIPTS

The manuscripts that constitute this thesis are listed below and the contributions that I and other co-authors made to each manuscript are declared here:

Manuscript 1: Omolara Baiyegunhi, Funsho Ogunshola, Nasreen Ismail, Bruce Walker, Thumbi Ndung'u and Zaza M. Ndhlovu. 2018. Frequencies of circulating Th1-biased T follicular helper cells in acute HIV-1 infection correlate with the development of HIV-specific antibody responses and lower set point viral load. *Journal of Virology*. Published (doi:10.1128/JV100659-18).

Authors' contributions: Dr. Zaza M. Ndhlovu, Dr. Bruce D. Walker and Prof. Ndung'u initiated the study cohorts. Dr. Zaza Ndhlovu conceived the idea. Dr. Zaza Ndhlovu and I designed the experiments. Dr. Bongiwe Ndlovu conducted the Luminex assay and generated the IgG antibody titer values. I performed the rest of the laboratory experiments. I analyzed the data and wrote the manuscript under Dr. Ndhlovu's supervision. All the authors approved of the manuscript before it was submitted for publication.

Manuscript 2: Omolara Baiyegunhi, Faatima Laher, Daniel Muema, Funsho Ogunshola, Thandeka Nkosi, Anele Mbatha, Nasreen Ismail, Veron, Ramsuran, Bruce D. Walker, Krista Dong, Thumbi Ndung'u and Zaza M. Ndhlovu. 2018. Early initiation of antiretroviral therapy during hyperacute HIV-1 infection preserves T follicular helper cell function

Authors' contributions: Dr. Zaza Ndhlovu, Dr. Bruce D. Walker, Dr. Krista Dong and Prof. Thumbi Ndung'u initiated the study cohorts. Dr. Zaza Ndhlovu conceived the

idea; Dr. Zaza Ndhlovu and I, designed the experiments. Dr. Daniel Muema and I conducted the IgG ELISA assay. Dr. Veron Ramsuran, Faatima Laher, Funsho Ogunshola and I conducted the digital droplet PCR experiments. Faatima Laher and I conducted the class II tetramer analysis. I performed the rest of the laboratory experiments, data analysis and wrote the manuscript under the supervision of Dr. Zaza Ndhlovu.

Manuscript 3: Omolara Baiyegunhi, Funsho Ogunshola, Faatima Laher, Thandeka Nkosi, Trevor Khaba, Nasreen Ismail, Bruce D. Walker, Krista Dong, Thumbi Ndung'u and Zaza M. Ndhlovu. 2018. HIV RNA persists in $CXCR3^{+}CCR6^{+}$ GCTfh cells in the lymph nodes of HIV-infected individuals initiated on ART in Fiebig stage I.

Authors' contributions: Dr. Zaza Ndhlovu, Dr. Bruce D. Walker, Dr. Krista Dong and Prof. Ndung'u initiated the study-cohorts. Dr. Zaza Ndhlovu conceived the idea; Dr. Zaza Ndhlovu and I designed the experiments. I performed the rest of the laboratory experiments, data analysis and wrote the manuscript under the supervision of Dr. Zaza Ndhlovu.

Omolara Baiyegunhi (student): …… Date: ……

Dr. Zaza Ndhlovu (supervisor): ..…….…….. Date: ……

PRESENTATIONS AND SCHOLARSHIP AWARDS

Oral Presentations

1. **South Africa Immunology Society (SAIS) 2016, March 2016**. Evolution of Follicular helper CD4 T cells during hyperacute HIV-1 infection.
2. **Keystone Symposia conference-J6, HIV Vaccines, January 2018**. Early initiation of antiretroviral therapy during hyperacute HIV-1 infection preserves T follicular helper cell function.

Poster presentations

1. **IUIS-IDA 2015, October 2015**. T follicular helper (Tfh) cell dynamics in acute HIV-1 subtype C infection and relevance for T-helper cell-dependent antibody responses.
2. **Keystone Symposia conference-X8, HIV Vaccines, March 2016**. Phenotypic characterization of peripheral blood follicular helper T cells during hyperacute subtype C infection.
3. **Keystone Symposia conference-D2, B cells and Tfh cells, April 2017**. Circulating memory follicular helper CD4 T cells during acute HIV-1 subtype C infection influence the development of HIV binding antibodies.
4. **Conference on Retroviruses and opportunistic infections (CROI), March 2018.** HIV RNA persists long-term in lymph nodes of individuals initiated on ART in Fiebig I.

Scholarship awards

1. NRF Innovation Doctoral Scholarship (2014-2016).
2. HIV Pathogenesis Programme (HPP) Doctoral Fellowship (2014-2018).
3. South African Immunology Society (SAIS) student scholarship award (March 2016).
4. Keystone Symposia scholarship for X8, HIV Vaccines (March 2016).
5. Ragon Institute of MGH, MIT and Harvard International travel award (May 2016).
6. Connect Africa scholarship (March 2017).
7. College of Health Sciences Scholarship for consumables (2017).
8. Keystone Symposia scholarship for J6, HIV Vaccines (January 2018).
9. Conference on Retroviruses and Opportunistic Infections (CROI) young Investigator award (March 2018).

DEDICATION

To my husband

Lloyd Baiyegunhi

You make me happy and you inspire me to excel

ACKNOWLEDGEMENTS

All glory goes to the God and father of our Lord Jesus who makes all things possible.

My heartfelt thanks go to my mentor Dr. Zaza Ndhlovu, for his mentorship, and for all the learning opportunities he offered to me during my PhD studies. I would also like to thank our director Prof. Thumbi Ndung'u, who is my co-supervisor; for his mentorship and for running a fantastic PhD Program at HPP. I am grateful to all the study participants, the doctors and nurses at our study sites, Denise Margolis from the optics and imaging unit and our many funders.

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I have enjoyed the encouragement and support of many friends, including, Doty, and Uchenna. Lastly, I want to thank my very own special "B cell", my husband Lloyd (I am his Tfh cell) for his amazing support, patience and love and my family, the Adekunle dynasty and the Baiyegunhi clan for believing in me. I love you all very much.

"I can do all this through Christ who gives me strength". Philippians 4:13

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ABBREVIATIONS

ART	Combination antiretroviral therapy
BCL-6	B cell lymphoma 6
bNAbs	Broadly neutralizing antibodies
CD	Cluster of differentiation
CM	Central memory
cTfh	Circulating T follicular helper
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
Early Tx	Early treated
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Effector memory
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FFPE	Formal fixed paraffin embedded
FFPE	Formalin fixed paraffin embedded
FRESH	Females rising through education, support and health
GC	Germinal center
GCTfh	Germinal center T follicular helper
gp	Glycoprotein
HIV-1	Human immunodeficiency virus type 1
HIVneg	HIV negative
HLA	Human leucocyte antigen
HPP	HIV pathogenesis programme
ICOS	Inducible T cell co-stimulator
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry

IL	Interleukin
IQR	Interquartile range
ISH	*In situ* hybridization
LMC	Lymph node mononuclear cells
LN	Lymph nodes
MFI	Median fluorescence intensity
mRNA	Messenger ribonucleic acid
nnAbs	Non-neutralizing antibodies
OPD	O-phenylenediamine dihydrochloride
PBMC	Peripheral blood mono nuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
RNA	Ribonucleic acid
RT	Room temperature
SEB	Staphylococcal enterotoxin B
SPVL	Set point viral load
TEMRA	Terminally differentiated effector memory
TET	Tetramer
Tfh	T follicular helper cells
Un Tx	Untreated
VL	Viral load

ABSTRACT

Affordable, efficacious, protective and safe HIV vaccine is the best strategy to end the HIV-1 epidemic. A protective vaccine will need to elicit high affinity antibodies and long-term B cell immunological memory. $CD4^+$ T cells are critical for the development of protective adaptive immune responses. Specifically, the subset termed T follicular helper (Tfh) cells are crucial for the establishment of germinal center (GC) reactions, the production of high affinity antibodies and the generation of memory B cells and long-lived plasma cells. However, the contribution of these cell subsets to the development of a vaccine is not well defined, probably due to their heterogeneity, susceptibility to HIV-1 infection and most importantly, the difficulty associated with access to human lymphoid tissues samples to study them.

Using a multipronged approach that included flow cytometry, HLA class II tetramers, immunohistochemistry (IHC) and immunofluorescence microscopy (IF) assays, ELISA, digital droplet PCR, RNA *in situ* hybridization (ISH) assays and *in vitro* co-culture techniques, this study sought to comprehensively characterize lymphoid tissue and peripheral blood Tfh cell subsets during clade C HIV-1 infection. We also endeavored to define virus persistence in the lymph nodes (LNs) of early treated individuals and identify cellular subsets that harbor residual virus in aviremic individuals on suppressive antiretroviral therapy (ART), which was initiated during hyperacute HIV infection.

Our studies generated several notable novel findings. Firstly, we showed that HIV-specific Th1-biased Tfh cells promote the development of Gag-specific antibodies,

which in turn reduce set point viral loads during acute HIV-1 infection. We further elucidated the function of Tfh cells in the lymph nodes and showed that highly functional GCTfh responses are induced in early treated individuals. In addition, these cells were superior at inducing B cell class switching and at secreting higher amounts of IL-21 compared to GCTfh cells from untreated individuals.

Secondly, we used IHC, IF and ISH technologies, to show that the persistence of Gag p24 antigens and HIV RNA in the GCs of early treated aviremic individuals. Thirdly, we also showed that LN viral loads were significantly higher than plasma viral loads. Fourthly, we showed that the cumulative plasma antigen load of early treated individuals predicted the magnitude of the GC response and the frequencies of GCTfh cells at the time of LN excision. Finally, we demonstrated HIV driven induction of $CXCR3^{+}CCR6^{+}$ GCTfh subsets in human LNs and showed that this subset contributes to virus persistence in the LNs.

Overall, the work included in this thesis highlights a cTfh cell subset that could slow down HIV replication by enhancing antibody generation and demonstrates that the early initiation of ART is beneficial to the development of superior functioning GCTfh responses. In addition, our results underscore the inefficiency of ART in eradicating persistent viremia in the GCs in secondary lymph nodes. These results will inform prophylactic or therapeutic HIV-1 vaccine studies directed at inducing long lasting antibody responses and highlights barriers that need to be surmounted to achieve sterilizing HIV-1 cure.

CHAPTER 1: INTRODUCTION

1.1 The HIV/AIDS epidemic

Acquired immunodeficiency syndrome (AIDS), a disease responsible for the deaths of millions of people worldwide, is caused by two lentiviruses, human immune deficiency virus type 1 (HIV-1) and HIV-2 (UNAIDS, 1999, Sharp et al., 2011). Human infections by these viruses originated from multiple cross-species transmissions of simian immunodeficiency virus (SIV) from primates to humans (Sharp et al., 2011). Since the clinical recognition of the disease in 1981, HIV/AIDS has been a significant public health problem all over the world especially in sub-Saharan Africa, the region most affected by the epidemic (Hahn et al., 2000). An estimated 37 million people globally were reported to be living with HIV in 2016, with almost 70% of that figure living in sub-Saharan Africa (Figure 1.1) (UNAIDS, 2017a). Although both HIV-1 and HIV-2 have similar modes of transmission, replication pathways and clinical consequences, they are genetically different viruses with different primate ancestral origins (Van Heuverswyn et al., 2007, Motomura et al., 2008, Nyamweya et al., 2013). Furthermore, HIV-1 is more widespread globally while HIV-2 is mostly confined in West Africa and in few European countries like Portugal and France, mainly because of their socio-economic links to West African countries (Nyamweya et al., 2013, Campbell-Yesufu et al., 2011). The present study was conducted in South Africa where HIV-1 is prevalent, thus the focus hereon will be on HIV-1 infection.

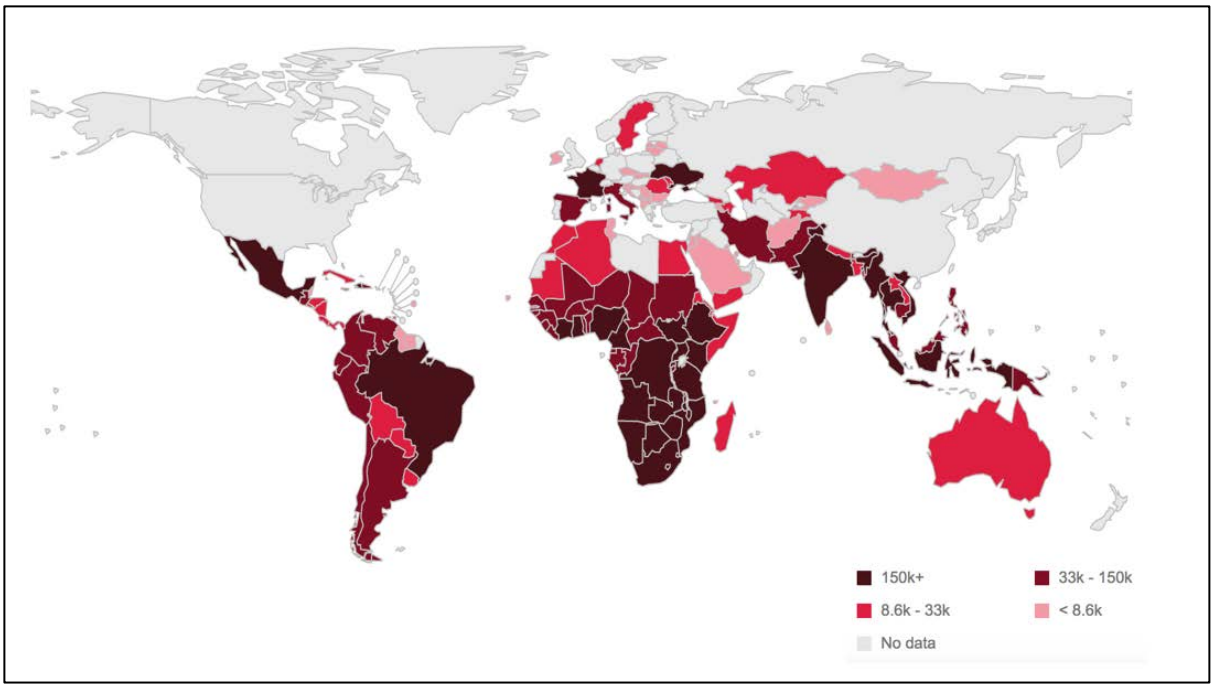

Figure 1.1: Global adult HIV prevalence in 2016. Source: (UNAIDS, 2016a)

1.2 Pathogenesis of HIV-1 infection

1.2.1 Acute HIV infection

The global spread of HIV-1 is due to its exceptional ability to effectively thwart the human immune system and to establish life-long infection (Simon et al., 2006). HIV-1 transmission in humans occurs primarily through sexual contact via penile, rectal or vagina routes, through exposure to HIV-contaminated blood, contaminated needles, syringes and surgical items or transmitted vertically from mother to child in-utero and post-partum (Simon et al., 2006, Patel et al., 2014, Lavoie et al., 2017, Al-Jabri, 2007). Following HIV-1 transmission, there is rapid virus replication mainly at the mucocutaneous surfaces of the reproductive tracts or gastro intestinal tract, the virus subsequently disseminates into the lymphatic system (Simon et al., 2006, Genesca et al., 2010, Cohen et al., 2011).

During the initial 1 to 14 days of infection commonly referred to as the window period, the infection is asymptomatic and the virus remains undetectable in the plasma by standard testing methods (Fiebig et al., 2003, Douek, 2003, Cohen et al., 2011). Qualitative or quantitative HIV viral RNA detection or amplification methods are typically used for diagnosis and early detection of virus during the window period, because HIV antigens or anti-HIV antibodies are undetectable in the blood. Recent advancements in nucleic acid amplification technologies have led to the development of more sensitive diagnostic assays that can detect low levels of viral RNA down to a threshold of 20 copies/ml (Busch et al., 2005, Cobb et al., 2011, Busch, 2015). In addition, the development of fourth generation serological tests that detect either antigens or antibodies have greatly reduced the window period down to 5 days after infection (Busch, 2015).

The asymptomatic stage of virus replication is followed by seroconversion, which denotes the appearance of HIV-1 specific antibodies in plasma (Cohen et al., 2011). The step-wise detection of viral markers and antibodies in the blood by different assays is used to define the clinical stages of acute HIV-1 infection (Cohen et al., 2011). The Fiebig staging system of classification, which defines six sequential stages of acute HIV infection, was published in 2003 and is widely adopted by HIV researchers and public health practitioners worldwide (Figure 1.2) (Fiebig et al., 2003, Busch, 2015). The eclipse phase is the period from virus infection to stage I where HIV RNA is first detected. Positivity to subsequent tests that detect Gag p24 antigen and anti-HIV antibodies defines the remaining stages (Figure 1.2).

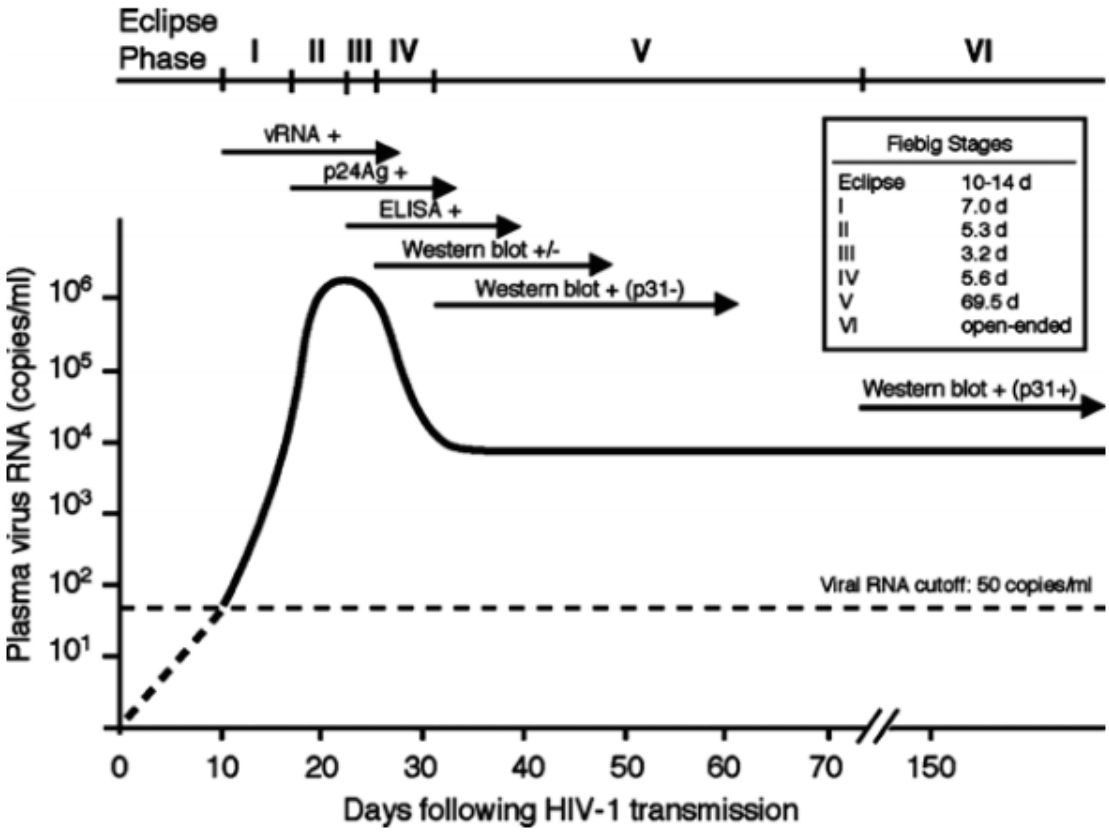

Figure 1.2: Staging of acute HIV infection. Acute HIV infection classification by the Fiebig staging system defines seven successful stages ranging from the “eclipse phase” before detectable viremia to progressive Viral RNA detection and subsequent “seroconversion” stages. Adapted from Busch (2015).

1.2.2 Chronic HIV infection

The clinical symptoms and the rate of HIV-1 disease progression are largely determined during the acute stage of infection (Hansasuta et al., 2001, Robb et al., 2016). Various host and virus factors contribute to the different rates of HIV disease progression observed in infected individuals (Pohlmeyer et al., 2013, Naif, 2013, Selhorst et al., 2017). The chronic exposure of the immune system in an untreated individual to actively replicating virus progressively damages the immune system, resulting in a dysregulated immune response (Boasso et al., 2009). The generalized perturbation of the immune system by chronic HIV infection, includes but is not limited to, impaired B cell responses, excessive antibody production, aberrant T cell

activation, T cell exhaustion, senescence and depletion, chronic inflammation and the disruption of lymphoid tissue architecture (Ng et al., 2013). Furthermore, the rapid depletion of the $CD4^+$ T cells with concurrent high plasma viral loads accelerates progression to AIDS (Moir et al., 2011, Naif, 2013). During this stage individuals could be susceptible to other opportunistic infection like *Mycobacterium tuberculosis* and *Pneumococcus meningitis* (Naif, 2013). The progression of HIV-1 disease can however, be curtailed through the administration of antiretroviral (ARV) drugs.

1.3 Therapeutic and preventative strategies for HIV-1 infection

1.3.1 Antiretroviral therapy

The inception of ARV drugs in mid 1980s was a breakthrough in the HIV/AIDS epidemic, turning an incurable disease into a chronic infection (McElrath et al., 2010). Antiretroviral therapy (ART) has since evolved from the administration of single ARVs to the combined administration of multiple drugs with different modes of action (Bhatti et al., 2016). The combination of ARV agents with different modes of action results in rapid virus suppression, minimizes drug resistance and limits treatment failure (Arts et al., 2012). The six mechanistic classes of ARV agents currently available are: Nucleoside reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), Integrase inhibitors (INSTIs), Fusion inhibitors (FIs) and Chemokine receptor antagonists (CCR5 antagonists) (Table 1) (Bhatti et al., 2016, Meintjes et al., 2017). Their mechanisms of action are summarized in table 1.1. Remarkable progress has been made in the simplification of ART to reduce side effects and improve adherence. Single-tablet regimens which contains 3 or more active ingredients, improved drug formulations with reduced toxicity, and novel dosage forms like slow release drug forms or long-acting

injectables and inserts are current innovations in the pipeline (Cihlar et al., 2016, HIVandHepatitis.com., 2015, Clark et al., 2015).

Previous ART initiation guidelines stipulated that HIV-infected individuals had to have a CD4 count of below 250 cells/µl, or having co-morbidities like tuberculosis and meningitis. With emerging evidence of the benefits of early initiation of ART to the immune system (INSIGHT START et al., 2015, TAS et al., 2015), the World Health Organization (WHO) guidelines now recommends the immediate initiation of ART for individuals that test positive for HIV regardless of the CD4 count (WHO, 2015, Meintjes et al., 2017). The global scale-up of ART has contributed greatly to a massive reduction in AIDS-related deaths and has significantly reduced HIV transmission rates globally (UNAIDS, 2017b, UNAIDS, 2016b). Of important mention is the huge investment by the United States President's Emergency Plan for AIDS Relief (PEPFAR) towards increased access to HIV care and treatment across sub-Saharan Africa (Chin et al., 2015, El-Sadr et al., 2012).

In spite of the many benefits of ART in preserving life-expectancy of HIV-1 infected individuals, lifelong ART has many drawbacks which include; toxicity, residual chronic inflammation, cumulative costs of drugs and increased onset of diseases associated with aging (Woldemedhin et al., 2012, Dekoven et al., 2016). Novel interventions are therefore required to take people off lifelong ART. The success of these strategies will require total eradication of residual virus from sanctuary sites such as lymph nodes, the gut and the gastrointestinal tract, which have disparate and suboptimal drug levels during ART (Deeks et al., 2016).

Table 1.1: Classes of antiretroviral agents

Class	Abbreviation	Mechanism of action	Specific action
Nucleoside and nucleotide reverse transcriptase inhibitors	NRTIs and NtRTIs	Reverse transcriptase inhibition	Nucleic acid analogues mimic the normal building blocks of DNA, preventing transcription of viral RNA to DNA
Non-nucleoside reverse transcriptase	NNRTIs	Reverse transcriptase inhibition	Alter the conformation of the catalytic site of reverse transcriptase and directly inhibit its action
Protease inhibitors	PIs	Protease inhibition	Inhibits the final maturation of stages of HIV replication, resulting in the formation of non-infective viral particles
Integrase inhibitors (also termed integrase strand transfer inhibitors)	InSTIs	Inhibition of viral integration	Prevent the transfer of proviral DNA strands into the host chromosomal DNA
Entry inhibitors	-	Entry inhibition	Bind to viral gp41 or host cell CD4+ or chemokine (CCR5) receptors

CCR5, C-C chemokine receptor type 5; NRTIs, nucleoside reverse transcriptase inhibitors; NtRTIs, nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; InSTIs, integrase inhibitors (integrase strand transfer inhibitors). Adapted from Meintjes et al. (2017)**.**

1.3.2 Pre-exposure prophylaxis

Despite the relative success of ART in the control of the HIV-1 epidemic and a reduction in mother to child transmission events globally, a combination of behavioral, biomedical and structural approaches are still required to contain the epidemic (Roxby

et al., 2014). Pre-exposure prophylaxis (PrEP) was recently recommended by the World Health Organization (WHO) for the prevention of HIV acquisition by high-risk individuals in combination with other methods (WHO, 2007, Karim et al., 2010, McElrath et al., 2010, WHO, 2013). Currently PrEP is administered orally as a single tablet fixed dose of Tenofovir and emtricitabine or Tenofovir only (Coutinho et al., 2013). Numerous trials have demonstrated the efficacy of PrEP which relies largely on adherence to the recommended dosing schedule, with the potential of non-adherence leading to drug resistance (Landovitz et al., 2009, Eakle et al., 2013). Currently PrEP is gaining widespread acceptance and incorporation into health care systems globally, alongside WHO guidelines (WHO, 2015) recommending the availability of PrEP on demand (Conniff et al., 2016, DoH, 2016, KSA, 2017).

1.3.3 HIV vaccine

The HIV research community has pursued the development of a safe and efficacious vaccine since the discovery of HIV in the early 1980s. If developed, a preventative vaccine for HIV will be the backbone of the integrated HIV prevention strategy (Dieffenbach et al., 2011). The expectation is that a vaccine will be able to protect against any form of HIV transmission with the ultimate goal of ending the epidemic (Dieffenbach et al., 2011, McElrath et al., 2012). Numerous factors, including, the broad genetic diversity of HIV-1, mutability of the HIV-1 target epitopes, the structural properties of its viral envelope, early establishment of reservoirs, viral evasion of host's immune responses and the limited knowledge of immune correlates of protection are obstacles in the development of an effective vaccine (Barry SM et al., 2014, Ahlers, 2014, Walker et al., 2008).

Several vaccine trials have been conducted with minimal to no success (Buchbinder et al., 2008, McElrath et al., 2008, Rerks-Ngarm et al., 2009, Gray et al., 2016). Initial trials in the 1990s vaccinating with recombinant gp120 induced narrow antibody responses specific only for the virus strain in the immunogen, which led to interest in immunogens that would induce cross clade responses (Gray et al., 2016). The STEP and Phambili trials were T cell based vaccines administered as an Adenovirus 5 (Ad5) virus vector but the trials were discontinued after failing the futility test, with increased HIV incidence rates observed in certain patient categories (Buchbinder et al., 2008, McElrath et al., 2008). The RV144 trial which used a canary pox prime ALVAC-HIV and AIDS-VAX booster regimen to vaccinate Thai adults, was the most promising trial so far with 31% efficacy (Rerks-Ngarm et al., 2009).

Recent efforts to build on the RV144 trial and induce long-lasting immune responses, redesigned the ALVAC vector with a clade C Env insert and administered it together with a bivalent subtype C recombinant gp120 and an MF59 adjuvant (Gray et al., 2016). The subsequent HVTN 702 phase III and IIb trial is currently in progress in multiple sites across South Africa (AVAC, 2016). Additionally, an efficacy study for a mosaic vaccine comprising Ad26.Mos4.HIV in combination with Clade C gp140 is currently being conducted in multiple sites, including South Africa, Malawi, Mozambique, Zambia and Zimbabwe (TAG, 2016, NIAID, 2016).

Further HIV vaccine efforts are directed at designing immunogens that can induce broadly neutralizing antibodies (bNAbs) against HIV (Kwong et al., 2011). HIV-1 bNAbs are antibodies that have cross-reactive neutralization of a broad array of HIV-1 strains *in vitro* (Corti et al., 2010). The proof of concept for designing such a vaccine lies in the ability of some people to develop bNAbs during untreated HIV-1 infection

(Borrow et al., 2017). In addition, the strength of this approach lies in the ability of bNAbs to neutralize multiple viral strains and providing coverage against most circulating HIV strains (Ahmed et al., 2017, Corti et al., 2010). Thus, there is considerable interest in the development of candidate immunogens as well as developing immunization strategies to elicit bNAbs by vaccination (de Taeye et al., 2016, Moore et al., 2017). Summarily, the consensus in the HIV vaccine research field is that an ideal vaccine candidate should induce coordinated B cell, $CD4^+$ and $CD8^+$ T cell responses (McElrath et al., 2010). Furthermore, there is a need for new immunogens to overcome diversity for T cell responses and to induce durable neutralizing antibody responses with great breadth (Haynes et al., 2014).

1.3.4 Antibody therapy

Monoclonal antibody therapies are potential approaches to prevent HIV acquisition. Studies involving non-human primates and humanized mice have shown that passively infusing antibodies to prevent virus infection or reduce levels of viremia have been efficacious (Horwitz et al., 2013, Halper-Stromberg et al., 2014). This was further supported by evidence from macaque studies. In one such study, the administration of $\alpha_4\beta_7$ antibody with ART effectively controlled viremia and reconstituted the immune system of SIV infected macaques (Byrareddy et al., 2016). A phase I clinical trial in humans demonstrated the safety and tolerance of 3BNC117 antibody monotherapy (Caskey et al., 2015). The antibody however had a faster decay rate in HIV-infected individuals and induced resistance in some individuals (Caskey et al., 2015). Conversely, the 3BNC117 monotherapy enhanced host antiviral immunity in another study (Schoofs et al., 2016). Lastly, in a Phase IIa clinical trial, four 30 mg Kg^{-1}

infusions of 3BNC117 delayed viral rebound for up to 19 weeks in ART mediated fully suppressed HIV-infected individuals (Scheid et al., 2016).

The efficacy of VRC01 antibody at preventing HIV infection in different target populations is currently being tested by the Antibody Mediated Prevention (AMP) studies (NIAID, 2016, Clinicaltrial.gov, 2015, HVTN, 2017). Taken together, results from these trials will potentially answer important questions relating to antibody-based therapy for HIV prevention. Furthermore, combination of antibodies with different specificity and potency will be explored in upcoming trials to prevent the development of antibody resistance or virus escape and to validate the potency and breadth of antibody therapies (Zhang et al., 2016, Julg et al., 2017).

1.4 Host immune responses to HIV infection

As with all infections, effective anti-viral responses are required by the host's immune system to control HIV-1 infection and prevent the development of AIDS. Both the innate and adaptive immune system; comprising T-cell dependent and humoral immune responses, contribute to the partial suppression of HIV (Mogensen et al., 2010). Importantly, the cytotoxic $CD8^+$ T cell (CTL) response is critical for virus control during acute HIV-1 infection (Streeck et al., 2010). Evidence for the role of CTL in HIV control include; the association between the initial decline in peak viral load with the emergence of $CD8^+$ T cell responses in HIV infected individuals, immune selection pressure in autologous viral sequences which manifest in key CTL epitopes resulting in abrogation of CTL recognition, and the experimental depletion of $CD8^+$ T cells in SIV disease models resulted in increased plasma viral loads (Koup et al., 1994, Walker et al., 2012, Jin et al., 1999, Matano et al., 1998).

Numerous studies have demonstrated the capacity of cytotoxic $CD8^+$ T cells to kill HIV infected cells (Migueles et al., 2008, Streeck et al., 2010). Characteristics like CTL proliferation, cytokine production, degranulation and cytolysis of infected cell targets have been used to define the quality of the CTL response (Cao et al., 2003, Streeck et al., 2009, Ndhlovu et al., 2012). Furthermore, the host genetic determinants of CTL responses and antiviral characteristics of CTLs are progressively being described (Walker et al., 2012, Ndhlovu et al., 2015, Reuter et al., 2017).

Until recently, little or no attention was paid to the contribution of HIV-specific $CD4^+$ T cell responses and viral control (Streeck et al., 2010). Some of the reasons for the paucity of information on this topic include; HIV induced $CD4^+$ T cell loss, the domination of T cell responses by $CD8^+$ T cells and lack of sensitive assays to detect low frequency HIV-specific $CD4^+$ T cells (Ramduth et al., 2005, Mattapallil et al., 2005, Sant et al., 2012). The importance of HIV-specific $CD4^+$ T cell responses for the development of robust CTL responses and improved antibody neutralization breadth has been described by several studies (Kalams et al., 1999, Ranasinghe et al., 2015, Schultz et al., 2016). One such study analyzed HIV-specific $CD4^+$ T cell responses in a cohort of HIV-infected controllers with and without neutralization breadth found elevated breadth and magnitude of Gag- and gp41-specific $CD4^+$ T cells responses in controllers with neutralizing antibodies (Ranasinghe et al., 2015). Additionally, the magnitude of Gag-specific responses correlated with the neutralization breadth, suggesting a role for these $CD4^+$ T cell responses in the development of neutralization breadth (Ranasinghe et al., 2015).

In multiple studies, IL-21 secretion by HIV-specific $CD4^+$ T cells and the expansion of $IL21^+$ $CD4^+$ T cells were associated with enhanced virus suppression by CTLs and

lower viral load set points (Porichis et al., 2011, Chevalier et al., 2011a). Furthermore, HIV specific $CD4^+$ T cells help to $CD8^+$ T cells was examined in *in vitro* co-culture assays and the expression levels of cytolytic enzymes; granzyme B and perforin were elevated in $CD8^+$ T cells co-cultured in that study (Schultz et al., 2016). All these lines of evidence demonstrate the importance of HIV specific $CD4^+$ T cells in HIV control and with the deployment of more sensitive detection methods for HIV-specific $CD4^+$ T cells, their role in HIV control will be better delineated (Laher et al., 2017). This information will be critical for HIV vaccine strategies.

1.5 $CD4^+$ T helper cell subsets and acute HIV infection

$CD4^+$ T helper cells are important mediators of adaptive immunity through performing panoply of functions, including, 'helping' B cells and $CD8^+$ T cells by producing cytokines and chemokines, which coordinate the full array of immune responses to infections (Zhu et al., 2008, Luckheeram et al., 2012). Historically, $CD4^+$ T cells were classified into T helper (Th) 1 and Th2 cells based on the predominant cytokine, IFN-γ or IL-4 respectively, produced by each cell type (Eyerich et al., 2014). With the emergence of new technologies to study $CD4^+$ T cells, the dichotomous paradigm of Th1 and Th2 cells has since evolved to recognize multiple other subsets, which are identified by their lineage-defining transcription factor or by the migration marker expressed (Eyerich et al., 2014, Luckheeram et al., 2012). These subsets include the Th9, Th17, Th22, T regulatory (Treg), and T follicular helper (Tfh) cells (Figure 1.3) (Zhu et al., 2008, Luckheeram et al., 2012).

The polarization of naïve $CD4^+$ T cells into these subsets with distinct effector function occurs through an integration of T cell receptor, co-receptor and cytokine receptor signaling. In addition, the type of antigen, the priming action of antigen presenting cells

(APCs), the member of the signal transducer and activator of transcription (STAT) family that is induced or the soluble factors present, also contribute to effector subset differentiation (O'Shea JJ et al., 2010, Zhang et al., 2013b, Tangye et al., 2013). The transcription factors that drive $CD4^+$ T helper cell differentiation include, GATA-binding protein 3 (GATA-3) for Th2, T-bet for Th1, retinoid-related orphan receptor gamma t (RORγT) for Th17 and the forkhead box protein 3 (Foxp3) for the Tregs and the B cell CLL lymphoma 6 (BCL-6) for Tfh cells (Figure 3) (O'Shea JJ et al., 2010).

The effector lineages of T helper cells specialize in the following: the Th1 cells secrete the cytokines IFN-γ and IL-2 and mediate immune responses against intracellular pathogens (Zhu et al., 2008, London et al., 1998), while the IL-4 and IL-10 secreting Th2 cells are responsible for immunity against extracellular parasites like the helminths (Figure 3) (Zhang et al., 2013b). The Th9 cells which also produce anti-inflammatory IL-10 and IL-9 are involved in providing defenses against nematodes (Mackay, 2000, Schmitt et al., 2014a) and the Th17 facilitate antimicrobial immunity and protection at mucocutaneous sites (Bettelli et al., 2008). Treg cells regulate immune responses and prevent autoimmunity while the Tfh cells interact with antigen-specific B cells to support B cell functions (Mackay, 2000).

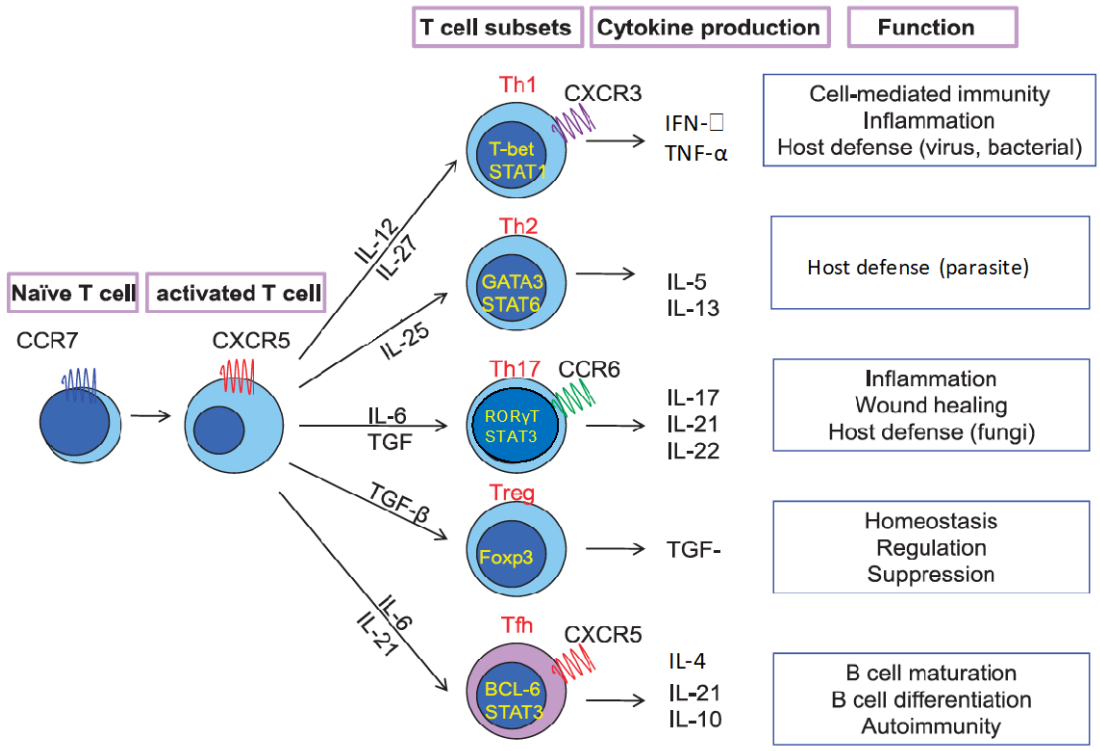

Figure 1.3: T helper cell lineage development and functions. T helper cell lineages comprise Th1, Th2, Th17, Treg and Tfh cells that mediate various immune functions. Also, the prevailing cytokine environment during the differentiation of activated $CD4^+$ T cells reinforces these respective lineages on activated $CD4^+$ T cells. Sourced from Zhang et al. (2013b)

1.6 T follicular helper (Tfh) cells

T follicular helper cells are important components of the adaptive immune system, whose primary function is to support B cell responses (Nurieva et al., 2010, Ma et al., 2012). The role of $CD4^+$ T cell help in the development of T-cell dependent antibody responses has long been known but it was only until the early 2000s that Tfh cells were identified as a distinct $CD4^+$ T helper subset specializing in helping B cell responses (Deenick et al., 2011). Tfh cells are characterized by a high expression of the follicle-homing chemokine receptor 5 (CXCR5), the transcription factor B cell lymphoma 6 (Bcl6), the inducible T cell co-stimulator (ICOS) and programmed cell death protein-1 (PD-1) (Breitfeld et al., 2000, Ma et al., 2012, Tangye et al., 2013).

1.6.1 Differentiation of Tfh cells

Numerous signals including T cell receptor signaling, surface molecules and the cytokine milieu contribute to the generation of Tfh cells (Deenick et al., 2011). Activated $CD4^+$ T cells upregulate the surface expression of CXCR5 thus promoting their migration to the B cell follicles (Figure 1.4).

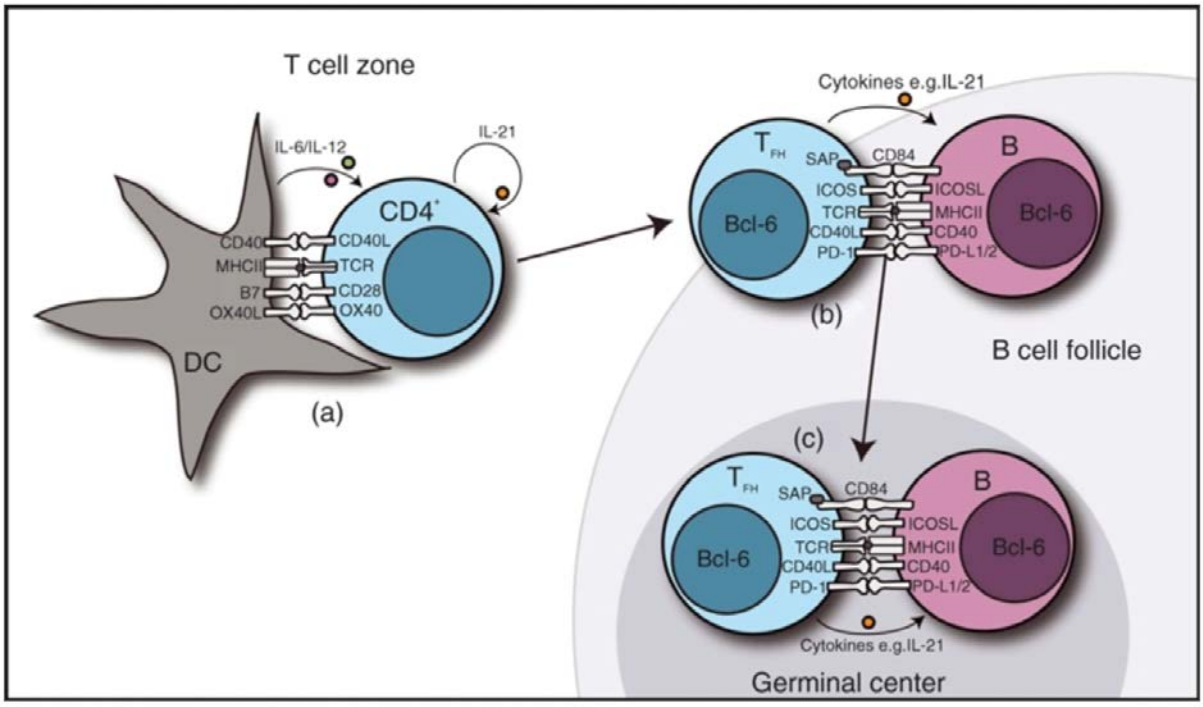

Figure 1.4: Generation of Tfh cells. TCR dependent interactions between $CD4^+$ T cells and dendritic cells (DCs) activate pre-Tfh cells in the T cell zones. Further interactions at the T-B border with antigen specific B cells promote their differentiation into matured Tfh cells, which upregulate CXCR5 and other Tfh associated markers and in turn facilitate B cells' affinity maturation in the germinal center. Adapted from Deenick et al. (2011).

Chemotactic signaling by the chemokine CXCL13 which is secreted by B cells and follicular stromal cells attract primed $CD4^+$ T cells through the ligand CXCR5 into the follicles of the secondary lymphoid tissues (Ramiscal et al., 2013, De Guinoa et al., 2011). Bcl6 is required for lineage differentiation of primed T cells as Tfh cells (Nurieva et al., 2009, Liu et al., 2013). It represses the expression of master transcription

factors; GATA-3, T-bet and RORγT of other $CD4^+$ T cell lineages, while promoting the expression of CXCR5, IL-21, IL-6 and other Tfh associated factors (Crotty, 2011).

These cellular and molecular signals mediated by specific cytokines including; IL-4, IL-6, IL-10 and IL-21, initiate the Tfh cell program which is enhanced by cell to cell interactions between the Tfh and B cells (Vogelzang et al., 2008, Vinuesa et al., 2005). Ongoing stimulation of Tfh cells by antigen-presenting B cells upregulates the expression of transcription markers, Bcl6 and c-Maf, which promote maintenance of the Tfh phenotype in the presence of cognate B cell interaction (De Guinoa et al., 2011). Thus, Tfh cell lineage differentiation is closely associated with its functional role in B cell differentiation and the development of long lived antibody responses (Pissani et al., 2014).

1.6.2 Tfh cells' help to B cells

Optimal B cell differentiation, survival, immunoglobulin class switching or class switch recombination and the production of long-lasting, high affinity antibodies after infection or vaccination requires the cognate help of Tfh cells (Hoffman et al., 2016, Crotty, 2011, Crotty, 2014). Naïve B cells activated in a T-cell-dependent manner present peptide-MHC-II complexes to early Tfh cells (Vinuesa et al., 2005, Vinuesa, 2012, Ramiscal et al., 2013). Cognate interactions between B cells and Tfh cells in lymphoid tissues at the T cell-B cell border (Mackay, 2000) through ligand-receptor engagements promote B cell differentiation and proliferation (Figure 5). Some of the B cells differentiate via the extra-follicular route to generate short lived plasma blasts that secrete low affinity antibodies while others migrate into the follicles and mature in germinal centers (Figure 1.5) (Hoffman et al., 2016). The master regulator of Tfh cells,

Bcl6 also modulates B cell differentiation by controlling cell cycle genes, DNA damage response genes and the B cell receptor signaling pathways (Crotty, 2011).

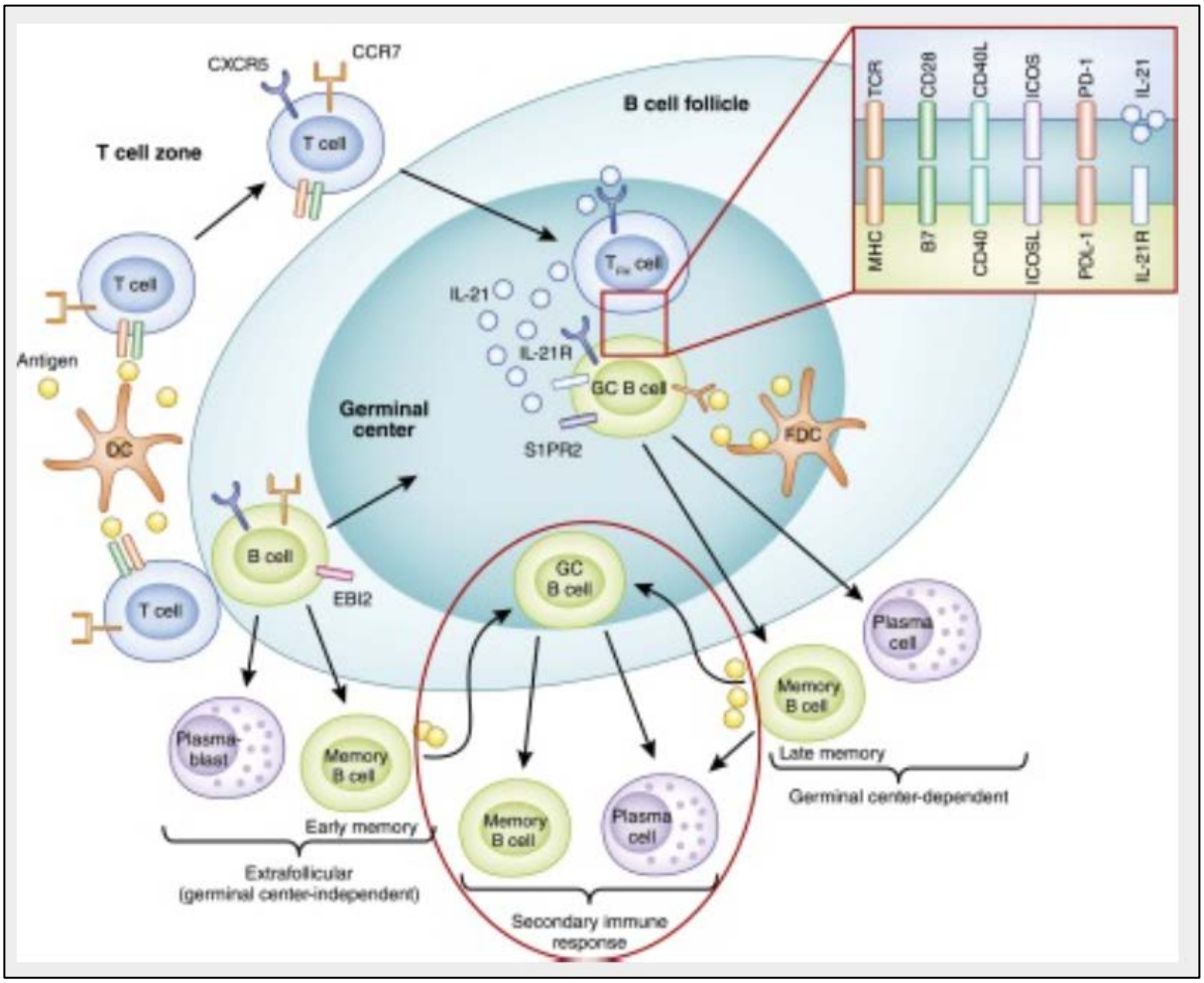

Figure 1.5: T follicular helper cell and B cell differentiation. $CD4^+$ T cells are primed by DCs in the T cell zones and migrate to the borders of the B cell follicles to interact with antigen primed B cells. Cognate interactions between Tfh cells and B cells initiate the germinal center reaction, which is maintained by T-B interactions and signaling cytokines like IL-21. Also, B cells differentiate in a germinal center dependent or independent manner into memory B cell and plasma cells or plasmablasts. Taken from Hoffman et al. (2016).

Signaling lymphocytic activation molecule (SLAM) family of receptors and SLAM-associated protein (SAP) adaptors sustain the close interaction of antigen-specific Tfh and B cells (Deenick et al., 2011). This interaction is re-enforced by the co-stimulatory signals of ICOS, PD-1 and CD40-ligand (CD40-L) and the secretion of soluble mediators and cytokines like IL-4 and IL-21 (Bryant et al., 2007, Vogelzang et al.,

2008, Pissani et al., 2014). The ligation of PD-1 by its ligand PDL-1 expressed on B cell, inhibits the suppressive signals of follicular regulatory T cells to promote the GC response (Pissani et al., 2014). Importantly, ligation of CD40 by CD40-L induces the expression of activation-induced cytidine deaminase (AID), which is a key enzyme that regulates somatic hyper mutation and immunoglobulin class switching (Pissani et al., 2014).

Tfh signaling by IL-21 is crucial for the formation and maintenance of GCs, which are specialized structures within the B-cell follicles of secondary lymphoid tissues for affinity maturation or class switching of antibodies (Bryant et al., 2007, Vogelzang et al., 2008, Ramiscal et al., 2013). During affinity maturation, pathogen-specific B cells acquire random point mutations within the immunoglobulin (Ig) V-region genes which changes the specificity of the B cell receptor (Ramiscal et al., 2013). Signals provided by Tfh mediate the positive selection of high affinity B cells over low affinity or self-reactive B cell clones (Vinuesa, 2012, Ramiscal et al., 2013). High affinity B cells may either undergo further rounds of affinity maturation, or alternatively develop into long-lived memory B cells or long-lived plasma cells (Borrow et al., 2017).

1.6.3 Circulating Tfh cells

Tfh cells primarily localize to the B cell follicles and in active GCs within lymphoid organs, however, recent literature has reported a population of $CD4^+$ T cells in the peripheral blood that possess similar phenotypic characteristics to the lymph nodes Tfh cells (Breitfeld et al., 2000, Morita et al., 2011, Locci et al., 2013). Circulating blood $CXCR5^+$ $CD4^+$ T cells have heterogeneous phenotypic characteristics (Hale et al., 2015). Unlike bonafide Tfh cells, circulating Tfh (cTfh) cells express lower levels of CXCR5, PD-1 and little or no Bcl6 (Pissani et al., 2014). PD-1 expressing cTfh cells

generally express ICOS and are activated and majority of cTfh are quiescent cells that have a central memory phenotype (Breitfeld et al., 2000, Locci et al., 2013, Schmitt et al., 2014b). Furthermore, cTfh cells comprise of Tfh1, Tfh1-17, Tfh2 and Tfh17 subsets, which are so named due to phenotypic similarities with Th1, Th2 or Th17 $CD4^+$ T cells (Schmitt et al., 2014b). The functional relevance of these subsets during HIV infection is not well understood (Pissani et al., 2014). In addition, because peripheral blood samples are easier to assess for research or vaccine monitoring than lymphoid tissues, there is interest in characterizing cTfh cells, understanding their similarities and differences to lymphoid tissue Tfh cells and defining their function during acute HIV infection (Locci et al., 2013, Boswell et al., 2014, Martin-Gayo et al., 2017).

1.7 Tfh cells in HIV infection

The role of Tfh cells in supporting efficient antibody responses has been described in the context of various autoimmune diseases and infectious diseases like malaria, influenza and HIV. Although Tfh cells are beneficial for the development of B cell responses during HIV infection, the regulation of Tfh cell numbers is critical because excessive expansion of Tfh cells during HIV infection has been associated with dysregulated antibody production and excessive B cell numbers (Lindqvist et al., 2012).

1.7.1 Tfh cells and HIV broadly neutralizing antibody responses

Extraordinarily high mutation rates of HIV-1 enables the virus to evade host antibody responses by producing resistant variants to any developing antibody (Nurieva et al., 2009). Interestingly, the resultant repeated cycles of GC antibody maturation and

somatic hyper mutation in some individuals brings about the development of anti-HIV broadly neutralizing antibodies (bNAbs). Longitudinal cohort studies following the development of bNAbs have allowed in-depth characterization of bNAbs. Structurally, HIV-1 bNAbs have a protruding complementarity-determining region (CDR) and H3s formed by VDJ recombination or insertions (Doria-Rose et al., 2014). These characteristics are acquired due to high levels of somatic hypermutations during the affinity maturation process and facilitated by repeated Tfh cells' interaction with GC B cells (Cubas et al., 2013). These characteristics also suggest a constant and prolonged activation of the enzyme AID whose activation and phosphorylation processes occurs through CD40-CD40L signaling mediated by Tfh cells (Pissani et al., 2014). Thus, suggesting that bNAbs are developed due to adequate B cell help offered by the Tfh cells within the GCs (Vinuesa, 2012).

The heightened interest in studying Tfh cells emanates from their critical role in the generation of bNAbs (Locci et al., 2013). Furthermore, the possibility that the magnitude of Tfh responses after vaccination could be a useful biomarker for successful bNAbs induction by the candidate vaccine was proposed (Streeck et al., 2013, Pissani et al., 2014, Ahlers, 2014). Nevertheless, the magnitude or quality of antigen-specific Tfh responses that is beneficial for the development of bNAbs is unknown. This knowledge is critical since excessive Tfh expansion has been associated with hypergammaglobulinemia; the generation of low quality antibodies in chronic HIV and autoimmunity (Lindqvist et al., 2012, Pratama et al., 2014).

1.7.2 Tfh cells and HIV persistence

The complete eradication of HIV by ART has not been possible due to HIV persistence in the latent reservoir comprising majorly of long-lived memory $CD4^+$ T cells (Murray

et al., 2016). Soon after HIV infection, a latent reservoir of HIV is established in the human body and these transcriptionally silent cells evade immune recognition or ART mediated killing of HIV within these cells (Ananworanich et al., 2016, Murray et al., 2016).

Tfh have been implicated in HIV persistence during ART for several reasons (Miles et al., 2016a). Studies have shown that Tfh cells are more permissive to HIV infection compared to other $CD4^+$ T cells subsets, with further studies indicating that Tfh cells harbor a higher percentage of HIV DNA compared to various other cell subsets (Perreau et al., 2013, Pallikkuth et al., 2015, Kohler et al., 2016). Particularly with Tfh cells being more abundant in lymphoid tissue GCs, which as previously mentioned, (Lorenzo-Redondo et al., 2016). Follicular dendritic cells can harbor HIV virions on their cell processes for prolonged periods of time and as Tfh are found in close contact with these cells, it increases their chances for HIV infection (Alexaki et al., 2008, Heesters et al., 2015, Miles et al., 2016a). Moreover, infected Tfh cells evade direct killing by immune cells since CTLs and natural killer cells are mostly excluded from GCs where Tfh cells interact with B cells (Fukazawa et al., 2015). It is generally believed that a distinct subset of GCTfh cells harbors the HIV reservoir and the identification of this subset is the focus of active investigations by various research groups.

1.8 Thesis outline

The need for an HIV vaccine has never been greater, yet this remains an elusive goal. Most investigators believe that an effective vaccine will require both broadly neutralizing antibodies and effective cytotoxic T cell responses and efforts are underway to develop vaccines to induce these responses (McElrath et al., 2010,

Haynes et al., 2016). However, one critical aspect of host immunity that has been persistently neglected is vaccine-mediated induction of HIV-specific $CD4^+$ T follicular helper (Tfh) cells. There have been concerns that the induction of such responses by a therapeutic vaccine would add “fuel to the fire”, since Tfh cells are targets for HIV and are cellular reservoirs for HIV persistence in infected persons (Kohler et al., 2016, Perreau et al., 2013). We however, hypothesize that these responses are critical to any successful preventive or therapeutic vaccine strategy. Moreover, we postulate that understanding the ontogeny and function of these responses will accelerate the path toward a successful vaccine.

Studies of primary HIV infection have been critical for understanding the components of an effective immune response to HIV-1 infection, that can be translated to vaccine studies (Cohen et al., 2001). However, with the recent guidelines specifying the administration of ART to all HIV-1 infected persons (WHO, 2015, Meintjes et al., 2017), as well as recent efforts to provide pre-exposure prophylaxis to high-risk individuals (WHO, 2007, Karim et al., 2010, McElrath et al., 2010, WHO, 2013), it has become clear that any candidate prophylactic or therapeutic vaccine will be administered to individuals taking ARV drugs. Thus, understanding how early treatment modulates immune responses is key to future interventions.

Over the past five years, our group has developed a unique longitudinal cohort of persons with “hyperacute” HIV infection termed FRESH for Females Rising through Education, Support and Health. This program at the heart of the HIV epidemic in KwaZulu-Natal, South Africa has successfully identified and treated persons at the onset of plasma viremia, in some cases, when plasma viral loads are less than 1000 RNA copies/ml (Dong et al., 2017). In addition to this unique cohort, we established

protocols for excisional lymph node biopsies, cell sorting, high-resolution multicolor immunofluorescence microscopy and class II tetramer technology and thus, set out to perform a detailed characterization of the role of Tfh cells in the setting of controlled infection. The overall goal of this project was to define the role of Tfh cells in controlling HIV disease progression, to determine how extremely early ART initiation modulates Tfh cell function and to define the contribution of Tfh subsets to persistent HIV infection during early ART.

The detailed aims of the present study are as follows:

Aim 1: To perform a comprehensive phenotypic and functional characterization of lymphoid tissue Tfh and peripheral blood Tfh cell subsets during hyperacute and chronic HIV-1 subtype C infection.

Aim 2: To determine the relationship between Tfh cell responses during hyperacute HIV-1 infection, and antibody levels or HIV-1 disease progression.

Aim 3: To define the anatomical localization of Tfh cell subsets in lymphoid tissue in relation to B cells and HIV-1 antigens and to define the contribution of Tfh cell subsets to virus persistence in the lymph nodes during HIV-1 infection.

Chapter 1 is the introduction of the thesis. It includes a review of relevant topics and defines the aims of the study.

In chapter 2, we report on the comprehensive phenotypic characterization of circulating T follicular helper (cTfh) cell subsets in the context of acute HIV-1 clade C infection using flow cytometry and HLA class II tetramers. We screened for plasma anti-gp41, -gp120, -p24 and -p17 antibodies using a customized multivariate Luminex

assay and further defined the contribution of cTfh subsets to the development of non-neutralizing antibodies and HIV-1 disease progression.

Chapter 3 was directed towards characterizing Tfh responses in lymphoid tissue samples. Specifically, we defined immune responses in HIV-1 infected individuals initiated on ART during the very early stages of acute infection. In addition, the phenotype and helper function of Tfh cells were defined *ex vivo* using flow cytometry, HLA class II tetramers, *in vitro* co-culture assays, ELISA and digital droplet PCR techniques. The localization of Tfh cells *in situ* was also defined using immunohistochemistry (IHC) and immunofluorescence (IF) microscopy assays.

In chapter 4 of the study, we investigated virus persistence in lymphoid tissues and determined how this influenced the induction of immune responses during early treated HIV-1 infection. The cellular distribution of HIV-1 was defined using IHC, IF microscopy, RNA *in situ* hybridization, digital droplet PCR and flow cytometry techniques.

Chapter 5 is a discussion of the overall implications of our findings and includes future directions for the study.

Chapter 2 Overview

From our review of existing literature, we have shown that T follicular helper (Tfh) cells are important for the development of efficient antibody responses to HIV-1 infection. We have also shown that circulating counterparts of lymph node Tfh cells have been described. In addition, we have discussed the importance of HIV-specific $CD4^+$ T cell responses for natural HIV-1 control. In Chapter 2, we present a study conducted to determine if circulating Tfh cells impact HIV-1 disease progression during untreated acute HIV-1 infection. In addition, we showed that circulating Tfh cells are heterogeneous in phenotype and the various subsets differentially influence the development of non-neutralizing antibodies. These results have been published in the Journal of Virology. (2018, volume 92, issue 15)

CHAPTER 2: FREQUENCIES OF CIRCULATING TH1-BIASED T FOLLICULAR HELPER CELLS IN ACUTE HIV-1 INFECTION CORRELATE WITH THE DEVELOPMENT OF HIV-SPECIFIC ANTIBODY RESPONSES AND LOWER SET POINT VIRAL LOAD

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Keywords: HIV, T follicular helper cells, non-neutralizing antibodies, Gag p24 IgG

2.1 Abstract

Despite decades of focused research, the field has yet to develop a prophylactic vaccine for HIV-1 infection. In the RV144 vaccine trial, non-neutralizing antibody responses were identified as a correlate for prevention of HIV acquisition. However, factors that predict the development of such antibodies are not fully elucidated. We sought to define the contribution of circulating T follicular helper (cTfh) cell subsets to the development of non-neutralizing antibodies in HIV-1 clade C infection. Study participants were recruited from an acute HIV-1 clade C infection cohort. Plasma anti-gp41, -gp120, -p24 and -p17 antibodies were screened using a customized multivariate Luminex assay. Phenotypic and functional characterizations of cTfh cells were performed using HLA class II tetramers and intracellular cytokine staining. In this study, we found that acute HIV-1 clade C infection skewed differentiation of functional cTfh subsets towards increased Tfh1 (p=0.02) and Tfh2 (p<0.0001) subsets, with a concomitant decrease in overall Tfh1-17 (that shares both Tfh1 and Tfh17 properties) (p=0.01) and Tfh17 subsets (p<0.0001) compared to HIV negative subjects. Interestingly, the frequencies of Tfh1 during acute infection (5.0-8.0 weeks post-infection) correlated negatively with set point viral load (p=0.03, r=-60) and were predictive of p24-specific plasma IgG titers at one year of infection (p=0.003, r=0.85). Taken together, our results suggest that the circulating Tfh1 subset plays an important role in the development of anti-HIV antibody responses and contributes to HIV suppression during acute HIV-1 infection. These results have implications for vaccine studies aimed at inducing long lasting anti-HIV antibody responses.

2.2 Introduction

A safe and effective prophylactic vaccine remains the most efficient way of ending the HIV/AIDS epidemic which affects over 36 million people worldwide (UNAIDS, 2017a). Although studies in non-human primate and animal models have demonstrated the efficacy of anti-HIV broadly neutralizing antibodies (bNAbs) in preventing HIV infection, human vaccine trials to date have been largely unsuccessful in inducing such responses (Rerks-Ngarm et al., 2009, Genesca et al., 2010, Kwong et al., 2011). Thus, an improved understanding of the mechanisms that underlie the development of functional and durable anti-HIV antibody responses in the context of a natural infection will be essential for optimal vaccine design efforts (Martin-Gayo et al., 2017). Moreover, with the quality of immune responses in early acute HIV infection predicting disease outcome (Pantaleo et al., 1997, Deeks et al., 2004), early acute HIV infection is a useful model to identify early correlates of HIV-1 control.

T follicular helper (Tfh) cells, a lineage of $CD4^+$ T cells that express the chemokine receptor CXCR5, are specialized for B cell help and the development of antibody responses (Crotty, 2011, Crotty, 2014). Tfh-B cell interactions in the B cell follicles promote germinal center (GC) formation, B cell differentiation, B cell survival, antibody affinity maturation and class switch recombination (Vinuesa et al., 2005, Crotty, 2011). The circulating memory counterparts of *bona fide* germinal center Tfh cells have been recently described (Morita et al., 2011, Hale et al., 2015). These cells display either an activated or quiescent phenotype based on the expression of PD-1 and ICOS or CCR7 receptors and can be further divided into subsets based on the expression of CXCR3 and CCR6 receptors (Schmitt et al., 2014b, Hale et al., 2015). The subsets; Tfh1, Tfh2, Tfh17 and Tfh1-17, were named due to their similarities to other T helper

cell lineages. Tfh1 cells express CXCR3 like Th1 cells, Tfh2 cells produce IL-4 like Th2 cells, Tfh17 cells express CCR6 similar to Th17 cells and Tfh1-17 cells have functional properties that are similar to both Th1 and Th17 cells (Morita et al., 2011, Schmitt et al., 2014b, Hale et al., 2015).

From the RV144 vaccine trial, which had a modest efficacy in preventing HIV acquisition, we learned that non-neutralizing antibodies (nnAbs) could protect against HIV acquisition (Haynes et al., 2012). Consistent with this observation, a recent study exploring the efficacy of nnAbs for blocking virus entry, showed that anti-Env nnAbs could modulate the transmission of simian HIV (SHIV) in macaques and reduce the number of transmitted/founder viruses establishing infection in the animals (Santra et al., 2015). Moreover, a humanized mouse model of HIV infection, reported near-complete clearance of adoptively transferred infected cells within 5 hours of nnAbs treatment (Horwitz et al., 2017) further demonstrating the potential for nnAbs in preventing HIV infection.

Specific Tfh subsets have been shown to help the induction of various antibody functions. For instance, a recent study correlated the frequencies of $CXCR3^-$ cTfh; which includes both Tfh2 and Tfh17 subsets, with the development of bNAbs against HIV infection (Locci et al., 2013), suggesting a potential role of these subsets as correlates for the induction of bNAbs in infection and possibly by vaccines. It is thus important to define specific Tfh subsets that contribute to nnAbs development in the context of natural HIV infection.

Here we investigated if the induction of cTfh responses during acute HIV infection contribute to initial HIV control and promote the development of anti-HIV nnAbs. We examined the role of HIV-specific cTfh cell subsets during acute HIV infection using

HLA class II tetramers and multiparametric flow cytometry. HIV-specific antibody responses were further measured using a customized multivariate Luminex assay. Our results showed that acute HIV infection induces significant expansion of HIV-specific memory Tfh1 cells (p=0.02), which correlated with lower set point viral loads. Moreover, the frequencies of Tfh1 cells during early infection were predictive of p24-specific IgG titers. These data suggest that circulating Tfh1 cells play a role in controlling viral replication during primary HIV infection by enhancing robust anti-HIV antibody production, which is desirable for a prophylactic HIV vaccine.

2.3 Materials and Methods

2.3.1 Study Participants

Study participants comprised of 16 acute and 5 chronic HIV-infected ART-naïve individuals from HIV Pathogenesis Programme (HPP) Acute Infection cohort, Durban, South Africa. Patients were chosen based on availability of acute infection samples. Acute infection classification and disease staging in this cohort was previously described (Wright et al., 2011). Briefly, at screening, patients had detectable HIV RNA but had not yet seroconverted, either by ELISA or Western blotting. The date of infection for the study participants was estimated to be 14 days prior to screening as previously described (Van Loggerenberg et al., 2008). One acute infection time point was selected per patient for the study based on sample availability. The time post-infection across the patients was a median of 7 weeks (interquartile range-IQR, 5.25-7.75). The CD4 count, viral load and other patient characteristics are summarized in table 1.

15 HIV uninfected individuals from the Females Rising Through Education, Support and Health (FRESH) cohort (Ndhlovu et al., 2015, Dong et al., 2017), also in Durban, South Africa were included as controls. The controls were chosen randomly based on sample availability at the time the study was conducted. The University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) and the Massachusetts General Hospital ethics review board approved the study. All study participants signed informed consent for participation in the study.

2.3.2 Immunophenotyping

For surface phenotyping, frozen PBMCs were thawed, rested and stained using the LIVE/DEAD Aqua dead cell staining kit (Thermofisher scientific, Waltham MA, USA) as per manufacturer's instructions, followed by staining with an antibody panel comprising: CD14 Horizon V500 [(HV500) BD Biosciences, San Jose, CA], CD19 HV500 (BD Biosciences), CD3 Brilliant Violet (BV) 711 (BioLegend, San Diego, CA, USA0, CD8 Qdot 800 (Life Technologies, Carlsbad, CA, USA), CD4 Qdot 655 (Life Technologies), CXCR5 Alexa fluor (AF) 488 (BD Biosciences), PD-1 BV421 (BioLegend), CCR6 phycoerythrin (PE) (BioLegend), CXCR3 BV605 (BioLegend), CD45RA PE-Cy7 (BioLegend), CCR7 Peridin-chlorophyll (PerCp) Cyanine (Cy) 5.5 (BioLegend) and CD27 APCH7 (BD Biosciences). For intracellular cytokine staining, peripheral blood mononuclear cells (PBMCs) were either left unstimulated or stimulated with HIV clade C overlapping peptide (OLP) pools spanning Gag, Nef, or Env proteins or staphylococcal enterotoxin B (SEB, 0.5 µg/ml) in the presence of GolgiStop and GolgiPlug protein transport inhibitors (BD Biosciences) for 16 hours at 37°C. Cells were surface stained, washed, fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. Cells

were subsequently stained intracellularly with IL-2 PE (BD Biosciences), IL-21 APC (BioLegend), TNF-α A700 (BD Biosciences), IL-4 BV605 (BioLegend) and IFN-γ PE-Cy7 (BioLegend) antibodies. Cells were acquired using an LSRFortessa cytometer (BD Biosciences) with FACSDiva™ software and fluorescence minus one controls were used to define gates for the different cell subsets. Data was analysed using the FlowJo version 10.0.8 (Flowjo, LLC).

2.3.3 HLA class II tetramer staining

HIV-specific cTfh responses were measured using HLA class II tetramers. The immunodominant Gag C41 epitope (Laher et al., 2017) was interrogated using DRB1*11:01 and DRB1*13:01 tetramers produced in the laboratory of Dr Søren Buus as previously described (Braendstrup et al., 2013). The design and validation of these tetramers by our group have also been described (Laher et al., 2017). Briefly, recombinant human DRB1*11:01 or DRB1*13:01 HLA molecules were complexed with clade C HIV-1 Gag 41 peptide (YVDRFFKTLRAEQATQDV). For the assay, PBMCs were stained for 1 hour at 37^{o}C with APC and PE conjugated HLA class II tetramer complexes, washed in 2% fetal calf serum (FCS) in phosphate buffered saline (PBS) and then stained with these antibodies: LIVE/DEAD Fixable Blue dead cell stain kit (Thermofisher Scientific), CD3 BV711 (BioLegend), CD4 BV650 (BD Biosciences), CD8 BV786 (BD Biosciences), CXCR5 AF488 (BD Biosciences), CXCR3 BV605 (BioLegend), PD-1 BV421 (BioLegend) and CD45RA AF700 (BioLegend); for 20 min at room temperature. Cells were washed and acquired on the LSRFortessa (BD Biosciences).

2.3.4 Customized multivariate Luminex assay

Plasma HIV-1 specific antibodies were measured using a customized multivariate Luminex assay as previously described (Brown et al., 2012). Carboxylated fluorescent polystyrene beads (Biorad, Hercules, CA, USA) were coated with HIV-1 specific proteins including gp120 clade C of strain ZA.1197MB, gp41 clade C of strain ZA.1197MB, C-terminal 6xHis tagged p24 subtype C and p17 HXBc2 (Immune Technology, New York, NY, USA). Plasma samples were incubated with antigen-coated beads in a 96 well plate and unbound antibodies were washed with 0.05% Tween-20 in PBS. HIV-1 specific IgG antibodies were detected with PE mouse IgG1 to IgG4 secondary antibodies.

2.3.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA). Statistical significance was assessed using Mann-Whitney U tests and Kruskal-Wallis H test, or two-way ANOVA with Dunn's multiple comparisons test. The correlations between two variables were done using Spearman's rank correlation. *P* values were considered significant if less than 0.05.

2.4 Results

2.4.1 Circulating CXCR5$^+$ cells in healthy donors have a predominantly central memory phenotype.

Recent studies have focused on characterizing circulating CXCR5$^+$CD4$^+$ T cells (cTfh) because of their similarities with germinal center Tfh cells and their potential role in the development of bNAbs (Locci et al., 2013, Cohen et al., 2014). The difficulty

associated with obtaining *bona fide* Tfh from lymphoid tissues has also stirred the interest in studying cTfh as surrogates. Although the phenotype of cTfh has not been clearly defined, the consensus is that they represent circulating memory Tfh (Schmitt et al., 2014b). To determine how HIV infection perturbs global frequencies and phenotypes of peripheral Tfh we began by establishing baseline characteristics of this cell population in our study cohort who are predominantly of the Zulu/Xhosa ethnicity. We used CCR7 and CD45RA, well-established memory markers to define four memory subsets. Specifically, we defined naïve (N) T cells by gating on $CCR7^+$ and $CD45RA^+$, central memory (CM) by $CCR7^+CD45RA^-$, effector memory (EM) by $CCR7^-CD45RA^-$, and terminally differentiated effector memory (TEMRA) by $CCR7^-CD45RA^+$ (Larbi et al., 2014) (Figure 2.1A).

Phenotypic analysis of total $CD4^+$ T cells from 12 HIV negative donors revealed that 34.0% (29.1-43.2) were naïve, 21.8% (19.1-28.0) were CM, 33.7% (30.4-44.4) were EM and 2.8% (2.1-3.3) were TEMRA (Figure 2.1B). Next, we measured the frequency of cTfh ($CXCR5^+CD4^+$) cells and found that they comprised 12% (10.1-14.3) of circulating $CD4^+$ T cells (Figure 2.1C). Memory phenotyping of Tfh cells showed that cTfh cells comprised 37.3% of CM $CD4^+$ T cells, 7.8 % of EM $CD4^+$ T cells and only a paltry 2.6% and 2.9% of the naïve and TEMRA $CD4^+$ T cell compartments respectively (Figure 2.1D). Consistent with studies in Caucasian populations (Chevalier et al., 2011b, Boswell et al., 2014), our data show that cTfh constitute a small fraction of circulating $CD4^+$ T cells and are predominantly of a central memory phenotype.

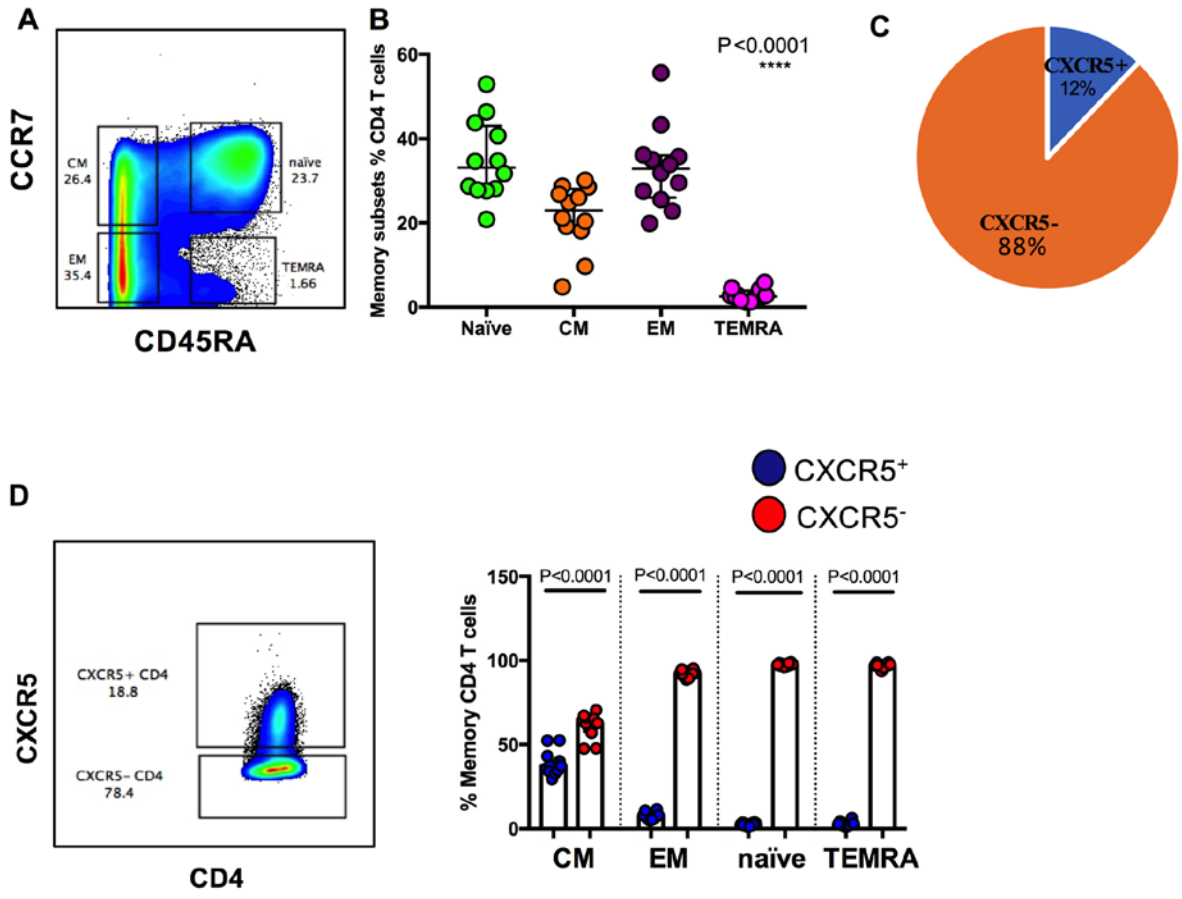

Figure 2.1: Memory distribution of CXCR5$^+$ cells within circulating CD4$^+$ T cell compartment in healthy donors. (A) Representative flow cytometry plot showing the gating strategy for CD4$^+$ T cell memory populations. (B) Summary dot plots showing proportions of CD4$^+$ T cells that are naïve, central (CM), effector (EM) and terminally differentiated (TEMRA) memory cells. (C) Pie chart showing median percentages of CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T cells. (D) Representative flow cytometry plots for CXCR5$^+$ and CXCR5$^-$ gating within bulk CD4$^+$ T cells and summary plots depicting the proportions of CXCR5$^+$ (blue) and CXCR5$^-$ (red) CD4$^+$ T cells within the CM, EM, naïve and TEMRA memory subsets. Statistical analysis was done using Kruskal-Wallis H test (B) and Mann-U Whitney tests (D).

2.4.2 Perturbation of circulating Tfh cells during acute HIV-1 infection.

Having established the normal frequencies and phenotypes of circulating Tfh cells, we next investigated how acute HIV infection alters the frequency and differentiation profiles of these cells. Samples obtained at a median of 6.9 weeks after HIV diagnosis were used for these studies (Table 2.1). As shown in (Figure 2.2A), HIV infection did not alter the overall frequencies of total circulating memory Tfh.

Table 2.1: Characteristics of study participants

	HIV negative	Acute HIV
n	12	14
Male	0	6
Female	12	8
CD4 Count (cells/µl)*	N/A	463 (422-561)
Viral load (copies/ml)*	N/A	121 000 (8 984-352 000)
Time post-infection (weeks)*	N/A	6.9 (5.0-8.0)

*Data are represented as median (IQR).

However, memory subset analysis revealed an increase in naïve Tfh (p=0.004) and TEMRA Tfh (p=0.02), whereas CM Tfh (p=0.13) and EM Tfh (p=0.16) remained unchanged (Figure 2.2B).

Next, we used CXCR3 and CCR6 chemokine receptor markers to characterize cTfh subsets in an effort to identify which subset most influences the generation of anti-HIV antibodies during acute HIV infection. CXCR3 and CCR6 chemokine receptor markers have been previously used to identify several functional subsets that exhibit distinct B cell helper functions namely: Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$), Tfh1-17 ($CXCR3^+CCR6^+$) and Tfh17 ($CXCR3^-CCR6^+$) (Schmitt et al., 2014b). A representative flow plot, as seen in Figure 2.2C, depicts the distribution of cTfh subsets in an acutely infected donor based on the expression levels of the two respective chemokine receptor markers. Interestingly, acute infection skewed the distribution of cTfh subsets towards the Tfh1 (p=0.02) and Tfh2 (p<0.0001) phenotypes with significant reduction

in the proportions of Tfh1-17 (p=0.01) and Tfh17 (p<0.0001) compared to HIV negative donors (Figure 2.2C).

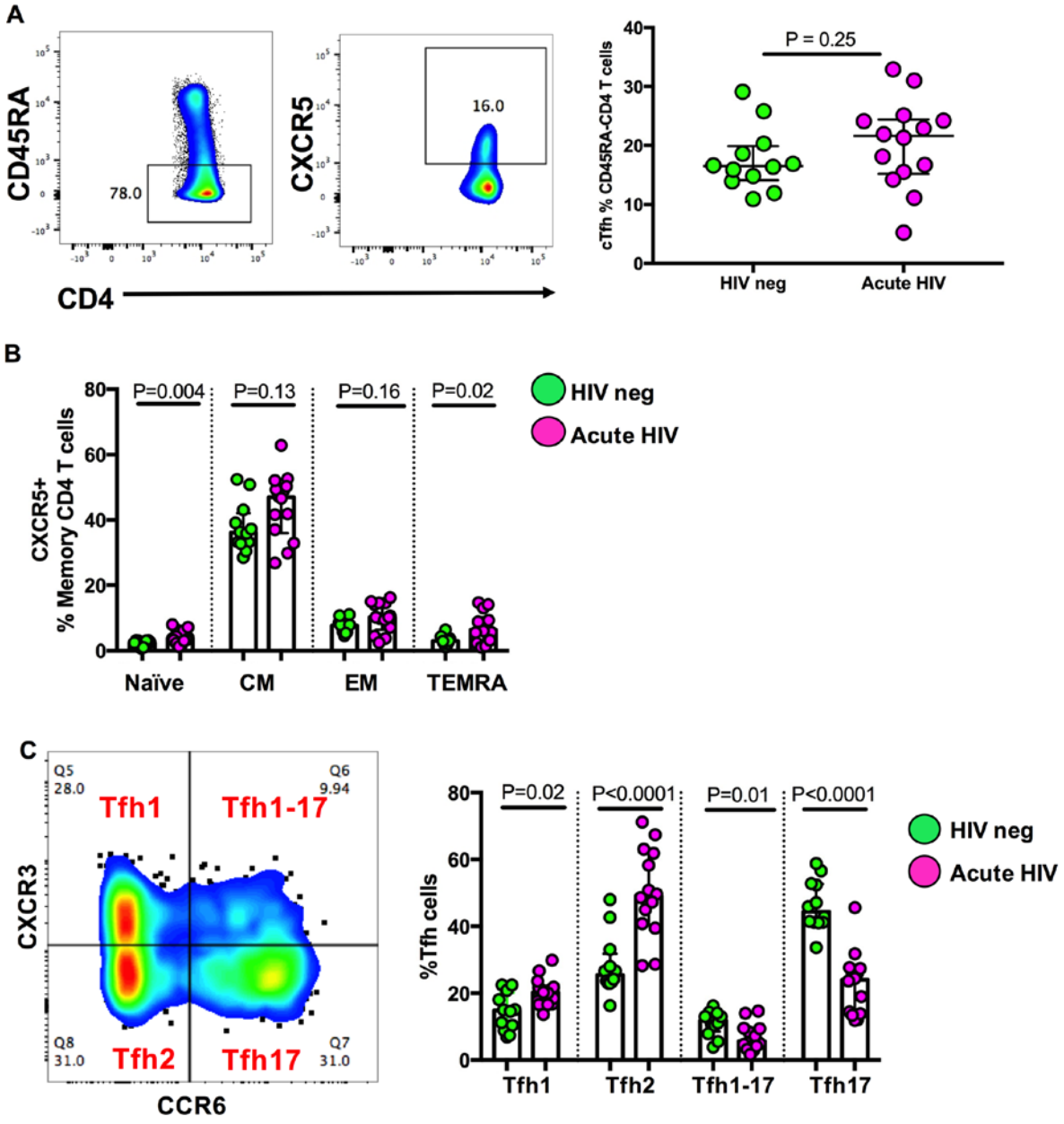

Figure 2.2: Heterogeneity within circulating Tfh compartment during acute HIV-1 infection. (A) Representative flow cytometry plots showing the gating strategy for bulk cTfh within CD45RA- CD4$^+$ T cells and summary proportions of cTfh cells in HIV negative and acute HIV groups. (B) Summary plot comparing the frequencies of naïve, CM, EM and TEMRA cTfh cells in HIV negative and acute HIV donors. (C) Gating strategy for Tfh1, Tfh1-17, Tfh2 and Tfh17 subsets. The proportions of Tfh1, Tfh2, Tfh1-17 and Tfh17 subsets are compared between HIV negative and acute HIV groups. *P* values are from Mann-U Whitney tests.

2.4.3 Frequency of Tfh1 cells during early acute HIV-1 infection correlates negatively with set point viral load.

Having observed a significant expansion of Tfh1 and Tfh2, we next investigated if there was a relationship between the expanded cTfh subsets and set point viral load (SPVL), which is a reliable predictor of the rate of HIV disease progression. We calculated SPVL as the average VL from 3 to 12 months' post-infection as previously reported (Wright et al., 2011) and correlated it to the frequencies of different cTfh subsets. Strikingly, Tfh1 frequencies correlated negatively with SPVL (p=0.03, r=-0.60) (Figure 2.3A) but there were no significant associations between Tfh2, Tfh1-17, Tfh17 or bulk cTfh cells and SPVL (Figures 2.3B, 2.3C, 2.3D and 2.3E). These results suggest that Tfh1 cells contribute to viral control during the first year of infection.

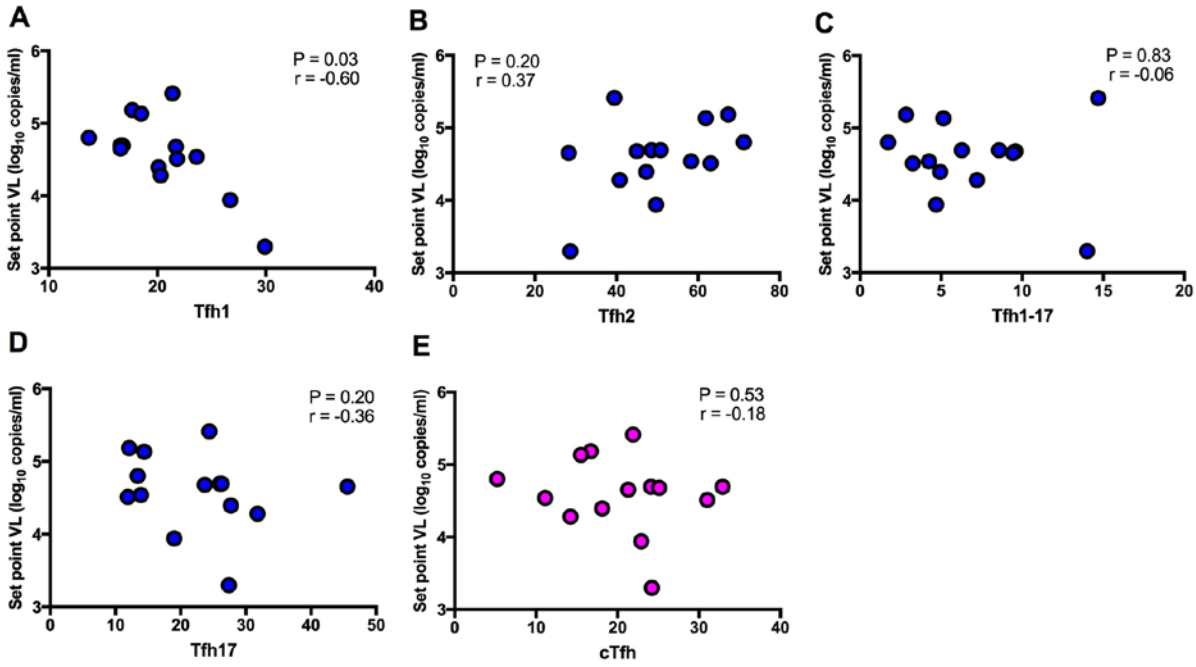

Figure 2.3: Tfh1 correlates negatively with set point viral load. Set point viral load was plotted against the frequency percentages of (A) Tfh1, (B) Tfh2, (C) Tfh1-17, (D) Tfh17 and (E) bulk cTfh cells determined by flow cytometry. Spearman rho (r) values and p values are reported.

2.4.4 Tfh1 responses during early acute infection correlate with p24 IgG responses detected at one year post-infection.

Numerous studies have associated slower disease progression with higher serum levels of HIV-1 Gag-specific IgG antibodies [reviewed in (French et al., 2013)]. We next, hypothesized that Tfh1 responses impact SPVL by driving the production of HIV-specific IgG antibodies. We measured plasma gp41, gp120, p17 and p24-specific IgG antibody titers at 12 months after infection for 10 study participants based on sample availability. Correlation analysis of IgG titers with SPVL revealed a negative correlation between SPVL and p24 IgG (p=0.007 r=-0.81) (Figure 2.4A) or gp41 IgG (p=0.009, r=-0.80) (Figure 2.4B) and no significant correlations between SPVL and p17 IgG (p=0.09, r=-0.58) (Figure 2.4C) or gp120 IgG titers (p=0.20, r=-0.44) (Figure 2.4D). We also examined the correlation of SPVL to the titers of p24 IgG isotypes; IgG1, IgG2, IgG3 and IgG4 and found no significant correlations between the p24 IgG isotypes and SPVL (Figure 2.4E and data not shown).

Lastly, we interrogated the relationship between Tfh1 frequencies and antibody titers. We found that Tfh1 frequencies during early infection (5.0-8.0 weeks) were directly correlated to the plasma titers of p24 IgG (p=0.003, r=0.85), p17 IgG (p=0.01, r=0.77), gp41 IgG (p=0.05, r=0.65) and p24 IgG1 (p=0.04, r=0.66) that were detected at 1 year post-infection (Figures 2.4F, 2.4G, 2.4H and 2.4I). There was however, no association between gp120 titers and Tfh1 frequencies (Figures 2.4J). These results suggest that the polarization of cTfh responses towards a Tfh1 phenotype can potentially impact the development of long-lasting antibody responses.

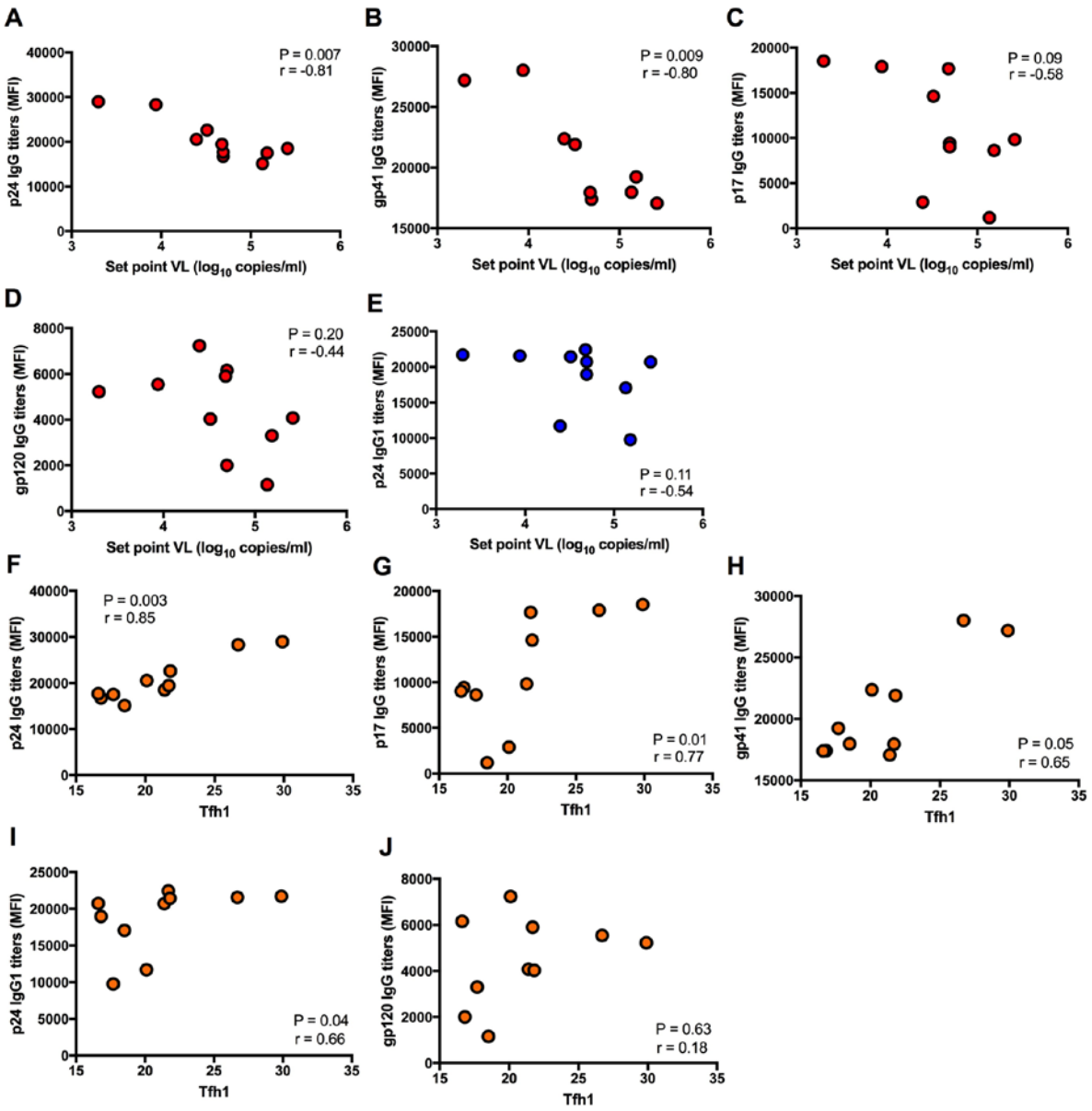

Figure 2.4: Early Tfh1 responses are predictive of p24 IgG responses at 1 year of infection. (A) p24 IgG titers, (B) gp41 IgG titers, (C) p17 IgG titers, (D) gp120 IgG titers and (E) p24 IgG1 titers, at 1 year time-point were determined using a customized multivariate Luminex assay and the values were inversely correlated to SPVL. (F) p24 IgG, (G) p17 IgG, (H) gp41 IgG, (I) p24 IgG1 and (J) gp120 IgG titers at 1 year time-point were correlated to the frequencies of Tfh1 at 6.9 (IQR, 5.0-8.0) weeks of infection. Mean Fluorescence Intensity (MFI) and viral load (VL). Spearman rho (*r*) values and *p* values are reported.

2.4.5 HIV-specific Tfh responses are induced during acute HIV-1 infection. Next, we investigated if the expanded cTfh in acute HIV infection were HIV-specific using intracellular cytokine staining (ICS) assay and MHC class II tetramers. Although HIV-specific $CD4^+$ T cells are important for viral control (Porichis et al., 2011), the presence of HIV-specific Tfh responses in circulation remains controversial (Locci et al., 2013, Morita et al., 2011). Therefore, we interrogated the cytokine expression pattern of cTfh cells after stimulation with HIV peptides. Figure 2.5A shows representative flow plots of unstimulated controls (top panel), cytokine secreting antigen specific $CD4^+$ cells responding to HIV peptide pools (middle panel) or SEB stimulation (bottom panel) in an ICS assay. Our group previously showed that most of the HIV-specific $CD4^+$ T cells in chronic clade C infection target the HIV Gag protein (Laher et al., 2017). Here we found no significant differences in Gag, Nef and Env responses (Figure 2.5B).

Further interrogation of the cytokine profile of cTfh cells revealed that unlike SEB-specific cells which abundantly secreted TNF-α and IFN-γ (Figure 2.5C), HIV-specific cTfh were biased towards the secretion of Tfh functional cytokines: IL-21 and IL-4, with lower proportions of cTfh cells secreting TNF-α and IFN-γ (Figure 2.5D). These differences however did not reach statistical significance after correcting for multiple comparisons (Figure 2.5D). Comparative analysis with non cTfh cells revealed that HIV-specific cTfh cells (blue) secreted more IL-21 (Figure 2.5E i, ii & iv) and IL-4 (Figure 2.5E ii & iii) whereas non cTfh cells (red) secreted significantly more IFN-γ (Figure 2.5E i & iv).

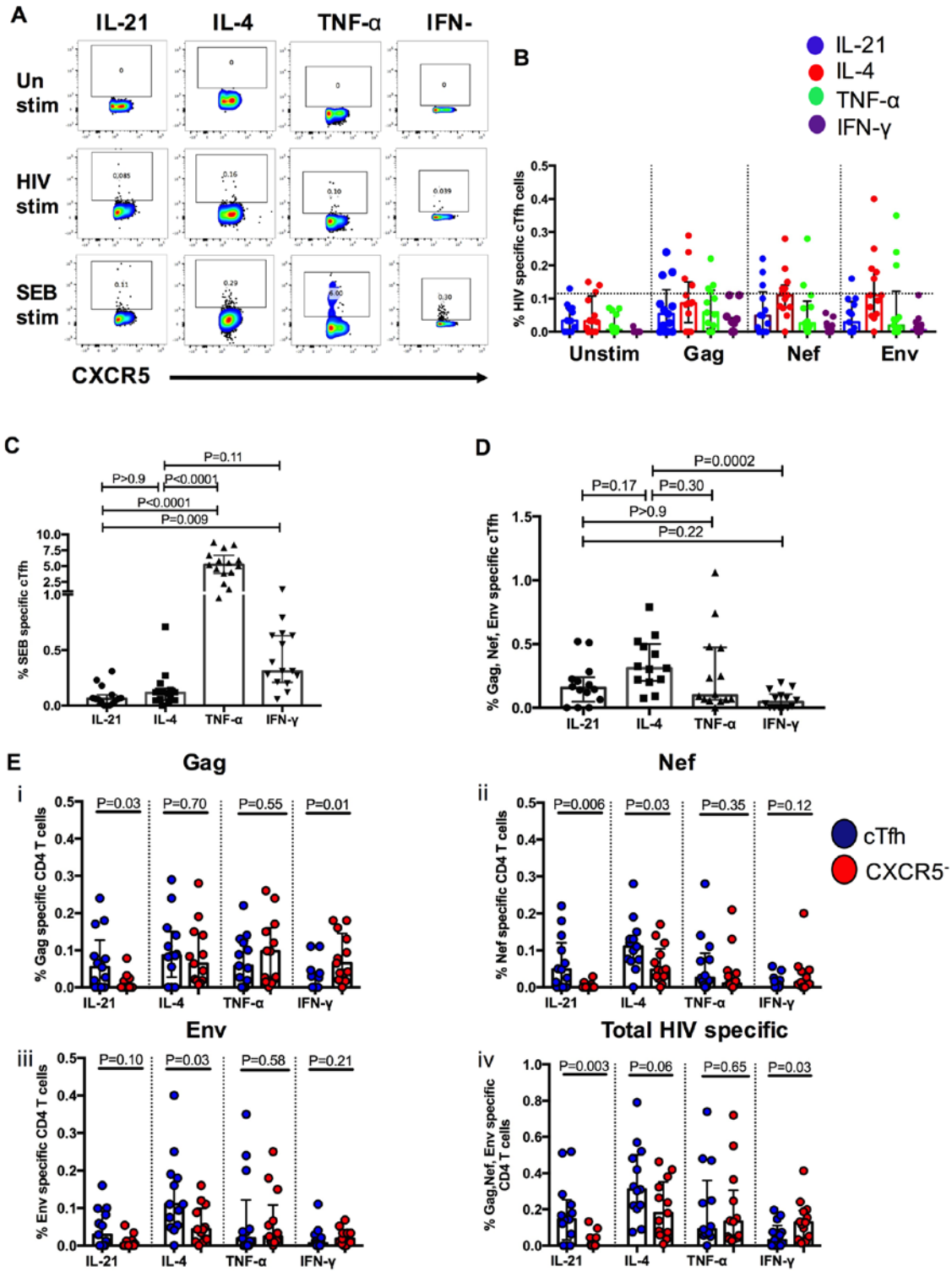

Figure 2.5: HIV-specific cTfh measurements using ICS assay. (A) Representative flow cytometry plots for cytokine secreting cTfh cells. PBMCs were unstimulated or stimulated with SEB or HIV OLP pools for Gag, Nef and Env for 16h in the presence of GolgiStop and GolgiPlug transport inhibitors (BD Biosciences), and the intracellular expression of IL-21, IL-4, TNF-α and IFN-γ respectively was measured. (B) Summary frequency plots for unstimulated, Gag, Nef and Env-specific cTfh cells (horizontal line denotes background threshold based on the responses in unstimulated control conditions). (C) Summary plots for SEB stimulated cells. (D) Total HIV-specific cTfh cells. IL-21$^+$, IL-4$^+$, TNF-α$^+$ and IFN-γ$^+$ cTfh cells were summed up for Gag,

Nef and Env. (E) Comparison of the cytokine secretion profiles of cTfh ($CXCR5^+$) and non-cTfh ($CXCR5^-$) cells. Frequencies for Gag (i), Nef (ii) and Env-specific (iii) cells were plotted separately or totaled (iv). *P* values are from Dunn's multiple comparisons test (C & D) and Mann-U Whitney test (E).

2.4.6 Persistence of Gag-specific Tfh responses during HIV-1 infection.

We further used MHC class II tetramers to confirm the presence of HIV-specific cTfh subsets. Samples from seven HIV infected participants (Table 2.2) expressing either the DRB1*11:01 (n=6) or the DRB1*13:01 (n=1) class II HLA haplotypes, were analyzed.

Table 2.2: Study participants for tetramer staining assay

	HIV negative	Acute HIV	Chronic HIV
N	3	2	5
Male	0	0	0
Female	3	2	5
CD4 Count (cells/µl)*	N/A	365 (351-433)	720 (537-1 022)
Viral load (copies/ml)*	N/A	18 760 (9 642-377 905)	2950 (455-30 975)

*Data are represented as median (IQR).

As shown in Figures 2.6A and 2.6B, dual tetramer positive (Tet++) $CD4^+$ T cells were detectable in HIV infected patients but not in class II HLA-matched HIV negative controls (p=0.02). Further phenotypic analysis revealed an enrichment of tetramer specific $CXCR5^-$ $CD4^+$ T cells compared to cTfh cells (p=0.006) (Figure 2.6C).

To define tetramer-specific cTfh cell subsets and to track their dynamics over time, we used longitudinal samples for one acute HIV participant (patient 1) who had strong a response to the Gag C41 epitope restricted by DRB1*11:01 HLA haplotype. An overlay of the double tetramer $CD4^+$ population onto $CXCR5^+CXCR3^+CD4^+$ cTfh showed that HIV-specific cTfh cells were predominantly $CXCR3^+$ (Tfh1 and Tfh1-17) cells and were detectable at 12, 14, 16 and 20 weeks post-infection (Figure 2.6D). This result was mirrored by another participant (patient 2) sampled at 6 weeks and 138 weeks post infection (Figure 2.6E). Combined data for the participants revealed significantly higher frequencies of Tfh1 and Tfh1-17 tetramer-specific cells compared to Tfh2 and Tfh17 tetramer-specific cells (p=0.0007) (Figure 2.6F). Together, these results demonstrate that HIV-specific cTfh cells persist during HIV infection.

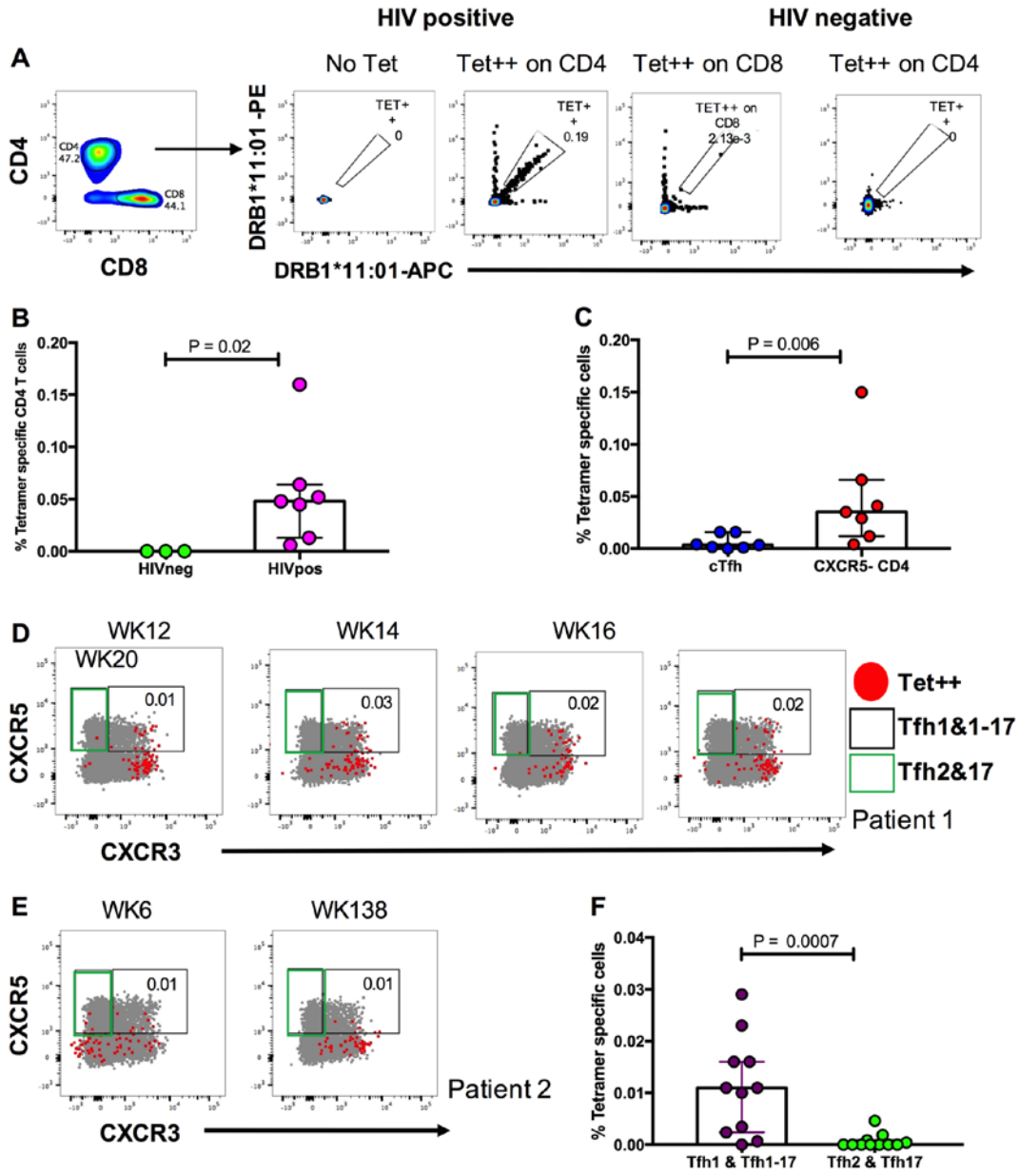

Figure 2.6: HIV-specific cTfh cells' detection by HLA class II tetramers.
(A) Representative flow cytometry plots showing gating strategy for tetramer double positive (Tet++) cells within $CD4^+$ T cells and $CD8^+$ T cells. (B) Frequencies of Tet++ $CD4^+$ T cells for HIV negative and HIV infected donors. (C) Percentages of Tet++ cTfh cells and Tet++ $CXCR5^-$ cells within $CD4^+$ T cells. (D and E) Overlay plots showing Tet++ cells (red dots) within cTfh subsets. (D) HIV-specific cTfh cells are detected at 12, 14, 16 and 20 weeks or at (E) 6 and 138 weeks post-infection. $CXCR3^+CXCR5^+$ gate (black) and $CXCR3^-CXCR5^+$ gate (green). (F) Summary plots showing the frequencies of tetramer-specific $CXCR3^+$ (Tfh1 & Tfh1-17) and $CXCR3^-$ (Tfh2 & Tfh17) cTfh cells. *P* values are from Mann-U Whitney test.

2.5 Discussion

The extreme genetic diversity of HIV is a significant obstacle in the development of an effective anti-HIV vaccine (Ahmed et al., 2017). Even with the identification and isolation of several potent bNAbs in recent years, induction of such antibodies *in vivo* by vaccination has been a challenge (Ahmed et al., 2017, McCoy et al., 2017). Furthermore, nnAbs have been associated with protection from HIV acquisition and could be easier to induce by immunization as compared to bNAbs (Corey et al., 2015). This study sheds new light on circulating $CD4^+$ T cell help that can impact the development of effective non-neutralizing anti-HIV antibody responses.

To understand how HIV modulates the frequency and function of circulating HIV-specific Tfh responses during primary HIV infection, we first established baseline frequencies of cTfh cells in HIV uninfected individuals. Comparative analysis between HIV infected and uninfected individuals showed there are similar frequencies of total memory cTfh cells across both groups. More in depth phenotypic characterization of cTfh cells revealed four distinct functional subsets namely Tfh1, Tfh2, Tfh1-17 and Tfh17 cells. We next showed that the increased frequency of Tfh1 cells positively correlated with p24 IgG antibody responses and negatively correlated with set point viral load. These data suggest that the Tfh1 subset plays an important role in the induction of anti-HIV antibodies and may contribute to control of HIV replication, consistent with murine model studies which have shown that cTfh cells can traffic into lymph nodes and interact with B cells in interfollicular zones and in germinal centers (Sage et al., 2014).

The differential induction of cTfh subsets has been described in the context of other infectious diseases. Consistent with our data, the early induction of circulating $CXCR3^+$ cTfh, which comprises Tfh1 and Tfh1-17 subsets, correlated with the emergence of protective responses to the Influenza vaccine (Bentebibel et al., 2013). In a subsequent study, the same investigators further demonstrated that $CXCR3^+$ Tfh cells promote the development of high avidity antibody responses to the H1N1 vaccine (Bentebibel et al., 2016). The aforementioned studies and our data suggest that Tfh1 cells might play an important helper role in the production of efficacious antiviral nnAbs. However, since studies using *in vitro* Tfh and B cell co-culture assays, have shown that $CXCR3^+$ Tfh cells are effective in providing help to memory B cells but deficient at offering naïve B cell help (Morita et al., 2011, Locci et al., 2013), more mechanistic work using animal models will be critical to delineating the intricacies of circulating Tfh1 cell helper capacity and providing clarity on the functional ability of Tfh1 subsets.

From our results, we also observed an expansion of the Tfh2 subsets during acute HIV-1 infection compared to the controls. The $CXCR3^-$ subset which comprises Tfh2 and Tfh17 subsets has been described as having superior helper capacity *in vitro* and the frequencies during acute HIV-1 infection was predictive of the ability to develop bNAbs in one study (Locci et al., 2013). However, another study did not see any relationship between this subset and the ability to develop bNAbs (Boswell et al., 2014). Although, we sought to determine the relationship between the Tfh2 subset and bNAbs development in our study, only one study participant developed bNAbs thus, we were unable to make any conclusions.

Several reports have implicated bulk $CD4^+$ T cells in immune mediated control of chronic HIV infection (Porichis et al., 2011, Schieffer et al., 2014, Laher et al., 2017), but little is known about the role of HIV-specific cTfh cells in HIV control mainly because of their very low frequency in circulation and the paucity of reliable tools to study them. Even though there were few numbers of cytokine secreting cTfh cells in response to stimulation by HIV peptide as previously shown (Lindqvist et al., 2012), our tetramer staining results provided conclusive evidence of the existence of HIV-specific cTfh cells during primary HIV infection. Notably, unlike bulk HIV-specific $CD4^+$ T cells, which mostly target Gag, our data show that cTfh responses during acute HIV infection are dynamic and comprise a broad repertoire of cells specific for HIV-1 Gag, Nef and Env proteins. Virus-specific cTfh cells targeting different HIV proteins may have synergistic antiviral effect via cross-talk through the so-called intrastructural help to promote a greater net antiviral effect.

This concept was first demonstrated in SIV_{MAC} Gag adenoviral vector immunized macaques and later validated by a murine model of SIV_{MAC} infection (Liu et al., 2009, Nabi et al., 2013). In the initial study, a faster onset and magnitude of antibody-dependent cell-mediated virus inhibition mediated by Env-specific antibodies, was observed in immunized animals compared to controls (Liu et al., 2009, Nabi et al., 2013). Human studies of cTfh cells comparing the effector profile of cTfh cells having different HIV-protein specificity, showed that Env-specific cTfh cells were superior at inducing class switching to IgG while Gag-specific cTfh cells were better at inducing B cell proliferation and maturation (Schultz et al., 2016). These assays were conducted *in vitro* but the microanatomy of immune responses *in vivo* might encourage interactions between cells of different specificities. Additionally, studies have alluded to some degree of promiscuity in Tfh cell help to B cells in the GCs. It has been shown

that the Tfh response is polyclonal (Vinuesa et al., 2016), also the egression of Tfh cells from their initial colonized GCs and migration into other GCs have been documented (Shulman et al., 2013, Vinuesa et al., 2016). These kinds of results argue for a less rigid Tfh help and highlight the dynamism of Tfh cell-B cell interactions, which are the subject of many studies.

As previously mentioned, our tetramer staining results give a strong indication that cTfh cells persist in circulation well into chronic HIV infection. Although there were significantly higher frequencies of Tfh2 cells compared to Tfh1 cells during acute HIV, there were higher proportions of tetramer-specific Tfh1 cells. The tetramers we tested were directed at the Gag C41 epitope and one possibility is that Tfh2 cells may be targeting a different epitope other than the Gag C41 epitope, which we interrogated. We however, consider the expansion of the Tfh2 subset as an interesting observation that warrants further studies.

Our data reveals important associations between nnAbs and viral load set point. The exact mechanism of how nnAbs influence HIV replication requires further investigation. Nevertheless, we speculate that the negative correlation between antibody titers and lower viral load set point may be attributable to antibody effector functions that have been associated with improved virus control (Ackerman et al., 2016) and slower HIV disease progression (Wren et al., 2013, Borrow et al., 2017). Fc effector functions like antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) have been described as important for virus control and these functions are mostly attributed to Env-specific IgG antibodies. (Wren et al., 2013, Borrow et al., 2017). Additionally, studies investigating the mechanisms for virus suppression by Gag-specific antibodies have described the ability of Gag-

specific antibodies to opsonize antigens and recruit conventional or plasmacytoid dendritic cells to phagocytose antibody-coated antigens (Tjiam et al., 2015, French et al., 2017, Tjiam et al., 2017). These opsonophagocytic IgG responses were associated with lower plasma HIV-RNA levels (Tjiam et al., 2015, Tjiam et al., 2016), thus, highlighting another potential mechanism of virus control. Interestingly, Gag p24 antibodies and gp41 antibodies independently correlated with viral load set point whereas gp120-specific antibodies did not. The reason gp41 but not gp120 IgG antibodies correlate with set point viral load remains an open question, but it could be because functional epitopes for Fc binding antibodies reside in gp41. Alternatively, it could be due to the reported differences in the kinetics of the two antibody specificities (Tomaras et al., 2008, Liu et al., 2011).

Early studies investigating the kinetics and magnitude of anti-Gag and anti-Env IgG antibodies observed that the decay of Gag-specific antibodies correlated with poorer disease outcomes and argued that Gag-specific antibodies are a surrogate for $CD4^+$ T cell help to Gag-specific $CD8^+$ T cells (Binley et al., 1997). $CD8^+$ T cells are important for virus control and robust IL-21 mediated Tfh help to $CD8^+$ T cells improves $CD8^+$ T cell cytolytic activity (Schultz et al., 2016), but we observed no correlations between the frequencies of IFN-γ^+ $CD8^+$ T cells and lower set point viral loads among our study participants. Additionally, a paper from our group showed that the association between Gag p24 IgG and viral control was still maintained even after controlling for Gag-specific $CD4^+$ and $CD8^+$ T cell responses suggesting a $CD8^+$ T cell independent anti-viral mechanism of these antibodies (Chung et al., 2018).

A notable limitation of the study is the small sample size due to difficulty in recruiting subjects with untreated acute HIV-1 infection in the present era of mass ART induction

in all HIV-1 infected patients. Nevertheless, despite the small sample size, we generated statistically significant results that provide new insight into the role of cTfh cells and their impact on the induction of antibody responses during primary HIV infection. Further studies to validate our findings in other acute infection cohorts are warranted.

In conclusion, the present study has identified a circulating Tfh1 subset whose frequency during acute HIV infection predicts the development of anti-p24 non-neutralizing antibodies. We also show that higher p24 IgG titers contribute to the control of HIV replication and have a beneficial effect on HIV disease progression. These results highlight the important role of HIV-specific cTfh cells in the generation of robust anti-HIV antibody responses, which are desirable for an HIV vaccine. Additionally, the identification of a cTfh subset that predicts the development of highly functional antibody responses might be useful to vaccine trials/studies as a potential biomarker to predict the development of robust antibody responses in vaccine responders or as a potential cell subset that can be manipulated to enhance vaccine responses (Locci et al., 2013).

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Chapter 3 Overview

Our results in chapter 2 demonstrate that Th1 biased cTfh cell responses during acute HIV-1 infection may be involved in promoting HIV suppression by Gag p24 and gp41 specific antibodies. In chapter 3 of this thesis we focused our investigations on the impact of early ART on the development of immune responses in the lymph nodes. These studies are necessitated by the WHO guidelines for test and treat. For instance, it is not known if early treatment initiation abrogates Tfh cell responses. To address this question, we investigated the impact of extremely early ART initiation on the induction and functional qualities of Tfh cell responses. Specifically, we investigated the induction of GCTfh and B cells' responses in early treated individuals and defined the functional capacity of GCTfh cells during early treated or untreated HIV-1 infection. Our results show that, in early treated individuals, there is a significant expansion of GCTfh cells, albeit not to the same level as in untreated infection. Importantly, these cells are less exhausted and are highly functional compared to Tfh cells in chronic untreated individuals. Our findings highlight the benefits of early ART initiation to the development and preservation of GCTfh cell responses.

CHAPTER 3: EARLY INITIATION OF ANTIRETROVIRAL THERAPY DURING HYPERACUTE HIV-1 INFECTION PRESERVES T FOLLICULAR HELPER CELL FUNCTION

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Keywords: T follicular helper cells, Tfh, early treatment, acute HIV infection, lymph node study, Fiebig stage I treated.

3.1 Abstract

Germinal center T follicular helper (GCTfh) cells are important for the development of anti-HIV antibody responses but the pathologic expansion of GCTfh cells in chronic HIV infection is associated with hypergammaglobulinemia and B cell dysfunction. Although ART reverses some HIV-induced immune perturbations, it is unclear if early treatment restores GCTfh's B cell helper function. Thus, we investigated the dynamics of GCTfh cells in early treated individuals and their ability to stimulate B cell antibody production. Paired peripheral blood and lymph node samples were collected from 16 individuals initiated on ART mostly during Fiebig stage I, 8 HIV negative and 8 untreated individuals. GCTfh cells and B cells were phenotyped by flow cytometry. Immunofluorescence microscopy was used to define the localization of these cell subsets in lymph nodes. HIV-specific responses were measured using HLA class II tetramers and Tfh's B cell helper function was assessed using T-B co-culture assay, ELISA and digital droplet PCR.

Higher frequencies of GCTfh cells were observed in early treated individuals compared to controls (p=0.05), but significantly lower than in chronic HIV-1 infection (p=0.01). The increase in GCTfh cells was directly correlated to the proportions of GC B cells (p=0.0003, r=0.9321). Longitudinal analysis of plasma samples in early treated patients further revealed the accumulation of gp41 and gp120 specific IgG antibodies which peaked at 12 weeks post-infection. Furthermore, GCTfh cells from early treated donors stimulated the production of higher amounts of IgG antibodies by co-cultured naïve B cells. Together, our results demonstrate that early initiation of ART results in the development of highly functional GCTfh cell responses. These results have

implications for therapeutic HIV vaccine design strategies seeking to induce durable antibody responses in early treated individuals.

3.2 Introduction

HIV-1 infection is primarily a disease of the lymphatic system and the induction of immune responses against HIV-1 is mainly directed from the secondary lymphoid tissue compartments (Estes, 2013). Most of the immune cells, reside in lymphoid tissues, and within the lymph nodes (LNs), germinal centers (GCs) are organized microanatomical compartments where B cell antibody maturation takes place (Victora et al., 2012). A subset of highly specialized $CD4^+$ T cells, known as T follicular helper (Tfh) cells interact with B cells at the border of T and B cell zones and migrate into GCs to interact with B cells (Hoffman et al., 2016). In addition to promoting GC reactions, the interactions between Tfh cells and B cells are critical for B cell differentiation, proliferation and survival (Crotty, 2011, Crotty, 2014). Additionally, bidirectional signals between Tfh and B cells reinforces the Tfh cell program (De Guinoa et al., 2011).

Numerous transcription factors and signaling pathways come into play in Tfh and B cell development amongst which BCL-6 has been identified as the so called “master-regulator” of the differentiation of GC B cells and GCTfh cells (Shlomchik et al., 2012). Also, Tfh cells express an array of cell surface receptors like CXCR5, PD-1, ICOS, OX-40 and CD40L which are important for Tfh cell migration, Tfh cell priming by antigen presenting cells like the dendritic cells or Tfh interactions with B cells (Rolf et al., 2010, Crotty, 2011). Cytokines like IL-21 and IL-6 also play a major role in Tfh cell helper function to B cells (Crotty, 2011). Though previously overlooked, the importance of $CD4^+$ T cells in the control of primary HIV-1 infection has been described

(Porichis et al., 2011). In addition, we and others have shown that Tfh cells contribute to HIV-1 control through promoting robust non-neutralizing or neutralizing antibody responses (Cohen et al., 2014, Baiyegunhi et al., 2018).

Antiretroviral therapy (ART), has been the best intervention for the care of HIV-1 infected people worldwide (Cihlar et al., 2016). It has improved the life expectancy of HIV-1 infected people through full viremia suppression, a reconstitution of immunity, reduced viral reservoirs and improved adaptive immune responses (Cihlar et al., 2016, Ananworanich et al., 2016, Dong et al., 2017). With increasing numbers of people being initiated on ART daily, the quality of antiHIV immune responses in such very early treated individuals remain unknown. These responses will be critical for post-treatment control of HIV-1.

To define the effect of early ART initiation on the induction and function of HIV-specific GCTfh responses, we recruited 24 HIV-infected individuals [(8 chronic untreated (Un Tx), 12 Fiebig stage I early treated individuals, and 2 each of Fiebig stage III and Fiebig stage V treated individuals (early Tx)] and 8 HIV negative (HIVneg) controls. Excisional LN biopsies and paired peripheral blood samples were obtained. Flow cytometry, immunohistochemistry (IHC), immunofluorescence microscopy (IF), HLA class II tetramers, T-B co-culture assays, ELISA and digital droplet PCR were used to define the phenotype, localization, function and B cell helper capacity of GCTfh cells.

Longitudinal analysis of HIV-specific antibodies in early Tx donors revealed that plasma gp41 and gp120 specific IgG were induced early, peaked at 12 weeks post infection and persisted long term. From flow cytometry characterization of cell subsets within the LNs, we observed an expansion of GCTfh cells in early Tx individuals compared to controls. Also, there was a direct correlation between the proportions of

GCTfh cells and GC B cells in these donors (p=0.0003, r=0.93). Although GCTfh cells expanded compared to HIV negative controls (p=0.05), the percentages of these cells in early treated individuals were significantly lower than chronic untreated individuals (p=0.01) and they expressed lower levels of the PD-1 receptor (p=0.05). Furthermore, GCTfh cells from early treated donors induced higher levels of IgG and IL-21 when co-cultured with naïve B cells. Together, these results document the induction of superior functioning Tfh cells in early treated individuals and highlight the importance of early treatment for the preservation of Tfh cell helper function during HIV-1 infection.

3.3 Materials and Methods

3.3.1 Study population and samples

The study participants were drawn from the HIV Pathogenesis Programme (HPP) lymph node study cohort, which is an on-going protocol that recruits' HIV-infected and uninfected individuals from multiple sites in Durban, South Africa, to freely consent to the donation of excisional LN biopsies and blood samples. A total of 32 individuals were studied here comprising; 8 HIV-uninfected and 16 early treated females recruited from Females Rising Through Education, Support and Health (FRESH) cohort [cohort characteristics were described by Dong et al. (2017)], 6 chronic untreated individuals recruited from the HPP Acute infection cohort (Wright et al., 2011) and 2 chronic untreated patients recruited from Prince Mshiyeni memorial hospital, Durban, South Africa. Axillary, cervical or inguinal LNs were excised in addition to 120 ml of peripheral blood. Global Clinical and Viral laboratories in Durban, South Africa carried out measurements of CD4 counts and viral loads. The University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) and the Institutional review board of

Massachusetts General Hospital granted ethics approval for the study. All study participants signed inform consent forms for participation in the study.

3.3.2 Lymph node and blood sample processing

Biopsied LN were sectioned into two; one section was fixed in 10% formal-saline (Sigma-Aldrich, St. Louis, Missouri, USA) for IHC studies, while, the second section was macerated to release lymph node mononuclear cells (LMCs) according to the method of Schacker et al. (2006). The cells were passed through a mesh screen and harvested by centrifugation [1 800 rpm, 6 minutes (min), room temperature (RT)]. Peripheral blood mono nuclear cells (PBMCs) were isolated from patient's blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in liquid nitrogen (McCoy, 1998).

3.3.3 Flow cytometry analysis

Freshly isolated or frozen LMCs and PBMCs were phenotypically and functionally characterized using flow cytometry analysis with standardized protocols. Briefly, for surface staining, cells were incubated for 30 min at RT in staining buffer [2% fetal calf serum (FCS) in Phosphate buffered saline (PBS) buffer] containing the following antibodies; LIVE/DEAD Fixable Blue dead cell stain kit (Thermofisher Scientific, Waltham MA, USA), CD19 Horizon V500 [(HV500) BD Biosciences, San Jose, CA], CD3 Brilliant Violet 711 [(BV711) BioLegend, San Diego, CA, USA], CD8 BV786 (BD Biosciences), CD4 BV650 (BD Biosciences), CXCR5 Alexa fluor 488 [(AF488) BD Biosciences], PD-1 BV421 (BioLegend), CCR6 phycoerythrin [(PE) BioLegend], CXCR3 BV605 (BioLegend), CD45RA AF700 (BioLegend), ICOS PE-Dazzle 594 (BioLegend), CD38 PE Cyanine-7 [(Cy7) BD Biosciences], IgD Allophycocyanin

[(APC) BD Biosciences], CCR7 Peridinin-chlorophyll proteins (PerCp) Cy5.5 (BioLegend) and CD27 APCH7 (BD Biosciences).

For intracellular staining of the transcription factor B cell lymphoma 6 (BCL-6), cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ kit according to manufacturer's instructions (BD Biosciences) and stained for 30 min, RT. To sort Tfh subsets, cells were stained at RT for 30 min with LIVE/DEAD Aqua dead cell staining kit (Thermofisher scientific) as per manufacturer's instructions, followed by an antibody panel comprising: CD3 BV711 (BioLegend), CD8 BV786 (BD Biosciences), CD4 BV650 (BD Biosciences), CXCR5 AF488 (BD Biosciences), PD-1 BV421 (BioLegend), CCR6 PE (BioLegend), CXCR3 BV605 (BioLegend) and CD45RA PE-Cy7 (BioLegend).

Stained cells were acquired using an LSRFortessa (BD Biosciences) with FACSDiva™ software or sorted using the FACS aria fusion (BD Biosciences). Data was analysed using the FlowJo version 10.0.8 (Flowjo, LLC, Ashland, Oregon).

3.3.4 HLA Class II tetramer studies

HIV-specific Tfh responses were defined using fluorochrome conjugated HLA class II tetramers. Briefly, cells were stained for 1 hour at 37^{o}C with APC and PE conjugated HLA Class II tetramer complexes, washed in 2% FCS-PBS and then stained with these antibodies: LIVE/DEAD Fixable Blue dead cell stain kit (Thermofisher Scientific), CD3 BV711 (Biolegend), CD4 BV650 (BD Biosciences), CD8 BV786 (BD Biosciences), CXCR5 AF488 (BD Biosciences), CXCR3 BV605 (Biolegend), PD-1 BV421 (Biolegend) and CD45RA AF700 (Biolegend); for 20 mins at RT. Cells were washed and acquired on the LSRFortessa (BD Biosciences).

3.3.5 T-B co-culture assay

Naïve B cells were firstly isolated using the human naïve B cell enrichment kit (negative selection) according to manufacturer's instructions (Stemcell technologies, Vancouver, Canada). FACS aria fusion (BD Biosciences) sorted Tfh cells (5 x 10^4 cells) were further co-cultured with autologous naïve B cells (5 x 10^4 cells) in the presence of staphylococcal enterotoxin B (SEB, 0.5 µg/ml) (Sigma-Aldrich) in 96-well U-bottom plates using AIM V medium (Thermofisher scientific) as previously described (Morita et al., 2011). Cells and supernatants were harvested on day 8 and used for IL-21 mRNA quantitation by digital droplet PCR and total IgG measurement by ELISA respectively.

3.3.6 Droplet digital PCR

Total RNA was extracted from co-cultured Tfh and naïve B cells using QIAzol lysis reagent (Qiagen, Hilden, Germany) and Qiagen RNeasy kit (Qiagen) according to manufacturer's instructions, and used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was used as a template for IL-21 mRNA quantification by Taqman digital droplet PCR assay using a pre-designed experiment (ThermoFisher Scientific, Assay ID: Hs00222327) in a two-step digital droplet PCR reaction. PCR thermal cycling was conducted following optimized cycling conditions: an initial denaturation at 95°C for 10 min, 40 cycles of 30 seconds at 94°C, 1 min at 60°C, followed by a final incubation at 98°C for 10 min and holding at 4°C until reading time. After PCR amplification, droplets were measured in the QX200 ddPCR Droplet Reader (Bio-Rad), and target gene copy number was analyzed using QuantaSoft analysis software (Bio-Rad) and recorded as mRNA copies/20µL.

Absolute IL-21 mRNA counts were normalized to the expression of the housekeeping gene B2M.

3.3.7 Total and HIV-specific IgG ELISA

Plasma HIV-specific IgG antibodies were measured by ELISA as previously described with minor modifications (Gach et al., 2014). 96 well plates (eBiosciences, Waltham MA, USA) were coated with monoclonal anti mouse IgG (eBiosciences), (10 µg/ml in PBS, 100 µl/well) for 16 hours at 4^{o}C. Plates were washed 3 times with wash buffer (0.05% Tween-20 in PBS) and incubated with blocking buffer (1% Bovine serum albumin in PBS, 200 µl/well, RT) for 1 hour. Plates were washed with wash buffer before and after incubation with dilutions of samples and standards in triplicates (100 µl/well, 2 hours, RT). Pooled plasma samples from chronically infected HIV patients were used to generate the standard curves for HIV-specific IgG antibodies. Secondary horseradish peroxidase-conjugated donkey anti-human IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). Horse radish peroxidase conjugated secondary antibodies diluted at 1 in 5 000 in reagent diluent (1% BSA in PBS, 100 µl/well, 1 hour), followed by o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, 100 µl/well) were used to detect IgG antibodies in samples and standards. The reaction was stopped by the addition of 2N Sulfuric acid (Sigma-Aldrich) and the OD values (490 nm) were measured using a BIOTEC plate reader. OD values were imputed into GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA) to plot standard curves and extrapolate IgG antibody concentrations in each sample.

3.3.8 Immunohistochemistry with chromogenic detection

Immunohistochemistry staining was performed on 0.4 µM sections of formalin fixed paraffin embedded (FFPE) lymph nodes using the Dako EnVision FLEX Mini kit, high pH (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. Summarily, target proteins on deparaffinized sections were exposed by heat induced epitope retrieval using Dako antigen retrieval solution [Tris/Ethylenediaminetetra acetic acid (EDTA) buffer, (pH9)] and treated with Dako peroxidase-blocking reagent (10 min, RT), prior to incubation with BCL-6 (Dako), CXCR5 (Abcam, Cambridge, MA, USA), CD4 (Dako), Ki67 (Dako), IL-21 (Abcam) or PD-1 (Abcam) primary antibodies.

Signal amplification was achieved by incubation in Dako EnVision FLEX Linker (15 min, RT) prior to incubation with Dako EnVision HRP (20 min, RT) and colorimetric detection using diaminobenzidine (DAB) detection system (Agilent). Tissues were counterstained with hematoxylin (Sigma-Aldrich) and mounted using DPX (Sigma-Aldrich). Imaging was done using the Axio Scope.A1 LED (Zeiss) or the Axio Observer with TissueFAXS imaging software (TissueGnostics, Vienna, Austria) and images were analyzed with the AxioVision Rel 4.8 software (Zeiss) or with TissueQuest analysis software (TissueGnostics).

3.3.9 Immunofluorescence (IF) microscopy

Multicolor immunofluorescence microscopy staining was conducted using the opal 4-color fluorescent IHC kit (PerkinElmer, Waltham, MA, USA) according to manufacturer instructions with minor modifications. Briefly, following antigen retrieval, two blocking steps (2 x 10 min, RT) were performed using the Dako peroxidase-blocking reagent (Agilent) and Bloxall block (Vector Laboratories, Burlingame, CA,

USA). The slide was washed with 0.05% Tween 20 in Tris-buffered saline (TBS-T) for 5 min, probed with the first primary antibody, BCL-6 [Dako, (30 min, RT)], washed with TBS-T (5 min), probed with Opal polymer HRP [20 min, RT (PerkinElmer)], washed (TBS-T, 2 X 5 min) and detected using the Opal polymer 520 (10 min, RT). This protocol was repeated for the second antibody [(CXCR5, CD4 or PD-1), Dako], followed by counterstaining with spectral DAPI (PerkinElmer) and mounting with Dako fluorescence mounting medium (Agilent). Images were acquired using the Axio Observer with TissueFAXS imaging software (TissueGnostics). Quantitative image analysis was conducted using TissueQuest (TissueGnostics). Threshold values were set using the negative control slides.

3.3.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA). Mann-Whitney U tests were used for the comparisons between any 2 groups. Variation across multiple groups was assessed using Kruskal-Wallis H test. Lastly, Pearson or Spearman's rank correlation was used to define the correlation between 2 variables. *P* values were considered significant if less than 0.05.

3.4 Results

GCTfh cells are a key component of the adaptive immune response to HIV-1 infection. They provide cognate help to B cells (Crotty, 2011, Crotty, 2014) and $CD8^+$ T cells (Chevalier et al., 2011a, Schultz et al., 2016). Massive expansion of GCTfh cells during chronic HIV infection is associated with B cell dysfunction and aberrant antibody production, but the effect of early ART initiation on GC responses has not been described. We thus, characterized GCTfh responses in 16 individuals initiated on ART

very early after infection, 12 of which were initiated on therapy in Fiebig stage I, 2 were initiated in Fiebig stage II and the other 2 in Fiebig stage V. In addition, 8 uninfected and 8 chronic untreated individuals were also studied. All study participants achieved full plasma viremia suppression after a median of 17 days on intensive ART. The ART regimen used and the clinical characteristics of the study participants are detailed in Appendix 2 and Table 3.1. Appendix 3 details the number of patients studied for each objective.

Table 3.1: Patient characteristics

	HIV negative	Early treated HIV	Untreated chronic HIV
n	8	16	8
Male	0	0	1
Female	8	16	7
Age (years)*	21 (20-22)	21 (19-22)	24 (22-31)
CD4 Count (cells/ul)*	NA	885 (663-1033)	357 (355-359)
Viral load (cps/ml)*	NA	<20	9000 (1193-21750)
Duration on treatment (days)*	NA	346 (34-466)	NA
Time to viremia suppression (days)*	NA	17 (8-24)	NA

* Data are represented as median (IQR). Abbreviations: NA, not applicable.

3.4.1 GCTfh cells are phenotypically different from nonGCTh cells and cTfh cells

Due to the paucity of LN Tfh studies in humans in general and the fact that prior to this study, no study has described immune responses in LNs in the Zulu/Xhosa population, we began by defining the baseline phenotypic and functional characteristics of LN and peripheral blood Tfh cell subsets in our cohort. Consistent with other Tfh studies

(Lindqvist et al., 2012), we defined GCTfh cells as $CXCR5^{hi}PD\text{-}1^{hi}$, extrafollicular Tfh (nonGCTfh) and circulating Tfh (cTfh) as $CXCR5^{+}PD1^{+}$ (Figure 3.1A) and determined the expression levels of Tfh transcriptional regulator, BCL-6 and some other previously described Tfh associated molecules (Nurieva et al., 2009, Pratama et al., 2014).

In comparison to nonGCTfh and cTfh cells respectively, we observed significantly higher median fluorescence intensities (MFI) of BCL-6 (p=0.008, p=0.02), ICOS (p=0.0002, p=0.0007) and programed cell death protein-1 (PD-1) (p=0.0002, p=0.0007) on GCTfh cells (Figure 3.1B). GCTfh cells did not express CCR7, a peripheral migratory receptor, while nonGCTfh and cTfh cells expressed similar levels of CCR7 (panel iv, Figure 3.1B). These results highlight previously described phenotypical differences between GCTfh, nonGCTfh and cTfh cells.

Having validated our definition of the different Tfh populations, we next determined the baseline GCTfh characteristics in our cohort. GCTfh cells constituted a very small population of 2% of total memory ($CD45RA^{-}$) $CD4^{+}$ T cells in the lymph nodes while nonGCTfh cells were 11% (panel i, Figure 3.1C). Furthermore, cTfh cells comprised 4% of total memory $CD4^{+}$ T cells (panel ii, Figure 3.1C). These results demonstrate that GCTfh cells are the most differentiated Tfh cell subset and comprise a very small proportion of memory $CD4^{+}$ T cells in the lymph nodes of HIV negative donors.

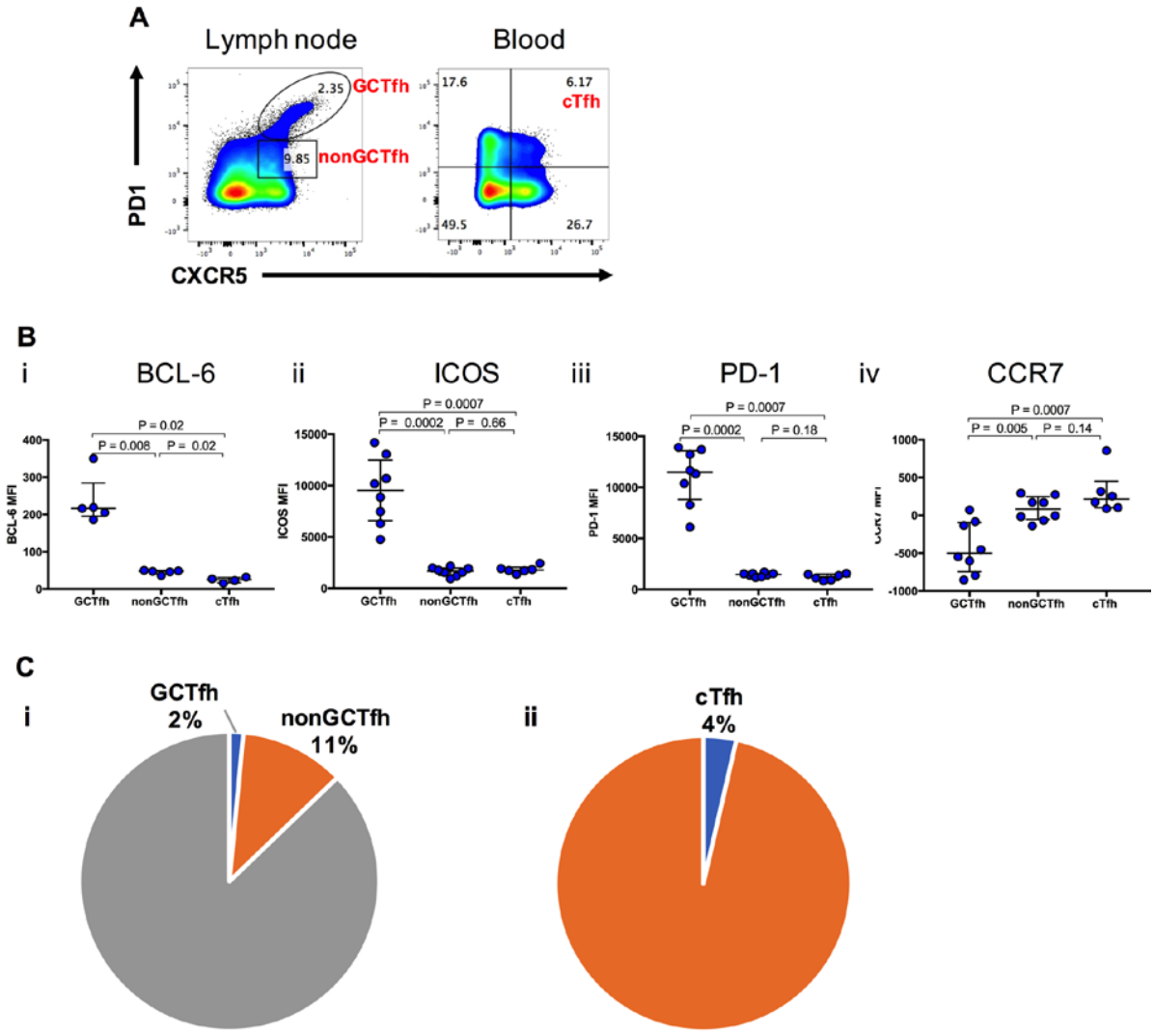

Figure 3.1: Phenotypic and functional characterization of Tfh subsets in the lymph nodes and peripheral blood of HIV negative donors. (A) Representative flow cytometry plots showing the gating strategy for GCTfh (PD-1^{hi}CXCR5^{hi}), nonGCTfh (PD-1^{+}CXCR5^{+}) and cTfh (PD-1^{+}CXCR5^{+}) within CD45RA^{-} CD4^{+} T cells. (B) Summary of median fluorescence intensity (MFI) values for BCL-6 (i), ICOS (ii), PD-1 (iii), and CCR7 (iv), markers on GCTfh, nonGCTfh and cTfh cells. (C) Pie charts showing the proportions of Tfh subsets within total memory (CD45RA^{-}) CD4^{+} T cells. Statistical differences were calculated using Mann U-Whitney tests.

3.4.2 Expansion of GCTfh cells in early ART treated individuals

The dysregulation of Tfh function during chronic HIV-1 infection has been documented (Lindqvist et al., 2012, Cubas et al., 2013), but it is known if the scenario of rapid viremia suppression in early Tx individuals prevents Tfh cell dysfunction. To address

this question, we first, interrogated how HIV-1 infection affects Tfh cell frequencies and found that there was a 4-fold increase in the median frequencies of GCTfh cells (6.7%, IQR 39%-7.9%) during chronic HIV-1 infection compared to healthy controls (p=0.0002) (Figures 3.2A & 3.2B). The median proportions of GCTfh cells in early treated individuals were also higher (2.4%, IQR 1.2%-4.3%) than the proportions in uninfected controls (p=0.05). However, the expansion of GCTfh cells in the early Tx group was attenuated compared to chronic infection (p=0.01) (Figures 3.2A & 3.2B). In contrast, the frequencies of nonGCTfh and cTfh cells were not significantly altered during chronic untreated or early treated HIV-1 infection (Figures 3.2A & 3.2B). These results show that early treatment initiation results in a mitigated but significant GCTfh response.

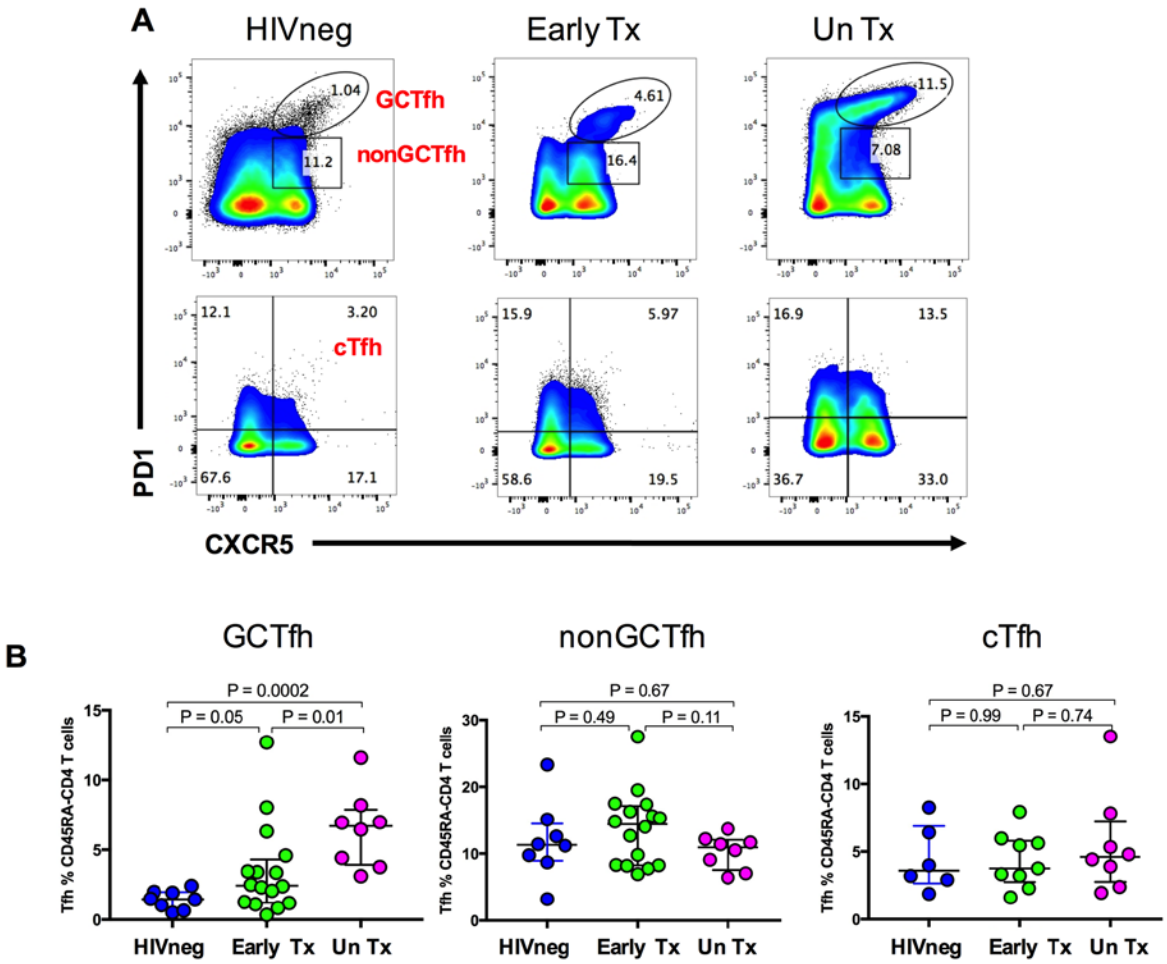

Figure 3.2: Expansion of the GCTfh cells during HIV-1 infection. (A) Representative flow cytometry plots showing the proportions of GCTfh ($CXCR5^{hi}PD\text{-}1^{hi}$), nonGCTfh ($PD\text{-}1^{+}CXCR5^{+}$) cells and cTfh cells ($PD\text{-}1^{+}CXCR5^{+}$) in HIV negative (HIVneg), early treated (Early Tx) and untreated HIV-infected (Un Tx) groups. (B) Summary plots comparing the frequencies of Tfh subsets in HIVneg, Early Tx and Un Tx donors. Statistical differences were calculated using Mann U-Whitney tests.

3.4.3 HIV-specific Tfh responses are induced during early treated HIV infection

Having demonstrated the expansion of GCTfh cells in early treated individuals, we next sought to determine if early ART initiation results in the induction of functional HIV-specific Tfh responses. We used DRB1*11:01 and DRB1*13:01 class II tetramers described in chapter 2 for our characterization of HIV-specific responses. Our tetramer staining strategy involved the dual tetramer PE and APC double positive staining approach (Figure 3.3A), which our group previously showed excludes non-specific

background signals (Laher et al., 2017, Baiyegunhi et al., 2018). We gated on HIV-specific CD4 T cells and overlaid that population on Tfh cell subsets (Figure 3.3B).

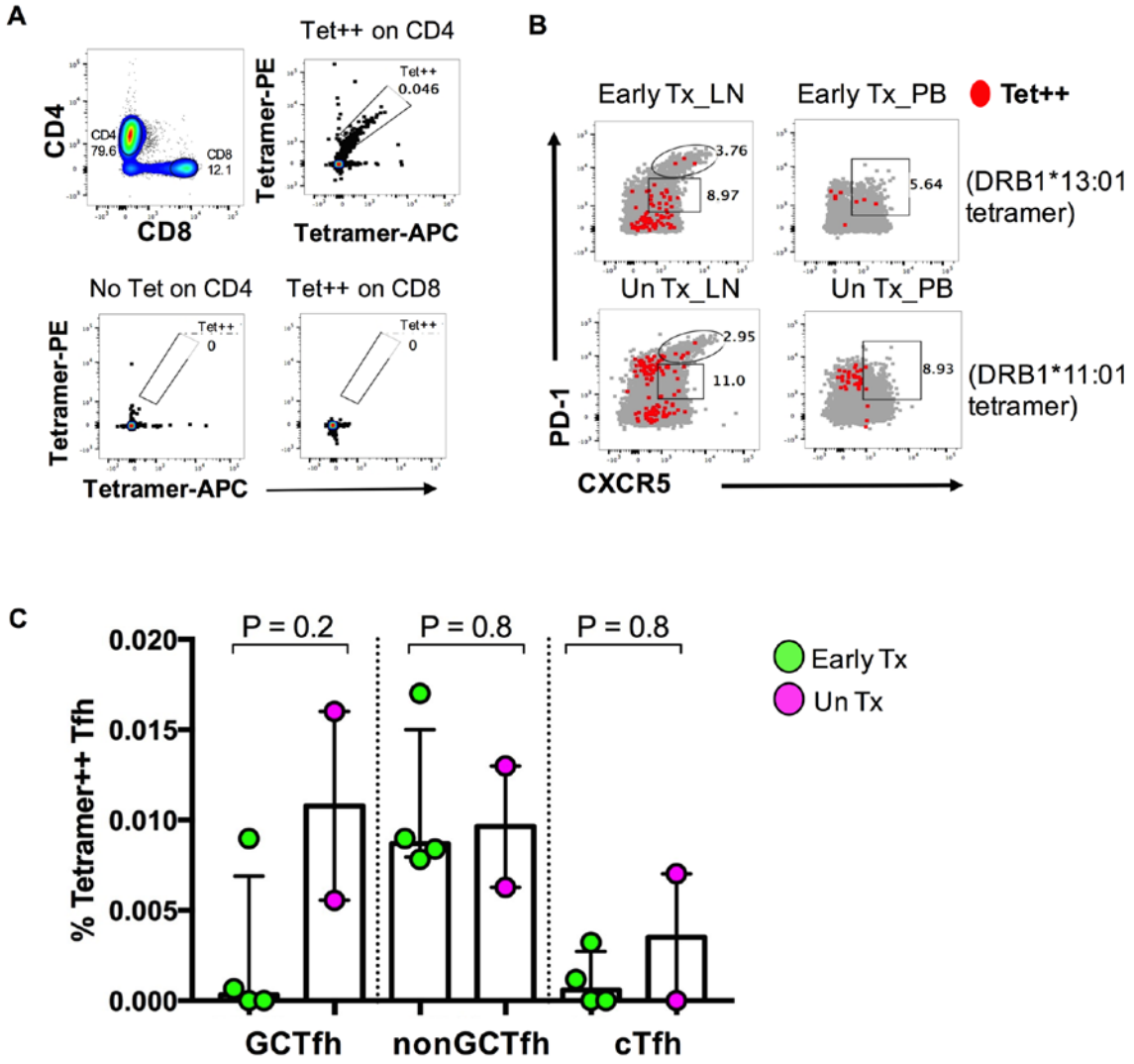

Figure 3.3: Detection of HIV-1 specific Tfh cells using HLA class II tetramers. (A) Gating strategy for identifying double-positive tetramer specific (Tet++) $CD4^+$ T cells with minimal background staining on $CD8^+$ T cells. (B) Overlay plots of Tet++ $CD4^+$ T cells (red dots) on Tfh subsets. (C) Summary plot comparing proportions of Tet++ Tfh subsets in early treated (Early Tx) and untreated HIV-infected (Un Tx) individuals.

Summary data from the analysis of 4 early treated and 2 untreated donors within our cohort expressing the class II DRB1*11:01 and DRB1*13:01 alleles, revealed that tetramer-specific GCTfh, nonGCTfh and cTfh cells were detected in our chronic un Tx and early Tx participants at similar frequencies (Figure 3.3C). These results show that

HIV-specific Tfh responses are induced in early treated subjects at comparable levels to untreated HIV infection.

3.4.4 Early ART initiation modulates the expression of Tfh related molecules on Tfh subsets

Having demonstrated that early treatment attenuates excessive HIV-1 induced GCTfh expansion, we next interrogated if there was an alteration in Tfh functional characteristics during early ART. As previously mentioned, BCL-6 is the master transcription factor that regulates Tfh differentiation and BCL-6 expression is up regulated with Tfh development (Liu et al., 2012), thus, we evaluated if BCL-6 expression on GCTfh cells is altered. Our results showed that, BCL-6 was constitutively expressed on GCTfh cells regardless of disease condition or treatment status (Figure 3.4A). However, BCL-6 expression in nonGCTfh and cTfh cells was increased during HIV-1 infection and significantly attenuated by early treatment (Figure 3.4A).

The inducible T cell co-stimulator (ICOS) is an important costimulatory molecule for Tfh differentiation and migration, among other functions (Crotty, 2014). We next, showed that ICOS expression on both GCTfh and nonGCTfh cells remained significantly higher during HIV-1 infection regardless of treatment status (Figure 3.4B). In peripheral blood, early treatment resulted in reduced expression of ICOS on cTfh cells compared to untreated infection (p=0.0006) (Figure 3.4B).

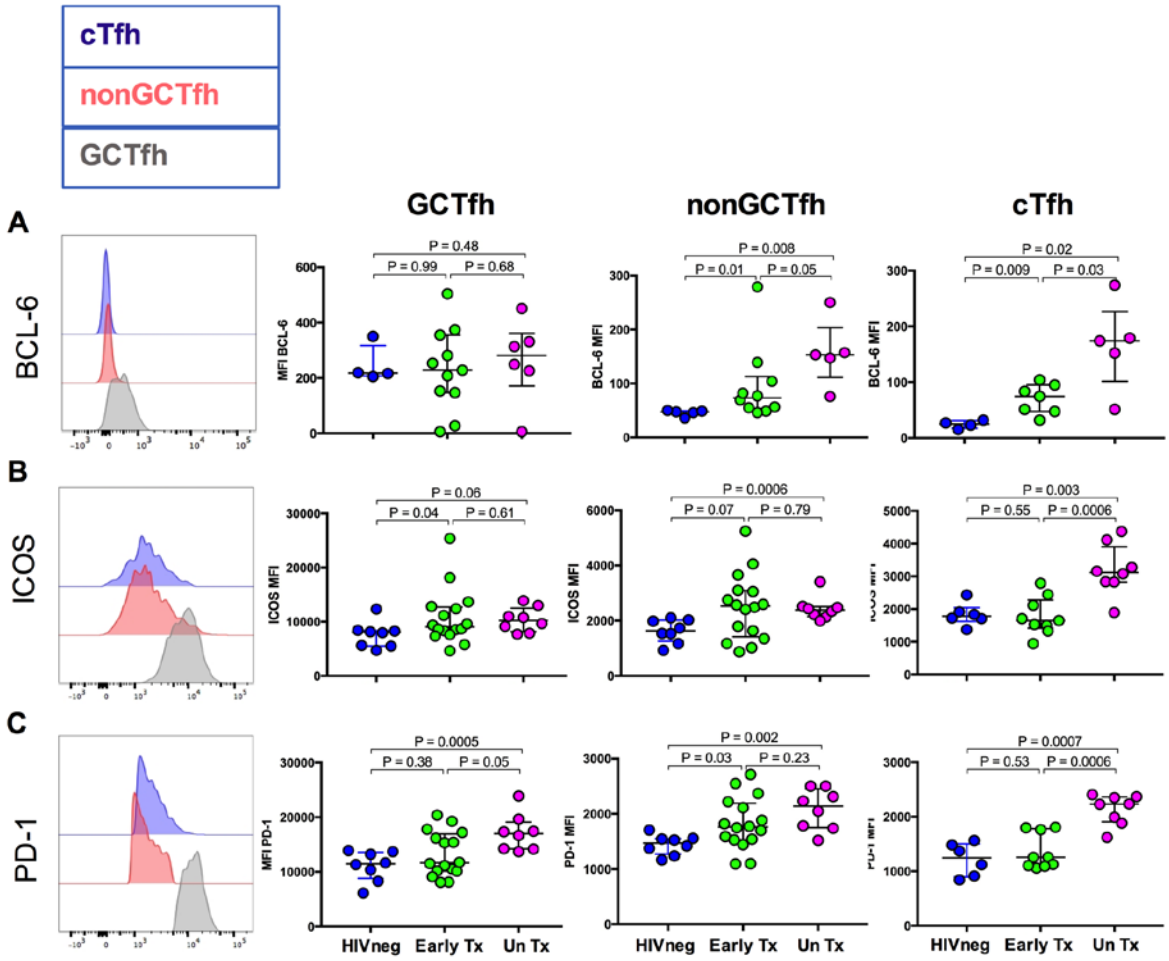

Figure 3.4: Expression of Tfh functional molecules by Tfh subsets. Samples from HIV negative (HIVneg), early treated (Early Tx) and untreated HIV-infected (UnTx) individuals were characterized by flow cytometry. (A to C) Representative histograms and summary plots of median fluorescence intensity (MFI) for BCL-6 (A), ICOS (B), and PD-1 (C) on GCTfh (grey), nonGCTfh (pink) and cTfh (blue) cells. Statistical differences were calculated using Mann U-Whitney tests.

Although PD-1 is important for Tfh function, high expression of PD-1 is associated with Tfh impairments during chronic HIV-1 infection (Cubas et al., 2013), so we also interrogated if early treatment modulates PD-1 expression on Tfh cell subsets. Our results showed that while, PD-1 expression on GCTfh (p=0.0005), nonGCTfh (p=0.0019) and cTfh (p=0.0007) cells was higher in the chronic un Tx group compared to controls, PD-1 expression on GCTfh (p=0.05) and cTfh (p=0.0006) cells from early treated individuals was lower compared to untreated individuals (Figure 3.4C). Taken together, these results demonstrate that the phenotype of nonGCTfh and cTfh cells

more closely resembles GCTfh cells during HIV-infection and that early treatment modulates the expression of PD-1 receptor on GCTfh cells.

3.4.5 Cumulative exposure to viremia drives lymph node germinal center (GC) responses in early treated individuals

In an effort to define the distribution of GCTfh cells in early treated individuals, we conducted immunohistochemistry studies on FFPE LNs of 17 study participants based on sample availability. Firstly, we defined GCs by immunostaining serial sections with BCL-6 and Ki67 markers (Figure 3.5A) (Goteri et al., 2011). Next, we quantified the mean percentage area staining for BCL-6 or Ki67 GC clusters in each image using the AxioVision Rel 4.8 software (Zeiss). We observed a strong positive correlation between the area percentages of BCL-6 and Ki67 (Figure 3.5B), demonstrating that both markers identified GCs with similar accuracy but we chose to use BCL-6 for our subsequent studies because in our hands, it was cleaner with minimal background staining.

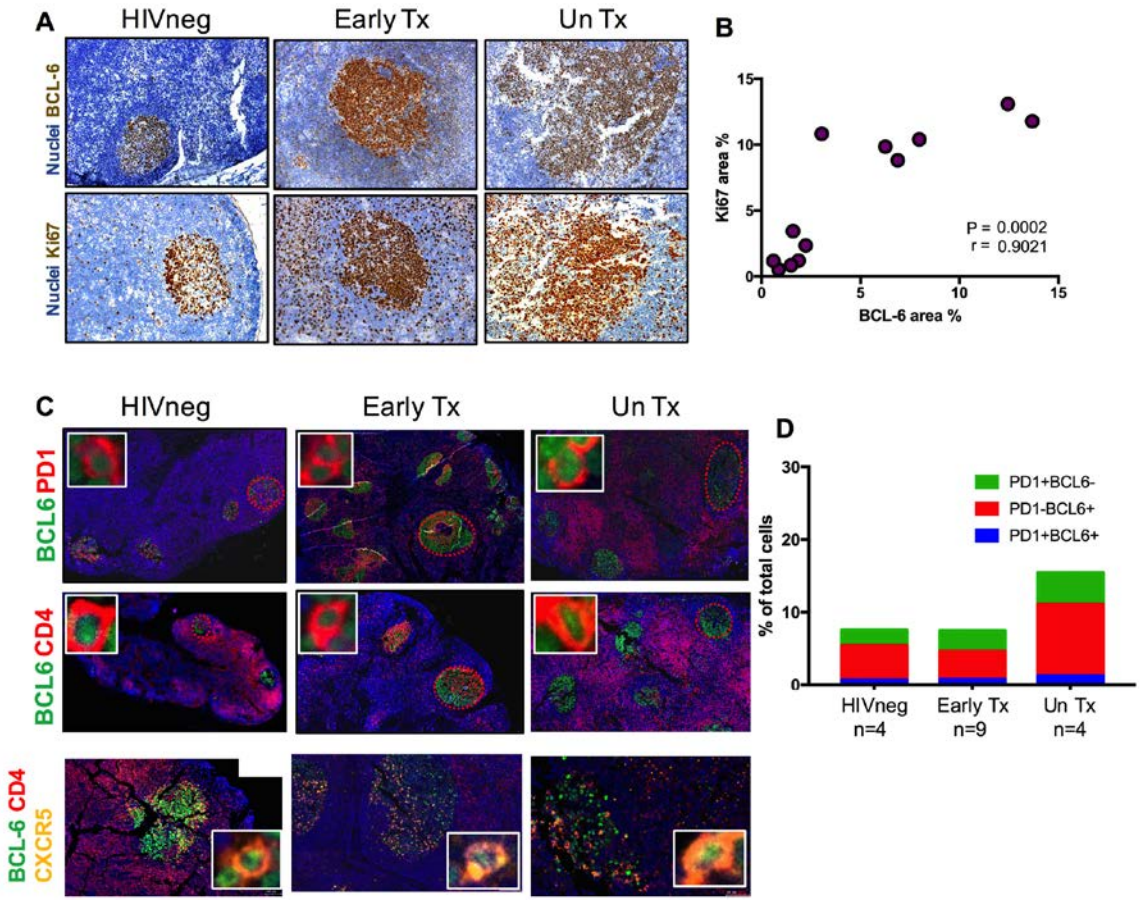

Figure 3.5: *In situ* localization of Tfh cells in the lymph nodes using IF microscopy. (A) Representative micrographs showing chromogenic detection of intranuclear BCL-6 and Ki67 staining. (B) Correlation between average area percentage staining of BCL-6 and Ki67 in GCs. Area percentages were quantified using AxioVision Rel 4.8 software. (C) Lymph node sections were stained with BCL-6 (green) to define germinal centers (GCs) and stained with (i) PD-1 (red), or (ii) CD4 (red) and (iii) CXCR5 (yellow) to localize Tfh cells. (D) Summary plots showing the distribution of PD-1 and BCL-6 expressing cells in the GCs. TissueQuest (TissueGnostics, Vienna) was used to compute the cell frequencies.

Fluorescent multiplex IHC assays provide a unique advantage of *in situ* characterization and quantification of immune cell interactions. Thus, we employed this method to localize Tfh cells to GCs, by multiplexing the BCL-6 marker with either PD-1 or CD4 (Figure 3.5C, top and middle panels). We also combined CXCR5, CD4 and BCL-6 markers to confirm co-localized membrane staining of both receptors on Tfh cells (Figure 3.5C, bottom panel). The expression levels and the overlapping mean intensities for BCL-6 and PD-1 markers defined as PD-1^{+}BCL-6^{+} or BCL-6 only (PD-1^{-}BCL-6^{+}) or PD-1 only (PD-1^{+}BCL-6^{-}), were calculated using TissueQuest software

(TissueGnostics). We observed a trend of higher BCL-6^{+} cells in chronic untreated LNs by IF (Figure 3.5D). This result confirms our flow cytometry data showing the expansion of Tfh cells during chronic HIV infection.

3.4.6 HIV-1 induced changes in lymph node B cell subset distribution

One of the cardinal functions of Tfh cells is to promote B cell differentiation, antibody class switching and antibody affinity maturation. To determine if Tfh cell helper capacity to B cells was impacted by the early initiation of ART, we first, characterized the frequencies of B cell subsets across the study groups (4 HIVneg, 9 Early Tx and 4 Un Tx) and the induction of class switched antibodies in the early treated study participants. We used CD38 and IgD markers to define four subsets within total CD3^{-} CD19^{+} B cells and naïve B cells were defined as CD38$^{lo/-}$IgD^{+} cells, pre-GC B cells were CD38^{+}IgD^{+}, GC B cells were CD38^{+}IgD^{-} and plasmablasts/ plasma cells were CD38^{hi}IgD^{-} (Figure 3.6A). The frequencies of naïve and pre-GC B cells were not significantly impacted by HIV-1 infection (Figures 3.6B & 3.6C), however, the proportions of GC B cells and plasmablasts/plasma cells were significantly higher during chronic untreated HIV-1 infection compared to controls (Figures 3.6D & 3.6E).

We next conducted independent correlation analysis of the different B cell subsets and GCTfh cells and found a strong positive correlation between the percentage of GC B cells and GC Tfh cells only (p=0.0003, r=0.9321) (Figure 3.6F and results not shown). Taken together, these results demonstrate that similar to the GCTfh compartment, chronic untreated HIV-1 infection induces a significant expansion of B cell subsets, which is blunted by early treatment. Also, the induction of GC B cells is associated with GC Tfh cell numbers in early treated individuals.

To further interrogate B cell responses in the early treated participants, we investigated if HIV-specific class switched antibodies are produced especially since plasmablasts/plasma B cell responses were significantly expanded in early treated individuals compared to controls. Plasma HIV-1 gp41 and gp 120 envelope glycoprotein-specific IgG titers were determined in early treated donors at baseline and at weeks 1, 12, 24, 36, 48, 60, 72, 96 and 108 post-diagnosis of HIV infection. We found that plasma gp41 (Figure 3.7G) and gp120 (Figure 3.7H) antibodies were induced early and peaked at approximately 12 weeks post-infection in most participants. Although gp120 IgG rapidly declined to almost undetectable levels by 60 weeks post-infection, gp41 IgG remained higher than the threshold of detection in more than half of the participants tested. These results demonstrate the persistent production of gp41- and to a lesser degree, gp120-specific class switched antibodies in early treated donors despite rapid plasma viremia suppression after a median of 17 days on ART.

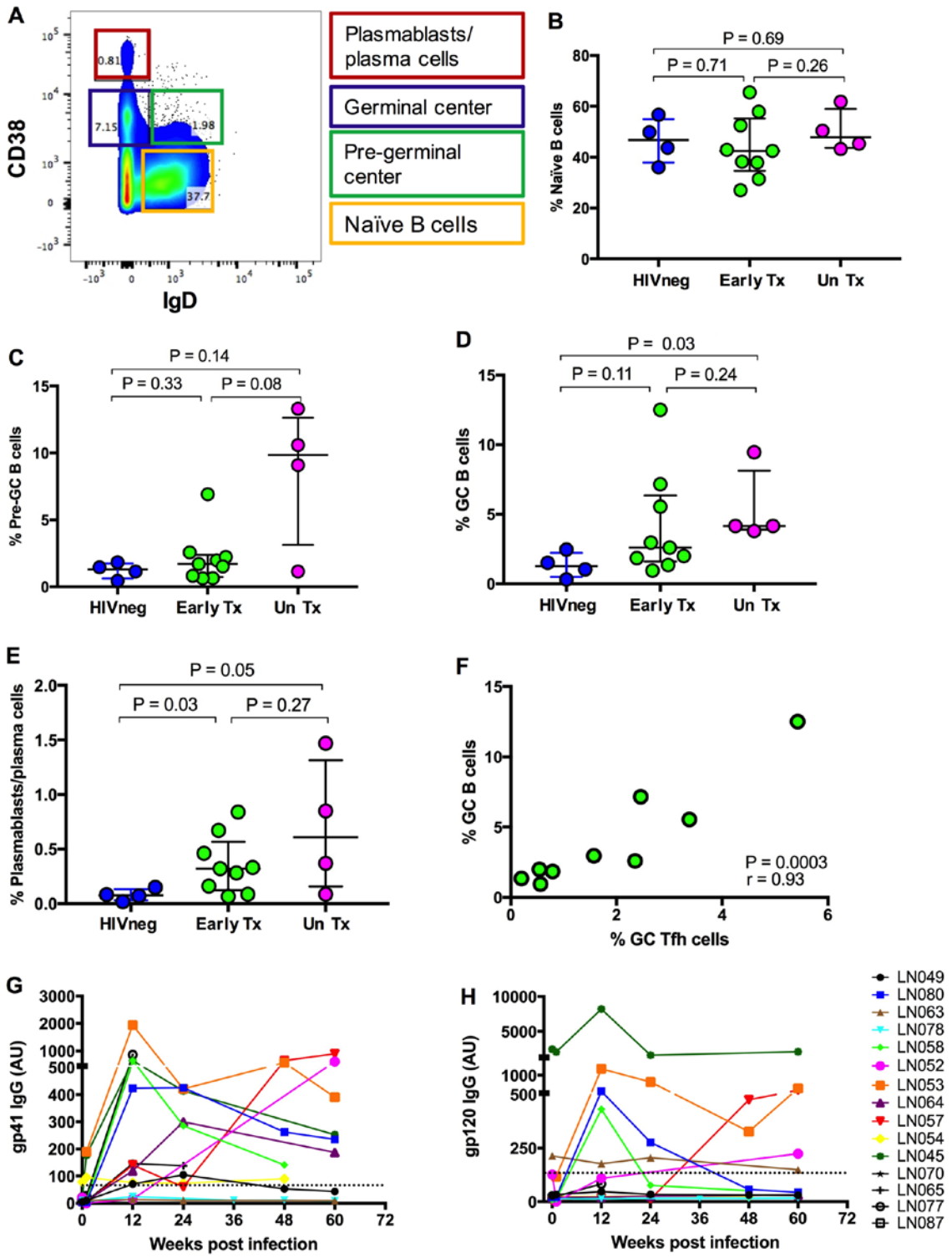

Figure 3.6: B cell responses are induced in early treated individuals and correlate with Tfh responses. (A) Representative flow cytometry plot showing the gating strategy for B cell subsets in the lymph nodes. (B to E) Summary plots comparing the proportions of naïve B cells (B), pre-GC B cells (C), GC B cells (D) and plasmablasts/plasma cells (E) within total CD19^{+} B cells from HIV negative (HIVneg), early treated (Early Tx) and untreated HIV-infected (Un Tx) donors. (F) Correlation between the frequencies of GC B cells and GCTfh cells in Early Tx donors. (G & H) Titers for gp41 IgG (G) and gp120 IgG (H) antibodies were

determined at baseline and at weeks 1, 12, 24, 36, 48 and 60 post-infection for Early Tx donors. *P* values from the Mann-U Whitney test (B to E) and Pearson rho (*r*) and *p* values (F) are reported.

3.4.7 Early initiation of antiretroviral therapy is associated with superior GCTfh helper capacity

Lastly, we directly interrogated GCTfh help to naïve B cells in an *in vitro* co-culture assay. To increase cell numbers for the assay, we sorted total LN CXCR5$^+$ CD4$^+$ T cells and co-cultured them with autogous naïve B cells in quadruplicates. Co-cultures for early treated individuals yielded 2-fold higher concentrations of total IgG (Figure 3.7A) and 3-fold higher IL-21 mRNA trancripts (Figure 3.7B) than co-cultures using cells from chronic untreated individuals. These results demonstrate that Tfh responses in early treated individuals are more efficient at supporting IgG production by secreting high amounts of the cytokine IL-21 than Tfh cells from chronic untreated individuals.

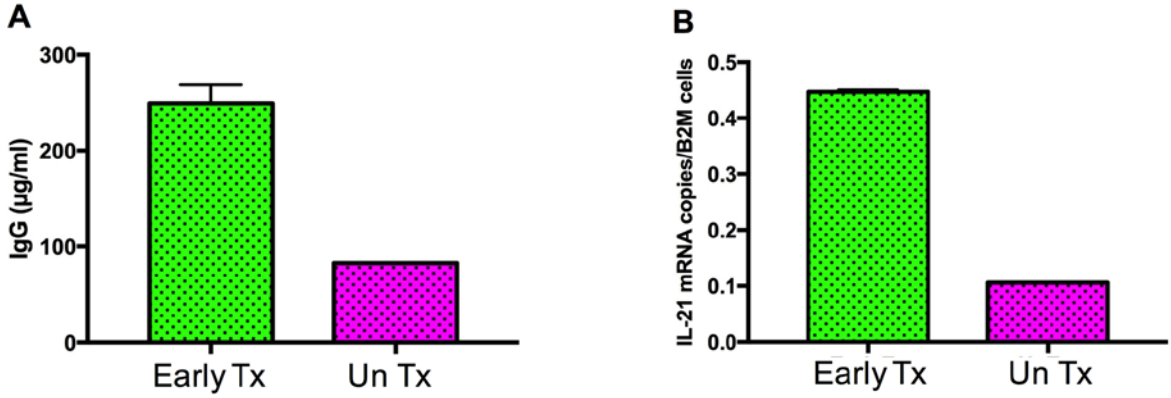

Figure 3.7: Tfh cells from early treated donors provide more efficient B cell help than Tfh cells from donors with untreated HIV infection. (A) Sorted CXCR5$^+$ CD4$^+$ T cells were incubated with purified naïve B cells, in the presence of Staphylococcal enterotoxin B for 8 days. IgG concentration was determined in day 8 culture supernatants by ELISA. (B) IL-21 mRNA was quantified in co-cultured CXCR5$^+$ CD4$^+$ T cells and naïve B cells using digital droplet PCR. Early treated, Early Tx; untreated HIV-infected, Un Tx.

3.5 Discussion

A therapeutic vaccine for HIV-1 infection would need to induce robust anti-HIV immune responses in ART suppressed individuals to mediate post-treatment viral control. Tfh cells are critical for the induction of effective T cell-dependent antibody responses, but there is a paucity of LN Tfh studies especially in early treated HIV-infected individuals. Since lymphoid tissues are the primary sites for the induction of anti-HIV T cell-dependent immune responses *in vivo*, the present study used paired peripheral blood and LN samples from well-characterized cohorts of hyperacute and chronic HIV-1 infection to phenotypically and functionally define and localize HIV-induced GCTfh responses.

We have previously shown that the total circulating Tfh cell compartment described, as total memory cells $CXCR5^+$ $CD4^+$ T cells are a heterogeneous population with many subsets (Baiyegunhi et al., 2018). To focus our studies here on cells with similar phenotypic characteristics to *bona fide* GCTfh cells, we restricted our definition of cTfh cells to PD-1 expressing $CXCR5^+$ cells. Consistent with previous studies, our results revealed that GCTfh cells are phenotypically distinct from their extrafollicular and circulating counterparts by the high expression of key functional molecules like BCL-6, ICOS, PD-1 and CCR7 (Morita et al., 2011, Locci et al., 2013, Lindqvist et al., 2012). In addition, we showed that HIV-1 infection resulted in the upregulation of BCL-6, ICOS and PD-1 on cTfh cells, suggesting that cTfh cells acquire an effector 'GCTfh-like' phenotype during HIV-1 infection.

It is assumed that the rapid withdrawal of HIV antigens by early ART could completely abrogate GCTfh responses in the LNs. Hence, there is a notion that early treated individuals would be unable to develop affinity matured antibody responses against

HIV. Using several lines of evidence, we demonstrated the induction of GC responses in early treated individuals. Firstly, we observed significant expansion of bulk GCTfh cells in early treated donors compared to HIV negative controls. Using HLA class II tetramers, we demonstrate the induction of HIV-specific GCTfh cells in early treated individuals. Thirdly, active GCs were visualized in these participants using IF microscopy techniques and plasma HIV-specific antibodies persisted up to 60 weeks post infection. Lastly, we observed significantly increase in plasmablasts and plasma cells in early treated individuals compared to uninfected controls. Together these results demonstrate a robust induction of GC responses in early treated individuals.

One of the underlying mechanisms of Tfh dysfunction during chronic HIV-1 infection is the high expression of PD-1, whose ligation by PD-L1 or PD-L2 on B cells results in decreased IL-21 and ICOS production by Tfh cells (Miles et al., 2016b, Cubas et al., 2013). Of note we reported significantly lower expression levels of the PD-1 receptor on GCTfh and cTfh cells from early treated individuals compared to chronic untreated individuals. The role of PD-1 on Tfh function is controversial. Some studies associate PD-1 expression with Tfh dysfunction while other studies show that, PD-1 signaling impacts IL-21 production by Tfh cells and plasma cell differentiation by B cells (Victora et al., 2012). Our data suggest that the balance between over expression and optimal expression of this receptor might be a determining factor for Tfh dysfunction or qualitative Tfh function.

Using evidence from *in vitro* co-culture assays, to answer the question of Tfh functionality in early treated individuals, we showed that Tfh cells could induce IgG production by naïve B cells than Tfh cells from untreated individuals. The impaired capacity of GCTfh cells from untreated individuals to support B cells' IgG production

in vitro has been previously described (Cubas et al., 2013). In that study, IgG production in the co-culture was rescued by the addition of exogenous IL-21. Which is reflected with our results that showed there was increased IL-21 production in co-cultures of cells from early treated individuals. IL-21 is a key cytokine that drives germinal center formation and antibody class switch recombination (Pissani et al., 2014, Morita et al., 2011). Thus, impaired IL-21 production by Tfh cells during chronic HIV infection contributes to the dysregulation of the GC response and the excessive production of low affinity antibodies during chronic HIV infection.

Another factor that contributes to a dysregulated GC response during untreated chronic HIV infection or an impaired GC response to vaccination, is very high GCTfh cell numbers (Miles et al., 2016a). It has been described that Tfh cell numbers need to be tightly regulated in the GCs so that the B cell affinity maturation process can be optimal (Pratama et al., 2014). Our study shows that early treated individuals have superior functioning Tfh cells, which could be attributable to a more mitigated Tfh response. These data suggest that early treated individuals have the potential to respond more robustly to prophylactic HIV vaccination.

The persistence of Tfh cells and anti-HIV antibody responses in aviremic early treated individuals' warrants further investigation. We however, speculate that there is persistent low-level viremia within the lymph nodes, which is driving these responses. Our hypothesis is based on reports that the concentrations of many antiretroviral drugs are lower in tissue sites like the LNs than in peripheral blood (Fletcher et al., 2014). Thus, lower drug concentrations will lead to slower clearance of HIV in LNs hence promoting ongoing GC reactions. Future investigations on this cohort will be directed at interrogating virus persistence in the lymphoid tissues. A major limitation of our

study is limited sample availability to interrogate IgG responses in untreated individuals and to conduct *in vitro* co-culture assays for the direct evaluation of Tfh cell helper function in all our study participants. However, the results from the few participants tested together with evidence from flow cytometry characterization of Tfh and B cell subsets make a compelling case on the benefits of early induction of ART to the Tfh compartment. In conclusion, our results here demonstrate that early ART preserves the function of Tfh and B cells, which has implications in the overall immune function of HIV infected individuals in response to other pathogenic infections or vaccinations.

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Chapter 4 Overview

In chapter 3 of this thesis, we reported results outlining phenotypic and functional differences between cTfh cells and GCTfh cells. We also showed that early ART initiation results in the induction of highly functional HIV-1 specific Tfh responses and these responses have a potent helper effect on B cell responses and antibody production. Several studies have implicated Tfh cells in HIV-1 persistence in people taking ART because of their proximity to follicular dendritic cells that carry potentially infectious virions on their surfaces. The limited tissue penetration of antiretroviral drugs in GCs coupled with exclusion of cytotoxic $CD8^+$ T cells from this site further support the notion that GCTfh cells may be more vulnerable to HIV-1 infection during ART. Moreover, the few studies that have looked at persistent infection have mainly been conducted in individuals who initiated therapy in chronic infection and tend to have a larger HIV reservoir. Therefore, studies in chapter 4 of this thesis were aimed at determining if there is persistence of virus in the lymph nodes of people who initiate therapy extremely early, mostly in Fiebig stage I. The studies describe the architectural and cellular localization of persistent virus infection in lymph nodes and report on the impact of persistent infection on $CD4^+$ T helper function and B cell responses.

CHAPTER 4: HIV RNA PERSIST IN CXCR3^{+}CCR6^{+} GCTfh CELLS IN THE LYMPH NODES OF HIV-INFECTED INDIVIDUALS INITIATED ON ART IN FIEBIG STAGE I

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Keywords: T follicular helper cells, GCTfh, early treatment, acute HIV infection, lymph node study, HIV persistence, Germinal centers, HIV sanctuaries.

4.1 Abstract

One of the major barriers to achieving HIV remission through combination antiretroviral therapy (ART) is the persistence of virus in reservoirs and tissue sanctuaries. B cell follicles and germinal centers (GC) within lymph nodes (LNs) are immune privileged sites with the potential to support low-level virus replication. However, the persistence of virus within this tissue compartment in individuals who initiated ART during hyperacute HIV-1 infection is not well described. Here, we used excised LNs and paired peripheral blood samples from 18 HIV-1 infected subjects from the well-characterized FRESH cohort who mostly initiated ART during Fiebig stage I, 8 chronic untreated and 8 HIV negative individuals, to investigate virus persistence and to identify cell subsets that harbor residual virus during early ART. LN viral load was quantified using the Cobas® AmpliPrep HIV-1 test and HIV-infected cells in LNs were detected by staining for Gag p24 capsid protein using immunohistochemistry (IHC), immunofluorescence (IF) microscopy and RNAscope *in situ* hybridization (ISH) techniques. LN cell subsets were also phenotyped by flow cytometry.

Viral load assessment after a median of 403 days on treatment revealed a significantly higher median viral load in the LNs compared to plasma (p=0.02). IF imaging and RNAscope ISH showed the presence of Gag p24 protein and *gag-pol* RNA predominantly in the GCs even after 228 days of ART mediated viral suppression. Phenotypic analysis of GCTfh cell subsets by flow cytometry showed an expansion of $CXCR3^{+}CCR6^{+}$ GCTfh cells which harbored higher levels of Gag p24 antigen compared to other subsets. Together, our results demonstrate that despite early initiation of ART, HIV persists in the LNs, in sufficient quantities to drive persistent GC

reactions. This study demonstrates the huge difference in viral load decay kinetics between peripheral blood and LNs during early ART and underscores the need for future interventions directed at eliminating residual virus in tissue sanctuaries.

4.2 Introduction

Antiretroviral therapy (ART) does not eradicate HIV largely because of virus persistence in cellular and tissue reservoirs (Murray et al., 2016, Puertas et al., 2016, Chun, 2013). Although, the exact nature and size of the reservoir is not precisely determined, memory $CD4^+$ T cells and other cell subsets like macrophages (Abbas et al., 2015, Rose et al., 2016), T follicular helper (Tfh) cells (Kohler et al., 2016, Pallikkuth et al., 2015), regulatory T cells (McGary et al., 2017) and CD32a positive $CD4^+$ T cells (Descours et al., 2017), have all been implicated in virus persistence during suppressive ART (Miles et al., 2016a, Abdel-Mohsen et al., 2018). More so, peripheral lymphoid tissues have been implicated in virus persistence, specifically, in microanatomical structures termed germinal centers (GCs), which have been described as immune privileged or virus sanctuary sites.

Germinal centers primarily function for the maturation of B cell antibody responses and this process is initiated and sustained by the cognate interactions of CD4+ T cells by T follicular helper cells which also promote B cell differentiation and survival (Crotty, 2011). Others and we have described phenotypic heterogeneity within the circulating Tfh (cTfh) compartment and the relationship between the frequencies of different cTfh subsets and the induction of robust antibody responses to HIV infection or vaccination (Locci et al., 2013, Martin-Gayo et al., 2017, Baiyegunhi et al., 2018). However, heterogeneity of Tfh cells in the lymph nodes (LNs) has not been comprehensively evaluated.

One of the few studies that have examined the heterogeneity of GC Tfh cells from lymphoid tissues was a study of SIV infection in macaques (Velu et al., 2016). In that study, there was an expansion of $CXCR3^{+}$ GCTfh cells within the follicles and these cells contained higher amounts of SIV DNA compared to $CXCR3^{-}$ GCTfh cells (Velu et al., 2016). Thus, demonstrating that the heterogeneity of GCTfh cells impacts latent reservoir accumulation but heterogeneity within human GCTfh cells or its impact on active reservoirs or persistent virus production during early ART has not been defined.

Persistent HIV reservoirs have been widely accepted as a barrier to HIV cure. However, there is no consensus on the definition of HIV reservoirs, there are no reliable phenotypic markers for the cells that constitute the reservoir and there is no consensus as to whether HIV continues to replicate in sanctuary sites during ART (Lorenzo-Redondo et al., 2016, Rose et al., 2016, Puertas et al., 2016, Brodin et al., 2016). For example, a study of simian immunodeficiency virus (SIV) infection in macaques found no evidence of virus evolution or *de-novo* viral replication during ART (Oue et al., 2013) but Fletcher et al. (2014) showed that sub-optimal drug penetration into the lymphatic sites, allowed for virus replication. This was further corroborated by another study using deep sequencing and phylogenetic analysis of lymph node and blood samples collected longitudinally at day 0 and after 3 and 6 months of treatment (Lorenzo-Redondo et al., 2016). Paradoxically, a more recent study did not find evidence of virus evolution during ART (Vancoillie et al., 2017).

The optimal outcome for an HIV cure would be the complete eradication of all replication competent viruses within an individual. The prerequisite to achieving such a cure, will be to conclusively determine whether ongoing virus replication occurs during ART, define all cellular sources of productive infection during ART and develop

strategies to selectively eliminate the cells harboring residual virus (Ryscavage et al., 2014, Tobin et al., 2005).

In this study, we set out to investigate the persistence of virus in the LNs of individuals initiated on ART during Fiebig stage I and defined the phenotypic features of cells that harbor persistent virus infection within GCs. The excisional LNs and paired blood samples used for these studies were obtained from 18 early treated, 8 healthy controls and 8 chronic untreated HIV-1 infected individuals. Flow cytometry and immunofluorescence microscopy (IF) technologies were used to define the phenotype and localization of GCTfh subsets respectively and the Cobas® AmpliPrep HIV-1 test was used to measure LN viral loads. Immunohistochemistry (IHC) and RNAscope *in situ* hybridization (ISH) techniques were further used to define virus persistence and cellular distribution of HIV RNA *in situ*.

Our results show that LN viral loads were significantly higher than contemporaneous plasma viral loads after a median of 403 days on treatment. Despite ART-mediated complete plasma viral suppression, HIV Gag p24 capsid protein and *gag-pol* RNA were persistently detectable almost exclusively in the GCs. We further showed that GCTfh cells exhibited very high expression of CXCR3 and CCR6 as measured by IF microscopy as well as by flow cytometry and these cells together with FDCs harbored the detected Gag p24 antigens. These results show that HIV persists in the form of Gap p24 protein and as viral RNA in the lymph nodes of early treated individuals. These findings highlight the need to target the $CXCR3^{+}CCR6^{+}$ GCTfh cell subset for selective elimination in future HIV-1 cure strategies.

4.3 Methods

4.3.1 Study population and samples

The study participants were drawn from the HIV Pathogenesis Programme (HPP) lymph node study cohort in Durban, South Africa. The lymph node study cohort and recruitment procedures were previously described (Chapter 3, section 3.2.1). Briefly, excisional lymph node biopsies and blood samples were collected from 8 HIV-uninfected and 18 early treated (Early Tx) females recruited from Females Rising Through Education, Support and Health (FRESH) cohort [cohort characteristics were described by Dong et al. (2017)]. In addition, 6 chronic untreated (Un Tx) individuals were recruited from the HPP Acute Infection cohort (Wright et al., 2011) and 2 chronic untreated patients were recruited from Prince Mshiyeni memorial hospital, Durban, South Africa. Axillary, cervical or inguinal lymph nodes were surgically excised and 120 ml paired peripheral blood was drawn from each participant. Global Clinical and Viral laboratories, Durban, South Africa, measured absolute CD4 counts and viral loads. The University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) and the Institutional review board of Massachusetts General Hospital granted ethical approval for the study. All study participants signed inform consent forms for participation in the study.

4.3.2 Lymph node and blood sample processing

Biopsied lymph nodes were sectioned into two; one section was fixed in 10% formal-saline (Sigma-Aldrich, St. Louis, Missouri, USA) for IHC studies, while, the second section was macerated to release lymph node mononuclear cells (LMCs) according to

the method of Schacker et al. (2006). The cells were passed through a mesh screen and harvested by centrifugation (1 800 rpm, 6 min, RT).

Peripheral blood mononuclear cells (PBMCs) were isolated from patient's blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in liquid nitrogen (McCoy, 1998).

4.3.3 Viral RNA quantification in lymph node mononuclear cells

Cryopreserved LMCs (10 million cells/ml) were lysed and viral RNA was quantified using the Cobas® AmpliPrep HIV-1 test (Roche, Mannheim, Germany) by Global Clinical and Viral laboratories, Durban, South Africa using standardized protocols.

4.3.4 Immunohistochemistry (IHC) with chromogenic detection

IHC staining was performed on 0.4 µM sections of formalin fixed paraffin embedded (FFPE) lymph nodes using the Dako EnVision FLEX Mini kit, high pH (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol and as previously described (Chapter 3, section 3.2.8). Following antigen retrieval, slides were sequentially incubated with p24 (Dako), primary antibody (20 min, RT), Dako EnVision FLEX Linker (15 min, RT) and Dako EnVision HRP (20 min, RT). The signal was detected using the diaminobenzidine (DAB) detection system (Agilent), counterstained with hematoxylin (Sigma-Aldrich) and mounted using DPX (Sigma-Aldrich). Imaging was conducted using the Axio Scope.A1 LED (Zeiss) or the Axio Observer with TissueFAXS imaging software (TissueGnostics, Vienna, Austria) and analyzed with AxioVision Rel 4.8 software (Zeiss).

4.3.5 Immunofluorescence (IF) microscopy

IF microscopy staining was conducted using the opal 4-color fluorescent IHC kit (PerkinElmer, Waltham, MA, USA) as described in Chapter 3, section 3.2.9. Slides were stained with a total of 3 antibodies and counterstained with DAPI (PerkinElmer) to make a total of 4 different fluorochromes. Primary antibodies used in these combinations included BCL-6 (Dako), CCR6 (Thermofisher Scientific, Waltham MA, USA), CD4 (Dako), CXCR3 (Thermofisher Scientific), CXCR5 (Abcam, Cambridge, MA, USA), FDC (Dako), Ki67 (Dako), and PD-1 (Abcam). Slides were imaged with the Axio Observer and TissueFAXS imaging software (TissueGnostics). Quantitative image analysis was conducted with TissueQuest (TissueGnostics) and negative control slides were used to set the threshold values.

4.3.6 Flow cytometry analysis

Freshly isolated or frozen LMCs and PBMCs were characterized using flow cytometry analysis with standardized protocols. As described in section 3.2.3, cells were stained with LIVE/DEAD Fixable Blue dead cell stain kit (Thermofisher Scientific), CD19 Horizon V500 [(HV500) BD Biosciences], CD3 Brilliant Violet (BV) 711 (Biolegend), CD8 BV786 (BD Biosciences), CD4 BV650 (BD Biosciences) CXCR5 Alexa Fluor (AF) 488 (BD Biosciences), PD-1 BV421 (Biolegend), CCR6 Phycoerythrin [(PE) Biolegend], CXCR3 BV605 (Biolegend), CD45RA PE-Cyanine (Cy)-7 (Biolegend), ICOS PE-Dazzle 594 (Biolegend), CCR7 Peridinin-chlorophyl protein (PerCp) Cy5.5 (Biolegend) and CD27 APCH7 (BD Biosciences) for 30 mins at RT, then acquired on the LSRFortessa (BD Biosciences, San Jose, CA).

4.3.7 RNAscope in situ hybridization (ISH)

RNAscope ISH was conducted using the RNAscope® 2.5 HD assay kit [Advanced Cell Diagnostics (ACD), Newark, CA, USA] and the RNAscope® multiplex fluorescent kit v2.0 (ACD) as per manufacturer's instructions. Briefly, pre-treated samples were hybridized with the clade C HIV-1 *gag-pol* probe at 40^{o}C for 16 hours. Next, the samples were incubated with signal amplification probes and horseradish peroxidase conjugated secondary antibodies. The signal was detected with either DAB for the RNAscope® 2.5 HD assay (ACD) or with opal polymers (PerkinElmer) for the multiplex fluorescent assay. Slides were imaged with Axio Observer and TissueFAXS imaging software (TissueGnostics).

4.3.8 Statistical analysis

All statistical analyses were conducted with GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA) and p values were considered significant if less than 0.05. Specifically, the Mann-Whitney U test was used to compare two groups, the Kruskal-Wallis H test was used for comparing multiple groups and correlations between variables were defined by the Spearman's rank correlation test.

4.4 Results

4.4.1 Identification of hyperacute HIV-1 infection allowed for early ART initiation in study participants

Hyperacute HIV-1 infection was detected in 18 females (Table 4.1) and they were initiated on ART (FCD pill plus raltegravir) at a median of 1 day (min:max, 1:3 days) after virus detection (see Appendix 2) and treated for a median of 228 days (min:max,

11:926 days) before LN excision (Figure 4.1, Appendix 4). All Early Tx patients but one had achieved full virus suppression at the time of LN excision (Appendix 4) and the cumulative HIV antigen load was calculated for each patient as the area under the viral load curve (Appendix 5). In addition to the 18 early treated participants (Early Tx), 8 HIV negative (HIVneg) and 8 chronic untreated (Un Tx) individuals were studied. Most of the study participants were females and other characteristics are detailed in Table 4.1.

Table 4.1: Patient characteristics

	HIV negative	Early treated HIV	Untreated chronic HIV
n	8	18	8
Male	0	0	1
Female	8	18	7
Age (years)*	21	22	24
	(20-22)	(19-22)	(22-34)
CD4 Count (cells/ul)*	N/A	919	357
		(709-1075)	(355-359)
Viral load (cps/ml)*	N/A	<20	9000
			(1193-21750)
Treatment duration (days)	N/A	228	N/A
		(43-440)	

* IQR values are reported in parenthesis under median values.

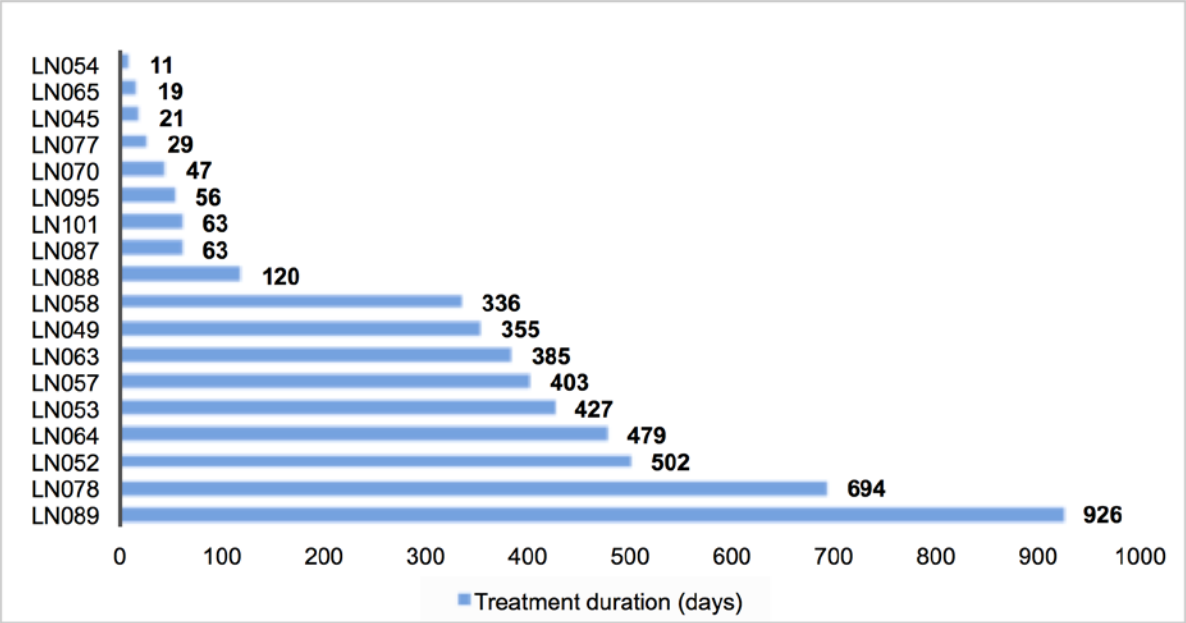

Figure 4.1: Kinetics of treatment duration across study participants. The number of days on treatment prior to lymph node excision was plotted for each participant in the early treated group.

4.4.2 HIV Gag p24 persists long term in lymph node sections obtained from individuals initiated on ART soon after HIV-infection

The HIV-1 Gag p24 is a capsid protein that constitutes the core of the virus. It is produced by cleavage of its precursor, Gag p55 by HIV-1 protease and is essential to produce infectious virions. Immunostaining of this antigen in lymph node biopsies has been used to define virus persistence (de Paiva et al., 2007). We used the presence of Gag p24 protein in lymph node tissues as a proxy for HIV persistence and it was undetectable in HIV negative donor samples (Figure 4.2A). However, clusters of Gag p24 protein were detected in lymph node sections from a Fiebig stage V treated individual after 427 days on ART and a Fiebig stage I treated individual after 502 days of treatment (Figure 4.2B). Samples from chronic untreated persons also stained positive for Gag p24 antigen (Figure 4.2C).

Comparative analysis of Gag p24 staining across the study groups revealed that significantly lower Gag p24 was detected in early Tx (n=13) subjects compared to UnTx participants (n=6) (*p*=0.003, Figure 4.2D). Furthermore, the quantified amount of Gag p24 antigen detected in the early treated participants directly correlated to the Fiebig stage classification at the time of treatment initiation (*p*=0.0001, *r*=0.87, Figure 4E) but was not associated with the treatment duration (Figure 4.2F). These results demonstrate that Gag p24 protein persists in the lymph nodes of early treated individuals with increasing magnitude based on the Fiebig classification of the participant.

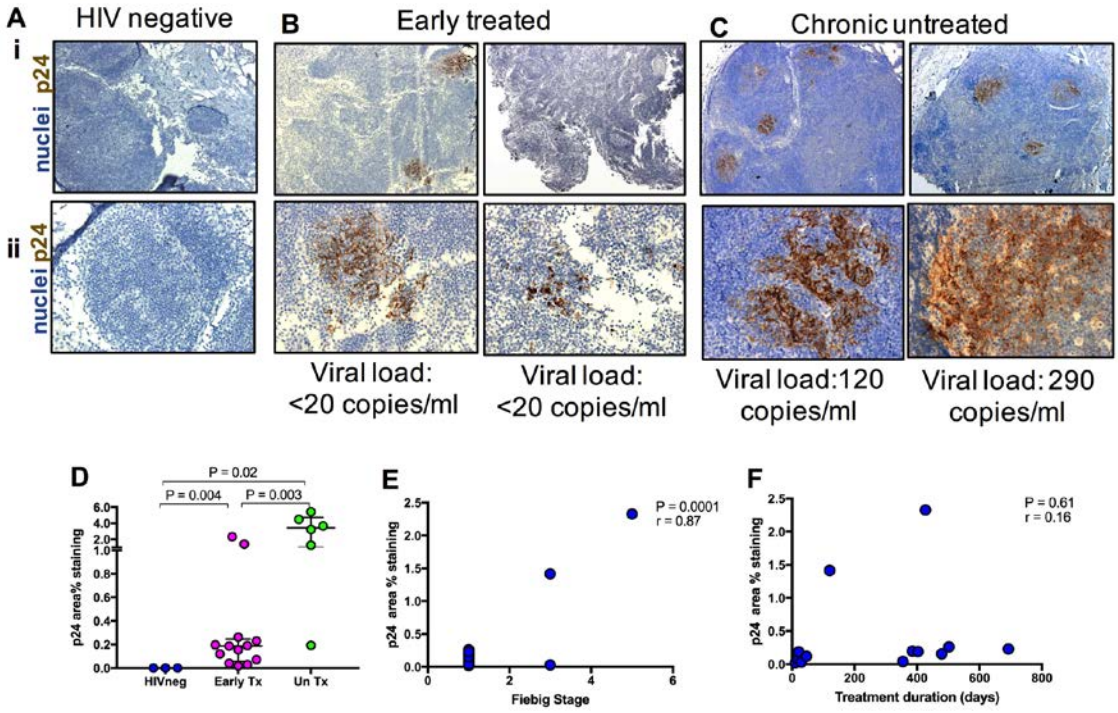

Figure 4.2: HIV Gag p24 detection in the lymph node of early treated HIV-1 infected patients. (A to C) Representative micrographs of Gag p24 staining in lymph node (LN) sections. The images in panel i are magnified 1000 times in panel ii. (D) Summary plots comparing the average area percentage staining of Gag p24 on LN sections for HIV negative (HIVneg), early treated (Early Tx) and untreated HIV-infected (Un Tx) individuals. (E and F) Correlation between Gag p24 average area percentage staining in LNs and Fiebig stage (E), or days on treatment (F). Mann U-Whitney p values, Spearman rho (*r*) values and *p* values are reported.

4.4.3 HIV Gag p24 persistence occurs almost exclusively in LN GCs of early treated HIV-infected individuals

Since Gag p24 antigen was detected in clusters in the LNs, we reasoned that these regions were GCs. To definitvely determine if GCs harboured most of the Gag p24 antigens, we used BCL-6 as a marker for GCs (Goteri et al., 2011), and multiplexed it with Gag p24 antibody. We observed co-localization of HIV Gag p24 antigen with BCL-6 (Figure 4.3A). Next, we wanted to determine if the observed GCs were being propagated by HIV infection, so we analysed our images using TissueQuest (TissueGnostics), which is a high throughput analysis software that allows for FACS-like analysis of fluorescently labelled samples (Schmid et al., 2015). Firstly, we measured the size of GCs across our study groups and we observed significantly larger GCs in UnTx (p=0.05) and early Tx (p=0.04) groups compared to the HIVneg group (Figure 4.3B). Next, the magnitude of GCs was defined as the cell counts within BCL-6^+ clusters and we found higher counts of BCL-6^+ cells in early Tx (p=0.01) and a trend of higher BCL-6^+ counts in UnTx (p=0.20) compared to HIVneg group (Figure 4.4C). These results demonstrate that the size and magnitude of GCs are increased during HIV-1 infection.

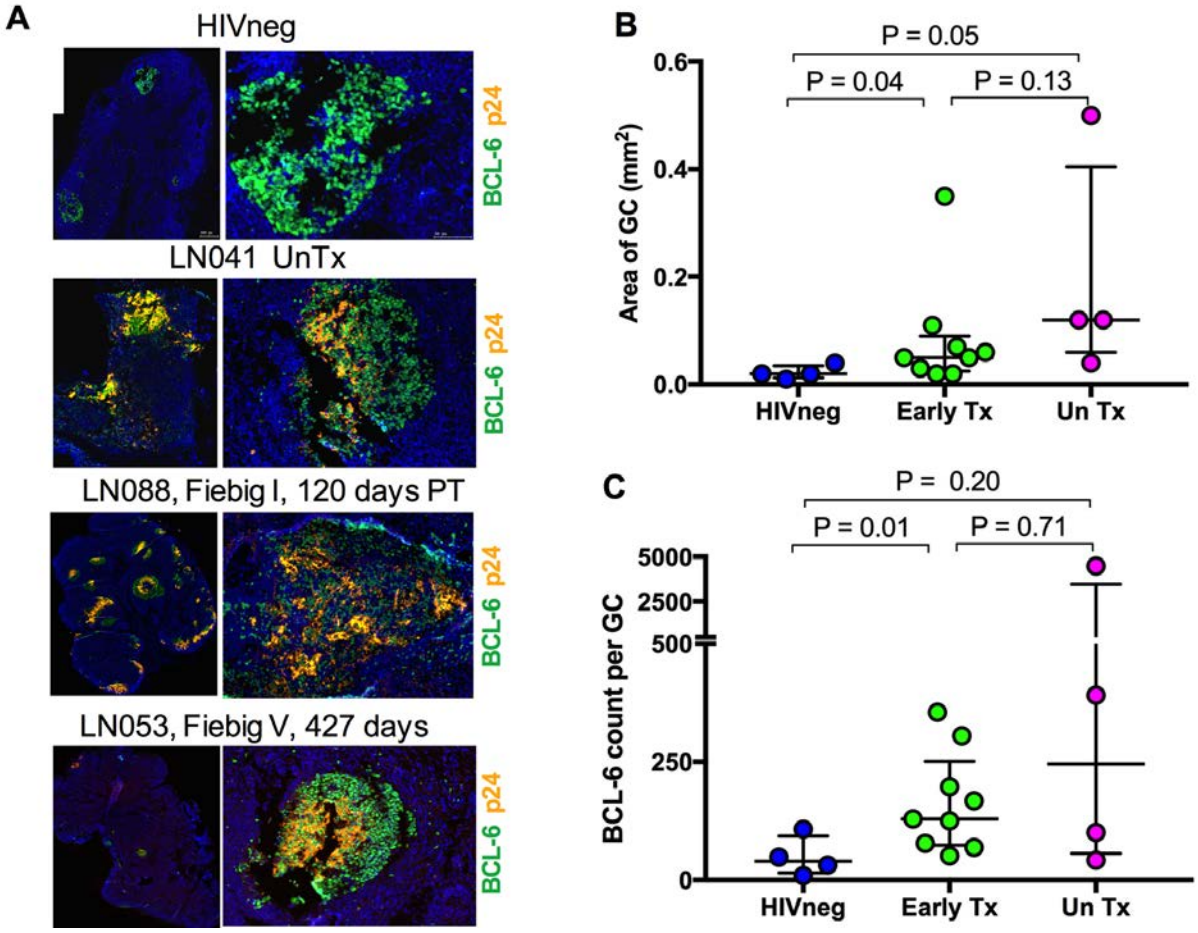

Figure 4.3: Persistence of Gag p24⁺ cells in BCL-6⁺ germinal centers during early treated and untreated HIV-1 infection. (A) Representative immunofluorescence images of multiplexed BCL-6 and Gag p24 staining on lymph node (LN) sections. Scale bars represent 200 µm and 50 µm on left and right panels respectively. (B & C) Summary plots comparing the area of GCs (B) or the mean GC BCL-6 count (C) in HIV negative (HIVneg), early treated (Early Tx) and untreated HIV-infected (UnTx) LN sections. TissueQuest (TissueGnostics, Vienna) was used to compute the area of GCs and BCL-6⁺ cell counts in each tissue section. *P* values were determined using Mann U-Whitney test.

4.4.4 HIV Gag p24 persistence in lymph nodes is accompanied by active GCs

Having demonstrated the presence of virus in LN GCs and the abundance of GCs in HIV infected individuals, we next investigated if the persistence of HIV Gag p24 in the LNs was driving the observed GC reactions. Active GCs were defined by staining for Ki67, a well defined proliferation marker. The area percentage staining of BCL6, Ki67 and p24 were determined in serial sections using the AxioVision Rel 4.8 software

(Zeiss). Correlation analysis showed a positive trend between Gag p24 antigens and BCL-6 (p=0.07, r=0.57) or Ki67 (p=0.09, r=0.51) area percentages (Figures 4.4A and 4.4B) which did not reach statistical significance.

To further validate our hypothesis, we correlated the cumulative antigen load of each early treated participant (area under the viral load curve, Appendix 5) to BCL-6$^+$ cell counts and the size of GCs determined in section 4.3.3. There was a direct correlation between the cummulative antigen load and the BCL-6$^+$ cell counts (p=0.003, r=0.96) in early treated individuals (Figures 4.4C). There was also a trend of larger GCs (p=0.09, r=0.71) in individuals with high exposure to viremia (Figures 4.4D). Taken together, these results suggest that the persistence of HIV Gag p24 was driving the observed GC reactions and the proliferation of cells within the GCs.

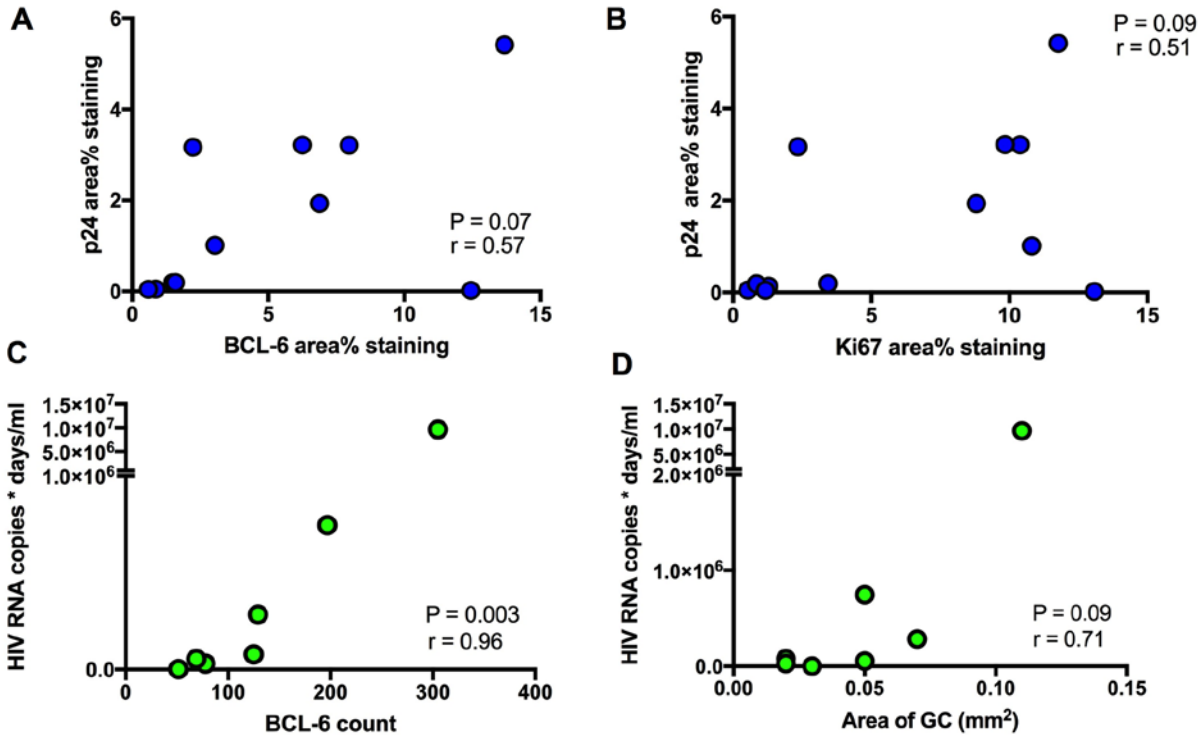

Figure 4.4: HIV persistence in lymph nodes drives germinal center formation and proliferation. (A & B) The area percentage staining intensity of Gag p24 is correlated to that of BCL-6 (A) or Ki67 (B) for each lymph node (LN) section. (C & D) Correlation between viremia copy days and BCL-6$^+$ cell counts in GCs (C) or area of GCs (D) in LN sections of early treated individuals. Area percentages were quantified using AxioVision Rel 4.8 software

(A & B) and TissueQuest (TissueGnostics) was used to compute the area of GCs and BCL-6 cell counts in each tissue section (C & D). Spearman rho (*r*) values and *p* values are reported.

4.4.5 Discordant HIV-1 RNA loads in plasma and lymph nodes

Having demonstrated the persistence of HIV structural proteins in lymph nodes, we next sought to investigate HIV RNA persistence in the lymph nodes by quantifying LN viral loads using the Cobas® Ampliprep HIV-1 test. Samples were available for 6 aviremic early treated study participants and viral load assessment revealed higher median viral loads of 1560 (copies/ 10 million LMCs) compared to the plasma viral loads which were below the limits of detection (Table 4.2 & Figure 4.5). The amplification of viral RNA from LMCs demonstrate the persistence of LN cell associated RNA despite early ART.

Table 4.2: Compartmental viral load analysis for the early treated group

Patient ID	Fiebig stage	Plasma VL at treatment initiation (copies/ml)	Time to complete suppression (days)	Treatment duration at excision (days)	Plasma VL at LN excision (copies/ ml)	PBMC VL at LN excision (copies/ ml)	LN VL (copies/ ml)
LN053	V	200000	22	427	<20	312	903
LN057	I	1100	13	403	<20	406	2240
LN088	III	110000	16	120	<20	935	2055
LN089	V	<100	7	926	<20	27	1560
LN095	I	5300	9	56	<20	107	20
LN101	III	76000	17	63	<20	178	2960
LN078	I	5900	13	694	<20	<20	<20

Abbreviations: LN, lymph node; VL, viral load; ID, identification number.

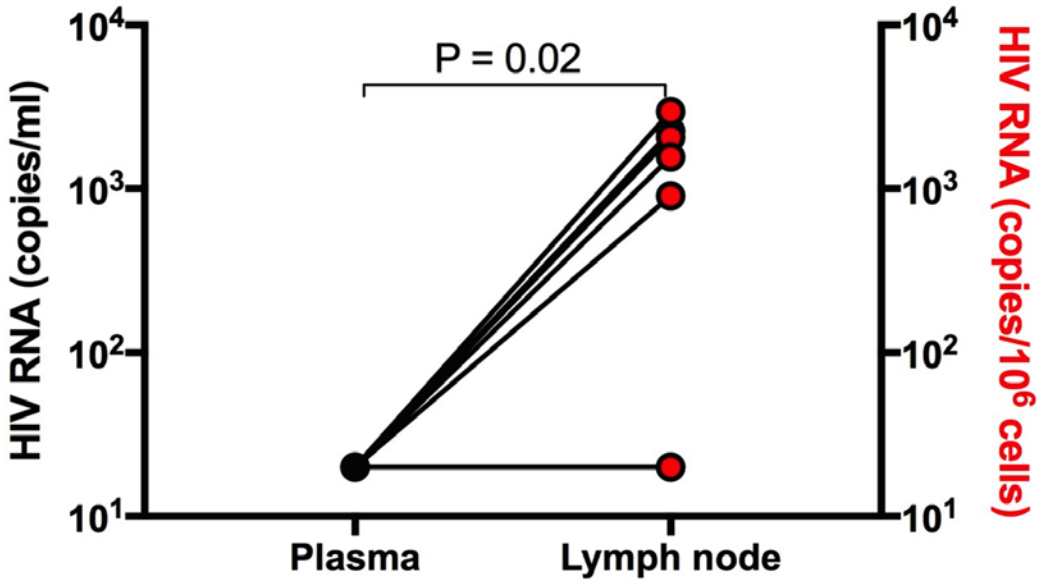

Figure 4.5: Viral loads in plasma and in lymph node cells of early treated participants. HIV RNA copies/ml were quantified in plasma and in 10 million lymph node (LN) cells for 7 early treated participants. Statistical differences were calculated using Mann U-Whitney tests and viral loads below the limits of detection were assigned a value of 20.

4.4.6 HIV RNA⁺ CD4⁺ T cells and follicular dendritic (FDC) bound virions are detected in the GCs despite early initiation of ART

We next investigated the localization of viral RNA and FDC bound virions using RNAscope ISH approach. Individual HIV virions/viral RNA$^+$ cells were detected in LN sections of all our early treated and untreated study participants (Figure 4.6A). Productively infected viral RNA$^+$ cells were identified as a densely spherical signal (red arrow) whereas, FDC bound virus particles were defined by a diffuse lattice-like pattern (green arrowhead) consistent with previous reports (Deleage et al., 2016) (Figure 4.6A). Combined RNAscope ISH *gag-pol* staining with IF staining for BCL-6 confirmed viral RNA localization in the GCs in early treated participants (Figure 4.6B, top panel). Co-localization of viral RNA (green) with CD4$^+$ T cell (red) staining identified CD4$^+$ T cells harboring HIV RNA (Figure 4.6B, bottom panel). Taken together, the

detection of HIV RNA by ISH confirms the persistence of HIV RNA in the LNs of early treated individuals.

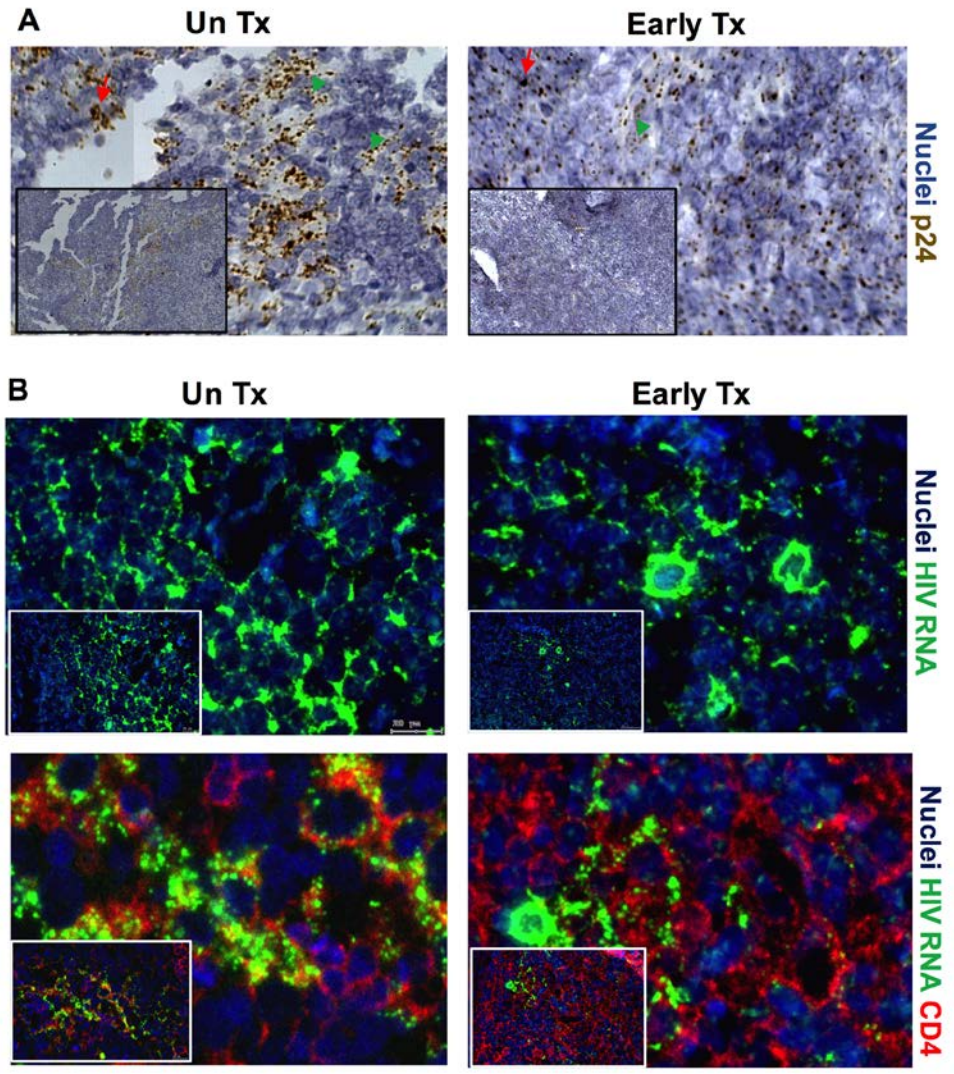

Figure 4.6: HIV-RNA$^+$ cells in the lymph node of untreated and early treated HIV-1 infected individuals. (A) RNAscope hybridization for HIV *gag-pol* RNA detected using 3, 3'-diaminobenzidene (brown). Representative images for early treated (Early Tx) and untreated HIV-infected (Un Tx) lymph node sections. Single RNA transcripts seen as punctate dots; and clusters of transcripts are also observed. Red arrows identify HIV RNA$^+$ cells and green arrowheads identify virions on follicular dendritic cells. (B) Images showing multiplexed RNAscope *gag-pol* hybridization (green) and IF staining for CD4$^+$ cells (red).

4.4.7 GCTfh cells predominantly comprises of CXCR3$^+$CCR6$^+$ cells.

Having identfied a subset of HIV RNA postive CD4$^+$ T cells in the GC, we next used flow cytometry to determine the phenotype of GCTfh cells (Hong et al., 2012, Kohler et al., 2016). We examined the relationship between GCTfh cells and GCs, and found a strong positive correlation between the frequencies of GCTfh cells measured by flow cytometry and the mean BCL-6 count (p=0.02, r=0.55) or the area of GCs (p=0.01, r=0.59) determined using IHC analysis (Figure 4.7B and 4.7C).

Our *in situ* staining revealed that only a small subset of CD4$^+$ T cells were positive for HIV RNA and/or HIV Gag p24 protein suggesting that only a subset of GCTfh are infected. Also, our prior flow cytometry staining showed that the vast majority of GCTfh cells expressed significantly higher levels of CXCR3 (p<0.0001) (Figure 4.7D) and CCR6 (p<0.0001) than circulating Tfh cells (Figure 4.7E). These two chemokine receptors have prevously been shown to be highly expressed on circulating Tfh subsets but have not yet been assessed on lymph node GCs (Schmitt et al., 2014b). Also a study on macaque lymph nodes reported phenotypic heterogeneity among GCTfh cells (Velu et al., 2016).

Therefore, we next investigated the expression pattern of the two chemokine receptors (CXCR3$^+$CCR6$^+$) and found that a sigificantly larger proportion of GCTfh coexpressed CXCR3$^+$CCR6$^+$ compared to nonGCTfh populations (Figure 4.7F). To our knowledge, this is the first description of this subset in human lymph nodes. Also, chronic untreated HIV infection had significantly higher frequencies of CXCR3$^+$CCR6$^+$ compared to HIV negative LNs (p=0.02) (Figure 4.7G). This phenotype was rarely detected in cells from human tonsils excised from HIV uninfected individuals (Figure 4.7H). Together, these results identify the CXCR3$^+$CCR6$^+$ GCTfh subset as the predominant GCTfh subset

induced in LNs during HIV infection and also highlights phenotypic differences between GCTfh cells from LNs and tonsils.

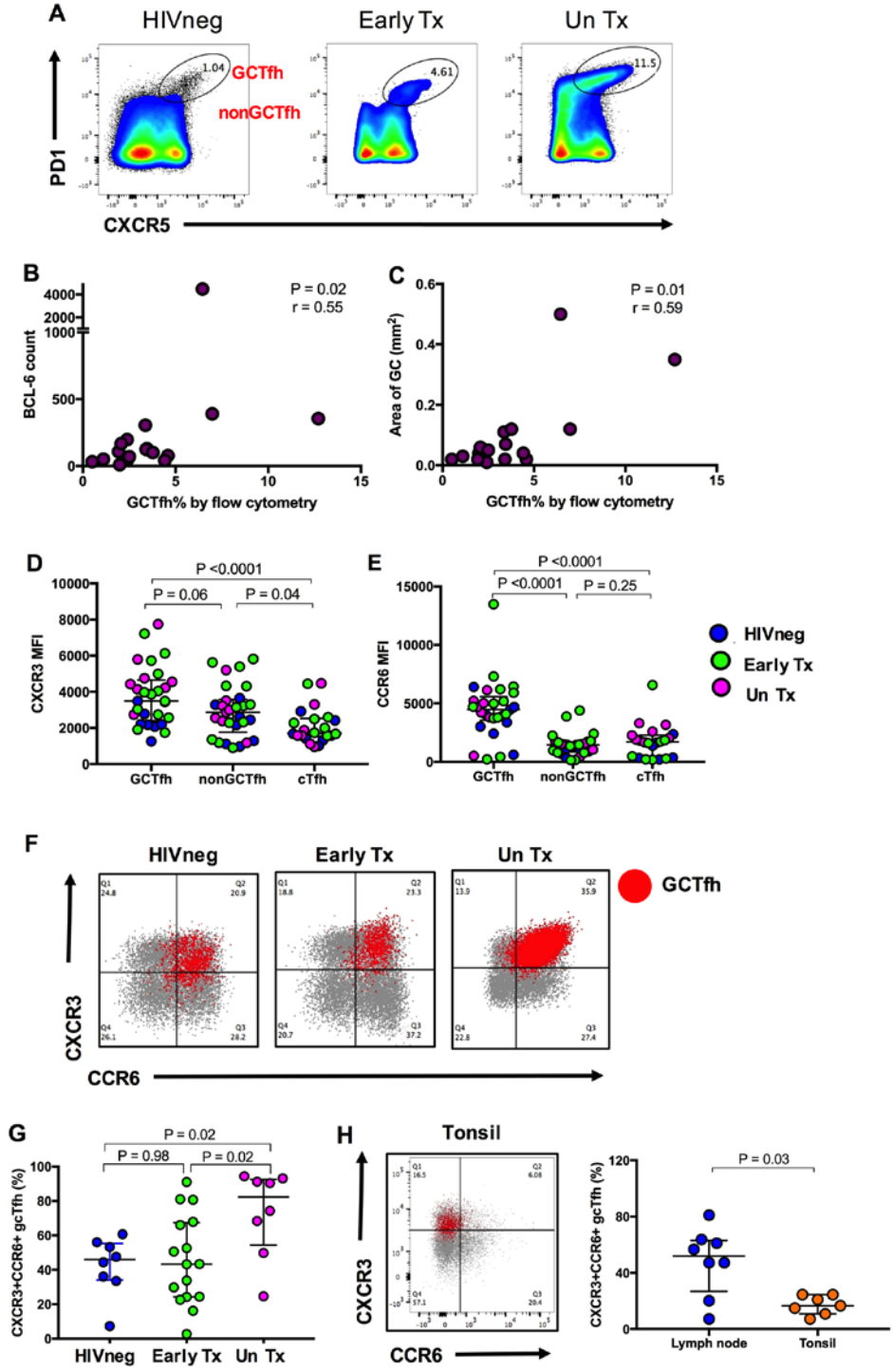

Figure 4.7: Heterogeneity within GCTfh cells. (A) Representative flow cytometry plots showing gating strategy for GCTfh ($CXCR5^{hi}PD\text{-}1^{hi}$) cells. (B & C) Correlation analysis of GCTfh cell frequencies and BCL-6^{+} cell counts (B) or area of germinal centers (GCs) (C) calculated from IHC studies. (D & E) Expression of CXCR3 (D) and CCR6 (E) chemokine receptors by Tfh subsets. (F) GCTfh cells are predominantly $CXCR3^{+}CCR6^{+}$. The GCTfh gate (red) was overlaid

on $CXCR3^+CCR6^+$ $CD4^+$ T cells representative flow plots. (G) Expansion kinetics of $CXCR3^+CCR6^+$ GCTfh subsets in HIV negative (HIVneg), early treated (Early Tx) and untreated HIV-infected (Un Tx) donors. (H) Representative flow plot showing the distribution of tonsil GCTfh cells (red) within $CXCR3^+CCR6^+$ $CD4^+$ T cells and summary plot comparing the frequencies of $CXCR3^+CCR6^+$ GCTfh cells in lymph node and tonsil cells. Statistical differences were calculated using Mann U-Whitney tests, Spearman rho (*r*) values and *p* values are reported for correlation analyses.

4.4.8 HIV p24 antigen persists in $CXCR3^+CCR6^+$ GCTfh cells in early treated HIV-infected individuals

Next we wanted to invetigate if $CXCR3^+CCR6^+$ GCTfh cells was the subset that habored most of the persisting virus. Co-localization analysis revealed that Gag p24 co-localized with $PD1^+$ cells (Figure 4.8, panel i), $CD4^+$ cells (Figure 4.8, panel ii), $CXCR3^+$ cells (Figure 4.8, panels iii) and $CCR6^+$ cells (Figure 4.8, panels iv) in early treated and chronic untreated LN sections. Gag p24 antigens also co-localized with FDCs and $CD4^+$ T cells (Figure 4.8, panel v). These results demonstrate that $CXCR3^+$ GCTfh cells contribute to HIV persistence during early ART.

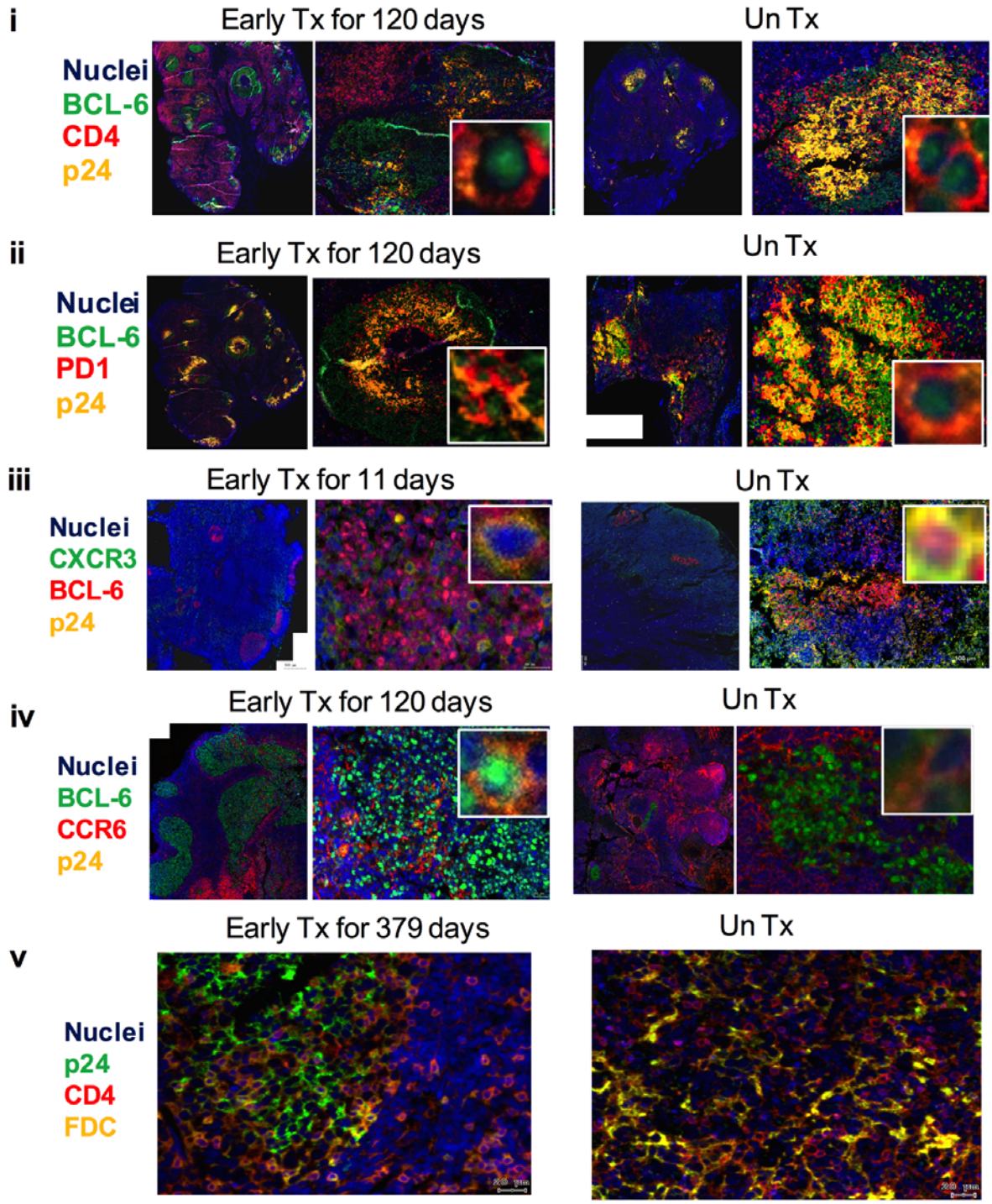

Figure 4.8: Anatomical distribution of HIV Gag p24 antigen. HIV Gag p24 co-localizes with (i) $CD4^+$, (ii) $PD1^+$, (iii) $CXCR3^+$, (iv) $CCR6^+$ and (v) follicular dendritic cells (FDCs) in LNs. Representative IF images characterizing HIV Gag p24 positive cells in the GC/follicles of early treated (Early Tx) and untreated HIV-infected (Un Tx) donors.

4.5 Discussion

The initiation of ART very early after infection reduces peak viremia (Dong et al., 2017), and decreases viral reservoirs and HIV DNA set-point (Ananworanich et al., 2016). It is thus conceivable that early initiation of ART and rapid plasma viremia suppression will significantly reduce residual viremia in tissue and cellular virus sanctuaries. Additionally, most HIV studies and HIV clinical investigations rely on plasma viral load kinetics to define the dynamics of persistence in patients (Deleage et al., 2016, Archin et al., 2014), however, viral load kinetics in tissue sites might be better indicators of HIV suppression in infected patients.

Measurement of viral RNA in peripheral blood and lymph nodes using the Cobas® Ampliprep test, revealed a discordant pattern of viral RNA detection in plasma and LNs of our early treated study participants. Plasma viral loads were below the detection limits but readily detectable in the LNs. Although, endpoint reverse transcription-PCR tests like the Cobas® Ampliprep test are traditionally used for plasma viral load quantification, these assays are also valuable for viral load estimation in other sample types for example cervical swab samples (Klein et al., 2018) and LMCs as we have reported here.

We further confirmed viral RNA persistence in the LNs using a highly sensitive and specific *in situ* hybridization assay, RNAscope (Deleage et al., 2016, Barton et al., 2016). Our results are consistent with a previous study in which they detected, transcriptionally active viral RNA in the LNs of ART treated patients using the traditional RNA *in situ* hybridization technique (Abdel-Mohsen et al., 2018). Viral RNA hybridization signals in the LNs were significantly reduced after 6 to 13 months of ART in another study (Popovic et al., 2005). Since we didn't collect serial LN samples in

this study, we can't make assertions as to whether ART reduced viral RNA detection in our study.

Our results also describe the persistence of Gag p24 antigen in the GCs of early treated individuals even after 926 days of treatment and the cellular distribution of Gag p24 within GCTfh subsets. Furthermore, in keeping with the notion that antigenemia drives GC formation (Hong et al., 2012), we showed that the total antigen burden was directly correlated to the magnitude of the GC response in the early treated individuals. It is important to note that the LNs for half of our study participants were obtained after a year of complete plasma viremia suppression, thus the correlation between total plasma viremia exposure and LN GC responses at the time of LN excision suggests that early seeding HIV antigens in the LN leads to long term persistence and slow clearance of viral antigens from the GCs. Although, our investigations are directed at virus persistence and not at reservoir estimations, these results are consistent with reports that the HIV reservoir is seeded in peripheral tissue sites very soon after HIV infection and decays slowly (Whitney et al., 2014).

Our results corroborate reports that the FDC network can trap and retain infectious HIV particles for long periods of time with a decay half-life of approximately 2 months, thus acting as a long-term source of replication competent virus in the GCs (Smith et al., 2001, Hufert et al., 1997, Zhang et al., 2013a). The proximity of Tfh cells to FDCs in the GCs increases vulnerability to HIV infection (Miles et al., 2016b, Klatt et al., 2013). However, although the preferential infection of Tfh cells by HIV has been demonstrated *ex-vivo* (Kohler et al., 2016), and the higher contribution of circulating Tfh cells to the HIV reservoir in treated individuals (Pallikkuth et al., 2015), the reason for the high permissiveness of Tfh to HIV infection remains unanswered. Tfh cells

generally express low levels of the CCR5 co-receptor but our results here demonstrate that some GCTfh cells express CCR6, which is an alternate co-receptor for HIV entry (Islam et al., 2013) and thus could be contributing to Tfh susceptibility to HIV infection. Future investigation on the potential role of CCR6 in HIV persistence is warranted.

Although we have shown that HIV persists in the LNs despite the initiation of ART extremely after infection, it is not clear from our studies if the observed persistent viremia is sustained by low-level virus replication and if the ongoing active replication leads to virus evolution. These studies are ongoing and are beyond the scope of this work. Taken together, our results emphasize the importance of very early initiation of ART and highlight the need for novel drug formulations and alternate routes of antiretroviral drug administration that will improve the distribution and retention of ART within tissue-privileged sites. We are planning to continue to monitor HIV persistence in our early treated study individuals for longer periods than we have reported here to get a better appreciation of how long HIV RNA transcripts persist in these individuals. Ongoing studies will continue to investigate if ART eventually resolves HIV persistence in LNs.

Our characterization of GCTfh subsets in the sanctuary sites of virus persistence, revealed a distinct phenotypic signature of dual expression of high levels of CXCR3 and CCR6, particularly during chronic HIV infection. Among $CD4^+$ T cell lineages, Th1 cells generally express CXCR3 receptor while Th17 cells majorly express CCR6. Heterogeneity of phenotypes or the expression of varied phenotypes among $CD4^+$ T cell lineages is a well described phenomenon (Crotty, 2018). Extensive characterization of peripheral Tfh cells by our group (chapter 2) and others have demonstrated that the circulating Tfh compartment is a heterogeneous collection of

Tfh cells, which share the same core Tfh characteristic CXCR5 expression (Crotty, 2018). Although, heterogeneity among lymphoid tissue Tfh populations has been less described in humans, mouse studies have described GCTfh cells as having gene expression programs similar to other Th lineages. This was observed during LCMV and SIV infection and was attributed to the prevailing Th1/Th2 polarizing conditions of these diseases that could be influencing Tfh differentiation patterns (Moukambi et al., 2015, Moukambi et al., 2017). This is in keeping with our results that showed an expansion of $CXCR3^{+}CCR6^{+}$ GCTfh cells in chronic untreated HIV infection, during which there are high levels of inflammatory cytokines produced (Biancotto et al., 2007). Additionally, the increased expression of the transcription factor T-bet, which is important for Th1 transcription was seen in Tfh cells during chronic SIV infection (Moukambi et al., 2015, Moukambi et al., 2017).

In our study, we also observed an absence of the $CXCR3^{+}CCR6^{+}$ phenotype in tonsil GCTfh cells, which highlight compartmental and micro environmental influences on Tfh differentiation patterns. The influence of tissue compartmentalization on Tfh differentiation dynamics in the spleen and peripheral blood of rhesus macaques has been documented in the context of SIV infection (Moukambi et al., 2015, Moukambi et al., 2017). Since CXCR3 and CCR6 receptors are very important for migration to peripheral inflamed tissue sites (Groom et al., 2011), it is unclear why GCTfh cells express these chemokine receptors and will warrant further investigation. In conclusion, our results demonstrate that despite ART mediated plasma viremia suppression in Fiebig stage I treated individuals, HIV structural proteins and HIV RNA persist in the LN GCs on FDCs and intracellularly within productively infected $CXCR3^{+}CCR6^{+}$ GCTfh cells. These results are relevant for HIV cure strategies seeking to eliminate infected cell subsets.

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CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

The HIV-1 epidemic is responsible for up to 1 million deaths worldwide in 2016 (UNAIDS, 2017a) and is a major global health challenge that still requires remedial measures. Two important goals of the global HIV research community are to (i) find an effective vaccine and (ii) to develop a cure for HIV. Although, there are enormous challenges hindering the achievement of both goals, significant progress has been made, and some candidate vaccines are currently being tested in clinical trials (Gray et al., 2016, NIAID, 2016, AVAC, 2016). Furthermore, it has become clear that HIV remission might be an easier goal to attain than complete HIV eradication (Dieffenbach et al., 2011). Key to developing a cure or a preventative HIV vaccine is to improve our understanding of host antiviral immune responses in lymphoid tissues. In addition, since the landscape of HIV-1 research has been drastically altered by the widespread use of ART either for HIV treatment or as pre-exposure prophylaxis, the improved understanding of immune responses in early treated individuals would be beneficial to future vaccination strategies or HIV cure interventions.

The FRESH cohort, which has been in existence for more than 5 years, was strategically designed to identify early HIV infection by longitudinal follow up and frequent testing for HIV acquisition in a high risk HIV negative study population (Dong et al., 2017). With the identification of hyperacute infection, these individuals are placed on ART very soon after infection and can also be recruited into the lymph node study cohort for the definition of immune responses in the lymph nodes. In addition,

due to long-term follow-up and longitudinal sampling, clinical characteristics of all participants are captured, and are invaluable for defining immune responses at the very early stages of HIV-1 infection and treatment initiation.

T follicular helper (Tfh) $CD4^+$ T cells are important mediators of anti-HIV humoral immunity but their potential role in HIV vaccine strategies have been largely unexplored due to limited information on their biology, subset distribution and localization in difficult to access lymphoid tissues. In addition, whether early initiation of ART modulates Tfh cell homeostasis and function is not fully defined. From a HIV cure perspective, all cellular and tissue reservoirs of HIV during ART need to be identified and eradicated by immunotherapies or other novel strategies. Tfh cells are preferential targets for HIV-1 infection and persistence (Perreau et al., 2013, Pallikkuth et al., 2015, Kohler et al., 2016), thus, we investigated the dual role of Tfh cells, as the prime orchestrator of B cell responses and as a major target of HIV infection in lymph nodes.

Taking advantage of access to the unique FRESH cohort and precious lymph node samples from early treated individuals, the current study endeavored to comprehensively characterize Tfh cells from lymph nodes and peripheral blood during untreated and early treated HIV-1 subtype C infection and defined the contribution of Tfh cells to the development of anti-HIV B cell antibody responses and HIV-1 disease progression. In addition, this study further described virus persistence in lymph nodes during ART and shed light on the phenotype of cells that are more likely to be persistently infected with HIV in the lymph nodes.

Study implications and future directions

Overall, our study highlights the usefulness of longitudinal study cohorts for defining Tfh cell responses during very early stages of HIV-1 infection and how these responses are modulated by early treatment. Our initial studies focused on characterizing Tfh responses in peripheral blood and we identified the circulating Tfh1 subset as a potential biomarker for functional antibody responses. Our results demonstrating a correlation between the frequencies of Tfh1 cells and nnAbs imply a functional role for Tfh1 cells during HIV-1 infection as potential B cell helpers for nnAbs development. We thus, postulate that designing immunogens that induce Tfh1 responses might be critical in vaccine efforts seeking to induce long-lasting non-neutralizing antibodies against HIV-1 infection.

The rest of our investigations focused on characterizing Tfh cells in lymph nodes, firstly, evaluating how early treatment modulates their effector functions and secondly, characterizing their role in persistent HIV infection during ongoing therapy. Our results from Tfh studies in the lymph nodes suggest that early treatment is beneficial for improving the quality of the Tfh response. More so, these primed responses have the potential to be boosted by a prophylactic vaccine. Although early treatment improved the quality of the GC response, it was unable to completely eradicate virus persistence in the lymph nodes for the duration that we examined in this study, which was a median of 282 days. We thus recommend that the study participants should be followed for longer periods of time than we have reported here, to be able to fully define the kinetics of full virus suppression in the lymph nodes after early initiation of ART. It’s important to note that virus persistence could be influenced by numerous factors like the magnitude of the initial viremia burden the individual is exposed to and the time to full

plasma viremia suppression. Among other factors, viral characteristics, which were not investigated by the present study, could also contribute to HIV persistence in GCs and should be investigated by future studies.

The direct correlation between the total plasma antigen burden and the magnitude of the GC response, suggests that the seeding of persistent virus in the lymph node occurs soon after infection. The early viral seeding of peripheral lymphoid organs has long-term implications on immune responses in the tissues. Thus, public health surveillance approaches designed at identifying acute HIV infection at the earliest possible time will be very important for patient management and overall immune reconstitution in HIV-1 infected individuals. In addition, there is a need for novel strategies to improve ART penetration and intensification in sanctuary sites like lymph nodes for improved virus clearance. Also, cytotoxic T cells can be engineered to enter the GCs to eradicate productive infections.

One controversial topic in the HIV research field is the question of active virus replication during ART. One side of the debate believe that there is no productive virus replication during suppressive ART and that the residual virus pool is replenished by clonal expansion of long-lived memory pools (Murray et al., 2016). However, since GCTfh cells comprise not only central memory cells, which are long lived, but also effector memory cells, which have a shorter life span. Productive infection within effector memory GCTfh cells challenges the dogma of clonal expansion of long-lived cells as the only source of HIV reservoir. Some other studies have made a case for ongoing virus replication during ART (Lorenzo-Redondo et al., 2016). Here, we have described the cell subsets that contribute to persistent virus in the lymph nodes. More

investigations are needed to look for evidence of sequence evolution in this subset during ongoing suppressive ART.

The retention of HIV virions on follicular dendritic cells in the GCs is a well-described phenomenon and is a major barrier to HIV eradication from such immune privileged sites. Also, the close contact of Tfh cells to follicular dendritic cells increases the chances of infection further contributing to virus replication and persistence in these sites. There is therefore, a need for interventions to reduce the retention rate of HIV virions on follicular dendritic cells. More research directed at understanding the formation of immune complexes and the processing of these virus complexes by follicular dendritic cells will be invaluable for innovations directed at virus clearance within the lymphoid tissue sites.

Although we defined the qualitative functional characteristics of GCTfh cells in early treated individuals compared to untreated individuals, additional information can be obtained from a study comparing GCTfh responses in individuals treated in chronic infection versus early treated individuals. These results will define if Tfh dysfunction is progressive and reversible by ART regardless of the time of ART initiation. Also, future studies should be designed to define the transcriptional and epigenetic signatures associated with improved GCTfh function, which can be harnessed for vaccine design studies desiring to manipulate Tfh cell and B cell interactions in GCs with the aim of improving the durability of anti-HIV antibody responses.

Concluding Remarks

One key novel observation of this study was the induction of $CXCR3^{+}CCR6^{+}$ GCTfh subsets during HIV-1 infection and the persistence of virus in these subsets. An

understanding of why GCTfh cells in the lymph nodes express CXCR3 and CCR6 receptors will be informative to approaches designed to eradicate persistent virus from these subsets. Our demonstration of prolonged persistence of HIV infection in the lymph nodes is another important finding that has significant implications on future structured treatment interruption strategies. The other important result is the identification of a potential biomarker for binding antibody responses. In chapter two of this thesis we identified the circulating Tfh1 subset as a potential biomarker for the development of anti-HIV antibody responses and antibody mediated suppression of HIV. Having a biomarker for immune responses induced in the lymph nodes is critical, given that frequent lymph node sampling for research purposes is not feasible.

In conclusion, the results of this study give important insights into Tfh biology, the preservation of Tfh cell function when HIV-1 replication is controlled by effective ART, and defined the phenotype of the Tfh subset that harbors persistent HIV-1 infection in aviremic patients. Our results support the notion that Tfh boosting strategies should be incorporated into novel anti-HIV therapeutic and vaccine approaches with the aim of inducing durable and highly functional antibody responses. Furthermore, strategies for inducing Tfh subsets that are resistant to viral infection in lymph nodes and approaches to eradicate infected cells in the GCs will be important to achieve HIV-1 cure.

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APPENDICES 1 - 4

Appendix 1: Ethics approval for the study

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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

12 December 2016

Mrs Omolara Baiyegunhi (211560569)
HIV Pathogenesis Programme
School of Laboratory Medicine and Medical Sciences
aral200@yahoo.com

Dear Mrs Baiyegunhi

Protocol: T follicular helper cell dynamics in HIV-1 subtype C infection and relevance for T helper cell-independent antibody responses.
Degree: PhD
***BREC reference* number:** BE069/16

Your letter dated 04 November 2016 submitting an Amendment Application to change Aim 2, Hypothesis 2, the inclusion and exclusion criteria and the study timeline in relation to the above study has been **noted and provisionally approved** by a sub-committee of the Biomedical Research Ethics Committee subject to response to the following queries:

1. Amendment two: Please provide reasons for this request.
2. Please provide the BREC reference for the HPP lymph node study from which samples will be accessed. Is this change an indication of sample availability?

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc supervisor: ndhlovuz@ukzn.ac.za
cc postgrad: dudhrajhp@ukzn.ac.za

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19 January 2017

Mrs Omolara Baiyegunhi (211560569)
HIV Pathogenesis Programme
School of Laboratory Medicine and Medical Sciences
aral200@yahoo.com

Dear Mrs Baiyegunhi

Protocol: T follicular helper cell dynamics in HIV-1 subtype C infection and relevance for T helper cell-independent antibody responses.
Degree: PhD
***BREC reference* number:** BE069/16

Your response dated 15 December 2016 to BREC letter dated 12 December 2016 has been noted by a sub-committee of the Biomedical Research Ethics Committee.

Your letter dated 04 November 2016 submitting an Amendment Application to change Aim 2, Hypothesis 2, the inclusion and exclusion criteria and the study timeline in relation to the above study has now been **approved** by a sub-committee of the Biomedical Research Ethics Committee.

This approval will be **RATIFIED** by a full Committee at its meeting taking place on **14 February 2017.**

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc supervisor: ndhlovuz@ukzn.ac.za
cc postgrad: dudhrajhp@ukzn.ac.za

Appendix 2: Detailed patients' characteristics

Study group	Patient ID	Sex	Age	Time to ART initiation (days)	ART Regimen	Fiebig stage	Absolute CD4 counts (cells/µl)	Plasma Viral loads (copies/ml)	Treatment duration (days)	Days post suppression to LN excision (days)	Time to viremia suppression (days)
HIV negative	LN002	F	66								
	LN020	F	22								
	LN022	F	21								
	LN028	F	19								
	LN031	F	22								
	LN032	F	20								
	LN034	F	21								
	LN035	F	21								
Early treated	LN045	F	18	1	FDC+RAL	I	1291	<20	21	12	10
	LN049	F	22	1	FDC+RAL	I	1104	<20	355	338	17
	LN052	F	19	3	FDC	I	652	<20	502	470	32
	LN053	F	19	1	FDC+RAL	V	885	<20	427	405	22
	LN054	F	22	1	FDC+RAL	I	940	1300	11	N/A	13
	LN057	F	21	1	FDC+RAL	I	724	<20	403	390	13
	LN058	F	22	1	FDC+RAL	I	663	<20	336	258	78
	LN063	F	24	1	FDC+RAL	I	898	<20	385	379	6
	LN064	F	21	2	FDC+RAL	I	782	<20	479	446	33
	LN065	F	19	1	FDC+RAL	I	628	<20	19	2	17
	LN070	F	20	1	FDC+RAL	I	1065	<20	47	22	24
	LN077	F	18	1	FDC+RAL	III	507	<20	29	6	23
	LN078	F	25	1	FDC	I	1042	<20	694	681	13
	LN087	F	20	1		I	966	<20	63	57	6
	LN088	F	22	1	FDC+RAL	III	813	<20	120	104	16
	LN089	F	25	3	FDC	V	1033	<20	926	919	7
	LN095*	F	23	1	FDC+RAL	I	1290	<20	56	47	9
	LN0101*	F	22	1	FDC+RAL	III	1139	<20	63	46	17
Chronic untreated	LN018	F	57					3900			
	LN037	F	24					12000			
	LN040	F	24					120			
	LN041	F	24					6000			
	LN042	M	37				359	21000			
	LN044	F	18					290			
	LN047	F	21					23000			
	LN062	F	24				355	22000			

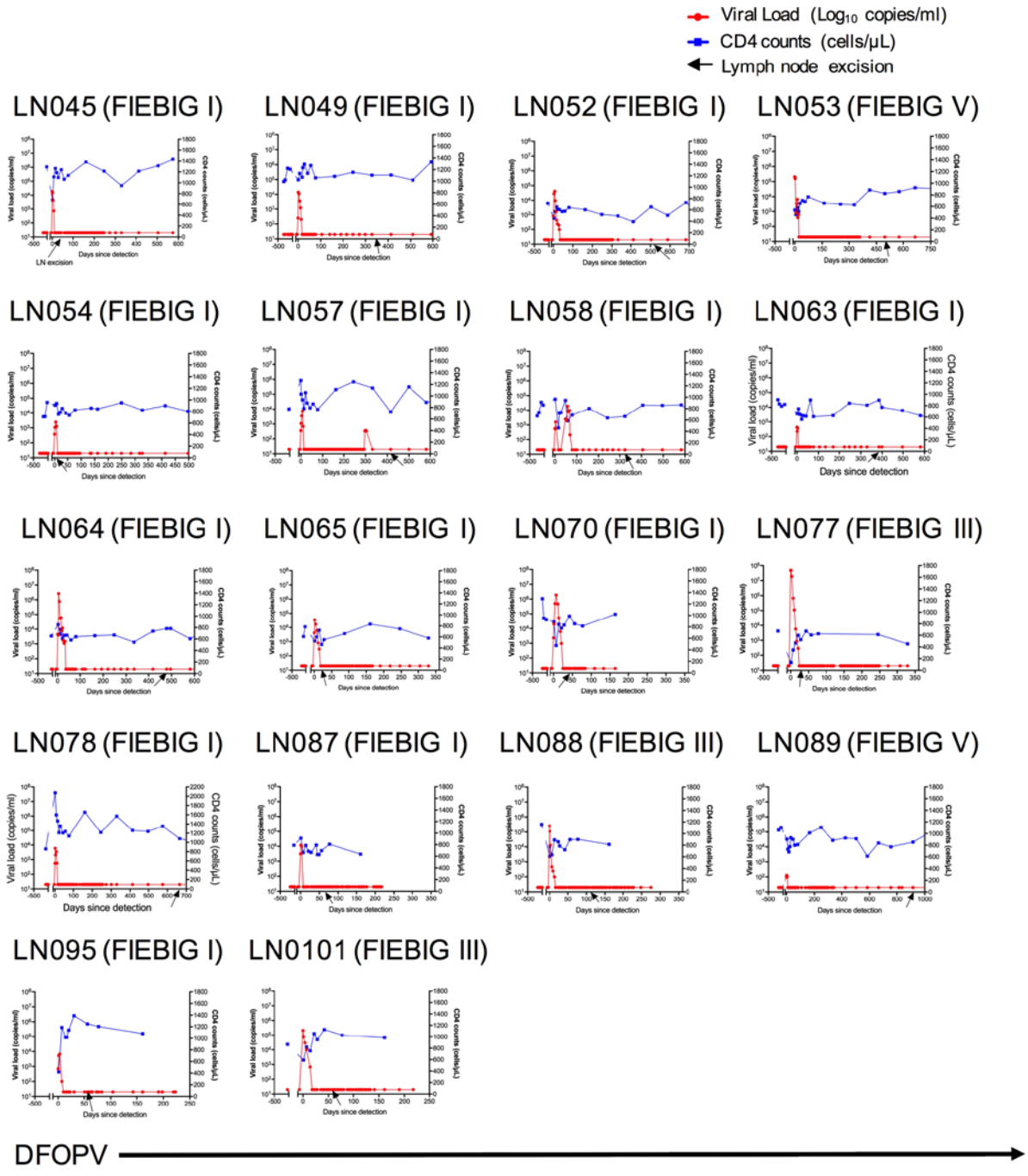
*Denotes patients that were studied only in chapter 4 but not in chapter 3.

Appendix 3: Study grouping and distribution of participants in Chapter 3

	BCL-6 characterization	GCTfh cell phenotyping	Non GCTfh cell phenotyping	cTfh cell phenotyping	B cell phenotyping	Tetramer staining	IF microscopy	IgG ELISA
HIV negative	5	8	8	6	4	N/A	3	N/A
Early treated HIV	11	16	16	9	9	4	10	15
Untreated HIV	6	8	8	8	4	2	5	N/A
	22	32	32	23	17	6	18	15

*Group sizes are indicated in each cell. Abbreviations: N/A, not applicable

Appendix 4: Kinetics of HIV-1 viral load decay and absolute CD4 counts during early combination antiretroviral therapy

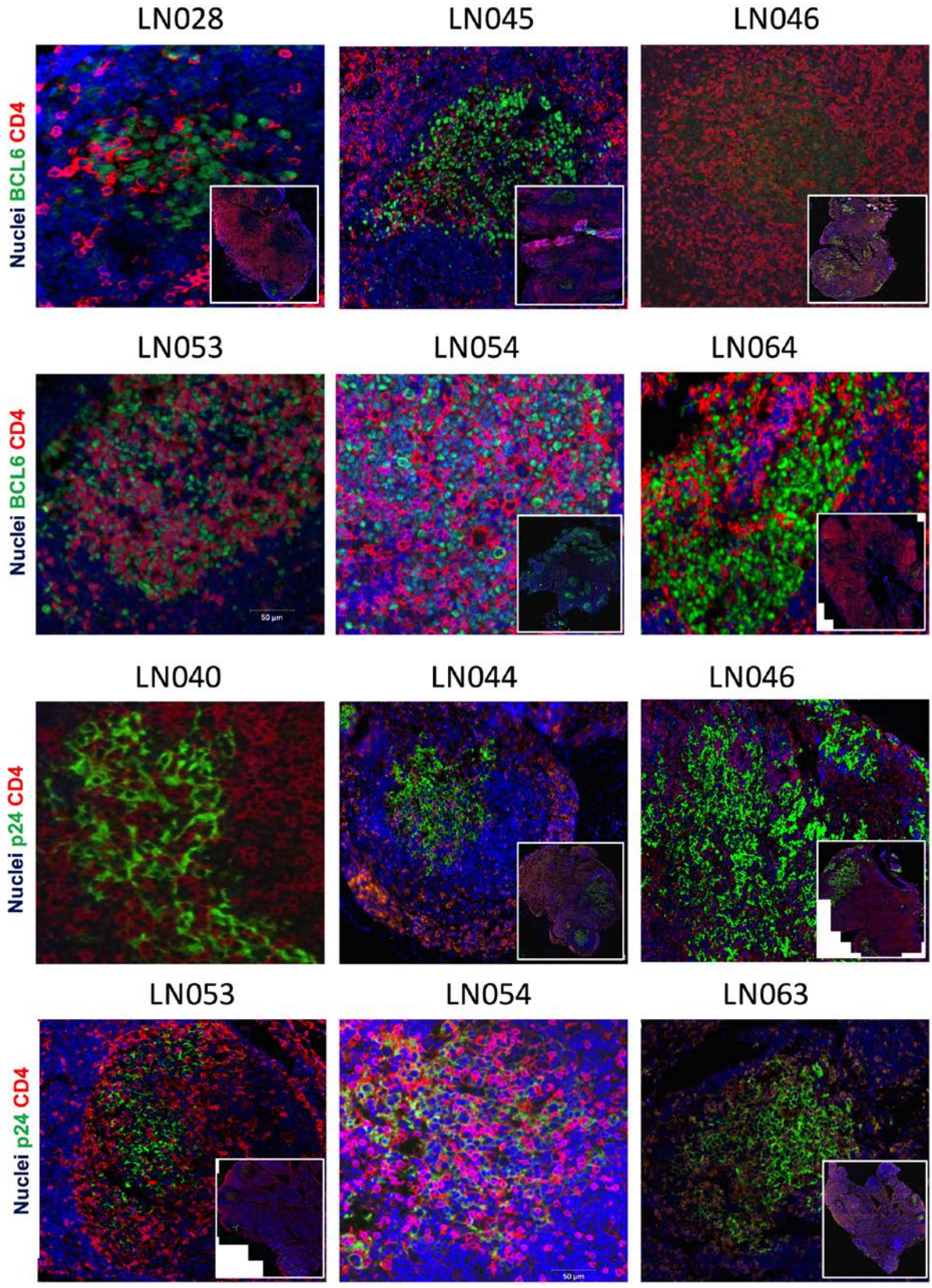


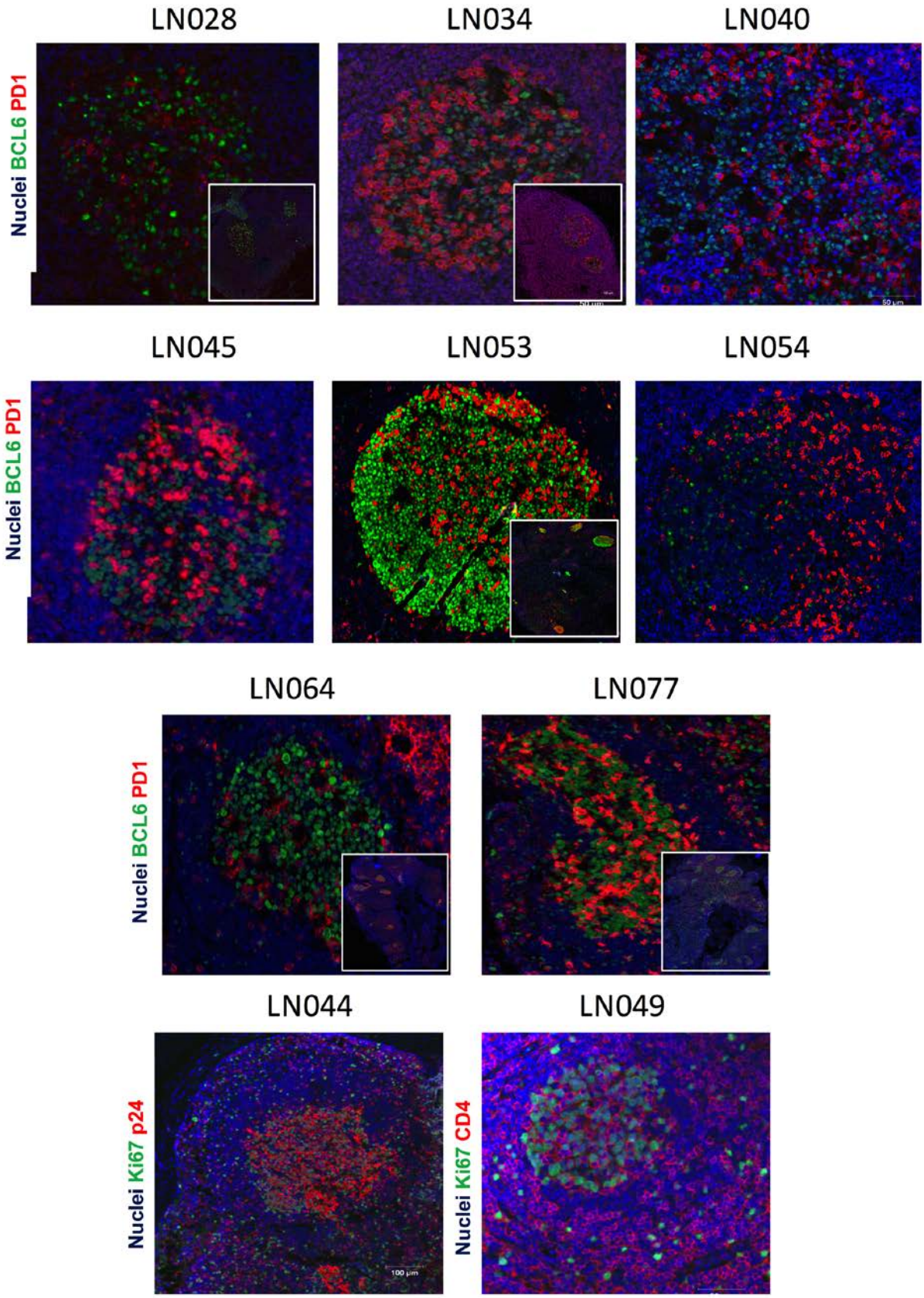
DFOPV means day of first positive viral load test.

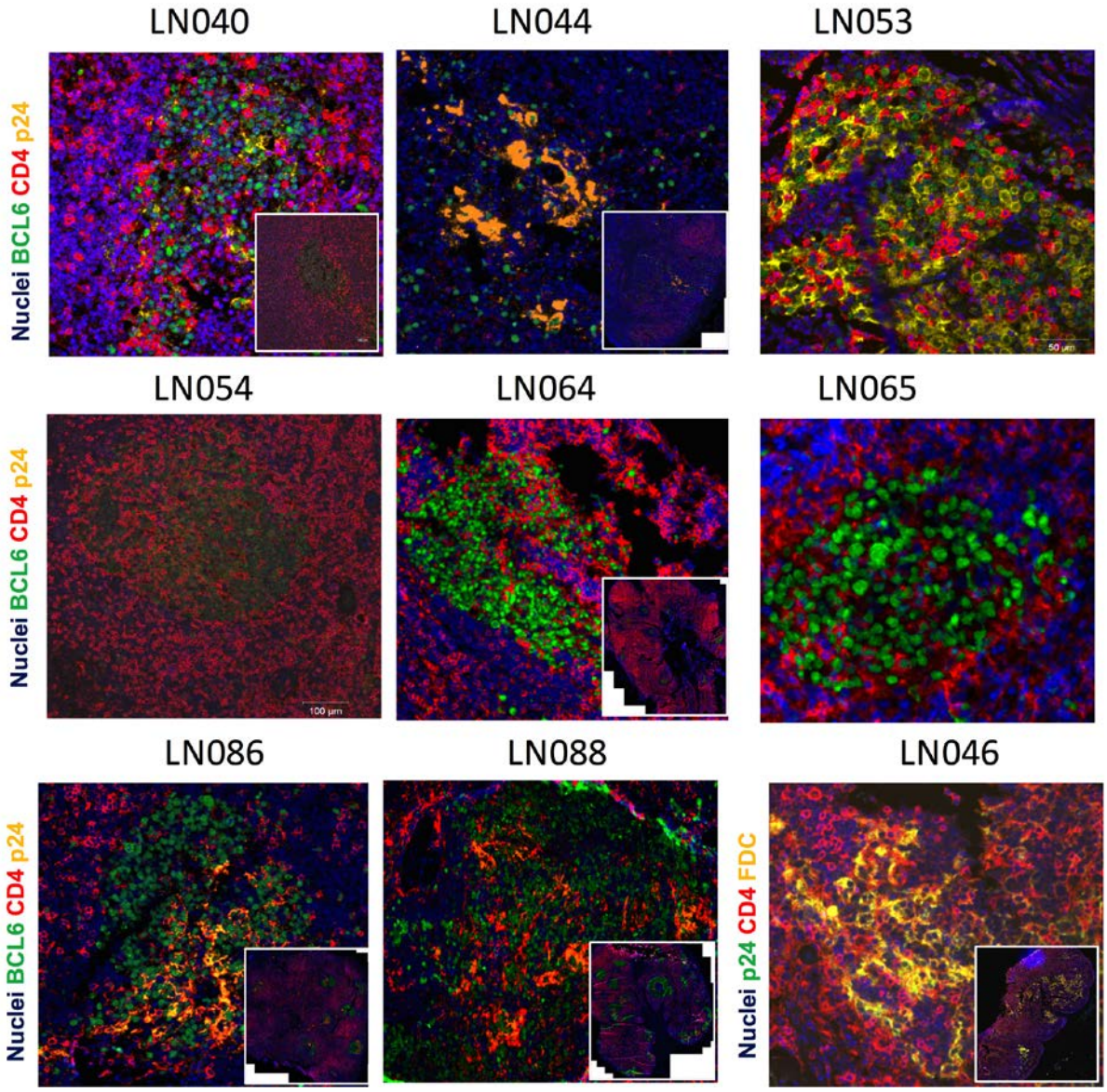
Appendix 5: Area under the viral load curve (AUC) values for participants in early treated group.

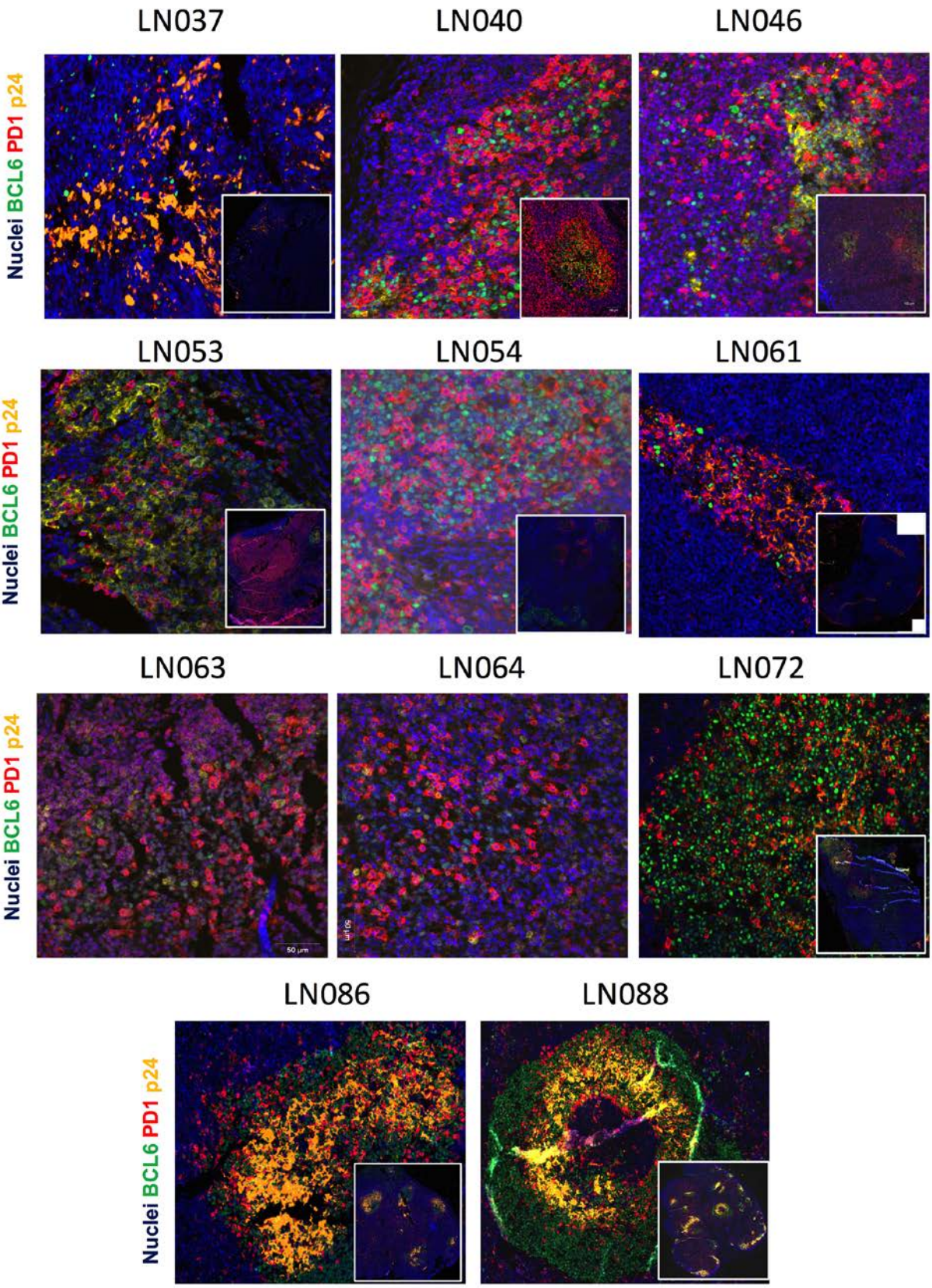
Patient ID	AUC (RNA copies*days/ml)
LN045	54170
LN049	78305
LN052	283947
LN053	744655
LN054	14260
LN057	36955
LN058	219935
LN063	1575
LN064	9696000
LN065	148085
LN070	10040000
LN077	255763265
LN078	29435
LN087	63275
LN088	865185
LN089	639
LN095	33340
LN0101	1140750

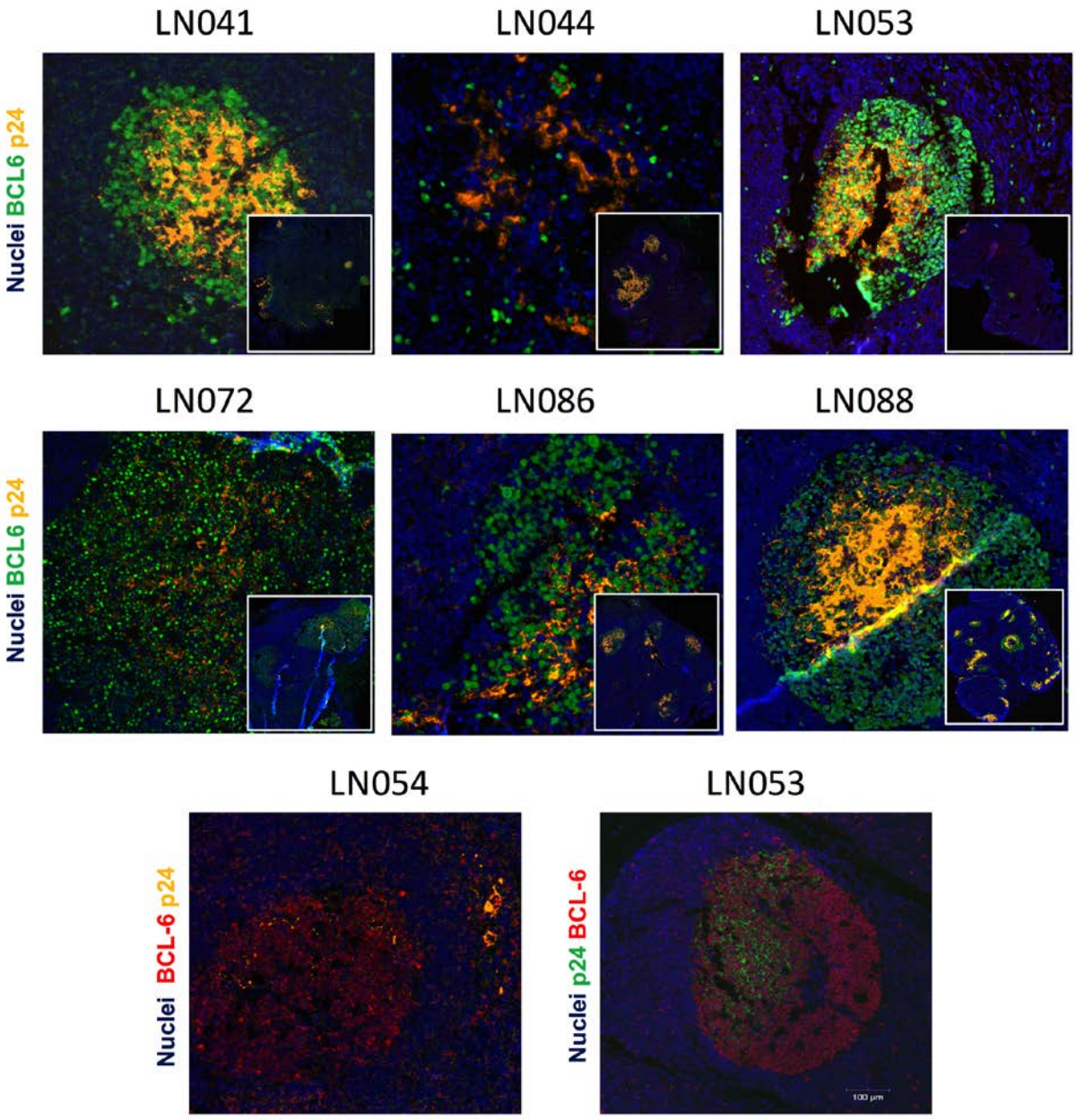
Appendix 6: Tfh phenotyping and localization with respect to HIV antigens using immunofluorescence microscopy imaging.

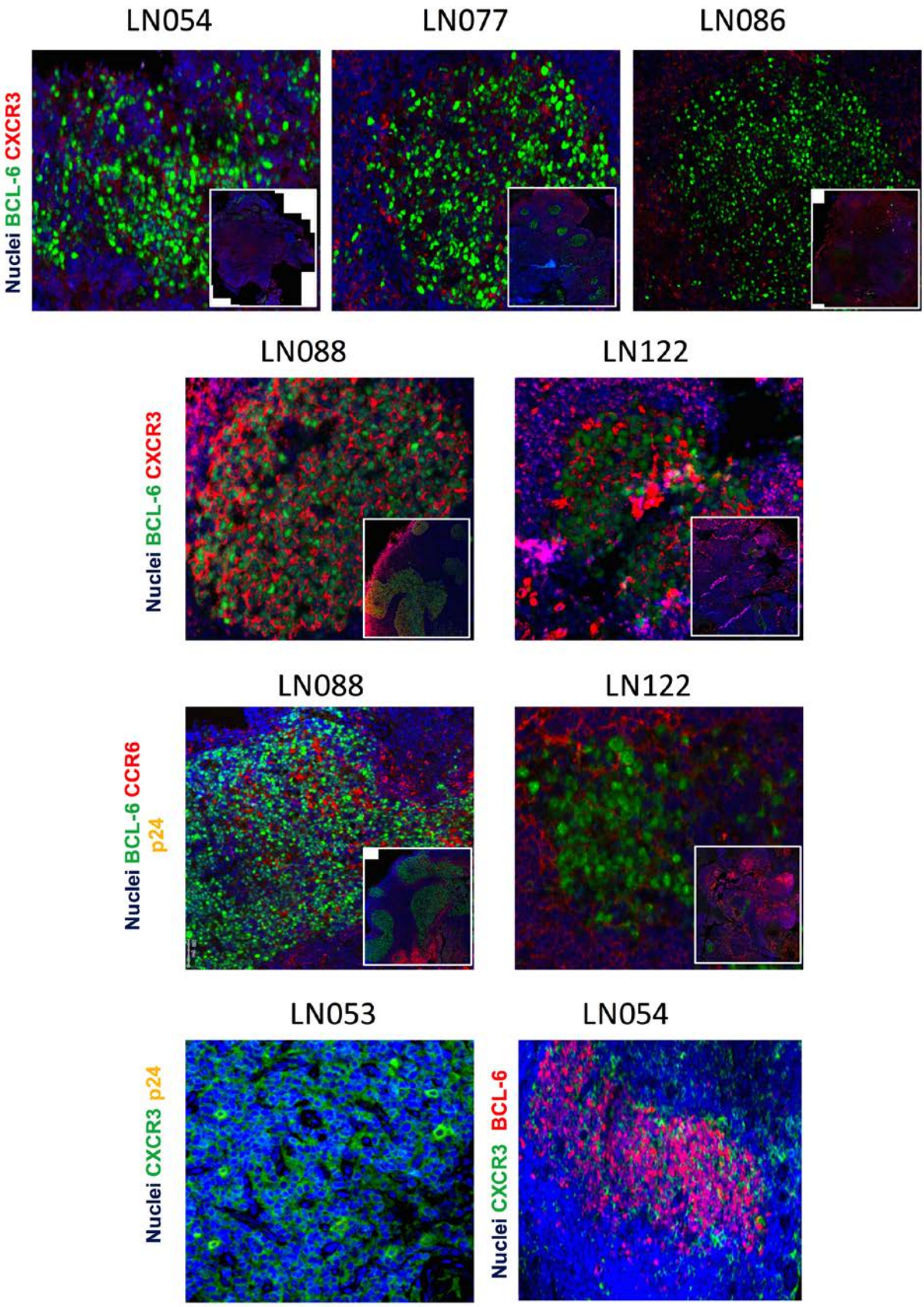


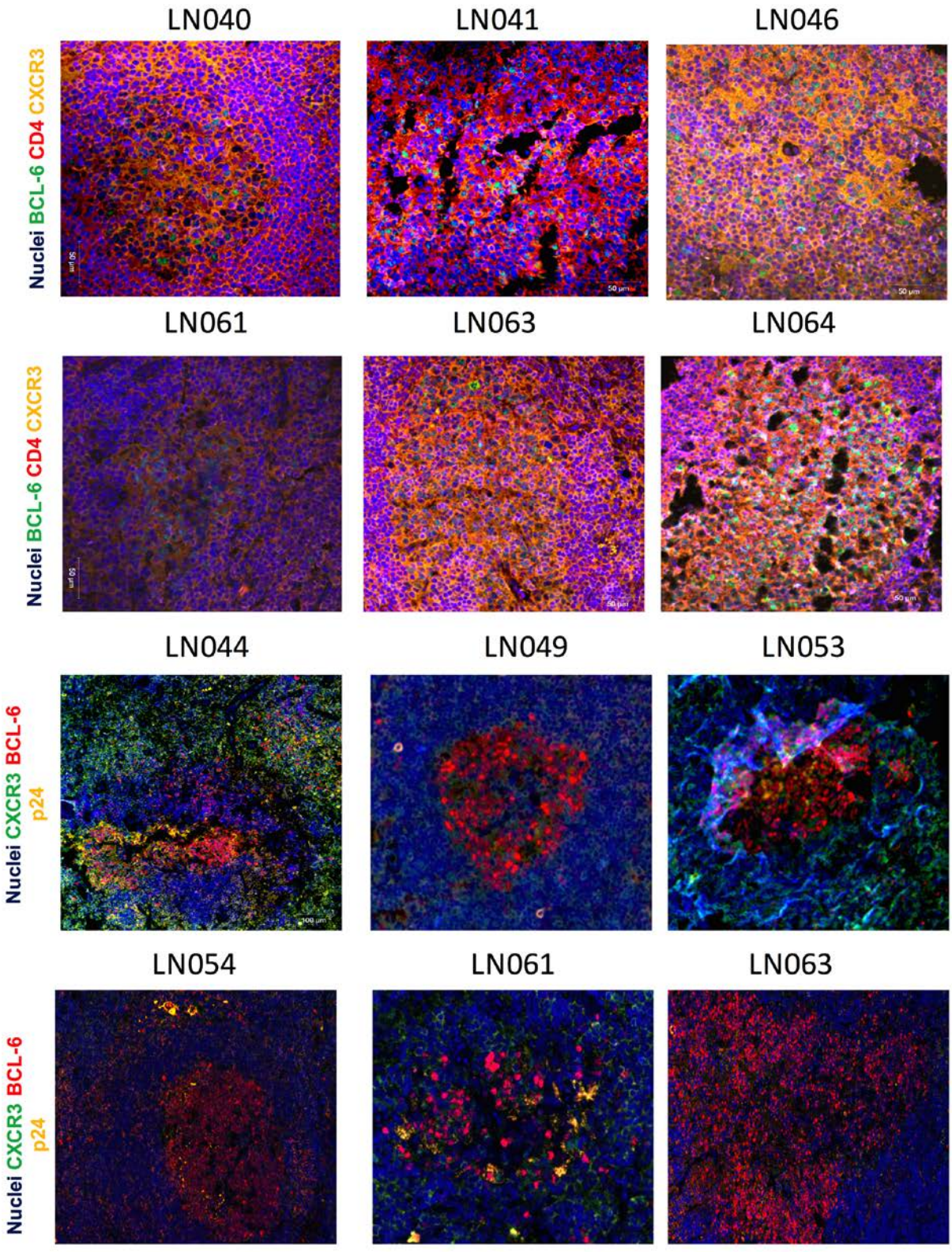

LN028
LN034
LN040
Nuclei BCL6 PD1
LN045
LN053
LN054
Nuclei BCL6 PD1
LN064
LN077
Nuclei BCL6 PD1
LN044
LN049
Nuclei Ki67 p24
Nuclei Ki67 CD4

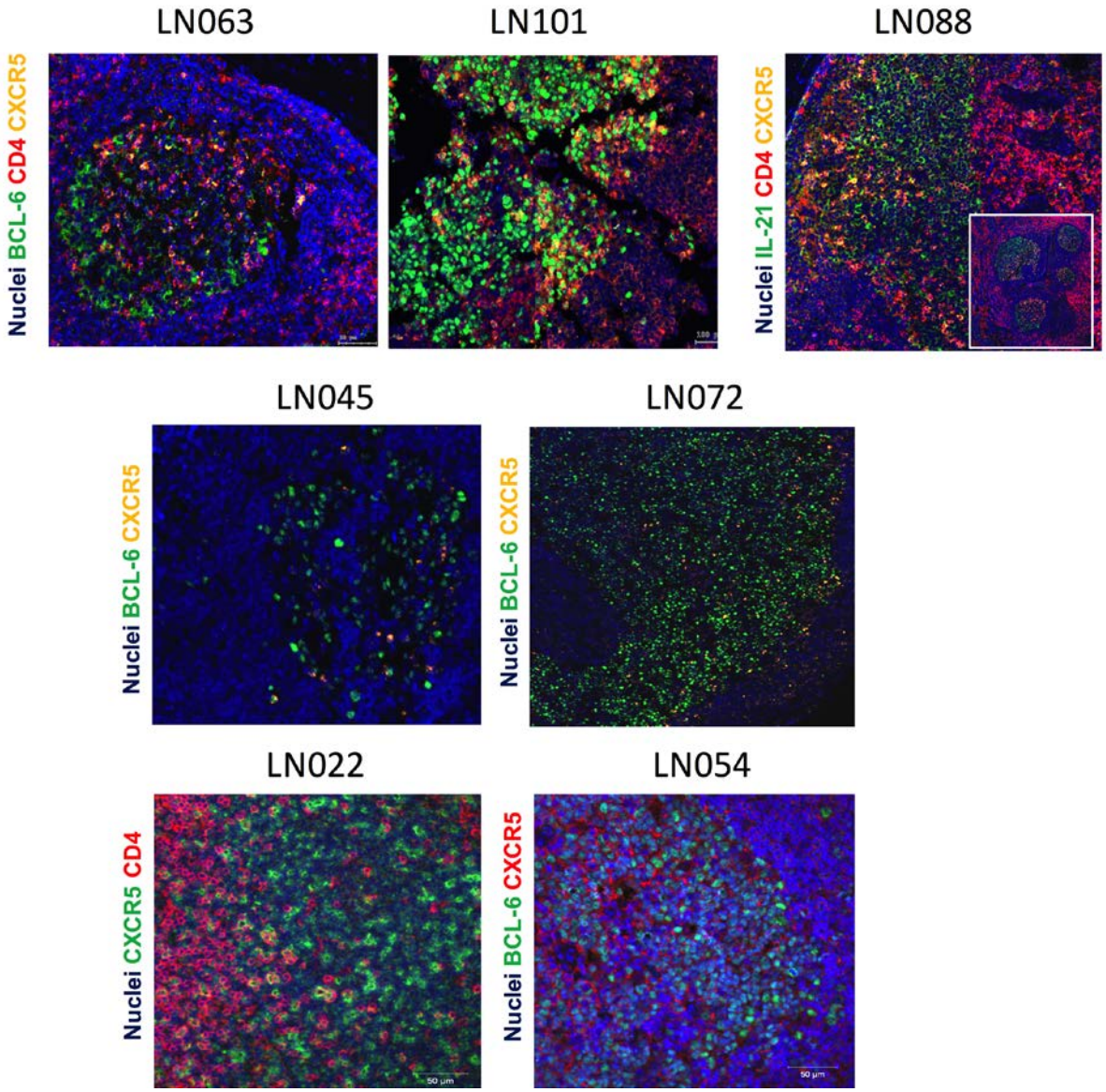

LN040
LN044
LN053
Nuclei BCL6 CD4 p24
LN054
LN064
LN065
Nuclei BCL6 CD4 p24
100 µm
LN086
LN088
LN046
Nuclei BCL6 CD4 p24
Nuclei p24 CD4 FDC


LN037
LN040
LN046
LN053
LN054
LN061
LN063
LN064
LN072
LN086
LN088
Nuclei BCL6 PD1 p24


LN041
LN044
LN053
Nuclei BCL6 p24
LN072
LN086
LN088
Nuclei BCL6 p24
LN054
LN053
Nuclei BCL-6 p24
Nuclei p24 BCL-6


LN054
LN077
LN086
Nuclei BCL-6 CXCR3
LN088
LN122
Nuclei BCL-6 CXCR3
LN088
LN122
Nuclei BCL-6 CCR6
p24
LN053
LN054
Nuclei CXCR3 p24
Nuclei CXCR3 BCL-6


LN040
LN041
LN046
Nuclei BCL-6 CD4 CXCR3
LN061
LN063
LN064
Nuclei BCL-6 CD4 CXCR3
LN044
LN049
LN053
Nuclei CXCR3 BCL-6 p24
LN054
LN061
LN063
Nuclei CXCR3 BCL-6 p24



Representative IF images characterizing T follicular helper cells in lymph node germinal centers. The staining combinations for the images on each row are displayed on the left.