Characterizing the role of CD4+ T cell immunoregulatory networks in peripheral blood and lymphoid tissue during HIV-1 clade C infection

By Faatima Laher

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy in Immunology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

Nelson R Mandela School of Medicine

University of KwaZulu-Natal

Durban, South Africa

Supervisor: Dr Zaza M. Ndhlovu

Co-supervisor: Prof Thumbi Ndung'u

2018

PREFACE

The experimental work described in this dissertation was conducted at the HIV Pathogenesis Programme, School of Laboratory Medicine and Medical Sciences, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, from March 2015 to November 2018, under the supervision of Dr Zaza M. Ndhlovu and Prof Thumbi Ndung'u.

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

Faatima Laher (candidate): _____ **Date ……..**

Dr Zaza M Ndhlovu (supervisor): _____ **Date ……..**

As the candidate's supervisor, I agree to the submission of this thesis:

DECLARATION 1: PLAGIARISM

I, **Faatima Laher** declare that

(i) The research reported in this dissertation/thesis, except where otherwise indicated, is my original research.

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DECLARATION 2: PUBLICATION AND MANUSCRIPTS

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

Publication 1:

The following publication has been included in this thesis (Chapter 2):

Laher F, Ranasinghe S, Porichis F, Mewalal N, Pretorius K, Ismail N, Buus S, Stryhn A, Carrington M, Walker BD, Ndung'u T, Ndhlovu ZM, 2017. HIV Controllers Exhibit Enhanced Frequencies of Major Histocompatibility Complex Class II Tetramer+ Gag-Specific CD4+ T Cells in Chronic Clade C HIV-1 Infection. Journal of Virology, 13;91(7). DOI: 10.1128/JVI.02477-16.

Authors' contributions: Prof. Thumbi Ndung'u and Dr. Bruce D. Walker initiated the study cohort. Dr. Zaza Ndhlovu conceived the idea. Dr. Zaza Ndhlovu, Dr. Srinika Ranasinghe and I designed the experiments. Dr Søren Buus and Dr Annette Stryhn assisted with synthesis of MHC class II tetramers. Dr Srinika Ranasinghe provided L cell lines for the HLA restriction assay. I performed all the laboratory experiments. I analyzed the data and wrote the manuscript under Dr. Ndhlovu and Dr Ranasinghe's supervision. All the authors approved of the manuscript before it was submitted for publication.

Manuscript 1:

Faatima Laher, Samuel W. Kazer, Toby Aicher, Omolara Baiyegunhi, Funsho Ogunshola, Thandeka Nkosi, Anele Mbatha, Trevor Khaba, Thandekile Ngubane, Ismail Jajbhay, Johan Pansegrouw, Nasreen Ismail, Krista Dong, Alex K. Shalek, Bruce D. Walker, Thumbi Ndung'u, Zaza M. Ndhlovu, 2018. Comprehensive phenotypic and functional characterization of regulatory CD4+ T cell subsets in peripheral blood and lymph node tissues in HIV-1 clade C infection.

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 and Dr. Zaza Ndhlovu and I designed the experiments. Samuel Kazer and Toby Aicher assisted with the Seq-Well assays and analysis. I performed the rest of the laboratory experiments, data analysis and wrote the manuscript under the supervision of Dr. Zaza Ndhlovu.

Manuscript 2:

Faatima Laher, Omolara Baiyegunhi, Funsho Ogunshola, Daniel Muema, Trevor Khaba, Veron Ramsuran, Thandeka Nkosi, Anele Mbatha, Thandekile Ngubane, Ismail Jajbhay, Johan Pansegrouw, Nasreen Ismail, Krista Dong, Bruce D. Walker, Thumbi Ndung'u, Zaza M. Ndhlovu, 2018. Localization and function of T follicular regulatory cells in lymph nodes of very early ART initiators in HIV-1 clade C infection.

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

and Dr. Zaza Ndhlovu and I designed the experiments. Funsho Ogunshola assisted with FACS sorting for digital droplet PCR and Dr. Veron Ramsuran assisted with running a part of the assay. Dr. Omolara Baiyegunhi and Dr. Daniel Muema performed ELISA assays. I performed the rest of the laboratory experiments, data analysis and wrote the manuscript under the supervision of Dr. Zaza Ndhlovu.

RESEARCH OUTPUT: PRESENTATIONS AND SCHOLARSHIP AWARDS

Oral Presentations

1. **KENBOP3: Kenya Biology of Pathogens, Pathogenesis & Parasitism Course at KEMRI/Wellcome Trust, Kilifi, Kenya, 2016.** Characterization of HIV-specific CD4 T cells using MHC class II tetramers in a clade C chronic HIV-1 infection cohort.

2. **University of KwaZulu-Natal, College of Health Sciences Research Symposium, Durban, South Africa, 2017.** Follicular Regulatory T cell (TFR) dynamics in peripheral blood and lymphoid tissue during HIV-1 clade C infection.

3. **HIV Persistence During Therapy: Reservoirs & Eradication Strategies Workshop in Miami, USA, 2017.** Follicular Regulatory T cell dynamics in peripheral blood and lymphoid tissue during very early treatment initiation in HIV-1 clade C infection.

Poster Presentations

1. **21St International AIDS Conference, Durban, South Africa, 2016.** Phenotypic and functional characterization of HIV-specific CD4 T cells using MHC class II tetramers in a clade C chronic HIV-1 infection cohort.

2. **KENBOP3: Kenya Biology of Pathogens, Pathogenesis & Parasitism Course at KEMRI/Wellcome Trust, Kilifi, Kenya, 2016.** Characterization of HIV-specific CD4 T cells using MHC class II tetramers in a clade C chronic HIV-1 infection cohort.

3. **HIV Research for Prevention 2016: AIDS Vaccine, Microbicide and ARV-based Prevention Science (HIVR4P), Chicago, USA, 2016.** HIV-specific CD4+

T Cell Responses Targeting the Immunodominant Gag41 Peptide are Associated with Reduced Viral Load in HIV-1 Clade C Infection.

Scholarship Awards

1. NRF Innovation Doctoral Scholarship (2015-2017).
2. HIV Pathogenesis Programme Doctoral Fellowship (2015-2018).
3. Harvard Medical School Translational Medicine Course full scholarship (June 2015).
4. Ragon Institute of MGH, MIT and Harvard international travel award (August 2015).
5. 21st International AIDS conference registration scholarship (July 2016).
6. KENBOP3 full scholarship (September 2016).
7. HIVR4P full scholarship (October 2016).
8. HIV Persistence During Therapy early-stage investigator scholarship (December 2017).

DEDICATION

To my parents

For their motivation, support and belief in me

ACKNOWLEDGEMENTS

All praises to God Almighty for making all things possible and for never letting me down.

I would like to thank the following people, without whom this journey would not have been possible…

I am eternally grateful to my family – Dad and Mum; who have undoubtedly been my source of strength, my sisters Naadhirah and Tasneem, my niece Yusayrah, my nephew Yahyaa and my brother-in-law Zakariyyaa – for their continued love, support, patience, encouragement, motivation and most importantly, belief in me.

My supervisor Dr Zaza Ndhlovu, for allowing me to work under his mentorship, and for all the opportunities that were provided during my studies. I would also like to thank Prof Thumbi Ndung'u, my co-supervisor for giving me the opportunity to work at HPP, as well as for his support.

All the wonderful HPP team members; lab and administration staff, for providing technical assistance, encouragement, advice, support and friendship. Furthermore, I am grateful to all the study participants for donating their precious samples, the doctors for volunteering their time and expertise and the nurses at our study sites. I am grateful to all the individuals that have been supportive during my studies and have assisted in every which way, including the Ndhlovu research group; Thandeka, Anele, Trevor, Lara and Funsho, with very special thanks to Karyn and Nikoshia for their continuous encouragement, generous advice and friendship. I would also like to thank Dr. Srinika

Ranasinghe, Dr. Kamini Gounder and Dr. Kavidha Reddy for their friendship and constant support.

I am grateful to the National Research Foundation (NRF) for providing financial support during my studies.

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ABBREVIATIONS

APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
BCIP	5-Bromo-4-Chloro-3'Indolyphophate p- Toluidine Salt
BCL-6	B cell lymphoma 6
bNAbs	Broadly neutralizing antibodies
BSA	Bovine Serum Albumin
CCR5	Chemokine Receptor 5
CD4+ T cells	Human T cells expressing CD4+ antigen
CD8+ T cells	Human T cells expressing CD8+ antigen
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CXCR4	CXC Chemokine Receptor 4
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
Early Tx	Early treated
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunosorbent spot assay
Env	Envelope
FACS	Fluorescent Activated Cell Sorting
FCS	Fetal Calf Serum
FFPE	Formalin fixed paraffin embedded

FRESH	Females rising through education, support and health
Gag	Group-specific antigen
GC	Germinal centre
GC TFH	Germinal centre T follicular helper
GC TFR	Germinal centre T follicular regulatory
GIT	Gastrointestinal Tract
gp	Glycoprotein
HAART	Highly Active Antiretroviral Treatment
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICOS	Inducible T cell co-stimulator
ICS	Intracellular Cytokine Staining
IF	Immunofluorescence
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
LMC	Lymph node mononuclear cells
LN	Lymph nodes
MAb	Monoclonal Antibody
MFI	Median fluorescence intensity
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
NBT	Nitro-Blue Tetrazolium Chloride

Nef	Negative regulatory factor
OLP	Overlapping Peptide
ORF	Open Reading Frame
P24	P24 Capsid Protein
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PHA	Phytohemagglutinin
Pol	Polymerase
Rev	Regulator of virion expression protein
RNA	Ribonucleic Acid
SEB	Staphylococcal enterotoxin B
SFC	Spot Forming Cells
SIV	Simian Immunodeficiency Virus
Tat	Transactivator of transcription
TCR	T-cell Receptor
TFH	T follicular helper cells
TFR	T follicular regulatory cells
Un Tx	Untreated
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

ABSTRACT

HIV eradication efforts have been unsuccessful due to virus persistence in cellular and tissue reservoirs. Recent evidence suggests that germinal centers (GCs) within lymph nodes (LN) contain a novel subset of regulatory T cells (TREGs), termed follicular regulatory T (TFR) cells. These cells control the magnitude and specificity of the GC response and like TREGs are essential for the maintenance of self-tolerance and immune homeostasis. However, the exact role of TFR cells in HIV infection and their contribution to viral control is not completely understood, possibly due to their low frequency, heterogeneity and more so, the difficulty in accessing human lymphoid tissue samples to fully study them.

Thus, we set out to comprehensively investigate TFR cells in LN and peripheral blood (PB) samples, using a multifaceted approach including flow cytometry, MHC class II tetramers, immunofluorescence microscopy (IF), ELISA, digital droplet PCR and single-cell RNA sequencing (SeqWell), in HIV-1 clade C infection. Furthermore, we aimed to determine the effect of very early treatment on the frequency and function of this cell subset.

Overall, our studies contributed various notable findings to the field. Firstly, we were able to develop MHC class II tetramers, specific in our HIV-1 clade C setting, as a more sensitive method of identifying very low cell frequency antigen-specific CD4+ T cells without relying on function. Tetramers eliminate the bias associated with *in vitro* stimulation required for functional assays and the limitation associated with only detecting subsets of cells capable of secreting a cytokine. Notably, we used class II

tetramers to demonstrate that HIV-specific CD4+ T cell responses restricted to DRB1*11-Gag41 are associated with immune control of HIV-1 infection.

We next focused on understanding the role of CD4+ regulatory cells during HIV-1 infection. Firstly, we showed that TFR cell frequencies were significantly higher in LN compared to PB samples. Secondly, TFR are a phenotypically and transcriptionally distinct subset compared to regulatory T cells (TREGs) and T Follicular Helper cells (TFH). Thirdly, we were able to detect HIV-specific TFR using our newly synthesized MHC class II tetramers, and showed higher frequencies observed in LNs during untreated HIV infection. Fourthly, as measured by both flow cytometry and IF, most of TFR localized outside of the GC, with very early ART initiators displaying larger proportions of TFR within the GC. Lastly, TFR cells exhibited a potential suppressive functional capacity as they produced IL-10, which is a canonical suppressive cytokine and they were also positively associated with gp41 IgG antibodies titers.

Overall, the data presented in this thesis highlights the advantage of MHC class II tetramers in evaluating HIV-specific CD4 + T cell responses in natural infections. More so, the results give important insights into regulatory cells within lymph nodes; their biology, function and their role in the setting of very ART initiation.

CHAPTER 1: INTRODUCTION

1.1 The HIV/AIDS epidemic

The first clinical observation of Acquired Immune Deficiency Syndrome (AIDS) was made in 1981 in the United States of America amongst injecting drug users and homosexual men with no known cause of weakened immunity. These individuals presented with symptoms of *Pneumocystis* pneumonia and Kaposi's sarcoma, with both diseases having been previously reported as rare infections, occurring only in people with severely compromised immune systems (1-4). A few years later, the Human Immunodeficiency Virus (HIV), discovered in 1983, was isolated and identified as the pathogen responsible for AIDS. HIV infection quickly became established as a global pandemic (5). The disease is caused by two lentiviruses, human immune deficiency virus type 1 (HIV-1) and HIV-2 (6) and is closely related to Human T Lymphotropic Virus (HTLV) (7). AIDS is characterized by a marked reduction of CD4+ T cells and ultimately a failure of the immune system to contain virus replication, leading to increased susceptibility to opportunistic infections and malignancies and finally death (8).

The origin of human infections traces back to multiple cross-species transmissions of simian immunodeficiency virus (SIV) from primates to humans (6). In humans it is primarily transmitted through sexual contact (most commonly heterosexual), exposure to HIV-contaminated needles, surgical items and bodily fluids or through vertical transmission from mother to child (9, 10). Since the clinical recognition of the disease,

HIV/AIDS has rapidly emerged as one of the world's deadliest infectious diseases. An estimated total of 36.9 million people were reported to be living with HIV-infection globally at the end of 2017, with 66% living in sub-Saharan Africa (Figure 1.1) (11). In that same year, an estimated 1.8 million people became newly infected with HIV, and 940 000 died of AIDS, including 110 000 children.

Although there is still no cure for HIV-1 infection, the introduction and implementation of antiretroviral therapy (ART) from the mid-1990s onwards has proven to be successful in prolonging the lives of those infected, preventing new transmissions and reducing mortality and morbidity rates (12). This is evident in the reduction in AIDS-related deaths by 51% since the peak in 2004. Furthermore, the number of new infections has declined by 47% from 3.4 million in 1996 to 1.8 million in 2017. In addition, as at 2017, 59% (21.7 million) people living with HIV were accessing ART (11). However, despite these advances made in combating HIV, the pandemic is still a global health concern. The increasing evidence of resistance to currently available treatment regimens, together with limited accessibility of ART in resource-limited countries and adherence are ongoing issues making it difficult to bring the epidemic under control. Additionally, HIV has a high mutation rate which contributes to the development of resistance to treatment (13). Therefore, it is widely believed that a successful HIV vaccine would be the best weapon to control the HIV pandemic.

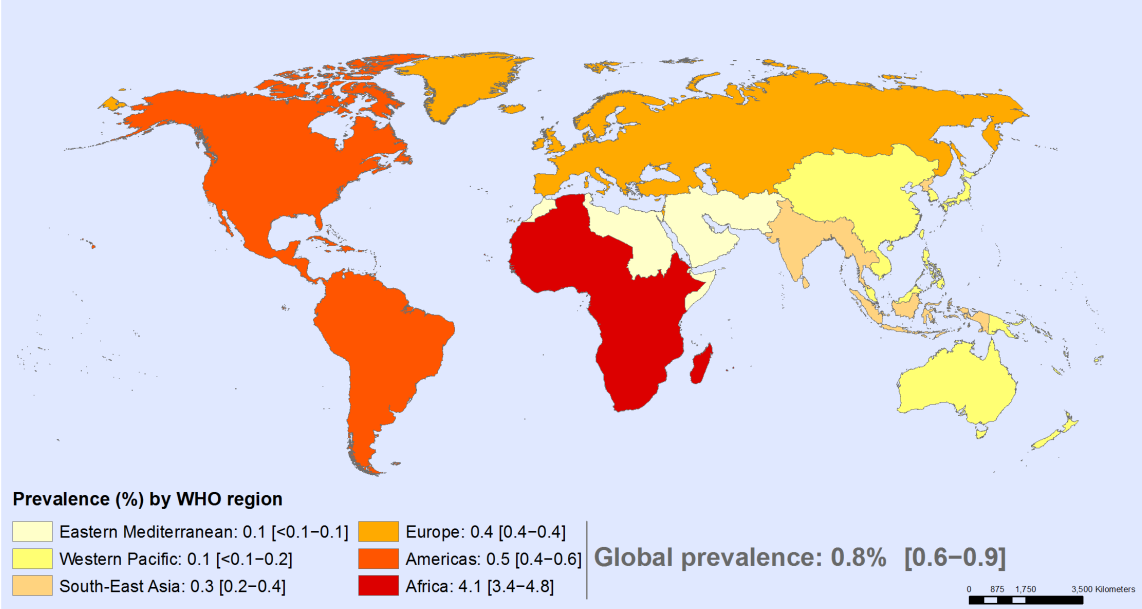

Figure 1.1 Global prevalence of HIV among adults aged 15-49 in 2017. Source: WHO (14).

1.2 Clinical course of HIV-1 infection

The natural history of HIV is well documented. It can be categorised as three distinct phases i.e. acute (primary) infection, asymptomatic phase and clinical AIDS-defining illness. The acute phase usually lasts a few months, followed by the early/clinically dormant phase that characteristically lasts for 3-10 years, and finally the characteristic immune collapse of AIDS (Figure 1.2) (15). Following HIV-1 infection, in the acute phase, HIV is detectable in the blood serum and plasma, but antibodies which is regularly used to diagnose infection have yet to form (16). HIV replication during the acute phase, usually results in high levels of viremia and shedding of the virus at mucosal sites. The onset of early virus-specific immune responses is coincident with a decrease in viremia (17, 18). Evidence in SIV-infected rhesus macaques indicate that resting memory CD4+ T cells expressing CCR5 are suggested to be the first cells infected. The virus

disseminates quickly into the lymphatic system and becomes detectable in local lymph nodes one week after infection (19). The virus spreads to various other anatomical sites after a week, with the gut-associated lymphoid tissue (GALT) being the most notable, with large numbers of CD4+ CCR5+ memory T cells residing within (20).

The initial two weeks of infection is often referred to as the window period, with asymptomatic infection and undetectable plasma virus by standard methods of testing (21, 22). Since anti-HIV antibodies are usually undetectable during this window period, more sensitive qualitative and quantitative diagnostic assays, such as qualitative or quantitative HIV viral RNA detection or amplification methods are used for diagnosis and very early HIV-1 detection (23). Fourth generation serological tests developed in recent years that detect either antigens or antibodies have considerably reduced the window period down to five days after infection (24).

Seroconversion follows the asymptomatic phase of virus replication and is denoted by the appearance of HIV-1 specific antibodies in plasma (25). The clinical stages of acute HIV-1 infection is defined by the step-by-step detection of antibodies and viral markers in the blood by different clinical assays. One of the most widely used system of classification is called Fiebig staging, which comprise of six sequential steps of defining acute HIV infection, based on detection of Gag p24 antigen and anti-HIV antibodies (Figure 1.3) (22).

A clinical latency phase, also known as the chronic stage, follows the acute period. It is at this point that viral and host factors merge to dictate a virologic set point (26). Towards

the middle or end of this period, viral load begins to increase and CD4 count begins to decrease, usually signalling the progression of HIV symptomatic disease, thus leading to the eventual and full blown AIDS (26). The course of infection is influenced by various host and virus factors and is therefore characteristically different between individuals (27). Most individuals, following primary infection, would experience an extended clinical latency period, with progression to clinically noticeable disease or an AIDS defining illness occurring within eight to ten years. Untreated infection leads to progressive damage to the immune system and susceptibility to opportunistic infections; with impaired humoral responses, T cell activation, exhaustion and depletion of CD4+ T cells, chronic inflammation and changes in lymphoid tissue architecture (28, 29). This overall dysregulation can be decreased drastically with the administration of antiretroviral (ARV) drugs, especially if initiated very early during the acute phase of the infection.

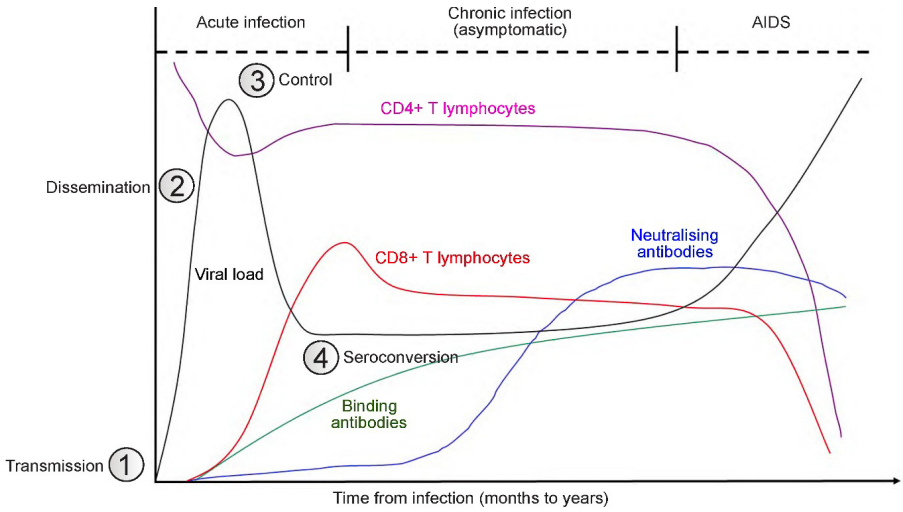

Figure 1.2 Stages of HIV infection from transmission to seroconversion and the immune events which occur at each stage (Immunopaedia.org, accessed October 2018).

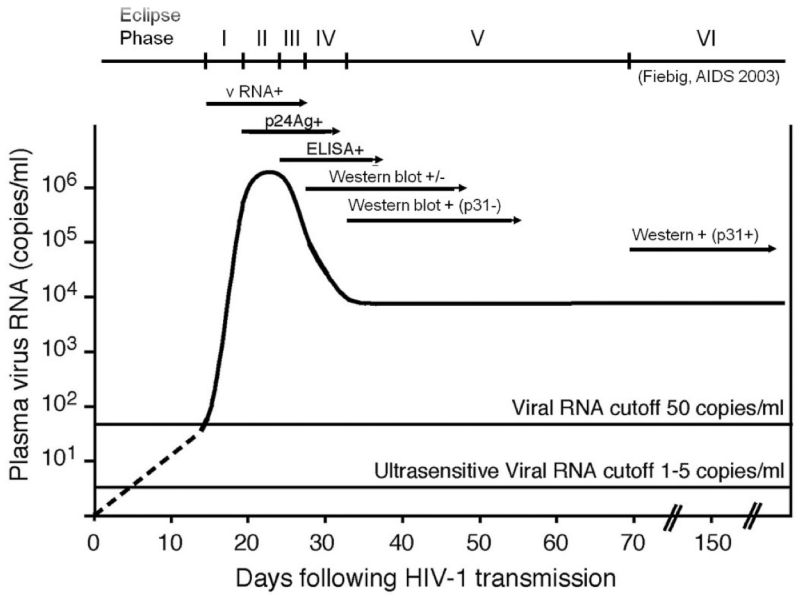

Figure 1.3 Natural history and fiebig staging of acute HIV infection. Source: Fiebig *et al*., (22).

1.3 Therapeutic strategies for HIV-1 infection

1.3.1 Antiretroviral treatment

Unprecedented efforts by various stakeholders have contributed to progressively turn HIV infection from an inevitably fatal condition into a chronic manageable disease, with the initiation of ARV drugs in the mid-1980s (30, 31). Standard antiretroviral treatment regimens have evolved from single ARVs to combinations of multiple drugs with different modes of action. The role of ART is to stop viral replication and restore immune function by inhibiting crucial HIV replication steps. Combination therapy rapidly suppresses the virus , minimizing drug resistance and limiting treatment failure (32).

The major goals of ART is to prevent the onset of AIDS and AIDS-related illnesses and to improve the quality of life. ART also prolongs the life expectancy of those infected and prevents further transmission to uninfected individuals. There are six different mechanistic classes of ARV agents currently approved by the US Food and Drug Administration (US-FDA) for the treatment of HIV infection are as follows (Figure 1.4):

a. **Nucleoside reverse transcriptase inhibitors (NRTIs)**: their mechanism of action is reverse transcription inhibition. Nucleic acid analogues mimic the normal building blocks of DNA, preventing transcription of viral RNA to DNA (33).

b. **Non-nucleoside reverse transcriptase inhibitors (NNRTIs)**: also target the reverse transcription step by binding to the RT at a site distant to the active site and therefore inducing a conformational change that alters the active site of the enzyme and limiting its activity (33).

c. **Protease inhibitors (PIs)**: function by inhibiting the final maturation stages of HIV replication, resulting in the formation of immature and non-infective viral particles (33, 34).

d. **Integrase inhibitors (IIs)**: function by inhibiting the transfer reaction of the proviral strands into host chromosomal DNA during the integration step through binding of the metallic ions in the enzyme's active site (32, 35).

e. **Fusion inhibitors (FIs)**: function by interfering with the HIV fusion step by competitively binding to the gp41 and preventing the conformational changes required for gp41 to complete the fusion process (36).

f. **Chemokine receptor antagonists (CRAs)**: also known as CCR5 antagonists, bind to the CCR5 co-receptor hence blocking the gp120-CCR5 interaction (37, 38).

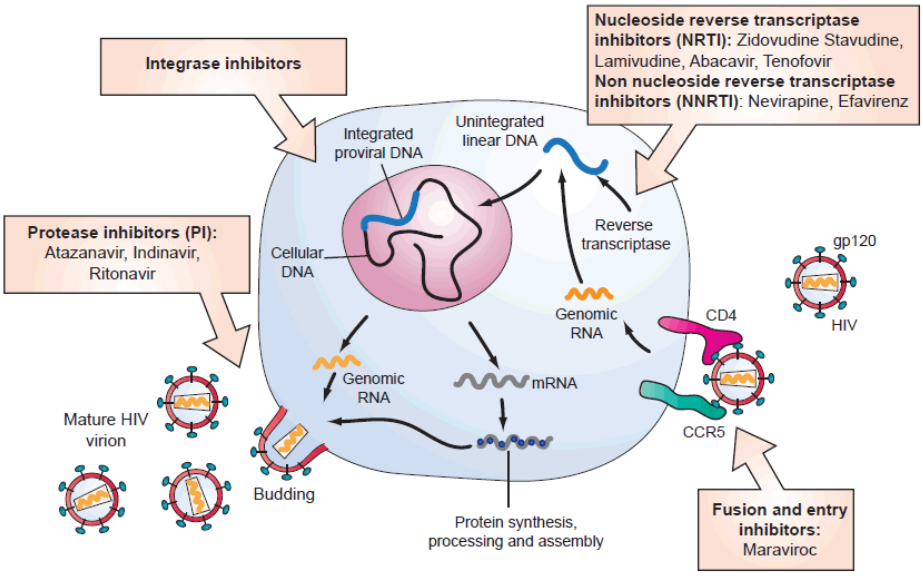

Figure 1.4 HIV life cycle: Targets for antiretroviral intervention. Approved drugs categorized according to their mechanism of action within the life cycle (39).

1.3.2 Treatment of primary HIV-1 infection

World Health Organization (WHO) has new recommendations that ART should be immediately initiated for individuals that test positive for HIV regardless of the CD4

count (40, 41). Previous guidelines stated that treatment be initiated at 500 CD4+ cells or fewer per mm^3 of blood of HIV infected individuals and before that infected individuals had to have a CD4 count of below 250 cells/µl, or present with co-morbidities. The scale-up of ART worldwide has resulted in significant reduction in AIDS-related deaths and has contributed to reduced HIV transmission rates (11).

Since the introduction of the very first antiretroviral agents, the question of when treatment should be initiated has not been fully answered. There has been heightened interest on the benefits and risks of rapid initiation of ART soon after HIV diagnosis is confirmed. As alluded to earlier, international guidelines now recommend early treatment initiation due to emerging data showing the beneficial impact of early ART (42, 43). The effect of the timing of ART initiation on both clinical and microbiologic outcomes has been debatable in terms of the benefit of therapy and the associated short-and long term costs, effects of drug toxicity, development of resistance and adverse effects on quality of life due to the longer duration of ART (44). There have been numerous studies providing strong evidence that initiation of ART at a threshold of <350 cells/µl CD4+ T cell count improves survival and delays disease progression (45), with additional studies suggesting a reduction of the risk of AIDS-defining illnesses and/or death with ART initiation at <500 CD4+ T cells/µl (46), and most recently data from randomized studies indicating that ART initiation immediately after HIV diagnosis, irrespective of CD4+ T cell count results in significant reductions of morbidity and mortality (47, 48). Results from these trials led to strengthening of international recommendations of ART for all HIV-infected individuals.

Various studies evaluating ART during acute HIV infection have shown the beneficial effects on laboratory progression markers. The positive effects of early ART initiation, during the acute phase of HIV infection, include reduction of viremia (49), lower viral load set point (50), lower probability of transmission (15, 51, 52), and overall a reduced number of infected cells thus limiting the size of the pool of latently infected CD4+ T cells (53, 54). Additionally, preservation of HIV-specific immune responses have been observed following initiation of ART in the initial phase of infection, by preserving the ability to control viral replication and avoiding early destruction of CD4+ T cells (55).

Despite having understood the overall immunological, virological and clinical benefits of early detection and treatment during acute HIV infection, it should be noted that diagnosis of acute HIV infection is quite challenging, with the clinical appearance in most cases being unspecific and resembling that of other viral infections (15).

Our group is uniquely positioned to assess acute HIV infection and the effects of very early ART initiation because of our access to the Females Rising through Education, Support, and Health (FRESH) study in KwaZulu-Natal, South Africa (56). FRESH is an acute HIV infection cohort that has overcome the challenges of detection of HIV during Fiebig stage I. The study uses the approach of testing HIV uninfected individuals who are at high risk of infection, adhering to a frequent surveillance schedule, allowing for detection during acute HIV infection. A socioeconomic empowerment programme, designed to coincide with sampling before and after infection, is conducted alongside the basic science research. Investigation of the earliest immunological and virological events after HIV infection is essential for novel development of preventive vaccines as well as

for HIV cure research. In this thesis work, we utilized the FRESH cohort to obtain matched peripheral blood and lymph node samples to answer valuable questions surrounding the impact of very early ART initiation on HIV-specific CD4+ T cell responses.

1.4 The need for an HIV cure

With the major strides that have been made in improving life expectancy and overall quality of life, lifelong ART has major side effects which result in toxicity, malignancies, increased age-related diseases, chronic inflammation among others. Moreover, ART is not readily accessible and free to everyone especially in low and middle-income countries and therefore cumulative costs of therapy is also a major concern (57, 58). Therefore, it is crucial to explore novel interventions that can enable people to control the virus in the absence of therapy. These strategies would need to completely eliminate residual virus from sanctuary sites within the body, which include lymph nodes and the gastrointestinal tract, which have been shown to harbour HIV reservoirs and have suboptimal drug penetration (59-61). The search for a curative strategy for HIV is a goal that is of utmost importance, with this area now being defined as a key priority in HIV research (61, 62).

It is important for the term “cure” to be well defined amongst the broader interested individuals i.e. people living with HIV, researchers and clinicians. The most favourable outcome would be to achieve a sterilizing cure, which would be complete eradication of replication-competent HIV within an individual. This however has proven extremely challenging with the current knowledge and technology. Therefore, a more practical outcome would be to achieve long-term remission, which is defined as the absence of

viral rebound after ART interruption for an undefined period of at least several years. The concept of remission is increasingly being described and utilized in the field of HIV research to indicate the overall goal of long-term undetectable viremia in the absence of ART (63-65). The idea of disease remission is already well established in medical settings and is known to denote improvement, albeit with some uncertainty (66).

The primary barrier to a cure for HIV is the existence of a stable reservoir of silenced fully integrated HIV DNA in long-lived cellular reservoirs, which besides CD4+ T cells include cells of the monocyte-macrophage lineage, as well as hematopoietic progenitor cells (HPCs) amongst others (67). This small population of memory T cells that lack activation markers have been repeatedly isolated from peripheral blood despite durable, successful ART, and are found to harbour integrated HIV DNA that are capable of producing replication-competent virus. These cells have been shown to persist for many years, with little changes in their frequency despite prolonged therapy (59, 60, 67, 68). These cells are enriched in lymphoid tissues, particularly secondary lymph nodes (69), the spleen and mucosal surfaces (70). The viral rebound from these reservoirs upon treatment interruption represent the major challenge in achieving a cure for HIV. There have been a few encouraging cases over the years that indicate a cure and sustained HIV remission is possible in the absence of ART (Figure 1.5). Sustained periods of aviremia in the absence of therapy was achieved in an aggressively treated infant (The Mississippi Child) (71, 72) , in at least two individuals who have received allogeneic stem cell transplant (The Boston patients) (73), in adults who received several years of ART initiated soon after infection (The Visconti cohort) (65), and a teenager showing viral remission more than 12 years after ART interruption from the same group (74). But, the

most notable cure case is Timothy Ray Brown, most commonly known as the Berlin Patient, who received a stem cell transplant using donor stem cells that were homozygous for CCR5Δ32 and is considered to be the only person known to be effectively cured of HIV (75).

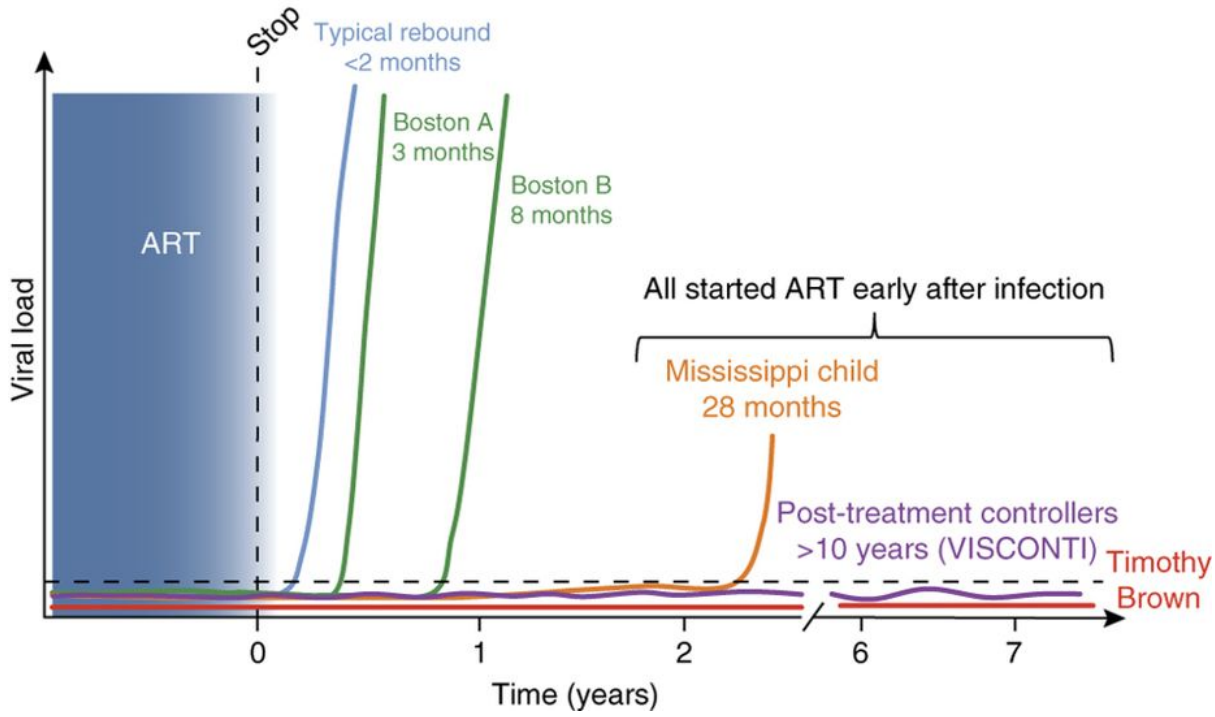

Figure 1.5 Viral rebound following cessation of ART. Typical rebound usually occurs within 2-3 weeks. In some instances viral rebound has been significantly delayed in the setting of stem cell transplantation (Boston Patients) or very early ART in an infant (the Mississippi Child). In some individuals, long-term post-treatment control (PTC) off ART has been achieved. In these PTC, ART was nearly always initiated in acute infection and virus is usually detected at low levels in plasma. Timothy Brown (the Berlin Patient) remains the only HIV infected individual off ART with no virus detected in blood or tissue. Source: Deeks *et al*., (61).

There are many elements that limit our ability to define the factors that would favour control off ART. These would include, dynamics of viral reservoirs as well as their size, cellular and anatomic locations. Furthermore, of particular importance is the limited knowledge on the impact of the timing of ART initiation on reservoirs and the immunologic and virologic factors that influence reservoir formation. Unless complete viral eradication is achieved, immune control through native responses or immune-based therapies will likely be necessary for maintaining remission after ART interruption (76). Many questions remain unanswered in the setting of HIV persistence on ART. These include the antigen-specificity during ART and if this is similar to untreated disease, the CD4+ T cell populations that HIV persist in and the changes in distribution and frequency of these cells over time. Ultimately, it would be important to discover non-virologic biomarkers that detect with high sensitivity the persistence of HIV in an infected individual as well as identify mechanisms of enhancing the capacity of the immune system to clear or control HIV. Immune-based therapeutics that are informed by the immunology of HIV persistence are likely be a major focus of cure research in the future (77).

1.5 Host immune responses to HIV infection

HIV infection is associated with robust immune responses. In untreated HIV-1 infection, a partial immune response is essential to prevent disease progression to full blown AIDS. Both the innate and adaptive immune systems (which comprise of humoral and T-cell dependent responses) are required to suppress HIV-1 infection (78).

HIV-specific T cell responses play an important role in the decline of viral load during primary infection as well as in being able to influence viral load set point (79-81). The role of cytotoxic CD8+ T cells (CTL) has been well established in immune mediated control of HIV. One of the key observations arguing for the role of CTLs in the initial control of virus replication is the temporal association between the emergence of CD8+ T cells and the rapid decrease in viral load during the acute phase of viral replication (82-84). The loss of protective HIV-specific CD8+ T cell responses has been associated with a swift progression to full blown AIDS (85), with additional evidence in rhesus macaque models in SIV infection where depletion of CD8+ T cells resulted in a subsequent rise in SIV viral load and progression to disease (84, 86, 87). Furthermore, immune selection pressure and the detection of viral escape mutations in CTL-targeted epitopes further demonstrated the important role of CD8+ T cells in mounting a strong immune response against the virus (88).

The progressive dysfunction and loss of HIV-specific CD8+ T cells have been implicated in increasing viral load and disease progression over time, together with the ongoing loss of CD4+ T cells. Other key attributes of CD8+ T cells, apart from the cytolytic activity, include polyfunctionality and functional avidity. The ability of CD8+ T cells to release a mixture of cytokines and chemokines in tandem have been shown to enhance cytolytic activity (89). Interestingly, polyfunctional CD8+ T cells have been shown to be elevated in a rare group of HIV positive individuals who maintain undetectable viral loads in the absence of any treatment, commonly referred to as elite controllers (90).

Host genetic effects and MHC class I molecules have also been widely studied, with a strong association between the rate of disease progression and the HLA class I alleles being demonstrated. The expression of particular HLA class I alleles restricting HIV-specific CD8+ T cell responses are associated with different clinical outcomes. Various alleles such as HLA- B*57 or B*27 being referred to as 'protective' alleles, whilst the accelerated onset of AIDS is associated with 'risk' alleles such as HLA-B*35. Overall, strong associations between HLA class I expression and viral control indicate an important role for HLA class I-restricted CD8+ T cells in the setting of HIV-1 infection (91).

HIV-specific CD8+ T cells have been studied in great detail, with various studies elucidating the host genetic determinants and antiviral effects of HIV-specific CTLs (92-94). However, very little is known about the role and contribution of CD4+ T cells to immune mediated control of HIV. Amongst the reasons for the paucity of studies on HIV-specific CD4+ T cells and their contribution to viral control include; firstly, CD4+ T cells are the primary targets of HIV-1 infection (95) , secondly, unlike CD8+ T cells, CD4+ T cells circulate at very low frequencies, mostly below the sensitivity threshold of most current assays (20, 96).

The main function of HIV-specific CD4+ T helper cells is to help B cell and CD8+ T cell responses. For instance, CD4 helper cells have been shown to play an important role in generating long-lasting, robust antiviral memory of CD8+ T cells (97, 98). While antigen-specific CD8+ T cells have the ability to be primed without CD4+ T cell assistance, subsequent expansion following the re-encountering of the antigen is inefficient without

CD4+ T cell help (99-101). The advanced loss of general CD4+ T cells and HIV-specific CD4+ T cells during HIV infection has been assumed to lead to the dysfunction of virus-specific CD8+ T cells and the unsuccessful suppression of these chronic viral infections (102-104). More recently HIV-specific CD4+ T cell responses and have been shown to play an important role in helping B cells responses. CD4+ T cells are necessary to generate and maintain humoral immune responses by providing help to antigen-specific B cells for the production of antibodies (105, 106). Furthermore, CD4+ T cell responses are thought to provide superior helper activity to Env-specific B cells, promoting their differentiation, maturation, and secretion of neutralizing antibodies (105).

Currently virus-specific CD4+ T cell responses in HIV-1 infection are poorly defined and there is a lack of information on HLA class II restriction of HIV-specific responses. As previously alluded to, HLA class I alleles have been characterized, with their genetic association with HIV control being well defined (107, 108). However, recent studies have suggested that HLA class II alleles may also have an effect on the control of HIV (109-111). Several HLA class II DRB1*13 alleles and the DRB1*13-DQB1*06 haplotype have been shown to bestow a degree of protection in terms of HIV disease outcome. Studies have shown that individuals expressing theses alleles, display the strongest HIV-specific CD4+ T cell responses (112). The DRB1*1303 allele in particular has been associated with reduced viral loads in both subtype B and C populations (109, 112). The DRB1*13 allele has also been shown to confer protection in other infections, particularly hepatitis B, where presence of this allele has been associated with a greater frequency of clearance as well as better clinical outcome (109). Although not conclusive, the finding of strong associations between class II HLA alleles and breadth and magnitude of CD4+

T cell responses re-iterates the important role that CD4+ T cells have in immune mediated control of HIV.

The identification of the specificities and efficacies of HIV-specific CD4+ T cell responses is likely to be vital for HIV vaccine design. Indeed, it will be important to identify which HIV-specific CD4+ T cells are induced in natural HIV infection in order to successfully augment the efficacy of these responses in future vaccine and cure strategies.

1.6 CD4+ T helper cell subsets

T helper (Th) cells play important roles in coordinating adaptive immune responses. They carry out functions principally through the secretion of cytokines and chemokines that activate and/or recruit other immune cells including B cells, CD8+ T cells, macrophages and other effector cells (113). The complex biology and diversity in functions of CD4+ T cells are determined by their cytokine secretion and their tissue locations (Figure 1.6). Initially, CD4+ T cells were divided into two subsets by Mosmann *et al*., in 1986 (114), Th1 and Th2, which respectively produced the signature cytokines interferon (IFN)-γ and interleukin (IL)-4 and IL-13. Th1 cells are known to promote cytotoxic effector functions of natural killer (NK) cells, CD8+ T cells and macrophages and mediate immune responses against intracellular pathogens such as viruses, intracellular bacteria and protozoan parasites (113). This subset also promotes antibody-dependent cell-mediated cytotoxicity (ADCC) by supporting B cell production of IgG1 in humans (113). Th2 cells are important for the clearance of extracellular pathogens such as helminths and also promote humoral immunity, mediated by B cell-produced IgG4 and IgE in humans (113,

115). As new technologies have emerged, various other subsets of CD4+ T cells have arose to add to the classic Th1 and Th2. Each of the subsets are identified and characterized by their lineage-defining transcription factor or the migration markers they express (116, 117). Besides Th1 and Th2, other well defined CD4+ T cell subsets include; Th9, Th17, Th22, TFH and TREG. The various lineages are dependent on the expression of transcription factors, effector cytokines, and chemokine receptors (Figure 1.6) (113, 118). Th9 cells, which produce anti-inflammatory cytokines, IL-10 and IL-9 provide defences against nematodes (119, 120) and the Th17 facilitate antimicrobial immunity and protection at mucocutaneous sites (121). TFH cells specialize in helping B cell responses, while TREGs regulate immune responses and prevent autoimmunity (119, 122, 123).

It is important to note that most of CD4+ T cells reside within the gastrointestinal (GI) tract, lymph nodes (LNs) and other lymphatic tissues rather than in peripheral blood (124).

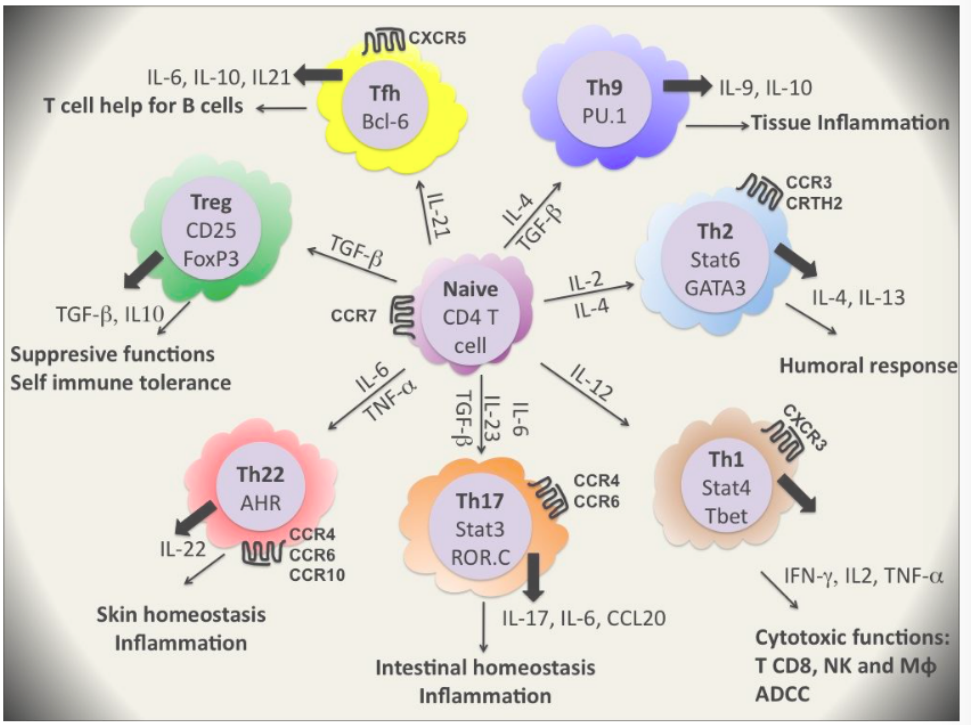

Figure 1.6 T helper cell lineage development and functions. T helper cell lineages can be divided into Th1, Th2, Th9, Th17, Treg, Tfh and Th22 cells that are involved in distinct immune functions (118).

1.7 T regulatory (TREG) cells

Regulatory T (TREGs) cells are a subset of circulating CD4+ T cells with suppressive properties implicated in immune tolerance (125), auto-immune diseases, cancer, transplantation, maternal-fetal tolerance and inflammation induced by chronic pathogens (126, 127). They are phenotypically defined minimally as CD4+CD25+FOXP3+, with their development, maintenance and function dependent on the expression of the master transcription factor FOXP3 (forkhead box P3) (128). Natural Tregs (nTreg) are generated in the thymus (129) and are detected in peripheral blood, mucosal and lymphoid tissues

where they prevent autoimmunity and decrease immune activation (130). Adaptive or induced Tregs (iTregs) develop in the periphery from mainstream peripheral αβ T cells using self (131) or foreign antigens (132). These are essential in mucosal immune tolerance and during normal homeostasis of the gut. There have been many conflicting reports of on the role of TREGs in HIV infection, with many considering these cells a double-edged sword (Figure 1.7) (133). The effects of TREG expansion, which has been observed in HIV infection, could be either beneficial, by suppressing generalized T-cell activation, or detrimental, by weakening HIV-specific responses and thus contributing to viral persistence (133). The mechanisms through which TREGs carry out their function are not fully understood, however studies have shown that TREG action is through the production of bioactive molecules, such as IL-10 and TGF-β as well as through cell-cell contact mediated regulation via the high affinity TCR and other co-stimulatory molecules such as CTLA-4; glucocorticoid-induced tumor necrosis factor-related receptor (GITR) and cytolytic activity (134, 135).

Most of the studies on TREGs have been in peripheral blood, despite the fact that HIV is a disease primarily of lymphatic tissues, with most viral replication occurring in CD4+ T cells within these compartments (136). It should be noted that peripheral blood only contains 2% of the total CD4+ T cell population in the body, with most of these being effector memory cells in transit. The impact of HIV replication is most reflective on the populations residing within secondary lymphoid organs (136).

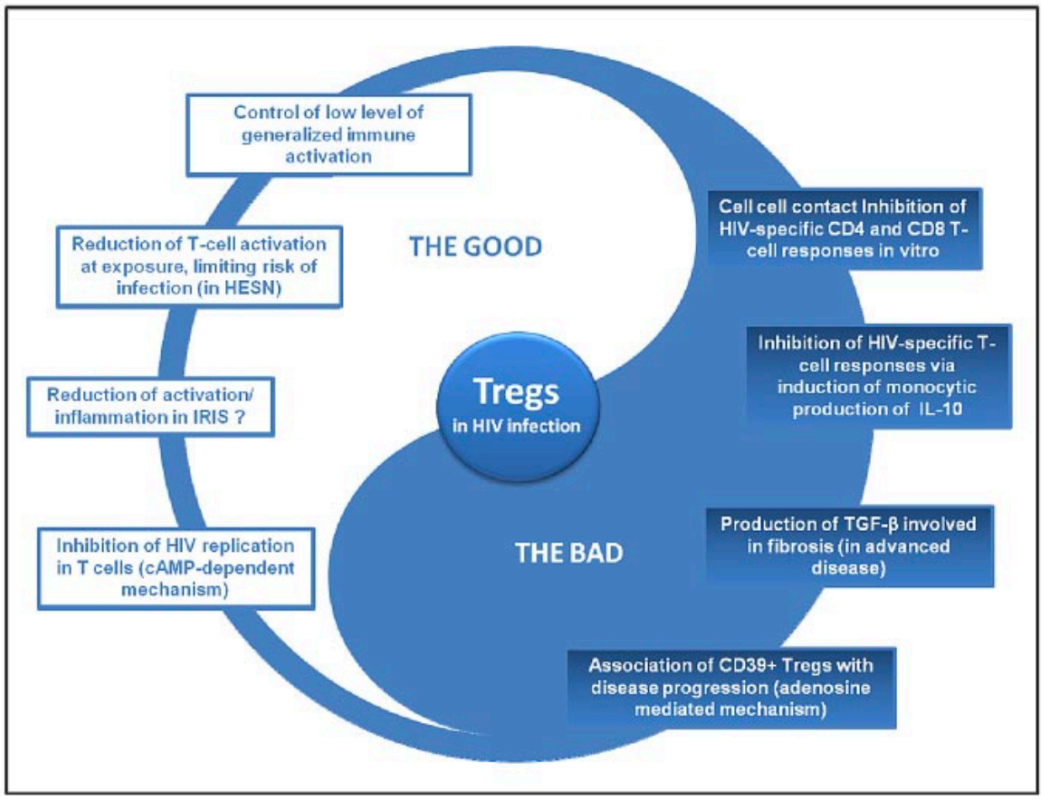

Figure 1.7 Dual role of TREGs in HIV infection. The presence and expansion of TREGs observed during HIV infection could be either beneficial or detrimental to the host immune system (133).

1.8 T follicular regulatory cells

As alluded to earlier, CD4+ T cells are also critical in both supporting and regulating the efficacy and longevity of humoral immune responses. T cells are known to migrate to germinal centres (GC) and B-cell-enriched follicles within secondary lymphoid compartments in order to provide help to B cells. Recent evidence suggests that B-cell follicles contain a novel subset of TREGs, termed follicular regulatory T cells (TFR) (137, 138). The transcriptional pattern of TFR overlaps with follicular T helper cells (TFH). TFH are critical initiators of GC responses, however studies have shown that

although an expansion of TFH cells is observed in HIV infection, this expansion does not necessarily correlate with improved GC responses. The factors driving this dysregulation remain unclear. One potential mechanism could be the presence of high frequencies of TFR cells in the lymph node (139). Like TFH, TFR cells express high levels of the follicle-homing chemokine receptor 5 (CXCR5), programmed cell death protein-1 (PD-1) and inducible T cell co-stimulator (ICOS) (140). TFR also express the transcription factor B cell lymphoma 6 (Bcl6), which is a canonical TFH transcription factor, albeit at lower levels. However, several studies have shown that TFR cells primarily originate from natural FOXP3+ Treg precursors (141, 142). TFR cells are also different from TFH in that they express FOXP3 and BLIMP-1.

1.8.1 Differentiation and regulation of TFR cells

Antigen exposure triggers the differentiation of TFR cells in a dendritic cell (DC)-dependant manner, which are found in the spleen, lymph nodes or other lymphoid organs such as Peyer's patches (143, 144). Like TFH, TFR cells are thought to have two phases of differentiation: firstly, the imprinting of the TFR program after interaction with DCs, and the second phase where the TFR signature is strengthened upon interaction with B cells within the follicle/germinal centre (140). The mTorc1 pathway has recently been shown to be a key regulator of TFR cells. The mTorc1 complex is essential in regulating the conversion of TREGs to TFR cells, which is potentially mediated through a Stat3-Tcf-1 – Bcl6 pathway (Figure 1.8) (145, 146). The precise antigens and signals that TREGs respond to in order to become TFR cells is not well understood, with studies indicating that TFR cells respond more strongly to self-antigens than foreign antigens,

with further work showing TFR cells develop in a polyclonal and antigen-independent manner from TREGs (144, 147). Much work remains to fully understand the specific genes and pathways that regulate human TFR cells.

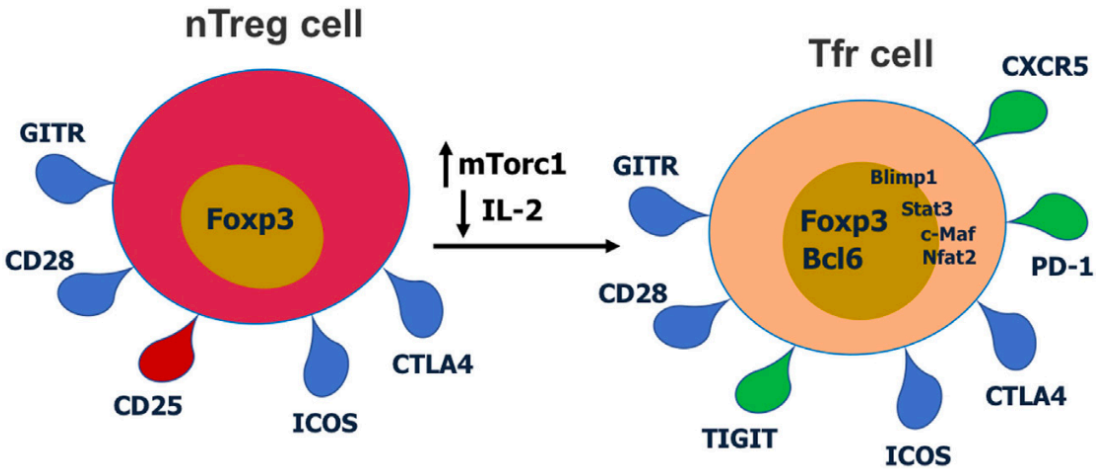

Figure 1.8 Cell surface receptors and transcription factors involved in TFR cell differentiation and function. Red receptors are downregulated and green receptors are upregulated during TFR cell differentiation (148).

1.9 T follicular regulatory cells and HIV infection

1.9.1 Suppressive functions of TFR cells

TFR have been mainly described in literature as suppressors of the GC reaction and antibody (Ab) response, shown to repress proliferation of TFH cells and GC B cells, thus limiting the generation of Ab-secreting cells and overall Ab responses (Figure 1.9). *In vitro* studies have shown that TFR can suppress proliferation and cytokine production of TFH cells as well as proliferation and Ig secretion of B cells, similarly to what has been described for TREGs (143, 147, 149-151). The precise mechanisms that is used by TFR to negatively regulate the GC reaction is still unknown. A major disadvantage is the fact

that *in vitro* studies cannot mimic the *in vivo* environment of the GC reaction and cannot be used to analyse affinity selection of GC B cells.

1.9.2 "Helper" functions of TFR cells

Although TFR are generally described as suppressor cells, there have been a few reports that suggest that under certain special circumstances, TFR can be deemed as having a "helper" function. Linterman *et al.,* in 2009 (152), was the first to show that TFR display a vital "helper" role by helping TFH in selecting high-affinity Ag-specific B cell clones. In a murine study by Wu *et al.,* (153), loss of TFR cells led to a significant decrease in IgG response and consistent with previous work, TFR cells were required to produce highest affinity Ag-specific Abs. Another murine study by Laidlaw *et al.*, (154) analysed the GC and Ab response in lymphocytic choriomeninigitis virus (LCMV). Their results showed that IL-10 drives the growth of GCs. In these studies, they observed a decrease in GC B cells numbers and LCMV-specific Ab in the absence of IL-10 producing TFR cells. Overall these studies point towards a major role of TFR in maintaining the GC reaction and acting as "helper" cells. This seemingly paradoxical role of TFRs warrants further detailed and mechanistic investigation.

There are many unanswered questions surrounding this newly identified subset of T suppressor cell. TFR cells have been analysed in a very small number of infectious and immunological disease models and testing the function of TFRs in various disease states is an important research area. A major consideration as the field progresses would be to determine the mechanisms and under which conditions TFR are able to switch between helper and suppressor functions within GCs. The signals that drive TFR responses and

the antigens that they recognize is also a very much unknown. Much work remains to fully understand the role of TFRs in humoral immune response in particular and in the overall immune response to infection. Therefore, this thesis is focused on understanding how this cell subset modulates induction of adaptive immune responses.

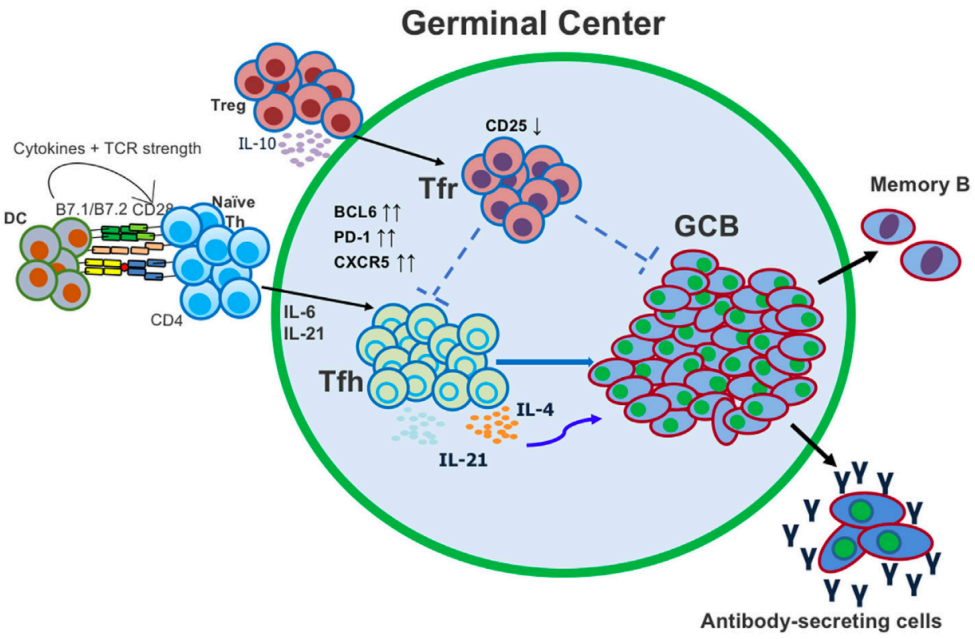

Figure 1.9 Follicular helper T (Tfh) and T follicular regulatory (Tfr) cells both act in the germinal centre (GC) to regulate the generation of antigen (Ag)-specific antibody-secreting cells (148).

1.9.3 TFR and HIV persistence

HIV-1 replication is mainly concentrated in TFH cells within B cell follicles of secondary lymphoid tissues during chronic asymptomatic disease (155-157). Virus replication within these sites could be attributed to various factors including paucity of virus-specific CTL (156, 158), heightened permissivity of TFH (157) and the presence of highly infectious virions on follicular dendritic cells (FDC) (159). TFH, within secondary

lymphoid follicles, have been shown to be highly susceptible to HIV infection (160), but, there is very little evidence on TFR susceptibility to HIV-1 infection. A recent study by Miller *et al*., in 2017 (161), was the first to show in tonsil cells from children at low risk of HIV-1 infection, that TFR were more permissive to R5-tropic HIV-1 infection *ex vivo* than TFH and extra-follicular CD4+ T cells. TFR were more susceptible to R5 viral fusion than other cells and expressed the highest levels of CCR5 and CD4. Furthermore, this study showed in lymph node (LN) cells from asymptomatic HIV-1-infected humans, TFR harboured the highest concentrations of HIV-1 RNA (161) . The authors suggested that from their previous work demonstrating that TFR were more resistant to apoptosis than TFH in *ex vivo* HIV-1 infection (139) and the current work on TFR permissivity, it seemed likely that TFR and TREGs may be more resistant than other cells to cell death, and able to survive despite HIV infection and therefore contribute to the latent viral reservoir in ART-treated patients. Further work on the establishment and maintenance of latent infection in TFR and TREGs is therefore warranted and could lead to new HIV cure strategies.

1.10 Appropriate tools to investigate HIV-specific CD4+ T cells

Antigen (Ag)- specific T cells play a crucial role in mediating specific immune responses as well as in the formation of immunological memory. Therefore, detailed information about their frequencies, phenotypes, and functional capacities is necessary to estimate the specific immune status of an individual, to understand the mechanisms of protective immunity or immunopathology and to predict immune protection or diagnose immune-related diseases (162). Evaluating antigen-specific T cells and their effector functions is vital to understanding overall T cell immunity in terms of assessing the effectiveness of

specific immune therapies (163). However, because of the low frequency at which these antigen-specific T cells occur in peripheral blood and lymphoid tissue, consistent and reliable measurement of T cell immunity is a major challenge. Commonly used assays to measure HIV-specific CD4+ T cells include the interferon (IFN)-γ ELISPOT, tetramer-staining for common HLA-restricted epitopes and intracellular cytokine staining (ICS) measured by flow cytometry. The ELISPOT is a rapid and cost-effective method used in the large-scale detection and mapping of T cell responses. One of the limitations of the assay is that it works under the assumption that all HIV-specific CD8- or CD4+ T cells are identifiable by their ability to secrete IFN-γ post peptide stimulation. However, some antigen-specific cells do not readily produce IFN-γ and are therefore more likely to be missed by this assay.

MHC class II tetramers have emerged as an important tool for characterization of the specificity and phenotype of CD4+ T cell immune responses, useful in a large variety of disease and vaccine studies (164). The analysis of Ag-specific T cells through the use of soluble MHC-peptide ligands that engage the αβTCR was originally developed for MHC class I recognition in the context of CD8+ T cells (165, 166). In recent years, similar approaches have been used for MHC Class II recognition of CD4+ T cells for various antigens. However, CD4+ T cell recognition by MHC class II tetramers has had several constraints because of numerous factors which include; firstly, difficulty in producing class II MHC-based reagents due to folding conditions that need to be optimized individually for species and allotype. Secondly, low frequency of CD4+ T cells of interest in many biological contexts; which would require expansion or enrichment prior to detection by tetramers. And lastly, complications caused by low T-cell receptor (TCR)-

MHC avidity; with some CD4+ T cells having too weak MHC-TCR affinity to support tetramer binding (167).

The major limitation for using tetramers is the requirement to have prior knowledge of the specific peptide-MHC components involved in the recognition events being studied, because these are necessary to construct the tetramer reagents. The antigenic epitope has to be well characterized i.e. a defined peptide restricted to a particular MHC haplotype (162) .

The initial part of this thesis therefore focused on developing the appropriate tools i.e. MHC class II tetramers for the analysis of antigen-specific CD4+ T cell populations within lymph nodes and peripheral blood in HIV-1 clade C infection. This tool, which provides information on cell frequency and specificity was used in conjunction with other immunological techniques to elucidate the role of helper and immunoregulatory cells within these two compartments.

1.11 Thesis outline

Increasing evidence suggests that virus-specific CD4+ T cells contribute to immune-mediated control of clade B HIV-1 infection. Yet, there is very little known about the role of HIV-specific CD4+ T cells in immune mediated control of HIV-1 clade C infection, which is responsible for majority of HIV infections worldwide (168). Specifically, CD4+ T regulatory cells have been shown to be important during various chronic infections, including HIV, but little is known about their biology and function during HIV-1 clade C

infection. Furthermore, the exact role of regulatory cells during HIV-1 infection remains controversial, with both beneficial and detrimental roles having been described (133, 169, 170). Regulatory cells have been shown to be beneficial because they suppress chronic immune activation and downregulate CD4+ T cells, thereby contributing to subsequent control of viral replication (169). But, they could also be detrimental by inhibiting anti-HIV immune responses, therefore promoting HIV persistence (171). Poor understanding of the role of TREGs in HIV pathogenesis is probably due to a number of reasons including but not limited to the shortage of tools to properly characterize these low frequency cells. Currently, developing assays that can fully quantify all the relevant epitope specific CD4+ T cell responses remains a major challenge in natural HIV infection and vaccine studies because of the tremendous complexity of CD4+ T cell biology.

This study focused on understanding the role of HIV-specific immunoregulatory cells in lymphoid tissue and peripheral blood, particularly during early HIV infection, where these cells are more likely to influence the evolution of adaptive immune responses. The improved elucidation of HIV-specific CD4+ T cell responses and enhanced understanding of how regulatory cells may promote the generation of protective CD8+ T cell and B cell responses or mediate a direct antiviral role is highly relevant for future HIV vaccine design. Understanding the mechanisms by which CD4+ T regulatory cells exert their influence is an important research area with broad implications for the development of therapeutic strategies for many disease processes including immune mediated diseases.

We chose to investigate the role of HIV-specific CD4+ T cells in HIV pathogenesis because our access to a primary HIV infection cohort (FRESH) that identifies individuals with “hyperacute” HIV infection and treats them at the onset of plasma viremia, with some being treated with plasma viral loads less than 1000 RNA copies/ml (56). In addition, our group has established protocols for excisional lymph node biopsies, cell sorting, multicolor immunofluorescence microscopy and class II tetramer technology. All these resources uniquely position us to effectively carry out detailed characterizations of CD4+ T helper and regulatory cells in HIV-1 clade C infection.

Goal of study

The overall goal of the study was to comprehensively characterize antigen-specific CD4+ T cells in peripheral blood and lymphoid tissue in HIV-1 clade C infection in KwaZulu-Natal, South Africa. In particular, we sought to examine how regulatory CD4+ T cell subsets alter helper T cell frequency and function in the lymph nodes and how this interaction modulates adaptive immune responses, during early treated and untreated HIV-1 infection.

Hypothesis

Antigen-specific CD4+T cell immunoregulatory cells present in secondary lymphoid organs play a key role in modulating effective adaptive immune responses against HIV-1 infection.

Specific aims

1. Develop class II tetramers to interrogate low frequency HIV-specific CD4+ T cells in HIV-1 clade C infection.

2. Phenotypically and functionally characterize antigen-specific CD4+ T cells in the immune regulation of HIV-1 within lymph nodes and peripheral blood.

3. Determine the effect of early treatment on the frequency and function of regulatory CD4+ T cell populations in lymph nodes and peripheral blood.

Chapter 1 is the introduction and provides an overview of relevant literature.

Chapter 2 describes MHC class II tetramers designed to characterize Gag-specific $CD4^+$ T cell responses in HIV-1 clade C infection.

Chapter 3 defines the phenotype, transcriptional signature and antigen-specificity of CD4+ helper and regulatory T cell subsets within lymph nodes and peripheral blood using flow cytometry, single cell RNA-sequencing and MHC class II tetramers.

Chapter 4 assesses changes in regulatory T cell frequencies within the lymph node in terms of localization and function, particularly in the setting of early antiretroviral therapy initiation, using flow cytometry, immunofluorescence microscopy, droplet digital PCR and ELISA.

Chapter 5 details the overall implications and future research directions of the research within this thesis.

Chapter 2 Overview

As alluded to in our literature review, collective evidence suggests that virus-specific CD4+ T cells contribute to immune-mediated control of HIV-1 infection. Due to the low frequency of these cells, there is increased difficulty in measuring antigen-specific CD4+ T cells without prior manipulation, which massively alters their phenotypic and functional properties. Furthermore, only a small fraction of HIV-specific CD4+ T cell responses have been identified, with many studies relying solely on measuring IFN-γ as a readout for CD4+ T cell function. Having noted the complexity of these cells, it is evident that this would be an inaccurate representation of CD4+ T cell responses in the setting of HIV infection. With MHC class II tetramer technology now advancing, more studies are using this method of directly detecting antigen-specific CD4+ T cells without relying on function.

In Chapter 2, we present a study conducted to develop the appropriate tools, i.e. MHC class II tetramers to better understand the role of HIV-specific CD4+ T cell responses in HIV control and disease progression.

These results have been published in the Journal of Virology (Volume 91, Issue 7, 2017).

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PATHOGENESIS AND IMMUNITY

HIV Controllers Exhibit Enhanced Frequencies of Major Histocompatibility Complex Class II Tetramer$^+$ Gag-Specific CD4$^+$ T Cells in Chronic Clade C HIV-1 Infection

Faatima Laher,[a] Srinika Ranasinghe,[b,c] Filippos Porichis,[b,c] Nikoshia Mewalal,[a] Karyn Pretorius,[a] Nasreen Ismail,[a] Søren Buus,[d] Anette Stryhn,[d] Mary Carrington,[b,e] Bruce D. Walker,[a,b,f] Thumbi Ndung'u,[a,b,g,h] Zaza M. Ndhlovu[a,b]

HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa[a]; Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, Massachusetts, USA[b]; Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery, The Scripps Research Institute, La Jolla, California, USA[c]; Department of Immunology and Microbiology, University of Copenhagen, Copenhagen N, Denmark[d]; Cancer and Inflammation Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA[e]; Howard Hughes Medical Institute, Chevy Chase, Maryland, USA[f]; KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa[g]; Max Planck Institute for Infection Biology, Berlin, Germany[h]

ABSTRACT Immune control of viral infections is heavily dependent on helper CD4$^+$ T cell function. However, the understanding of the contribution of HIV-specific CD4$^+$ T cell responses to immune protection against HIV-1, particularly in clade C infection, remains incomplete. Recently, major histocompatibility complex (MHC) class II tetramers have emerged as a powerful tool for interrogating antigen-specific CD4$^+$ T cells without relying on effector functions. Here, we defined the MHC class II alleles for immunodominant Gag CD4$^+$ T cell epitopes in clade C virus infection, constructed MHC class II tetramers, and then used these to define the magnitude, function, and relation to the viral load of HIV-specific CD4$^+$ T cell responses in a cohort of untreated HIV clade C-infected persons. We observed significantly higher frequencies of MHC class II tetramer-positive CD4$^+$ T cells in HIV controllers than progressors ($P = 0.0001$), and these expanded Gag-specific CD4$^+$ T cells in HIV controllers showed higher levels of expression of the cytolytic proteins granzymes A and B. Importantly, targeting of the immunodominant Gag41 peptide in the context of HLA class II DRB1*1101 was associated with HIV control ($r = -0.5$, $P = 0.02$). These data identify an association between HIV-specific CD4$^+$ T cell targeting of immunodominant Gag epitopes and immune control, particularly the contribution of a single class II MHC-peptide complex to the immune response against HIV-1 infection. Furthermore, these results highlight the advantage of the use of class II tetramers in evaluating HIV-specific CD4$^+$ T cell responses in natural infections.

IMPORTANCE Increasing evidence suggests that virus-specific CD4$^+$ T cells contribute to the immune-mediated control of clade B HIV-1 infection, yet there remains a relative paucity of data regarding the role of HIV-specific CD4$^+$ T cells in shaping adaptive immune responses in individuals infected with clade C, which is responsible for the majority of HIV infections worldwide. Understanding the contribution of HIV-specific CD4$^+$ T cell responses in clade C infection is particularly important for developing vaccines that would be efficacious in sub-Saharan Africa, where clade C infection is dominant. Here, we employed MHC class II tetramers de-

Received 29 December 2016 **Accepted** 9 January 2017

Accepted manuscript posted online 11 January 2017

Citation Laher F, Ranasinghe S, Porichis F, Mewalal N, Pretorius K, Ismail N, Buus S, Stryhn A, Carrington M, Walker BD, Ndung'u T, Ndhlovu ZM. 2017. HIV controllers exhibit enhanced frequencies of major histocompatibility complex class II tetramer$^+$ Gag-specific CD4$^+$ T cells in chronic clade C HIV-1 infection. J Virol 91:e02477-16. https://doi.org/10.1128/JVI.02477-16.

Editor Guido Silvestri, Emory University

Address correspondence to Zaza M. Ndhlovu, zndhlovu@mgh.harvard.edu.

CHAPTER 2: HIV CONTROLLERS EXHIBIT ENHANCED FREQUENCIES OF MHC CLASS II TETRAMER+ GAG-SPECIFIC CD4+ T CELLS IN CHRONIC CLADE C HIV-1 INFECTION

Faatima Laher[1], Srinika Ranasinghe[2,3], Filippos Porichis[2,3], Nikoshia Mewalal[1], Karyn Pretorius[1], Nasreen Ismail[1], Søren Buus[4], Anette Stryhn[4], Mary Carrington[2,5], Bruce D. Walker[1,2,6], Thumbi Ndung'u[1,2,7,8], Zaza M. Ndhlovu[1,2]

[1]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

[2]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA, USA

[3]Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery, The Scripps Research Institute, La Jolla, CA, USA

[4]Department of Immunology and Microbiology, University of Copenhagen, 2200-Copenhagen N, Denmark

[5]Cancer and Inflammation Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA

[6]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA

[7]KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

[8]Max Planck Institute for Infection Biology, Charitestraße 1, Berlin, Germany

Keywords: HIV, CD4 T-helper cells, MHC class II tetramers

2.1 Abstract

Immune control of viral infections is heavily dependent on helper $CD4^+$ T cell function. However, understanding the contribution of HIV-specific $CD4^+$ T cell responses to immune protection against HIV-1, particularly in clade C infection remains incomplete. Recently, MHC class II tetramers have emerged as a powerful tool for interrogating antigen specific $CD4^+$ T cells without relying on effector functions. Here, we defined the MHC class II alleles for immunodominant Gag $CD4^+$ T cell epitopes in clade C virus infection, constructed MHC class II tetramers, and then used these to define the magnitude, function, and relation to viral load of HIV-specific $CD4^+$ T cell responses in a cohort of untreated HIV clade C infected persons. We observed significantly higher frequencies of MHC class II $tetramer^+$ $CD4^+$ T cells in HIV controllers compared to progressors ($p=0.0001$) and these expanded Gag-specific $CD4^+$ T cells in HIV controllers showed higher expression of the cytolytic proteins, Granzymes A and B. Importantly, targeting of the immunodominant Gag41 peptide in the context of HLA class II DRB1*1101 was associated with HIV control ($r=-0.5$, $p=0.02$). These data identify an association between HIV-specific $CD4^+$ T cell targeting of immunodominant Gag epitopes and immune control, particularly the contribution of a single class II MHC-peptide complex to the immune response against HIV-1 infection. Furthermore, these results highlight the advantage of class II tetramers in evaluating HIV-specific $CD4^+$ T cell responses in natural infections.

2.2 Importance

Increasing evidence suggests that virus-specific $CD4^+$ T cells contribute to immune-mediated control of clade B HIV-1 infection. Yet, there remains a relative paucity of data regarding the role of HIV-specific $CD4^+$ T cells in shaping adaptive immune responses in clade C infection, which is responsible for majority of HIV infections worldwide. Understanding the contribution of HIV-specific $CD4^+$ T cell responses in clade C infection is particularly important for developing vaccines that would be efficacious in sub-Saharan Africa, where clade C infection is dominant. Here, we employed MHC class II tetramers designed to immunodominant Gag epitopes and used them to characterize $CD4^+$ T cell responses in HIV-1 clade C infection. Our results demonstrate an association between the frequency of HIV-specific $CD4^+$ T cell responses targeting an immunodominant DRB1*11-Gag41 complex and HIV viral control, highlighting the important contribution of a single class II MHC-peptide complex to the immune response against HIV-1 infections.

2.3 Introduction

Detailed characterization of immune cells that contribute to suppression of HIV replication is critical to both vaccine design and therapeutic approaches. Although both $CD4^+$ and $CD8^+$ T cells mediate cellular immune responses to HIV, most research efforts have focused on understanding the role of $CD8^+$ T cells. In contrast, the role of $CD4^+$ T cells is less well defined. $CD4^+$ T cells contribute to immune control of many viral infections (1-6), implying that they may be similarly important for HIV immunity. Indeed, recent studies in HIV clade B infection have highlighted the important role of

HIV-specific $CD4^+$ T cells as effectors of viral immunity (7, 8), in addition to being orchestrators of $CD8^+$ T cell and B cell responses via helper signals (9).

Although $CD4^+$ T cell responses remain relatively understudied, emerging evidence suggests that they play a more active role in controlling HIV disease progression. A recent SIV study provided the first direct evidence of $CD4^+$ T cell driven escape mutation. They identified a unique post-breakthrough mutation in the Gag region only targeted by CD4 responses, which abrogated $CD4^+$ T cell recognition (10). In humans indirect evidence generated by a computational approach identified HLA class II associated polymorphisms and linked predicted epitopes within adaptation sites to $CD4^+$ T cell driven immune escape (11). Furthermore, recent studies conducted in clade B HIV infection in predominantly Caucasian populations showed that targeting Gag by $CD4^+$ T cell responses restricted by some HLA-DRB1 alleles such as DRB1*15:02, 13:01 and DQB*:06 was associated with low viremia whereas CD4 responses restricted by DRB1*03:01 allele were linked to high viremia. The plausible explanation for the difference was that DRB1 alleles associated with low viremia had the capacity to present multiple epitopes at low functional avidity compared to alleles associated with high viremia (12-14). Taken together, these data underscore the impact of HIV-specific $CD4^+$ T cell responses on the control of HIV replication.

In contrast to these studies in clade B virus infection, much less is known about the role of $CD4^+$ T cell responses in clade C HIV infection. Improved understanding of immune responses to clade C is particularly important for developing vaccines that would be efficacious in sub-Saharan Africa, where subtype C viruses which account for more than

50% of infections globally, are most dominant (15). A few clade C studies have identified immunodominant class II restricted $CD4^+$ T cell epitopes, but the restricting alleles for these responses remain unknown (16, 17). The lack of knowledge about restricting alleles has made it difficult to synthesize class II tetramers that could be used to conduct more comprehensive $CD4^+$ T cell studies without relying on specific effector functions. Most clade C studies have used IFN-γ intracellular cytokine staining (ICS) or ELISPOT assays to study $CD4^+$ T cell responses (17). This approach is limited in scope because it can only detect IFN-γ secreting $CD4^+$ T cell responses and is therefore more likely to miss other $CD4^+$ T cell subsets such as Th-2, Th-17, T follicular helper cells and regulatory T cells that are defined by different signature cytokines.

To investigate if virus-specific $CD4^+$ T cells play an active antiviral role in the pathogenesis of clade C HIV infection, we first screened the entire HIV-1 clade C proteome for immunodominant HIV-specific $CD4^+$ T cell epitopes and performed class II restriction studies to identify the most frequent class II DRB-1 alleles in an initially untreated, predominantly Zulu/Xhosa ethnic study population in a clade C endemic region. The screening data were used to synthesize MHC class II tetramers to the most immunodominant $CD4^+$ T cell epitopes. The tetramers were then used to thoroughly examine the frequency and function of HIV-specific $CD4^+$ T cell responses during chronic untreated HIV-1 clade C infection. Our data demonstrate that the frequency of immunodominant Gag-specific $CD4^+$ T cells as measured by tetramers is inversely associated with contemporaneous viral load. These data highlight the important contribution of HLA class II-restricted epitope-specific $CD4^+$ T cells to the immune

response against HIV-1 clade C infection and indicate that MHC class II tetramers are a sensitive tool for interrogating HIV-specific $CD4^+$ T cells responses in natural infections.

2.4 Materials and Methods

2.4.1 Cohort characteristics

A total of 80 subjects, chronically infected with HIV-1 clade C (76.3% female, n=61 and 23.8% male, n=19) from the local Zulu/Xhosa population recruited as part of the Sinikithemba cohort (18) in Durban, South Africa, were studied. The Biomedical Research Ethics (BREC) of the University of KwaZulu-Natal and the institutional review board (IRB) of Massachusetts General Hospital approved this study. All study participants were antiretroviral naïve at the time of enrollment. Socio-demographic characteristics, plasma viral load measurements, and $CD4^+$ T cell counts were obtained at baseline. Follow-up $CD4^+$ T cell counts, and plasma viral load measurements were performed at 3-month and 6-month intervals. Individuals recruited in this study were stratified into two major groups: 34 HIV controllers defined here as individuals who maintained lower viral loads of <2,000 HIV RNA copies/ml for >1 year and 46 HIV chronic progressors (viral loads of >2000 HIV RNA copies/ml) based on contemporaneous viral load (Table 1). HLA-DRB1 typing was performed for all participants, as described (16). Cryopreserved peripheral blood mononuclear cells (PBMCs) samples from each individual were utilized for all assays. An initial number of 72 participants were screened for HIV-specific $CD4^+$ T cell responses using the ELISPOT assay. Individuals that made a $CD4^+$ response were then screened using the HLA restriction assay to determine restricting alleles and epitopes. For additional comparison

between controllers and progressors, an additional 8 individuals that were not utilized in the initial screening were added to the data set. Hence, the total number of 80 participants in the study (Table 1). MHC class II tetramers were synthesized and tested in approximately one quarter (21/80) of study subjects in this clade C infection cohort who expressed the matching HLA class II DRB1 alleles.

2.4.2 $CD8^+$ T cell depletion and modified IFN-γ Elispot Assay

A modified IFN-γ ELISPOT assay was used to screen all chronically infected subjects. Cryopreserved PBMCs were thawed and $CD8^+$ T cells were depleted using Miltenyi MACS CD8 MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The initial screening was carried out using a "megamatrix" approach (19, 20) using clade C consensus HIV-1 peptides custom-produced at the Massachusetts General Hospital Core facility. The megamatrix involved the use of 410 18-mer overlapping peptides (OLPs), overlapping by 10 amino acids that spanned the entire HIV-1 proteome. These were arranged into 72 pools of between 10 to 12 peptides in each pool such that an individual peptide was uniquely represented in two different pools. Each megamatrix peptide pool was co-cultured with 100,000 CD8-depleted cells per well in 96-well polyvinylidene plates (MAIP S45, Millipore, MA, USA) pre-coated with 100µl MAb1-D1k anti-IFN-γ monoclonal antibody (0.5µg/ml) (Mabtech, Stockholm, Sweden) overnight at 4ºC. Positive responses from initial megamatrix screening were confirmed using a separate ELISPOT assay at the single peptide level. For screening at the single peptide level, a total of 100,000 CD8-depleted PBMC per well were plated in 96-well polyvinylidene plates (MAIP S45, Millipore, MA, USA), pre-coated with 100µl MAb1-D1k anti-IFN-γ

monoclonal antibody (0.5µg/ml) (Mabtech, Stockholm, Sweden) overnight at 4ºC, in the presence or absence of specific HIV-1 derived OLPs in a final volume of 200 µl R10 medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml of penicillin, 50 µg of streptomycin/ml, and 10 mM HEPES). Each well contained a single OLP (10 µl) at a final concentration of 2 µg/ml for each individual peptide. Cells in medium without antigenic stimuli represented negative controls. The addition of phytohemagglutinin antigen (PHA) (Sigma-Aldrich, St Louis, MO, USA), at a concentration of 5µg/ml, served as a positive control for both cell viability and functionality of the immunoassay. The plates were incubated for 40 hours at 37°C, 5% CO_2 to elicit maximum cytokine secretion. The ELISPOT plates were then processed as previously described (21). An antigen-specific $CD4^+$ T cell response was considered positive only if it was at least ≥3 times the mean background and also ≥3 times the standard deviation of the SFC (spot forming cells) within the negative controls. The breadth of responses was defined as the sum of IFN-γ-positive peptide responses within a given individual across the entire HIV proteome or within a specified protein. Positive responses to two adjacent overlapping peptides were relatively rare in our cohort (2/30 responders), but where they occurred, the two overlapping peptides were treated as a single epitope to ensure that the breadth of $CD4^+$ T cell responses was not over-estimated.

2.4.3 HLA genotyping

High-resolution (4-digit allele) class I/II genotyping was carried out using standard sequence-based typing protocols (16). In brief, HLA class I genes were amplified by PCR, with primers spanning exons 2 and 3, with HLA class II typing based on exon 2.

Sequencing results were interpreted using the ASSIGN 3.5 software developed by Conexio Genomics (Fremantle, Western Australia, Australia).

2.4.4 HIV-specific $CD4^+$ T cell lines and HLA-DR restriction assay

A modified HLA-DRB1 restriction assay was utilized as previously described (12) to define restricting HLA class II alleles. In brief, frozen CD8-depleted PBMC samples from subjects with known $CD4^+$ T cell responses were used to generate $CD4^+$ T cell lines. CD8-depleted PBMC was stimulated with 10 μg/mL of peptide at a concentration of 2 million cells on a 24-well plate in R10 medium. The cells were incubated at 37°C and 5% CO_2. After 2 days, the cells were washed and fresh R10 medium supplemented with 100 U/mL recombinant interleukin-2 (IL-2) was added. The $CD4^+$ T cell lines were fed twice weekly with regular media replenishment. After 14 days, the T cell lines were simultaneously assessed for their specificity and HLA-DR restriction using a large panel of L cell lines (LCLs), which are mouse fibroblasts, each stably transfected with a single recombinant human HLA-DR molecule, as previously described (22). Each LCL was pulsed with 10 μg/mL peptide for 90 minutes at 37°C and 5% CO_2 and washed five times to remove free peptide. Clade C consensus sequence HIV-1 overlapping peptides were used in the assay. 10,000 peptide-pulsed LCL were co-cultured in triplicate with 50,000 of each respective $CD4^+$ T cell line per well on a pre-coated IFN-γ plate. As a negative control, each $CD4^+$ T cell line was co-cultured in triplicate with the appropriate LCL in the absence of peptide. As a positive control, PHA was added at 2μg/mL. The plates were incubated for 18 hours at 37°C and 5% CO_2 and processed as per usual ELISPOT protocol described above. The AID ELISpot reader (Autoimmun Diagnostika, Germany) was used

to determine the number of spot-forming cells (SFC) per 50,000 of the $CD4^+$ T cell line. HLA-DR restriction was considered positive if it was at least ≥3 times the mean background and also ≥3 times the standard deviation of the negative control wells.

2.4.5 MHC Class II tetramers

HIV p24 clade C Gag41 peptide (YVDRFFKTLRAEQATQDV) was complexed to recombinant human DRB1*11:01 or DRB1*13:01. In addition, HIV p24 clade C Gag37 peptide (WIILGLNKIVRMYSPVSI) was complexed to recombinant human DRB1*13:01 and DRB1*03:01. These tetramers are referred to as the specific HLA together with the peptide designation hereafter (e.g. DRB1*11:01-Gag 41). All tetramers were PE- and APC-conjugated at a concentration of either 454nM or 432nM. The tetramers were produced in the laboratory of Dr Søren Buus under previously described conditions (23).

2.4.6 Tetramer and surface staining

Unless stated otherwise, PBMC were incubated with MHC class II tetramers with a final staining concentration of 30 nM for one hour at 37°C in media. Post washing with cold 2% FCS/PBS buffer, antibodies to cell surface molecules were added and incubated at 4°C for 30 minutes. The following anti-human-conjugated antibodies were used: CD3-BV785-OKT3 (BioLegend, San Diego, CA, USA, Cat No. 317330), CD4-AF700-RPA-T4 (BD, San Jose, CA, USA, Cat No. 557922), CD8-BV605-SK1 (BD, Cat No. 564116).

PBMC's were also stained with live/dead fixable aqua cell viability dye (Invitrogen, L34957). Stained cells were washed with 2% FCS/PBS buffer and fixed with fixation medium A (Invitrogen, GAS001S-100). Data was acquired on a LSRFortessa™ (Serial # H647794E6049, BD). Flow data were analyzed using FlowJo software (Treestar FlowJo, version 10.1, Ashland, OR, USA).

2.4.7 Intracellular cytokine staining

Briefly, PBMC were incubated overnight with a clade C consensus Gag peptide pool or Gag41 as specific antigen/stimulus, and Staphylococcal enterotoxin B (SEB) (Sigma) as a positive control, in the presence of Golgi stop protein transport inhibitor (BD Biosciences) and Golgi plug protein transport inhibitor (BD Biosciences), according to manufacturer's instructions. Anti-CD107a-PECy5-H4A3 (BD, 555802) was also added at the beginning of the stimulation period. Post the stimulation, intracellular cytokine staining was performed according to the BD Biosciences ICS protocol. Unstimulated cells were used as a control. Cells were stained with live/dead fixable aqua dead cell viability dye (Invitrogen, L34957) and fluorescent antibodies against CD3-BV711-OKT3 (BioLegend, 317328), CD4-BV650-SK3 (BD, 563875), CD8-AF700-RPA-T8 (BD, 557945). Cells were permeabilized and fixed with BD intracellular staining reagents according to the manufacturer's instructions (BD Bioscience) and stained with cytokine-specific antibodies against IFN-γ – PECy7- B27 (BD, 557643), IL-21 – PE – 3A3-N2.1 (BD, 560463) and IL-2– FITC– 5344.111 (BD, 340448). Cells were acquired on an LSRFortessa™ (Serial # H647794E6049, BD). Flow data was analyzed using FlowJo software (Treestar FlowJo version 10.1).

2.4.8 *Ex vivo* expansion of HIV-specific CD4 T cells

MHC class II tetramer staining was performed prior to culture. Cells were then cultured at 37°C, 5% CO_2 for 14 days in the presence of HIV p24 clade C Gag 37 and 41 OLPs, as described above for HIV-specific $CD4^+$ T cell lines. On day 14, cells were washed three times with fresh R10 media and rested at 37°C, 5% CO_2 overnight in fresh R10 media. The following day, MHC class II tetramer staining was performed to determine the levels of expansion. The cells were then stimulated for 6 hours with the same peptides used for expansion, and cytolytic activity of the expanded cells was assessed by ICS staining forgranzymeA-AF488-CB9 (BioLegend, 507212), granzyme B-AF700-GB11 (BD, 560213) and CD107a-PECy5-H4A3 (BD, 555802).

2.4.9 Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Spearman's Rank correlation was used to assess the relationship between immune responses and viral load. Statistical analysis of significance was calculated using Kruskal Wallis test with Dunn's post hoc analyses for multiple comparisons. Significance of the non-random associations (contingency) between controllers and progressors were computed using Fisher's exact test, additionally Mann-Whitney U test was utilized to compare differences between the two groups. Statistical significance was set at $p < 0.05$.

2.5 Results

To better understand the role of HIV-specific $CD4^+$ T cell responses in HIV viral control and disease progression, we comprehensively investigated the specificity and functional properties of these responses in the setting of chronic clade C HIV-1 infection using IFN-γ ELISPOT and multi-parameter flow cytometry with MHC class II tetramers. Modified IFN-γ ELISPOT assays were used to identify immunodominant HIV-specific $CD4^+$ T cell epitopes and restricting alleles in our chronic infection cohort to inform the production of appropriate MHC class II tetramers. MHC class II tetramers were then utilized to detect low frequency populations of antigen-specific $CD4^+$ T cells (that may be missed in conventional peptide-stimulus based assays). This approach enabled the direct *ex vivo* characterization of antigen-specific HIV-specific $CD4^+$ T cell responses targeting immunodominant Gag epitopes.

2.5.1 Immunodominance hierarchy of $CD4^+$ T cell responses in chronic clade C infection.

Here, we evaluated a cohort of 72 clade C untreated, chronically HIV-infected individuals. HIV-specific $CD4^+$ T cell responses were initially screened against a panel of 410 pooled peptides spanning the entire HIV-1 clade C consensus sequence using the IFN-γ ELISPOT megamatrix assay. Results from the initial megamatrix screening were validated using confirmatory IFN-γ ELISPOT assays at the single peptide level. Our data demonstrate that HIV-specific $CD4^+$ T cell responses in chronic clade C infection dominantly target the Gag protein (Figure 2.1A). The most commonly targeted region in Gag was the p24 sub-protein (20/63 peptides), while the p17 and p15 regions of Gag were

sub-dominantly targeted by $CD4^+$ T cells (12/63 peptides each). The p24 region of Gag has also been shown to be immunodominant for HIV-specific $CD8^+$ T cell responses and these responses have previously been associated with viral control (24). However, no correlation was observed between the breadth of Gag-specific $CD4^+$ T cell responses (Spearman r= -0.17, p=0.42) , as well as the magnitude of these responses (Spearman r= 0.22, p=0.30), as measured by ELISPOT, and contemporaneous viral load. At the epitope level, our data showed that Gag41 within the p24 sub-unit is the most immunodominant peptide, with over 40% of subjects in our cohort showing a detectable response to this peptide (Table 2.2). A previous study found Gag6 in p17 to be the most dominant epitope (17). The difference may be due to differing proportions of controllers and progressors between the two studies.

The second most commonly targeted epitope was Gag40 (23%). Interestingly, only two responders targeted both the adjacent peptides, indicating the presence of distinct epitopes rather than targeting of the shared overlap represented in each peptide. Gag6, 25 and 37 were targeted independently by 20% of the 30 responders (Table 2.2). Additional immunodominant epitopes were located within the Nef region of the proteome (7/63 peptides). The results highlight a large number of detectable HIV-specific $CD4^+$ T cell responses across the HIV proteome, dominantly targeting Gag and Nef, consistent with previous studies (17). We observed that Gag-specific $CD4^+$ T cell responses are immunodominant and are directed at multiple distinct epitopes, with very few detectable $CD4^+$ T cell responses to Env or accessory proteins. This is in contrast to studies in chronic clade B infection, which have detected $CD4^+$ T cell responses targeting multiple epitopes within Env gp120 and rarely target OLP 25 in Gag (21), suggesting that the

immunodominance hierarchy of HIV-specific $CD4^+$ T cell targeting may be influenced by the infecting clade (25). Moreover, our study has identified 22 peptides that have not been described as immunodominant epitopes in previous clade C studies.

As the cohort was stratified into controllers and progressors, we next evaluated whether HIV-specific $CD4^+$ T cell responses within these two groups may be directed at different epitopes. However, there was no significant difference between the two groups (p=0.65) (Figure 2.1B). A few epitopes were exclusively targeted by each group, at low frequencies of 3%. There were particular epitopes that, albeit targeted by both groups, were more targeted by progressors, such as Gag25, which was targeted by only 3% of controllers and 13% progressors (Fischer's exact test p=0.01). Notably, the highly immunodominant Gag41 epitope was targeted by both controllers and progressors (at 23% and 17% responders, respectively), thus providing sufficient coverage of both groups for in-depth quantitative and qualitative evaluation of the Gag-41-specific $CD4^+$ T cell responses by MHC class II tetramers and the relationship of these responses to viral load.

Table 2.1 Summary of clinical characteristics of study participants[a]

Strata definition	No. of subjects	Median viral load (copies/ml) (range)	Median CD4 count (cells/µl)(range)	Screening (Elispot + HLA restriction)	Tetramer staining	Intracellular cytokine staining
Controllers <50- 2,000 copies/ml	34	400 (20-2,000)	684.5 (340-1,162)	30	11	6
Progressors 2,001 - >50,000 copies/ml	46	26800 (2,100-4,540000)	415.5 (225,934)	42	10	8
	80			**72**	**21**	**14**

[a]Patient demographics were as follows: Sex (76.3% female, n=61 and 23.8% male, n=19) and Race (97.5% African, 1.2% each Asian and Caucasian). All study participants included in tetramer and intracellular cytokine staining studies maintained viral control for a minimum of one year including at time point of analysis in the case of controllers and had comparable CD4 cell counts (p = 0.87). Individuals utilized for the initial screening and HLA restriction assays were not necessarily the same individuals used to perform tetramer and intracellular cytokine staining.

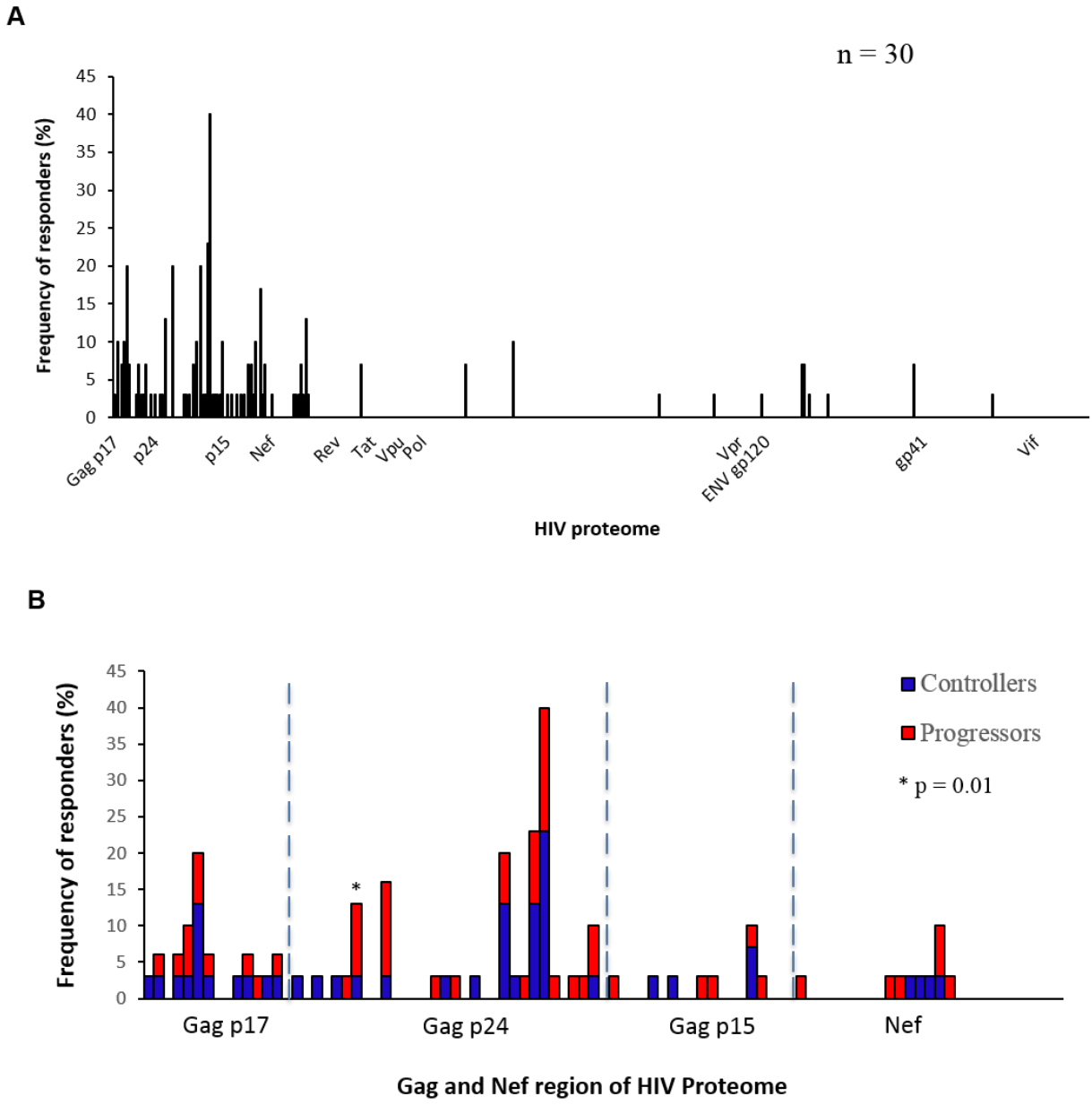

Figure 2.1 (A) Percentage frequency targeting of HIV-specific CD4+ T cell responses to overlapping peptides across the HIV-1 proteome. HIV-specific $CD4^+$ T cell responses were screened against a panel of 410 overlapping peptides (OLPs) spanning the entire HIV proteome. Labels on X-axis indicate the start of the relevant HIV protein or sub-protein. The frequencies of responders (30/72 individuals screened) with epitope-specific $CD4^+$ T cell responses are shown. (B) Percentages of epitope-specific CD4+ T cell responses targeting respective OLPs across the Gag and Nef proteins between controllers (blue bars; n=13) and progressors (red bars; n=17) from a chronically infected cohort. No significant differences were observed between the two groups (p=0.65; based on non-parametric two-tailed t-test). Further analysis for each individual response indicated a significant predominant targeting of OLP-25 by progressors compared to controllers (marked with an asterisk) in the Gag p24 region (Fisher's exact test, p = 0.01).

Table 2.2 Immunodominant peptides targeted by study participants[a]

Peptide	Protein	HXB2 location	OLP sequence	% Targeting in responders
41	p24	164-181	YVDRFFKTLRAEQATQDV	40
40	p24	156-173	GPKEPFRDYVDRFFKTLR	23
6	p17	37-51	ASRELERFALNPGLL	20
25	p24	46-62	GATPQDLNTMLNTVGGH	20
37	p24	133-150	WIILGLNKIVRMYSPVSI	20
62	p15	85-102	FLQSRPEPTAPPAESFRF	17
22	p24	23-40	WVKVIEEKAFSPEVIPMF	13
81	Nef	104-121	KKRQEILDLWVYHTQGYF	13
2	p17	9-26	RGGKLDKWEKIRLRPGGK	10
5	p17	32-46	KHLVWASRELERFAL	10
35	p24	117-134	WMTSNPPVPVGDIYKRWI	10
46	p24	200-217	TILRALGPGASLEEMMTA	10
60	p15	72-89	GKIWPSHKGRPGNFLQSR	10
168	RT	15-32	GMDGPKVKQWPLTEEKIK	10
4	p17	25-41	GKKHYMLKHLVWASREL	7
7	p17	42-58	ERFALNPGLLETSEGCK	7
11	p17	70-86	TGTEELRSLYNTVATLY	7
14	p17	92-110	IEVRDTKEALDKIEEEQNK	7
34	p24	109-126	STLQEQIAWMTSNPPVPV	7
57	p15	51-68	WKCGKEGHQMKDCTERQA	7
58	p15	59-75	QMKDCTERQANFLGKIW	7
64	p15	103-123	RFEETTPAPKQEPKDREPL	7
79	Nef	88-105	SFFLKEKGGLEGLIYSKK	7
104	Rev	72-88	PLQLPPIERLHIDCSES	7
148	protease	22-40	RANSPTSRELQVRGDNPR	7
289	gp120	1-7	MRVMGIQRNCQQWWRW	7
290	gp120	7-27	QRNCQQWWRWGILGFWML	7
336	gp120	351-371	EHFPNKTIKFAPSSGGDLEI	7

[a] Immunodominance hierarchy of frequently targeted HIV-specific $CD4^+$ T cell responses in chronic clade C HIV infection cohort. Percentages are calculated according to those individuals that demonstrated an HIV-specific $CD4^+$ T cell response.

2.5.2 HLA-DRB1*13:01 and DRB1*11:01 are the most frequent alleles among CD4$^+$ T cell responders.

Having identified immunodominant epitopes targeted in chronic clade C infection, we next performed class II HLA-typing as described in the methods to determine HLA-class II allele frequencies among the subjects with detectable responses by IFN-γ ELISPOT assay (n=30) (Figure 2.2A). Our data suggest that individuals with the strongest HIV-specific CD4$^+$ T cell responses possessed the class II HLA-DRB1*13:01 allele (n=12, 21% of responders). The next most common alleles were DRB1*11:01 and DRB1*03:02, expressed by 14% and 9% of responders, respectively. Interestingly, the most common DRB1 alleles among the responders in our study were also the most frequent in the larger chronic clade C cohort (n=439) whereby DRB1*03:02 was the most common allele observed (n=146, 33%) followed by DRB1*11:01 (n=111, 25%) and DRB1*13:01 (n=102, 23%) (16) . It is important to note that DRB1*13 alleles has been reported to confer a protective effect (13), with DRB1*13:03 in particular having been shown to mediate protection independent of ethnicity, sex, and viral clade (16).

2.5.3 HLA-DRB1 allele restriction characteristics of HIV-specific CD4$^+$ T cell responses in clade C infection.

Having defined the frequencies of these class II alleles in this chronic infection cohort, we next identified DRB1 restricting alleles using the HLA-restriction assay previously described by Ranasinghe *et al*. (12). We observed high levels of peptide promiscuity, which is known to be a distinctive feature of antigen-specific CD4$^+$ T cell recognition (22), with the presentation of a single peptide on numerous HLA class II variants. Gag41 in the Gag p24 region, previously identified as the most frequently targeted epitope by

HIV- specific $CD4^+$ T cells in clade B (17, 21), and shown in this cohort to also be the most frequently targeted, exhibited the highest levels of promiscuity, restricted by 7 different HLA-DRB1 variants (Figure 2.2B). The promiscuity in peptide-binding is likely mediated by the open conformation of HLA class II, allowing for long peptides recognized by $CD4^+$ T cells to extend beyond the HLA binding groove (12). Despite the high degree of HLA-DRB1 binding promiscuity, marked differences were observed in the number of peptides that were restricted by each DRB1 variant. Variants such as DRB1*14:01 and DRB1*04:01 had no detectable contribution to overall peptide restrictions as compared to DRB1*13:01 and DRB1*11:01 which had 9 and 4 HIV-specific peptides restricted, respectively.

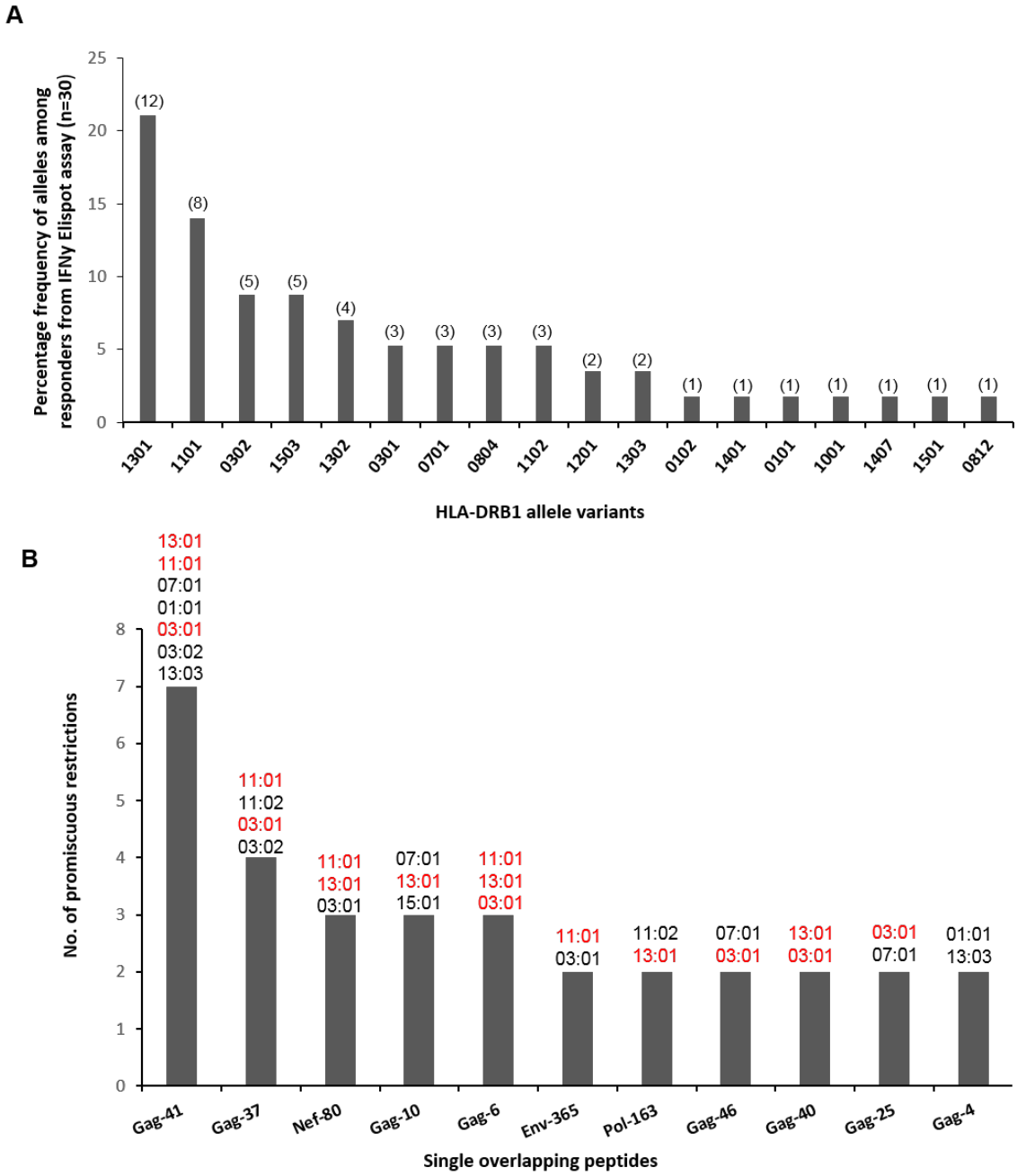

Figure 2.2 HLA class II restriction characteristics of HIV-specific CD4+ T cell responses in a chronic clade C infection cohort. (A) Percentage frequency of various HLA-DRB1 allele variants in $CD4^+$ T cell responders (n=30). The number of responders possessing each allele is indicated in parenthesis above each bar. (B) HLA-DRB1 allele restriction characteristics of HIV-specific $CD4^+$ T cell responses in clade C infection (promiscuous

epitopes). The restricting HLA alleles for each overlapping peptide are indicated above each bar. Alleles that are highlighted in red were used to generate MHC class II tetramers.

2.5.4 Use of HLA class II tetramers for sensitive detection of HIV-specific $CD4^+$ T cells.

Having identified immunodominant $CD4^+$ T cell epitopes and the most common restricting HLA DRB1 allele variants in a large number of clade C responders (30/72 individuals tested), the information was used to successfully generate and validate the following tetramers; DRB1*03:01 (Gag37), DRB1*11:01 (Gag41), and DRB1*13:01 (Gag37 and Gag41), each conjugated to both PE and APC (Table 2.3). The newly synthesized tetramers were then used to evaluate the role of HIV-specific $CD4^+$ T cell responses in the pathogenesis of clade C HIV-1 infection. To our knowledge, these particular MHC class II tetramers have not been synthesized before in the setting of clade C infection. Furthermore, our study is the first to generate and utilize the DRB1*11:01 tetramer to Gag41 to study $CD4^+$ T cell responses.

Given the low frequency of HIV-specific $CD4^+$ T cell responses (26), a dual PE and APC tetramer staining strategy gating on double staining cells was used to increase our ability to detect genuine $tetramer^+$ cells and minimize non-specific background staining (Figure 2.3A). Class II tetramer staining was also assessed on $CD8^+$ T cells for each sample to ensure that there was minimal non-specific tetramer binding (Figure 2.3A). The specificity was confirmed by staining 3 HIV negative samples, none of which stained positive (representative data shown in Figure 2.3B). Low frequency class II DRB1*03:01, *11:01 and *13:01 tetramer binding $CD4^+$ T cells were readily detectable in 11 controllers

expressing requisite DRB1 alleles at a median frequency of 0.31% (IQR 0.045% to 0.41%) of the total $CD4^+$ T cells. Notably, the median frequency of class II tetramer binding cells in 10 chronic progressors expressing the requisite DRB1 was 0.036% (IQR 0.015% to 0.27%, p=0.0001) which is 10 fold lower than in controllers (Figure 2.3C). Analysis of the DRB1*11-Gag41 tetramer binding responses also showed higher frequency in controllers than progressors (p=0.0005, Figure 2.3D). The controllers and progressors were matched for expression of DRB1*11, with an equal number of each expressing this allele. These data suggest that HIV-specific $CD4^+$ T cell responses targeting immunodominant Gag41 in the context of DRB1*11 expression are associated with maintenance of low viremia during chronic clade C HIV infection.

Table 2.3 MHC Class II tetramers[a] used for analysis of tetramer$^+$ $CD4^+$ T cells

HIV Tetramer	Sequence	OLP	Clade
HLA-DRB1*03:01	WIILGLNKIVRMYSPVI	Gag-37	C
HLA-DRB1*11:01	YVDRFFKTLRAEQATQDV	Gag-41	C
HLA-DRB1*13:01	WIILGLNKIVRMYSPVI	Gag-37	C
HLA-DRB1*13:01	YVDRFFKTLRAEQATQDV	Gag-41	C

[a]MHC class II tetramers were produced according to allele variants associated with most frequently targeted peptides and highest number of responders.

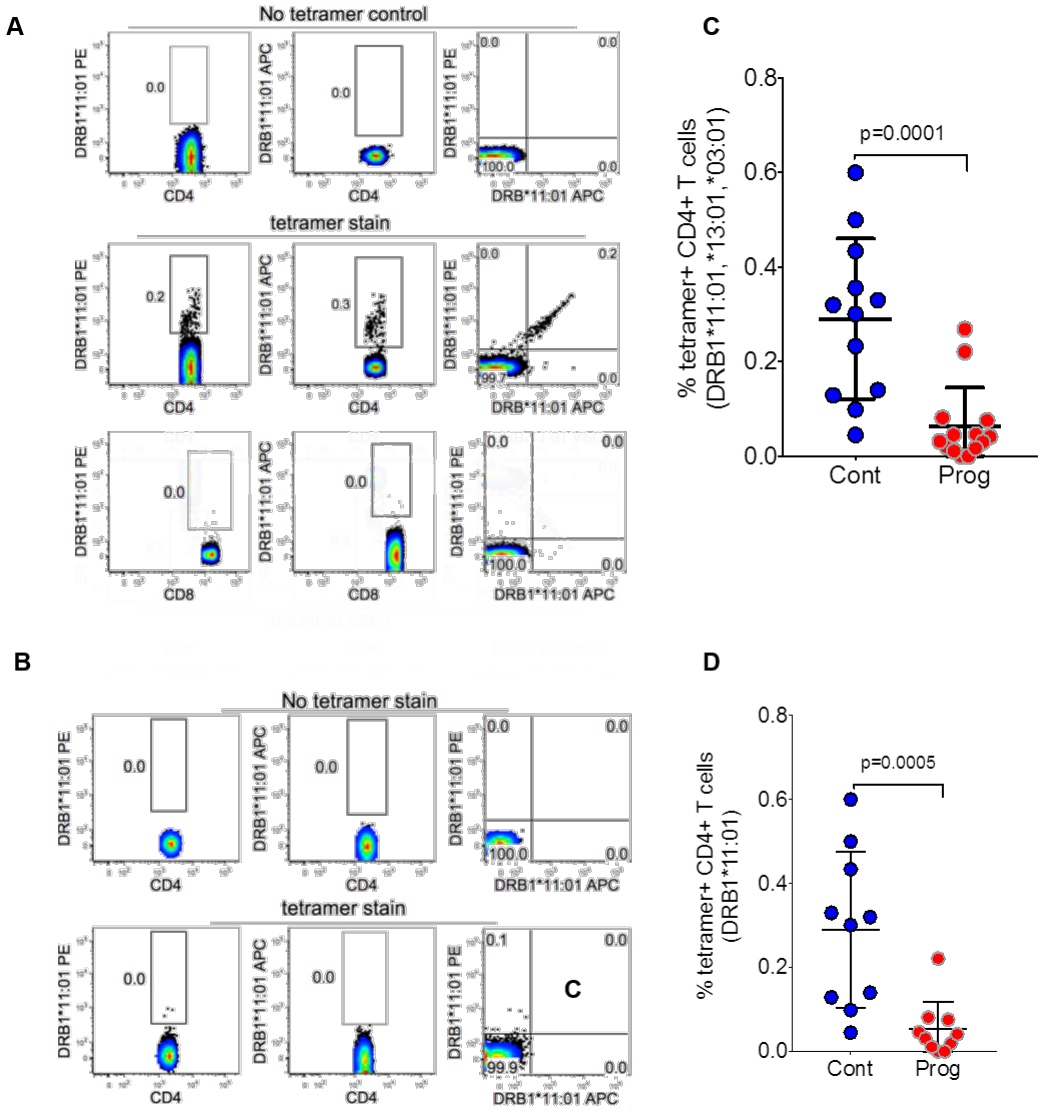

Figure 2.3 Frequency of tetramer positive CD4+ T cells in controller and progressor chronically infected individuals and HIV negative subjects. (A) Representative flow plots indicating a dual PE and APC tetramer staining strategy gating on double stained cells, which was used to increase the ability to detect genuine tetramer+ cells and minimize non-specific background staining on $CD8^+$ T cells, from an HIV infected individual and (B) from a HIV negative individual. (C) Differences in the percentage of $CD4^+$ $tetramer^+$ T cells between controllers (blue) and progressors (red) for all class II DRB1*03:01, *11:01 and *13:01 tetramers utilized (p= 0.0001) and (D) for only DRB1*11:01 tetramer (p= 0.0005).

2.5.5 Dominance of mono-specific HIV-specific CD4$^+$ T cell responses in controllers and progressors.

Given that tetramers can bind antigen-specific cells irrespective of function, we next examined the range of CD4$^+$ T cell functionalities that constitute tetramer$^+$ responses. PBMCs were stimulated withHIV clade C Gag pool or Gag 41 or 37 OLPs. Multicolour flow cytometry was used to detect and enumerate key effector CD4$^+$ T cell functions in individuals with tetramer$^+$ responses. We measured markers that define key CD4$^+$ T cell functional subsets. Our analysis included the degranulation marker CD107a, used to enumerate cytolytic CD4$^+$ T cells (7), IFN-γ and IL-2 used to identify Th-1 helper cells (27), and IL-21 was included to identify cells that augment B cell and CD8$^+$ T cell effector functions (9, 28). To investigate if any of the measured functions impact virus suppression, we compared the expression level of each marker in controllers and progressors. HIV controllers' responses exhibited higher mean/median expression of each of the four functions compared to progressors in response to stimulation with a mix of Gag peptides (Figure 2.4A,B), or in response to the immunodominant Gag41 (Figure 2.4C), or in response to SEB (Figure 2.4D), yet only SEB reached statistical significance after correction for multiple comparisons. Previous studies show that the ability to simultaneously elaborate multiple functions is associated with protective immunity against several viral infections (Reviewed by Harari *et al*., (29)), moreover, multifunctional CD4$^+$ T cell responses were associated with protection in the RV144 trial (30-32). Therefore, we next examined if certain combinations of functions were associated with slow HIV disease progression. Combinatorial polyfunctionality analysis showed a preponderance of mono-specific responses for both controllers and progressors (Figure 2.4E, F). The high frequency of mono-functional cells highlights the limitation

of assays which rely on a single function as a surrogate for the entire HIV-specific CD4^{+} T cell response.

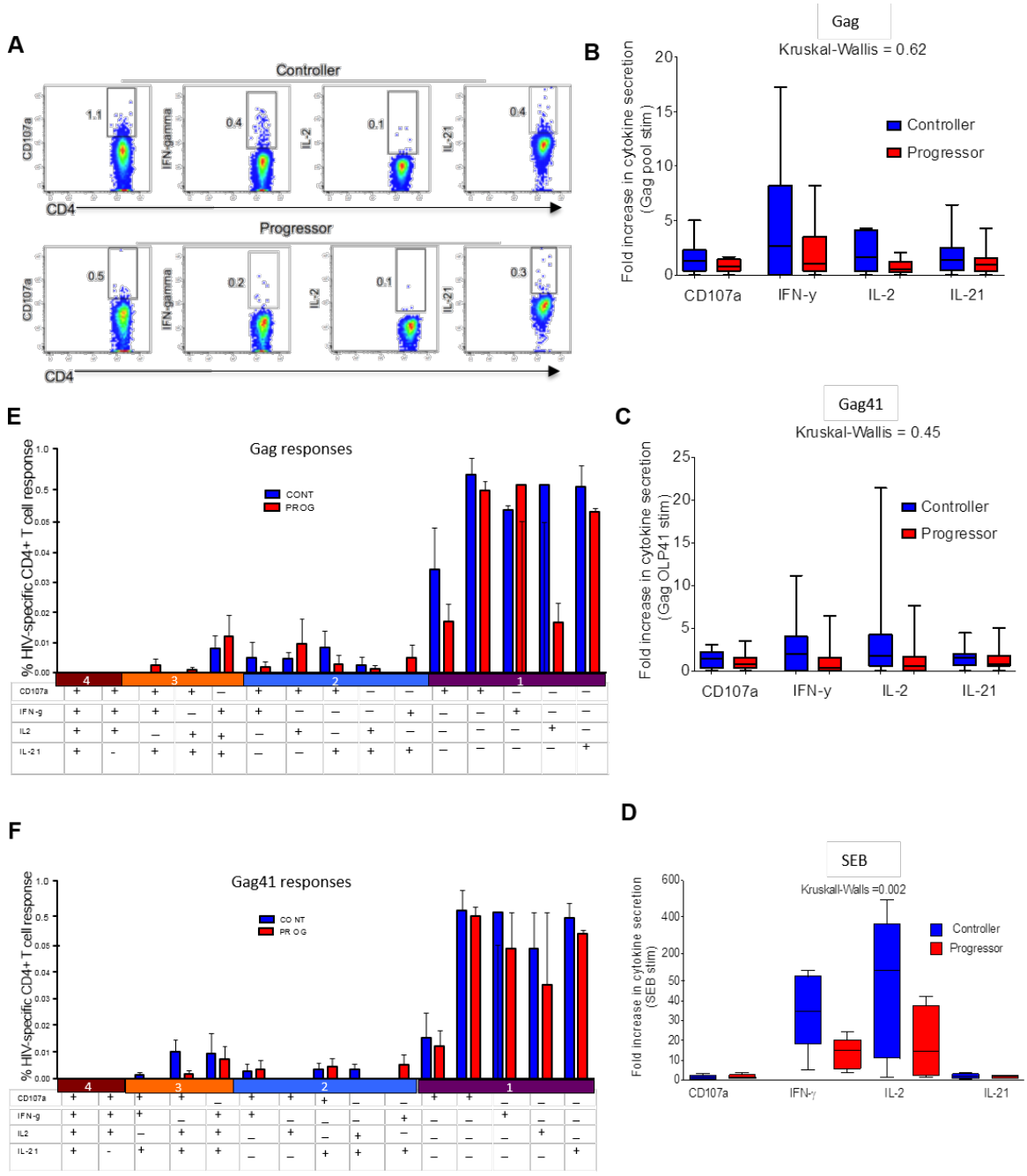

Figure 2.4 HIV-specific CD4+ T cell polyfunctional responses (A) Graphical representation of intracellular cytokine staining of controller and progressor individuals based on CD107a stimulation. Expression of CD107a, IFNγ, IL-2 and IL-21 were

measured in 14 chronically infected subjects, divided into controllers (blue) and progressors (red) in responses to Gag pool stimulation (B), to Gag41 stimulation (C) and to SEB stimulation (D). (E, F) The bars depict the frequency of $CD4^+$ T cells expressing a combination of functions indicated below the x-axis.

2.5.6 Gag-specific tetramer$^+$ CD4$^+$ T cell responses inversely correlated with HIV viral load.

Given the observed link between $CD4^+$ T cells responses and maintenance of low viremia, we next interrogated whether maintenance of Gag-specific (tetramer$^+$) $CD4^+$ T cell responses during chronic HIV infection influence disease progression. The frequency of Gag-specific $CD4^+$ T cells measured by four MHC class II tetramers negatively correlated with contemporaneous viral load (Spearman r=-0.5, p=0.005) when both controllers and progressors were analyzed together (Figure 2.5A). However, separate analysis of controllers (Spearman r= 0.3, p=0.3) and progressors (Spearman r= -0.01, p=0.9) responses did not reach statistical significance, which may be due to the small sample size. Furthermore, a negative correlation was also observed between the frequency of tetramer$^+$ $CD4^+$ T cells targeting DRB1*11-Gag41 and contemporaneous viral load (Spearman r=-0.5, p=0.02) (Figure 2.5B). Interestingly IFN-γ secretion measured by ICS exhibited a similar negative correlation with viral load (Spearman r=-0.7, p=0.003) (Figure 2.5C). However, IL-2, IL-21 and CD107a did not correlate with contemporaneous viral load (Data not shown). This observation prompted us to examine the relationship between tetramer$^+$ $CD4^+$ T cells and cytokine$^+$ cells following stimulation with Gag peptides. Interestingly, only IFN-γ secreting cells correlated with tetramer$^+$ cells (Spearman r=0.7, p=0.001) (Figure 2.5D). There was no correlation between IL-2 or IL-

21 and frequency of tetramer$^+$ responses (Figure 2.5 E, F) and only borderline significance of an association with the CD107a degranulation marker (p=0.04, Figure 2.5G). The plausible explanation for the lack of positive correlation between tetramer$^+$ cells and these cytokine$^+$ cells may be because some IL-2 and IL-21 secreting cells recognized different epitopes within Gag or alternatively, were restricted by other class II alleles.

Overall, these data highlight the heterogeneity of the effector functions of HIV-specific CD4$^+$ T cells and illustrates the advantage of using tetramers to identify antigen-specific cells without relying on function. Furthermore, these data suggest that immunodominant Gag-specific CD4$^+$ T cell responses are linked with better control of HIV, indicating that these responses may play a role in suppressing HIV replication by antiviral effector functions.

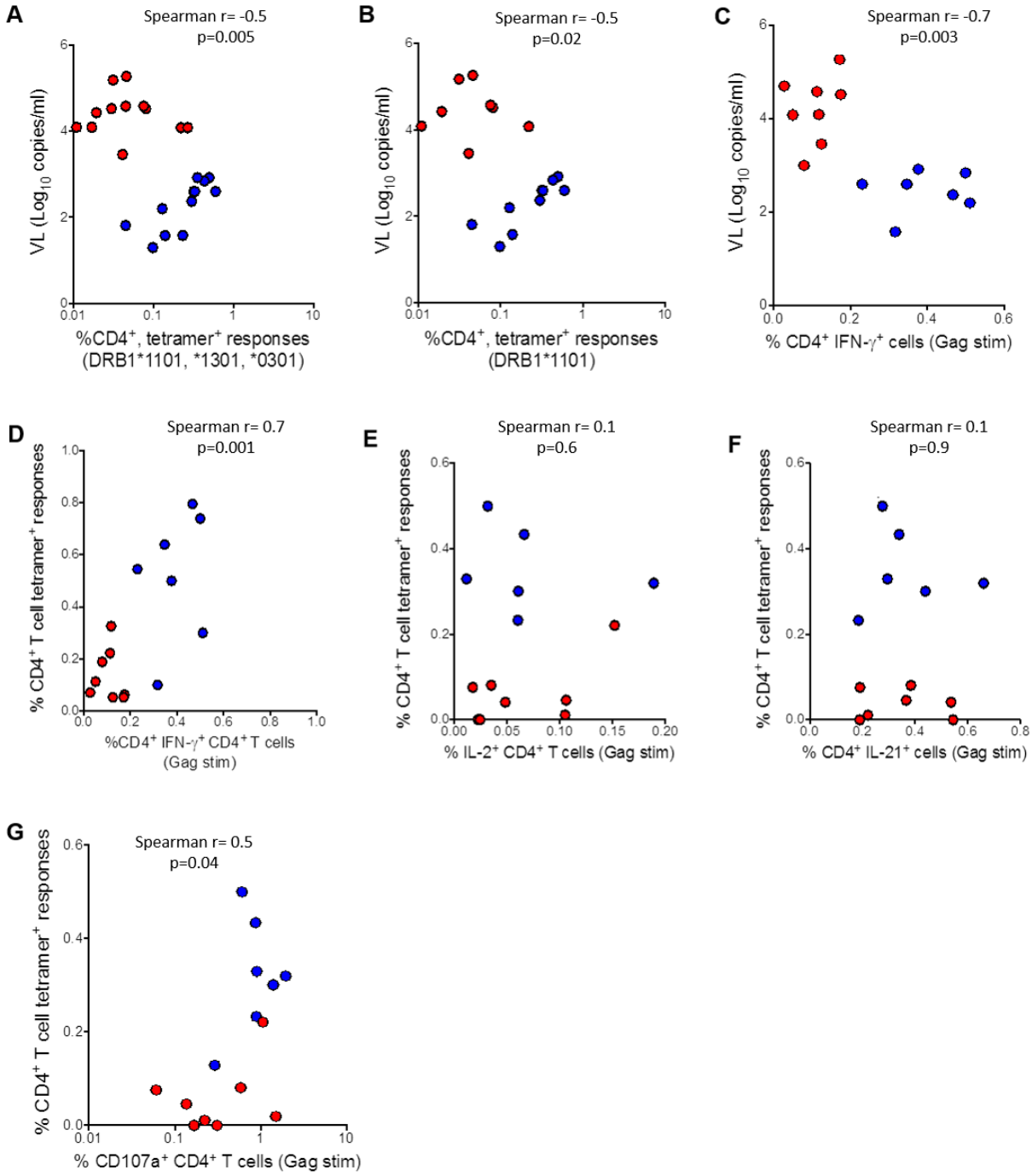

Figure 2.5 Gag-specific tetramer+ CD4+ T cell responses correlates with markers of HIV disease progression. (A) The frequency of HIV-specific CD4+ T cells measured by four class II tetramers negatively correlated with contemporaneous viral load (Spearman –r =0.7, p=0.005). (B) A negative correlation was also observed between the most dominant

Gag41 response (DRB1*11:01) and contemporaneous viral load (C) IFN-γ secretion measured by ICS exhibited a similar negative correlation with viral load (Spearman r=-0.7, p=0.003). In addition, the relationship between tetramer$^+$ CD4$^+$ T cells and cytokine$^+$ cells following stimulation with HIV peptides was analysed (D-G).

2.5.7 *Ex vivo* expanded HIV-specific CD4$^+$ T cells display increased cytolytic activity in controllers.

The low frequency HIV-specific CD4$^+$ T cells detected by class II tetramers may be mostly low frequency memory responses, which have low potential to secrete cytokines *ex vivo*. Moreover, several studies in HIV-specific CD8$^+$ T cell responses have demonstrated that *ex vivo* expansion is needed to accurately measure the cytolytic potential of memory responses (33, 34). We reasoned that this might also be true for HIV-specific CD4$^+$ T cell responses. Thus, we next performed stimulated expansion of low frequency HIV-specific CD4$^+$ T cells that were detected by tetramers and evaluated the cytolytic potential of these expanded cells. Cultured stimulation of HIV-specific CD4$^+$ T cells from controllers resulted in significant expansion of tetramer$^+$ populations compared to baseline levels (p=0.03, Figure 2.6A and B), whereas class II tetramer$^+$ populations from progressors expanded poorly (p=0.2) (Figure 2.6C and D). Assessment of the cytolytic activity of *ex vivo* expanded HIV-specific CD4$^+$ T cells by intracellular cytokine staining (Figure 2.6E) showed that controllers had significantly higher proportion of HIV-specific cells expressing granzyme A (p=0.03) and granzyme B (p=0.01) compared to progressors (Figure 2.6F). No significant differences were observed for CD107a between the groups. Taken together these data suggest that HIV-specific CD4$^+$ T cells from controllers have higher proliferative and cytolytic functions when compared to

progressors, suggesting their potential contribution to direct antiviral activity during chronic HIV infection.

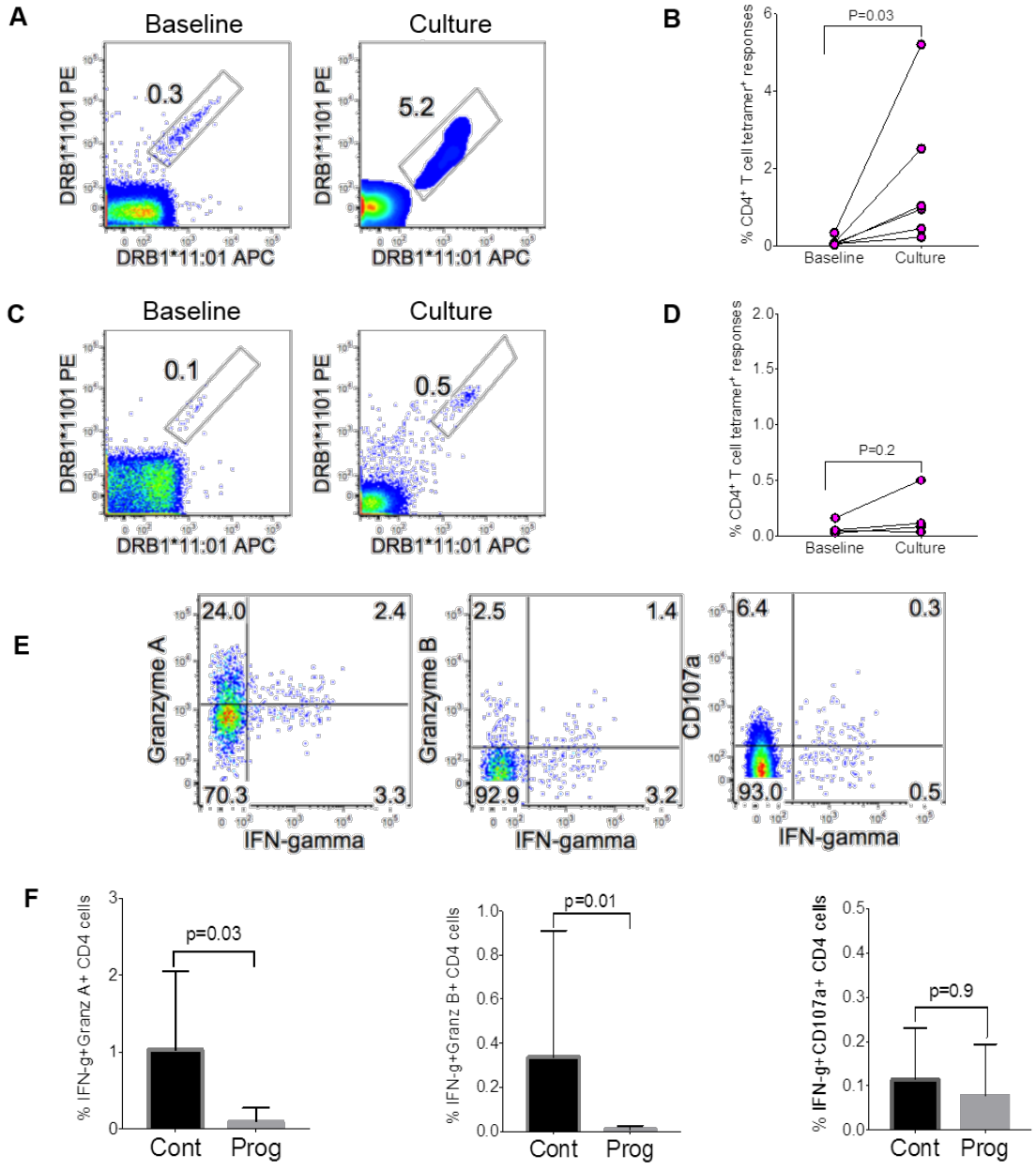

Figure 2.6 Frequency of tetramer positive CD4+ T cells in controller and progressor chronically infected individuals following cell expansion. (A) Representative flow plots indicating a dual PE and APC tetramer staining strategy gating on double stained cells at

baseline and 2 weeks following expansion of peptide specific T cell lines, from an HIV controller and (C) from an HIV progressor (B) Differences in the percentage of $CD4^+$ $tetramer^+$ T cells in 3 controllers, indicating responses at baseline and 2 weeks after expansion in culture (red) for all class II DRB1*03:01, *11:01 and *13:01 tetramers utilized ($p= 0.02$) and (D) for 3 progressors, using the same principle. (E) Representative flow plots indicating functional responses by IFNγ – specific $CD4^+$ T cells to Granzyme A, Granzyme B and CD107a following 2 weeks of expansion and (F) Comparison of IFNγ – specific $CD4^+$ T cells between controllers and progressors to Granzyme A ($p= 0.03$), B ($p= 0.01$) and CD107a ($p=0.9$).

2.6 Discussion

Together these data demonstrate an association between the frequency of HIV-specific $CD4^+$ T cell responses targeting distinct immunodominant epitopes in Gag and immune control of clade C HIV viremia. In particular, we show that $CD4^+$ T cells targeting Gag41 in the context of HLA-DRB1*11 are associated with immune control of HIV (in HLA-matched individuals). To our knowledge, this is the first study to demonstrate that $CD4^+$ T cells directed against a single peptide-HLA class II specificity are associated with low HIV viremia. Indeed, although $CD8^+$ T cell targeting of HLA class I-peptide complexes such as B*27-KK10 are well-known to associate with spontaneous control of HIV infection (35, 36), previous studies assessing the contribution of HIV-specific $CD4^+$ T cells to HIV immune control have focused either on the expression of distinct HLA-DRB1 alleles (12-14, 16), or identified particular $CD4^+$ T cell epitopes such as Gag41 targeted at different stages of disease (17, 21, 25, 37). The finding that there is an association between the frequency of HIV-specific $CD4^+$ T cells targeting the DRB1*11-

Gag41 complex and low HIV viremia (in our assessment of 20 HIV-positive HLA-matched individuals) is consistent with our hypothesis that the maintenance of robust HIV-specific $CD4^+$ T cell responses to the Gag p24 region may contribute to immune control of chronic HIV replication, rather than to fuel disease progression through infection of activated HIV-specific $CD4^+$ targets.

It is important to note that although the frequency of HIV-specific $CD4^+$ T cells measured by four MHC class II tetramers negatively correlated with contemporaneous viral load when controllers and progressor were analyzed together, the correlation did not reach statistical significance when each group was analyzed separately. This may due to smaller sample size. Nevertheless, the fact that controllers maintained higher frequencies of class II tetramer$^+$ CD4 T cells compared to progressors, suggests an association between higher frequencies of virus-specific $CD4^+$ T cells responses and maintenance of lower viral loads.

Although our analysis of peptide-stimulated HIV-specific $CD4^+$ T cells found that they predominantly secreted IFN-γ directly *ex vivo*, we speculate that *in vivo* these epitope-specific $CD4^+$ T cells may provide superior helper activity to HIV-specific $CD8^+$ T cells that enhance suppression of HIV replication through a cocktail of cytokine signals, or they may directly kill HIV-infected $CD4^+$ T cell and macrophage targets. Indeed, the finding that our *ex vivo* expanded $CD4^+$ T cell lines expressed granzyme A and B, were significantly enhanced in HIV controllers, support the hypothesis that $CD4^+$ T cells targeting immunodominant Gag peptides may exhibit functions that contribute to immune control of HIV (7, 38). However, since it is not possible to determine cause versus

consequence in our cross-sectional analysis, it remains unclear whether weak HIV-specific T cell responses are a result of ongoing viral replication, or alternatively whether the superior frequency and function of these responses either contributes to effective immune control of the virus, or that some T cell specificities may simply be better preserved in individuals with low viremia and low immune activation.

The improved elucidation of HIV-specific $CD4^+$ T cell responses and enhanced understanding of how such $CD4^+$ T cells may promote the generation of protective $CD8^+$ T cell and B cell responses or mediate a direct antiviral role is highly relevant for future HIV vaccine design. Currently, developing assays that can fully quantify all the relevant epitope specific responses remains a major challenge in natural infection and vaccine studies because of the tremendous complexity of $CD4^+$ T cell biology. This study employed MHC class II tetrameric complexes to enumerate and characterize Gag p24-specific $CD4^+$ T cell populations *ex vivo* in HIV infected individuals bearing HLA DRB1*11:01, DRB1*13:01 and DRB1*03:01 alleles. Overall, our study shows that MHC class II tetramers are very sensitive methods for detecting very low cell frequencies of HIV-specific $CD4^+$T cells directly *ex vivo*. Our approach provides an alternative method of identifying antigen-specific $CD4^+$ T cell without relying on function and removes the bias associated with *in vitro* stimulation required for functional assays and the limitation associated with only detecting subsets of cells capable of secreting cytokines. Yet, a notable limitation of our study is the lack of availability of a wide range of class II tetramers complexed with other MHC class II DRB1 alleles, or the less-well characterized DP and DQ alleles. Notwithstanding this limitation, the ELISPOT screening data show that we were able measure most of the immunodominant responses, which are restricted

by DRB1 alleles pertinent to this African population for which we successfully generated a subset of the relevant class II tetramers. Taken together, these data demonstrate that HIV-specific $CD4^+$ T cell responses restricted to DRB1*11-Gag41 are associated with immune control of HIV-1 infection and highlight the advantages of class II tetramer technology in evaluating HIV-specific $CD4^+$ T cells responses in future natural infection and vaccine studies.

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Chapter 3 Overview

Our results in chapter 2 demonstrated that usage of MHC class II tetramers is very sensitive for directly detecting *ex vivo* HIV-specific CD4+ T cells present at very low frequencies. Our approach provides an alternative method of identifying antigen-specific CD4+ T cells without relying on function and removes the bias associated with *in vitro* stimulation of cells for functional assays and the limitation of only detecting cell subsets capable of secreting cytokines.

Having validated these tetramers in peripheral blood, in our clade C chronic infection cohort, we next wanted to further our investigations and utilize these MHC class II tetramers in interrogating low frequency HIV-specific regulatory T cell populations in lymph nodes and peripheral blood. Among the CD4+ T cell populations, regulatory T cells (TREGs), have been shown to be essential for the maintenance of self-tolerance and immune homeostasis. More recently, evidence suggests that B-cell follicles and germinal centers within secondary lymphoid tissue contain a novel subset of TREGs, termed follicular regulatory T cells (TFR). Much of the work conducted on CD4+ T cell subsets within lymph nodes focuses on T follicular helper (TFH) cells, with limited knowledge available on the cells that regulate the functional capacity of these cells.

In chapter 3 of this thesis, we focus on phenotypically, transcriptionally and functionally characterizing TFR as a distinct subset from TFH in peripheral blood and lymphoid tissue. Furthermore, we assess the antigen-specificity and impact of disease on these subsets.

CHAPTER 3: COMPREHENSIVE PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF REGULATORY CD4+ T CELL SUBSETS IN PERIPHERAL BLOOD AND LYMPH NODE TISSUES IN HIV-1 CLADE C INFECTION

Faatima Laher[1], Samuel W. Kazer[2,3], Toby Aicher[2,3], Omolara Baiyegunhi[1], Funsho Ogunshola[4], Thandeka Nkosi[4], Anele Mbatha[1], Trevor Khaba[1], Thandekile Ngubane[1], Ismail Jajbhay[4], Johan Pansegrouw[4], Nasreen Ismail[1], Krista Dong[2], Alex K. Shalek[2,3], Bruce D. Walker[1,2,6], Thumbi Ndung'u[1,2,5,7], Zaza M. Ndhlovu[1,2,5]

[1]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

[2]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA, USA

[3]Department of Chemistry and Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA

[4]Prince Mshiyeni Memorial Hospital, KwaZulu-Natal Department of Health, Durban, South Africa

[5]Africa Health Research Institute (AHRI), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

[6]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA

[7]Max Planck Institute for Infection Biology, Berlin, Germany

Keywords: HIV, T regulatory cells, T follicular regulatory cells, human lymph nodes

3.1 Abstract

Regulatory T cells (TREGs) play a fundamental role in the maintenance of tolerance as well as in the control of immune activation, particularly during chronic infections. The role of TREGs during HIV infection is still poorly understood, with both detrimental and beneficial roles having been proposed. More recently TREGs have been shown to be precursor cells for T follicular regulatory (TFR) cells, a subset of regulatory cells shown to enter the B cell follicles and modulate T follicular helper (TFH) cells and B cells to control the germinal center (GC) reaction. Being a relatively newly identified subset, much remains to be discovered regarding TFR biology, phenotype and function in HIV-1 infection. Thus, we set out to elucidate the phenotypic and transcriptional markers that better define TFR and distinguish them from regulatory (TREG) and T follicular helper cells (TFH cells), using lymph nodes (LN) and peripheral blood (PBMC) samples.

We studied 31 individuals, grouped into HIV uninfected and HIV infected early ART initiators and untreated individuals, from a clade C HIV infection cohort in Durban, South Africa. TREG (CD4+FOXP3+), TFH (CD4+CXCR5+PD-1) and TFR (CD4+CXCR5+PD-1+CD25+CD127-) phenotypes were defined using flow cytometry. Transcriptional signatures and unique transcriptional markers of TFH and TFR were assessed using single cell RNA-sequencing Seq-Well assays and their antigen-specificity were determined using MHC class II tetramers.

Frequencies of total TREG, TFH and TFR cells were comparable in LNs of HIV uninfected individuals, with increased TFH compared to TFR observed in PBMCs of the same individuals (p=0,002). TFR shared phenotypic and transcriptional markers with

both TREGs and TFH as determined by both flow cytometry and Seq-Well assays. However, these cells are a distinct subset of CD4+ T cells as seen by unique identifying transcriptional signatures determined by Seq-Well. Antigen-specific TFR were identified in LN of HIV-infected individuals, with higher frequencies observed during untreated HIV infection. Lastly, regulatory subsets, TREGs and TFR did not show any changes in frequencies upon HIV infection or treatment initiation.

Our results show TFR are indeed a distinct CD4+ regulatory T cell subset within lymph nodes and peripheral blood. Better understanding of regulatory subsets in terms of phenotypic heterogeneity and antigen-specificity, particularly within LN, will enhance the ability to identify and track these cells, and will open up opportunities for manipulation for immunotherapies advantageous to the HIV-infected host.

3.2 Introduction

CD4+ T cell responses are essential in the development of effective cellular and humoral immune responses against viral infections. Amongst the CD4+ T cell populations, regulatory T cells (TREGs) have been shown to play a crucial role in the maintenance of tolerance as well in the control of immune activation, more so during chronic infections (1-3). TREGs have been associated with various roles during HIV infection and their function may be affected by the initiation of/or ongoing therapy (4-6). Within the regulatory networks, T follicular regulatory cells (TFR) have recently been described as a novel subset of TREGs which has an overlapping transcriptional pattern with T follicular helper cells (TFH) (7-9). A recent study showed that CXCR5 expressing TFR

originate from TREG precursors and regulate GC responses through interactions with TFH (10). Furthermore, studies in SIV have further described the importance of the TFR: TFH ratio in regulating autoreactive antibody production and a decreased ratio could possibly lead to unchecked expansion of TFH and GC B cells (11, 12).

Antiretroviral therapy (ART) has been the most successful intervention for improving life expectancy of HIV-1 infected individuals, allowing for reconstitution of immunity and improved adaptive immunity amongst numerous other benefits (13, 14). ART has been shown to have a significant influence on TREG cell numbers in HIV-infected subjects, decreasing or even normalizing their frequencies to comparable levels with healthy controls (15, 16). With other studies reporting that decreased TREG cell numbers were found in blood and lymphoid tissues of treated compared to untreated HIV-1 infected subjects (1, 17).

The role of TREGs in HIV-1 immune pathogenesis has been widely studied in peripheral blood, but much less is known about the role of these regulatory subsets in secondary lymphoid organs, where they more likely influence B cells and CD8+ T cell functions (18). Understanding the mechanisms by which regulatory CD4+ T cells, particularly TFR, exert their influence in lymphoid tissues is an important research area with broad implications for the development of therapeutic strategies for many diseases including immune mediated diseases, such as HIV-1. Therefore, in-depth understanding of how this cell subset modulates induction of adaptive immune responses during HIV infection is warranted.

To assess heterogeneity and antigen-specificity of regulatory cells within lymph nodes (LN) and peripheral blood (PBMC), we recruited 31 individuals (8 HIV negative and 16 HIV-infected early treated and 7 HIV-infected chronic untreated). Excisional LN and paired PBMC were obtained. Flow cytometry, single cell RNA-sequencing (Seq-Well) and MHC class II tetramers were used to define the phenotype, transcriptional signature, and antigen-specificity of TREG, TFH and TFR cells.

Based on a combination of flow cytometric and transcriptional analyses, TFR cells were identified as a distinct subset from TREG and TFH cells. Antigen-specific CD4+ T cells were detected, with higher frequencies in LNs compared to PBMC. Furthermore, comprehensive analysis of these CD4+ T cells showed TREG, TFH and TFR cells were low frequency populations in LN/PBMC. Additionally, analysis of the impact of HIV infection and treatment initiation showed no significant changes in frequencies in regulatory subsets within LNs or PBMCs. Taken together, these results document three distinct CD4+ T cell subsets in human LNs and peripheral blood. Furthermore, these data set a foundation for future detailed characterization on the role of TFR in HIV-1 infection, where these cells could be used as a therapeutic target to enhance anti-viral humoral immunity and vaccine efficacy.

3.3 Materials and Methods

3.3.1 Cohort characteristics

A total of 31 participants were used from the HIV Pathogenesis Programme (HPP) lymph node study cohort. Informed consent was obtained from all study participants prior to

enrolment in the study. Excisional lymph nodes (LN) and paired blood samples were obtained from study participants recruited in an on-going protocol approved to recruit HIV uninfected and HIV-infected individuals from study sites based in the Umlazi Township, Durban, South Africa. In this study, we used 8 HIV negative and 16 early treated donors recruited from the FRESH cohort (14) and 7 chronic untreated participants recruited from the HPP Acute infection cohort (19). Inguinal, axillary or cervical LN were excised and paired peripheral blood samples were collected from study participants. Measurements of CD4+ T cell counts, and viral loads were performed by Global Clinical and Viral laboratories (Durban, South Africa). The Biomedical Research Ethics (BREC) of the University of KwaZulu-Natal and the institutional review board (IRB) of Massachusetts General Hospital approved this study.

3.3.2 Lymph node and peripheral blood sample processing

Excised LN were sliced into two pieces; approximately 1/3 of the LN was fixed in 10% formal-saline (Sigma-Aldrich, St Louis, Missouri, USA) for downstream microscopy studies, with the remaining 2/3 mechanically processed to release LN mononuclear cells (LMCs) as described by Schacker *et al*., (20). In brief, the macerated LN was passed through a 70uM cell strainer (BD, 352350) into a collection tube containing R10 media. Cells were pelleted and collected by centrifugation [1800 RPM, 6 minutes (min), room temperature (RT)]. Peripheral blood mononuclear cells (PBMCs) were isolated from patient blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). LMCs and PBMCs were cryopreserved in liquid nitrogen.

3.3.3 Flow cytometric analysis

Freshly isolated and frozen LMCs and PBMCs were phenotypically and functionally characterized using multi-parameter flow cytometry. Standardized protocols using the transcription factor diluent buffer set (BD Biosciences, San Jose, CA, 562574) were utilized. In brief, for surface staining, cells were incubated for 20 min at RT in the dark in staining buffer [2% fetal calf serum (FCS) in phosphate buffered saline (PBS) buffer] containing the following antibodies: CD3-AF700-UCHT1 (BD Biosciences, 557943), CD4-BV650-SK3 (BD Biosciences, 563875), CD8-PE- RPA-T8 (BD Biosciences, 555367), CD25- PE-Cy5 -M-A251 (BD Biosciences, 555433), CD127-BV785-A019D5 (BioLegend, San Diego, CA, USA, 351329), CXCR5-AF488-RF8B2 (BD Biosciences, 558112), PD-1 – BV421-EH12.2H7 (BioLegend, 329920), CXCR3-BV605-G025H7 (BioLegend, 353728), ICOS-AF647-C398.4A (BioLegend, 313516) and CCR7- PerCP-Cy5.5 – T68 (BioLegend, 335605). Cells were also stained with live/dead fixable aqua cell viability dye (Invitrogen, L34957). After the incubation period, cells were washed with 2% FCS/PBS buffer and thereafter incubated for 20 min at 4°C with transcription factor fix/perm buffer 4X according to manufacturer's instructions (BD Biosciences, 51-9008100). Subsequently, fixed and permeabilized cells were washed again with perm wash buffer (BD Biosciences, 519008102). Cells were thereafter incubated for 20 min at RT with perm wash buffer containing FOXP3- PE-CF594- 259D/CY antibody (BD Biosciences, 562421). Cells were acquired on an LSRFortessa™ (Serial # H647794E6049, BD). Flow data was analyzed using FlowJo software (FlowJo, LLC version 10.1).

3.3.4 Seq-Well assay

Single-cell RNA-sequencing analysis was conducted using a recently developed Seq-well technology (21). The experimental design has been previously described by Gierahn *et al.*, (21) and was conducted and analyzed with assistance from Dr. Alex Shalek's laboratory. Briefly, two early treated LN samples were sorted to obtain the following cell subsets i.e. germinal center (GC) TFH ($CD4^{+}CXCR5^{hi}PD\text{-}1^{hi}$) and TFR ($CD4^{+}CXCR5^{+}PD\text{-}1^{+}CD25^{+}CD127^{-}$). Sorted cell populations were thereafter taken through the Seq-Well experimental and analysis pipeline (21).

3.3.5 Tetramer staining

HIV-specific CD4+ T cell responses were defined using fluorochrome-conjugated MHC class II tetramers as previously described in Laher *et al.,* (22). The following antibodies to cell surface molecules were used: Live/dead fixable blue dead cell stain kit (Invitrogen, L23105), CD3-BV711-OKT3 (BioLegend, 317328), CD4-BV650-SK3 (BD BioSciences, 563875), CD8 - HV500 – RPA-T8 (BD Biosciences, 560774), CXCR5-AF488-RF8B2 (BD Biosciences, 558112), CXCR3-BV605-G025H7 (BioLegend, 353728), CD25- PE-Cy5 -M-A251 (BD, 555433), CD127-BV785-A019D5 (BioLegend, 351329), PD-1–BV421-EH12.2H7 (BioLegend, 329920), CCR7- PerCP-Cy5.5-T68 (BioLegend, 335605) and CD45RA-AF700-HI100 (BioLegend, 304120). Cells were acquired on an LSRFortessa™ (Serial # H647794E6049, BD). Flow data was analyzed using FlowJo software (FlowJo, LLC version 10.1).

3.3.6 Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 7.0 software (GraphPad Software Inc., La Jolla, CA, USA). Mann-Whitney U

test was utilized to compare differences between any two groups and Spearman's Rank correlation was used to define the correlation between two variables. Statistical analysis of significance was calculated using Kruskal Wallis test with Dunn's post hoc analyses for multiple comparisons. Statistical significance was set at $p < 0.05$.

3.4 Results

T regulatory cells have been shown to play a pivotal role in chronic viral infections by limiting immune activation and specific immune responses (23). TFR, which was recently discovered in the LNs has not been well characterized and there are no well-defined markers to distinguish it from the conventional TREGS in circulation and in the LN and from the TFH cells, which is the predominant CD4+ T cell subset in the LN, thus we sought to undertake a comprehensive phenotypic characterization of these cells. With access to precious LN samples for our studies, we used multi-parameter flow cytometry, single-cell RNA sequencing and MHC class II tetramer technologies. A total of 31 individuals, 8 of whom were HIV negative, 16 early treated and 7 chronic untreated individuals were analyzed. The clinical characteristics of study participants are detailed in Table 3.1.

Table 3.1 Summary of clinical characteristics of study participants[a]

	HIV negative	Early treated	Untreated
n	8	16	7
Age (years)	20 (19-23)	21 (18-25)	26 (24-28)
CD4 count (cells/ul)	N/A	782 (507-1248)	856 (355-1229)
Viral load (copies/ml)	N/A	<20 (<20-55,000)	1900 (<20-59,000)

[a] All participants were female

3.4.1 Expression of CD25 and FOXP3 define regulatory T cells.

To identify TFR, we firstly needed to define the parent population which is TREGs. There are however numerous definitions of this regulatory subset having been described based on the expression of FOXP3, CD25 and CD127. Also, various phenotypic combinations have been used to define these regulatory cells, these include: $CD4^{+}FOXP3^{+}$ (24, 25), $CD4^{+}CD25^{+/hi}FOXP3^{+}$ (5, 26, 27), $CD4^{+}CD25^{+}CD127^{low}$ (6, 28) or $CD4^{+}CD25^{+}CD127^{low}FOXP3^{+}$ (4, 29). To assess the similarity among these cell populations, we began by evaluating 8 healthy participants using flow cytometry. We assessed the following three phenotypes; $CD4^{+}FOXP3^{+}$, $CD4^{+}CD25^{+}CD127^{-}$ and $CD4^{+}FOXP3^{+}CD25^{+}$ (Figure 3.1 A). Results showed that these phenotypes were interchangeable as we observed that indeed CD25+ cells overlap with FOXP3+ cells (Figure 3.1 B) and there was a strong correlation between $CD4^{+}CD25^{+}CD127^{-}$, $CD4^{+}FOXP3^{+}CD25^{+}$ and $CD4^{+}FOXP3^{+}$ T cells (Spearman r=0,9648; p<0,001) (Figure 3.1 C). For our study we thus defined TREGs as CD4+FOXP3+, TFR as CD4+CXCR5+PD1+CD25+CD127- and TFH as CD4+ CXCR5+PD1+, but since

FOXP3 is an intracellular marker, it could not be used for sorting live cell populations, we thus defined TREGS using the CD25+CD127- phenotype while sorting these cells. Together these results illustrate the importance in finding a more universally utilized regulatory phenotype for comparable analysis.

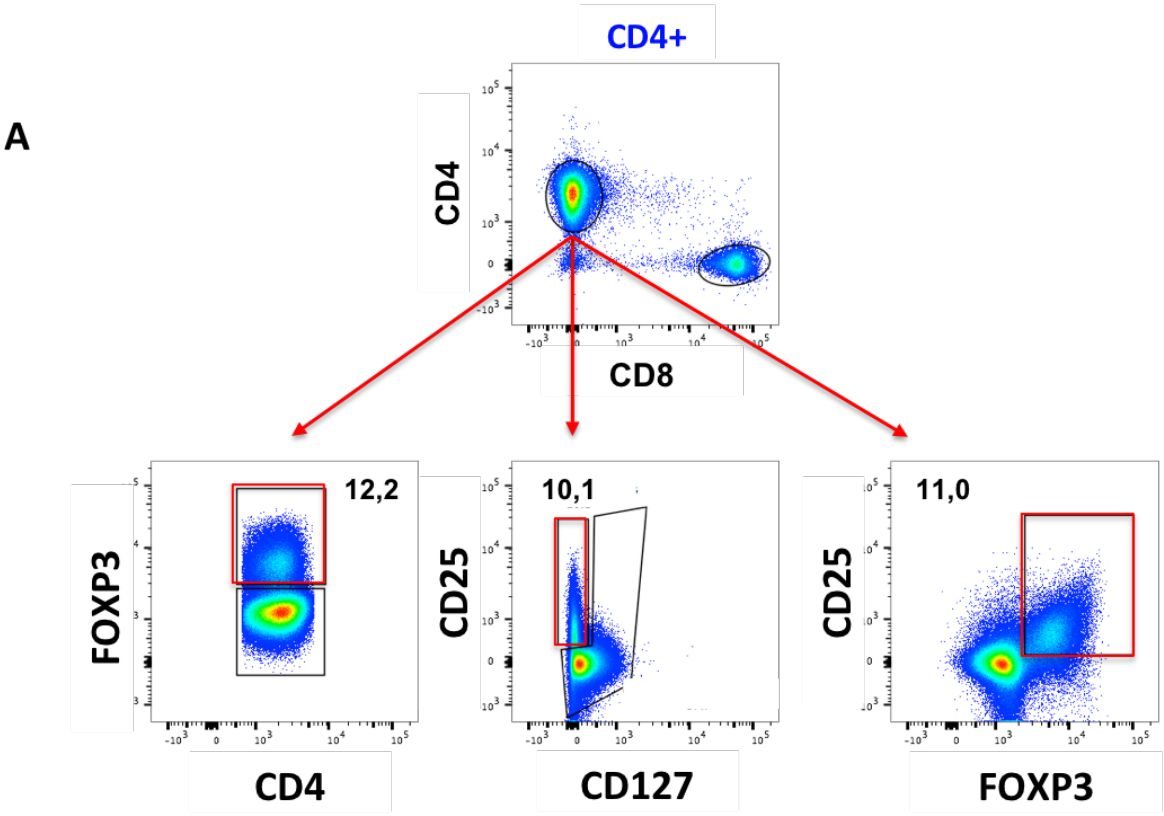


Figure 3.1. Phenotypic characterization of regulatory T cells in the lymph nodes of healthy donors. (A) Gating strategy for defining subsets of regulatory CD4+ T cells using FOXP3, CD25 and CD127. Plots shown are gated on lymphocytes, then CD3+ and CD4+ cell populations. (B) Representative plot demonstrating overlay of CD25+ CD127- populations and FOXP3+ populations together with CD25-CD127+ and FOXP3- populations. (C) Correlation of the frequency of CD4+CD25+CD127- and CD4+CD25+FOXP3+ cells (y-axis) and CD4+FOXP3+ populations (x-axis) within the same individuals (Spearman r = 0,9448, p <0,001).

3.4.2 TFR are phenotypically different from TFH and TREGs.

Having defined TREGs, we next sought to discriminate this subset from our cell subset of interest in this study, which are TFR. We also wanted to distinguish TFR from its closely linked counterpart, the TFH cells. The biology of TFR is not fully understood because of the difficulty in obtaining human LN samples and the lack of reliable markers that best defines this population. It is necessary to more clearly define this cell subset to better understand its biology. To understand the phenotypic differences of these 3 cell subsets, TREGs, TFH and TFR, we performed flow cytometry on LN and peripheral blood samples to assess the expression of the following canonical markers [measured as median fluorescence intensity (MFI)] of CXCR5, PD-1, ICOS, CD25 and FOXP3, markers used by other studies to define these cells. These markers of interest were chosen as they have been previously described as important in defining helper and regulatory T cells. The lymphoid homing marker, CXCR5, was expressed in comparable levels by TREGs, TFH and TFR in the LN, however TREGs had the lowest MFI in peripheral

blood (Figure 3.2 A). Both TFH and TFR cell commitment require programmed cell death protein-1 (PD-1) and inducible T-cell costimulatory (ICOS) amongst other transcriptional signaling (30). We next assessed these two co-stimulatory molecules between TREGS, TFH and TFR. In comparison to TREGs (p=0,0002; p=0,0002) and TFR (p=0,02; p=0,01) cells, TFH had the highest MFI of PD-1 in both LN and peripheral blood respectively (Figure 3.2 B). ICOS expression however, was highest in TFR in LNs compared to TREGs (p=0,003) and TFH (p=0,01), concordant with previous reports (Sage et al., 2015). However, no difference was observed in peripheral blood (Figure 3.2 C). As previously described, CD25 and FOXP3 expression are known to distinguish regulatory subsets. Higher levels of CD25 and FOXP3 were observed on the regulatory subsets i.e. TREG and TFR compared to TFH which was almost negligible (Figure 3.2 D and E respectively). These results demonstrate that the expression of key phenotypic markers can differentiate regulatory subsets from helper subset and within themselves.

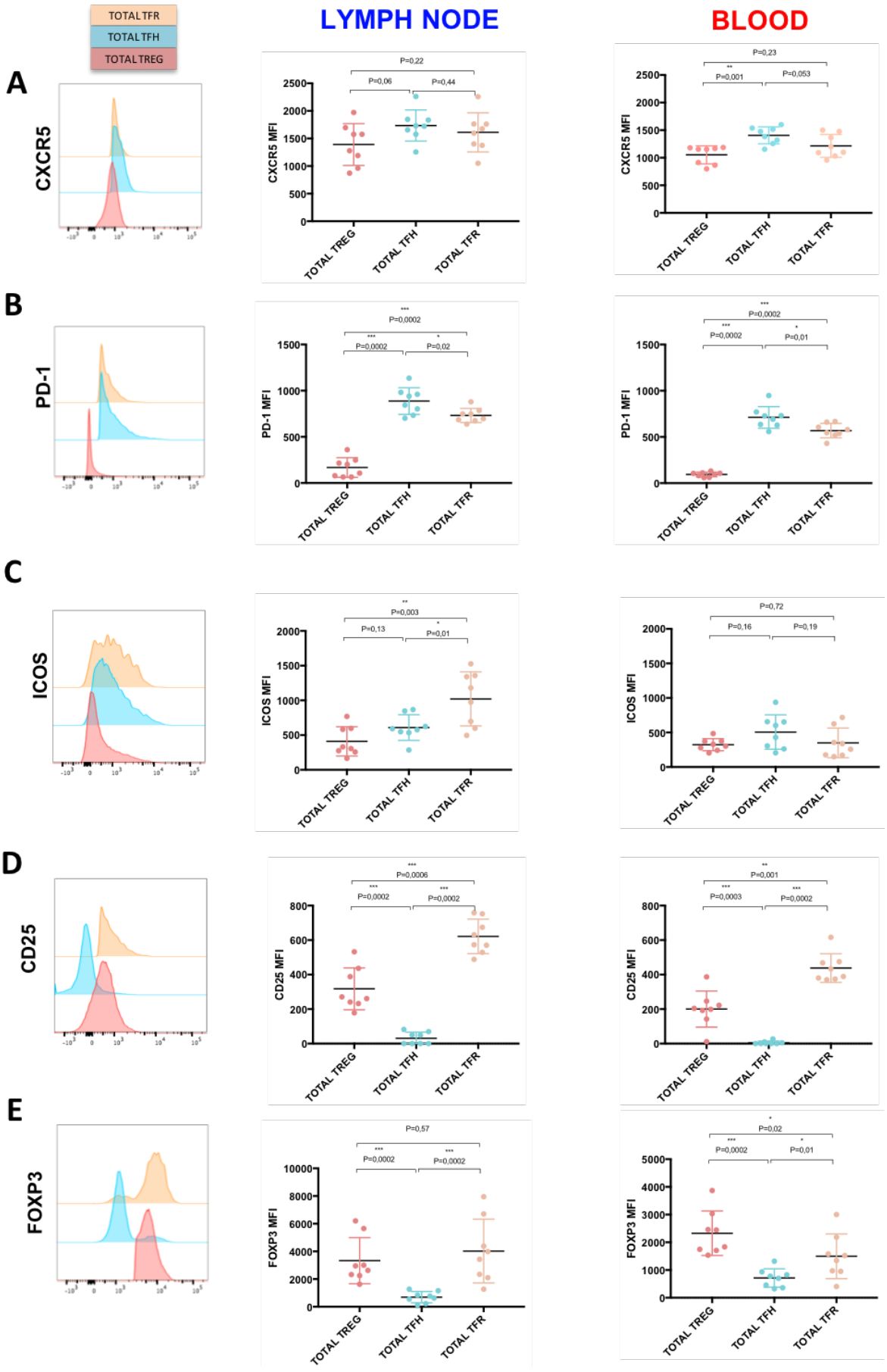
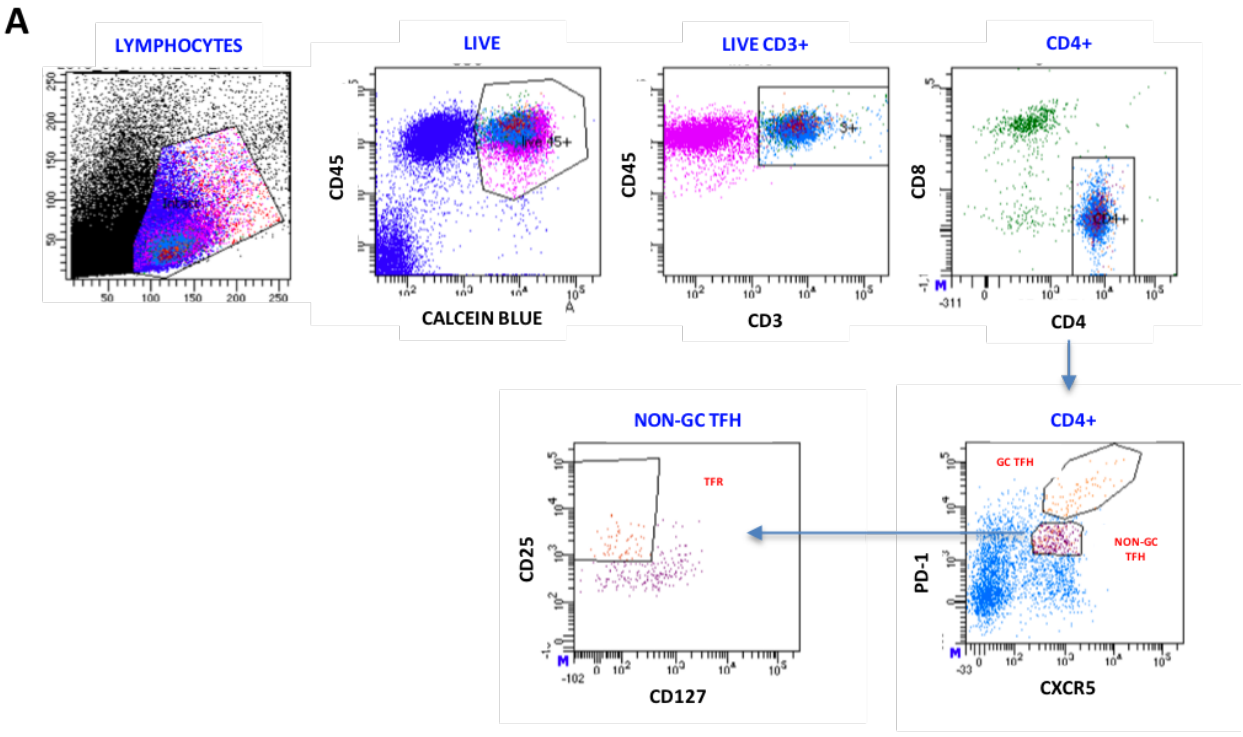

TOTAL TFR
TOTAL TFH
TOTAL TREG
LYMPH NODE
BLOOD
A
CXCR5
CXCR5 MFI
P=0,22
P=0,06
P=0,44
P=0,23
P=0,001
P=0,053
B
PD-1
PD-1 MFI
P=0,0002
P=0,0002
P=0,02
P=0,0002
P=0,0002
P=0,01
C
ICOS
ICOS MFI
P=0,003
P=0,13
P=0,01
P=0,72
P=0,16
P=0,19
D
CD25
CD25 MFI
P=0,0006
P=0,0002
P=0,0002
P=0,001
P=0,0003
P=0,0002
E
FOXP3
FOXP3 MFI
P=0,57
P=0,0002
P=0,0002
P=0,02
P=0,0002
P=0,01
TOTAL TREG
TOTAL TFH
TOTAL TFR

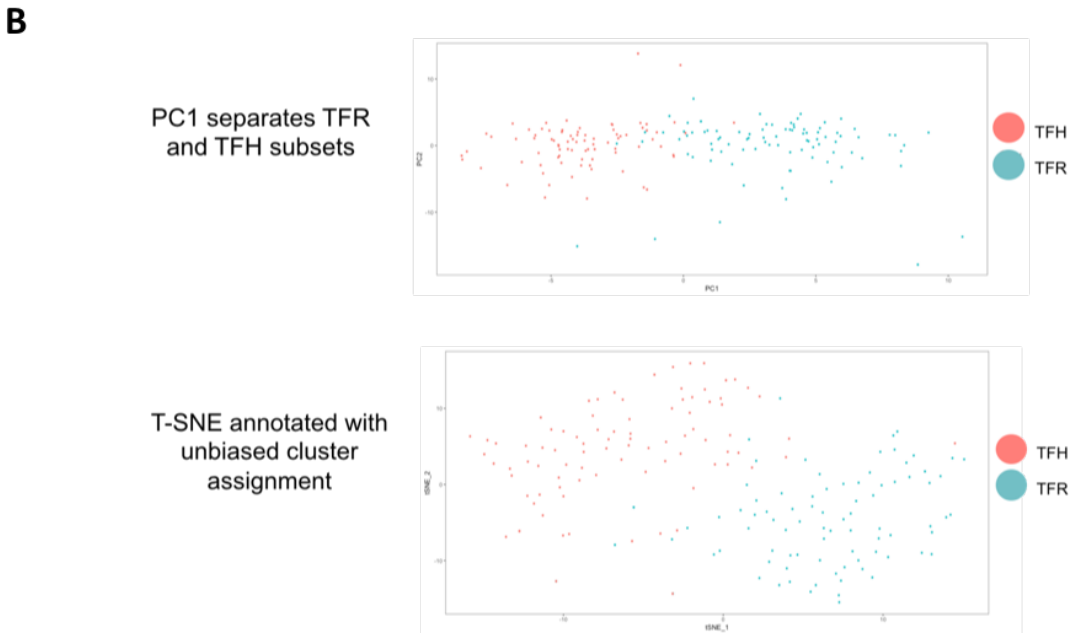
Figure 3.2. Immunophenotypical features of TREG, TFH and TFR in lymph nodes and peripheral blood of HIV uninfected individuals. Representative histograms and summary plots of median fluorescence intensity (MFI) values for (A) CXCR5, (B) PD-1, (C) ICOS, (D) CD25 and (E) FOXP3 on TREGs (pink), TFH (blue) and TFR (orange). Statistical significance was determined using Mann–Whitney U tests.

3.4.3 TFR cells are transcriptionally distinct from TFH cells.

To further define the phenotypic and functional features of TFR and to possibly identify novel markers that can be used to discriminate these subsets, our next approach was to examine the transcriptional profiles of TFR and TFH using Seq-Well single cell RNA-sequencing technology. Seq-Well allows for single cell capture by barcoded mRNA capture beads in an array of sub-nanoliter wells, enabling efficient cell lysis and transcript capture (21). Single cell sequencing further allows detailed and comprehensive studies of individual cells with a starting material from as few as 1000 cells. This technique was ideal for our cell of interest the TFR which is a low frequency population. The GC TFH and TFR were sorted from LMCs based on the phenotypic markers shown in Figure 3.3 (A) and sequenced as described by Gierahn *et al.,* (21). To examine the transcriptional profile of TFR relative to TFH we first performed principal component analysis and the transcriptomes of each subset were clearly distinct and grouped by subset (Figure 3.3 B). This was confirmed by unbiased clustering indicated by T-SNE analysis (Figure 3.3 B). An unbiased gene expression profiling was conducted, and we identified several distinct markers that were specific for the TFR subset compared to TFH cells (Figure 3.3 C). Specific markers like CCR7 (p=0,004), NINJ1 (p<0,0001), LTB (p<0,0001) and HAP1(p=0,001) were significantly expressed on TFR compared to TFH (Figure 3.3 C).

CCR7 has been shown to be a requirement for the *in vivo* functioning of CD4+ CD25+ regulatory T cells (31). NINJ1's role in regulatory T cells is relatively understudied, however it has been shown to mediate cell communication and enhance entry, migration, and activity of leukocytes during developmental and inflammatory processes (32). Studies show that regulatory T cells use lymphotoxin beta (LTB) receptor to migrate to lymph nodes. This novel form of TREG migration in tissues may provide a unique target for modulating Treg-mediated suppression (33). Our results demonstrate transcriptional signature differences between TFR and TFH which could be used for *in vitro* and mechanistic studies assessing TFR and potential targeted manipulation.


A
LYMPHOCYTES
LIVE
LIVE CD3+
CD4+
CD45
CALCEIN BLUE
CD3
CD8
CD4
NON-GC TFH
TFR
CD25
CD127
CD4+
GC TFH
NON-GC TFH
PD-1
CXCR5


B
PC1 separates TFR and TFH subsets
TFH
TFR
T-SNE annotated with unbiased cluster assignment
TFH
TFR

C

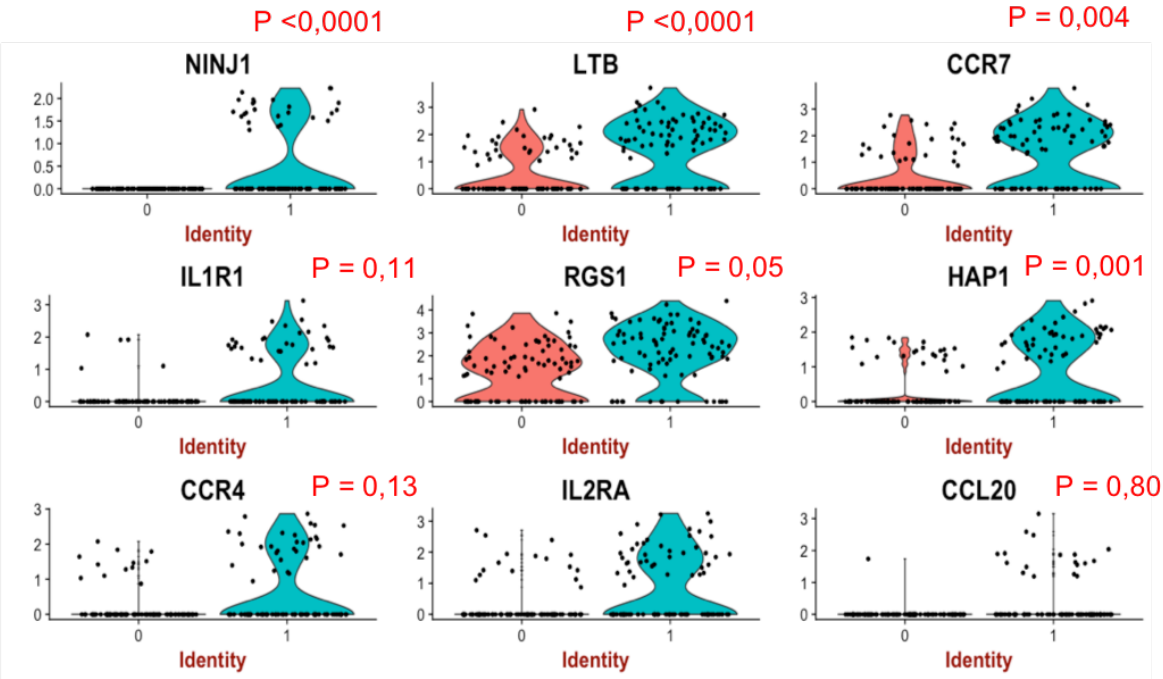


Figure 3.3. Transcriptional signature of follicular T cell subsets. (A) Representative flow cytometry plots showing gating strategy for two sorted populations i.e. GC TFH and TFR. (B) Principal component analysis (PCA) and t-SNE visualization of clusters identified among Seq-Well LNs single-cell transcriptomes recovered from loaded arrays. (C) Violin plots indicating probability density of markers defining the TFR cluster (p values in red indicate differences between expression between GC TFH and TFR).

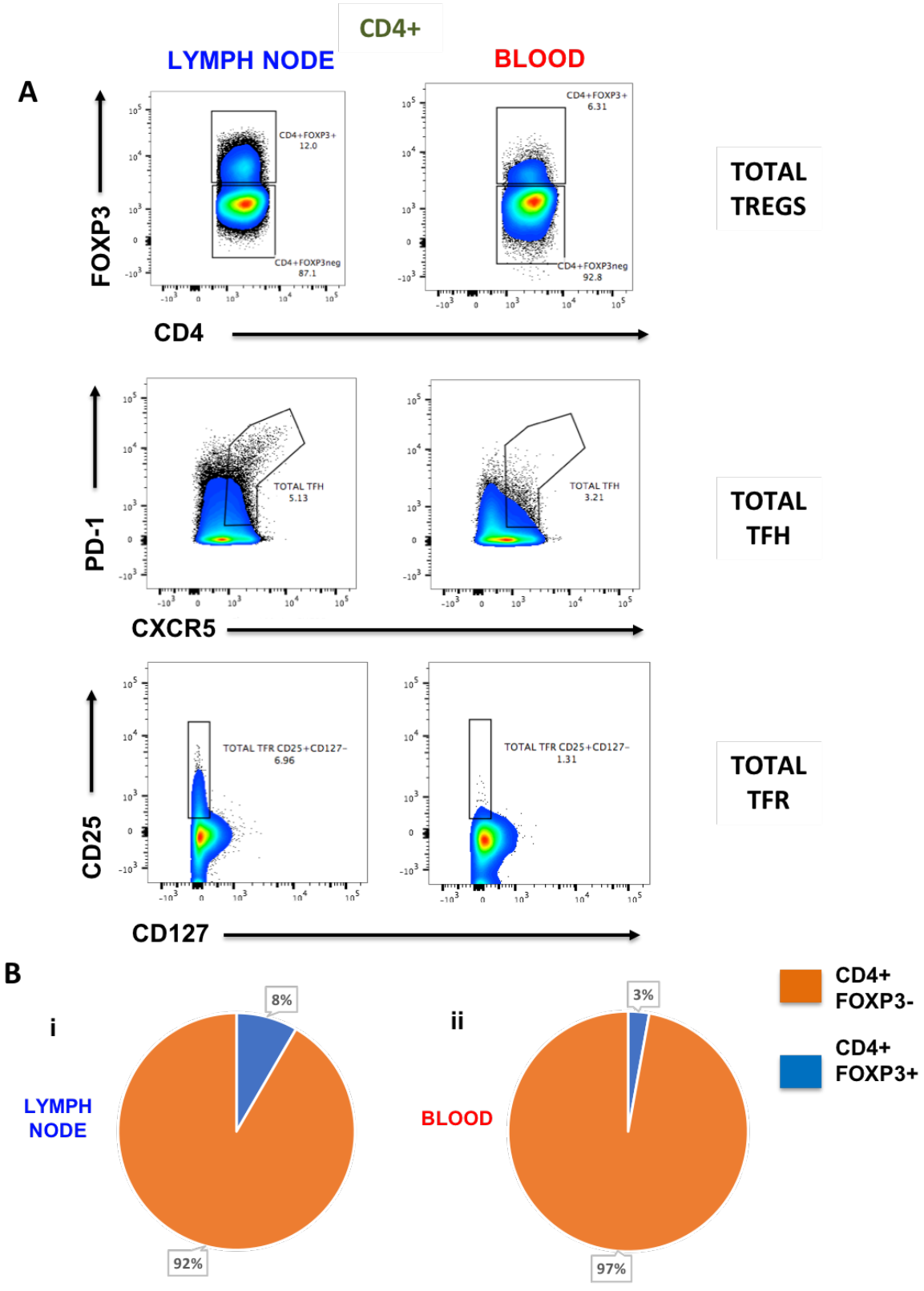
3.4.4 Effect of HIV infection or treatment status on global TFR proportions in the LN and in peripheral circulation.

CD4+ T cells are known to migrate to GCs and B cell follicles within secondary lymphoid compartments to provide help to B cells (34). Having been able to use previously described phenotypes to detect TREGs, we next wanted to answer the question on what

proportion of CD4+ T cells from within LN and peripheral blood constituted TFR, TREGS, or TFH. We utilized matched peripheral blood and LN from healthy participants to conduct this analysis. Using flow cytometry, we began by first characterizing these cell subsets with the following phenotypes: total TREGs ($CD4^+FOXP3^+$), total TFH ($CD4^+CXCR5^+PD\text{-}1^+$) and total TFR ($CD4^+CXCR5^+PD\text{-}1^+CD25^+CD127^-$) (Figure 3.4 A). We observed that FOXP3+ cells made up only 8% of the total CD4+ T cell population in LN (Figure 3.4 B, panel i) and 3% of the total CD4+ T cell population in peripheral blood (Figure 3.4 B, panel ii). Less than 10% of total FOXP3+ cells expressed chemokine receptor CXCR5, the chemokine receptor required for cells to migrate into GCs (Figure 3.4 C). The lack of CXCR5 expression on the majority of FOXP3+ cells in lymph node samples suggest that regulatory CD4+ T cells predominantly localize in extra-follicular areas of the lymph node. We next determined the global frequencies of TREGS, TFR and TFH subsets in HIV uninfected individuals and observed higher proportions of TFH in PBMC compared to TFR (p=0,002). However, no significant differences between the three subsets in the LN were observed (Figure 3.4 D). These results suggest that higher frequencies of TREG, TFR and TFR are observed in LN compared to PBMC. Furthermore, frequencies of TREG, TFR and TFH cells are similar in healthy donors, particularly in LN.

Alteration in regulatory subsets and the impact of ART during HIV-1 infection have long been documented with conflicting reports regarding an increase or decrease in overall frequency of TREGs in response to infection and treatment initiation (4, 15, 24, 25, 35). Having identified these subsets in healthy individuals and shown that they are indeed distinct subsets by phenotype and transcriptional pattern, we next want to determine the

impact of HIV-1 infection and initiation of therapy (very early) on these cell subsets. Using flow cytometry, we assessed changes in frequencies of total TREG, TFH and TFR in HIV negative individuals and compared them to HIV-infected individuals, subdivided into early treated and untreated. Summary plots for total TREGS (Figure 3.4 F), TFH (Figure 3.4 G) and TFR (Figure 3.4 H) are shown for both lymph nodes (i) and peripheral blood (ii). Significant increases in total TFH frequencies were observed in lymph nodes of early treated (p=0,04) and untreated (p=0,02) HIV infected individuals compared to HIV uninfected individuals (Figure 3.4 G). Total TFR and TREG cells showed no significant changes in both lymph nodes and peripheral blood in response to HIV infection or treatment initiation. These results imply, in individuals assessed, HIV infection and treatment initiation did not have an impact on regulatory subsets within lymph nodes and peripheral blood.


CD4+
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CD4+FOXP3neg 92.8
TOTAL TREGS
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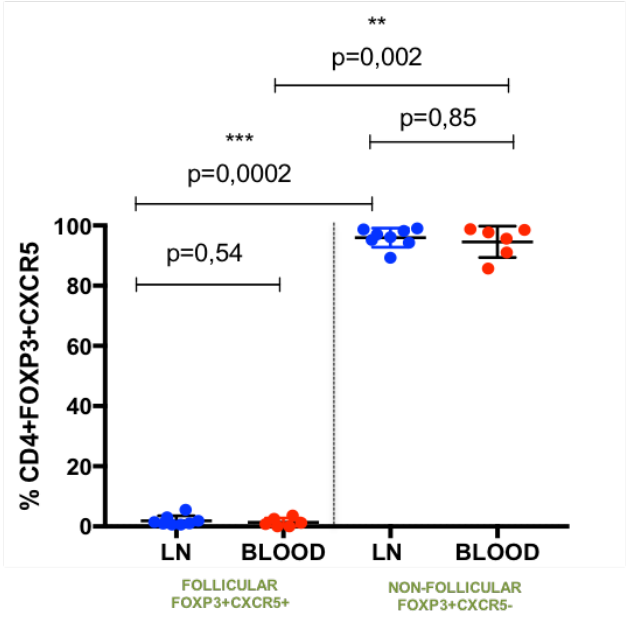

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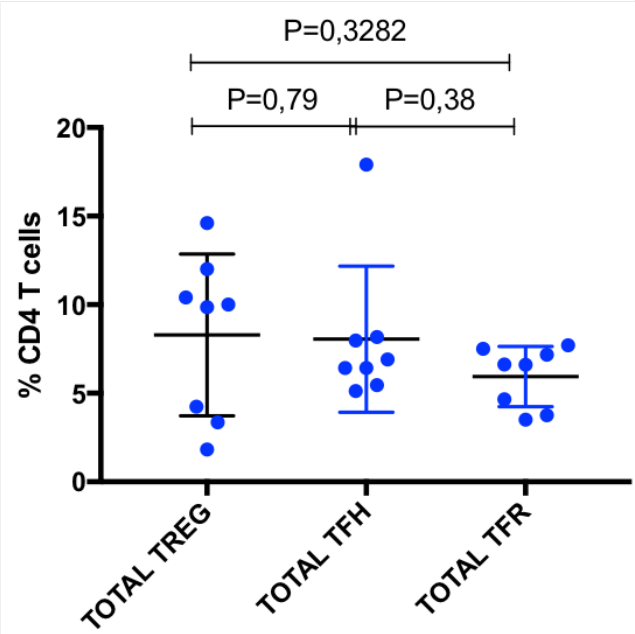

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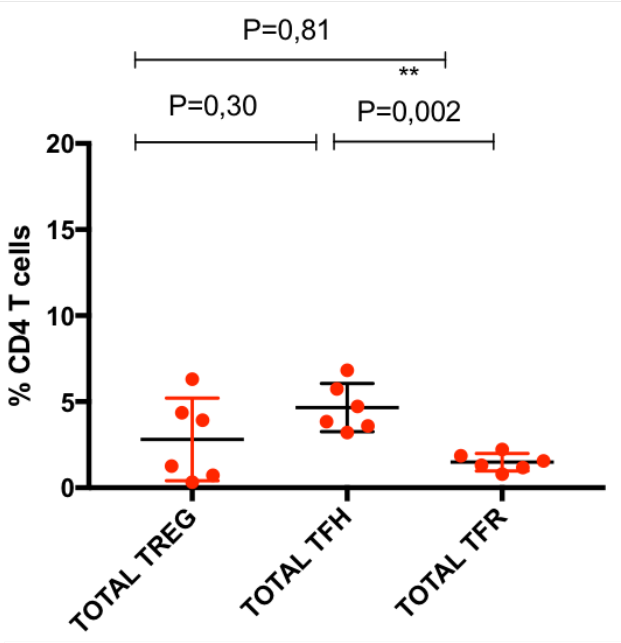

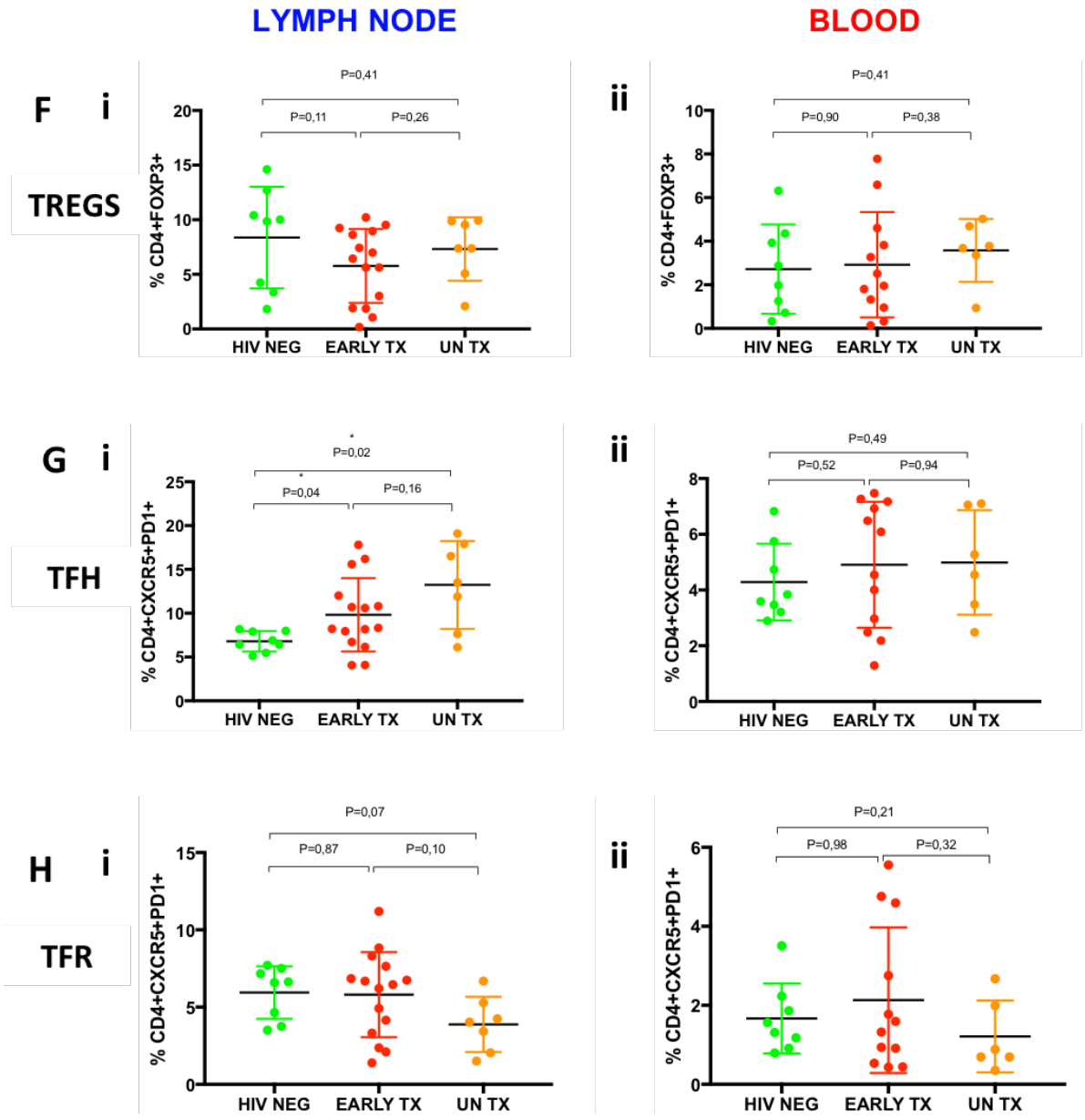

Figure 3.4. Effect of HIV infection or treatment status on global TFR proportions in the LN and in peripheral circulation. Representative flow cytometry plots showing gating for TREGs (FOXP3+), TFH (CXCR5+PD1+) and TFR (CXCR5+PD1+CD25+CD127-) within CD4+ T cells. (B) Pie charts indicating proportions of CD4+ T cells that are FOXP3+ and FOXP3- within (i) lymph nodes and (ii) peripheral blood. (C) Frequency of follicular (FOXP3+CXCR5+) and non-follicular (FOXP3+CXCR5-) CD4+ T cells within lymph nodes and peripheral blood. Summary

plot showing frequencies of CD4+ T cell subsets in (D) lymph nodes and (E) peripheral blood. Samples from HIV negative (HIV neg), early treated (Early Tx) and untreated HIV-infected (Un Tx) individuals were characterized by flow cytometry. (F to H) Summary plots of frequencies of (F) TREGS, (G) TFH and (H) TFR in ((i) lymph nodes and (ii) peripheral blood. Statistical significance was determined using Mann–Whitney U tests.

3.4.5 Frequency of antigen-specific TFR and TREG cells decrease during ART treatment.

Having demonstrated that TREG, TFR and TFH cells are distinct subsets of cells phenotypically and transcriptionally, we next wanted to determine whether these subsets exhibited functional HIV-specific responses. Despite the extensive study of "bulk" regulatory cell populations in the context of HIV-1 infection, there is limited data available on HIV-specificity of regulatory T cells and the induction of these cells in infected individuals. Furthermore, one of the key questions of TFR biology remaining unanswered is the antigen specificity of these cells. In order to screen HIV-specific responses within regulatory and helper populations, we utilized the MHC class II tetramers described in chapter 2 for the characterization of HIV-specific responses. These included the DRB1*11:01-Gag 41, DRB1*13:01-Gag 41, DRB1*13:01-Gag 31 and DRB1*03:01-Gag 41 tetramerized complexes. The same dual tetramer staining strategy utilizing a PE and APC double positive staining approach as in chapter 2 was used (22). We screened a total of 17 individuals, 3 HIV negative as controls and 14 HIV-infected treated or untreated individuals, matching PBMC samples were assessed for only 8 HIV infected donors. We were able to identify HIV-specific responses in TREG, TFH and TFR cells (Figure 3.5 A) in untreated infection and only HIV-specific responses in TFH cells during treated infection. Summary data from the analysis of 2 untreated (2 tetramers

used for 1 patient) and 12 treated (2 tetramers used for 1 patient) donors within our cohort expressing class II DRB1*11:01, DRB1*13:01 and DRB1*03:01 alleles on bulk CD4+ T cells are shown in Figure 3.5B. Twelve individuals demonstrated HIV-specific CD4+ T cells ranging from frequencies of 0,01 to 0,42 % of total CD4+ T cell population (Figure 3.5 B). Further analysis into the specific phenotype of these HIV-specific cells revealed low frequency tetramer+ TREGs (Figure 3.5 C), TFH (Figure 3.5 D) and TFR (Figure 3.5 E). Combined, lymph node cells indicated higher frequencies of tetramer+ compared to peripheral blood, as did untreated individuals as compared to treated individuals as seen in TFH (Figure 3.5 D) (P=0,01) and TFR cells (Figure 3.5 E). Tetramer specific TFR and TFH were identified, with higher frequencies of tetramer specific TFH compared to TFR in LN suggesting that TFR do not necessarily act as a response to specific antigen exposure. Furthermore, tetramer specific cells were more frequently detected during untreated infection than in treated donors. Together, these data demonstrate the advantage of using MHC class II tetramers in detecting low frequency antigen specific CD4+ T cell subsets.

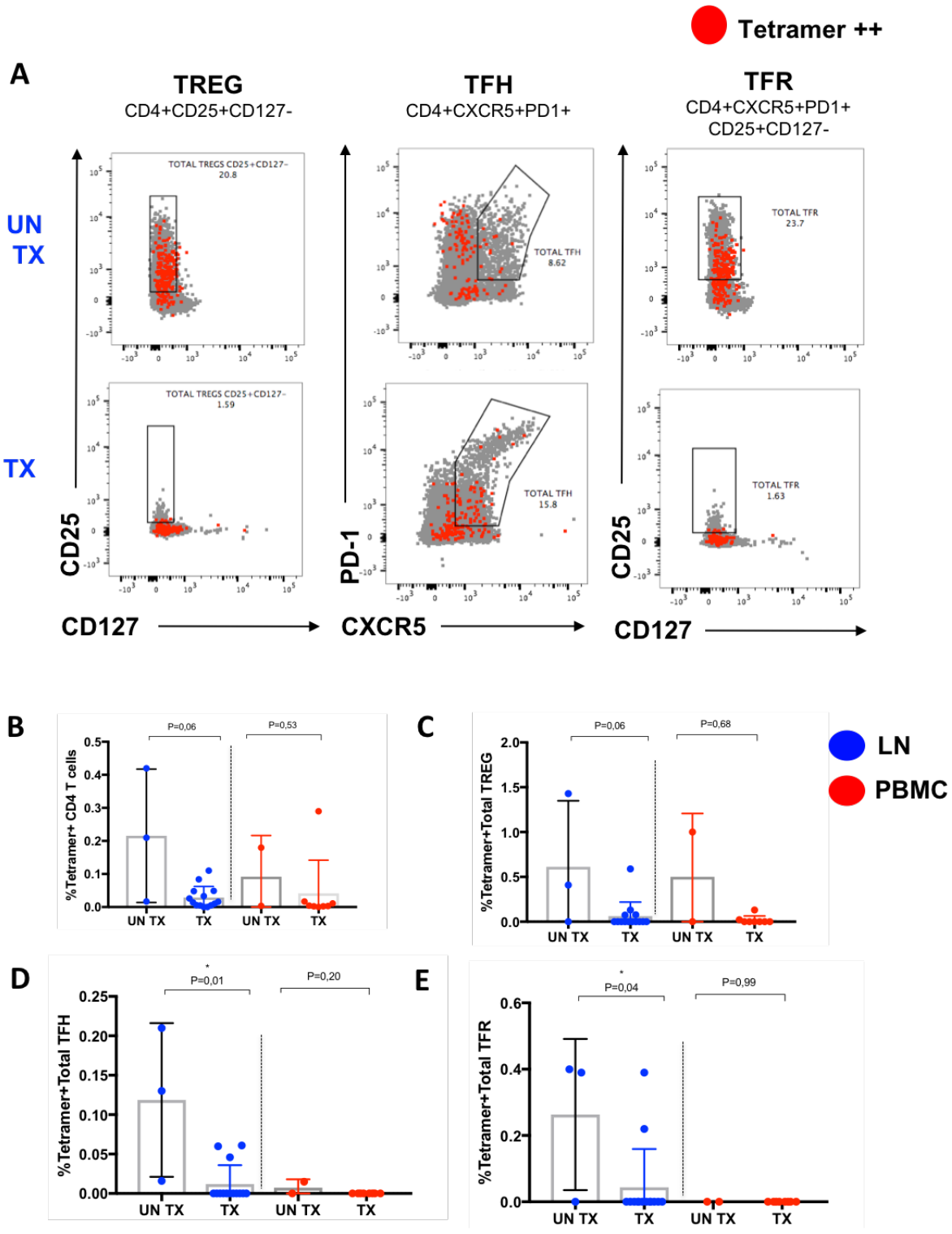

Figure 3.5. Antigen specificity of CD4+ T cell subsets using MHC Class II tetramers.

(A) Overlay plots of dual-tetramer positive (Tet++) CD4+ T cells (red) on total TREG, TFH and TFR subsets in a lymph node. (B) Summary plot of total Tet ++ CD4 T+ cells

in lymph nodes of untreated (UN TX) and treated (TX) individuals. (C-E) Summary plots comparing proportions of total (C) TREGS, (D) TFH and (E) TFR Tet ++ cells to total CD4+ Tet++ populations.

3.5 Discussion

Many unanswered questions related to the role of regulatory subsets in the context of HIV-1 immunopathogenesis remain. In the present study, we sought to identify and elucidate the function of the different regulatory and helper CD4+ T subsets present in LNs and peripheral blood in HIV uninfected and HIV infected individuals. The results from this study allowed for further detailed analysis of TFR present in lymph nodes as detailed in chapter 4 of this thesis.

It is well known that molecular markers are essential tools for defining and analyzing subpopulations of immune cells. We utilized flow cytometry and transcriptional tools to identify TFR cells in lymph nodes and peripheral blood and differentiated them from TREGs and TFH. Identification of cellular markers that distinguish human TFR cells from other TREGs and from TFH cells is essential for future *in vitro* functional analysis. TFR as previously reported, despite being a distinct subset, shared transcriptional patterns with TFH cells, such as CXCR5 and were further distinguished by PD-1 and ICOS expression (36, 37). Unique transcriptional signatures identified using SeqWell will allow for better distinguishing of TFR as a regulatory CD4+ subset within the LN.

Several studies have shown increases in TREG and TFR frequency in individuals with chronic progressive HIV infection in circulating blood and lymphoid tissues (3, 10, 24). Contradictory to these various reported increases in infected individuals, others suggest decreased levels of TREGs (1, 3, 38). In this study we observed no significant changes in regulatory T cells, both TREG and TFR cells in chronic, untreated HIV infection or during treatment initiation, in both LN and peripheral blood. These discrepancies could be due to different phenotypic markers used for analysis, sample size, inconsistence in reporting either frequencies or absolute number and time during infection among numerous other parameters. Ultimately, depending on the phase of infection, TREGs frequency and tissue distribution change; and therefore, their impact on HIV pathogenesis may vary accordingly.

The antigen-specificity of TFR cells has been a question of interest since their initial characterization. TFR cells were initially proposed to be specific for self-antigen because they were thought to derive exclusively from thymic-derived TREGs (7-9). In this study, we show low frequency of total TFR and TREG cells that are HIV-specific. This observance does not suggest that regulatory cells are predominantly not antigen-specific, rather that the specificity of these cells were not specific for the immunodominant peptide-MHC complex tested against. Limitations in the reagents for detecting all cells could be addressed by synthesis of more tetramers targeting a wider variety of specificities to allow for better recognition of antigen-specificity. These results also suggest that these regulatory subsets are much more diverse than what is currently known. Together, these data demonstrate the advantage of using the appropriate tools i.e. MHC class II tetramers in detecting low frequency antigen specific CD4+ T cell subsets.

Furthermore, it highlights the importance of developing additional MHC class tetramers with various peptide-MHC complexes, allowing for more widespread screening. Identification and further functional characterization of HIV-1 specific regulatory subsets will be important for further therapeutic and vaccine strategies aimed at targeting these cells in immunotherapies.

The importance of the contribution of regulatory cells in HIV pathogenesis is increasingly recognized. Future studies that examine new and reliable phenotypical regulatory cell definitions, their trafficking and homing in different lymphoid tissue and the immunomodulatory functions of TREG subsets are fundamental to further understanding the role of regulatory cells in HIV pathogenesis.

In conclusion, our study showed that TFR are different from TREGs and TFH by the expression of PD-1 and ICOS molecules, in addition to unique transcriptional markers such as NINJ1, LTB and HAP1. Moreover, we showed that HIV infection does not significantly impact this cell population, however a trend of less abundant TFR in untreated HIV infection was observed, with this possibly contributing to immune perturbations during chronic HIV infection. These results have 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 phenotypically, transcriptionally and functionally characterized TFR in lymph nodes and peripheral blood and assessed the impact of HIV disease on total TFR frequencies. We showed that TFR are distinct from TREGs and TFH and we identified low frequency antigen-specific TFR, particularly during untreated HIV infection.

Although numerous studies have revealed that TFR cells can suppress TFH and B cells, the localization of this subset within the lymphoid tissues and how they modulate humoral immune responses remains poorly understood. To better understand the localization and function of TFR during treated and untreated HIV-1 infection, the studies in chapter 4 analyzed the localization, phenotype and function of TFR in human lymph node tissues. The study provides further insights into the impact of very early treatment initiation on immunoregulatory cells within lymph nodes.

CHAPTER 4: LOCALIZATION AND FUNCTION OF T FOLLICULAR REGULATORY CELLS IN LYMPH NODES OF VERY EARLY ART INITIATORS IN HIV-1 CLADE C INFECTION

Faatima Laher[1], Omolara Baiyegunhi[1], Funsho Ogunshola[2], Daniel Muema[2], Trevor Khaba[1], Veron Ramsuran[3], Thandeka Nkosi[2], Anele Mbatha[1], Thandekile Ngubane[1], Ismail Jajbhay[4], Johan Pansegrouw[4], Nasreen Ismail[1], Krista Dong[5], Bruce D. Walker[1,5,6], Thumbi Ndung'u[1,2,5,7], Zaza M. Ndhlovu[1,2,5]

[1]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

[2]Africa Health Research Institute (AHRI), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

[3]KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

[4]Prince Mshiyeni Memorial Hospital, KwaZulu-Natal Department of Health, Durban, South Africa

[5]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA, USA

[6]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA

[7]Max Planck Institute for Infection Biology, Berlin, Germany

Keywords: HIV, T follicular regulatory cells, early antiretroviral therapy, lymph nodes, acute HIV infection

4.1 Abstract

Background: HIV eradication efforts have been unsuccessful mainly due to virus persistence in immune sanctuary sites such as germinal centers (GCs) within lymphoid tissues. Recent evidence suggests that these GCs contain a novel subset of regulatory T cells (TREGs), termed follicular regulatory T (TFR) cells. The exact function of these cells has yet to be fully elucidated. Most studies indicate that these cells control the magnitude and specificity of the GC response and they are essential for the maintenance of self-tolerance and immune homeostasis. However, detailed investigations are needed to fully understand the role of TFR cells in the overall humoral immune response and their contribution to viral persistence. Thus, we sought to investigate the localization of TFR cells during HIV infection in lymph node (LN) tissues and determine how early treatment impacts their functional roles.

Methods: We analyzed 30 individuals, grouped into HIV-uninfected, HIV-infected early ART initiators (median of 1-day post detection of HIV) and untreated individuals, from a clade C HIV infection cohort in Durban, South Africa. TFR phenotype ($CD4^+CXCR5^+PD\text{-}1^+CD25^+CD127^-$) and localization were defined using flow cytometry and immunofluorescence microscopy (IF). TFR regulatory cytokine; IL-10, and T helper cytokine; IL-21 were quantified using digital droplet PCR (ddPCR), and plasma antibodies were measured by ELISA.

Results: TFR cell frequencies were significantly higher in LN compared to peripheral blood (PB) samples ($p \leq 0.0001$). Very early ART initiators displayed larger proportions of TFR in the GC ($PD\text{-}1^{hi}$) compared to HIV negative and untreated participants, who had a $PD\text{-}1^+$, follicular phenotype ($p=0.0001$, $p=0.001$ respectively). This result was confirmed by IF ($p=0.0001$). Interestingly, TFR and TFH cells displayed comparable

levels of IL-10 and IL-21 mRNA transcripts as measured by ddPCR. Lastly, non-GC TFR frequencies showed a positive correlation with gp41 IgG antibodies at 12 weeks post-infection.

Conclusions: Together, our data show that very early ART results in increased TFR cell numbers in the GC, where they likely influence the GC response through interactions with TFH cells. Furthermore, the data suggest an additional function of TFR as "helper" cells as opposed to only their canonical function of immune suppression. These results could have implications for immunotherapeutic interventions aimed at using TFR and TFH cells as potential targets to induce durable antibody responses in early treated individuals.

4.2 Introduction

High affinity antibodies and overall humoral immunity is dependent on interactions between B cells and T follicular helper (TFH) cells in germinal centers (GCs). The CD4+ T cell helper function to B cells via secretion of cytokines and expression of costimulatory molecules is well characterized (1). The GC response is tightly regulated and requires adequate TFH and B cell interactions, however, the regulatory mechanisms underlying this process are not fully understood. Recent studies have identified a subset of regulatory T cells (TREGs), termed follicular regulatory T (TFR) cells, that express CXCR5, a follicular migratory receptor (2, 3). Much of the studies aimed at elucidating the biology and function of TFR cells have been conducted in murine models and peripheral blood (2-5), despite being first identified in human tonsils, where they were shown to control the magnitude of the GC response (6). Several studies suggest that although TFR

primarily differentiate from Foxp3+ TREG precursor cells (2, 4, 7) they display a mixed TFH/TREG phenotype because their transcriptional profile overlaps with TFH (3, 8, 9).

Numerous studies have revealed that TFR cells have the ability to suppress TFH and B cells (10, 11). However, localization and function of the TFR cells is not very well understood (2-4, 12, 13). Importantly, determining the role TFR cells play in GC reactions may have implications on the design of therapeutic interventions. The impact of TREGs on HIV infection remains controversial and much less is known about TFR in HIV-1 infection. Furthermore, with increasing numbers of individuals being initiated on antiretroviral therapy (ART) daily, the impact of ART-treatment on these regulatory cell subsets will provide useful information for rational design of immunotherapeutic innervations. The development of interventions that could manipulate immune responses capable of maintaining HIV suppression post ART cessation is desirable for the millions of individuals on life-long therapy. Studies have recently suggested a new paradigm of TFR cell function in the GC reaction, where they may promote the antibody response and even the magnitude of the GC response, rather than just having a suppressive role alone (14).

To gain insight into the localization and function of TFR during treated and untreated HIV-1 infection, we analyzed the localization, phenotype and function of TFRs in human lymph node tissues using flow cytometry, immunofluorescence microscopy (IF), droplet digital PCR (ddPCR) and Ig ELISA. These studies were conducted in a cohort of 8 HIV uninfected and 22 HIV-infected individuals (15 early treatment initiators and 7 chronic untreated).

Analysis of TFR cell frequencies using flow cytometry and immunofluorescence microscopy within and outside of the GCs of lymph nodes revealed greater proportions of TFR cells where localized in the extra-follicular region. Paradoxically, early treated individuals displayed increased frequencies of GC TFR as compared to HIV negative ($p=0.0001$) and untreated ($p=0.001$) individuals, although no difference in non-GC TFR frequencies were observed between HIV uninfected and infected individuals, as well as between treatment statuses. In addition, when total TFR frequencies were assessed against markers of disease progression, a correlation was observed between total TFR frequencies and plasma viral load in HIV infected, untreated participants ($p=0.004$, $r=0.95$). Functional assessment of TFR and TFH, indicated comparable levels of IL-10 and IL-21 mRNA expression between both CD4+ subsets within the lymph node. Lastly, further functional analysis of TFR, determined by correlations to HIV-specific antibodies in early treated individuals revealed that non-GC TFR frequencies correlated with plasma gp41 IgG antibodies. Together, these data provide further insight into the impact of very early treatment initiation on TFR cell frequencies and localization within lymph nodes. Elucidating HIV-specific CD4+ responses in lymphoid compartments will provide a greater understanding of the underlying mechanisms by which TFR cells exert a regulatory role in HIV-1 infection.

4.3 Materials and Methods

4.3.1 Study population

Study participants were enrolled from the HIV Pathogenesis Programme (HPP) lymph node study cohort in Durban, South Africa. The lymph node study and recruitment

protocols were previously described in Chapter 3, section 3.3.1. In brief, excisional lymph node biopsies and peripheral blood samples were collected from a total of 30 participants.

Inguinal, axillary or cervical lymph nodes were excised and in addition 120 ml of peripheral blood was obtained from study participants. Measurements of CD4 counts and viral loads were performed by Global Clinical and Viral laboratories (Durban, South Africa). Informed consent was provided by all study participants as a criterion for enrollment and participation in the study. The Biomedical Research Ethics (BREC) of the University of KwaZulu-Natal and the Institutional Review Board (IRB) of Massachusetts General Hospital approved this study.

4.3.2 Lymph nodes and peripheral blood samples

Excised lymph nodes were divided into two pieces; approximately 1/3 of the LN was fixed in 10% formal-saline (Sigma-Aldrich, St Louis, Missouri, USA) for downstream microscopy studies. The 1/3 formal-saline fixed tissue were paraffin embedded by Lancet Laboratories (Durban, South Africa). Tissue blocks were thereafter stored and cut using a microtome (Leica) for staining on individual slides. The remaining 2/3 was mechanically processed to release lymph node mononuclear cells (LMCs) as described by Schacker *et al.*, (15). In brief, the macerated LN was passed through a 70uM cell strainer (BD, 352350) into a collection tube containing R10 media. Cells were pelleted and collected by centrifugation (1800 RPM, 6 minutes (min), room temperature (RT). Peripheral blood mononuclear cells (PBMCs) were isolated by HPPs core processing facility from patient blood samples by density-gradient centrifugation using Histopaque-

1077 (Sigma-Aldrich). Lymph nodes and peripheral blood samples were obtained and processed on the same day and cryopreserved in liquid nitrogen.

4.3.3 Viral RNA quantification in lymph node mononuclear cells

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

4.3.4 Flow cytometry analysis

Freshly isolated and frozen LMCs and PBMCs were phenotypically and functionally characterized using multi-parameter flow cytometry. For phenotypic characterization experiments, fixation and permeabilization were performed using the transcription factor diluent buffer set (BD, 562574). Briefly, for surface staining, cells were incubated for 20 min at RT in the dark in staining buffer [2% FCS in PBS buffer] containing the following antibodies: CD3-AF700-UCHT1 (BD, 557943), CD4-BV650-SK3 (BD, 563875), CD8-PE- RPA-T8 (BD, 555367), CD62L-BV711 (BD, 565040), CD25- PE-Cy5 -M-A251 (BD, 555433), CD127-BV785-A019D5 (BioLegend, 351329), CXCR5-AF488-RF8B2 (BD, 558112), PD-1 – BV421-EH12.2H7 (BioLegend, 329920), CXCR3-BV605-G025H7 (BioLegend, 353728), ICOS-AF647-C398.4A (BioLegend, 313516) and CCR7-PerCP-Cy5.5 – T68 (BioLegend, 335605). Cells were also stained with live/dead fixable aqua cell viability dye (Invitrogen, L34957). After the incubation period, cells were washed with 2% FCS/PBS buffer and thereafter incubated for 20 min at 4°C with transcription factor fix/perm buffer 4X according to manufacturer's instructions (BD, 51-

9008100). Subsequently, fixed and permeabilized cells were washed again with perm wash buffer (BD, 519008102). Cells were thereafter incubated for 20 min at RT with perm wash buffer containing FOXP3- PE-CF594- 259D/CY antibody (BD, 562421). Cells were acquired on an LSRFortessa™ (Serial # H647794E6049, BD). Flow data was analyzed using FlowJo software (Treestar FlowJo version 10.1).

4.3.5 Immunofluorescence (IF) microscopy

Multicolor immunofluorescence microscopy staining was conducted on 0.4 µM sections of formalin fixed paraffin embedded (FFPE) lymph nodes using the opal 4-colour fluorescent IHC kit (PerkinElmer, Waltham, MA, USA) according to manufacturer instructions with minor modifications. Briefly, following deparaffinization, rehydration and antigen retrieval, two blocking steps (2 x 10 min, RT) were performed using the Dako peroxidase blocking reagent (Agilent, S202386) and Bloxall block (Vector Laboratories, SP-6000). The slide was then washed with Flex 20X wash buffer (Dako, K800721) for 5 min, followed by incubation with first primary antibody, BCL-6 (Dako, IR62561) for 30 min at RT, washed with wash buffer for 5 min, thereafter probed with Opal polymer HRP (PerkinElmer, ARH1001EA) for 20 min at RT, washed with wash buffer twice (5 min) and detected using the Opal polymer 520 (10 min, RT) (PerkinElmer, FP1487). This procedure was repeated for the second and third antibodies CD4 (Dako, R64961) and FOXP3 (AbCam, ab22510) detected on Opal 570 (PerkinElmer, FP1488) and Opal 670 (PerkinElmer, FP1489) respectively. Slides were counterstained with spectral DAPI (PerkinElmer, FF1490) and mounted with Dako fluorescence mounting medium (Agilent, S3023). Images were acquired using the Axio Observer with TissueFAXS imaging

software (TissueGnostics). Quantitative image analysis was conducted using TissueQuest (TissueGnostics).

4.3.6 Droplet Digital PCR (ddPCR)

Populations of interest i.e. GC TFH (CD4+CXCR5^{hi}PD-1^{hi}) and TFR (CD4+CXCR5+PD-1^{+}CD25+CD127-) were sorted. Total RNA was extracted from sorted populations using the QIAzol Lysis Reagent (Qiagen, Cat # 79306) and RNeasy Mini Kit (Qiagen, Cat # 74106) as per manufacturer's instructions, and used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Cat # 1708891). The cDNA was used as a template for IL-10 (ThermoFischer Scientific, Assay ID: Hs00961622_m1) and IL-21 (ThermoFischer Scientific, Assay ID: Hs00222327_m1) mRNA quantification by droplet digital PCR assays using pre-designed qPCR kits (ThermoFischer Scientific, 4331182) on a FAM/MGB fluorescence.

Briefly, PCR droplets were generated a using QX200 Droplet Generator (Bio-Rad) from a mastermix of cDNA, ddPCR supermix for probes (No dUTP) (Bio-Rad, 186-3025), pre-designed probes and droplet generation oil. PCR thermal cycling was conducted following optimized cycling conditions: an initial incubation at 95°C for 10 min, 40 cycles of 30s 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, and target gene copy number was analyzed using QuantaSoft analysis software (Bio-Rad) and recorded as mRNA copies/20µL. Absolute IL-10 and IL-21 mRNA counts were normalized to the expression of the housekeeping gene B2M (ThermoFischer Scientific, Assay ID: Hs00187842_m1).

4.3.7 Total and HIV-specific IgG and IgM ELISA

Plasma HIV-specific IgG and IgM antibodies were measured by ELISA as previously described with minor modifications (16). Briefly, 96 well plates (eBiosciences, Waltham MA, USA) were coated with either monoclonal anti mouse IgG or IgM (eBiosciences), (10 µg/ml in PBS, 100 µl/well) for 16 hours at 4°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 and IgM antibodies. 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 substrate (Sigma-Aldrich, 100 µl/well) were used to detect IgG and IgM 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 and IgM antibody concentrations in each sample.

4.3.8 Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 7.0 software (GraphPad Software Inc., La Jolla, CA, USA). Mann-Whitney U test was utilized to compare differences between any two groups. Spearman's Rank correlation was used to define the correlation between variables. Statistical analysis of

significance was calculated using Kruskal Wallis test with Dunn's post hoc analyses for multiple comparisons. Statistical significance was set at $p < 0.05$.

4.4 Results

Follicular helper T (TFH) cells are required for GC formation and provide important signals to B cells needed for their differentiation and secretion of high-affinity and isotype-switched antibodies (1, 17). T follicular regulatory (TFR) cells are a specialized subset of FOXP3+ cells that have also been shown to regulate the differentiation of Ab-secreting cells in GCs. TFR cell localization and function is not well understood, with the dominant paradigm being that they repress excessive TFH and B cell proliferation and thus could be implicated in the dysregulation of the immune response within sanctuary sites (18). To better characterize this immunoregulatory cell subset present in lymph nodes, we comprehensively investigated their phenotype, localization, association with markers of disease progression, response to early ART and function in the setting of HIV-1 clade C infection using multi-parameter flow cytometry, immunofluorescence microscopy (IF), droplet digital PCR (ddPCR) and Ig ELISA. A total of 30 females were studied. The detailed clinical characteristics are listed in Table 4.1.

Table 4.1 Summary of clinical characteristics of study participants[ab]

	HIV negative	Early treated	Untreated
n	8	15	7
Male	0	0	0
Female	8	15	7
Age (years)	20 (19-23)	21 (18-25)	26 (24-28)
CD4 count (cells/ul)	N/A	782 (507-1248)	856 (355-1229)
Viral load (copies/ml)	N/A	<20 (<20-55,000)	1900 (<20-59,000)
Treatment duration (days)	N/A	120 (8-926)	N/A

[a] 100% of participants were female

[b] Range values are reported in parentheses under median values

4.4.1 TFR cells localize outside of the GC in human lymph nodes.

Having previously comprehensively investigated the phenotype, transcriptional profile and antigen-specificity of TFR in chapter 3, we next wanted to assess the localization of this cell subset. There have been a limited number of studies that assessed the frequency and location of TFR (19-21), particularly within human secondary lymphoid tissue, with no study having described this subset previously in African populations in the context of clade C HIV-1 infection. We began, by analyzing total TFR frequencies in lymph nodes and blood of 8 HIV uninfected and 22 HIV infected individuals. We observed that TFR exist at extremely low frequencies in peripheral blood compared to lymph node tissues (Figure 4.1 A) ($p<0.0001$), therefore, subsequent studies were all conducted on lymph node tissue samples. To determine the baseline features of this population, we began by using previously described phenotypic flow panel to identify GC TFH ($CXCR5^{hi}PD\text{-}1^{hi}$), non-GC TFH ($CXCR5^{+}$ $PD\text{-}1^{+}$), GC TFR ($CXCR5^{hi}PD\text{-}1^{hi}$CD25+CD127-) and non-GC

TFR (CXCR5^{+} PD-1^{+}CD25+CD127-) within the CD4+ T cell population (20) (Figure 4.1 B). We observed higher frequencies of non-GC TFR compared to GC TFR ($p<0.0001$) (Figure 4.1 D). Next, we investigated the localization of TFR within lymph node tissues using IF imaging, staining with CD4+FOXP3+ as previously described by Miles *et al*., (20), we determined localization within the GC by immunostaining with BCL-6 (Baiyegunhi *et al.,* 2018 unpublished) (Figure 4.1 C). The results showed that most of the TFR were outside the GCs, ($p<0.0001$) (Figure 4.1 E), consistent with flow cytometry data. These results demonstrate that TFR predominantly localized out of the B cell follicles suggesting that TFR cells are more likely exert their functions outside of the GC (21).

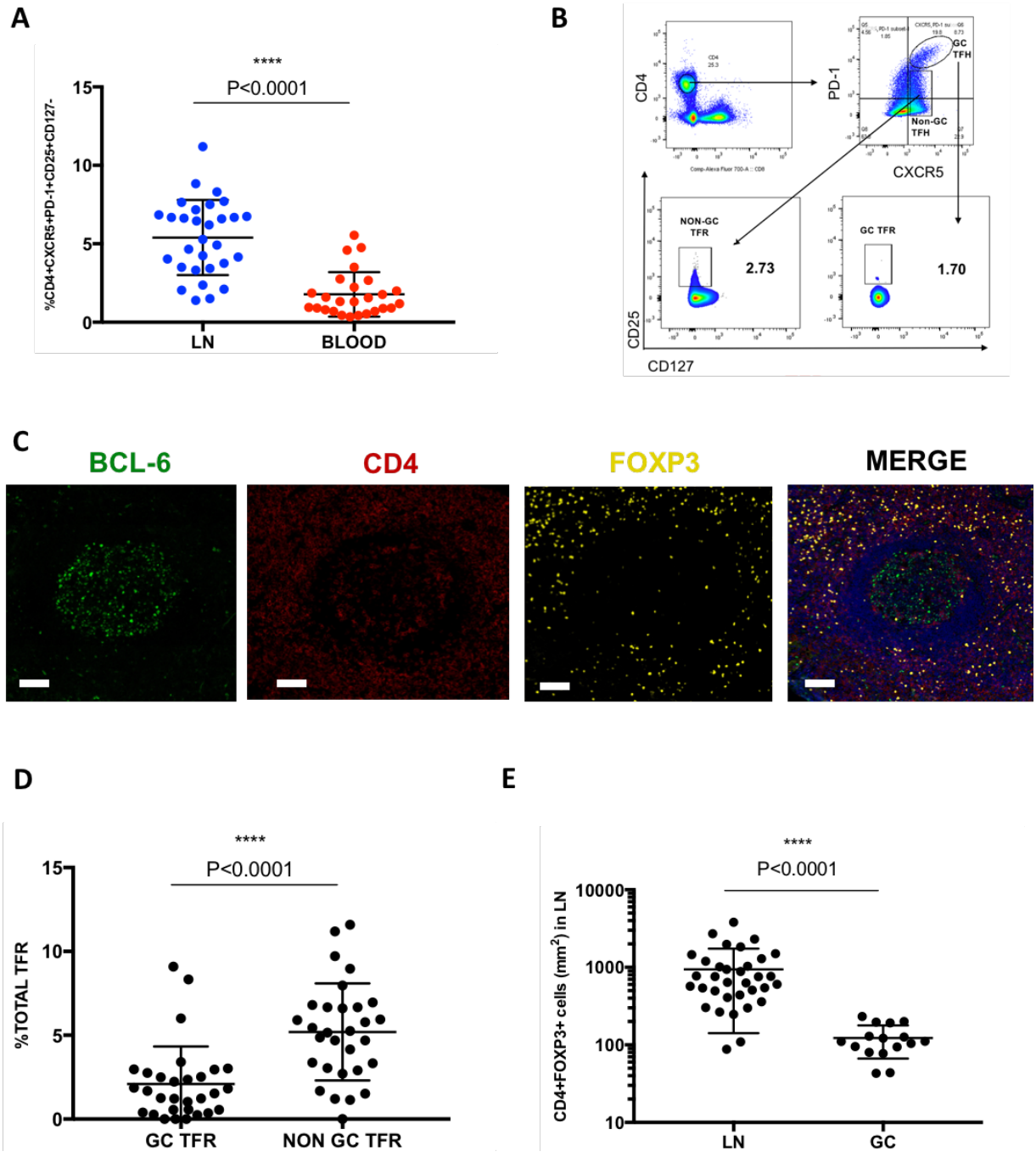


Figure 4.1 Majority of TFR localize outside of the germinal center (GC). (A) Proportion of TFR cells found in lymph nodes (LNs) compared to PBMC ($p<0.0001$). (B) Representative flow cytometry plots showing gating strategy for GC TFH (PD-1^{hi}CXCR5^{hi}), non-GC TFH (PD-1^{+}CXCR5^{+}), GC TFR (PD-1^{hi}CXCR5^{hi}CD25^{+}CD127^{-})

and non-GC TFR ($PD1^+CXCR5^+CD25^+CD127^-$) within CD4+ T cells. (C) Representative images of immunoflouresecntly stained LN sections from an HIV infected subject, LNs were stained with antibodies to BCL-6 (green), CD4 (red) and FOXP3 (yellow). Images were scanned at x20 magnification and scale bars equal 100μm. (D) Flow cytometry summary plots comparing GC TFR and non-GC TFR ($p<0.0001$). (E) CD4+FOXP3+ cells were quantified in the entire LN cross-section and within GCs of LNs from 6 HIV uninfected and 28 HIV-infected subjects. TissueQuest (TissueGnostics, Vienna) was used to compute CD4+FOXP3+ density in each tissue section. p values were determined using Mann-Whitney U test.

4.4.2 Plasma viral load correlates with TFR frequency during untreated HIV infection.

To begin to delineate the role of TFR in HIV infection and how treatment modulates this population, we first assessed the relationship of these cells with markers of HIV disease progression. Previous studies have reported an inverse relationship between TREG frequency and CD4 cell counts (22-30), with contradictory findings. Furthermore, TREG frequency and plasma viral load were shown to be unrelated in some studies (25, 29, 31) and positively correlated in others (23, 24, 26, 28). Secondly, to clarify some of these apparent contradictions, particularly in lymphoid tissue, we analyzed the relationship between total TFR frequencies (%) with peak viral load (copies/ml) and viremia copy days (cumulative antigen load, calculated as area under the viral load curve) in HIV infected early treated individuals and plasma viral load (copies/ml) at the time of lymph node excision in untreated individuals and absolute CD4 count (cells/μl) in both groups. We observed a direct correlation between plasma viral load (copies/ml) and total TFR

frequencies in untreated HIV infection ($p=0.004$, $r=0.95$) (Figure 4.2 B). In contrast, the frequencies of total TFR in early treated individuals did not correlate with peak viral load (Figure 4.2 A), neither with viremia copy days (Figure 4.2 E), nor with plasma viral load at treatment initiation (data not shown). There was no correlation observed between TFR frequencies and absolute CD4 counts in either early treated (Figure 4.2 C) or untreated individuals (Figure 4.2 D). Our results are consistent with studies that report a positive relationship between regulatory T cell frequencies and plasma viral loads in chronic untreated HIV infection (23, 24).

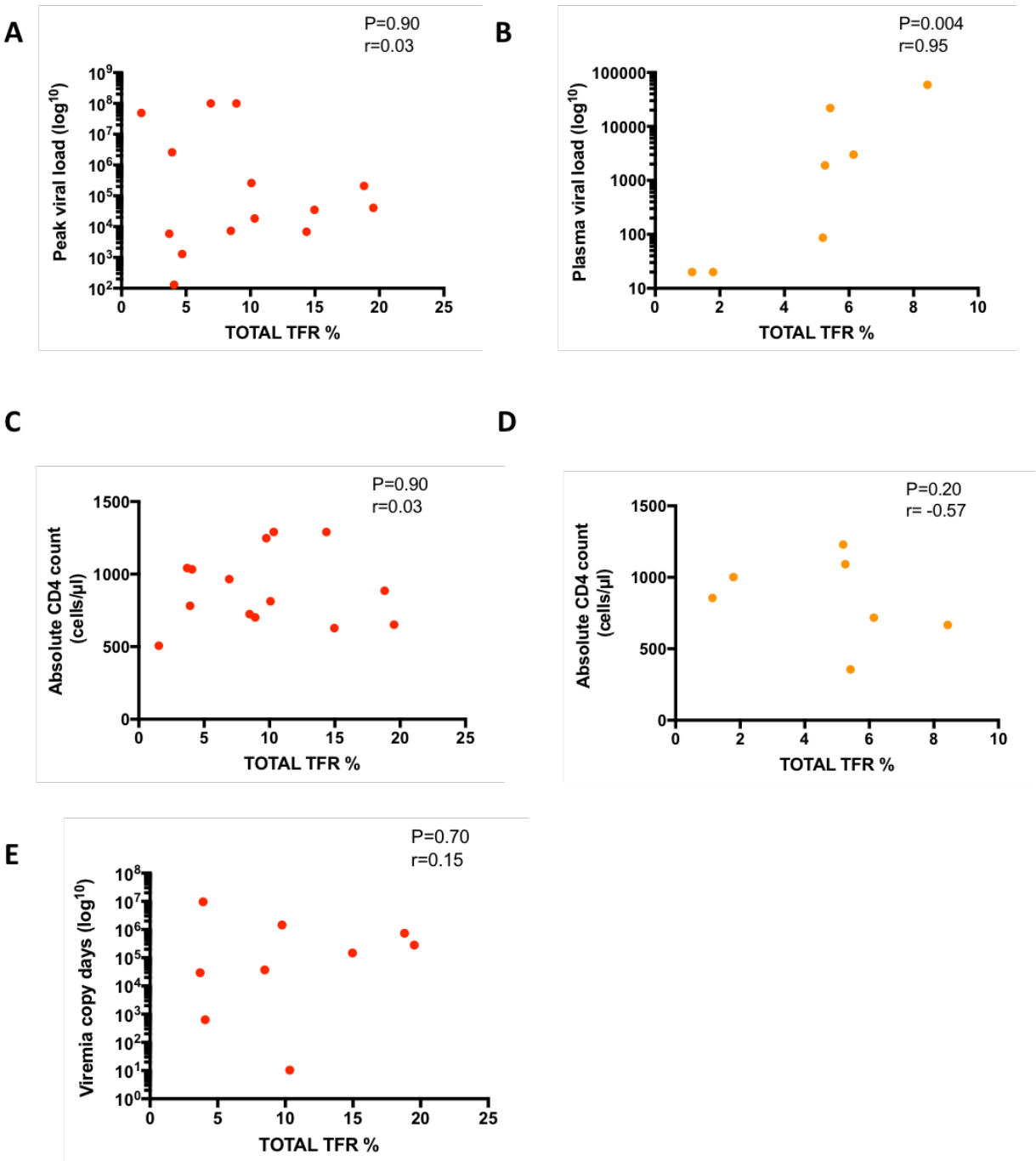


Figure 4.2 TFR frequency during untreated HIV infection correlate with plasma viral load. Correlation analysis between total TFR frequencies and peak viral load (copies/ml) (A), absolute CD4 count (C) and viremia copy days (E) in HIV infected early treated individuals and plasma viral load at the time of LN excision in untreated

individuals (B) and absolute CD4 count (D) in HIV infected untreated individuals. Spearman's rank correlation used to compute P and rho (r) values.

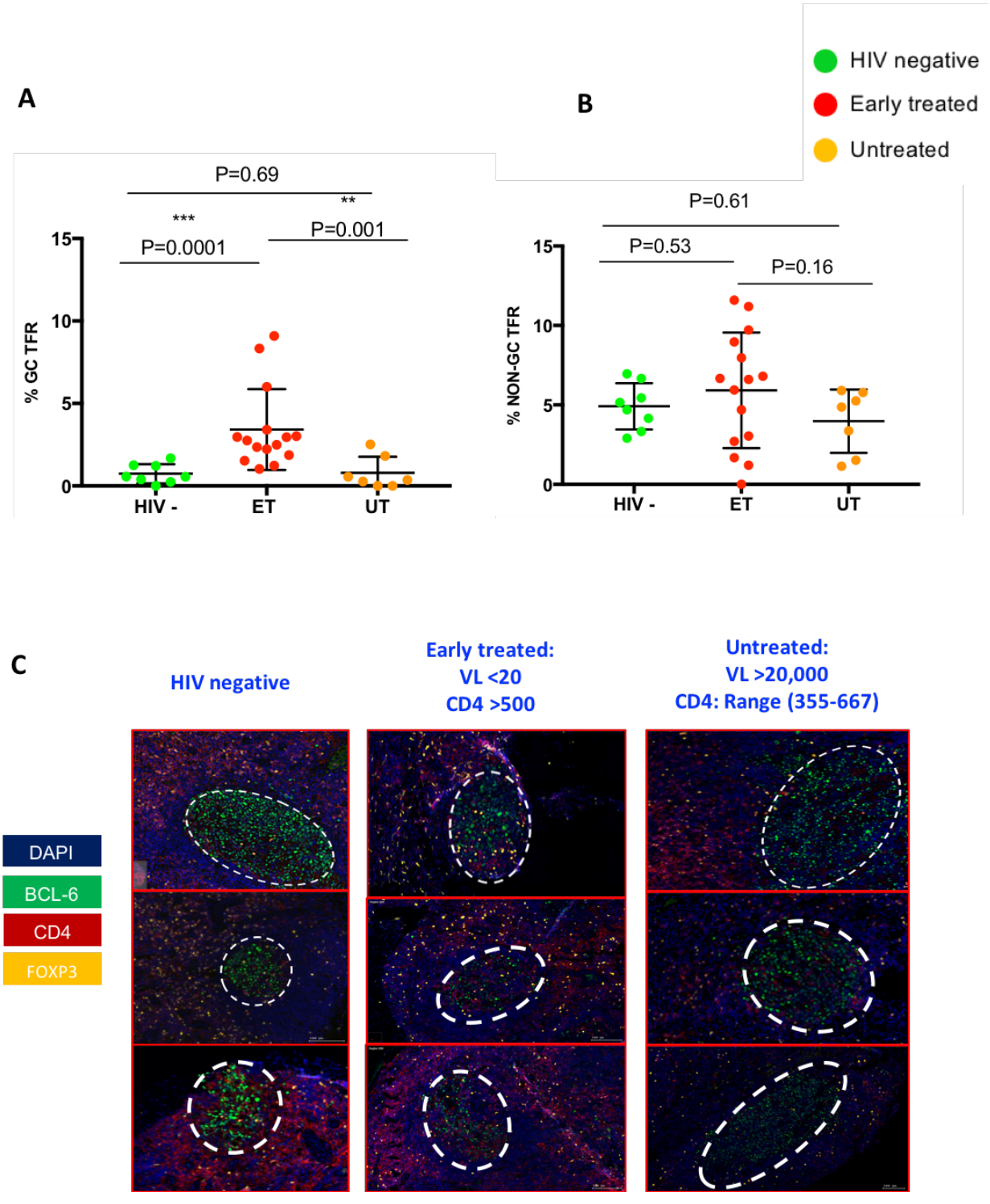
4.4.3 Increased frequencies of GC TFR during early treatment of HIV-1.

Having observed increased frequencies of total TFR cells outside of the GC, we next wanted to assess if TFR localization is affected by HIV infection and therapy status. Most previous studies assessing the impact of ART on TREG frequencies have suggested that successful treatment significantly decreases or normalized TREG frequencies to similar levels of healthy donors (22, 24, 32). Moreover, peripheral TREG frequency was shown to be lower in ART-treated individuals compared to chronic, untreated individuals (33). To our knowledge, there are no studies that have assessed changes in TFR frequencies during very early ART initiation, more so in human lymph nodes. Determining the effect of early treatment on the preservation of regulatory CD4+ T cell populations in lymph nodes is important in providing insight into the impact of sustained viral suppression by very early therapy on immune regulation within lymphoid tissue.

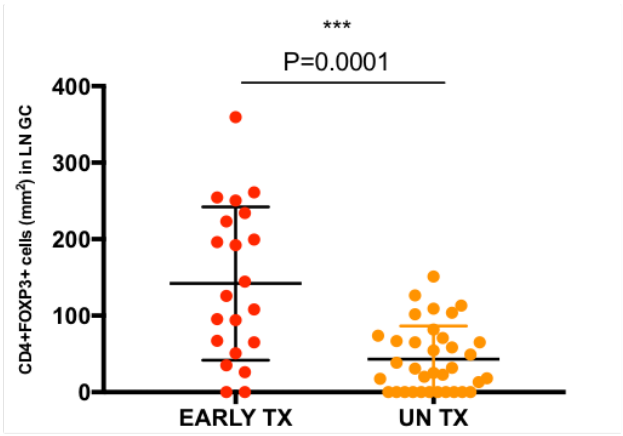
Using flow cytometry, we first compared TFR frequencies within the GCs (GC TFR) and outside of the GC (non-GC TFR) using the previously described phenotype in chapter 3 of this thesis. We found that very early initiation of ART resulted in a significant increase in GC TFR frequencies compared to untreated individuals ($p=0.001$) as well as HIV negative individuals ($p=0.0001$) (Figure 4.3 A). Similarly, IF imaging using BCL-6 as a marker for the GC and CD4+FOXP3+ as TFR cells (Figure 4.3 C), revealed increased infiltration of TFR into GCs in very early treated individuals compared to untreated individuals (Figure 4.3 D) ($p=0.0001$). The number of GCs in HIV negative individuals

by IF staining were negligible to warrant comparison. No differences in non-GC TFR frequency was observed between HIV negative and HIV infected early treated (p=0.53) and untreated individuals (p=0.61) (Figure 4.3 B). We also did not find differences between early treated and untreated HIV infected individuals (p=0.16) (Figure 4.3 B).

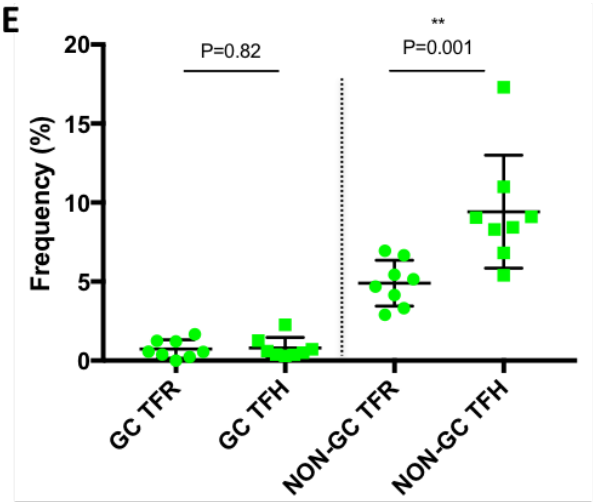
We next compared TFR to TFH frequencies by flow cytometry. Understanding changes in TFH and TFR cells during HIV infection is important because both cell types directly interact with cognate B cells and control antibody production, with many studies alluding to their role in controlling TFH function (2-5, 20). TFR and TFH phenotypes have been previously described (Chapter 3 of thesis). There were higher frequencies of non- GC TFH in HIV negative (p=0.001) (Figure 4.3 E), HIV infected early treated (p=0.003) (Figure 4.3 F) and chronic untreated (p=0.0006) (Figure 4.3 G) as compared to non-GC TFR. Moreover, early ART initiation was associated with increased GC TFR with a concomitant decrease in GC TFH (p=0.0004) (Figure 4.3 F). GC TFR and GC TFH frequencies were comparable in HIV negative (p=0.82) (Figure 4.3 E) and chronic untreated individuals (p=0.12) (Figure 4.3 G). To our knowledge, there have been no previous reports of increased TFR frequencies within the GC during very early ART initiation. This result warrants further exploration into the effect of very early ART initiation on cells within the GC and alterations in their frequency and localization.


A
B
HIV negative
Early treated
Untreated
P=0.69
**
***
P=0.001
P=0.0001
% GC TFR
15
10
5
0
HIV -
ET
UT
P=0.61
P=0.53
P=0.16
% NON-GC TFR
15
10
5
0
HIV -
ET
UT
C
HIV negative
Early treated:
VL <20
CD4 >500
Untreated:
VL >20,000
CD4: Range (355-667)
DAPI
BCL-6
CD4
FOXP3

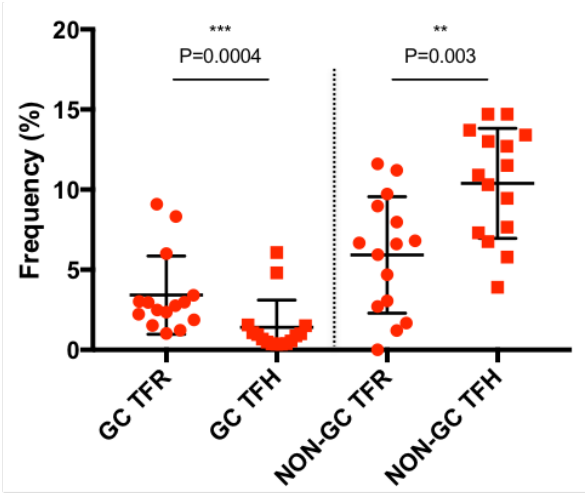
D


***
P=0.0001
CD4+FOXP3+ cells (mm²) in LN GC
400
300
200
100
0
EARLY TX
UN TX

E


P=0.82
**
P=0.001
Frequency (%)
20
15
10
5
0
GC TFR
GC TFH
NON-GC TFR
NON-GC TFH

F


***
P=0.0004
**
P=0.003
Frequency (%)
20
15
10
5
0
GC TFR
GC TFH
NON-GC TFR
NON-GC TFH

G

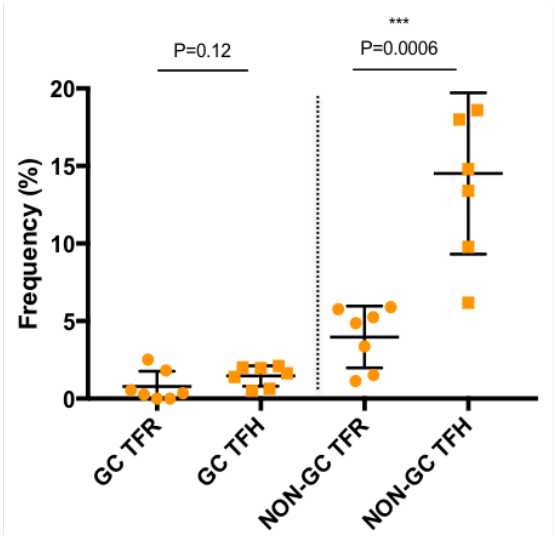
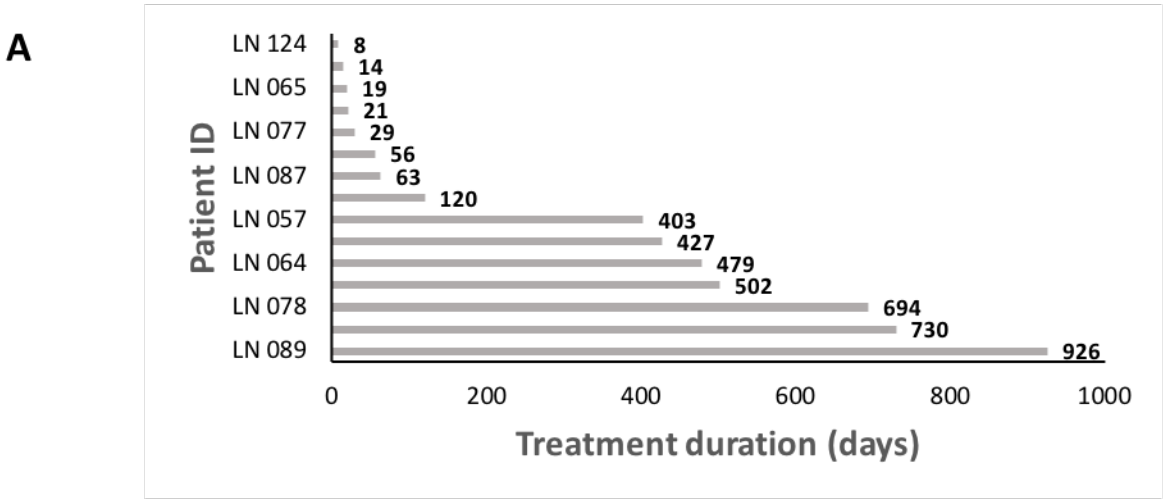

P=0.12
***
P=0.0006
Frequency (%)
20
15
10
5
0
GC TFR
GC TFH
NON-GC TFR
NON-GC TFH

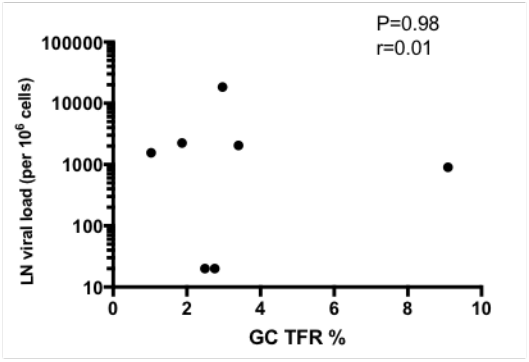
Figure 4.3 Increased GC TFR during early treatment initiation. Summary plots comparing the frequencies of GC TFR (A) and non-GC TFR (B) between HIV negative (n=8), HIV infected early treated (n=15) and untreated (n=7) subjects. (C) Representative immunofluorescence images of multiplexed BCL-6, CD4 and FOXP3 for 3 each HIV negative, early treated and untreated LN cross-sections. (D) Summary plots comparing CD4+FOXP3+ density in GCs of early treated and untreated subjects (p=0.0001). (E-G) Summary plots indicating frequency of GC and non-GC TFR to GC and non-GC TFH for (E) HIV negative, (F) early treated and (G) untreated subjects. P values were determined using Mann-Whitney U test.

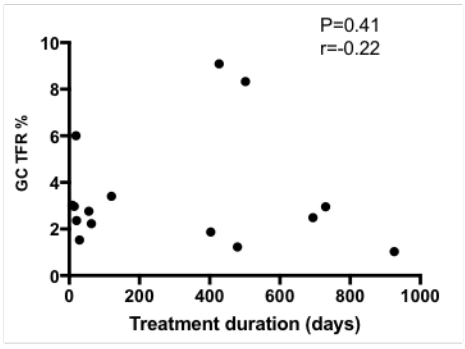
4.4.4 Increased GC TFR during early treatment correlates with Gag p24 persistence.

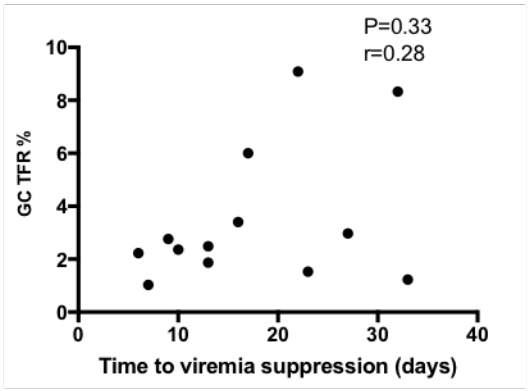
Having observed increased frequencies of GC TFR in the 15 early treated individuals (who were detected during hyperacute HIV-1 infection and initiated treatment at a median of 1 day after virus detection) (Figure 4.4 A), we next wanted to investigate their role during early treated HIV-1 infection. We assessed various parameters including lymph node viral load (Figure 4.4 B), treatment duration prior to lymph node biopsy (Figure 4.4 C), time to viremia suppression (Figure 4.4 D), Fiebig staging of participants (Figure 4.4 E) and HIV Gagp24 density within the lymph node (Figure 4.4 F). Lymph node viral load was assessed, despite having previously found no relationship with peak viral load and TFR frequencies in early treated individuals, as data from our group has shown discordant HIV-1 RNA load in plasma and lymph nodes, with higher median viral loads in lymph node cells compared to undetectable plasma viral loads. Access to early treated individuals with different kinetics in terms of treatment duration, time to viremia

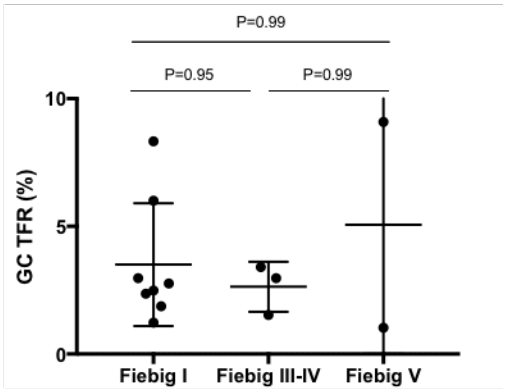
suppression and Fiebig staging allowed for detailed analysis of the observed increase in GC TFR frequencies. We observed no correlation of GC TFR frequency to any of these parameters except for HIV Gagp24 antigen density ($p=0.03$, $r=0.82$) (Figure 4.4 F). Immunostaining of the HIV-1 Gag p24 antigen in lymph node biopsies has been used to define virus persistence (34). Our group has previously shown that HIV Gag p24 persists long term in lymph nodes of individuals that have initiated ART soon after HIV infection (Baiyegunhi *et al*., 2018; data unpublished). These results warrant further investigation into molecular mechanisms that drive TFR expansion within the GC, even in the face of very early treatment initiation.


A
Patient ID
LN 124
LN 065
LN 077
LN 087
LN 057
LN 064
LN 078
LN 089
8
14
19
21
29
56
63
120
403
427
479
502
694
730
926
0
200
400
600
800
1000
Treatment duration (days)


B
P=0.98
r=0.01
LN viral load (per 10^6 cells)
GC TFR %


C
P=0.41
r=-0.22
GC TFR %
Treatment duration (days)


D
P=0.33
r=0.28
GC TFR %
Time to viremia suppression (days)


E
P=0.99
P=0.95
P=0.99
GC TFR (%)
Fiebig I
Fiebig III-IV
Fiebig V

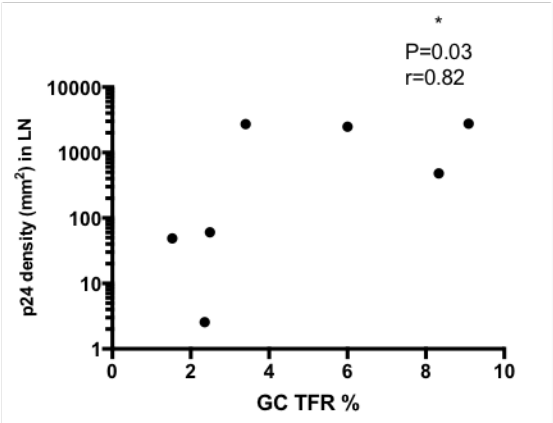

F
*
P=0.03
r=0.82
p24 density (mm²) in LN
GC TFR %

Figure 4.4 HIV p24 antigen correlates with GC TFR. (A) Kinetics of treatment duration across early treated participants. Correlation of GC TFR frequencies from flow cytometry staining and (B) LN viral load, (C) treatment duration, (D) time to viremia suppression and (F) HIV p24 antigen. (E) Distribution of GC TFR frequencies according to fiebig staging of participant upon treatment initiation. Spearman's rank correlation used to compute P and rho (r) values. Comparison of fiebig staging P value was determined using Mann-Whitney U test.

4.4.5 TFR play a dual helper and immunoregulatory role during HIV infection.

Having determined the localization and changes in TFR frequency according to disease and therapy status, we next wanted to assess function of these cells. We began by first analyzing function of TFR by cytokine secretion. We adopted droplet digital PCR (ddPCR) to detect IL-10 and IL-21 mRNA expression levels, due to the inability of other conventional methods such as flow cytometry to reliably measure these low-level cytokines, without manipulation or high background staining. IL-10 and IL-21 were chosen as they have been previously described as canonical cytokines secreted by regulatory subsets and T follicular helper cells respectively. IL-10 has been previously described as the master regulator of immunity to infection (35) and IL-21 has been shown to be secreted by TFH cells to provide help to B cells for the generation of affinity-matured antibody during the GC reaction (36). The ddPCR method is based on water-oil emulsion droplet technology, where a 20 µl sample is fractionated into 20,000 droplets and PCR amplification of the template molecules occurs in each individual droplet (Bio-Rad). The readout allows for separation of positive (for target gene) and negative

droplets. The technology provides an absolute count of target DNA copies per input sample without the need for running standard curves. We analyzed ddPCR on a total of 17 individuals (4 HIV negative, 8 HIV-infected early treated and 5 HIV-infected chronic untreated) (Table 4.2). To allow for true quantification of selected subsets, we sorted three populations of interest i.e. double negative (CD4+CXCR5-PD-1-), GC TFH (CD4+CXCR5^{hi}PD-1^{hi}) and TFR (CD4+CXCR5+PD-1+CD25+CD127-). Due to sorting and sample constraints, we were unable to sort GC TFR. The expectation was that the double negative population would show lowest levels of each cytokine. We utilized a housekeeping gene, B2M (Figure 4.5 B), which is found in diploid on all human cells to normalize expression levels of IL-10 and IL-21 (Figure 4.5 A) and determine input cell number.

We confirmed the production of IL-10 by TFR, interestingly, we found that they also produce IL-21 mRNA. Furthermore, we observed no significant differences in mRNA expression levels of both IL-10 (Figure 4.5 C) and IL-21 (Figure 4.5 D) between GC TFH and non-GC TFR. Previous work has reported that TFH cells, like TFR can produce IL-10 (37). Intriguingly, a previous study in LCMV showed that compared to IL-21-single producing TFH cells, IL-10+IL-21+co-producing TFH cells exhibited an enhanced germinal center (GC) TFH-like profile. Additionally, IL-10-producing TFH cells have an increased capacity to form stable TFH-B cell conjugates compared to their IL-10^{-} TFH counterparts (38). Furthermore, IL-10 secretion via TFR has been shown to promote the GC reaction (39). These studies suggest that TFR play a multi-faceted role in fine-tuning of the GC reaction. The results also suggest a role for TFH-derived IL-10 in promoting humoral immunity during viral infection.

Table 4.2 Study participants for ddPCR assay

	HIV negative	Early treated	Untreated
n	4	8	5
Age (years)	21 (20-22)	22 (21-25)	26 (18-29)
CD4 count (cells/ul)	N/A	986 (650-1290)	511 (251-1229)
Viral load (copies/ml)	N/A	<20 (<20-130,000)	22,000 (87-400,000)

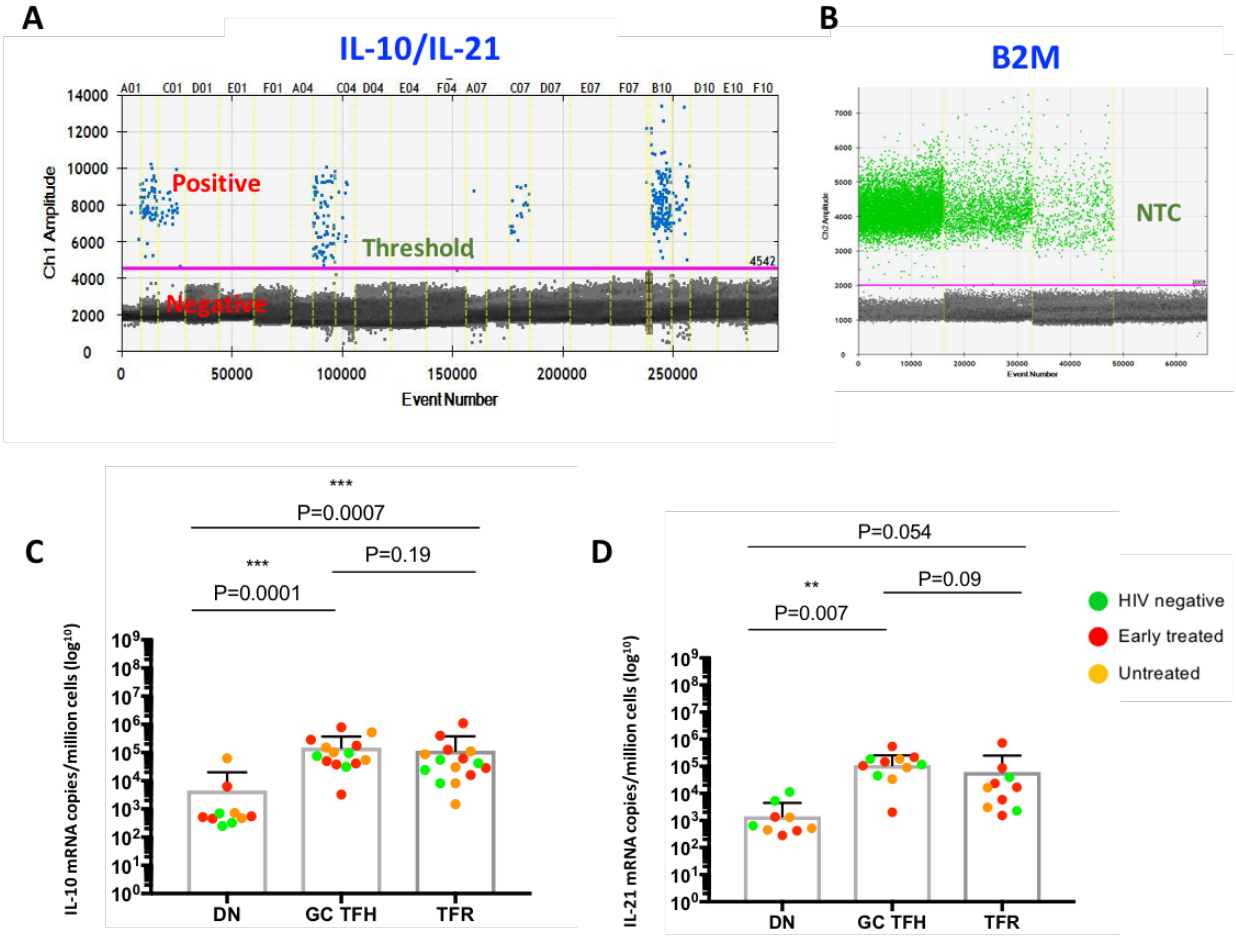


Figure 4.5 IL-10 and IL-21 mRNA expression. (A) Representative plots indicating positive IL-10/IL-21 and (B) B2M mRNA expression computed by QX200 digital droplet PCR machine. Threshold (pink line) manually set to stringently divide negative and positive droplets. (C-D) Summary plots indicating mRNA quantification of DN (double negative, CD4+CXCR5-PD-1^{-}), GC TFH (CD4+CXCR5^{hi}PD-1^{hi}) and TFR

(CD4+CXCR5+^{i}PD-1^{+}CD25+CD127-) for IL-10 (C) and IL-21 (D) in HIV negative (n=4), HIV-infected early treated (n=8) and HIV-infected untreated (n=5) subjects.

4.4.6 Non- GC TFR frequency in lymph nodes correlates with gp41 IgG responses.

While it is evident that TFR play an important role in the control of antibody production, the question remains on whether they primarily control GC B-cell numbers or have an indirect role in the control of the quality and specificity of the antibody response. We next wanted to assess an alternate function of TFR, by determining whether TFR frequencies inside and outside of the GC indicated a relationship to B cell responses by production of HIV-specific class switched antibodies. Previous animal studies have shown that TFR cells were required to produce high-affinity antigen-specific Abs (9) and loss of TFR cells led to significantly decreased IgG responses. We analyzed non-GC TFR as well due to a recent study that suggested that TFR-mediated suppression is primarily mediated outside of the GC (21), and we assumed this would be true for "helper" functions as well. We measured plasma HIV-1 gp41 and gp120 glycoprotein-specific IgG and IgM titers in early treated individuals at 12 weeks post-infection because previous data from our group indicated that plasma gp41 and gp120 antibodies were induced early and peaked at approximately 12 weeks post-infection in these cohort of early treated individuals (Baiyegunhi *et al.,* 2018; data unpublished). We observed a significant positive correlation between non-GC TFR frequency and gp41 IgG (Figure 4.6 F), which was not observed with GC TFR (Figure 4.6 B). However, we did not observe a correlation of GC TFR and non- GC TFR frequencies to either gp120 IgG (Figure 4.6 A and E), gp41 IgM (Figure 4.6 C and G) or gp120 IgM (Figure 4.6 D and H) respectively. These data suggest localization of TFR cells outside the GCs could be influencing gp 41 IgG production in

our cohort, supporting the interpretation by Sayin *et al.,* 2018 (21), that TFR cells do not need to physically reside in the GC to modulate antibody production.. Furthermore, the magnitude of the TFR response could be a reaction to the magnitude of antibody response. These results could suggest that during antibody production, the role of TFR could be dual functional as inhibitors or promoters.

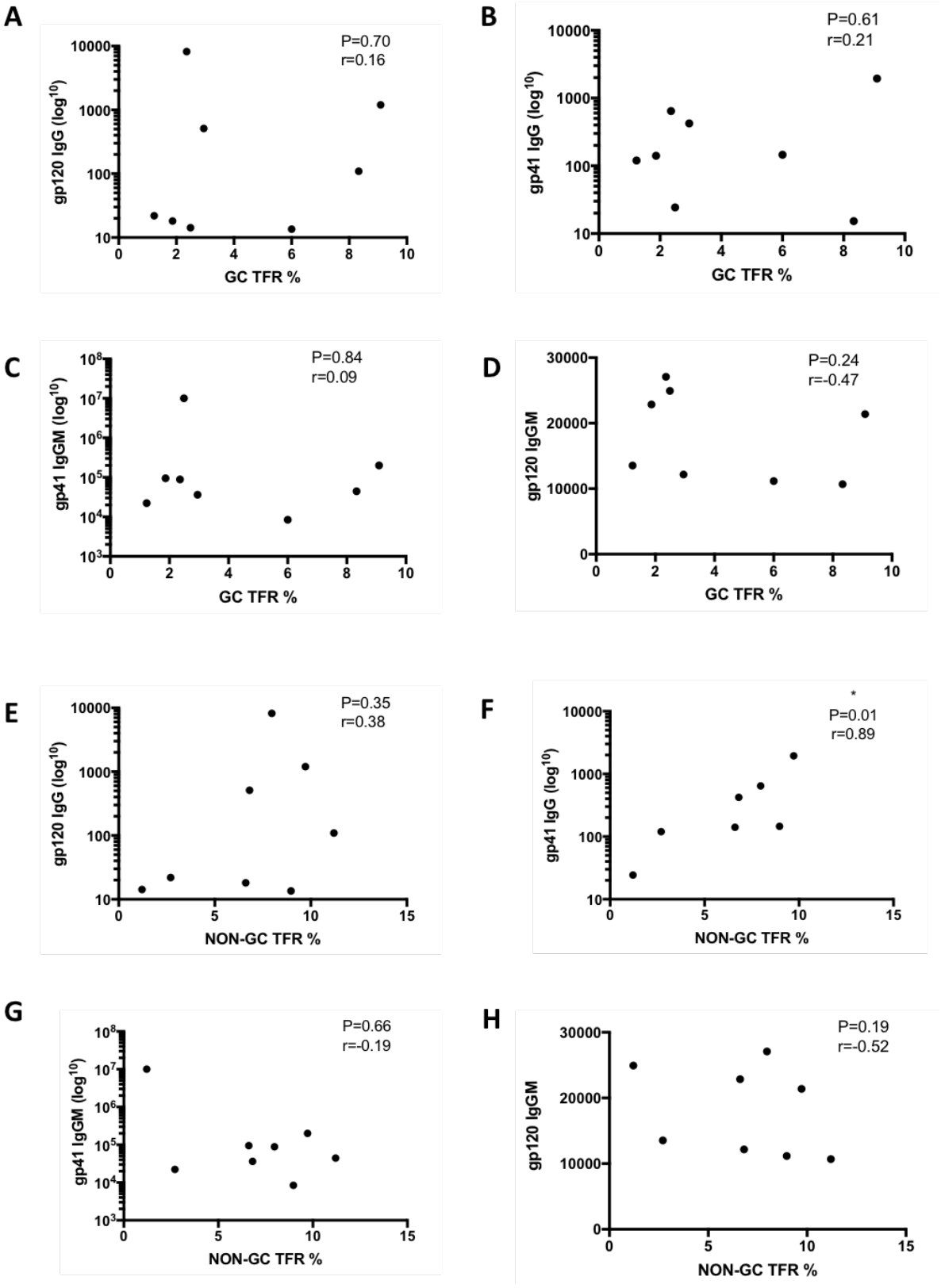

A
P=0.70
r=0.16
gp120 IgG (log10)
GC TFR %
B
P=0.61
r=0.21
gp41 IgG (log10)
GC TFR %
C
P=0.84
r=0.09
gp41 IgGM (log10)
GC TFR %
D
P=0.24
r=-0.47
gp120 IgGM
GC TFR %
E
P=0.35
r=0.38
gp120 IgG (log10)
NON-GC TFR %
F
*
P=0.01
r=0.89
gp41 IgG (log10)
NON-GC TFR %
G
P=0.66
r=-0.19
gp41 IgGM (log10)
NON-GC TFR %
H
P=0.19
r=-0.52
gp120 IgGM
NON-GC TFR %

Figure 4.6 non- GC TFR frequency in LN correlates with gp41 IgG responses at 12 weeks post infection. Correlation between frequencies of GC TFR and non-GC TFR to titers of gp120 IgG (A, E), gp41IgG (B), gp41 IgM (C, G) and gp120IgM (D, H) respectively. Titers for these antibodies were determined at 12 weeks post-infection for early treated individuals (n=8). Spearman's rank correlation P and rho (r) values reported.

4.5 Discussion

Animal studies have shown that TFR is a specialized TREG subset which potentially regulates GC B and T cells during the GC reaction, however due to their relatively recent discovery, the role of TFR in human disease remains to be fully elucidated. In this study, we provide a comprehensive analysis of the localization and function of TFR cells in HIV-1 infection, particularly in the setting of very early treatment initiation. We show that despite the widely-accepted paradigm that TFR cells function within the GC, we find that they mostly localize in the extra-follicular region, where they likely influence the humoral immune response from an "outside in" concept described by Sayin *et al.,* (21). This proposed concept suggests various methods by which TFR could exert their function. These include, preventing long-lived interactions between cognate T and B cells at the T-B border, which has been shown to be an important site for TFH differentiation (40, 41), preventing activated TFH cells from entering pre-existing GC reactions (42), or as widely believed, directly suppressing B cells, thus preventing differentiation into memory B cells, GC B cells and long-lived plasma cells (18, 19, 43).

Similar to TREGs, the role of TFR on HIV pathogenesis is also unclear. Furthermore, the impact of prompt antigen reduction by very early ART initiation on TFR frequency,

function and localization has not been explored. Our data showed no significant differences in TFR frequencies between chronic untreated individuals as compared to HIV negative individuals for both GC and non-GC TFR. A similar result was observed in a SIV macaque model indicating an apparent reduction in TFR frequency in the lymph nodes during the chronic phase of infection measured both by flow cytometry and immunohistochemistry, which was also observed in acute infection (44). This was opposed to different studies that showed an expansion of TFR in both chronic HIV and SIV infection (20). The conflicting results pertaining to the dynamics of TFR cells might be due to numerous factors including sample size, sampling time points and lack of consensus of phenotypic markers and anatomical locations sampled (peripheral blood versus tissues) and differences in methods of quantification (frequencies vs absolute numbers) (45). All of these discrepancies contribute to a lack of consensus on the important roles played by regulatory cells during the course of HIV-1 infection. With access to a unique cohort of paired LN and peripheral blood samples, we have been able to shed some light on the dual role of suppressor and helper of regulatory cells within lymphoid tissue.

Although, previous work has described the impact of ART on TREG frequencies as significantly decreasing or normalizing TREG frequencies to similar levels of healthy donors (24), we found that very early ART initiation results in increased TFR frequencies, particularly within the GC, this in comparison to HIV negative and chronic untreated. TFH cell numbers need to be well regulated in the GCs to have optimal B cell affinity maturation (46), with very high GC TFH cell numbers during untreated chronic HIV infection contributing to a dysregulated GC response (47). TFR could be playing a role

in this regulation. Also, a previous study from our group showed that early treated individuals have superior functioning TFH cells, which could be attributed to a more mitigated TFH response (Baiyegunhi *et al*., 2018, unpublished). Increased TFR frequencies within the GC, observed during very early ART initiation, could be contributing to the better functional output of TFH.

There has been much discussion on the function of TFR, with literature mainly describing them as suppressors of the GC reaction and the antibody response. We assessed the function of TFR in two ways, firstly by measuring IL-10 and IL-21, canonical regulatory and helper cytokines respectively and secondly, by determining the relationship between TFR frequency and HIV-specific antibody titers. Interestingly, we found no differences in mRNA expression of IL-10 and IL-21 between GC TFH and TFR. As mentioned, consensus considers IL-10 an inhibitory cytokine, however it has been reported that TFH, like TFR cells, can produce IL-10 (37). Furthermore, IL-10 secretion has also been shown to promote and maintain the GC reaction in LCMV infection (39). IL-21 has been shown to be key cytokine that drive germinal center formation and antibody class switch recombination (Pissani et al., 2014, Morita et al., 2011), and notably IL-10^{+}IL-21^{+}co-producing TFH cells have been shown to exhibit an enhanced germinal center (GC) TFH-like profile in LCMV again (38). These results suggest that the cells within the GC, particularly TFR cells exert different functions under different conditions, which needs further exploration. Also, further studies are needed to assess the signals that drive TFR cells to switch between help and suppression.

The observation of a positive correlation between gp41 IgG titers and non-GC TFR frequency warrants further study. In conjunction with the view of TFR as suppressors more evidence is emerging that they could play a helper role. A murine model by Wu *et al*., (9) showed that the loss of TFR cells led to a significantly decreased IgG response, with TFR cells being required to produce the highest affinity Ag-specific antibodies. Furthermore, in a peanut food allergy model by Xie and Dent (14), TFR cells have been shown to maintain peanut-specific GC responses and IgE response. Thus, highlighting the key role TFR could play as novel targets for various immunotherapies.

Overall, these data provide further detailed insight into the localization and function of TFR cells in HIV-1 infection. The study suggests a role of TFR in regulating GC TFH cell numbers during early treatment, which could culminate to better functioning of TFH cells and preventing a dysregulated GC response.

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CHAPTER 5: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

The HIV/AIDS pandemic represents one of the most important global health challenges. There have been tremendous advances in biomedical research and the implementation of effective antiretroviral treatment (ART), which enabled control of the virus resulting in dramatic improvement in the health and life expectancy of people living with HIV. Nonetheless, current treatment strategies have various limitations. Drug toxicities, persistent immune dysfunction, inflammation, excessive risk of co-morbidities and psychological issues pose important health consequences, over and above the immense logistical and operational challenges that come with delivering life-long care (44). These factors highlight the importance of identifying an effective means of controlling the virus in the absence of therapy. Therefore, the search for a curative strategy for HIV, together with finding an effective vaccine are key priorities for the future of HIV research. Despite the enormous challenges faced in achieving the goals of an effective vaccine and development of a cure, there has been significant progress made. Candidate vaccine trials are currently being tested (172) and the definition of a cure has shifted from finding a sterilizing cure to a more practical outcome of long-term remission, generally defined as absence of viral rebound after ART cessation, rather than complete eradication within an individual of all replication-competent HIV (63-65).

Central to development of a cure or a preventative HIV vaccine is to improve our understanding of host antiviral immune responses, particularly in very important tissue sites. These tissues have been studied in animal models, but these models have limitations

such as use of animal-specific viruses, shorter duration and lower effectiveness of ART (173). In addition, widespread use of ART for both HIV treatment and pre-exposure prophylaxis has dramatically altered the direction of HIV research. Effective ART reverses many abnormalities caused by HIV's profound impact on the immune system, however a state of persistent inflammation and immune dysfunction typically persists. Chronic low-level inflammation within the adaptive and innate immune systems, CD4+ and CD8+ T cell dysfunction, lymphoid fibrosis and elevated immunoregulatory responses characterize the immune state during ART (174-176). This compromised immune state is thought to contribute to HIV persistence, with efforts to clear the reservoir requiring a reversal of some or all of these immunologic abnormalities (61, 77). Improved understanding of immune responses in early treated individuals would be valuable to future HIV cure interventions or vaccination strategies.

Our group is uniquely positioned to contribute to gaps in knowledge due to access to a hyperacute HIV infection cohort. The FRESH cohort was purposefully designed to identify early infection by carrying out longitudinal follow-ups and frequent testing for HIV acquisition in a high-risk HIV negative study population (56). Individuals identified in hyperacute infection are placed on ART very soon after infection, with their immune responses being able to be defined in peripheral blood and lymph node samples. The cohort is an invaluable resource in enabling longitudinal characterization of HIV-specific immune responses in very early stages of HIV-1 infection and during very early ART initiation.

A growing body of literature has shown that effector CD4+ T cells are an important factor in immune control of HIV-1 infection. The role of CD4+ T cell responses in the control of numerous chronic viral infections has been well characterized, yet little is known about the role of these responses in HIV-1 infection. Previous studies have suggested that the preservation of HIV-1 specific CD4+ T cell responses might be critical for the control of viral replication (177, 178). A CD4+ T cell subset, T regulatory cells (TREGs) have been shown to be a key component of the immune system, to maintain the delicate balance between overactive responses and immunosuppression. In HIV infection, TREGs play both a beneficial and detrimental role (133). They regulate the immune system such that inflammation and spread of virus through activated T cells in suppressed. However, the suppression of immune activation also limits viral clearance and promotes reservoir formation (179). The apparent importance of TREGs in the pathogenesis and control of HIV makes them an interesting target for therapeutic manipulation in the search for an HIV cure.

The majority of HIV replication occurs in secondary lymphoid organs, with HIV RNA particularly concentrated in B cell follicles during chronic untreated infection, prior to progression to AIDS (155, 158). Data during chronic HIV disease show that follicular CD4+ cells are approximately 30-40-fold more likely to harbor HIV RNA compared to extra-follicular counterparts (156, 180). A reduction in the frequency of HIV RNA+ cells in lymph nodes and B cell follicles is observed during ART and lymphoid architecture is partially restored (181). TFR have been shown to limit the GC reaction and antibody production to prevent autoimmunity (138). The discovery of TFR has led to a revised view of the regulation of anti-HIV humoral immunity. However, many questions

regarding their biology, localization and function remain unanswered particularly as most of the previous work carried out on TFR has been carried out in non-human models or non-tissue samples. Identifying the factors that influence TFH function during HIV infection would be important and could lead to improved immune reconstitution in ART-treated individuals and potentially augment strategies to cure HIV infection.

The current study comprehensively characterized regulatory cells, particularly TFR from lymph nodes and peripheral blood during untreated and very early treated HIV-1 clade C infection and defined the phenotype, localization, antigen-specificity and function of these cells. The study showed that TFR are important players within the controlled sanctuary sites of LNs and early initiation of ART changes the localization and function of these cells.

Study implications and future directions

Overall, these studies highlight the important contribution of CD4+ T cells to immune protection against HIV-1 infection. Our initial study directed at developing tools to identify low frequency HIV-specific CD4+ T cells in the setting of clade C infection demonstrated an association between the frequency of HIV-specific CD4+T cell responses targeting an immunodominant DRB1*11-Gag41 complex and HIV viral control, thus highlighting the important contribution of a single class II MHC-peptide complex to the immune response against HIV-1 infections. The data obtained further underlined the use of MHC class II tetramers as a sensitive tool for interrogating HIV-specific CD4+ T cells responses in natural infections.

The rest of the investigations focused on characterizing immunoregulatory CD4+ T cells within peripheral blood and lymph node compartments. We identified TREGs, TFR and TFH cell subsets in HIV negative, HIV infected early treated and untreated individuals. We further used the class II tetramers developed to answer an important outstanding question in the field: do TREGs/TFR need to be antigen-specific to carry out their role? Regulatory cells, like all CD4+ T cells are able to specifically recognize antigenic peptides in the context of MHC class II molecules, due to a somatically-rearranged TCR. Conventional T helper cell activation requires specific antigen recognition by the TCR and it is expected that TREGs would follow this pattern (182). It has been shown that antigen exposure triggers the differentiation of TFR cells (141, 144). However, the signals that TREGs respond to in order to become TFR cells is not well understood. Studies have shown that TFR cells respond more strongly to self-antigens than foreign antigens (144, 147). Although TFR cells have been found to have specificity for the immunizing antigen (144), another study on TCR specificity of TFH and TFR indicated that TFR cells do not respond well to cognate antigen after immunization (183). In our study we were able to detect antigen-specific (HIV) TFR, however predominantly in lymph nodes and more so during untreated HIV infection. There were a few limitations regarding this analysis including: sample size and number of tetramers tested. Future studies should include synthesizing MHC class II tetramers that represent majority of the test population to ensure wider coverage HIV-specific CD4+ T cell responses. Indeed, it would be important to identify the signals that drive TFR responses to the GC and ultimately what antigen they recognize.

The identification of TFR predominantly outside of the GC warrants further investigation on how TFR cell activity is mediated. Furthermore, the role of ART in directing TFR within the GC is another important observation, particularly as early treatment has been shown to be beneficial for improving the quality of the TFH response in our cohort. The key observation of comparable IL-10 secretion by TFH and TFR as well as the link between non-GC TFR frequencies and gp41 IgG point to the number of unanswered questions regarding the role of TFR in promoting GC B cell responses and the GC-dependent high-affinity Ab response. An interesting concept of TFR being able to maintain a key balance between help by GC maintenance, antibody response and antibody affinity and suppression by controlling TFH cell numbers, GC B cell numbers and TFH cell cytokines (148) further highlights the complexity of these cells as well as the scope of knowledge that still has to be explored.

Ultimately, regulatory CD4+ T cells may play a central role in shaping the HIV reservoir and compromising the HIV-specific immune responses. Future work should focus on further refining the effects of various TREG manipulation techniques on the reservoir, which could be used in combination with other immunotherapies. Depletion of TREGs has the ability to directly target a small fraction of the reservoir, reactivate the virus and boost cell-mediated immune responses, all of which could be a desirable strategy for cure research. Future studies assessing the suggested increased permissiveness of TFR (161) are important as their permissiveness to infection enhances the beneficial aspects of targeting TFR.

In conclusion, the results of this study give important insights into regulatory cells within lymph nodes; their biology, function and their role in the setting of very ART initiation. Our results support the notion that TFR are important in contributing to the overall humoral response and could be interesting targets for therapeutic manipulation for an HIV cure.

CHAPTER 6: REFERENCES

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APPENDICES

Appendix 1: Ethics approval of study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

27 March 2017

Ms F Laher (209509632)
HIV Pathogenesis Programme
School of Laboratory Medicine and Medical Sciences
Laher.f@gmail.com

Dear Ms Laher

Protocol: CD4+ T cell immunoregulatory networks in peripheral blood and lymphoid tissue during HIV-1 clade C infection.
Degree: PhD
BREC reference number: BE046/17

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 20 January 2017.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 10 March 2017 to BREC letter dated 09 March 2017 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 27 March 2017.

This approval is valid for one year from **27 March 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **11 April 2017.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor Joyce Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: ndhlovuz@ukzn.ac.za
cc postgraduate administrator: dudhraip@ukzn.ac.za

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za

Appendix 2: Additional ethics approval of published study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

29 August 2013

Ms F Laher
719 Umbilo Road
Doris Duke Medical Institute
Level 1
Nelosn R Mandela School of Medicine Congella
Durban,4013
laher.f@gmail.com

PROTOCOL: Mapping Immunodominant Patterns of HIV-specific CD4 T cell responses in acute and chronic HIV-1 subtype C infection. REF: BE190/13

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 25 April 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 15 August 2013 to queries raised on 07 August 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 29 August 2013.

This approval is valid for one year from **29 August 2013.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **08 October 2013.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

Professor D Wassenaar (Chair)
Biomedical Research Ethics Committee
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban, 4000, South Africa
Telephone: +27 (0)31 260 2384 **Facsimile:** +27 (0)31 260 4609 **Email:** brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

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