DEFINING HIV PERSISTENCE AND HOST IMMUNE RESPONSES IN LYMPH NODES OF COMBINED ANTIRETROVIRAL THERAPY (cART) SUPPRESSED INDIVIDUALS AND THE DETERMINATION OF THE IMPACT OF HIV INFECTION ON SARS-CoV-2 SPECIFIC T CELL RESPONSES IN SOUTH AFRICA.

By

CAROLINE CHASARA

Submitted in fulfillment of the requirements for the

Doctoral of Philosophy Degree

in the

School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Nelson R. Mandela School of Medicine
University of KwaZulu-Natal
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Supervisor: Professor Zaza M. Ndhlovu

PREFACE

The experimental work described in this thesis was carried out at the Africa Health Research Institute (AHRI) and HIV Pathogenesis Programme (HPP), School of Laboratory Medicine and Medical Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, from July 2019 to June 2023, under Professor Zaza M. Ndhlovu's supervision.

This thesis comprises of two independent research projects including Coronavirus disease of 2019 (COVID-19) and Human Immunodeficiency Virus (HIV) research and the original aim of this study which was centered around understanding the HIV reservoir. When the COVID-19 pandemic hit South Africa in 2020, subsequent lockdowns and restrictions were implemented. This compelled us to change the original PhD proposal to incorporate COVID-19 research. My thesis, therefore, is a combination of the COVID-19 research work carried out during the first half of my PhD studies and the HIV reservoir research work carried out during the second half of my PhD studies.

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

Caroline Chasara (Candidate): [redacted] Date: 16/04/2024

Prof. Zaza Ndhlovu (Supervisor): [redacted] Date: 22/04/2024

DECLARATION 1: PLAGIARISM

I, **Caroline Chasara** declare that:

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

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other person's data pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.

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(v) Where other written sources have been quoted, their words have been re-written, but the general information attributed to them has been referenced.

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Signe  **Date:** 16/04/2024 ----------

DECLARATION 2: PUBLICATIONS AND MANUSCRIPTS

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

Manuscript 1: Caroline Chasara[†], Thandeka Nkosi[†], Andrea O Papadopoulos *, Tiza L Nguni, Farina Karim, Mahomed-Yunus S Moosa, Inbal Gazy, Kondwani Jambo, COMMIT-KZN-Team, Willem Hanekom, Alex Sigal, Zaza M Ndhlovu*. Unsuppressed HIV infection impairs T cell responses to SARS-CoV-2 infection and abrogates T cell cross-recognition.

Authors' contributions: A.S, W.H and COMMIT-KZN initiated the study-cohorts. Z.M.N conceived the study and designed the experiments. F.K processed the samples. M.Y, I.G and A.S provided the samples and clinical data. **C.C** performed some of the experiments with T.N, T.L.N under the supervision of Z.M.N. T.J.N, Z.M.N, K,J. Z.M.N, T.N, and **C.C** analyzed the data and wrote the manuscript. A.P, W.H, A.S and K,J edited the manuscript. **CC** and TN shared first authorship. This paper has been published in eLife Sciencies (DOI: 10.7554/eLife.78374).

Manuscript 2: Caroline Chasara, Trevor Khaba, Bongiwe Mahlobo, Leonard Mvaya, Thandekile Ngubane, Ismail Jajbhay, Johan Pansegrouw, Zaza M. Ndhlovu. Detailed characterization of the phenotype, spatial localization, and function of macrophages within human lymph nodes.

Authors' contributions: Z.M.N conceived the idea. Z.M.N and **C.C** designed the experiments. T.K assisted with imaging studies. L.M assisted with data analysis. **C.C** performed the rest of the laboratory experiments, analyzed the data, and wrote the manuscript under Z.M.N's supervision. This manuscript is in advanced stages of preparation for submission to a peer reviewed journal.

Manuscript 3: Caroline Chasara, Trevor Khaba, Bongiwe Mahlobo, Thandekile Ngubane, Ismail Jajbhay, Johan Pansegrouw, Zaza M. Ndhlovu. Investigating the role of lymph node macrophages in HIV persistence.

Authors' contributions: Z.M.N conceived the idea. Z.M.N and **C.C** designed the experiments. T.K assisted with imaging studies. **C.C** performed the rest of the laboratory experiments, analyzed the data, and wrote the manuscript under Professor Zaza Ndhlovu's supervision. This manuscript is in advanced stages of preparation for submission to a peer reviewed journal.

Caroline Chasara (Candidate): -  Date: 16/04/2024----------

Prof. Zaza Ndhlovu (Supervisor):  Date: 22/02/2024----------

PRESENTATIONS AND SCHOLARSHIP AWARDS

Poster presentations

1. Gordon Research Conference, Barcelona, Spain, June 2022. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.
2. Gordon Research Seminar, Barcelona, Spain, June 2022. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.
3. IUIS-FAIS-SANTHE-Immuno-Zambia Workshop, Lusaka, Zambia, December 2022. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.
4. Welcome Trust poster presentation, Durban, South Africa, January 2023. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.

Oral presentation

1. IUIS-FAIS-SANTHE-Immuno-Zambia Workshop, Lusaka, Zambia, December 2022. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.

Travel awards

1. IDA Symposium and Flow cytometry workshop, CHIL (HVTN), Cape Town, South Africa, October 2019.
2. Gordon Research Conference, Barcelona, Spain, June 2022. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.
3. Gordon Research Seminar, Barcelona, Spain, June 2022. Spatial localization of HIV-infected macrophages in lymph nodes using multicolor Tissue FAX imaging.
4. Imaging Africa Workshop, Cape Town, South Africa, October 2022.
5. IUIS-FAIS-SANTHE-Immuno-Zambia Workshop, Lusaka, Zambia, December 2022.

Scholarship Awards

1. Africa Health Research Institute PhD scholarship award (June 2019).

DEDICATIONS

This project is wholeheartedly dedicated to God,
for His abundant grace, love, and provision throughout my PhD programme
He has made everything beautiful in its time (Ecclesiastes 3:11).

To my beloved parents,
for being my source of inspiration and for continually providing moral, spiritual, and financial support. You have always given me the freedom to aim for the sky. You are my role models.

To my sister, and two brothers,
For being the sweetest and best siblings. I am extremely grateful for all the care and support.

And finally, to my mentors
who have provided guidance and motivation throughout my studies.

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LIST OF ABBREVIATIONS

AIDS	**A**cquired **I**mmunodeficiency **S**yndrome
cART	**C**ombined **a**ntiretroviral **t**herapy
LN	**L**ymph **n**ode
LMC	**L**ymph **n**ode **m**ononuclear **c**ells
PBMC	**P**eripheral **b**lood **m**ononuclear **c**ells
HIV	**H**uman **I**mmunodeficiency **V**irus
SIV	**S**imian **I**mmunodeficiency **V**irus
SA	**S**outh **A**frica
GC	**G**erminal **c**enter
BCL-6	**B** cell **l**ymphoma **6**
ET	**E**arly **t**reated
LT	**L**ate **t**reated
COVID-19	**C**oronavirus **d**isease of **2019**
SARS-CoV-2	**S**evere **A**cute **R**espiratory **S**yndrome **C**oronavirus **2**
PLWH	**P**eople **l**iving with **H**IV
ROI	**R**egions of interest
CD68	**C**luster of **d**ifferentiation **68**
CD11B	**C**luster of **d**ifferentiation **11B**

CD206	**C**luster of **d**ifferentiation **206**
HIV Gag p24	**G**roup **s**pecific **a**ntigen core **p**rotein 24 kDa
FFPE	**F**ormalin-**f**ixed **p**araffin-**e**mbedded
RT	**R**oom **t**emperature
PBS	**P**hosphate **b**uffered **s**aline
RNA	**R**ibonucleic **a**cid
DNA	**D**eoxyribonucleic **a**cid
IF	**I**mmunofluorescence
PID	**P**atient **i**dentifier
H and E	**H**ematoxylin and **e**osin
FACS	**F**luorescence-**a**ctivated **c**ell **s**orting

ABSTRACTS

Abstract 1

People living with HIV (PLWH) who have unsuppressed HIV are at a greater risk of acquiring infectious diseases such as Coronavirus disease of 2019 (COVID-19). More recent data has shown that unsuppressed HIV is associated with severe COVID-19 symptoms, but the mechanisms underpinning this susceptibility are still unclear. In our study we used flow cytometry and culture T lymphocyte expansion to assess the impact of HIV infection on the quality and epitope specificity of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) T cell responses in the first wave and second wave of the COVID-19 epidemic in South Africa. We observed that HIV-seronegative individuals had significantly greater $CD4^+$ T cell responses against the Spike protein compared to the viremic individuals living with HIV. In addition, there was diminished T cell cross-recognition between the two waves, which was more pronounced in individuals with unsuppressed HIV infection. Importantly, we identified four mutations in the *Beta variant* that resulted in the abrogation of T cell recognition. These findings partly explain the increased susceptibility of PLWH to diseases such as COVID-19 and highlight their vulnerability to emerging SARS-CoV-2 variants of concern.

Abstract 2

The major keys to developing an HIV cure is through understanding HIV reservoir dynamics. The role of tissue macrophages in HIV reservoirs is complex and not yet fully understood. However, their ability to support viral replication, longevity, localization in immune sanctuaries, and potential for viral latency all contribute to the persistence and resilience of HIV reservoirs in various tissues throughout the body. Understanding and targeting these reservoirs is a critical area of research in the quest for an HIV cure. To gain insight into the macrophage reservoir, we used a combination of flow cytometry and immunofluorescence microscopy to characterize and investigate HIV persistence in lymph node (LN) macrophages. We detected pro-inflammatory ($CD68^+$) macrophages harboring HIV Gag p24 and HIV-1 RNA in the germinal centers of HIV positive early and late treated individuals suggesting their potential role as an HIV reservoir. In contrast, anti-inflammatory ($CD206^+$) macrophages were localized along lymphatic vessels and outside the germinal centers. Importantly, we show the presence of long-lived $CD4^+$TIM-4^+ macrophages in LNs. The data reported in this thesis will go a long way in furthering our understanding of macrophage HIV reservoirs in lymph node macrophages.

ISIZULU ABSTRACTS

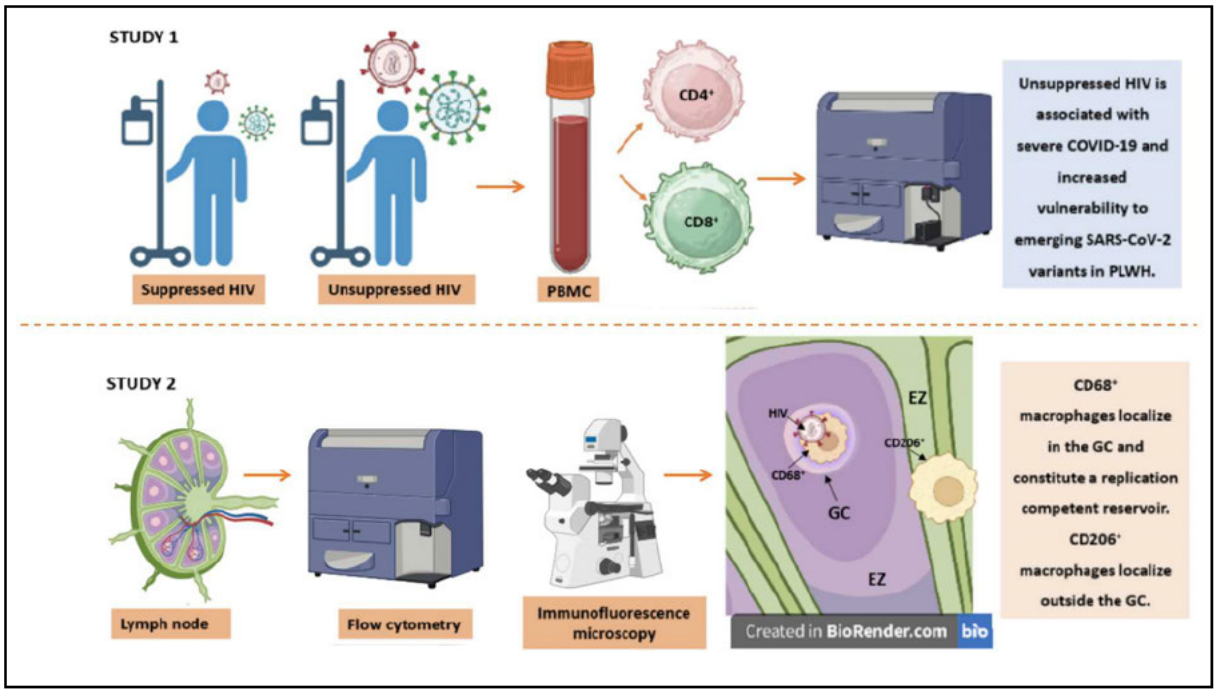
Abstract 1

Abantu abaphila ne-HIV (PLWH) abane-HIV engacindezelwe basengozini enkulu yokuthola izifo ezithathelwanayo njenge-Coronavirus ka-2019 (COVID-19). Idatha yakamuva ibonise ukuthi i-HIV engacindezelwe ihlotshaniswa nezimpawu ezinzima ze-COVID-19, kodwa izindlela ezisekela lokhu kuba sengozini azikacaci. Lapha, siqale sasebenzisa i-flow cytometry kanye nokwandiswa okuthuthukisiwe ukuhlola umthelela wokutheleleka nge-HIV kukhwalithi nokucaciswa kwe-epitope ye severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) T cell izimpendulo kumagagasi okuqala kanye negagasi lesibili. Siqaphele ukuthi abantu abangenayo i-HIV babene-$CD4^+$ T cell impendulo enkulu kakhulu ngokumelene ne-Spike protein uma kuqhathaniswa nabantu abane-HIV abaphila ne-HIV. Ukwengeza, kube nokuncipha kokuqashelwa kwe-T cell phakathi kwamagagasi amabili, okwakubonakala kakhulu kubantu abane-HIV engacindezelwanga. Okubalulekile, sihlonze izinguquko ezine kokuhlukile kwe-*Beta* okuholele ekuhoxisweni kokubonwa kweseli le-T. Lokhu okutholakele kuchaza ngokwengxenye ukwanda kwe-PLWH ezifweni ezinjenge-COVID-19 futhi kugqamisa nokuba sengozini kwezinhlobonhlobo ezisafufusa ze-SARS-CoV-2 zokukhathazeka.

Abstract 2

Enye yezinkinobho ezinkulu zokuthuthukisa ikhambi le-HIV ngokusebenzisa ukuqonda amandla we-HIV reservoir. Iqhaza lama-macrophage ezicubu kumithombo ye-HIV iyinkimbinkimbi futhi ayikaqondwa ngokugcwele. Kodwa-ke, ikhono labo lokusekela ukuphindaphinda kwegciwane, ukuphila isikhathi eside, ukwenziwa kwasendaweni ezindaweni ezivikela amasosha omzimba, kanye namandla ngokubambezeleka kwegciwane konke kunomthelela ekuphikeleleni nasekuqineni kwemithombo ye-HIV ezicutshini ezihlukahlukene emzimbeni wonke. Ukuqonda nokukhomba lezi zindawo zokugcina amanzi kuyingxenye ebalulekile yocwaningo ekufuneni ikhambi le-HIV. Ukuthola ukuqonda nge-macrophage reservoir, sisebenzise inhlanganisela yokugeleza kwe-cytometry, i-immunofluorescence microscopy, ne-RNAscope ukukhombisa nokuphenya ukuphikelela kwe-HIV kuma-macrophages e-lymph node (LN). Sithole i-pro-inflammatory ($CD68^+$) ama-macrophages aphethe i-HIV-Gag p24 ne-HIV-1 RNA ezikhungweni zamagciwane ze-HIV e-ET nabantu be-LT abaphakamisa iqhaza labo njenge-HIV reservoir. Ngokuphambene nalokho, ama-macrophages alwa nokuvuvukala ($CD206^+$) ama-macrophages enziwa endaweni ngemikhumbi ye-lymphatic nangaphandle kwezikhungo zamagciwane. Ngokubalulekile, sibonisa ukuba khona kwama-$CD4^+TIM\text{-}4^+$ ama-macrophages ama-LNs. Idatha ebikwe kule thesis izohamba ibanga elide ekuqhubekiseleni phambili ukuqonda kwethu amarezebe e-HIV e-macrophage kuma-lymph node macrophages.

GRAPHICAL ABSTRACTS



KEY: Peripheral blood mononuclear cells (PBMC), Coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), people living with HIV (PLWH), Germinal center (GC), Extrafollicular zone (EZ), Cluster of Differentiation 68 (CD68), Cluster of Differentiation (CD206). This figure was created using BioRender (https://biorender.com/).

CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

SECTION A

1.1 The Coronavirus disease 2019 (COVID-19) pandemic

COVID-19 is a highly contagious and infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1, 2]. Sadly, the COVID-19 pandemic remains a global challenge due to the unanticipated emergence of evolving SARS-CoV-2 variants. These variants have brought devastating effects including loss of lives and a dramatic strain on public health systems and economies [3, 4]. Additionally, several studies have shown that SARS-CoV-2 variants lead to an increase in COVID-19 severity, viral transmissibility, and mortality [5-8]. These devastating COVID-19 pandemic outcomes necessitated the need for the rapid design of diagnostic methods to rapidly detect and manage SARS-CoV-2 infections [9]. Unprecedented collaborative efforts among researchers, pharmaceutical industries, and governments around the world led to the rapid design and deployment of highly efficacious preventive vaccines and drugs that helped to bring the ragging epidemic under control [10].

1.1.1 COVID-19 burden in South Africa

To date, the impact of COVID-19 has been felt globally and the unprecedented spread of the virus has negatively impacted economies and health sectors [4]. Approximately 519 million cases and 6.26 million mortalities were recorded globally as of 11 May 2022, causing a strain on most global health systems [11]. On March 5, 2020, the first COVID-19 patient was confirmed in South Africa followed by the implementation of a national lockdown on the 27th of March 2020 [12]. Thereafter, the emergence of new variants led to repeated pandemic waves and increased transmissibility [13]. Like many places, by February 2022, South Africa had experienced four distinct pandemic waves caused by the ancestral SARS-CoV-2, Beta, Delta, and Omicron BA.1 variants. The emergence of variants negatively impacted South Africa's economy as well as its healthcare system [14] leading to reprogramming of hospital care units to accommodate COVID-19 patients and closure of selected health facilities. Nevertheless, primary vaccination and booster vaccines were urgently introduced leading to virus containment [15]. In addition, vaccination reduced virus transmission and reinforced protection against severe viral strains [16].

1.2 SARS-CoV-2 structure.

SARS-CoV-2 is a single-stranded positive sense RNA-enveloped virus belonging to the subfamily Coronavirinae [8, 17]. Its genome size is larger than most RNA viruses (approximately 29 kb) and it encodes approximately 9860 amino acids [18]. SARS-CoV-2 contains four main structural proteins including the spike glycoprotein (S), membrane protein (M), nucleocapsid protein (N), and envelope protein (E) (**Figure 1.1**). The spike protein facilitates virus entry into host cells by binding to angiotensin-converting enzyme 2 (ACE2), a host cell receptor. Immediately after the spike protein binds to ACE2, transmembrane protease serine 2 (TMPRSS2) promotes SARS-CoV-2 viral entry into the host cell [19]. Subsequently, viral RNA is released followed by transcription, translation, and virus release from the infected cell to other host cells [20]. The membrane protein is one of the most abundant viral structural proteins. Additionally, it plays a significant role in virus assembly by interacting with other structural proteins. Unlike the membrane protein, the nucleocapsid protein packages viral RNA into ribonucleoprotein (RNP) complexes located on the viral membrane. Lastly, the envelope protein facilitates virus assembly and release [21].

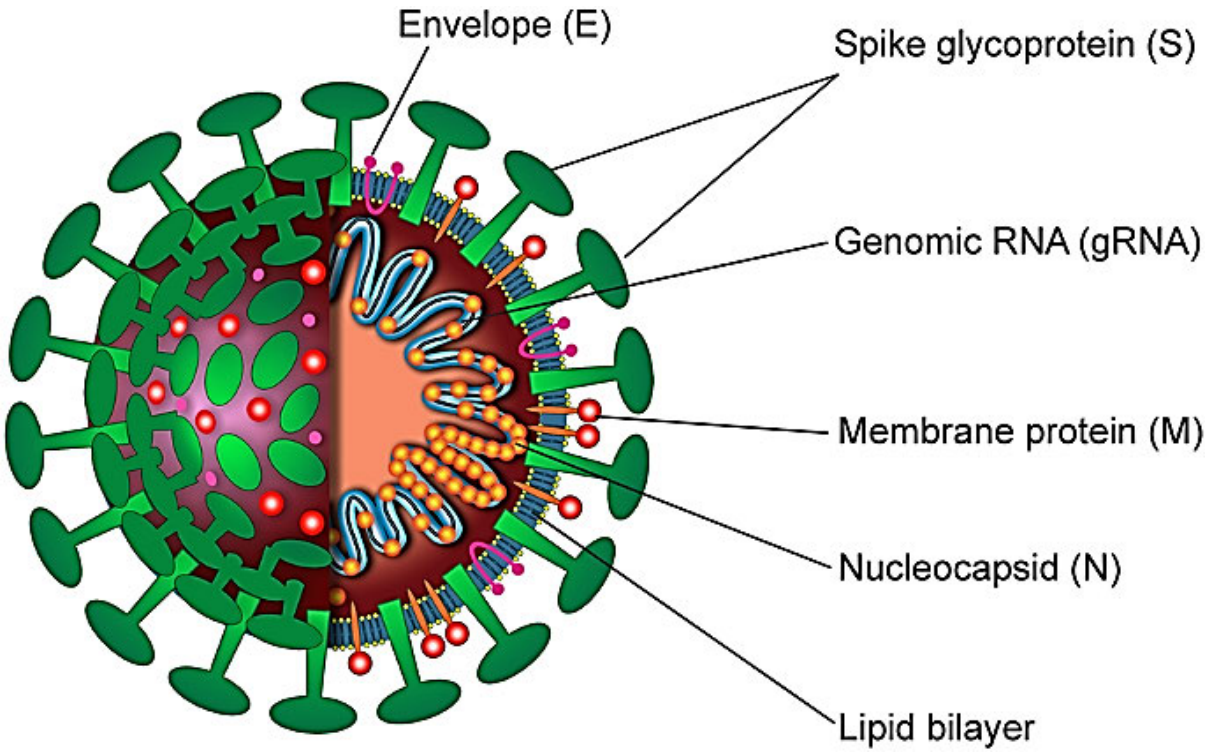


Figure 1.1: SARS-CoV-2 structure.

SARS-CoV-2 structure showing the four structural proteins including M, N, S, and E proteins. Image adapted from Yang et al. [21].

1.3 COVID-19 pathogenesis

Upon infection by SARS-CoV-2, the virus is transported through the naso-oral cavity to the lungs [22]. During this phase, virus replication occurs in the upper respiratory tract including the trachea, nasal passage, pharynx, and larynx. Within a few days (~2-14) early symptoms of COVID-19 such as dry cough, pharyngitis, fever, shortness of breath, and joint pain begin to appear. Additionally, nosocomial transmission of viral infection may occur during this phase, thereby increasing the possibility of spreading viral infection. Once the virus moves to the lower respiratory tract, a strong immune response is mounted [23]. Cumulative reports have shown that immunocompromised individuals are at higher risk of SARS-CoV-2 infection and usually have worse COVID-19 outcomes compared to healthy individuals [24].

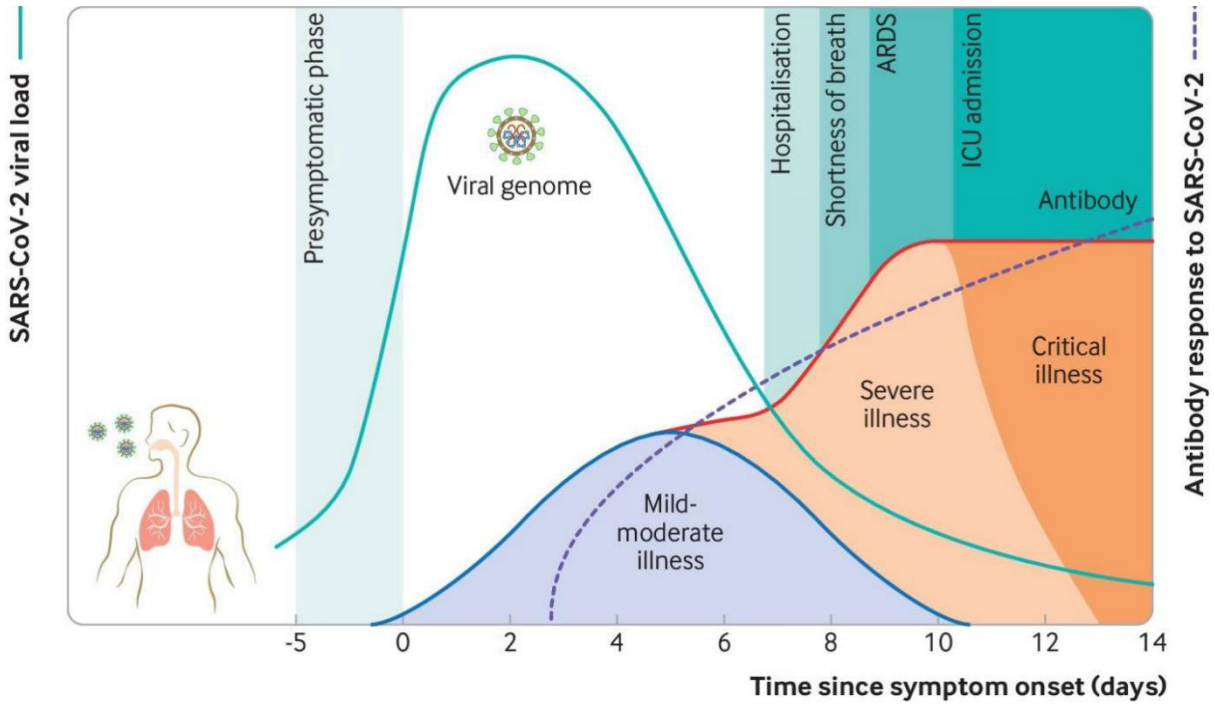


Figure 1.2: SARS-CoV-2 pathogenesis.

Upon exposure to SARS-CoV-2, patients develop mild symptoms within approximately 5 days, known as the incubation period, shortly followed by SARS-CoV-2 clinical manifestations such as mild infection, severe disease, or death. Mild infection can be readily controlled by initial host immune responses. However, severe COVID-19 takes longer to manage and may be associated with exacerbated immune responses, ultimately causing organ damage and mortality [25]. The viral load peak occurs during the first week of infection and gradually declines thereafter. Antibody responses increase to detectable levels by day 14. Image adapted from Cevik et al. [26].

1.3.1 Immune responses against SARS-CoV-2

The adaptive immune responses are important determinants of the clinical outcomes associated with SARS-CoV-2 infection [27, 28]. Recently, there has been heightened interest in understanding cellular immunity during acute SARS-CoV-2 infection. Moreover, research has shown that T cells may be one of the major mediators of COVID-19 control. Despite the high SARS-CoV-2 mortality rate, most individuals infected with the virus survive during acute infection. It is also important to note that SARS-CoV-2-specific T cells develop in most individuals despite their immune state [29, 30].

Many studies have sought to understand the complexity of T cell responses against SARS-CoV-2. These include the sequencing of SARS-CoV-2 in 2020 followed by a study by Peng et al. to assess the breadth of T cell responses in individuals with mild and severe infection [31]. This study reported the presence of stronger T cell responses in individuals with severe viral infection compared to individuals with mild infection. Additionally, the use of peptide pools to identify specific T cell responses against most viral proteins has improved the understanding of SAS-CoV-2 T cell responses [32, 33]. The magnitude of the SARS-CoV-2-specific $CD4^+$ and $CD8^+$ memory T cell response is typically low (around 0.2% and 0.6%), although a characteristic feature is heterogeneity between donors. The breadth of response within individual donors has been estimated at approximately 19 and 17 epitope-specific responses in most people. All the major proteins are targeted by T cell responses. SARS-CoV-2 infection is also associated with polyfunctional responses dominated by interleukin-2 (IL-2) and interferon-gamma (IFN-γ) [34]. A comprehensive understanding of immune responses against SARS-CoV-2 is needed for the design of next-generation vaccines and cures.

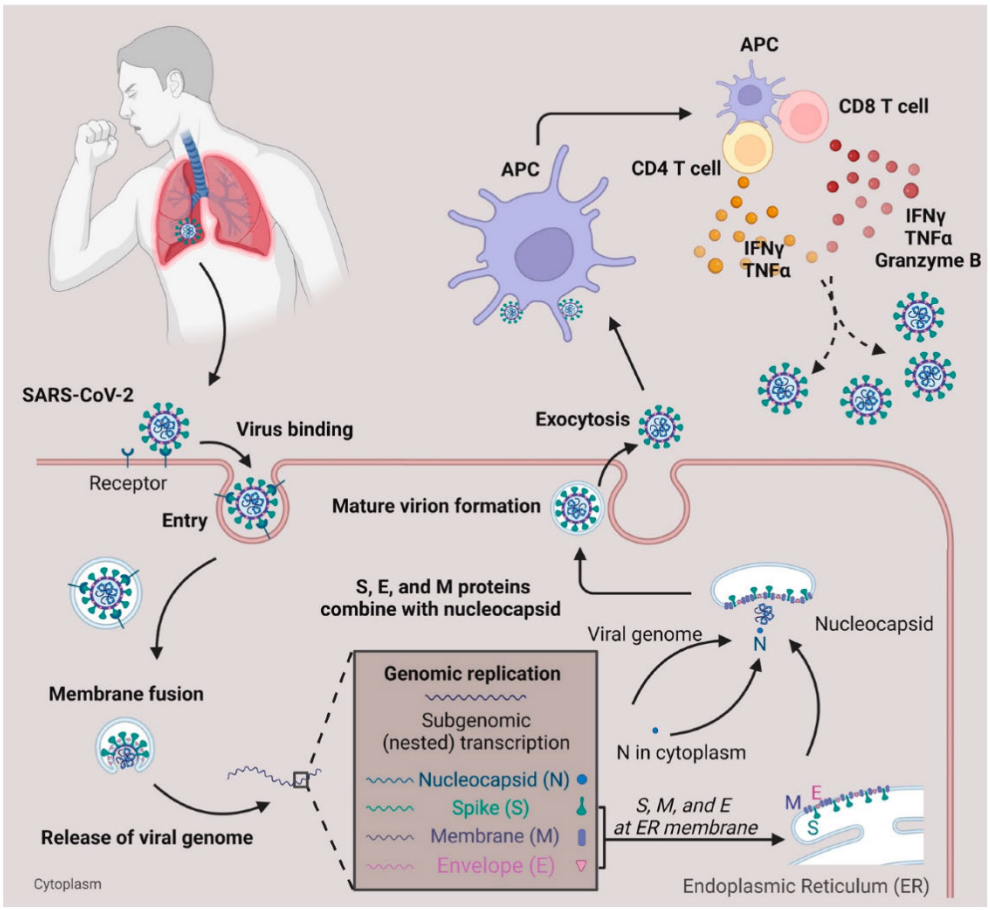


Figure 1.3: SARS-CoV-2 infection and T cell responses.

Summary of T cell responses mounted against SARS-CoV-2 after viral infection. When SARS-CoV-2 is captured and processed by antigen-presenting cells, $CD4^+$ and $CD8^+$ T cells are activated and cytokines such as IFN-γ, TNF-α, and granzyme B are released to fight the viral infection. Image adapted from Wang et al. [35].

1.4 COVID-19 prevention strategies (vaccines)

Initial vaccine rollout programs against SARS-CoV-2 were implemented approximately nine months after COVID-19 was declared a global pandemic. Since then, several vaccines have been developed and distributed. These include messenger RNA-based vaccines such as Sinopharm, adenovirus-based vector vaccines, and inactivated vaccines such as the Pfizer-BioNTech, Janssen, Moderna, and AstraZeneca vaccines [36, 37]. Messenger RNA vaccines have become more favorable for human use because they have low manufacturing costs, have high potency, and are considered safer than vector-based vaccines [38]. Subsequent vaccine studies have shown that most commercially available vaccines elicit robust SARS-CoV-2 T cell responses. Generally, the adaptive immune response underpins vaccine efficacy and determines the clinical outcome after SARS-CoV-2 infection. However,

discrepancies in the number of individuals eliciting T cell responses differ across the various vaccine types [39, 40].

Generally, most COVID-19 vaccines have been extremely effective in reducing hospitalization and preventing COVID-19 severe disease [41]. Nonetheless, significant challenges and concerns surrounding vaccine efficacy and ensuring equitable vaccine access around the globe still exist. Some of these concerns include the inability of current vaccines to protect against emerging viral variants and the side effects associated with these vaccines [42, 43]. Measuring the efficiency of T cell and antibody responses to Covid-19 vaccines is vital to power the rapid release of novel vaccines for public health interventions during the Covid-19 pandemic and beyond.

1.5 SARS-CoV-2 in people living with HIV (PLWH)

Immunocompromised individuals are generally considered a high-risk population due to weakened immune systems [44]. Currently, there is no consensus on the contribution of HIV to COVID-19 disease outcomes. However, research has shown that PLWH are at a higher risk of contracting SARS-CoV-2 infection and currently there is insufficient data and no substantive evidence on the association between HIV and SARS-CoV-2 disease outcomes [45]. Our study reported in chapter 2 of this dissertation demonstrated deleterious effects of unsuppressed HIV infection on T cell immunity to SARS-CoV-2.

SECTION B

1.6 HIV/AIDS

Acquired immune deficiency syndrome (AIDS) remains a global health burden and continues to cause numerous deaths across the globe. To date, approximately 74.9 million individuals have been infected globally, with nearly half of the infected population succumbing to AIDS disease [46, 47]. In 2018, over 400 000 AIDS-related deaths were recorded in Africa. Progressive research has shown that approximately 16 Southern Africa Development Community (SADC) countries remain the epicentre of the HIV/AIDS epidemic with the largest number of people living with HIV/AIDS [48]. The use of combination antiretroviral therapy (cART) has significantly reduced HIV-associated deaths and has undoubtedly improved the quality of life in PLWH. However, antiretroviral therapy is lifelong and may cause side effects such as drug toxicity or resistance when not taken optimally [49]. Moreover, ART interruption results in viral rebound revealing the persistence of HIV in sanctuary sites such as the brain and lymph nodes [50, 51].

One of the major barriers to an HIV cure is the persistence of latently infected cells which contain transcriptionally silent HIV DNA. Upon ART interruption, the transcriptionally silent HIV genome is activated leading to virus production [52, 53]. $CD4^+$ T cells remain one of the well characterized HIV reservoirs in virally suppressed individuals on cART. However, besides $CD4^+$ T cells, various other immune cells have been implicated as HIV reservoirs. These include cells of the myeloid lineage such as dendritic cells and macrophages [54, 55] where HIV remains sequestered for prolonged periods. Initially, researchers presumed that macrophages lack the potential to self-renew. However, more recent evidence challenges this dogma. There is now broad scientific consensus that a subset of macrophages has self-renewing capacity, a long-life span, and resist the cytopathic effects of HIV infection [56, 57]. This knowledge, combined with animal model studies, has led to a resurgence of interest in investigating HIV persistence in macrophages during cART.

1.7 Monocytes as macrophage precursors

A thorough understanding of monocytes and macrophages is critical for the specific targeting of the macrophage reservoir which will enrich the scientific HIV cure development strategies [58]. During infection and inflammation, monocytes travel to peripheral sites through the lymph and bloodstream and differentiate into macrophages in tissues [59, 60]. Evidence has shown that monocytes appear in the peripheral blood at different times suggesting that monocyte populations transition sequentially in the blood [61]. According to traditional nomenclature, human monocytes can be divided into three distinct populations including, intermediate monocytes ($CD14^{++}CD16^+$), classical monocytes

($CD14^{++}CD16^{-}$), and non-classical monocytes ($CD14^{+}CD16^{++}$) [62, 63]. Classical monocytes differentiate into pro-inflammatory (M1) macrophages [64]. Nonclassical monocytes differentiate into anti-inflammatory (M2) macrophages [65]. While monocytes are vital for phagocytosis and pathogen clearance, they potentially have negative effects on the pathogenesis of some inflammatory and degenerative diseases [61].

1.7.1 Macrophage plasticity

Macrophages detect and clear pathogens in the blood and tissues by triggering a series of immuno-inflammatory reactions [59]. In addition, macrophages are highly heterogenous cells, and they demonstrate high plasticity. Their phenotypic and functional diversity is influenced by various factors such as the presence of pathogens, the presence of a wound, and their interaction with various immune cells [61]. The polarization of macrophages into M1 and M2 macrophages is enhanced by various signaling molecules and transcription factors [66]. For instance, polarization of M1 macrophages occurs by lipopolysaccharides and Th1 cytokines such as IFN-γ. As a result, M1 macrophages secrete pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12). On the contrary, M2 macrophages are mainly polarized by Th2 cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) and they secrete anti-inflammatory signals including interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) [67-69].

Macrophages have been assessed in a wide range of diseases and cumulative evidence has shown that M1 and M2 macrophages have distinct functions and transcriptional profiles [70]. However, it is important to note that the M1/M2 macrophage polarization balance influences the fate of an organ during inflammation and injury [71]. For instance, when inflammation is high and severe enough to damage the organ, macrophages release anti-inflammatory cytokines against the stimulus and vice versa [72]. Despite the known role of chemokines as the major mediators of macrophage chemotaxis, the mechanisms underlying how they regulate M1 and M2 macrophages remain unclear.

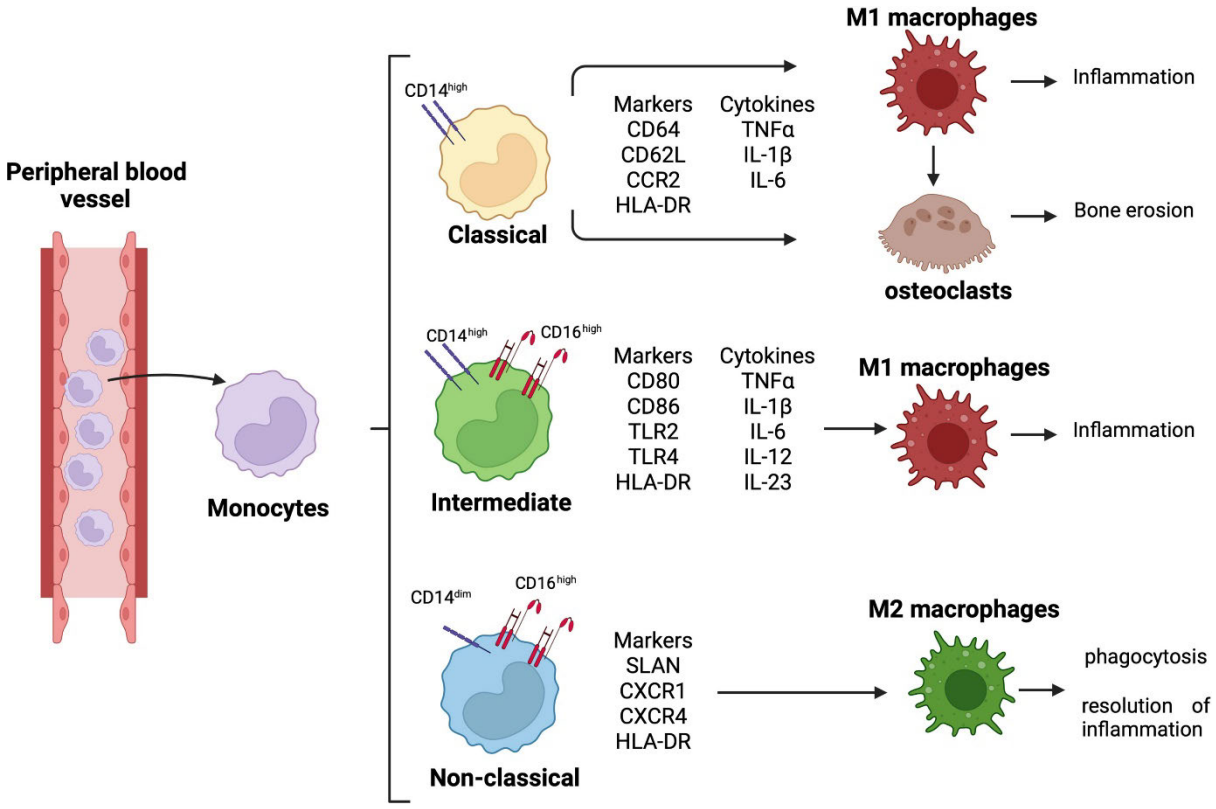


Figure 1.4: Monocyte differentiation into M1 and M2 macrophages

Human monocytes can be classified as classical, intermediate, and non-classical monocytes. Classical and intermediate monocytes differentiate into pro-inflammatory (M1) macrophages and non-classical monocytes differentiate into anti-inflammatory (M2) macrophages. Image adapted from Cutolo et al. [65].

Another notable factor is the ability of macrophages to change their phenotype in response to various signals in-vitro. The macrophage stimulating factor (M-CSF) and granulocyte-macrophage stimulating factor (GM-CSF) have been used to polarize macrophages into distinct phenotypes by manipulating various pathways in-vitro [73-75]. Markers such as cluster of differentiation 68 (CD68) and cluster of differentiation 206 (CD206) have been extensively used to phenotype M1 and M2 macrophages respectively [76].

The relevance of macrophage polarization is seen during infection. A shift of macrophage phenotype from a pro-inflammatory state to an anti-inflammatory state is vital for host defence [77]. In addition, macrophage polarization into different phenotypes is also relevant in HIV-associated inflammation, specifically during late and chronic HIV and simian immunodeficiency virus (SIV) infection [78]. Extensive macrophage in-vitro infection studies demonstrate that M1 and M2 macrophages have impaired function during both acute and chronic HIV infection [79, 80]. Although there is limited

evidence to support this notion, macrophage polarization likely has significant effects on HIV disease outcomes.

1.8 HIV reservoirs in tissues

HIV persists in tissues for prolonged periods leading to reservoir establishment in tissues such as the liver, gut, brain, and lymph nodes [20]. The persistence of replication-competent virus in tissues is a major challenge to the development of an HIV cure. Moreover, the mechanisms underlying viral persistence in tissues are not clear. Partly, this is caused by limited access to tissue samples and techniques to measure the size of the reservoir. Understanding HIV dynamics in tissues is crucial to inform HIV cure development and the specific targeting of HIV-infected cells.

The gut is one of the major sites of viral infection as it contains a large proportion of lymphocytes [81, 82]. HIV infection has been detected in gut $CD4^+$ T cells and myeloid cells in ART-suppressed individuals. A study by Poles et al. showed that HIV-RNA and DNA levels remain stable for over a year in rectal tissues [83]. Moreover, HIV-DNA and RNA have also been detected in brain tissues of ART-treated individuals. The virus was mostly localized to perivascular macrophages, microglial cells, and astrocytes [84]. Several tissues including kidneys, thymus, and lymph nodes have been implicated in HIV persistence. Despite extensive studies in HIV-infected tissues, much remains unclear about tissue reservoirs and their persistence during ART. However, lymph nodes are known to be a key reservoir site for HIV-1 persistence [85, 86].

1.8.1 Lymph nodes and their role in HIV infection

The presence of HIV has also been detected in lymph node $CD4^+$ T cells, with a few studies suggesting the presence of persistent HIV in macrophages [85, 87]. Lymph nodes are secondary lymphoid organs that play a role in filtering foreign particles through lymphatic vessels [88]. Some of the most important lymph node structures include the germinal center (GC), lymphatic vessels, and lymphatic sinuses. GCs are temporary structures formed in the B cell follicle within lymph nodes in response to infection [89, 90]. Afferent and efferent lymphatic vessels serve as immunologic communication highways by mediating the transport of leukocytes and antigens to draining lymph nodes [91]. Generally, B-lymphocytes are localized in the nodular lymphatic tissue, while T-cells and myeloid cells are present in regions such as the diffuse lymphatic tissue, sinus, and GC.

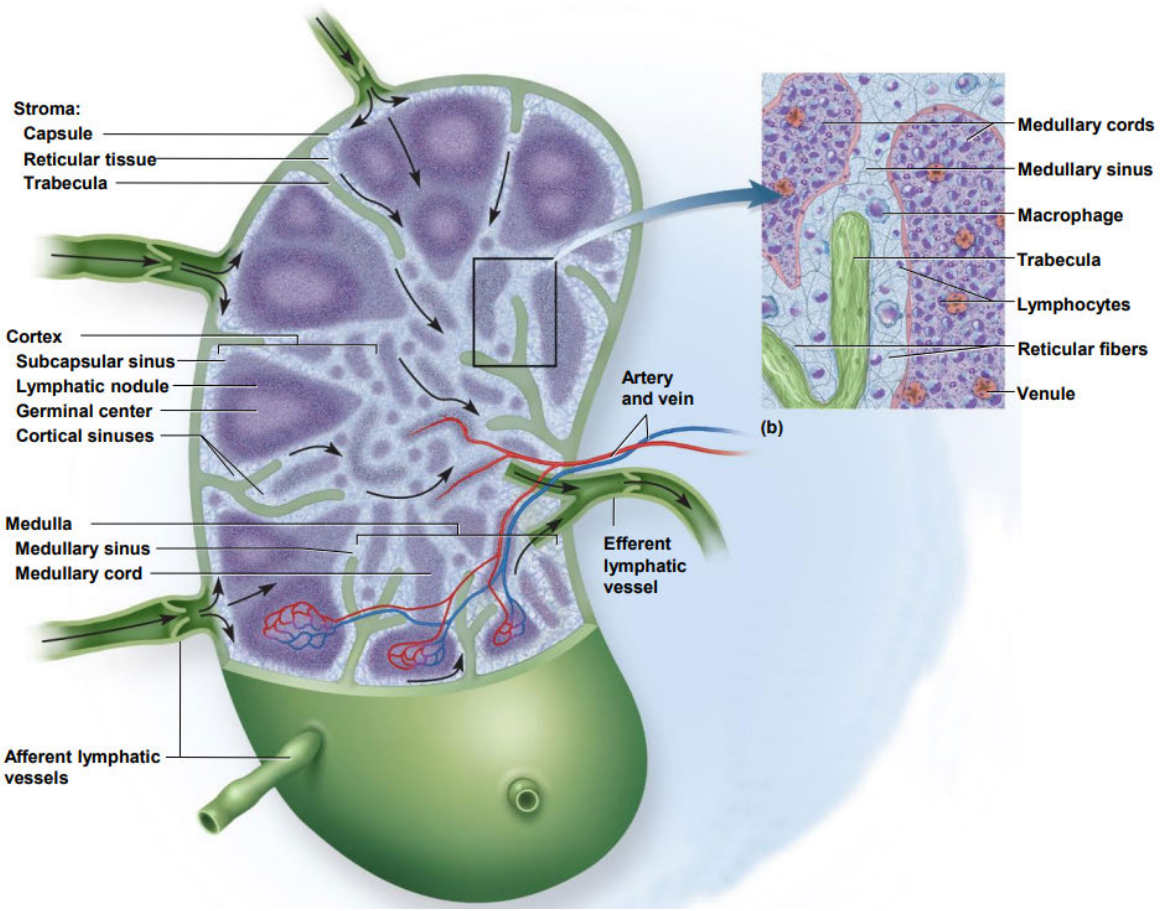


Figure 1.5: Lymph node structure

Lymph nodes are widely distributed throughout the body where they filter foreign antigens and fight infections. Image adapted from SlideServe (https://www.slideserve.com/mariah/chapter-21-lymphatic-and-immune-systems).

HIV infection leads to a disruption in the lymph node (LN) function and impairment of humoral as well as cellular immune responses. SIV studies have shown that GCs and LN follicular regions are primary sites of viral replication and viral reservoir establishment [87, 92]. Although some of the well-recognized anatomical reservoirs include the genitourinary (GU) tract [93], the lungs [55], the gut [94], and the central nervous system (CNS) [95], lymph nodes have received much attention in recent years. There is convincing evidence that lymph nodes are the major anatomical reservoirs of HIV infection [85]. Additionally, the continuous circulation of HIV-infected macrophages and their trafficking in lymph nodes represents an avenue for reservoir establishment. An in-depth understanding of the biology and function of immune cells in HIV-infected lymph nodes will better inform the specific targeting of infected cells needed to eradicate HIV in tissues.

1.8.2 HIV infection in macrophages

Macrophages are widely distributed throughout the body in most tissues including the liver, gut, and lymph nodes where they play various immunological roles. For instance, mononuclear phagocytes play a role in the construction of the vessel wall as well as in maintaining cardiac rhythm. In addition, liver macrophages (Kupffer cells) contribute to liver homeostatic functions [96]. Similar to monocytes, [97] the implications of tissue macrophage function and origin have not been fully explored. Scientific evidence suggests that macrophages play a critical role in the establishment and persistence of the HIV reservoir due to numerous factors [98]. First, research has shown that HIV production in macrophages occurs intracellularly in specific compartments known as virus-containing compartments (VCCs) [99]. These compartments play a role in sheltering virions, allowing for the retainment of their infectious potential for extended periods. Second, HIV-infected macrophages resist HIV-induced apoptosis and cytotoxic T cell killing [100, 101]. Moreover, some HIV-infected macrophages have self-renewing capacity and a long half-life, allowing them to reside in tissues for prolonged periods [102].

A recent study by Ganor et al. revealed that macrophages are a key HIV reservoir in penile urethral tissue in cART-suppressed individuals. In addition, a series of fluorescent in-situ hybridization (FISH) assays detected the presence of $CD68^+$ HIV-infected macrophages in the penile urethra [93]. Replication-competent SIV has also been detected in brain macrophages of ART-suppressed SIV-infected pig-tailed macaques [103]. Although the role of macrophages in HIV infection has been documented over the years, their role in HIV persistence is still controversial. Contrary to what other studies have reported, some researchers suggest that myeloid cells are not a major source of virus in SIV-infected nonhuman primates. They propose that macrophages merely ingest HIV-infected T cells during phagocytosis, which explains the presence of HIV nucleic acids and proteins in macrophages [104] [105]. Understanding the mechanisms underlying HIV persistence in LN macrophages is warranted for the design of strategies targeting the elimination of macrophages which may be different from $CD4^+$ T cell targeting strategies because of the inherent differences in the cellular biology of the two subsets.

1.8.3 Long-lived tissue resident macrophages

Tissue-resident macrophages originate from the fetal liver during embryonic development and from the bone marrow after birth [106]. Recently long-lived macrophages have been identified in certain organs such as the gastrointestinal tract, liver, lung, and brain [107], where they contribute to tissue homeostasis and immune surveillance. T cell immunoglobulin and mucin domain containing 4 (TIM-4), a phosphatidylserine receptor that is highly expressed on resident peritoneal macrophages [108]. Two independent studies have shown that long-lived macrophages express TIM-4 and CD4 [109, 110].

Additionally, HIV infection of long-lived macrophages elevates the potential of this subset to be an important reservoir in tissues.

1.8.4 Techniques and animal models used to study the myeloid reservoir.

Studies aimed at characterizing HIV reservoirs in HIV-infected individuals have mainly focused on peripheral blood, with a few studies focused on tissues. Generally, the tissue reservoir is understudied. Given the hurdles associated with obtaining human tissues, animal models such as SIV-infected macaques have become valuable models for studying HIV infection and replication in macrophages [111]. Similar to HIV, SIV infects macrophages in macaques, making SIV-macaque models useful for understanding HIV reservoir establishment during cART. In addition to SIV-infected macaques, humanized mice models have also been used to study the macrophage reservoir. A study by Arainga et al. revealed that humanized mice sustain viral infection and respond effectively to cART [112]. Interestingly, like humans, antiretroviral treatment in mice significantly lowers viral load, but it does not eliminate HIV infection in reservoirs. However, the use of mice models to study HIV reservoirs is hampered by the short half-life of mice [113].

The use of non-human primate models (NHPs) for HIV pathogenesis and HIV reservoir studies has led to significant advances in HIV cure development [114, 115]. Some of the advantages of using NHPs include easy access to tissue samples [116]. Additionally, NHPs allow researchers to carry out numerous procedures, most of which may be difficult to carry out in humans. Generally, many NHP species are naturally infected by SIV making them a good model to study HIV pathogenesis [117]. However, these models are limited in their ability to replicate the effects of HIV on various tissues and to recreate basic features of HIV disease in humans [118]. Although there is no perfect animal model, each animal model has its benefits and limitations and has provided the tools to explore HIV pathogenesis and HIV reservoirs.

One of the major challenges for HIV reservoir research is the lack of accurate and reliable methods that can be used to reproducibly measure HIV reservoirs. Currently, techniques such as DNAscope and RNAscope have been used to detect SIV-infected macrophages [78]. For instance, DiNapoli et al. used DNAscope to investigate macaques with undetectable viral load after receiving cART for five months. They detected the presence of SIV DNA in macrophages localized in the spleen [119]. Additionally, assays such as the quantitative viral outgrowth assay (qVOA) have been successfully used in mice studies to measure the frequency of HIV-infected cells [120]. However, the qVOA requires large blood volumes which may be difficult to obtain in some cases. Overall, the development of more sensitive techniques to detect HIV infection and the use of various animal models can significantly improve understanding of macrophage biology and the macrophage reservoir.

1.9 THESIS OUTLINE

Combined antiretroviral therapy has played a significant role in the suppression of plasma viremia in HIV-infected individuals [121]. However, the complete eradication of HIV-1 has been hampered by the formation of viral reservoirs. These reservoirs are established during early infection, and they can remain stable for extended periods in sanctuary sites. Immune dysfunction is well known to contribute to the establishment and maintenance of HIV reservoirs but the mechanisms underlying viral persistence under suppressive ART remain unclear.

The HIV reservoir was originally described as a homogeneous pool of resting memory $CD4^+$ T cells. An ever-growing body of in vitro and in vivo studies have demonstrated that memory $CD4^+$ T cells play a key role in HIV persistence. In addition, $CD4^+$ T cells can proliferate and contribute to the longevity of the reservoir in ART-treated individuals [122-124]. Over the years, multiple $CD4^+$ T cell subsets have been identified as a source of HIV persistence. The heterogeneity of these reservoirs suggests that numerous other cell types may contribute to viral persistence.

More recently, there has been a resurgence of interest in the myeloid reservoir. There is broad consensus that macrophages may contribute to HIV persistence due to their self-renewing capacity, long life span, and ability to resist the cytopathic effects of HIV infection. However, contrary to this notion, nonhuman primate studies suggest that myeloid cells are not a major source of HIV during suppressed infection [125]. They speculate that, unlike $CD4^+$ T cells, myeloid lineage populations are not readily permissive to HIV infection, that most of the seemingly infected macrophages are merely a result of ingested HIV-infected T cells during phagocytosis and not productive infection [126].

In addition to resisting cytotoxic T cell killing during HIV infection, macrophages are highly heterogeneous cells. They differentiate into pro-inflammatory and anti-inflammatory macrophages in response to environmental stimuli. Studies have reported a constant shift in macrophage states during HIV infection. However, the mechanism by which this polarization occurs remains unclear. Detailed investigation of macrophage phenotypes, distribution, and function will inform a better understanding of HIV pathogenesis in macrophages. On the other hand, unsuppressed HIV places HIV patients at a higher risk of acquiring infectious diseases such as COVID-19 although the mechanisms underpinning this susceptibility are not fully understood. Understanding the mechanisms underlying viral persistence is essential for managing HIV disease as well as the development of an HIV cure.

This thesis seeks to address the following questions:

- Research Question 1: How does unsuppressed HIV affect COVID-19 pathogenesis?
- Research Question 2: What macrophage phenotypes are found in lymph nodes?

- Research Question 3: What is the spatial distribution of lymph node macrophages?
- Research Question 4: What is the role of macrophages in HIV pathogenesis and reservoir establishment?

By addressing these study questions, we hope to provide information that will further our understanding of HIV pathogenesis and the persistence of HIV in lymph node macrophages.

The aims of the present studies are as follows:

Aim 1: To determine the impact of HIV infection on SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses in the first wave and second wave of the COVID-19 epidemic in South Africa.

Aim 2: To perform a comprehensive phenotypic and functional characterization of lymph node macrophages.

Aim 3: To investigate the role of lymph node macrophages in HIV persistence during cART.

Chapter 1 is the introduction of the thesis consisting of the relevant topics relating to the defined aims of the study.

In chapter 2, we assessed the impact of HIV infection on the quality and epitope specificity of SARS-CoV-2 T cell responses in the first and second wave of the COVID-19 epidemic in South Africa.

In chapter 3, we conducted a detailed characterization of the phenotype, spatial localization, and function of macrophages within human lymph nodes.

In chapter 4, we extended our study and investigated the role of macrophages in HIV persistence.

Chapter 5 is a general discussion including overall implications and future directions of our studies.

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CHAPTER 2

Aim: Investigate the impact of HIV infection on the quality and epitope specificity of SARS-CoV-2 T cell responses in the first wave and second wave of the COVID-19 epidemic in South Africa.

Chapter 2 Overview

HIV and SARS-CoV-2 are global pandemics and their co-action in humans is still a subject of growing research interest and clinical importance. Currently, the search for the most appropriate and relevant therapy for both HIV and SARS-CoV-2 continues, and the emergence of more transmissible and evasive variants is of great concern. Although several studies have demonstrated that unsuppressed HIV is associated with severe COVID-19, the mechanisms underpinning this susceptibility are still unclear. In Chapter 2, we present a study conducted to assess the $CD4^+$ T cell responses to SARS-CoV-2 using a combination of flow cytometry and culture T lymphocyte expansion during the first wave and second wave of the COVID-19 epidemic in South Africa. We observed that HIV-seronegative individuals had significantly greater $CD4^+$ T cell responses against the Spike protein compared to the viremic PLWH. In addition, we observed diminished T cell cross-recognition between the two waves, which was more pronounced in individuals with unsuppressed HIV infection. Notably, we identified four mutations in the *Beta variant* that resulted in abrogation of T cell recognition. These findings may partly explain the increased susceptibility of PLWH to severe COVID-19 and highlights their vulnerability to emerging SARS-CoV-2 variants of concern.

RESEARCH ARTICLE

Unsuppressed HIV infection impairs T cell responses to SARS-CoV-2 infection and abrogates T cell cross-recognition

Thandeka Nkosi[1†], Caroline Chasara[1†], Andrea O Papadopoulos[1*], Tiza L Nguni[1], Farina Karim[1], Mahomed-Yunus S Moosa[2], Inbal Gazy[3], Kondwani Jambo[4,5], COMMIT-KZN-Team[1], Willem Hanekom[1,6], Alex Sigal[1], Zaza M Ndhlovu[1,2,7*]

[1]Africa Health Research Institute, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal, Durban, South Africa; [2]HIV Pathogenesis Program, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa; [3]KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [4]Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi; [5]Liverpool School of Tropical Medicine, Liverpool, United Kingdom; [6]Division of Infection and Immunity, University College London, London, United Kingdom; [7]Ragon Institute of MGH, MIT and Harvard, Cambridge, United States

***For correspondence:** andrea.papadopoulos@ahri.org (AOP); zndhlovu@mgh.harvard.edu (ZMN)

[†]These authors contributed equally to this work

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Abstract In some instances, unsuppressed HIV has been associated with severe COVID-19 disease, but the mechanisms underpinning this susceptibility are still unclear. Here, we assessed the impact of HIV infection on the quality and epitope specificity of SARS-CoV-2 T cell responses in the first wave and second wave of the COVID-19 epidemic in South Africa. Flow cytometry was used to measure T cell responses following peripheral blood mononuclear cell stimulation with SARS-CoV-2 peptide pools. Culture expansion was used to determine T cell immunodominance hierarchies and to assess potential SARS-CoV-2 escape from T cell recognition. HIV-seronegative individuals had significantly greater $CD4^+$ T cell responses against the Spike protein compared to the viremic people living with HIV (PLWH). Absolute CD4 count correlated positively with SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses (CD4 r=0.5, p=0.03; CD8 r=0.5, p=0.001), whereas T cell activation was negatively correlated with $CD4^+$ T cell responses (CD4 r=−0.7, p=0.04). There was diminished T cell cross-recognition between the two waves, which was more pronounced in individuals with unsuppressed HIV infection. Importantly, we identify four mutations in the Beta variant that resulted in abrogation of T cell recognition. Taken together, we show that unsuppressed HIV infection markedly impairs T cell responses to SARS-Cov-2 infection and diminishes T cell cross-recognition. These findings may partly explain the increased susceptibility of PLWH to severe COVID-19 and also highlights their vulnerability to emerging SARS-CoV-2 variants of concern.

Editor's evaluation

This paper provides important descriptive evidence that untreated HIV infection has important negative effects on T cell responses to SARS-CoV-2, particularly in regards to cross recognition of new variants. Treatment of HIV with ART appears to partially reverse suppression of SARS-CoV-2 specific cellular immune responses.

Introduction

Despite measures to contain the spread of SARS-CoV-2 infection, the pandemic is persisting, with a devastating impact on healthcare systems and the world economy (**Verma et al., 2021**). The research community rapidly mobilized and developed vaccines and therapeutics at unprecedented speed (**Polack et al., 2020**; **Ball, 2021**). COVID-19 vaccines have prevented serious illness and death and have in some cases interrupted chains of transmission at community level (**Kampf, 2021**). However, the COVID-19 pandemic remains a major concern in Africa due to dismal vaccine coverage (**WHO, 2021a**) and the emergence of variants of concern that may be more transmissible, cause more severe illness, or have the potential to evade immunity from prior infection or vaccination (**Shinde et al., 2021**).

The interaction of HIV-1 infection, common in sub-Saharan Africa (**H. The Lancet, 2020**), with COVID-19 remains understudied. Initial small studies reported that people living with HIV (PLWH) had similar or better COVID-19 outcomes (**Calza et al., 2020**; **Lee et al., 2021**). Larger epidemiological studies have demonstrated increased hospitalization and higher rates of COVID-19-related deaths among PLWH compared with HIV-negative individuals (**Davies, 2020a**; **Geretti et al., 2020**; **Bhaskaran et al., 2021**; **Vizcarra et al., 2020**). Other studies have linked HIV-mediated CD4$^+$ T cell depletion to suboptimal T cell and humoral immune responses to SARS-CoV-2 (**Riou, 2021**). A recent study showed prolonged shedding of high titer SARS-CoV-2 and emergence of multiple mutations in an individual with advanced HIV and antiretroviral treatment (ART) failure (**Karim et al., 2021b**).

Although B cells have repeatedly been shown to play a pivotal role in immune protection against SARS-CoV-2 infection and antibody responses and are typically used to evaluate immune responses to currently licensed COVID-19 vaccines (**Sahin et al., 2020**; **Khoury et al., 2021**), mounting evidence suggest that T cell responses are equally important. For instance, strong SARS-CoV-2-specific T cell responses are associated with milder disease (**Riou, 2021**; **Sette and Crotty, 2021**; **Liao et al., 2020**; **Schub et al., 2020**; **Rydyznski Moderbacher et al., 2020**). Moreover, T cell responses can confer protection even in the absence of humoral responses, given that patients with inherited B cell deficiencies or hematological malignancies are able to fully recover from SARS-CoV-2 infection (**Bange et al., 2021**). In some instances, COVID-19 disease severity has been attributed to poor SARS-CoV-2-specific CD4$^+$ T cell polyfunctionality potential, reduced proliferation capacity, and enhanced HLA-DR expression (**Riou, 2021**). Importantly, a recent study identified nonsynonymous mutations in known MHC-1-restricted CD8$^+$ T cell epitopes following deep sequencing of SARS-CoV-2 viral isolates from patients, demonstrating the capacity of SARS-CoV-2 to escape from CTL recognition (**Agerer et al., 2021**). Regarding vaccine-induced T cell responses, it was recently shown that mRNA vaccines can stimulate Th1 and Th2 CD4$^+$ T cell responses that correlate with post-boost CD8$^+$ T cell responses and neutralizing antibodies (**Painter et al., 2021**). The cited examples, herein, highlight the need to gain more insight into T cell-mediated protection against COVID-19 (**Altmann and Boyton, 2020**).

This study used a cohort of PLWH and HIV-seronegative individuals diagnosed with COVID-19 during the first wave dominated by the wild-type (wt) D614G virus (**Tegally et al., 2021c**), and the second wave dominated by the Beta variant. Peripheral blood mononuclear cells (PBMCs) were used to determine the impact of HIV infection on SARS-CoV-2-specific T cell responses and to assess T cell cross-recognition. Our data showed impaired SARS-CoV-2-specific T cell responses in individuals with unsuppressed HIV infection and highlighted poor cellular cross-recognition between variants, which was more pronounced than those with unsuppressed HIV. The muted responses in unsuppressed HIV infection may be attributable to low absolute CD4 count and immune activation. Importantly, we identified mutations in the Beta variant that could potentially reduce T cell recognition. Taken together, these data highlight the need to ensure uninterrupted access to ART for PLWH during the COVID-19 pandemic.

Results

Study participants were drawn from a longitudinal observational cohort study that enrolled and tracked patients with a positive COVID-19 qPCR test presenting at three hospitals in the greater Durban area. Study participants were recruited into this study based on HIV status and sample availability. They include 25 participants recruited during the first wave (wt) of the pandemic in KwaZulu-Natal from June to December 2020 (**Karim et al., 2021a**). Twenty-three second wave (Beta variant) participants were

Table 1. Donor characteristics stratified by HIV status.

	All (n=48)	HIV-neg (N=17)	HIV+suppressed (n=17)	HIV+viremics (N=14)	Statistics
Demographics					
Age years, median (IQR)	40.5 (30–51.75)	45 (27–53.5)	45 (39.5–54)	31.5 (26.5–42)	0.036* (KW)
Male sex, n (%)	14 (29.16)	8 (47.05)	3 (17.64)	3 (21.42)	0.2 (0.82–10) (F)
HIV-associated parameters					
HIV viral load copies/ml				19,969 (2335–43,568)	
CD4 cells/µl median (IQR)	661 (398.5–836.5)	834.5 (739.3–1029)	661 (494–789.5)	301 (113.8–568)	0.0002** (KW)
Disease severity					
Asymptomatic, n (%)	9 (18.75)	4 (23.52)	3 (17.64)	2 (14.28)	0.6 (0.32–9.53) (F)
Mild	29 (60.42)	12 (70.59)	10 (58.82)	7 (50)	0.01* (0.13–0.84) (F)
Severe/oxygen supplementation	8 (16.67)	1 (5.88)	4 (23.52)	3 (21–42)	0.33 (0.48–49.67) (F)
Death, n (%)	1 (2.1)	0	0	1 (7.1)	0.46 (F)

P values calculated by Kruskal-Wallis test for unpaired three groups (KW) or Fischer's exact test (F).

recruited from January to June 2021. All study participants were unvaccinated because the COVID-19 vaccine was not readily available in South Africa at the time. Study participants were stratified into three groups, namely HIV-seronegative (HIV-neg), people living with HIV (PLWH) with viral load below 50 copies/ml, here termed (suppressed), and PLWH with detectable viral load of ≥1000 copies/ml (viremic). Study participants included HIV-seronegative (HIV-neg) (n=17). PLWHs (n=31) were subdivided into suppressed (n=17) and viremic (n=14). The male-to-female ratio and age distribution were comparable between PLWH and HIV-seronegative groups (***Table 1***). The median CD4 count for PLWH (suppressed 661 and viremic 301) (p=0.0002, ***Table 1***). Study participants had predominantly mild COVID-19 disease that did not require supplemental oxygen or ventilation (***Table 1***).

Unsuppressed HIV infection is associated with altered SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses

Immunity to SARS-CoV-2 typically induces robust T cell responses, but the impact of HIV infection on these responses has not been fully elucidated (***Bange et al., 2021***; ***Riou et al., 2021***; ***Grifoni et al., 2020***). Thus, we sought to determine the impact of HIV infection on SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses. PBMCs were stimulated with PepTivater 15 mer megapools purchased from Miltenyi Biotec. The pools contained predicted CD4 and CD8 epitopes spanning the entire Spike coding sequence (aa5-1273). Intracellular cytokine staining of peptide-stimulated PBMCs was followed by flowcytometric analyses described in the Materials and methods section. The samples used for these analyses were collected between 2 and 4 weeks after COVID-19 PCR positive diagnosis. The time points were selected based on longitudinal T cell analysis that showed SARS-CoV-2-specific T cell responses peaked between 14 and 30 days after PCR positive diagnosis (data not shown), consistent with other studies (***Keeton et al., 2021***; ***Keeton et al., 2022***). Representative flow plots for each group and aggregate data show viremic PLWH had significantly lower frequencies of SARS-CoV-2-specific IFN-γ/TNF-α-producing CD4⁺ T cells compared to suppressed PLWH (p=0.002) and HIV-seronegative individuals (p=0.0006) (***Figure 1B***). There was no significant difference in SARS-CoV-2-specific IFN-γ/TNF-α-producing CD8⁺ T cells among the groups (***Figure 1B***), and no significant differences in SARS-CoV-2-specific CD4⁺ or CD8⁺ T cell frequencies were observed between the suppressed PLWH and HIV-seronegative individuals (***Figure 1B***).

Simultaneous production of cytokines, commonly referred to as polyfunctionality, which is regarded as a measure of the quality of the T cell response, has been shown to correlate with viral control (***Betts et al., 2006***). Thus, we evaluated the quality of the CD4⁺ and CD8⁺ T cell responses among the groups by enumerating cells producing three (IFN-γ, TNF-α, and IL-2) cytokines in various

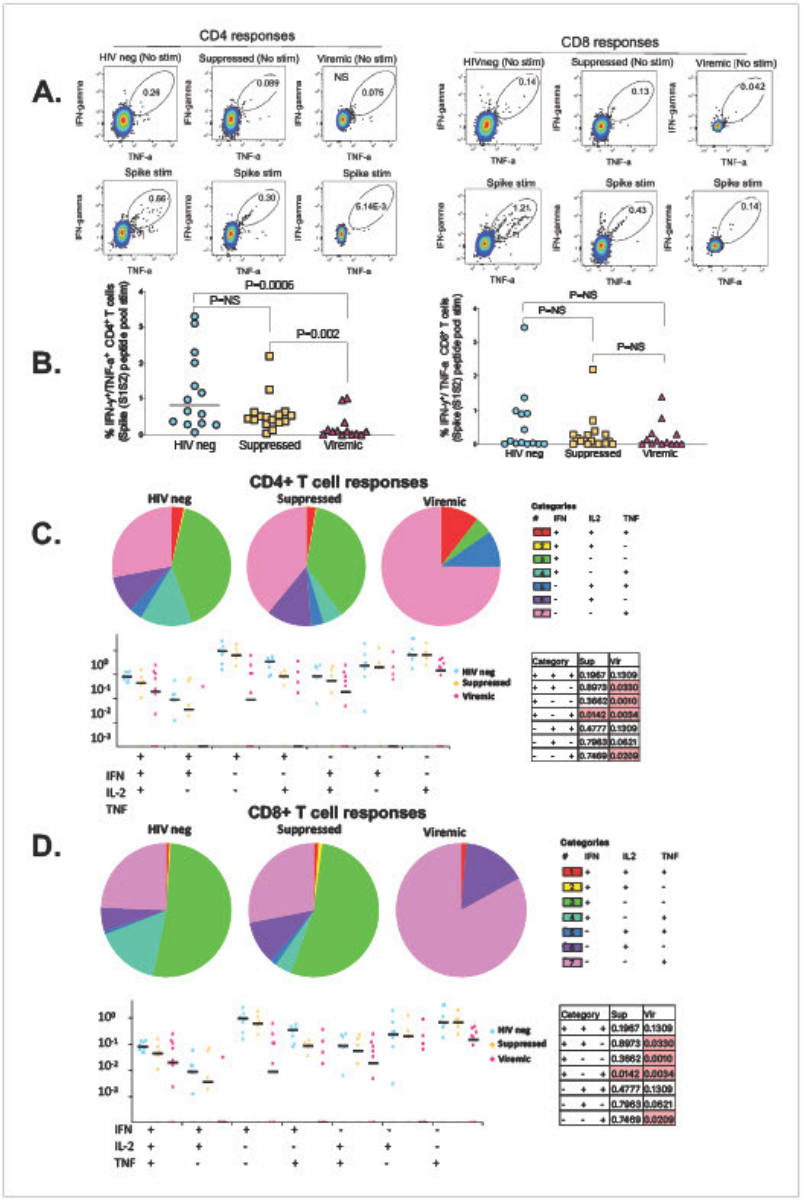

Figure 1. The impact of unsuppressed HIV infection on SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses. (A) Representative flowplots gated on IFN-γ/TNF-α dual positive CD4⁺ and CD8⁺ T cells. (B) Aggregate data for IFN-γ/TNF-α dual positive CD4⁺ and CD8⁺ T cells are shown (HIV-neg, n=14; suppressed, n=16: viremic, n=13). SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells producing IFN-γ, TNF-α, and IL-2 cells in various combinations are

Figure 1 continued on next page

Figure 1 continued

shown. Pie chart and dot plots for (C) SARS-CoV-2-specific CD4⁺ and (D) CD8⁺ T cells. Pie chart represents the mean distribution across subjects of mono-functional, bi-functional, and poly-functional cytokine producing SARS-CoV-2-specific T cells. Size of each pie segment relates to the frequency of a mono-functional, bi-functional, and triple-functional response. Dot plot represents the frequency of combinations of cytokines produced. Wilcoxon test was done among the dot plots using SPICE software (significant p values are highlighted).

combinations. Consistent with dual IFN-γ, TNF-α cytokine secretion data (***Figure 1B***), the patterns of cytokine production of HIV-seronegative was mostly similar to HIV suppressed individuals (pie charts, ***Figure 1C and D***). Analysis of single cytokine production revealed that HIV-seronegative individuals and suppressed PLWH predominantly produced IFN-γ responses (green sectors of the pie chart, ***Figure 1C and D***), whereas viremic PLWH predominantly produced TNF-α responses for both CD4⁺ and CD8⁺ T cells (magenta sectors of the pie chart, ***Figure 1C and D***). Cells co-producing all three cytokines were very rare regardless of HIV status (red sectors of the pie chart, ***Figure 1C and D***). Nonetheless, HIV-seronegative had greater frequencies of dual cytokine secreting cells compared to viremic PLWH (p=0.0330 for CD4, ***Figure 1C***; p=0.0330 for CD8, ***Figure 1D***). Taken together, the data show that uncontrolled HIV infection lowers the magnitude and alters the quality of SARS-CoV-2 T cell responses. Importantly, complete plasma HIV suppression preserves the capacity to mount high magnitude, dual-functional SARS-CoV-2-specific T cell responses.

T cell responses against the major SARS-CoV-2 structural proteins

Having observed differences in magnitude and quality of SARS-CoV-2 spike-specific T responses, we next measured responses directed against major structural proteins, the nucleocapsid (N), the membrane (M), and Spike (S), again using PepTivater peptide pools from Miltenyi biotec. Our data show all three major SARS-CoV-2 proteins are targeted by SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells (***Figure 2A and B***), with a preponderance for greater S-specific CD8⁺ T cell responses relative to M (***Figure 2A***). These data suggest that most SARS-CoV-2 structural proteins can be targeted by T cells, consistent with previous reports (***Tarke et al., 2021***).

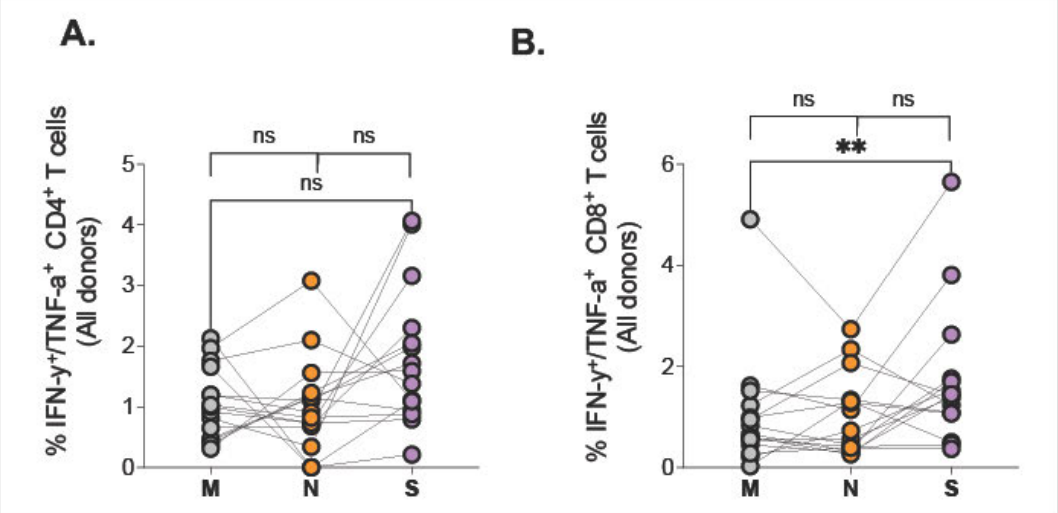

Figure 2. Comparison of SARS-CoV-2 protein targeting by T cell responses among HIV-negatives, suppressed and viremic donors. Magnitude of (A) CD4⁺ T and (B) CD8⁺ T cell responses targeting the Membrane (M), Nucleocapsid (N), and Spike (S) SARS-CoV-2 proteins among study groups. P values for differences among the groups are *<0.05; as determined by the Wilcoxon matched-pairs signed rank test (GraphPad Prism version 9.3.0).

Uncontrolled HIV infection abrogates SARS-CoV-2 T cell cross-recognition between wild-type D614G and Beta variant

To evaluate the impact of uncontrolled HIV infection on cross-reactive T cell responses between wt and the Beta variant, we compared the breadth of responses and the ability to cross-recognize SARS-CoV-2 Beta variant peptides among the three study groups. These studies were conducted using two sets of 15 mer overlapping peptides (OLPs). Set 1 was comprised of 16 wt peptides, spanning the receptor-binding domain (RBD) and non-RBD regions of spike (S) that are known hotspots for mutations (***Tegally et al., 2021c***). Set 2 consisted of corresponding peptides that included all the major mutations that define the Beta variant lineage (***Wibmer et al., 2021***). A detailed description of the peptides is contained in ***Supplementary file 1***.

We first sought to determine cross-reactivity of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells induced following infection with the wt (D614G, wave 1) and Beta variant (wave 2), between each other. We found that wave 1 donors had significantly lower $CD8^+$ (p=0.0312) and $CD4^+$ T cell responses (p=0.0078) to Beta variant relative to corresponding wt responses (***Figure 3A***). Wave 2 donors had no significant differences in T cells responses to Beta and wt (***Figure 3B***). Using a 12-day cultured stimulation assay, we were able to massively expand the magnitude of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells (***Figure 3C***) and (***Figure 3—figure supplement 1***), and this allowed us to hone in on single peptide responses (***Table 1***). Representative data for a wave 1 donor shows three $CD8^+$ and two $CD4^+$ wt responses (red circles), that did not cross-recognize corresponding Beta variants (blue bars) (***Figure 3D***). Contrariwise, a representative wave 2 donor had one $CD8^+$ and one $CD4^+$T cell response to the Beta variant that did not cross-react to the wt version of the peptide (***Figure 3E***). Intra-donor comparison revealed significantly more $CD8^+$ (p=0.0156) and $CD4^+$ T cell responses (p=0.0312) to wt peptides compared to the corresponding Beta variant peptides in wave 1 donors (***Figure 3F***). Conversely, unlike the ex vivo data (***Figure 3B***), wave 2 donors had significantly more $CD8^+$ T cell responses to Beta variant peptides relative to wt peptides (p=0.0312), and a trend toward increased $CD4^+$ T cells against Beta peptides (p=0.0625), highlighting the increased sensitivity of expanded cells (***Figure 3G***). Taken together, these data show poor cross-recognition of wt and Beta variant epitopes.

We then assessed the impact of HIV infection on cross-recognition of wt and Beta variant epitopes. Representative data for an HIV-seronegative individual from the first wave had six wt and five Beta variant $CD8^+$ T cell responses, one was cross-recognized (circled) (***Figure 4A***). The same individual had five wt and five Beta variant $CD4^+$ T responses, one was cross-recognized (***Figure 4B***). Similarly, a representative suppressed wave 1 donor had five wt and two Beta variant $CD8^+$ T cell responses, one of which was cross-recognized (***Figure 4C***). This same donor had six wt and zero Beta variant $CD4^+$ T cell responses (***Figure 4D***). A representative viremic individual had four weak wt $CD8^+$ T cell responses and three borderline CD4 responses, none of which were cross-recognized (***Figure 4E and F***). Summary data showed viremic PLWH had significantly narrow breadth of SARS-CoV-2-specific $CD8^+$ (p=0.039) and $CD4^+$ T cell responses (p=0.033) compared to suppressed PLWH and HIV-seronegative individuals (***Figure 4G and H***). Collectively, these data show that SARS-CoV-2-specific T cell responses in viremic PLWH have limited breadth and subsequently poor cross-recognition potential.

Identification of mutations in the Beta variant that are associated with reduced cross-recognition

Having shown poor T cell cross-recognition of SARS-CoV-2 epitopes between wt and Beta variant, we next sought to identify mutations that might be responsible for the loss of recognition. We combined all the T cell data for the 12 (4 HIV-negatives, 4 HIV-suppressed, and 4 HIV-viremics) donors used for cultured epitope screening studies. All the samples were culturally expanded using wt peptides from the first wave. This analysis identified four Beta variant peptides (listed in ***Supplementary file 1***) that had significant reduction in $CD8^+$ T cell recognition relative to wt peptides (***Figure 5A***). Three of these peptides were also poorly recognized by $CD4^+$ T cells (***Figure 5B***). The amino acid sequences for wt and corresponding mutations include the E484K mutation, a key Beta variant spike residual change also associated with loss antibody binding (***Wibmer et al., 2021***). Taken together, these data identified mutations in the Beta variant that may abrogate T cell recognition, suggesting that they may be potential T cell escape mutations and warrant further investigation.

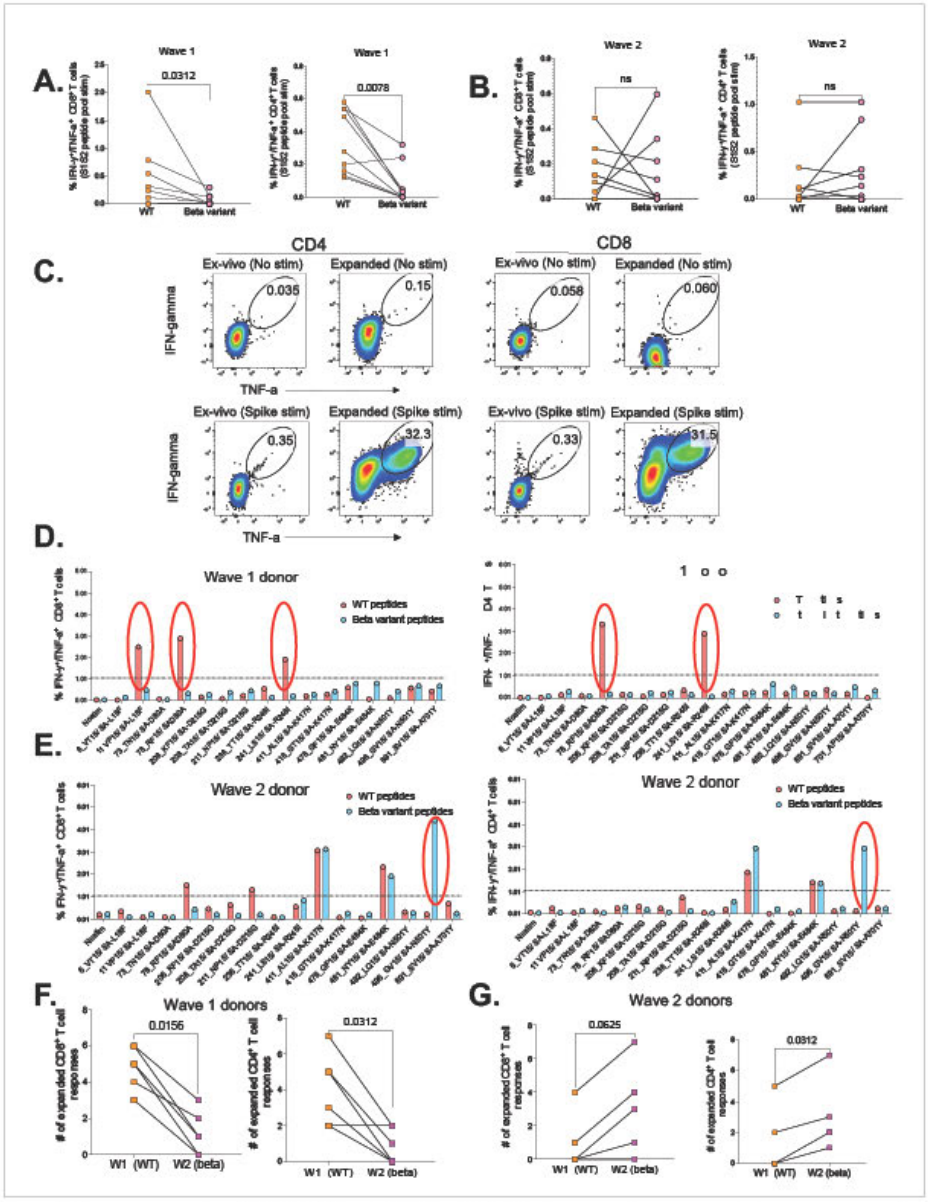

Figure 3. Poor cross-recognition of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cell responses between wt and beta variants in wave 1 and wave 2 COVID-19 participants. Ex vivo assessment of T cell cross-recognition between the two waves. (A) Intra-donor SARS-CoV-2-specific T cell responses to wt and corresponding Beta variant peptides by wave 1 participants. (B) Intra-donor SARS-CoV-2-specific T cell responses to wt and corresponding Beta variant peptides in wave 2 participants. Next, PBMCs were expanded for 12 days in the presence of S1S2 SARS-CoV-2 peptide pools and tested against wt

Figure 3 continued on next page

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and corresponding Beta variants at single peptide level. (**C**) Representative flow plots showing the frequency of SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells before and after cultured expansion. (**D**) T cell responses to single wt (red bars) and corresponding Beta (blue bars) peptide stimulation for a representative donor from wave 1. (**E**) T cell responses to single wt and corresponding Beta peptide stimulation for a representative donor from wave 2 (positive responses are circled). A response was deemed positive if ≥1% or higher. (**F**) Number of expanded wt and corresponding Beta responses for each wave 1 donor. (**G**) Number of expanded wt and corresponding Beta responses for each wave 2 donor. P values calculated using Wilcoxin matched-pairs signed rank T test. PBMC, peripheral blood mononuclear cell; wt, wild-type.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Cross-recognition of SARS-CoV-2 CD4⁺ T cell responses between wt and Beta variants in wave 1 and wave 2 COVID-19 donors: PBMCs were expanded for 12 days in the presence of S1S2 SARS-CoV-2 peptide pools.

Immunodominance hierarchy of SARS-CoV-2 CD8⁺ and CD4⁺ T cell responses targeting the spike protein

Virus-specific CD8⁺ and CD4⁺ T cells typically target viral epitopes in a distinct hierarchical order (***Streeck et al., 2009***; ***Laher et al., 2017***). Identifying SARS-CoV-2 epitopes that are most frequently targeted by T cells is important for the design of vaccines that can induce protective T cell responses. To determine the immunodominance hierarchy of SAR-CoV-2 specific T cell responses targeting the spike protein, OLPs were ranked based on magnitude and frequency of recognition. This analysis revealed the most immunodominant wt peptides targeted by CD8⁺ T cell responses (***Figure 6A***). The Beta variant resulted in dramatic shift in the immunodominance hierarchy whereby, three of five most dominant wt CD8⁺ T cell responses (***Figure 6A***), their Beta variant versions were subdominant (downward arrows) (***Figure 6B***). Contrariwise, three subdominant wt responses were among the most dominant Beta variant responses (upward arrows) (***Figure 6B***). A similar trend was observed for CD4⁺ T cell responses (***Figure 6C and D***). These data demonstrated a shift in the immunodominant hierarchy between wt and Beta variant responses, which partly explains poor T cell cross-recognition between successive SARS-CoV-2 variants.

The impact of HIV markers of diseases progression on SARS-CoV-2-specific T cell responses

To gain more insight into why viremic PLWH responded poorly to SARS-CoV-2 infection, we investigated if T cell activation defined here as co-expression of CD38 and HLA-DR, absolute CD4 count and plasma viral load, impacted immune responses (***Du et al., 2009***). The proportion of activated (CD38/HLA-DR) CD4⁺ T cells was higher in viremic PLWH compared to suppressed (p=0.02) and HIV-seronegative individuals (p=0.002; ***Figure 7A***). Moreover, proportion of activated (CD38/HLA-DR) CD4⁺ T cells among viremic PLWH negatively correlated with absolute CD4 counts (r=–0.7, p=0.04; ***Figure 7B***), and positively correlated with HIV plasma viral loads (r=0.9, p=0.0004; ***Figure 7C***). Similarly, proportion of activated (CD38/HLA-DR) CD8⁺ T cells were significantly higher in viremic PLWH relative to suppressed PLWH (p=0.04) and HIV-seronegative individuals (p=0.0008; ***Figure 7D***). The negative relationship between proportion of activated (CD38/HLA-DR) CD8⁺ T cells and CD4 counts did not reach statistical significance (***Figure 7E***), but proportion of activated (CD38/HLA-DR) CD8⁺ T cells were positively correlated with HIV plasma viral loads among viremic PLWH (r=0.8, p=0.0006; ***Figure 7F***).

Taken together, these data suggest that hyper immune activation driven by uncontrolled HIV infection impacts CD4⁺ and CD8⁺ T cell responses.

Finally, we interrogated the relationship between SARS-CoV-2-specific responses and disease severity, stratified into asymptomatic, mild, and severe diseases requiring oxygen supplementation, as previously defined (***Karim et al., 2021a***). We found no significant differences between the magnitude of CD4⁺ or CD8⁺ T cell responses and disease severity among the groups (***Figure 7—figure supplement 1A,B***). We next, examined sex differences and found no difference in CD4⁺ and CD8⁺T cell responses to SARS-CoV-2 infection (***Figure 7—figure supplement 1C,D***). Age is a risk factor for severe COVID-19 (***WHO, 2021a***); thus, we examined the relationship between age and T cell responses. There was a negative relationship between age and magnitude of CD8⁺ T cell responses (CD8 r=−0.6, p=0.002) (***Figure 7—figure supplement 1E***), and a similar trend for CD4⁺ T cell responses (CD4 r=−0.3, *P*=0.15) (***Figure 7—figure supplement 1F***). These data show that younger

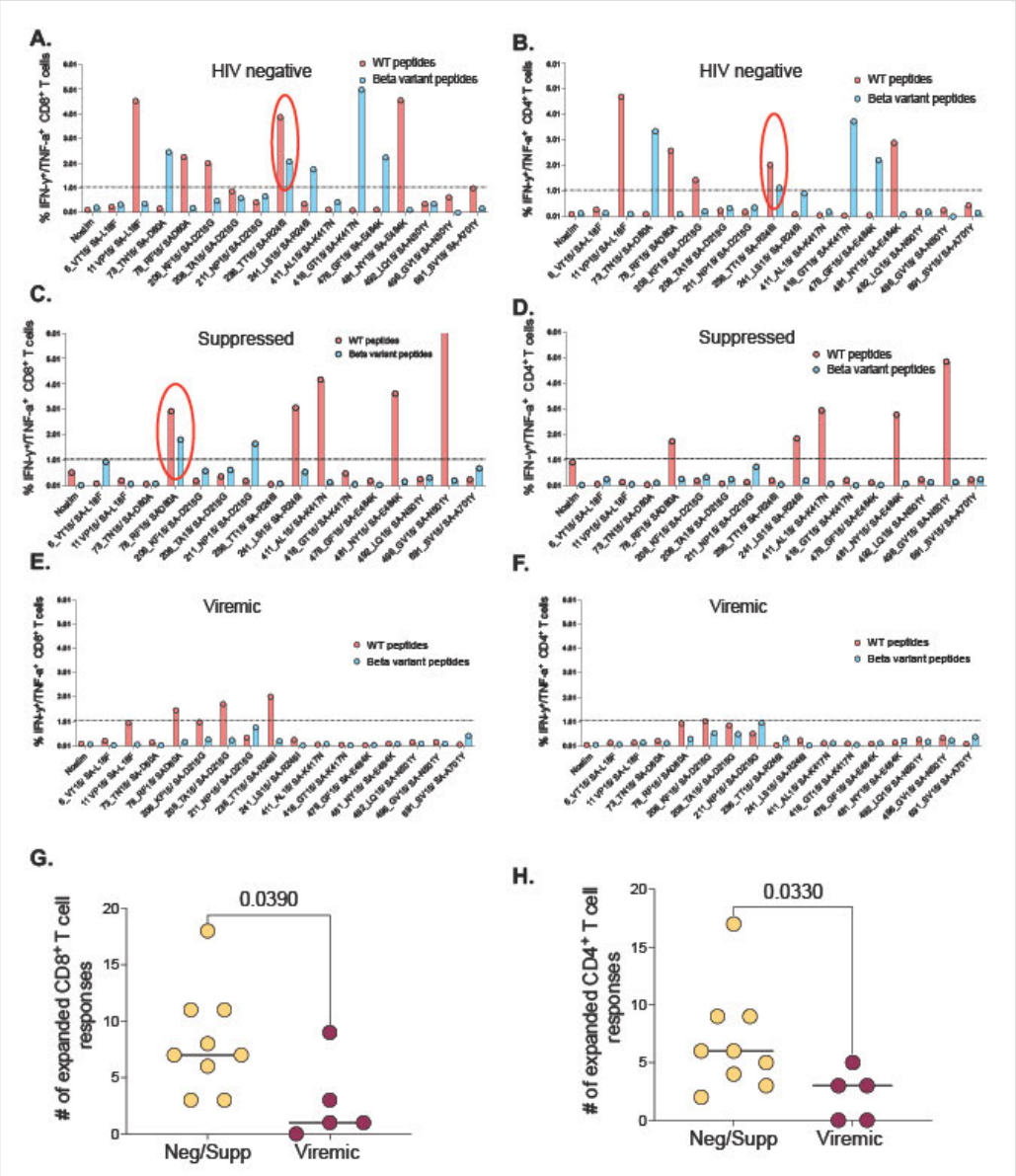

Figure 4. The effects of unsuppressed HIV infection on T cell breadth and ability to cross-recognize the Beta variant. Representative data for a negative donor showing greater, (**A**) CD8+ and (**B**) CD4+ T cell breadth. A cross-recognized responses between wt and Beta is circled. Representative data for a suppressed donor showing greater, (**C**) CD8+ and (**D**) CD4+ T cell breadth. A cross-recognized response is circled. Representative data for a viremic donor showing greater, (**E**) CD8+ and (**F**) CD4+ T cell breadth. (**G**) Aggregate data comparing breath of SARS-CoV-2-specific CD8+, and (**H**) CD4+ T

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cell response between HIV-negative and suppressed versus viremics. Breadth here is simply the number of positive responses among the individual peptides tested.

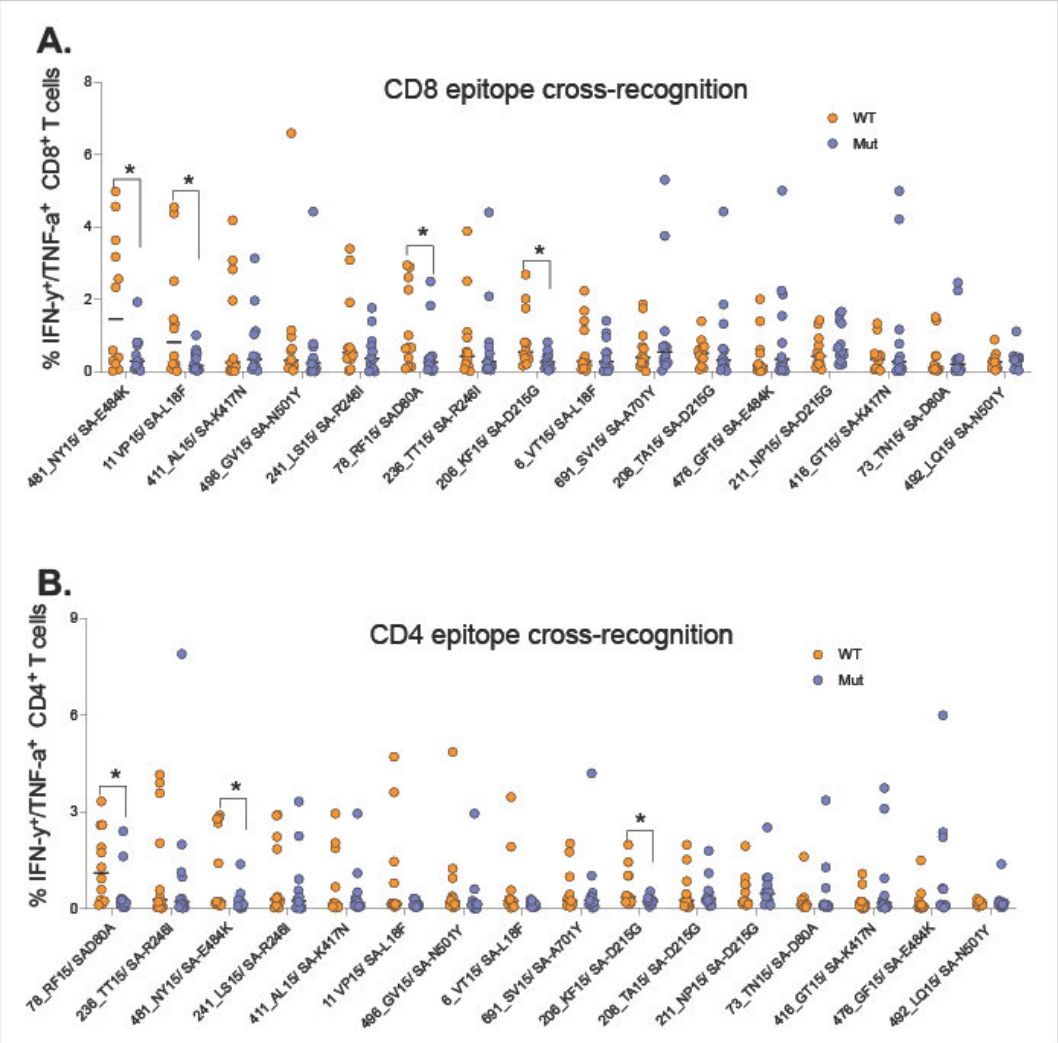

Figure 5. Identification of Beta mutations associated with reduced cross-recognition between wt and Beta variant. (A) Side-by-side comparison of SARS-CoV-2-specific CD8+ T cell response between wt and Beta. (B) Side-by-side comparison of SARS-CoV-2-specific CD4+ T cell response between wt. The analysis combined all the 12 participants. P values calculated by Mann-Whitney U-test. wt, wild-type.

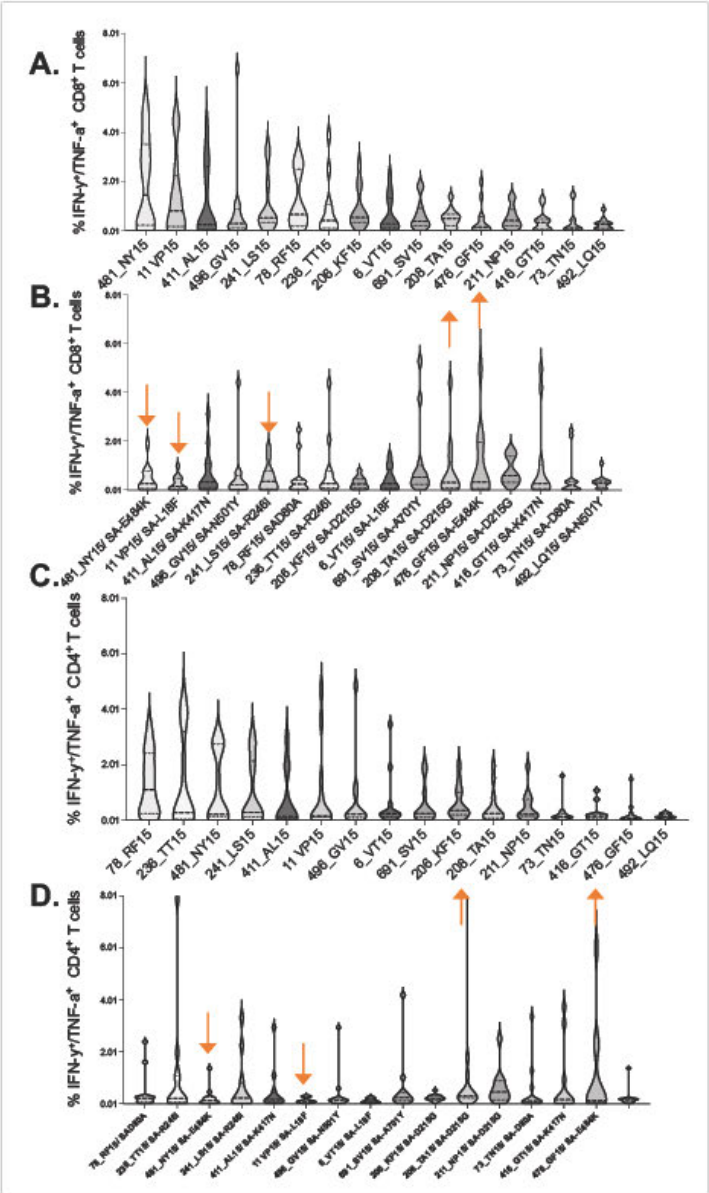

Figure 6. Immunodominance hierarchy of SARS-CoV-2 CD8⁺ and CD4⁺ T cell responses targeting wt and Beta. Immunodominance hierarchy of CD8⁺ T cell responses to, (A) wt and (B) the corresponding Beta variant peptides. Similarly, Immunodominance hierarchy of CD4⁺ T cell responses to, (C) wt and (D) the corresponding Beta variant. Arrows indicate responses that changed hierarchical position (among the six most dominant responses) between

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the two waves. Data arranged in descending order of magnitude of responses to wt peptide stimulation. wt, wild-type.

people had greater responses compared to older people, whereas disease severity and sex did not have discernible effect on SARS-CoV-2 T cell responses.

Discussion

The greater burden of HIV in sub-Saharan Africa makes investigating the impact of HIV infection on COVID-19 immunity and disease outcomes critical for bringing the epidemic under control in the region. Recent studies have documented strong cellular responses following SARS-CoV-2 infection and vaccination, but the effects of HIV on SARS-CoV-2-specific T cell responses are not well characterized. Here, we investigated the antigen-specific CD4⁺ and CD8⁺ T cell responses in a cohort of SARS-CoV-2- infected individuals with and without HIV infection. Our results show that unsuppressed HIV infection is associated with reduced cellular responses to SARS-CoV-2 infection. We also show that low absolute CD4 count and hyper immune activation are associated with diminution of SARS-CoV-2-specific T cell responses. Importantly, we identify spike mutations in the Beta variant that abrogate recognition by memory T cells raised against wt epitopes. Similarly, immune responses targeting Beta variant epitopes poorly cross-recognize corresponding wt epitopes. These data reveal the potential

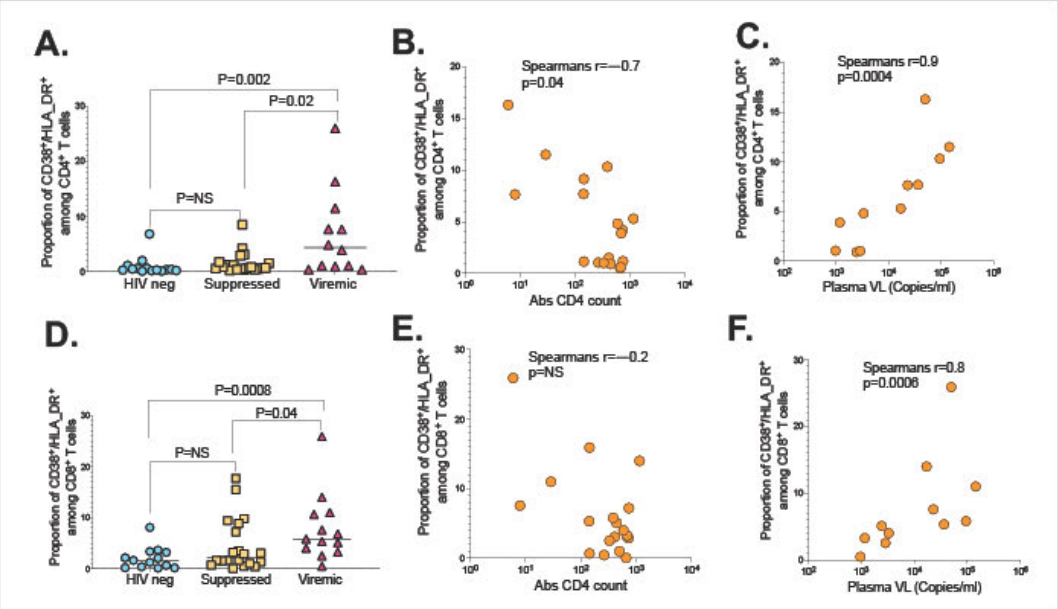

Figure 7. The impact of HIV markers of diseases progression on SARS-CoV-2 T cell immunity. (**A**) CD4⁺ T cell activation graphed based on the frequency of CD38/HLA-DR co-expressing cells. (**B**) Correlation between CD4⁺ T cell activation and absolute CD4 counts of viremic PLWH. (**C**) Correlation between CD4⁺ T cell activation and HIV plasma viral load of viremic PLWH. (**D**) CD8⁺ T cell activation measured by CD38/HLA-DR. (**E**) Correlation between CD8⁺ T cell activation and absolute CD4 counts of viremic PLWH. (**F**) Correlation between CD8⁺ T cell activation and HIV plasma viral load of viremic PLWH. P values calculated by Mann-Whitney U-test and Pearson correlation test. PLWH, people living with HIV.

The online version of this article includes the following figure supplement(s) for figure 7:

Figure supplement 1. Assessment of the effect of COVID-19 disease severity on, (**A**) SARS-CoV-2-specific CD4⁺, and (**B**) CD8⁺ T cell responses.

for emerging SARS-CoV-2 variants to escape T cell recognition. Importantly, our data highlight the potential for unsuppressed HIV infection to attenuate vaccine-induced T cell immunity.

HIV-induced immune dysregulation is well documented (**Klatt et al., 2013**). Unsuppressed HIV infection is associated with profound dysfunction of virus-specific T cell immunity partly caused by immune activation (**Klatt et al., 2013**; **Ndhlovu et al., 2015**). Recent studies have reported strong association between unsuppressed HIV infection and poor COVID disease outcomes, for instance, a large cross-section study found a link between severe HIV disease and poor COVID-19 outcomes including COVID-19-associated death (**Chanda et al., 2020**). This study showed that individuals with unsuppressed HIV infection mount weak responses to SARS-CoV-2 infection and poorly recognize SARS-CoV-2 Beta variant mutations. We also examined several mechanisms by which unsuppressed HIV can impact SARS-CoV-2-specific T cell responses and found that HIV-induced immune defects such as low $CD4^+$ T cell counts, higher HIV plasma viral loads, and elevated immune activation were invariably associated with diminished SARS-CoV-2 responses. These findings are consistent with several recent reports, such as a case of one HIV-positive patient with low CD4 count that had prolonged CIVID-19 disease (**Wang et al., 2020**). The ability of unsuppressed HIV to cause severe immune activation was also recently documented by others (**d'Ettorre et al., 2020**; **Sharov, 2021**). Taken together, these data suggest that HIV-induced immune dysregulation negatively impacts the potential to mount robust T cell responses to SARS-CoV-2 infection.

Furthermore, although ART-mediated HIV suppression rarely results in complete immune reconstitution (**Henrich et al., 2017**), sustained complete plasma HIV suppression was associated with robust SARS-CoV-2 responses that were mostly similar in magnitude and quality to responses mounted by HIV-seronegative individuals. Given reduced levels of CD38 and HLA-DR dual positive cells and near normal absolute CD4 counts in suppressed individuals, it is reasonable to speculate that reduced immune activation and superior $CD4^+$ T helper function were partly responsible for improved immune responses in suppressed individuals.

The emergence of several SARS-CoV-2 variants with mutations in the viral Spike (S) protein such as mutations in the RBD, N-terminal domain (NTD), and furin cleavage site region (**Tarke, 2021**) continue to fuel the epidemic. These mutations have been shown to directly affect ACE2 receptor binding affinity, infectivity, viral load, and transmissibility (**Tarke, 2021**; **Greaney et al., 2021**; **Starr et al., 2021**). The variants of concern identified since the start of the COVID-19 pandemic include the Alpha (**Davies, 2020b**), Beta (**Tegally et al., 2021b**), Gamma (**Voloch et al., 2020**), and Delta (**Mallapaty, 2021**), and now the Omicron variant. Most of these have been shown to attenuate neutralization but the impact of these mutations on T cell responses has not been extensively explored (**Riou et al., 2022**). However, a recent report demonstrating the potential for SARS-CoV-2 to evade cytolytic T lymphocyte (CTL) surveillance, highlight the need for more investigations regarding the potential CTL-driven immune pressure to shape emerging variants (**Agerer et al., 2021**). To this end, our study provides new evidence that SARS-CoV-2 has the potential to evade T cell recognition. Moreover, our data suggest that spike mutations in the Beta variant that were associated with antibody escape may also escape T cell recognition.

Southern Africa has had at least four epidemic waves of COVID-19. The first was a mixture of SARS-CoV-2 lineages (with D614G), the second wave was driven by the Beta variant (**Tegally et al., 2021a**), and the third by the Delta variant (**Callaway, 2021**). The fourth wave dominated by the highly mutated Omicron variant (**WHO, 2021b**; **Viana et al., 2021**). Intriguingly, there was some evidence that PLWH in South Africa had increased disease severity in the second wave compared to the first wave (**Karim et al., 2021a**). The precise mechanisms responsible for increased severity are not fully understood, but low $CD4^+$ T cell counts and high neutrophil-to-lymphocyte ratio (NLR) showed strong association with disease severity (**Karim et al., 2021a**). Our data suggest that diminished T cell responses to the Beta variant even in previously exposed individuals may have contributed to severe disease in the second wave.

Here, we report poor cross-recognition of the Beta variant by individuals infected with wt and vice versa, which was exacerbated by unsuppressed HIV infection. However, others have reported better cross-recognition between variants and vaccines. Possible explanation for the apparent discrepancy include, (1) unlike other studies that compared responses to the entire spike protein using peptide pools to stimulate cells (**Keeton et al., 2022**; **Gao et al., 2022**), our cross-recognition studies focused on head-to-head comparisons of single wt peptides with corresponding variants peptides

containing a lineage defining mutation (**Keeton et al., 2022**). We may have picked up fewer cross-reactive responses because we used dual section of IFN-γ and TNF-α as a readout for antigen-specific responses, which is more stringent than single cytokine producing cells. (3) We used cultured expansions prior to ICS assays which amplifies the response several folds above background and therefore more specific. In fact, our ex vivo cross-recognition data are comparable to other studies which also showed diminution of responses across variants (**Keeton et al., 2022**). Future studies should apply our cultured expansion and the dual cytokine secretion readout to assess cross-recognition among other variants and different vaccine regimens.

Although, we repeatedly showed robust in vitro T cell expansion following ex vivo peptide stimulation but limited expansion against mutant versions of the peptides, there is need to identify optimal peptides that were targeted by $CD8^+$ and $CD4^+$ T cells in the context of restricting MHC class I and II alleles. SARS-CoV-2 responses are generally very broad (**Grifoni et al., 2020**); thus, it is not clear from these studies how the loss of T cell cross-recognition in Spike affects the overall protective immunity. Furthermore, investigating if the observed poor T cell cross-recognition between wave 1 and wave 2 is generalizable to the Delta and the Omicron variants is clearly warranted. Importantly, our data raise the question of whether CTL selection pressure plays a significant role in shaping emerging variants. This concept should be investigated using larger longitudinal studies with longer durations of follow-up.

Previous work in this cohort examined the relationship T cell and B cell responses and found a positive association between $CD8^+$ T cells frequency and several CD19 B cell subsets, which was attenuated in PLWH (**Karim et al., 2021a**), suggesting that both arms of the immune system are impacted by HIV/SARS-CoV-2 coinfection. However, the current study did not examine this relationship at antigen-specific level due to sample limitations. Future work is required to understand the relationship between T cell and humoral immunity and the impact of unsuppressed HIV infection on long-term protection.

In conclusion, we show that uncontrolled HIV infection is associated with low magnitude, reduced polyfunctionality, and diminished cross-recognition of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses. Importantly, fully suppressed PLWH had comparable SARS-CoV-2-specific T cell responses with HIV-seronegative individuals. These findings may partly explain high propensity for severe COVID-19 among PLWH and also highlight their vulnerability to emerging SARS-CoV-2 variants of concern, especially those with uncontrolled HIV infection. Hence, there is need to ensure uninterrupted access to ART for PLWH during the COVID-19 pandemic.

Materials and methods

Ethical declaration

The study protocol was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) (approval BREC/00001275/2020). Consenting adult patients (>18 years old) presenting at King Edward VIII, Inkosi Albert Luthuli Central Hospital, and Clairwood Hospital in Durban, South Africa, between July 29 and August November 2021 with PCR confirmed SARS-CoV-2 infection were enrolled in the study.

Sample collection and laboratory testing

Blood samples used in this study were collected between 1 and 3 weeks after COVID-19 PCR positive diagnosis. HIV testing was done using a rapid test and viral load quantification was performed from a 4-ml EDTA by a commercial lab (Molecular Diagnostic Services, Durban, South Africa) using the RealTime HIV-negative1 viral load test on an Abbott machine. CD4 counts were performed by a commercial lab (Ampath, Durban, South Africa). PLWHs were categorized into suppressed and unsuppressed based on viral load measurements of <50 and >1000 copies/ml, respectively, at the time of sample collection.

T lymphocyte phenotyping

PBMCs were isolated from blood samples by density gradient method and cryopreserved in liquid nitrogen as previously described (**Karim et al., 2021b**). Frozen PBMCs were thawed, rested, and stimulated for 14 hr at 37°C, 5% CO_2 with either staphylococcal enterotoxin B (SEB, 0.5 µg/ml), SARS-CoV-2

wt peptide pool (8 µg/ml), 501Y.V2 variant peptide pool (4 µg/ml), or the Control Spike peptide pool (Miltenyi, Bergisch Gladbach, Germany, 2 µg/ml). Brefeldin A (BioLegend, CA) and CD28/CD49d (BD Biosciences, Franklin Lakes, NJ) were also added ahead of the 14-hr incubation at 5 and 1 µg, respectively. The cells were stained with an antibody cocktail containing: Live/Dead fixable aqua dead cell stain, anti-CD3 PE-CF594 (BD), anti-CD4 Brilliant Violet (BV) 650, anti-CD8 BV 786 (BD), anti-CD38 Alexa Fluor (AF) 700 (BD), anti-human leukocyte antigen (HLA) – DR Allophycocyanin (APC) Cy 7 (BD), and anti-programmed cell death protein 1 (PD) BV 421 (BD). After a 20-min incubation at room temperature, the cells were washed, fixed, and permeabilized using the BD Cytofix/Cytoperm fixation permeabilization kit. Thereafter, the cells were stained for 40 min at room temperature with an intracellular antibody cocktail containing: anti-IFN-γ BV 711 (BD), anti-IL-2 PE (BD), and anti-TNF-α PE-Cy 7 (BD). Finally, the cells were washed and acquired on an LSR Fortessa and analysed on FlowJo v10.7.2. Differences between groups were considered to be significant at a p value of <0.05. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc, San Diego, CA).

Ex-vivo cultured expansion of SARS-COV-2-specific T cells

PBMCs at a concentration of 2 million cells per well in a 24-well plate in R10 medium were stimulated with 10 µg/ml of SARS-COV-2 of OLP pools spanning the entire spike protein. The cells were incubated at 37°C in 5% CO_2. After 2 days, the cells were washed and fresh R10 medium supplemented with 100 U/ml recombinant IL-2 was added. Cultured cells were fed twice weekly with regular medium replenishment. On day 14, the cells were washed three times with fresh R10 medium and rested at 37°C in 5% CO_2 overnight in fresh R10 medium. On the following day, the cells were restimulated with individual peptides for 16 hr followed by ICS. Peptides that induced IFN-γ/TNF-α dual production above background (No stimulation control) were deemed reactive. Meaning that the expanded cells contained a subset of cells that were specific for that particular peptide.

Statistical analyses

All statistical analyses were conducted with GraphPad Prism 9.3.1 (GraphPad Software, La Jolla, CA) and p values were considered significant if less than 0.05. Specifically, the Mann-Whitney U- and Kruskal-Wallis H-tests were used for group comparisons. Additional post hoc analyses were performed using the Dunn's multiple comparisons test. Correlations between variables were defined by the Spearman's rank correlation test. Categorical data were analysed using the Fisher's exact test.

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Additional information

Competing interests

COMMIT-KZN-Team: The other authors declare that no competing interests exist.

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Author contributions

Thandeka Nkosi, Investigation, Writing - original draft, Writing – review and editing; Caroline Chasara, Investigation, Writing - original draft; Andrea O Papadopoulos, Resources, Formal analysis, Investigation, Writing - original draft, Project administration, Writing – review and editing; Tiza L Nguni, Investigation, Methodology, Writing - original draft; Farina Karim, Inbal Gazy, Data curation, Project administration; Mahomed-Yunus S Moosa, Resources, Investigation, Project administration, Writing – review and editing; Kondwani Jambo, Formal analysis, Writing – review and editing; COMMIT-KZN-Team, Data curation, Investigation, Project administration; Willem Hanekom, Conceptualization, Funding acquisition, Writing – review and editing; Alex Sigal, Data curation, Project administration, Writing – review and editing; Zaza M Ndhlovu, Conceptualization, Formal analysis, Validation, Investigation, Writing - original draft, Project administration, Writing – review and editing

Author ORCIDs

Caroline Chasara http://orcid.org/0000-0001-6860-6111
Andrea O Papadopoulos http://orcid.org/0000-0001-5317-1418
Farina Karim http://orcid.org/0000-0001-9698-016X
Mahomed-Yunus S Moosa http://orcid.org/0000-0001-6191-4023
Alex Sigal http://orcid.org/0000-0001-8571-2004
Zaza M Ndhlovu http://orcid.org/0000-0002-2708-3315

Ethics

Human subjects: Ethical Declaration: The study protocol was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) (approval BREC/00001275/2020). Consenting adult patients (>18 years old) presenting at King Edward VIII, Inkosi Albert Luthuli Central Hospital, and Clairwood Hospital in Durban, South Africa, between 29 July to August November 2021 with PCR confirmed SARS-CoV-2 infection were enrolled into the study.

Decision letter and Author response

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Additional files

Supplementary files

- Supplementary file 1. Wild-type (wt) Spike overlapping peptides and corresponding Beta variant peptides. The table contains a list of peptides spanning the receptor-binding domain (RBD) and non-RBD regions of spike with known hotspots for mutations, and a corresponding list of peptides with Beta variant lineage defining mutations. The Beta variants mutations are highlighted in red. The two sets of peptides were used for cultured expansion studies.
- MDAR checklist

Data availability

All source data files for the figures are now publicly available on our institutional website (Africa Health Research Institute database). The data can be accessed using this link: https://doi.org/10.23664/AHRI.SARS.CoV.2.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Nkosi K, Charasa C, Papadopoulos AO, Nguni TZ, Karim F, Moosa MYS, Gazy I, Jambo K, Hanekom W, Sigal A, Ndhlovu ZM	2022	Unsuppressed HIV infection impairs T cell responses to SARS-CoV-2 infection and abrogates T cell cross-recognition	https://doi.org/10.23664/AHRI.SARS.CoV.2	AHRI Data Repository, 10.23664/AHRI.SARS.CoV.2

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CHAPTER 3

Aim: Determine the phenotype, spatial location, and function of macrophages within human lymph nodes (LNs).

Chapter 3 Overview

Macrophages are dynamic cells that play a crucial role in host defence against pathogen invasion [1, 2]. In addition, macrophages exhibit extreme heterogeneity in response to physiological and pathological stimuli. According to traditional nomenclature, macrophages can be polarised into pro-inflammatory (M1) and anti-inflammatory (M2) macrophages in vitro. M1 and M2 macrophages exhibit diverse phenotypes and functions, and their characterization has been extensively conducted in mice. However, only a few studies have characterized macrophages in human lymph nodes (LNs). Additionally, the role of macrophage polarization during HIV infection is also of relevant importance but it has not been fully explored. In vitro studies have highlighted changes in macrophage states during acute and chronic HIV infection leading to impaired macrophage function. Given the heterogeneous nature of human macrophages, it is vital to further investigate and understand the distinct lymph node macrophage populations to ultimately manage HIV pathogenesis and macrophage reservoir dynamics.

Chapter 3 presents a study conducted to identify the different macrophage phenotypes found in human LNs using a combination of immunofluorescence microscopy and multi-color flow cytometry. In addition, the spatial location of these macrophage populations was determined and and the effect of HIV infection on the expression and frequency of these macrophage populations was investigated. $CD68^+$ (M1) macrophages localized inside and outside germinal centers (GCs) in HIV negative and HIV positive tissues. In contrast, $CD206^+$ (M2) and $CD68^+CD206^+$ macrophages (an intermediate phenotype) localized along lymphatic vessels (LV). Interestingly, an increase in $CD68^+$, $CD206^+$, and $CD68^+CD206^+$ macrophage frequency during HIV infection was observed. Flow cytometry was used to identify similar macrophage populations in lymph node mononuclear cells. These findings may partly explain the establishment and maintenance of the macrophage reservoir in germinal centers of human LN tissues. Additionally, the alteration of macrophage frequency during HIV infection may affect macrophage function leading to HIV persistence.

CHAPTER 3: DETAILED CHARACTERIZATION OF THE PHENOTYPE, SPATIAL LOCALIZATION, AND FUNCTION OF MACROPHAGES WITHIN HUMAN LYMPH NODES

Caroline Chasara[1], Trevor Khaba[1], Bongiwe Mahlobo[1], Leonard Mvaya[5], Thandekile Ngubane[3], Ismail Jajbhay[3], Johan Pansegrouw[3] , Zaza M. Ndhlovu[1,2,4]

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

[2]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

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

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

[5]Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi

Address correspondence to Zaza Ndhlovu (ZNDHLOVU@mgh.harvard.edu)

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3.1 ABSTRACT

Pro-inflammatory (M1) and anti-inflammatory (M2) macrophages exhibit distinct phenotypes and functions. The role of macrophages in HIV pathogenesis has been extensively characterized in mice. However, their characterization in humans, specifically in lymph nodes (LNs), has lagged because of the inability to access LN tissues. In human studies, polarization of macrophages into M1 and M2 subtypes has been shown to impact HIV pathogenesis. It has also been established that macrophage states switch during HIV infection hence influencing disease outcomes. A critical understanding of macrophage populations in LNs will inform the development of effective cure strategies. To gain insight into the various macrophage populations found in human LNs, we used immunofluorescence microscopy and multi-color flow cytometry to phenotypically characterize LN macrophages. We identified $CD68^+$ (M1) macrophages inside and outside the germinal centers (GCs). In addition, we identified $CD206^+$ (M2) and $CD68^+CD206^+$ (double positive) macrophages localized along lymphatic vessels (LV). We further investigated if HIV infection influences the frequency of these macrophages. Notably, immunofluorescence microscopy results revealed that HIV infection leads to a significant increase in both M1 and M2 macrophage frequencies. Lastly, we confirmed these data by flow cytometry and show that M1 and M2 macrophage frequencies are increased during HIV infection. Overall, this work identified spatial localization of different macrophage subsets within human LNs and highlight their potential role during HIV infection in lymphoid tissues.

3.2 INTRODUCTION

Monocytes and macrophages are myelomonocytic cells that play an important role in innate immunity. They provide protection by detecting and engulfing pathogens in the blood and tissues [3]. Once these myelomonocytic cells detect a pathogen, they trigger an immune inflammatory reaction, ultimately leading to homeostasis reinstating as well as pathogen clearance [3, 4]. Monocytes are derived from monocyte progenitors in the bone marrow. Thereafter, they enter the blood stream and use the blood as a transport system to migrate into tissues where they differentiate into macrophages [5].

Human monocytes are subdivided into three populations defined as classical ($CD14^{++}CD16^{-}$), non-classical ($CD14^{+}CD16^{+}$), and intermediate ($CD14^{++}CD16^{+}$) monocytes [6, 7]. Over the years, research has shown that macrophages are dynamic cells that differentiate under the influence of cytokines such as interleukin-10 (IL-10) into either pro-inflammatory (M1) or anti-inflammatory (M2) macrophages [8]. [9]. Some of the common markers that have been reliably used to identify M1 and M2 macrophages in mice include CD68 and CD206 respectively [10, 11]. However, the selection of the most appropriate markers for the identification of human macrophages will involve continuous screening as only a few studies have been attempted to define human M1 and M2 macrophages [12, 13].

$CD4^{+}$ T cells are the main targets of HIV-1 infection but circulating monocytes and tissue macrophages are also infected by HIV. Certain subsets of tissue macrophages have the potential to contribute to the establishment of viral reservoirs due to their long half-life, their resistance to cytopathic effects of virus replication, and their ability to produce mature HIV virions in intracellular compartments [14]. The persistence and outcome of HIV infection has been attributed to macrophage polarization [15, 16]. However, the specific mechanism of macrophage polarization in humans is not fully understood.

Alternatively activated $CD206^{+}$ (M2) macrophages have been reported to produce cytokines, such as IL-10, and help with the resolution of inflammation. Moreover, an increased frequency of $CD206^{+}$ macrophages has been associated with decreased inflammation during HIV infection. In contrast, classically activated $CD68^{+}$ (M1) macrophages have been reported to contribute to the viral reservoir in urethral tissue [17]. Currently, there is no consensus on the distinct macrophage markers that best define M1 and M2 macrophages. In addition, the frequency of M1 and M2 macrophages in HIV-infected individuals remains a subject of debate. Identifying the macrophage phenotypes in human lymph nodes and understanding the mechanisms underlying the changes in macrophage frequency during HIV infection is vital to inform HIV pathogenesis and macrophage reservoir formation.

Here, we postulate that the phenotype and location of macrophages play a significant role during HIV infection in human lymph nodes. Using immunofluorescence microscopy, we identified $CD68^{+}$ (M1)

macrophages localized inside and outside the germinal centers (GCs) in HIV negative and HIV positive individuals. In contrast, $CD206^+$ (M1) and $CD68^+CD206^+$ (double positive) macrophages localized along lymphatic vessels and outside the GCs. Several studies have combined flow cytometry and immunofluorescence microscopy to investigate changes in macrophage phenotype and frequency as well as the potential effect of this alteration. Despite the information known to date, it is still not fully understood whether HIV infection impacts macrophage polarization and/or induces a shift in macrophage frequency. Therefore, to address this, we used immunofluorescence microscopy and multicolour flow cytometry to investigate the effect of HIV on macrophage frequency. Our results show that HIV infection results in increased frequencies of M1 ($CD68^+$) and M2 ($CD206^+$) macrophages. Increased macrophage frequencies elevate their potential to contribute to the HIV reservoirs in LNs.

3.3 MATERIALS AND METHODS

3.3.1 Study approval

All study participants provided written informed consent before inclusion in the study. This study is a follow up study. Ethical approval for the primary study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (protocol number BF298/14) and the Institutional Review Board of Massachusetts General Hospital (protocol number 2015-P001018).

3.3.2 Study cohort

A total of 31 LN samples (20 LN tissue biopsies obtained from late treated (LT) individuals (**Table 3.1; supplementary Table 3.1**) and 10 mononuclear cell samples isolated from LNs (**Table 3.1; supplementary Table 3.2**)) were used in this study. Late treatment was defined as treatment initiated after the acute phase of HIV infection (3 months post infection). These samples were selected based on availability. Matched LN tissue biopsies and mononuclear cells were used. However, in cases where matched samples could not be obtained, samples from different timepoints were used instead. Study participants were drawn from the HIV Pathogenesis Programme (HPP) lymph node study (LNS) cohort in Durban, South Africa (SA). Recruitment was conducted at a study site in Umlazi, Durban, SA as part of the Females Rising through Education, Support and Health (FRESH) program. The FRESH program was designed to minimize HIV progression by implementing early and frequent HIV testing and treatment [18].

3.3.3 Sample collection and experimental procedures

Inguinal and mesenteric LNs were surgically excised at Prince Mshiyeni Hospital in Umlazi, and 120 ml paired PB was also obtained from each participant. Viral load measurements were performed by HIV-1 RNA testing using the NucliSens EasyQ v2.0 assay (BioMérieux Clinical Diagnostics, Marcy-l'Étoile, France), through a certified commercial laboratory. $CD4^+$ T cell counts were enumerated by Tru-Count technology and analyzed on a FACSCalibur flow cytometer (Becton Dickinson (BD) New Jersey, USA). Sample processing and laboratory studies were performed at the Africa Health Research Institute in Durban, South Africa.

3.3.4 Lymph node and blood sample processing

Excised LNs were divided into two sections. One section was fixed in 10% formal-saline (Sigma-Aldrich, St. Louis, Missouri, USA) for immunofluorescence microscopy studies while the second section was macerated to release lymph node mononuclear cells (LMCs) as described by *Schacker et al* [19]. Thereafter, cells were passed through a mesh screen and harvested by centrifugation (625 × g, 6 min, room temperature (RT). Peripheral blood mononuclear cells (PBMCs) were isolated from patients' blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in liquid nitrogen.

3.3.5 Hematoxylin and Eosin (H and E) staining

Briefly, tissue sections were deparaffinized using xylene (Honeywell research chemicals) and rehydrated prior to staining with the Harris' hematoxylin solution for 10 min at 37 °C. Next, the sections were rinsed in water followed by differentiation using 10% acetic acid and 85% ethanol. After differentiation, the slides were placed in the blueing agent for 3 min at 37 ºC, dipped in ammonia water until the sections turned blue, and rinsed under running tap water. Thereafter, the slides were stained with eosin Y ethanol solution for 3 min at RT. Slides were then mounted with Dako fluorescence mounting medium (Agilent Technologies) and imaged with the Axio Observer, ×20 objective lenses, a Hamamatsu C13440- 20C camera, and TissueFAXS imaging software (TissueGnostics, Vienna, Austria).

3.3.6 Immunofluorescence (IF) microscopy

IF microscopy staining was performed on 4 µM tissue sections of formalin-fixed paraffin-embedded (FFPE) LNs using the Opal 4-color fluorescent immunohistochemistry (IHC) kit (PerkinElmer, Waltham, MA, USA). Sections were deparaffinized using xylene (Honeywell research chemicals) and rehydrated before antigen retrieval using AR6 buffer (20 min, 100 °C, (PerkinElmer)). Next, two blocking steps (2 × 10 min, RT) were performed with the Dako peroxidase-blocking reagent (Agilent Technologies, Glostrup, Denmark) and Bloxall block (Vector Laboratories, Burlingame, CA, USA). The slides were washed with 0.05% Tween 20 in Tris-buffered saline (TBS-T) for 5 min, sequentially probed with the primary antibody (30 min, RT), and Opal polymer HRP (20 min, RT (PerkinElmer)) and detected using the Opal polymer 520 (10 min, RT). This protocol was repeated for the second and third antibodies with Opal polymers 570 and 690 respectively, followed by counterstaining with spectral DAPI (PerkinElmer) to make a total of four different fluorochromes. Primary antibodies used in these combinations include anti-human BCL-6 ((clone PG-B6p) Dako/Agilent Technologies), CD68 ((clone KP1) Dako/Agilent Technologies), and CD206 ((clone 685645) R&D Systems. After staining, slides

were mounted with Dako fluorescence mounting medium (Agilent Technologies) and imaged with the Axio Observer, ×20 objective lenses, a Hamamatsu C13440- 20C camera, and TissueFAXS imaging software (TissueGnostics, Vienna, Austria).

3.3.7 Quantitative image analysis

Quantitative image analysis was conducted using TissueQuest (TissueGnostics). Statistical analysis and graphical presentation were performed using GraphPad Prism version 9.0 software (GraphPad Software Inc., La Jolla, CA, USA). The Mann-Whitney U test was utilized to compare differences between any two groups. Differences between groups were significant at a P value of <0.05.

3.3.8 Flow cytometry analysis

Lymph node mononuclear cells (LMCs) were characterized using multi-parameter flow cytometry analysis. Briefly, cells were stained with LIVE/DEAD Fixable Blue dead cell stain kit (Thermo Fisher Scientific, Waltham, MA, USA), CD3-BV711 (BD Biosciences, San Jose, CA), CD4-BV650 (BioLegend, San Diego, CA, USA), CD19-PE-Cy5 (BioLegend), HLA-DR-APC-CY7 (BioLegend), CD45-BV786 (BD Biosciences), CD11B-BV421 (BioLegend), and TIM-4-APC (BioLegend). For intracellular staining, cells were washed with PBS and incubated for 20 min with cytofix/cytoperm (BD Biosciences) according to the manufacturer's instructions. After fixation, cells were washed with perm wash buffer (BD Biosciences) and incubated for 20 min at RT with perm wash buffer containing CD68 (BioLegend) and CD206 (BD Biosciences) antibodies. Fluorescence minus one (FMO) or unstained cells were used as a control. Stained cells were acquired using an LSRFortessa (BD Biosciences) with FACSDiva™ software. Data were analyzed using FlowJo version 10.6.0 (FlowJo, LLC, Ashland, Oregon).

3.4 RESULTS

Macrophage spatial localization, distribution, and function within lymph node tissues.

Macrophages are a heterogenous population and their polarization into diverse phenotypes has been shown to impact HIV progression [20]. However, the interplay between their polarization and disease progression has not been fully explored. To gain more insight into the phenotypes, distribution, and function of macrophages in human lymph nodes, we used a total of 31 HIV negative and HIV positive late treated (LT) samples to phenotype and spatially localize lymph node macrophages.

Table 3.1: Donor characteristics stratified by HIV status.

	All (n=31)	**HIV negative (n=11)**	**HIV positive (LT) (n=20)**
Age years (median)	27 (24-61)	27 (25-29)	27 (24-61)
Male	6	0	6
Female	25	11	14
HIV viral load copies/ml	275(20-6000)	N/A	275 (20-6000)
CD4 count cells/µl (median)	737 (375–406)	789 (659–1143)	737 (225–1369)

** Abbreviations: not applicable (NA), late treated (LT)*

3.4.1 Lymph node morphology

HIV infection causes cells to undergo clonal expansion. These cells rapidly expand over time in peripheral blood and LNs and may ultimately contribute to viral rebound [21]. The hematoxylin and eosin staining technique has been widely used as a standard technique to analyze tissue morphology and alteration during disease. To gain insight into the LN morphology and structure during HIV infection, we used H and E staining to study LN morphology in HIV negative and HIV positive LN tissues. We observed that follicles were expanded in HIV-infected tissues (**Figure 3.1C**) compared to the HIV negative tissues (**Figure 3.1A-B**). Our results confirm that HIV infection leads to an expansion of LN follicles due to HIV induced inflammatory responses.

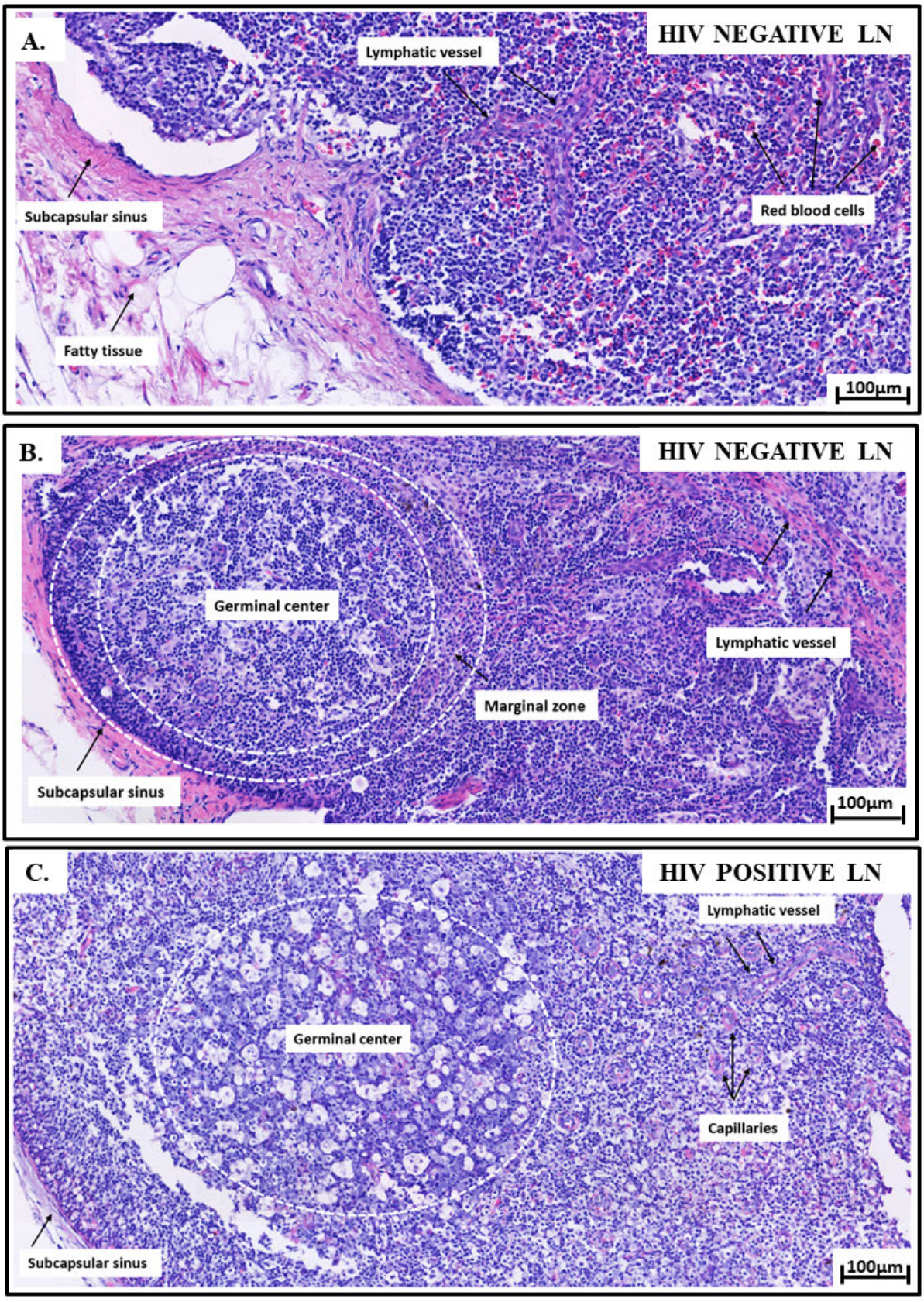

A.
HIV NEGATIVE LN
Lymphatic vessel
Subcapsular sinus
Red blood cells
Fatty tissue
100µm
B.
HIV NEGATIVE LN
Germinal center
Lymphatic vessel
Marginal zone
Subcapsular sinus
100µm
C.
HIV POSITIVE LN
Lymphatic vessel
Germinal center
Capillaries
Subcapsular sinus
100µm

Figure 3.1: Hematoxylin and eosin staining of lymph nodes.

(A) Lymph node biopsy obtained from an HIV negative individual showing red blood cells, subcapsular sinus, fatty tissue, and lymphatic vessels. Lymphatic vessels form part of the lymph node transport system. (B) Lymph node biopsy obtained from an HIV negative individual showing a normal germinal center (GC). (C) Lymph node biopsy obtained from an HIV positive individual showing expansion of GC cells during HIV infection. GCs are major sites of HIV replication in LN tissues. Images were scanned at x40 magnification and scale bars equal 100μm.

3.4.2 $CD68^+$ (M1) macrophages localize inside and outside germinal centers (GCs) in lymph node tissues.

Although H and E staining is a quick and versatile technique used to study tissue morphology, it does not provide phenotypic information needed to identify the different macrophage subsets. To address this, we used immunofluorescence imaging, a technique that simultaneously permits quantitative assessment of cellular phenotype and localization in tissues. We used the macrophage marker CD68 to define pro-inflammatory (M1) macrophages in HIV positive individuals. CD68 has been widely used to identify M1 macrophages in tonsils and brain tissues [22, 23]. BCL-6 was used to identify active GCs (**Figure 3.2A**), the regions of interest were selected (**Figure 3.2B**), and the frequency of $CD68^+$ macrophages were measured using the image analysis software TissueQuest (TissueGnostics) (**Figure 3.2C**). We identified $CD68^+$ macrophages localized inside and outside GCs of HIV positive tissues (**Figure 3.2A; 3.4**) with a higher frequency of these cells localized outside the GCs *(p*=0.04; **Figure 3.2C**). The presence of $CD68^+$ macrophages inside GCs**,** which are primary sites of viral replication and reservoir establishment suggests that this macrophage population may play a role in trapping HIV for prolonged periods or may potentially be a reservoir harboring subset.

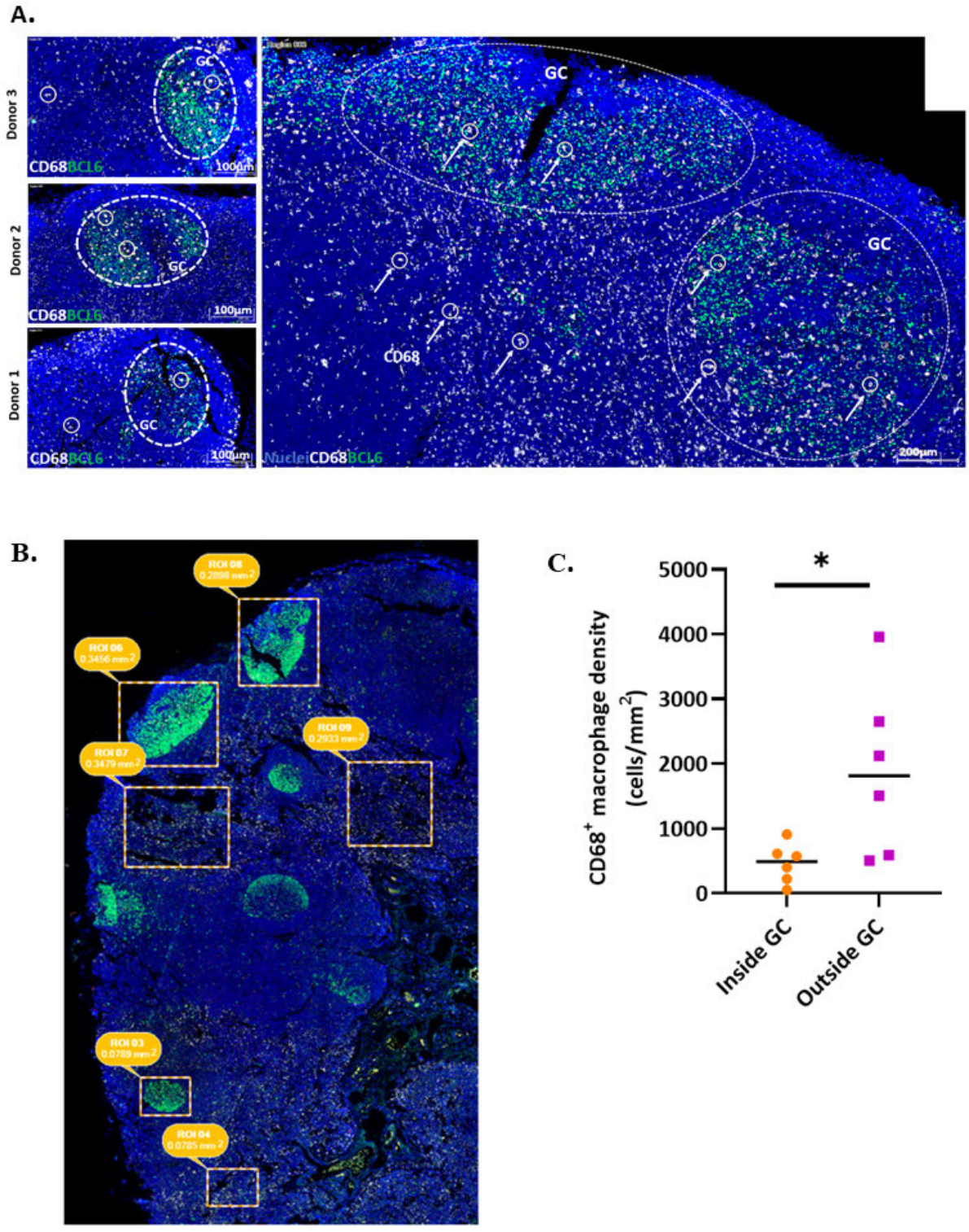


Figure 3.2: Localization and distribution of CD68^{+} macrophages

(A) Images showing staining for CD68 (white), BCL-6 (green), and DAPI (blue) in LN sections obtained from three HIV positive individuals. Images were scanned at x40 magnification and scale bars equal 200µm and 100µm for zoomed in images. The white circle defines the germinal center (GC). (B) Illustration of histology region (ROI) selection. The area of each box is dependent on the area of the

different germinal center regions. (C) Frequency of $CD68^+$ macrophages localized inside and outside the GC. P values were determined using the Mann-Whitney U test.

3.4.3 $CD206^+$ (M2) macrophages localize along lymphatic vessels and outside germinal centers (GCs) in lymph node tissues.

Macrophages are found in virtually every tissue in the body where they take on specific properties that allow them to perform unique functions required in their tissue of residence [24]. In addition, the bulk of LN macrophages are directly exposed to the lymphatic vessel where they constantly flush out antigens. To further characterize lymph node macrophages, we used the macrophage marker CD206 to localize anti-inflammatory (M2) macrophages. Similarly, the regions of interest were selected (**Figure 3.3B**) and the frequency of $CD206^+$ macrophages were measured using the image analysis software TissueQuest (TissueGnostics) (**supplementary Figure 3.6**). In contrast to $CD68^+$ macrophages, $CD206^+$ macrophages were localized along the lymphatic vessels (**Figure 3.3A**; **supplementary Figure 3.2-3.4**) and outside the GCs (**Figure 3.4**). We observed a similar pattern of distribution in HIV negative (HIV neg) and HIV positive (HIV pos) individuals (p=0.033; p<0.000; **Figure 3.3C**). Interestingly, we also identified another macrophage population expressing both CD68 and CD206 localized along the lymphatic vessels and outside of the GCs.

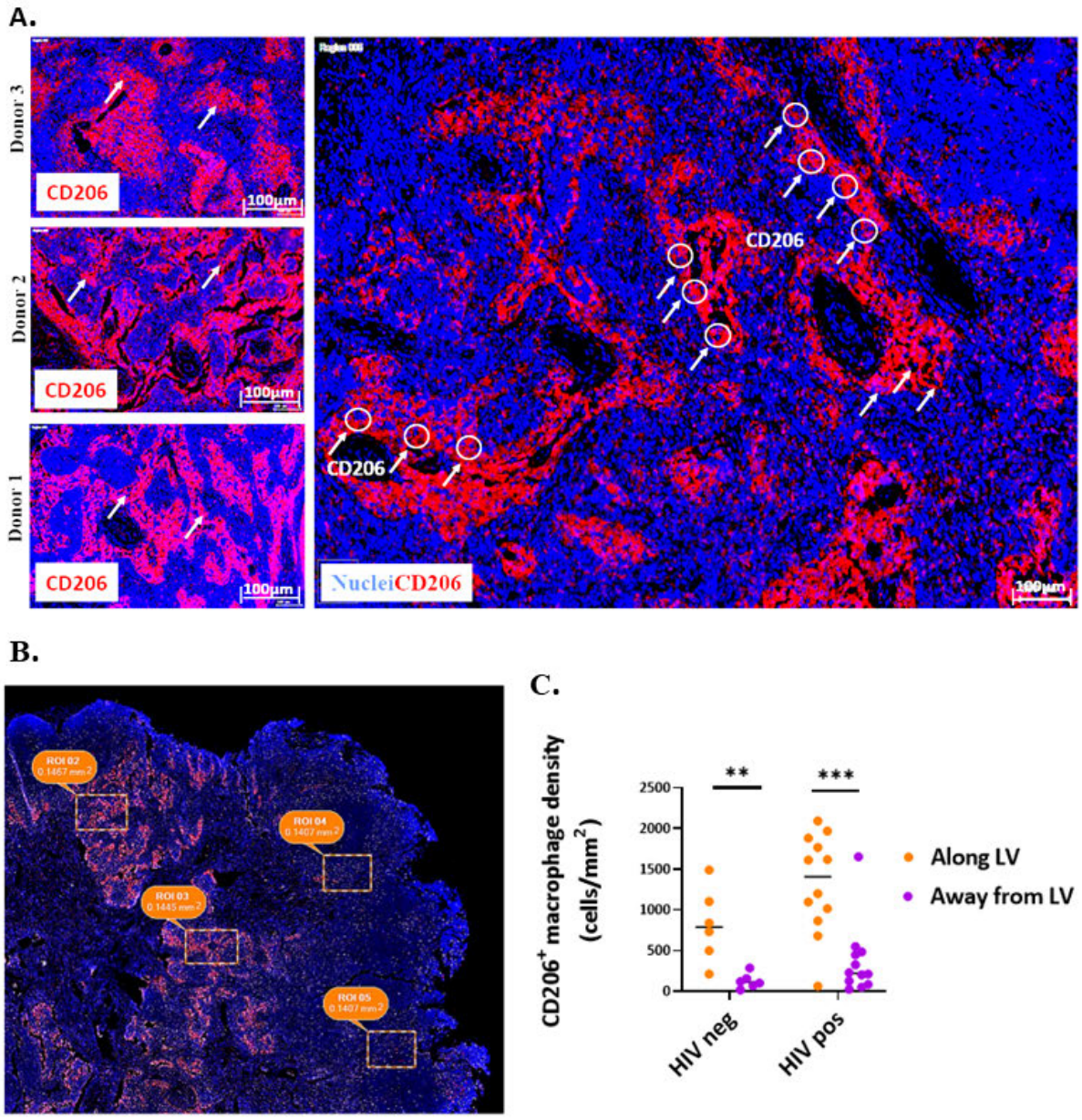


Figure 3.3: Localization and distribution of CD206$^+$ macrophages along lymphatic vessels

(A) Images showing staining for CD206 (red) and DAPI (blue) along lymphatic vessels in LN sections obtained from three HIV positive individuals. Images were scanned at x40 magnification and scale bars equal 100µm. (B) Representative image illustrating region of interest (ROI) selection. (C) Comparison of the frequency of CD206$^+$ macrophages localized along the LV and away from the LV in HIV negative and HIV positive individuals. P values were determined using the Mann-Whitney U test.

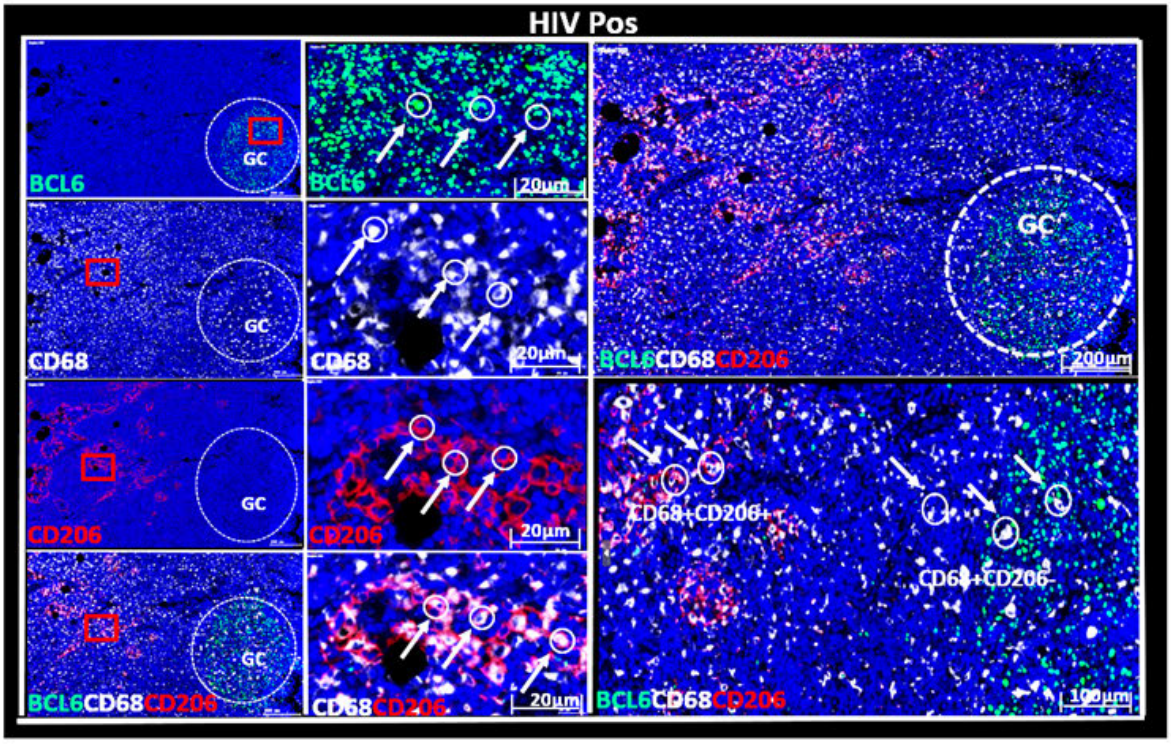


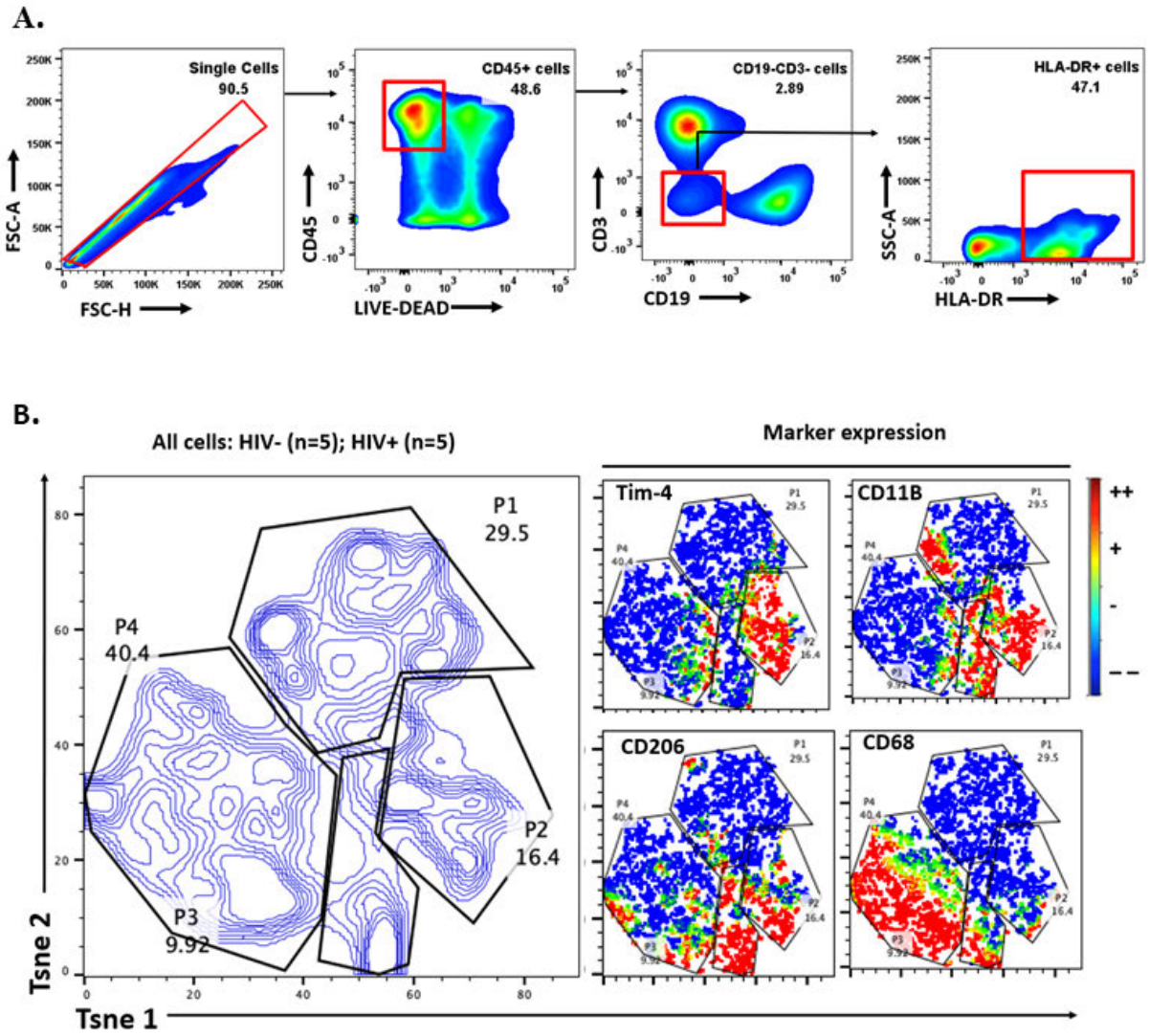
Figure 3.4: Merged image showing the localization of CD68$^+$, CD206$^+$, and CD68$^+$CD206$^+$ macrophages.

(A) Representative image showing staining for CD68 (white), CD206 (red), and DAPI (blue) in a LN section obtained from an HIV positive (LT) individual. CD68$^+$ cells were detected inside and outside the GC while CD206$^+$ and CD68$^+$CD206$^+$ cells were localized outside the GC.

3.4.4: Phenotypic characterization of LN macrophages using flow cytometry.

To validate our imaging (immunofluorescence) results, we used mononuclear cells isolated from LNs to perform ten colour flow cytometry on LN samples obtained from HIV negative and HIV positive individuals. Sequential gating analysis was performed using FlowJo (**Figure 3.5A)** and dimensional reduction and visualization was conducted using the t-SNE algorithm (**Figure 3.5B)**. After the exclusion of doublets and debris, immune cells were identified using CD45 (a pan-hematopoietic marker). Dead cells were also excluded at this step using live/dead staining. CD68$^+$, CD206$^+$, CD11B$^+$, and TIM-4$^+$ macrophage populations were readily identified based on the expression of HLA-DR. Double-positive cells were enumerated based on their expression of the detected macrophage markers. Our analysis revealed four distinct macrophage populations (**Figure 3.5B**). Similarly, we detected CD68$^+$ and CD206$^+$ macrophage subsets in LN. Interestingly, TIM-4 (a marker used to define tissue-resident long-lived macrophages) was detected in one of the macrophage populations P3 (**Figure 3.5C**). The expression of a broad range of macrophage markers in LNs reveals macrophage heterogeneity, a

quality that allows macrophages to polarize into various subsets, some of which play a role in the establishment of the HIV reservoir [15].



C.

	CD68	CD206	CD11B	TIM-4
P1	-	-	+	-
P2	-	+	+	++
P3	+	++	+	-
P4	++	-	-	-

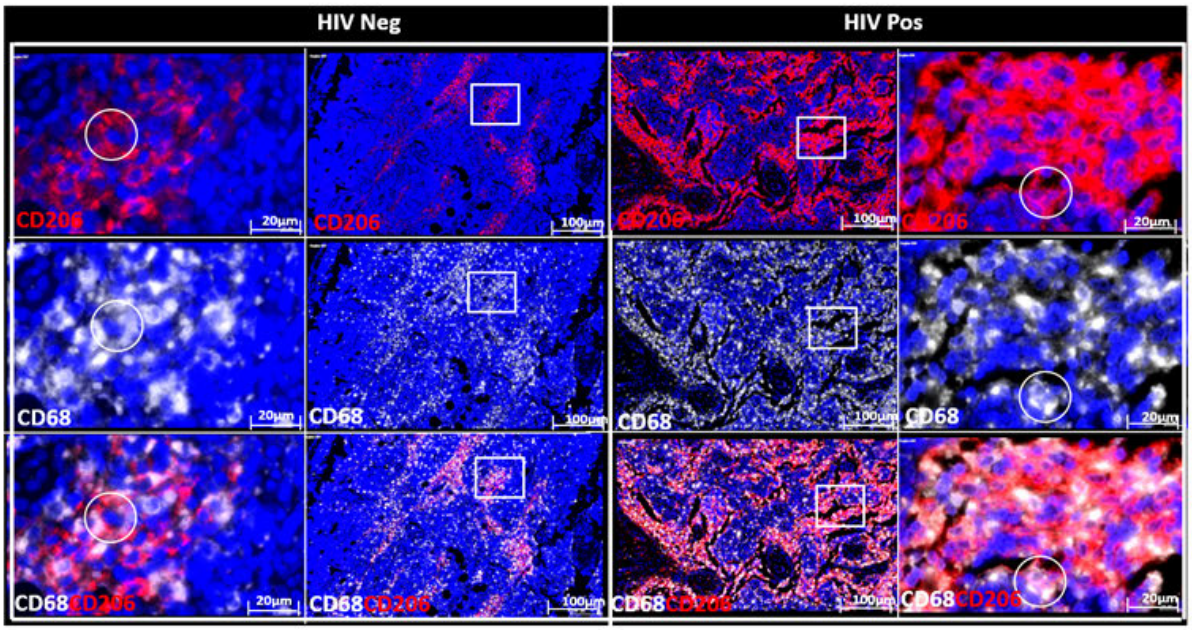
Figure 3.5: Macrophage subsets identified in LN using flow cytometry.

(A) Gating strategy used to identify HLA-DR$^+$ macrophage subsets in LN. Cells were stained as described in the methods section and analysis was done using t-SNE. (B) t-SNE plots showing all macrophage markers classified into four clusters. (C) Phenotypic expression of macrophage markers CD68, CD206, CD11B, and TIM-4. Using flow cytometry, we identified 4 distinct LN macrophage populations including **P1** (CD68$^-$CD206$^-$CD11B$^+$TIM-4$^-$), **P2** (CD68$^-$CD206$^+$CD11B$^+$TIM-4$^-$), **P3** (CD68$^+$CD206^{++}CD11B$^+$TIM-4$^-$), and **P4** (CD68^{++}CD206$^-$CD11B$^-$TIM-4$^-$).

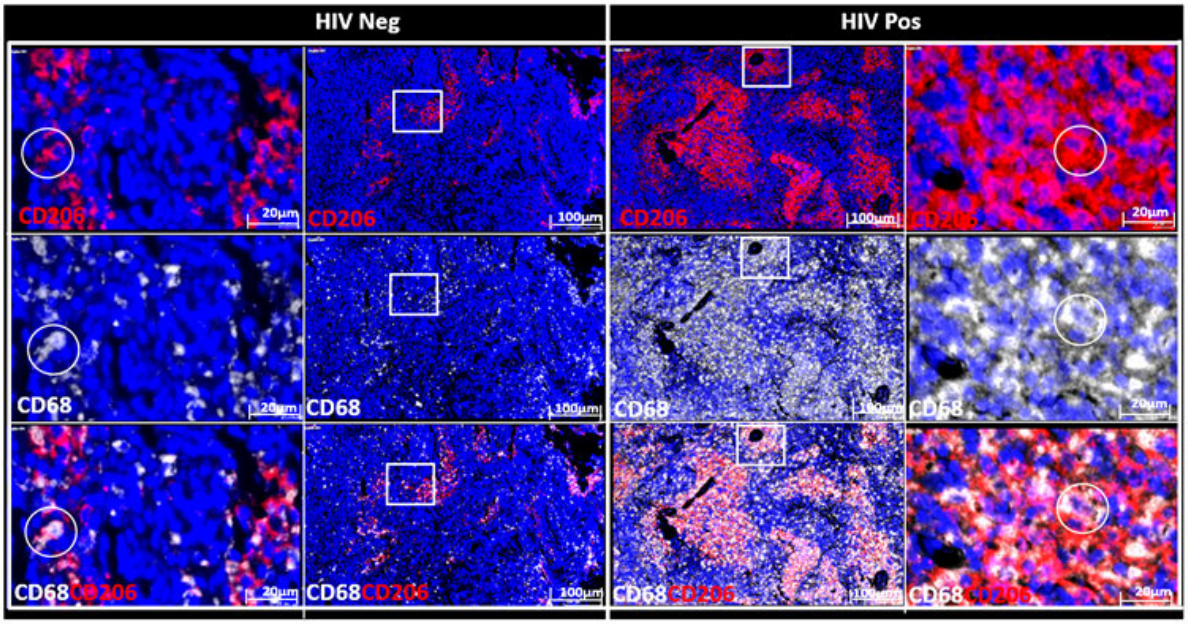
3.4.5: HIV infection alters macrophage frequency.

HIV infection is characterized by persistent inflammation and chronic immune activation [25]. Over the years, the use of ART has helped reduce inflammation and manage disease outcomes. [26]. Unlike CD4$^+$ T cells, the interplay between HIV infection and macrophage function has not been fully established. Previous studies have demonstrated that HIV infection primes macrophage polarization toward a pro-inflammatory (M1) phenotype [16]. This phenotype gradually shifts to an anti-inflammatory (M2) phenotype during chronic HIV infection. In addition, the accumulation of macrophages in organs such as the lungs and liver has been observed during suppressed HIV infection (Nowlin et al., 2015). However, how HIV infection impacts lymph node macrophage phenotype, frequency, and function is not well understood. We, therefore, hypothesized that unsuppressed HIV infection increases lymph node macrophage frequencies that have the potential to form part of the HIV reservoirs. To test this hypothesis, we compared the frequency of CD68$^+$, CD206$^+$, and CD68$^+$CD206$^+$ macrophages using immunofluorescence microscopy (**Figure 3.6A-F**) and flow cytometry (**Figure 3.7A-E**). The frequency of CD68$^+$, CD206$^+$, and CD68$^+$CD206$^+$ macrophages were measured using the image analysis software TissueQuest (TissueGnostics) (**supplementary Figure 3.6**). We observed elevated levels of CD68$^+$, CD206$^+$, and CD68$^+$CD206$^+$ macrophages in HIV-infected individuals compared to uninfected individuals ($p=0.0039$; $p=0.0061$; $p=0.0194$; **Figure 3.6D-F**). We speculate that CD68$^+$ macrophages are recruited to sites of active viral infection and may be continuously recruited to these sites during active viral infection. However, since CD68$^+$ macrophages amplify the pro-inflammatory response, their presence in sites of viral infection increases the likelihood of direct infection. Furthermore, increased HIV infection may prompt the recruitment of anti-inflammatory CD206$^+$ (M2) macrophages to dampen the pro-inflammatory response during HIV infection. It is possible that CD68$^+$ macrophages enter the vessel wall during acute inflammation and recruit CD206$^+$ macrophages during the inflammatory process.

A.


HIV Neg
HIV Pos
CD206
CD68
CD68 CD206
20µm
100µm

B.


HIV Neg
HIV Pos
CD206
CD68
CD68 CD206
20µm
100µm

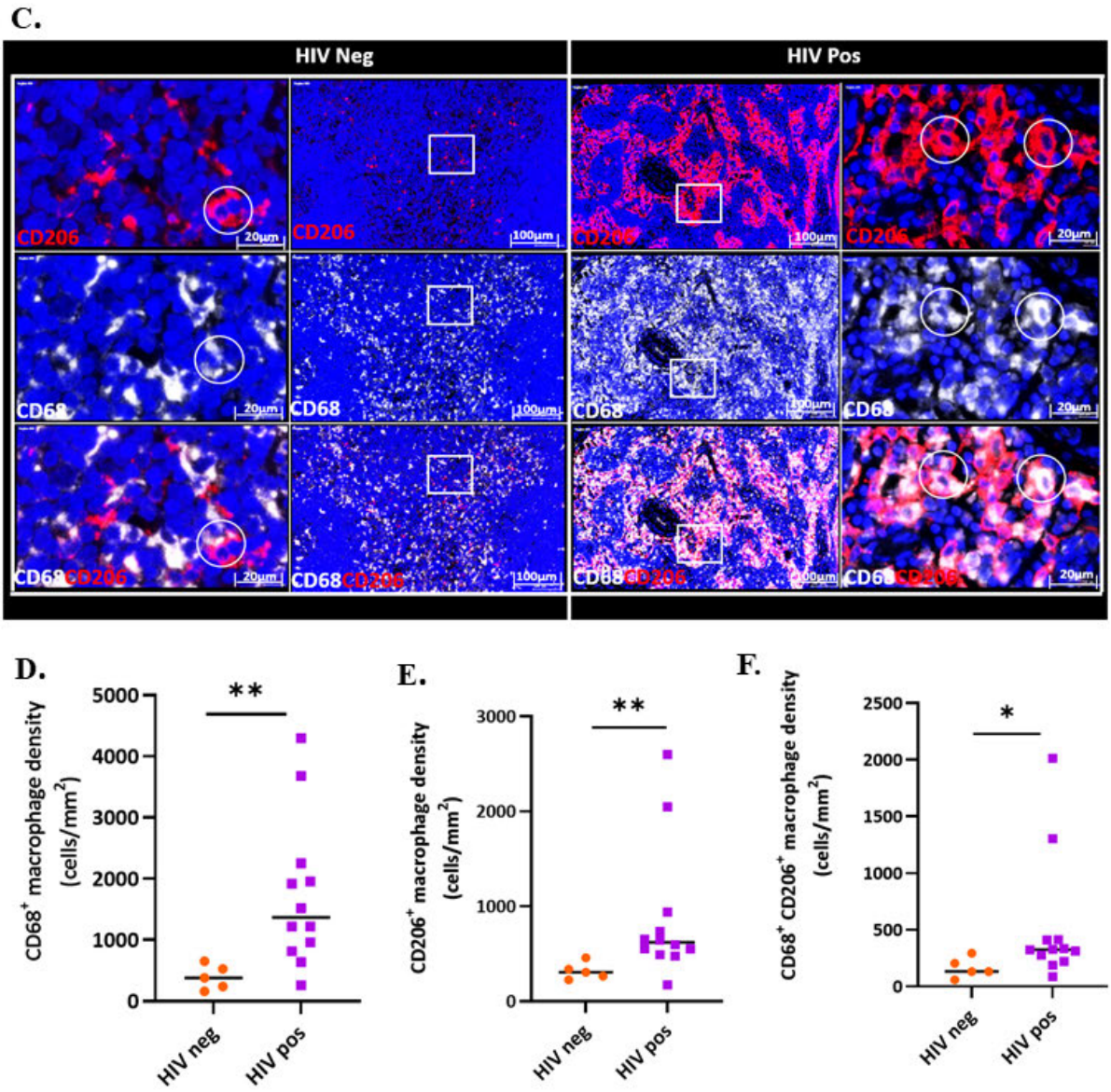
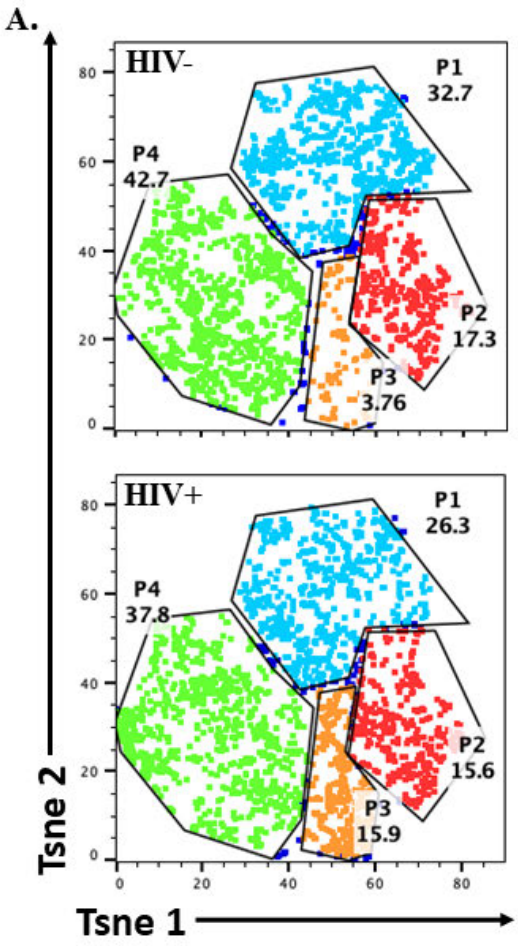


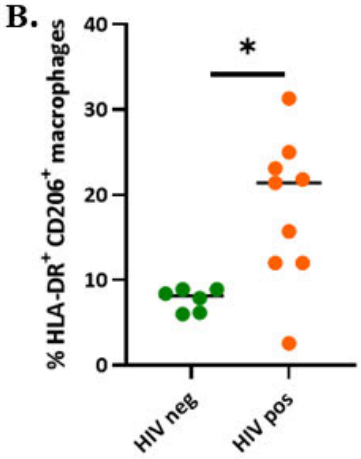
Figure 3.6: Comparison of macrophage frequency in HIV negative and HIV positive individuals

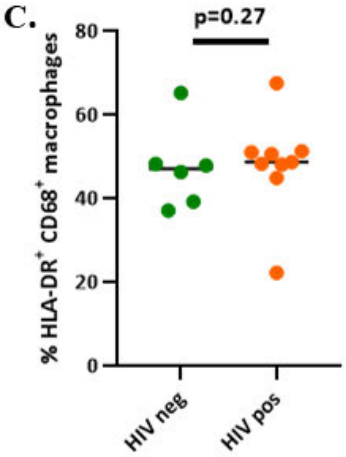
(A-C) Immunofluorescence staining showing CD206 (red), CD68 (white), and DAPI (blue) in LN sections of three HIV negative and three HIV positive (LT) individuals. Images were scanned at x40 magnification and scale bars equal 100µm and 20µm. (D) Frequency of $CD68^+$ macrophages in HIV negative and HIV positive individuals. (E) Frequency of $CD206^+$ macrophages in HIV negative and HIV positive individuals. (F) Frequency of $CD68^+CD206^+$ macrophages in HIV negative and HIV positive individuals. Quantitative image analysis (D-F) was conducted using TissueQuest (TissueGnostics).

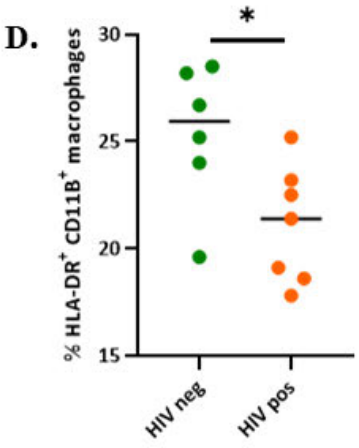
To further investigate how HIV infection impacts lymph node macrophage phenotype and frequency we used flow cytometry to measure lymph node macrophage cell frequency in HIV negative and HIV positive individuals. We used t-SNE (**Figure 3.7A**) and GraphPad Prism (**Figure 3.7B-E**) to visualize and measure macrophage cell frequency. HIV infection resulted in a 2-fold increase in the cell frequency of HLA-DR^{+}CD206^{+} macrophages (p=0.1; **Figure 3.7B**). Surprisingly, this effect was not observed in HLA-DR^{+}CD68^{+} (p=0.27; **Figure 3.7C**). The phosphatidylserine receptor T-cell transmembrane immunoglobulin and mucin domain containing 4 (TIM-4) is expressed on antigen-presenting cells [27]. Recent studies have confirmed that TIM-4 partially mediates exosome-mediated trafficking leading to enhanced HIV entry into human immune cells [28]. In addition, upcoming studies reveal that CD4^{+}TIM4^{+} cells are long-lived and may contribute to HIV persistence [29]. To investigate how HIV infection impacts TIM-4 macrophage frequency, we measured the frequency of HLADR^{+}TIM-4^{+} cells in HIV negative and HIV positive individuals. However, we did not observe any significant difference in the frequency of HLA-DR^{+}TIM-4^{+} cells in HIV negative and HIV positive individuals (p>0.99; **Figure 3.7E**). Furthermore, HIV infection resulted in a 2-fold decrease in cell frequency of HLA-DR^{+}CD11B^{+} cells (p=0.02; **Figure 3.7D**). Integrin αM (CD11B) is primarily expressed in monocytes, macrophages, basophils, eosinophils, and neutrophils. The decrease in the frequency of HLA-DR^{+}CD11B^{+} cells in peripheral blood could be attributed to co-morbidities during HIV infection or side effects associated with ART such as eosinophilia. Taken together, these data reveal macrophage heterogeneity and diversity of lymph node macrophages. We speculate that HIV infection is associated with increased lymph node macrophage frequencies thereby increasing the potential for macrophage infection and reservoir formation. In addition, given that macrophages undergo phenotypic changes during HIV infection, longitudinal studies that track these changes may provide direct evidence about the effect of HIV infection on macrophage evolution. We also recommend the use of more phenotypic markers to better define the different macrophage subsets in human tissues.



	CD68	CD206	CD11B	TIM-4
P1	-	-	+	-
P2	-	+	+	++
P3	+	++	+	-
P4	++	-	-	-







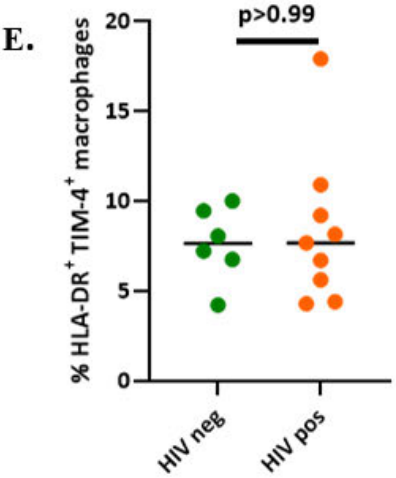


Figure 3.7: Multi-color flow cytometric analysis performed on LN cells from HIV negative and HIV positive (LT) individuals.

LN cells were identified as $CD45^{+}/CD3^{-}CD19^{-}/HLA\text{-}DR^{+}$ by flow cytometry. (A) t-SNE plots showing changes in macrophage frequency during HIV infection. (B) Summary plot showing percentages of $HLA\text{-}DR^{+}CD206^{+}$ macrophages. (C) Summary plot showing percentages of $HLA\text{-}DR^{+}CD68^{+}$ macrophages. (D) Summary plot showing percentages of $HLA\text{-}DR^{+}CD11B^{+}$ macrophages. (E) Summary plot showing percentages of $HLA\text{-}DR^{+}TIM\text{-}4^{+}$ macrophages. Data were analyzed using FlowJo. The Mann-Whitney U test was utilized to compare differences between any two groups. Differences between groups were significant at a P value of <0.05.

3.5 DISCUSSION

Macrophages are highly heterogenous cells that polarize to pro-inflammatory (M1) and anti-inflammatory (M2) subtypes under various environmental stimuli [5, 30]. Although macrophages have been extensively characterized in mice, many questions related to their phenotype and distribution in human tissues remain poorly understood. Moreover, their role in HIV pathogenesis and the interplay between HIV infection and macrophage polarization is still unclear. In this study, we investigated the phenotype, distribution, and function of macrophages in LNs of HIV negative and HIV positive individuals. We identified $CD68^+$ (M1) macrophages inside and outside the GCs. Although the bulk of $CD68^+$ macrophages were localized outside the GC, a small proportion were localized inside the GCs. Whether the GC and non-GC $CD68^+$ macrophages are biologically distinct from each other remains an open question. The HIV macrophage reservoir and HIV infection localize preferentially in distinct regions in lymph node tissues. Germinal centers are one of the established major HIV replication sites [31]. It is highly likely that GC $CD68^+$ macrophages harbor HIV and may potentially lead to the persistence of HIV in lymph node tissues. Additional work is required to determine the presence of HIV Gag p24 and replication competent virus in $CD68^+$ macrophages.

The mannose receptor CD206 has been successfully used in mice and human studies to identify M2 macrophages [32], hence we also used CD206 to localize M2 macrophages in lymph nodes. Contrary to $CD68^+$ macrophages, $CD206^+$ (M2) macrophages predominantly localized along lymphatic vessels and outside the GCs. We also identified another subset expressing both CD68 and CD206 (double positive cells). The biological relevance of this M1/M2 intermediate warrants further investigation. Cancer studies have shown that clonal expansion of stem progenitor cells may trigger the expression of macrophage surface markers leading to metastasis [33]. We therefore speculate that $CD68^+$ macrophages are significantly enhanced during HIV infection due to clonal expansion. Since $CD68^+$ macrophages promote inflammation, it is possible that these cells enter lymphatic vessels during the initial stages of HIV infection. Their migration into lymphatic vessels may lead to the recruitment of anti-inflammatory macrophages such as $CD206^+$ macrophages to dampen the inflammatory response.

The persistent expression of M1 macrophages may lead to tissue damage long-term [34, 35]. When tissues are injured during infection an inflammatory response is induced in response to molecules released by apoptotic cells [36]. These molecular triggers induce a complex inflammatory response characterized by the recruitment and proliferation of cells such as neutrophils and macrophages [36]. When the infection and inflammation is severe enough to affect an organ, macrophages first exhibit the M1 phenotype to release TNF-α, IL-1β, IL-12, and IL-23 against the stimulus [37]. Once the wound healing response is well organized and controlled, the inflammatory response quickly resolves leading

to the restoration of normal tissue architecture. However, if the wound healing response is dysregulated leading to the overexpression of M1 macrophages, normal tissue can be impaired ultimately causing organ failure. Studies aimed at targeting macrophage polarization states with the aim of striking a balance between M1 and M2 marker expression are currently being investigated in various diseases [35, 38]. It is therefore important to determine how macrophage frequency changes during HIV infection. To address this, we measured the frequency of $CD68^+$,$CD206^+$, and $CD68^+CD206^+$ macrophages in HIV negative and HIV positive individuals. We observed a rise in the frequency of $CD68^+$, $CD206^+$, and $CD68^+CD206^+$, macrophages in HIV positive individuals compared to HIV negative counterparts. Consistent with our results, Zanni et al. investigated $CD206^+$ macrophage dynamics during HIV infection in the aorta [39]. They reported a greater abundance of $CD206^+$ macrophages in the aortas of HIV-infected individuals compared to HIV-uninfected individuals. To gain more insight into the changes in macrophage frequency in the peripheral blood, we measured the frequency of HLA-DR^+ macrophages in HIV negative and HIV positive individuals. Flow cytometry results confirmed a significant increase in $CD206^+$ macrophages. However, this effect was not observed in $CD68^+$ macrophages. More sensitive experiments that can timeously track changes in macrophage states are required to inform macrophage dynamics during HIV infection. At this point, it is difficult to correlate the change in macrophage state and macrophage frequency during the various stages of HIV infection.

Integrin αM (CD11B) is an integrin molecule expressed on myeloid cells such as macrophages, dendritic cells, eosinophils, basophils, and neutrophils [40]. Although the expression of CD11B is not specific to one cell type, a series of humanised mice studies have used CD11B expression to identify M2 macrophages [41]. We also attempted to assess the changes in $CD11B^+$ cell frequency using flow cytometry and we observed a lower frequency of $CD11B^+$ cells in HIV positive individuals compared to HIV positive individuals. More work using techniques such as immunofluorescence microscopy and the quantitative viral outgrowth assay (qVOA) is needed to better understand and validate the role of $CD11B^+$ macrophages in HIV infection. However, the scarcity of suitable human LN biopsies remains a significant challenge.

In summary, our study suggests that lymph node tissues contain diverse tissue macrophage phenotypes highlighting macrophage heterogeneity and plasticity. As previously shown by other researchers, we confirm that HIV infection indeed alters macrophage frequency. The increased frequency of macrophages during HIV has been associated with impairment of macrophage function which in turn negatively affects adaptive immune responses and ultimately leads to HIV persistence in LNs. However, more studies are required to support this theory. Further elucidation of M1 and M2 macrophages using more markers will improve our understanding of their role in HIV pathogenesis in lymph nodes.

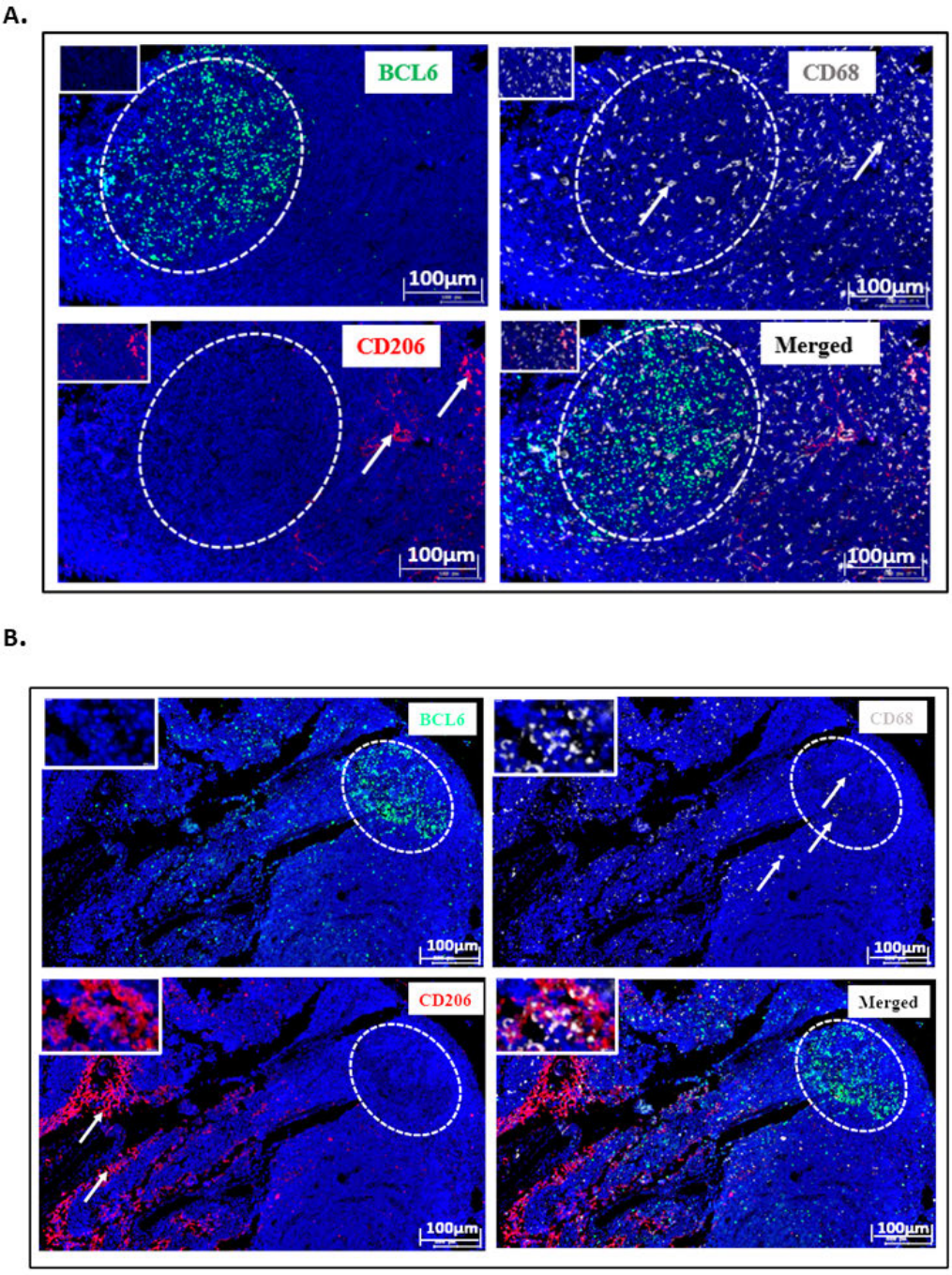
3.6 STUDY STRENGTHS AND LIMITATIONS

This study provides more insight into macrophage distribution and function in HIV negative and HIV positive LNs. Additionally, we assessed the expression of various macrophage markers in lymph nodes and provided a guide to macrophage subsets found in lymph nodes. However, the difficulties associated with obtaining lymph node biopsies due to the subsequent lockdown implemented during the COVID-19 pandemic hampered our efforts to use a larger sample size. Lastly, the number of male participants in this study was greatly outnumbered by female participants. Therefore, the study lacked gender diversity.

3.7 FUTURE RECOMMENDATIONS

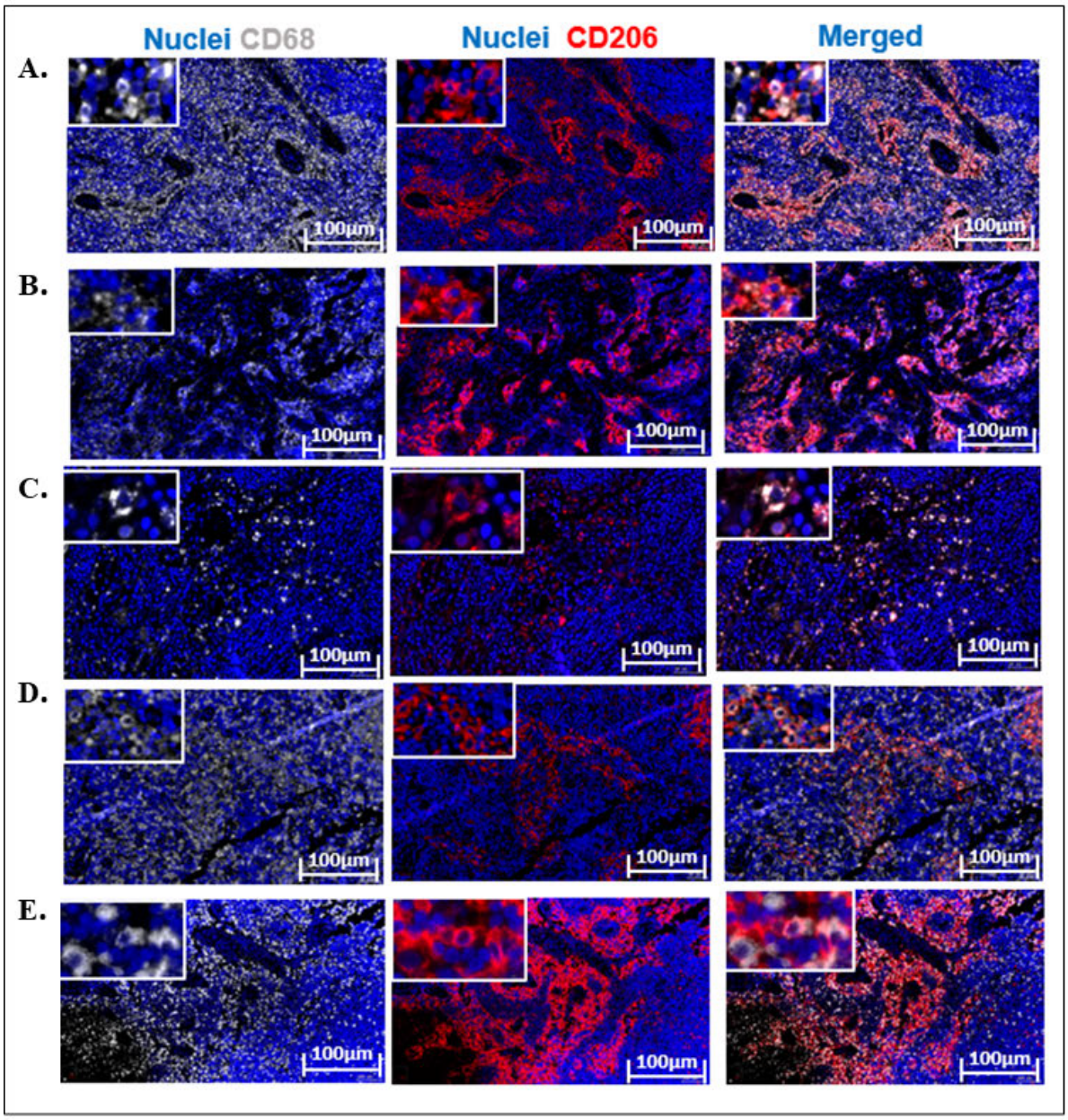
We recommend using a larger cohort including a balanced number of female and male participants. Despite the use of a combination of assays to characterize and localize LN macrophages, we highlight the need to perform longitudinal experiments to detect the shift in macrophage states and to further understand the interplay between HIV and macrophage polarization.

3.8 SUPPLEMENTARY DATA



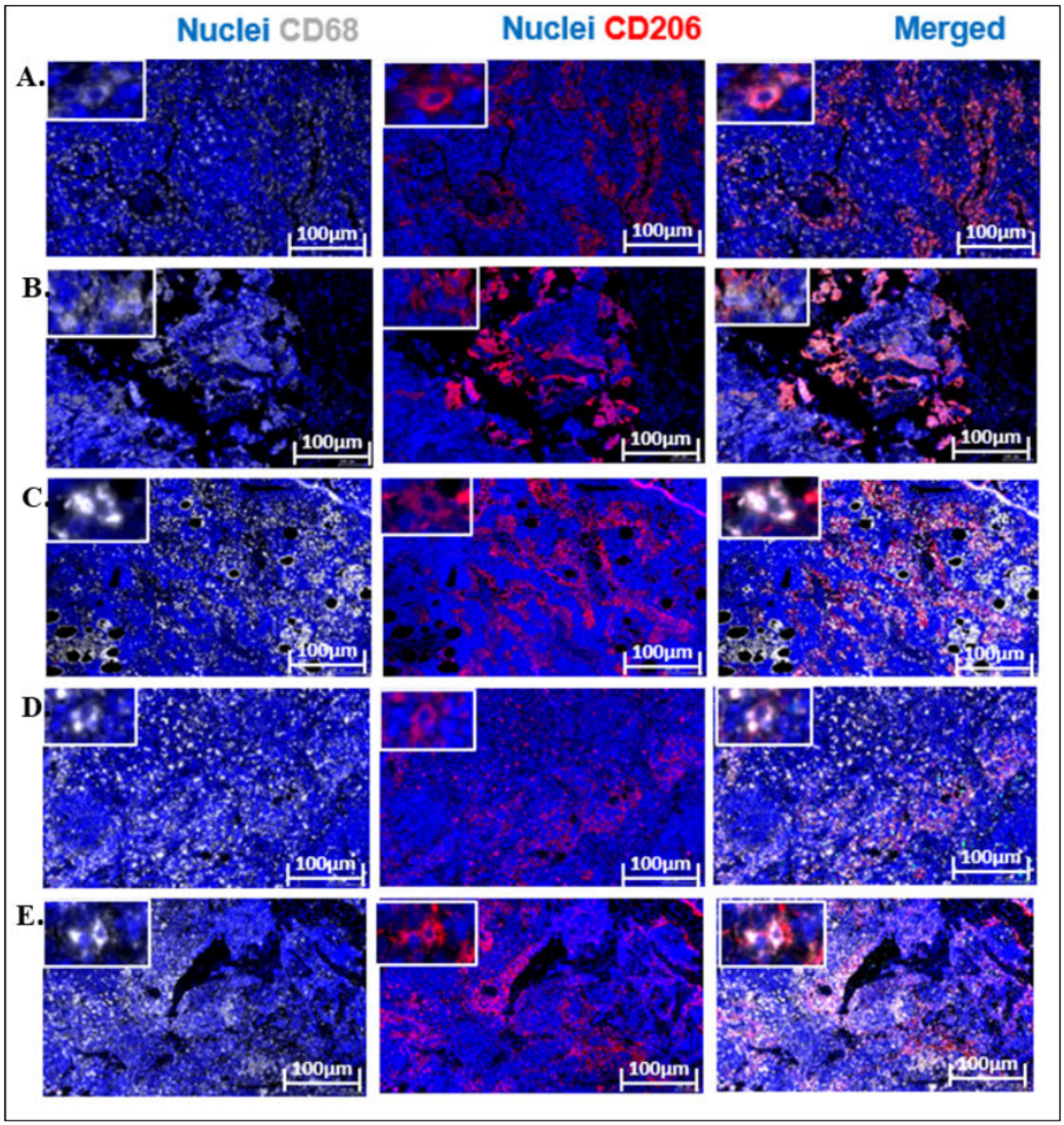
Supplementary Figure 3.1: Images showing the localization of CD68$^+$ macrophages inside and outside the GC and CD206$^+$ macrophages outside the GC along lymphatic vessels.

(A-B) Representative image showing staining for CD68 (white), CD206 (red), BCL6 (green), and DAPI (blue) in LN tissue sections from two HIV positive (LT) individuals. CD68$^+$ cells were detected inside and outside the GC while CD206$^+$ and CD68$^+$CD206$^+$ cells were localized outside the GC.



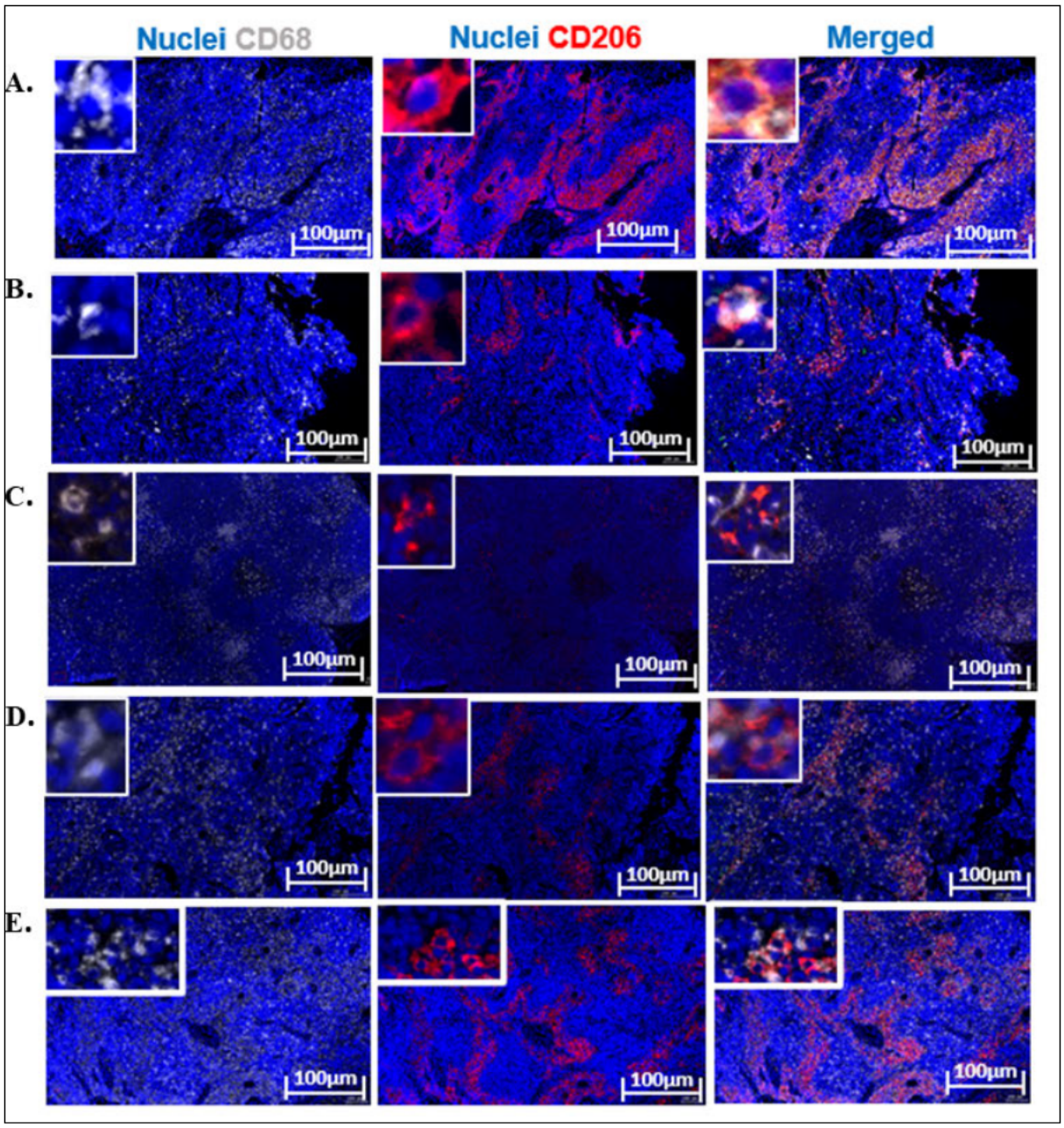
Supplementary Figure 3.2: Immunofluorescence staining showing $CD68^{+}$ and $CD206^{+}$ macrophage frequency in HIV positive tissue sections.

(A-E) Immunofluorescence staining showing CD206 (red), CD68 (white), and DAPI (blue) in LN tissue sections obtained from five HIV positive (LT) individuals. Images were scanned at x40 magnification and scale bars equal 100µm.



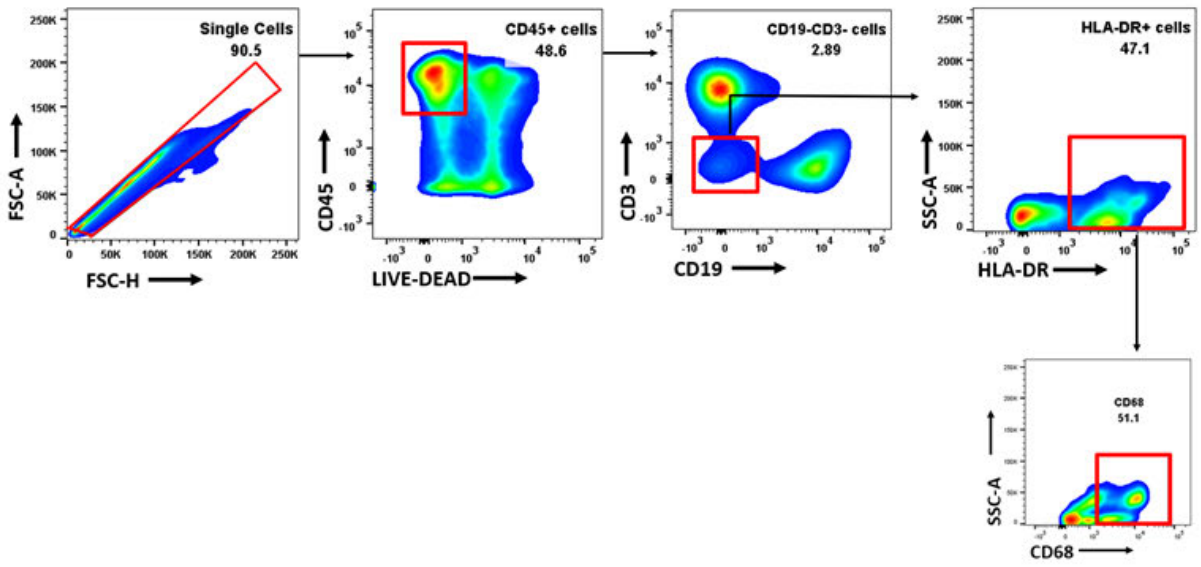
Supplementary Figure 3.3: Immunofluorescence staining showing $CD68^{+}$ and $CD206^{+}$ macrophage frequency in HIV positive tissue sections.

(A-E) Immunofluorescence staining showing CD206 (red), CD68 (white), and DAPI (blue) in LN tissue sections obtained from five HIV positive (LT) individuals. Images were scanned at x40 magnification and scale bars equal 100µm.



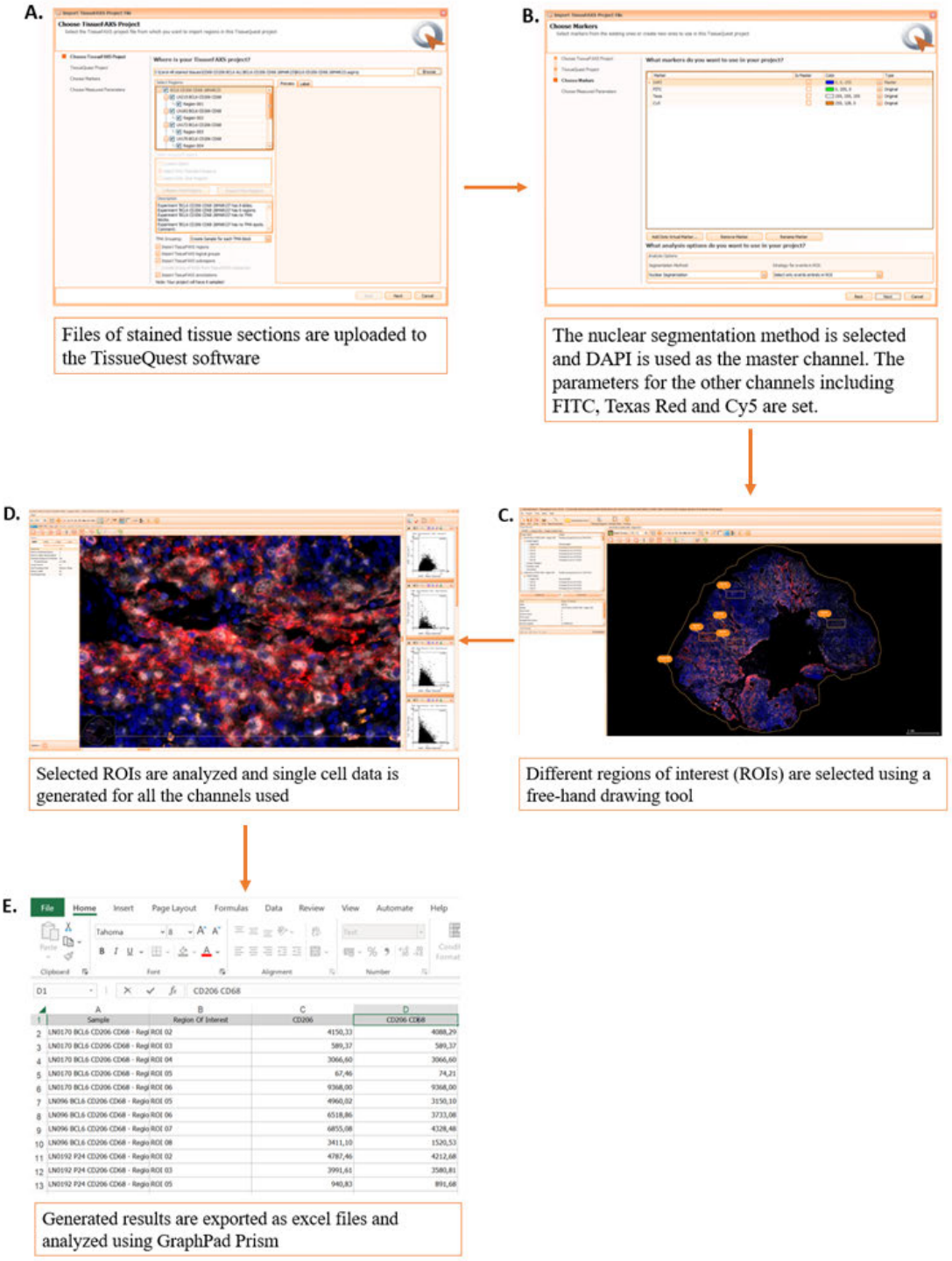
Supplementary Figure 3.4: Immunofluorescence staining showing $CD68^+$ and $CD206^+$ macrophage frequency in HIV negative tissue sections.

(A-E) Immunofluorescence staining showing CD206 (red), CD68 (white), and DAPI (blue) in LN tissue sections obtained from five HIV negative individuals. Images were scanned at x40 magnification and scale bars equal 100µm.



Supplementary Figure 3.5: Flow cytometry gating strategy.

LN cells were identified as $CD45^{+}/CD3^{-}CD19^{-}/HLA\text{-}DR^{+}$ by flow cytometry. Populations were further gated for SSC-A vs the different macrophage markers.



Supplementary Figure 3.6: Image cytometry pipeline used for immunofluorescence microscopy image analysis using TissueQuest software (TissueGnostics, Vienna, Austria).

(A) Files used for analysis are uploaded in TissueQuest for analysis. (B) The nuclear segmentation method is selected. (C) The regions of interest are specified, and appropriate parameters are defined using 4' 6-diamidino-2-phenylindole (DAPI) as the master channel. (D) Single cell information for Texas Red, fluorescein Isothiocyanate (FITC), cyanine 5 (Cy5) are displayed in scatter plots. (E) Summary results are exported in excel graphs.

Supplementary Table 3.1: Detailed patient characteristics (Immunofluorescence microscopy)

Study group	*PID*	*Type of node*	*Age*	*Gender*	*HIV status*	*Treatment status*	*Absolute CD4 count (cells/ul)*	*Plasma viral load (copies/ml)*
HIV negative	103	Inguinal	26	F	Neg	N/A	1143	N/A
HIV negative	106	Inguinal	29	F	Neg	N/A	928	N/A
HIV negative	111	Inguinal	29	F	Neg	N/A	667	N/A
HIV negative	116	Inguinal	27	F	Neg	N/A	874	N/A
HIV negative	168	Inguinal	25	F	Neg	N/A	-	N/A
Early treated	63	Inguinal	32	F	Pos	N/A	898	<20
Early treated	88	Inguinal	28	F	Pos	Yes	942	2055
Late treated	96	Inguinal	27	M	Pos	Yes	856	<20
Late treated	133	Inguinal	27	M	Pos	Yes	834	<20
Late treated	138	Inguinal	25	M	Pos	Yes	599	<20
Late treated	165	Inguinal	27	F	Pos	Yes	733	140
Late treated	170	Inguinal	26	F	Pos	Yes	472	180
Late treated	184	Inguinal	33	F	Pos	Yes	555	6000
Late treated	192	Inguinal	27	F	Pos	Yes	847	<20
Late treated	95025	Mesenteric	38	F	Pos	Yes	741	20
Late treated	95044	Mesenteric	61	F	Pos	Yes	375	20
Late treated	95069	Mesenteric	44	M	Pos	Yes	-	20

** Abbreviations: Patient identifier (PID), male (M), female (M), positive (Pos), negative (Neg)*

Supplementary Table 3.2: Detailed patient characteristics (Flow cytometry)

Study group	*PID*	*Type of node*	*Age*	*Gender*	*HIV status*	*Treatment status*	*Absolute CD4 count (cells/ul)*	*Plasma viral load (copies/ml)*
HIV negative	106	Inguinal	29	F	Neg	N/A	928	N/A
HIV negative	110	Inguinal	27	F	Neg	N/A	659	N/A
HIV negative	111	Inguinal	29	F	Neg	N/A	667	N/A
HIV negative	113	Inguinal	29	F	Neg	N/A	704	N/A
HIV negative	167	Inguinal	27	F	Neg	N/A	-	N/A
HIV negative	168	Inguinal	25	F	Neg	N/A	-	N/A
Late treated	126	Inguinal	28	F	Pos	Yes	406	<20
Late treated	133	Inguinal	27	M	Pos	Yes	834	7730
Late treated	171	Inguinal	25	F	Pos	Yes	1369	59
Late treated	172	Inguinal	27	F	Pos	Yes	624	370
Late treated	186	Inguinal	26	F	Pos	Yes	554	3000
Late treated	190	Inguinal	24	F	Pos	Yes	225	<20
Late treated	202	Inguinal	24	F	Pos	Yes	883	<20
Late treated	203	Inguinal	29	M	Pos	Yes	-	<20

** Abbreviations: Patient identifier (PID), male (M), female (M), positive (Pos), negative (Neg)*

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CHAPTER 4

Aim: Investigating the role of lymph node (LN) macrophages in HIV persistence.

Chapter 4 Overview

In Chapter 3, we phenotypically characterized and determined the spatial location of LN macrophages. We identified $CD68^+$, $CD206^+$, and $CD68^+CD206^+$ macrophage populations in HIV negative and HIV positive LN tissues. We also identified $CD4^+TIM\text{-}4^+$ macrophages in HIV negative and HIV positive lymph node mononuclear cells. The distribution and location of these macrophage populations differed in LN tissues. $CD68^+$ macrophages were localized inside and outside the germinal centers (GCs). Unlike $CD68^+$ macrophages, $CD206^+$ macrophages were localized outside the GCs, along lymphatic vessels. We also observed a significant increase in the frequency of $CD68^+$, $CD206^+$, and $CD68^+CD206^+$ macrophages during HIV infection. The localization of $CD68^+$ macrophages in GCs (which are major sites of HIV replication) makes them a target for persistent HIV replication in virally suppressed individuals on combined antiretroviral therapy (cART). Human and murine studies have identified transcriptionally active HIV in tissue macrophages. In these studies, they identified $CD68^+$ macrophages as a potential HIV reservoir. In chapter 4 of this thesis, we expanded our study to investigate the role of $CD68^+$ macrophages in HIV persistence using immunofluorescence microscopy and flow cytometry. We show that $CD68^+$ LN macrophages contain HIV Gag p24 protein. Moreover, we report the presence of HIV-1 RNA in $CD68^+$ macrophages in LN tissues of HIV positive individuals on cART.

CHAPTER 4: INVESTIGATING THE ROLE OF LYMPH NODE TISSUE MACROPHAGES IN HIV PERSISTENCE

Caroline Chasara[1], Trevor Khaba[1], Bongiwe Mahlobo[1], Thandekile Ngubane[3], Ismail Jajbhay[3], Johan Pansegrouw[3] , Zaza M. Ndhlovu[1,2,4]

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

[2]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

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

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

Address correspondence to Zaza Ndhlovu (ZNDHLOVU@mgh.harvard.edu)

Keywords: HIV, macrophages, CD68, lymph nodes

This manuscript is in advanced stages of preparation for submission to a peer reviewed journal.

4.1 ABSTRACT

A major obstacle to the complete eradication of HIV is the establishment of cellular HIV reservoirs that persist in secondary lymphoid organs for prolonged periods. It is well established that HIV persists in $CD4^+$ T cells, specifically in lymph node (LN) tissues. However, recent evidence suggests that myeloid cells such as macrophages are also infected by HIV, making them a potential source of viral rebound. A critical understanding of HIV infection in tissue macrophages is important to aid the development of effective cure strategies. Here, we investigated HIV infection in lymph node biopsies obtained from 16 HIV positive (5 early treated and 11 late treated) individuals. Using multicolor immunofluorescence microscopy, we detected the presence of HIV Gag p24 antigen in $CD68^+$ macrophages. We also detected HIV-1 RNA in HIV positive late treated individuals on cART. Notably, flow cytometry on 14 lymph node mononuclear cell samples revealed the presence of long-lived $CD4^+$TIM-4^+ macrophages. These cells have been shown to have a low turnover suggesting their potential role in long term HIV persistence. Overall, these results reveal HIV persistence in LN macrophages despite the initiation of ART in hyperacute infection and highlight the importance of understanding the macrophage reservoir to ultimately inform the specific targeting of HIV-infected macrophages and the development of an effective HIV cure.

4.2 INTRODUCTION

Combined antiretroviral therapy (cART) has successfully improved the life span and mortality of people living with HIV. However, many hurdles still hamper the complete eradication of HIV [1, 2]. These include the establishment and maintenance of HIV reservoirs in secondary lymphoid organs leading to persistent viremia [3, 4]. Lymph nodes have been established as one of the major anatomical reservoirs, where HIV persists in $CD4^+$ T cells [5, 6]. In addition to $CD4^+$ T cells (which are major cellular reservoirs) are macrophages, which can be found in all lymphoid and non-lymphoid tissues [7]. Some studies have demonstrated that macrophages may potentially harbor HIV in mucosal and brain tissues [2]. Moreover, a recent study reported the presence of replication competent HIV in penile tissues obtained from HIV-infected individuals on cART [8] suggesting that urethral macrophages are a principal HIV reservoir. Some macrophage subsets such as Kupffer cells in the liver and alveolar macrophages in the lungs are known to have longer lifespans. They also resist the cytopathic effects of viral infection and cytotoxic T cell killing [9, 10].

According to traditional nomenclature, macrophages can be distinguished into M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages [11-13]. Cumulative tissue and peripheral blood studies have detected varying frequencies of M1 and M2 macrophages. Of particular interest amongst the two subsets are $CD68^+$ (M1) macrophages. Highly phagocytosing $CD68^+$ macrophages are found in the germinal centers of active LNs [14] [15] making them a potential site for HIV persistence. On the other hand, macrophages are phagocytic cells and can orchestrate the elimination of HIV-infected $CD4^+$ T cells causing them to get infected in the process. Although the infection of macrophages has been demonstrated in various tissues, their role as a replication-competent reservoir remains controversial.

The inability of the field to accurately quantify changes in these tissue-resident reservoirs represents a major obstacle to the clinical development of approaches to shrink the reservoir. To date, several studies have been conducted to measure the size of the myeloid reservoir using a broad range of assays [16]. These include the quantitative viral outgrowth assay (QVOA), intact proviral DNA assay (IPDA), RNAscope, and fluorescence in situ hybridization (FISH) [17]. The success of these assays is based on their ability to estimate the size of the HIV reservoir before and after ART. However, there is no consensus on the actual reservoir size as each assay presents various limitations. More sensitive assays are urgently needed to ensure accurate measurement of the HIV reservoir in myeloid cells.

In this study, we report that $CD68^+$ LN tissue macrophages constitute an important cellular reservoir in lymph node tissues. We detected the presence of HIV Gag p24 antigen in HIV-infected individuals undergoing cART using immunofluorescence microscopy. Progress on elucidating the role of macrophages in human health and diseases has been hampered by the lack of suitable human tissue

samples for research. Consequently, we used RNAscope from 6 HIV-infected late treated tissues to confirm the presence of HIV RNA in $CD68^+$ macrophages. Importantly, our study identified $CD4^+$TIM-4^+ LN tissue macrophages, a macrophage subset, previously reported to have a slow turnover in blood monocytes. Taken together, these results suggest that macrophages may be a potential cellular reservoir in HIV-positive LN tissues despite effective ART.

4.3 MATERIALS AND METHODS

4.3.1 Study approval

All study participants provided written informed consent before inclusion in the study. This study is a follow up study. Ethical approval for the primary study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (protocol number BF298/14) and the Institutional Review Board of Massachusetts General Hospital (protocol number 2015-P001018).

4.3.2 Study cohort and sample collection

A total of 30 LN samples (6 LN tissue biopsies obtained from early treated (ET) and late treated (LT) individuals (**Table 4.1; supplementary Table 4.1**)) and 14 mononuclear cells isolated from LNs (**Table 4.1; supplementary Table 4.2**)) were used in this study. Early treatment was defined as treatment initiated during acute HIV infection, at a median of one day after virus detection. Late treatment was defined as treatment initiated after the acute phase of HIV infection (3 months post infection). Study participants were drawn from the HIV Pathogenesis Programme (HPP) lymph node study (LNS) cohort in Durban, South Africa. Recruitment into the HPP LNS cohort was from the FRESH cohort described before [18]. Inguinal LNs were surgically excised at Prince Mshiyeni Hospital in Umlazi, and 120 ml paired PB was also obtained from each participant. Viral load measurements were performed by HIV-1 RNA testing using the NucliSens EasyQ v2.0 assay (BioMérieux Clinical Diagnostics, Marcy-l'Étoile, France), through a certified commercial laboratory. $CD4^+$ T cell counts were enumerated by Tru-Count technology and analyzed on a FACSCalibur flow cytometer (Becton Dickinson (BD) New Jersey, USA). Sample processing and laboratory studies were performed at the Africa Health Research Institute in Durban, South Africa.

Table 4.1: Donor characteristics stratified by HIV and treatment status.

	All (n=30)	HIV negative (n=6)	HIV positive (ET) (n=5)	HIV positive (LT) (n=19)
Age years (median)	27 (23-33)	28 (25-29)	27 (25-31)	27 (23-33)
Male	3	0	0	3
Female	27	6	5	16
HIV viral load copies/ml	2055 (59-11000)	N/A	492 (59-614)	3000 (59-11000)
CD4 count cells/µl (median)	7275 (225-1369)	685.5 (659-928)	1168 (624-1800)	589.5 (225-1369)

** Abbreviations: NA, not applicable, early treated (ET), late treated (LT)*

4.3.3 Lymph node and blood sample processing

Excised LNs were divided into two sections. One section was fixed in 10% formal-saline (Sigma-Aldrich, St. Louis, Missouri, USA) for immunofluorescence microscopy studies while the second section was macerated to release LNMCs as described by *Schacker et al* [19]. Thereafter, cells were passed through a mesh screen and harvested by centrifugation (625 × g, 6 min, room temperature (RT). Peripheral blood mononuclear cells (PBMCs) were isolated from patients' blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in liquid nitrogen.

4.3.4 Immunofluorescence (IF) microscopy

IF microscopy staining was performed on 4 µM sections of formalin-fixed paraffin-embedded (FFPE) LNs using the Opal 4-color fluorescent immunohistochemistry (IHC) kit (PerkinElmer, Waltham, MA, USA). Sections were deparaffinized using xylene (Honeywell research chemicals) and rehydrated before antigen retrieval using AR6 buffer (20 min, 100 °C, (PerkinElmer)). Next, two blocking steps (2 × 10 min, RT) were performed with the Dako peroxidase-blocking reagent (Agilent Technologies, Glostrup, Denmark) and Bloxall block (Vector Laboratories, Burlingame, CA, USA). The slides were washed with 0.05% Tween 20 in Tris-buffered saline (TBS-T) for 5 min, sequentially probed with the primary antibody (30 min, RT), and Opal polymer HRP (20 min, RT (PerkinElmer)) and detected using the Opal polymer 520 (10 min, RT). This protocol was repeated for the second and third antibodies with Opal polymers 570 and 690 respectively, followed by counterstaining with spectral DAPI (PerkinElmer) to make a total of four different fluorochromes. Primary antibodies used in these combinations include anti-human BCL-6 ((clone PG-B6p) Dako/Agilent Technologies), CD68 ((clone KP1) Dako/Agilent Technologies), and p24 ((clone Kal-1) Dako/Agilent Technologies). Cell Sciences).

After staining, slides were mounted with Dako fluorescence mounting medium (Agilent Technologies) and imaged with the Axio Observer, ×20 objective lenses, a Hamamatsu C13440-20C camera and TissueFAXS imaging software (TissueGnostics, Vienna, Austria). Quantitative image analysis was conducted using TissueQuest (TissueGnostics).

4.3.5 Quantitative image analysis

Quantitative image analysis of HIV Gag p24 protein in whole tissue section scans was conducted with TissueQuest software (TissueGnostics). Total area measurements and nuclear segmentation analysis were performed on each whole tissue scan. The numerical data generated from the analysis are displayed in scattergrams. Statistical analysis and graphical presentation were performed using GraphPad Prism version 9.0 software (GraphPad Software Inc., La Jolla, CA, USA). The 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. Differences between groups were significant at a P value of <0.05.

4.3.6 RNAscope in situ hybridization (ISH)

RNAscope ISH was conducted using the RNAscope 2.5 HD assay kit (Advanced Cell Diagnostics (ACD), Newark, CA, USA, Cat No: 322300) and the RNAscope multiplex fluorescent kit v2.0 (ACD, Cat No: 323100) as per manufacturer's instructions. Briefly, pre-treated samples were hybridized with the clade C HIV-1 gag-pol probe (Cat No: 317691) at 40 °C or 16 h. Next, the samples were incubated with signal amplification probes and horseradish peroxidase conjugated secondary antibodies. The signal was detected with either diaminobenzidine for the RNAscope 2.5 HD assay (ACD) or with Opal fluorophores (PerkinElmer) for the multiplex fluorescent assay. Slides were imaged with Axio Observer and TissueFAXS imaging software (TissueGnostics).

4.3.7 Quantitative image analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 9.0 software (GraphPad Software Inc., La Jolla, CA, USA). The 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. Differences between groups were significant at a P value of <0.05.

4.3.8 Flow cytometry analysis

Lymph node mononuclear cells (LMCs) were characterized using multi-parameter flow cytometry analysis. Briefly, cells were stained with LIVE/DEAD Fixable Blue dead cell stain kit (Thermo Fisher Scientific, Waltham, MA, USA), CD3-BV711 (BD Biosciences, San Jose, CA), CD4-BV650 (BD Biosciences), CD19-PE-Cy5 (BioLegend, San Diego, CA, USA), HLA-DR-APC-CY7 (BioLegend), CD45-BV786 (BD Biosciences), CD11B-BV421 (BioLegend), and TIM-4-APC (BioLegend). For intracellular staining, cells were washed with PBS and incubated for 20 min with cytofix/cytoperm (BD Biosciences) according to manufacturer's instructions. After fixation, cells were washed with perm wash buffer (BD Biosciences) and incubated for 20 min at RT with perm wash buffer containing CD68 (BioLegend) and CD206 (BD Biosciences) antibodies. Fluorescence minus one (FMO) or unstained cells were used as a control. Stained cells were acquired using an LSRFortessa (BD Biosciences) with FACSDiva™ software. Data were analyzed using FlowJo version 10.6.0 (FlowJo, LLC, Ashland, Oregon). LN cells were identified as $CD45^{+}/CD3^{-}CD19^{-}/HLA\text{-}DR^{+}$ by flow cytometry. Further gating was used to identify specific macrophage phenotypes (**Supplementary Figure 4.7**).

4.4 RESULTS

Long-term persistence of HIV in lymph node macrophages

Cumulative studies have shown that $CD4^+$ T cells are primary targets of HIV infection. Although HIV persistence in $CD4^+$ T cell reservoirs has been well studied, the eradication of latent HIV reservoirs remains a major challenge. Increasing evidence now suggests that macrophages contribute to the HIV reservoir as they are readily infected by HIV and can persist for prolonged periods in human tissues. However, the specific role of lymph node tissue macrophages in HIV persistence remains unclear. To address this, we hypothesized that ongoing HIV replication occurs in macrophages within lymph nodes of cART-suppressed individuals leading to persistent HIV infection. We first used immunofluorescence microscopy to analyze tissues obtained from n=16 HIV-positive (5 ET and 11 LT) individuals. The clinical characteristics and demographic data of the participants included are outlined in **supplementary Table 4.1**.

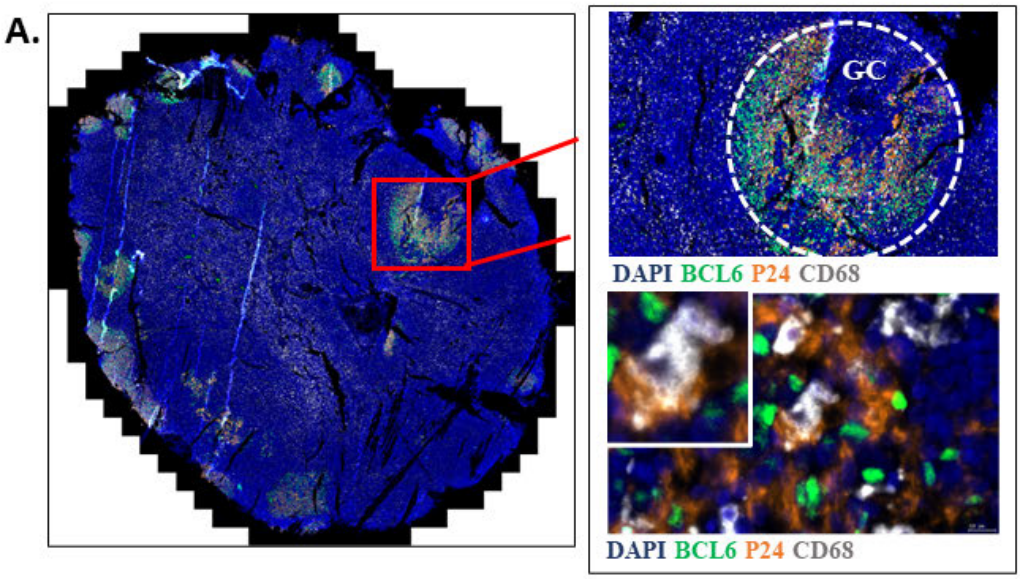
4.4.1 Long-term persistence of HIV Gag p24 antigen in $CD68^+$ macrophages of HIV positive early treated (ET) individuals

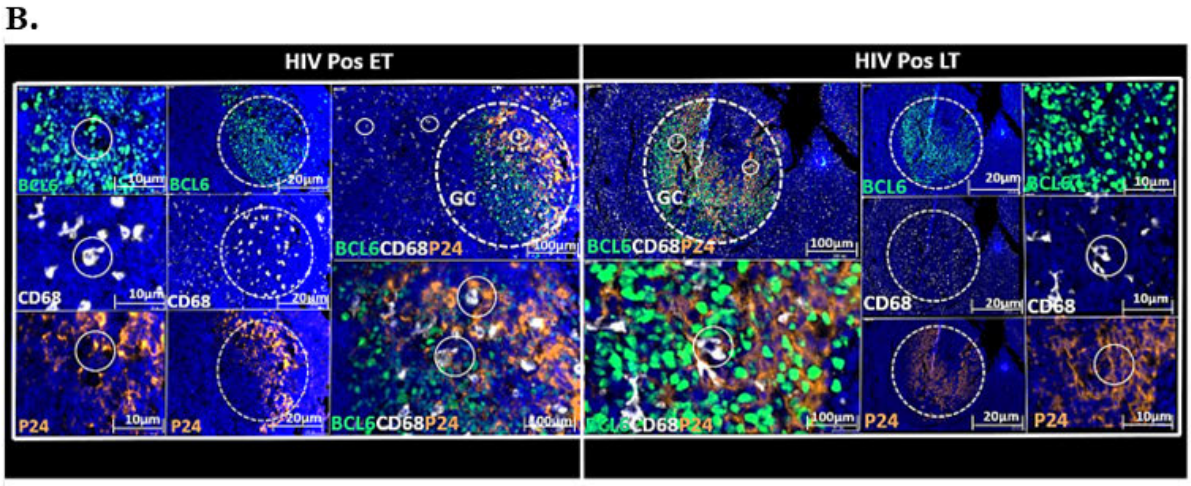
The early initiation of ART has been shown to limit the establishment of the HIV reservoir. However, early ART initiation does not prevent the establishment of the reservoir. Non-human pirate studies have shown that a replication-competent HIV reservoir can be established in the first few days of SIV infection [20]. To test this hypothesis in human LN tissues, we investigated HIV persistence in HIV-positive ET individuals (**Figure 4.1B**). We used multicolor immunofluorescence (IF) staining to measure HIV Gag p24 antigen in formalin-fixed paraffin-embedded (FFPE) LN sections. The transcription factor BCL-6 was used to identify active germinal centers (GCs) and the CD68 antibody was used to identify macrophages (**Figure 4.1B).** HIV Gag p24 detection was conducted on a total of 5 LNs from ET individuals (median viral load 492 copies/ml). All five stained tissues had detectable HIV Gag p24 in at least one GC. $CD68^+$ macrophages harboring HIV Gag p24 were detected in all the HIV positive ET individuals (**Figure 4.1B; supplementary Figure 4.1A-D**).

4.4.2 Long-term persistence of HIV Gag p24 antigen in $CD68^+$ macrophages of HIV positive late treated (LT) individuals

To gain further understanding into the macrophage reservoir, we investigated HIV persistence in HIV positive (LT) individuals. Similarly, we used multicolor immunofluorescence (IF) staining to measure HIV Gag p24 antigen in FFPE LN sections. The transcription factor BCL-6 was used to identify active GCs and the CD68 antibody was used to identify macrophages (**Figure 4.1A**). HIV Gag p24 detection

was conducted on a total of 7 LNs from LT individuals (median viral load 3000 copies/ml). All seven stained tissues had detectable HIV Gag p24 in at least one GC. $CD68^+$ macrophages harboring HIV Gag p24 were detected in all the HIV positive LT individuals (**Figure 4.1B; supplementary Figure 4.2A-F**). To compare the frequency of HIV Gag p24 staining cells between the study groups (ET vs LT), we conducted quantitative image analysis on all the tissues with detectable HIV Gag p24 using the image analysis software TissueQuest (TissueGnostics). Our analysis revealed a higher frequency of HIV Gag p24 staining macrophages in LT individuals compared to ET individuals ($p<0.02$; **Figure 4.1C**). There was a strong association between the frequency of GC $CD68^+p24^+$ macrophages and plasma viral load in LT individuals (**Figure 4.1D**). However, there was no correlation observed in ET individuals (**Figure 4.1E**). Additionally, there was no correlation between the frequency of GC $CD68^+p24^+$ and CD4 count in ET and LT individuals (**Figure 4.1E**).Taken together, these data demonstrate that early ART initiation limits the magnitude of HIV Gag p24 antigen in LN macrophages hence limiting the establishment of the macrophage reservoir. However, HIV Gag p24 persists for prolonged periods in LN macrophages despite early treatment initiation.


A.
GC
DAPI BCL6 P24 CD68
DAPI BCL6 P24 CD68


B.
HIV Pos ET
HIV Pos LT
BCL6
CD68
P24
GC
BCL6CD68P24

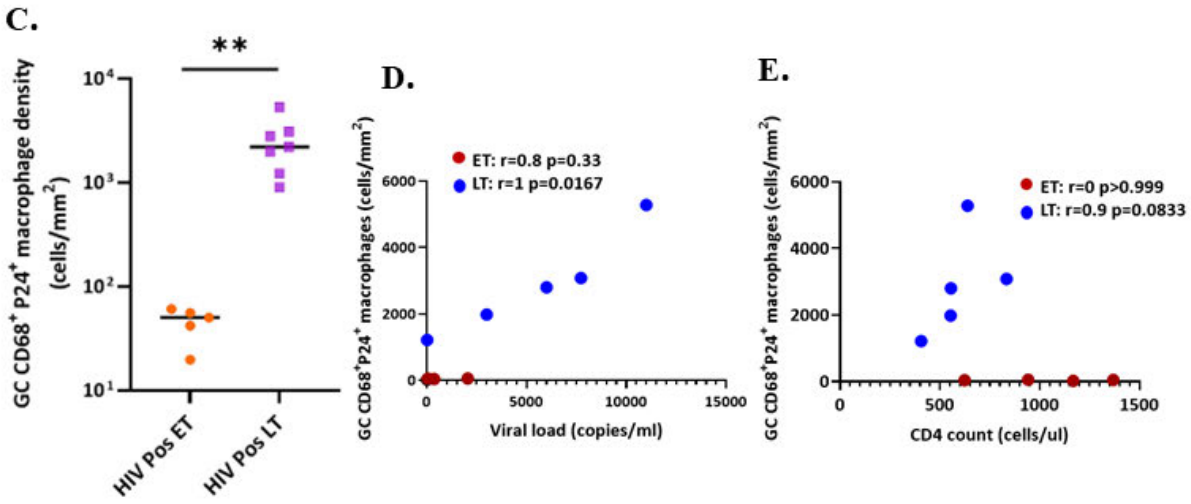

C.
**
GC CD68+ P24+ macrophage density (cells/mm2)
HIV Pos ET
HIV Pos LT
D.
ET: r=0.8 p=0.33
LT: r=1 p=0.0167
GC CD68+P24+ macrophages (cells/mm2)
Viral load (copies/ml)
E.
ET: r=0 p>0.999
LT: r=0.9 p=0.0833
GC CD68+P24+ macrophages (cells/mm2)
CD4 count (cells/ul)

Figure 4.1: HIV Gap p24 co-localizes with germinal center CD68$^+$ macrophages in lymph node (LN) tissue sections.

(A) Representative image of a whole LN section showing Gag p24 (yellow), BCL-6 (green), and CD68 (white). (B) Representative images of HIV positive ET and LT individuals. White circles depict germinal centers. Costaining of Gag p24 (yellow) and CD68 (white) was observed in both ET and LT individuals. (C) Frequency of Gag CD68$^+$p24$^+$ macrophages in ET and LT individuals. Quantitative image analysis was conducted using TissueQuest (TissueGnostics). (D) Correlation between GC C68$^+$ p24$^+$ cells and plasma viral load. (E) Correlation between GC C68$^+$ p24$^+$ cells and CD4 count.

4.4.3 CD68$^+$ LN macrophages harbor HIV-1 RNA

To date, the relevance of the macrophage HIV reservoir in humans has been subject to debate [21]. However, various techniques such as qPCR and quantitative viral outgrowth assays (qVOA) have been routinely used to investigate HIV infection in humans. More recently, a combination of techniques to comprehensively map the macrophage HIV reservoir have been developed. For instance, RNAscope, an in-situ hybridization technique has been used to detect viral RNA transcription. Coupled with flow cytometry, RNAscope has been previously used to detect HIV-1 RNA in macrophages expressing CD68 in human brain tissue during suppressive cART [22].

After detecting the presence of HIV Gag p24 protein in LN macrophages, we sought to determine if viral RNA transcription (which is paramount to facilitate the production of infectious virions) was occurring in these cells. We investigated the presence of HIV-1 RNA by in situ hybridization (ISH) in LN tissues of n=6 different HIV-1/cART individuals, using an antisense HIV-1 RNA probe followed by immunohistochemistry using the CD68 antibody to identify macrophages. Although the presence of HIV Gag p24 antigen was detected in both ET and LT LN tissues, we were unable to measure HIV-1 RNA in ET tissues because of the lack of tissue samples. Consequently, RNA scope experiments were conducted on LN tissues obtained from HIV positive LT individuals. CD68$^+$ macrophages harboring HIV-1 RNA were detected in 5 of the 6 HIV-positive LT tissues (**Figure 4.2A-B; supplementary Figure 4.3A**).

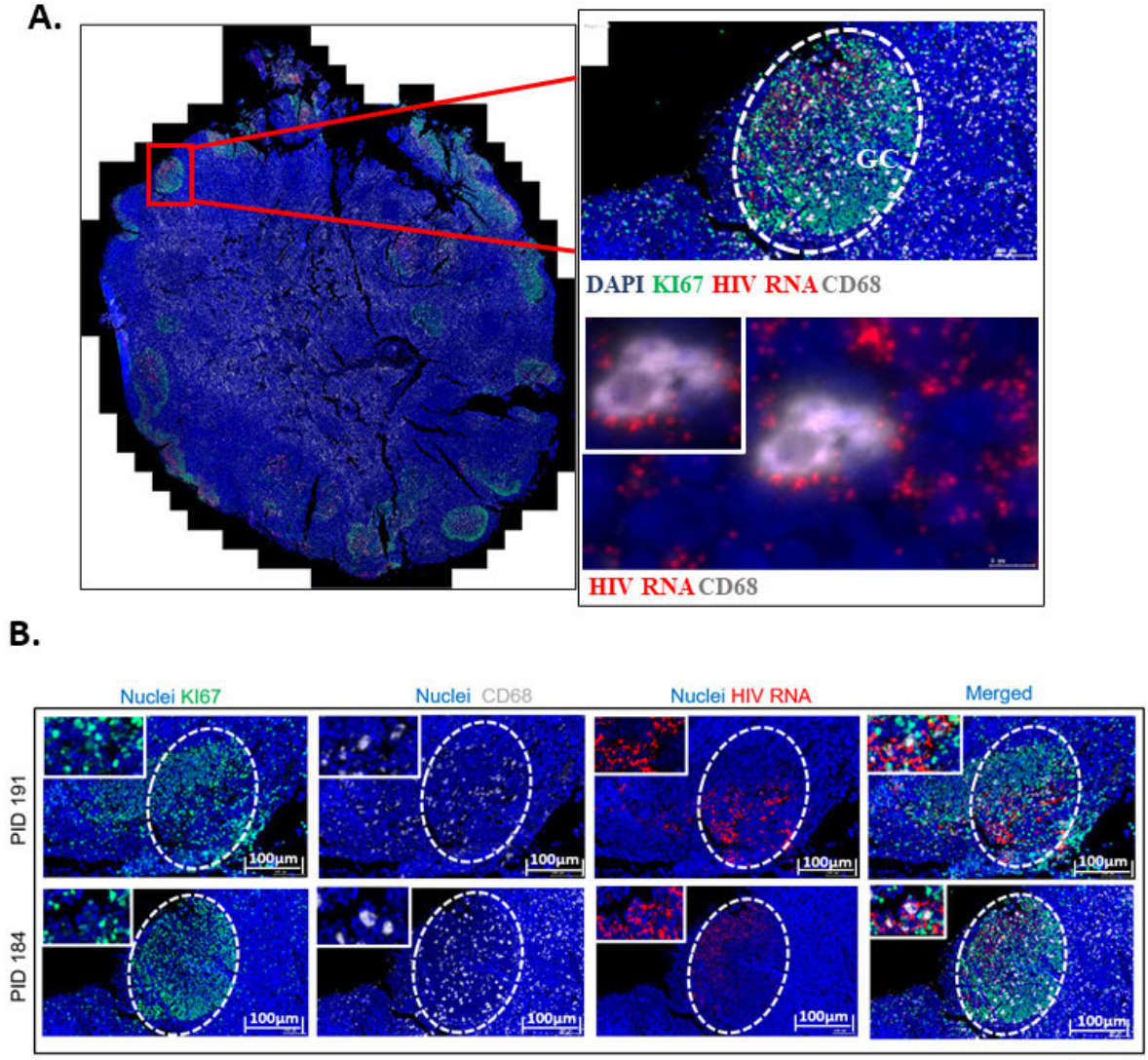


Figure 4.2: HIV-1 RNA persistence in lymph nodes of LT individuals.

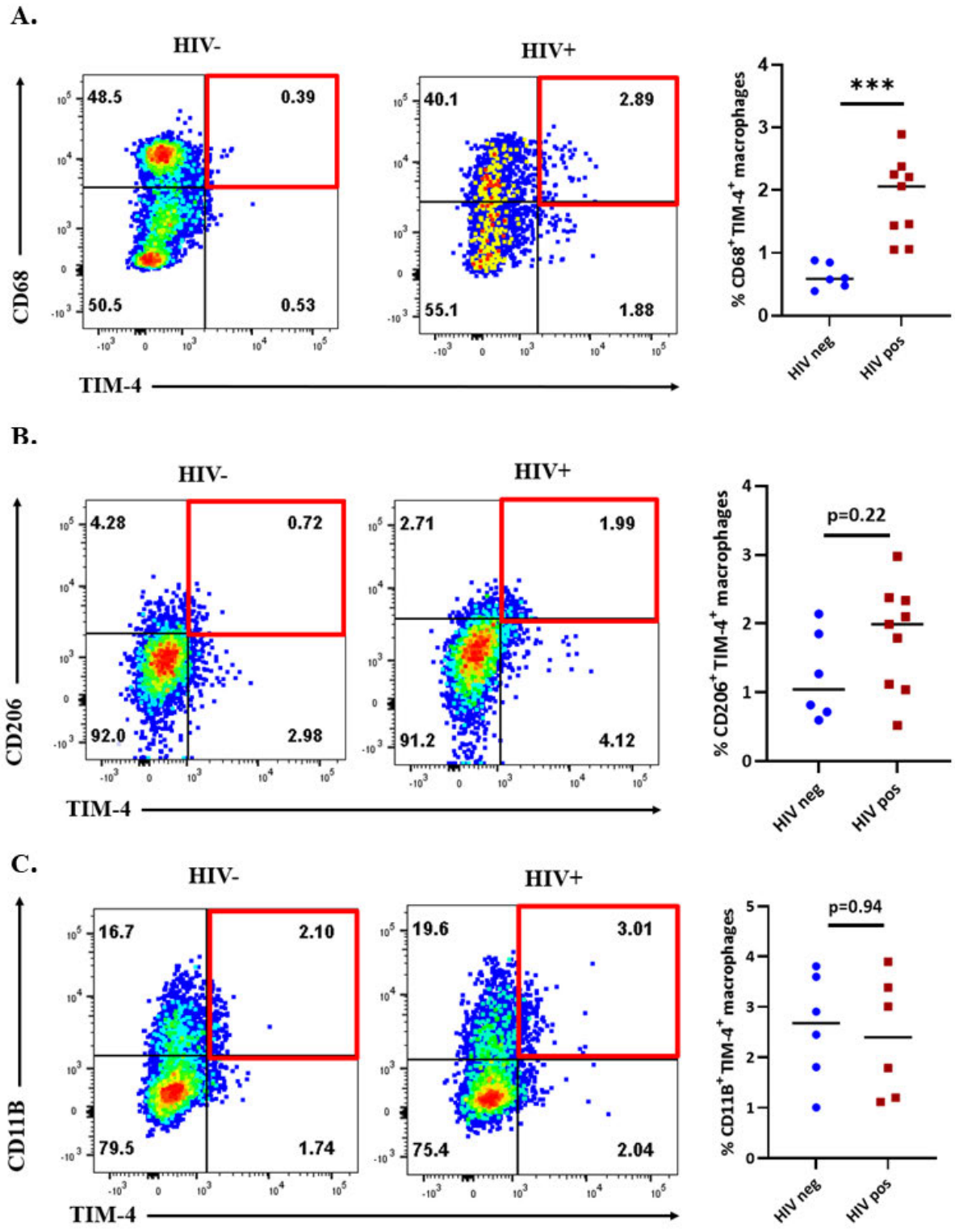
(A) Representative image of a whole LN section showing HIV-1 RNA (red), KI67 (green), and CD68 (white). (B) HIV-1 RNA detection in lymph nodes (LN) of 2 HIV positive LT treated individuals. RNAscope hybridization for HIV gag-pol RNA was detected using immunofluorescence microscopy. Single RNA transcripts are shown as punctate dots.

4.4.4: Multi-color flow cytometric analysis performed on LN cells from HIV negative and HIV positive LT individuals.

Humanized mice studies have previously used flow cytometry and PCR to study the macrophage reservoir. These studies used vigorous ART regimens to treat humanized mice but despite the use of these regimens, HIV-1 DNA was detected in spleen and bone marrow macrophages [23]. More recently, flow cytometry was used in combination with other assays to identify long-lived tissue-resident

macrophages based on the expression of TIM-4 and CD4 [24]. This study identified locally maintained $CD4^{+}TIM\text{-}4^{+}$ gut macrophages, which persist for prolonged periods and may consequently lead to the establishment of the HIV reservoir in the gut tissues.

Based on the established information on tissue-resident macrophages, we explored the presence of $CD4^{+}TIM\text{-}4^{+}$ cells and the co-expression of TIM-4 with the macrophage markers CD68, CD206, and CD11B. We used HIV negative (n=6) and HIV positive LT (n=8) LN mononuclear cells (LMCs) to analyze the frequency of the subsets of interest. The clinical characteristics and demographic data of the participants included are outlined in **supplementary Table 4.2**. Most of the LMC samples used were matched with PIDs used for immunofluorescence staining. In the case where the matched LMCs could not be obtained, different samples were selected based on viral load and suppression status. We used FlowJo to analyze the changes in the macrophage cell frequency during HIV infection and graphical presentations were performed using GraphPad Prism (**Figure 4.3A-D**). HIV infection resulted in a ~2-fold increase in cell frequency of $CD68^{+}TIM\text{-}4^{+}$ macrophages ($p=0.0004$; **Figure 4.3A**). However, this effect was not observed in $CD206^{+}TIM\text{-}4^{+}$ ($p=0.22$; **Figure 4.3B**) and $CD11B^{+}TIM\text{-}4^{+}$ ($p=0.94$; **Figure 4.3C**). To further investigate the presence of long-lived $TIM\text{-}4^{+}$ macrophages, we measured the frequency of $CD4^{+}TIM\text{-}4^{+}$ macrophages. Flow cytometry analysis identified a significantly higher frequency of $CD4^{+}TIM\text{-}4^{+}$ cells in HIV positive individuals compared to HIV negative counterparts ($p<0.03$; **Figure 4.3D**). Taken together these results confirm the existence of a small subset of $CD68^{+}TIM4^{+}$ macrophages that have the potential to persist for a long time in tissues and therefore contribute to LN tissue reservoirs.


A.
HIV-
HIV+
CD68
TIM-4
48.5
0.39
50.5
0.53
40.1
2.89
55.1
1.88
% CD68+TIM-4+ macrophages
***
HIV neg
HIV pos
B.
HIV-
HIV+
CD206
TIM-4
4.28
0.72
92.0
2.98
2.71
1.99
91.2
4.12
% CD206+TIM-4+ macrophages
p=0.22
HIV neg
HIV pos
C.
HIV-
HIV+
CD11B
TIM-4
16.7
2.10
79.5
1.74
19.6
3.01
75.4
2.04
% CD11B+TIM-4+ macrophages
p=0.94
HIV neg
HIV pos

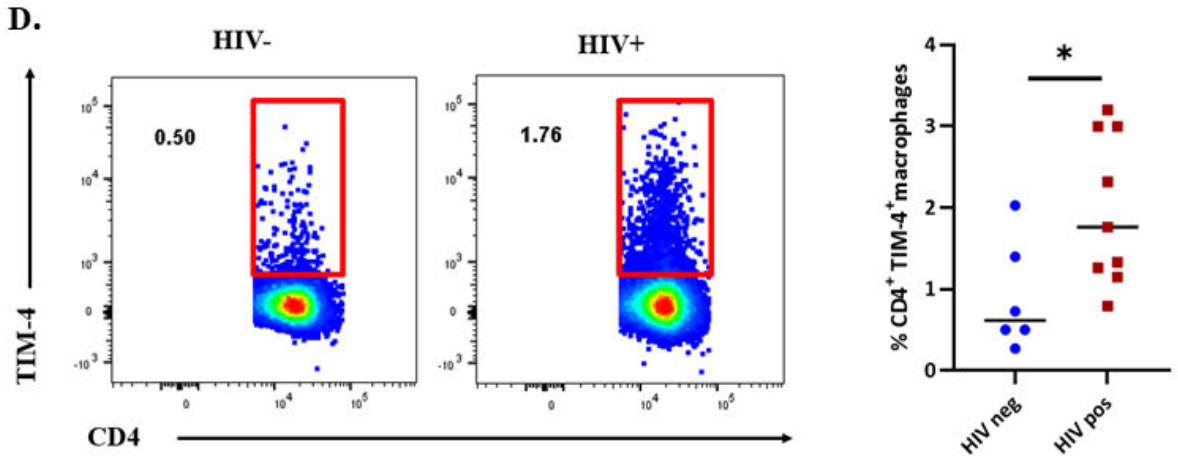


Figure 4.3: Multi-color flow cytometric analysis performed on LN mononuclear cells from HIV negative and HIV positive LT individuals.

LN cells were identified as $CD45^+/CD3^-CD19^-/HLA\text{-}DR^+$ by flow cytometry, as described in the methods section. (A) Representative flow cytometry and summary plot showing percentages of $CD68^+TIM\text{-}4^+$ macrophages. (B) Representative flow cytometry and summary plot showing percentages of $CD206^+TIM\text{-}4^+$ macrophages. (C) Representative flow cytometry and summary plot showing percentages of $CD11B^+TIM\text{-}4^+$ macrophages. (D) Representative flow cytometry and summary plot showing percentages of $CD4^+TIM\text{-}4^+$ macrophages. Data were analyzed using FlowJo. Mann-Whitney U test was utilized to compare differences between any two groups. Differences between groups were significant at a P value of <0.05.

4.5 DISCUSSION

Despite advances in antiretroviral treatment, the complete eradication of HIV remains elusive. Combined antiretroviral therapy (cART) effectively suppresses HIV, but it does not eliminate the latent HIV reservoir. One of the well-established sources of viral rebound are long-lived resting memory $CD4^+$ T cells. However, cumulative evidence now suggests that macrophages also contain persistent HIV. A thorough understanding of spatial localization and phenotype of the macrophage reservoir will allow for targeted elimination of lymph node reservoirs.

Macrophages may be a potential therapeutic target for HIV elimination. However, their potential role in HIV cure strategies has not been explored due to the paucity of information on their biology and function. Reports have implicated $CD68^+$ macrophages as an HIV reservoir in the gut and brain [2], with a few studies showing their relevance in lymph nodes [25]. In this study, we investigated HIV persistence in ET and LT human $CD68^+$ LN macrophages using flow cytometry and immunofluorescence microscopy. We detected the presence of HIV Gag p24 antigen in $CD68^+$ macrophages of HIV positive ET and LT individuals on cART, with a higher frequency observed in LT individuals. Our findings demonstrate the importance of early ART initiation to limit the magnitude of HIV Gag p24 antigen in LN macrophages hence limiting the establishment of the macrophage reservoir. Unfortunately, it has not been well established whether early ART initiation significantly affects reservoir establishment as HIV Gag p24 persists in LN macrophages despite early treatment initiation.

Determining the presence of HIV-1 RNA in tissue compartments is vital for understanding the dynamics of HIV infection and persistence. Using a highly specific ISH assay, RNAscope, we detected HIV-1 RNA in HIV positive LT germinal center macrophages indicating ongoing HIV transcription in $CD68^+$ macrophages. The presence of viral antigens and HIV transcription in LN macrophages in the face of cART supports previously reported data showing the persistence of HIV in macrophages. Besides weakening the immune system, we speculate that HIV persistence in lymph node macrophages may cause cell and tissue damage leading to impaired macrophage function during cART.

Recently long-lived macrophages have been identified in certain organs such as the liver, the lung, the brain, and the gastrointestinal tract. These long-lived macrophages contribute to tissue homeostasis, immune surveillance, and to immune responses in their respective tissue microenvironment. T cell immunoglobulin and mucin domain containing 4 (TIM-4) is a protein expressed by antigen presenting cells and macrophages. HIV infection of long-lived macrophages elevates the potential of this subset to be an important reservoir in tissues. Thus, we investigated the frequency and spatial localization of TIM-4 expressing macrophages in lymph node tissues using imaging approaches and flow cytometry. Using flow cytometry and LN mononuclear cells from HIV negative and HIV positive individuals, we

identified a small subset of potentially long-lived $CD68^+$ cells expressing TIM-4 reflecting their potential to persist for a long time in tissues and therefore contribute to LN tissue reservoirs.

In conclusion, our results demonstrate HIV persistence in LN macrophages despite early ART initiation. HIV structural proteins persist in LT individuals at lower levels compared to ET individuals. Additionally, we detected HIV-1 RNA in LT individuals showing the presence of transcriptionally active virus in LT germinal centers. Taken together, our results highlight the importance of early initiation of ART to reduce the levels of viral antigens and persistent HIV in the lymph nodes. Moreover, we emphasize the need for effective interventions to completely eradicate persistent virus or eliminate HIV-infected cells in immune-privileged sites.

4.6 STUDY STRENGTHS AND LIMITATIONS

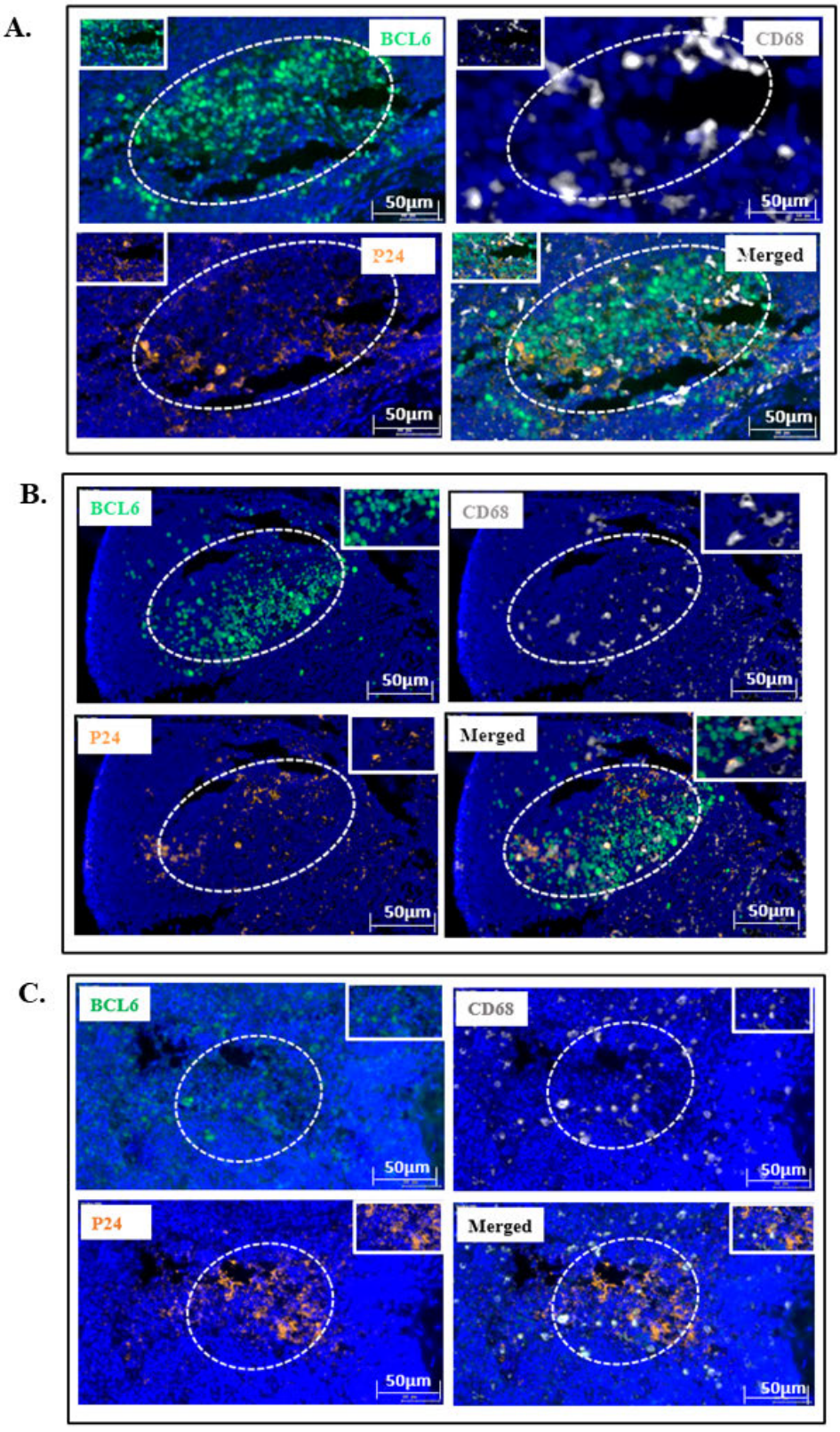
This study provided novel information including the detection of HIV Gag p24 protein and HIV-1 RNA in $CD68^+$ lymph node macrophages. Additionally, we highlight the importance of early ART initiation to minimize HIV persistence in LN macrophages. The use of flow cytometry and immunofluorescence microscopy combined was of paramount importance in addressing our study questions. However, we had limited access to lymph node samples due to the lockdown implemented during the COVID-19 pandemic. Consequently, we could not comprehensively quantify HIV Gag p24 protein and HIV-1 RNA in HIV positive ET individuals. Distinguishing HIV infected macrophages from macrophages that have engulfed HIV infected $CD4^+$ T cells is a major challenge since macrophages can internalize various cellular material including infected T cells. Moreover, the detection of HIV infected macrophages in vivo can be particularly challenging due to the lower levels of viral replication within these cells compared to $CD4^+$ T cells. Research in this area is ongoing and novel approaches may emerge to enhance the accuracy of distinguishing between productively infected macrophages and macrophages that have engulfed infected cells. That notwithstanding there is compelling evidence to suggest that indeed macrophages contribute to HIV persistence and reservoirs in human tissues, and strategies aimed at eradicating HIV in tissues should always take into consideration the macrophage reservoir.

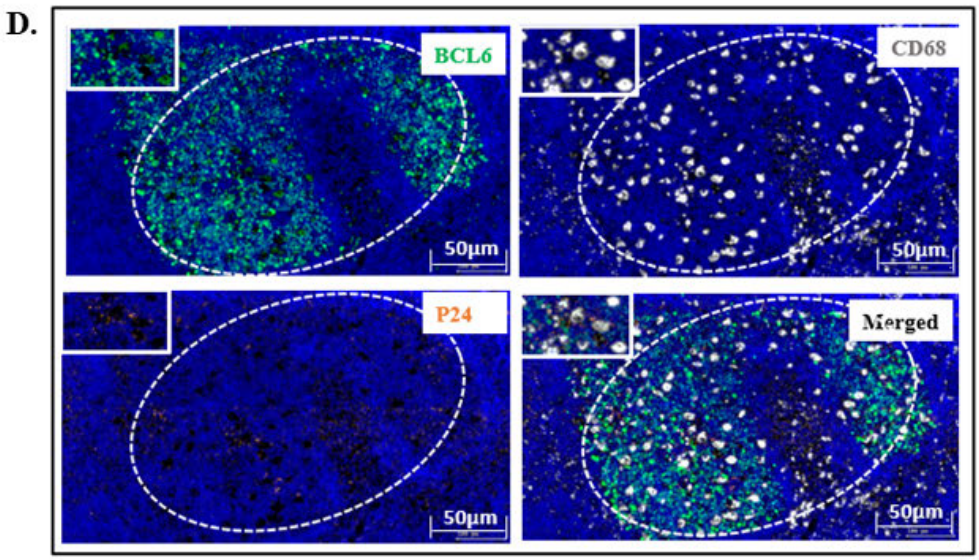
4.7. FUTURE RECOMMENDATIONS

We recommend the use of a larger sample size including a balanced number of ET and LT participants were applicable. In addition, we recommend the use of serial fine needle aspirates (FNA) to obtain

tissue samples as this procedure is quick, less invasive, and minimizes the loss of information downstream.

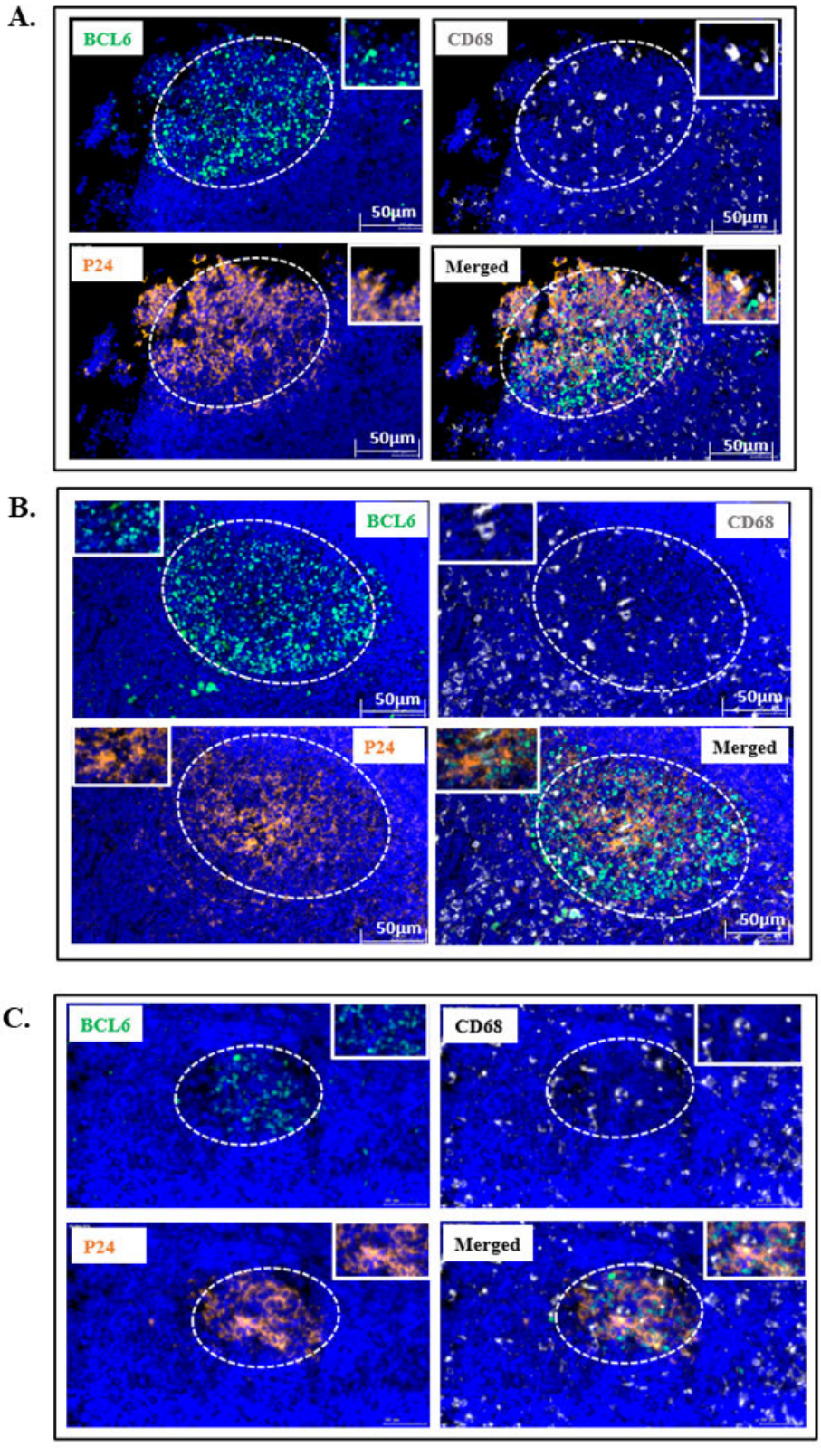
4.8 SUPPLEMENTARY DATA



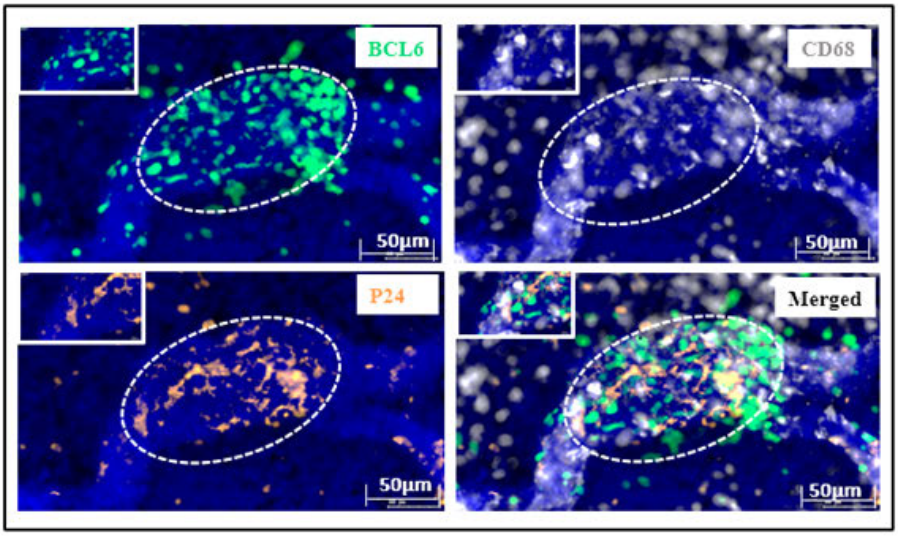


Supplementary Figure 4.1: HIV Gag p24 detection in CD68^{+} macrophages in lymph node (LN) sections obtained from HIV-positive ET individuals.

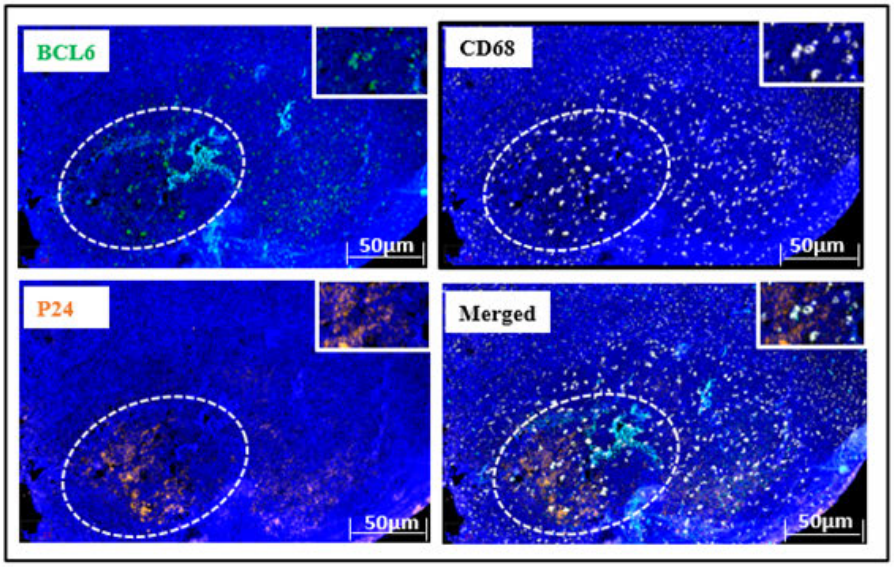
(A-D) Representative images of LN sections showing Gag p24 (orange), BCL-6 (green), and CD68 (white). (B) Costaining of Gag p24 (yellow) and CD68 (white) was observed in both ET and LT individuals.


A.
BCL6
CD68
P24
Merged
50µm
B.
BCL6
CD68
P24
Merged
50µm
C.
BCL6
CD68
P24
Merged

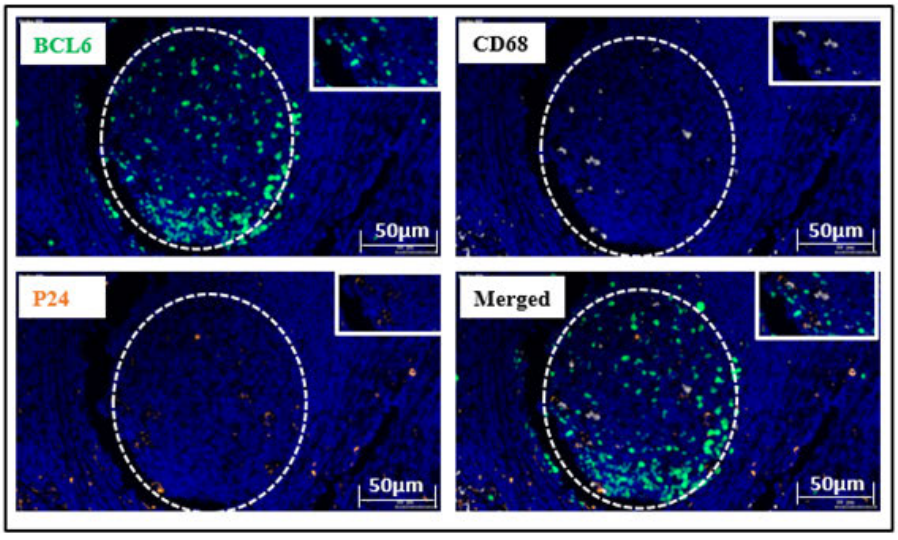
D.


BCL6
CD68
P24
Merged
50µm
50µm
50µm
50µm

E.

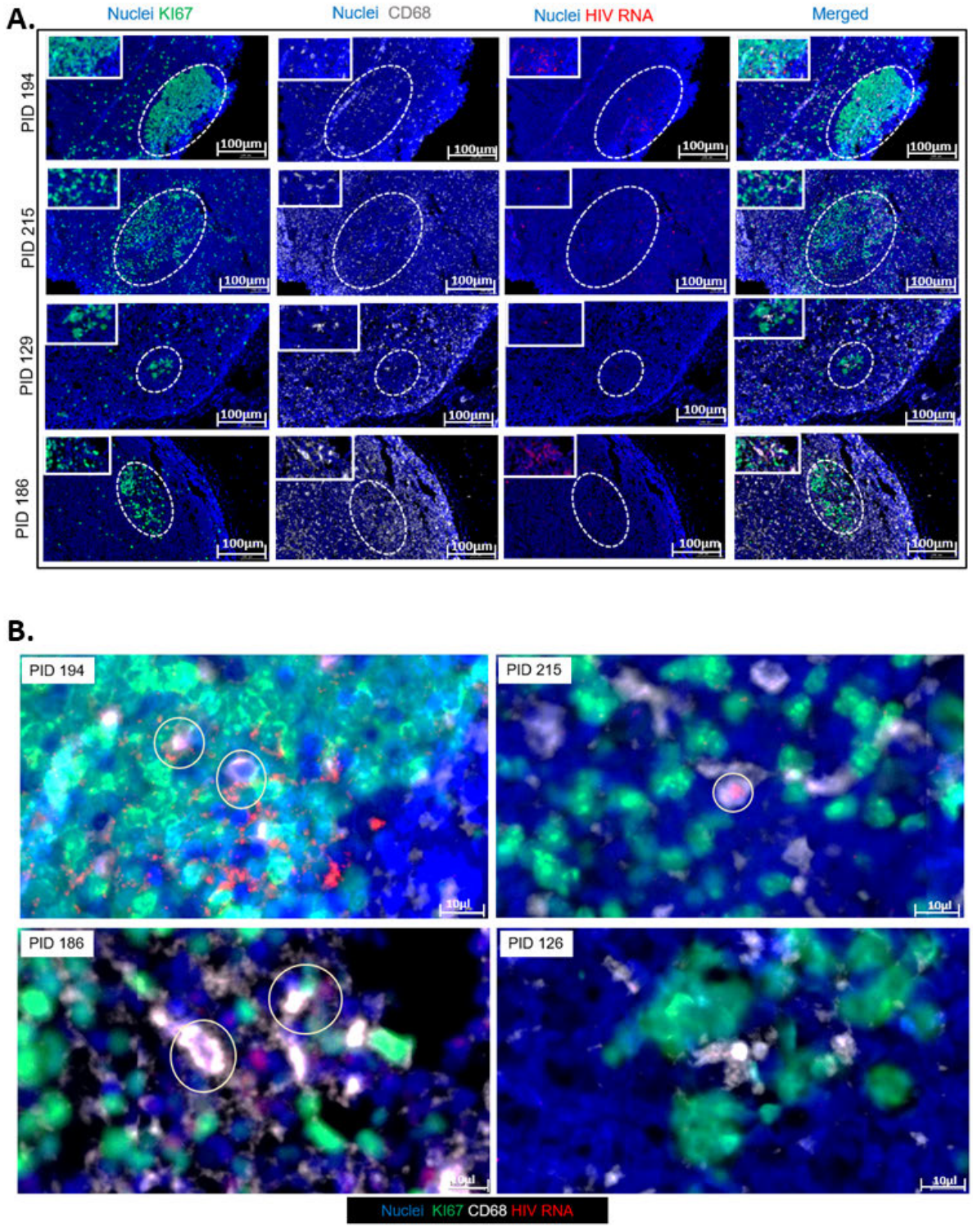

BCL6
CD68
P24
Merged
50µm
50µm
50µm
50µm

F.


BCL6
CD68
P24
Merged
50µm
50µm
50µm
50µm

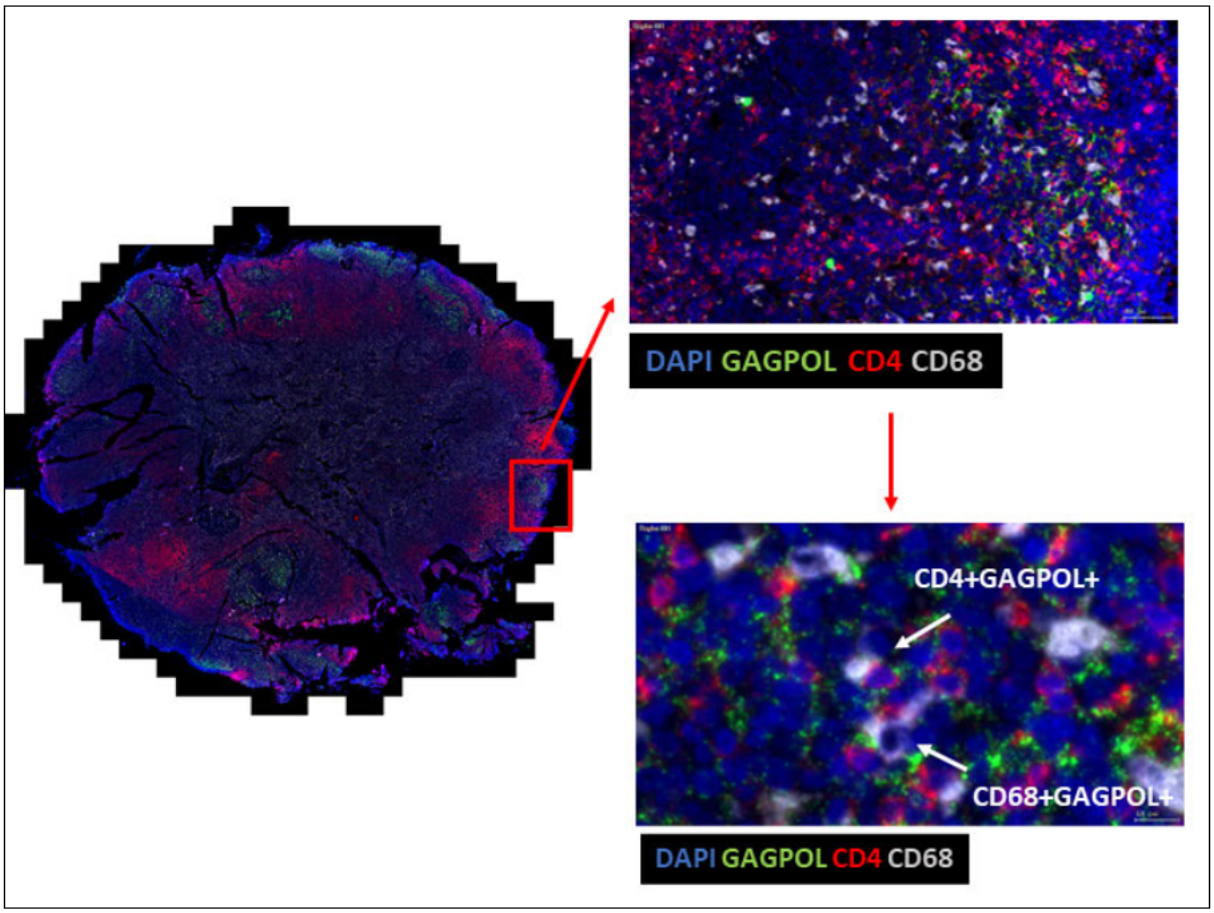
Supplementary Figure 4.2: HIV Gag p24 detection in CD68^{+} macrophages in lymph node (LN) sections from HIV-positive LT individuals.

(A-F) Representative images of LN sections showing Gag p24 (orange), BCL-6 (green), and CD68 (white). (B) Costaining of Gag p24 (yellow) and CD68 (white) was observed in both ET and LT individuals.



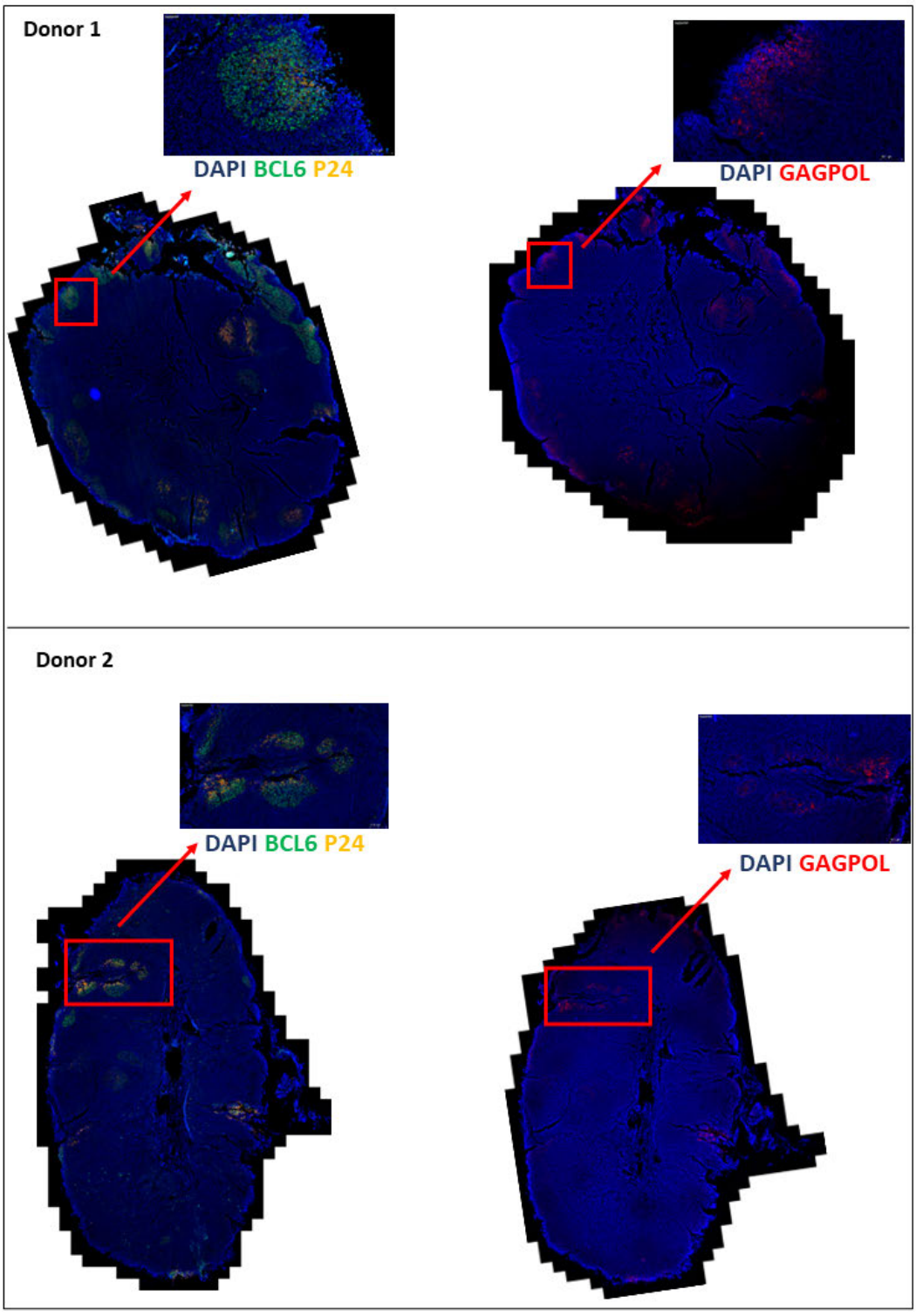
Supplementary Figure 4.3: Detection of HIV-1 RNA in lymph nodes of HIV positive LT individuals.

(A) Images of tissue sections obtained from 4 HIV positive LT individuals showing HIV-1 RNA (red), KI67 (green), and CD68 (white). (B) Zoomed in images of tissue sections shown in (A). Scale bars equal 10µm. RNAscope hybridization for HIV gag-pol RNA was detected using immunofluorescence microscopy. Single RNA transcripts are shown as punctate dots.



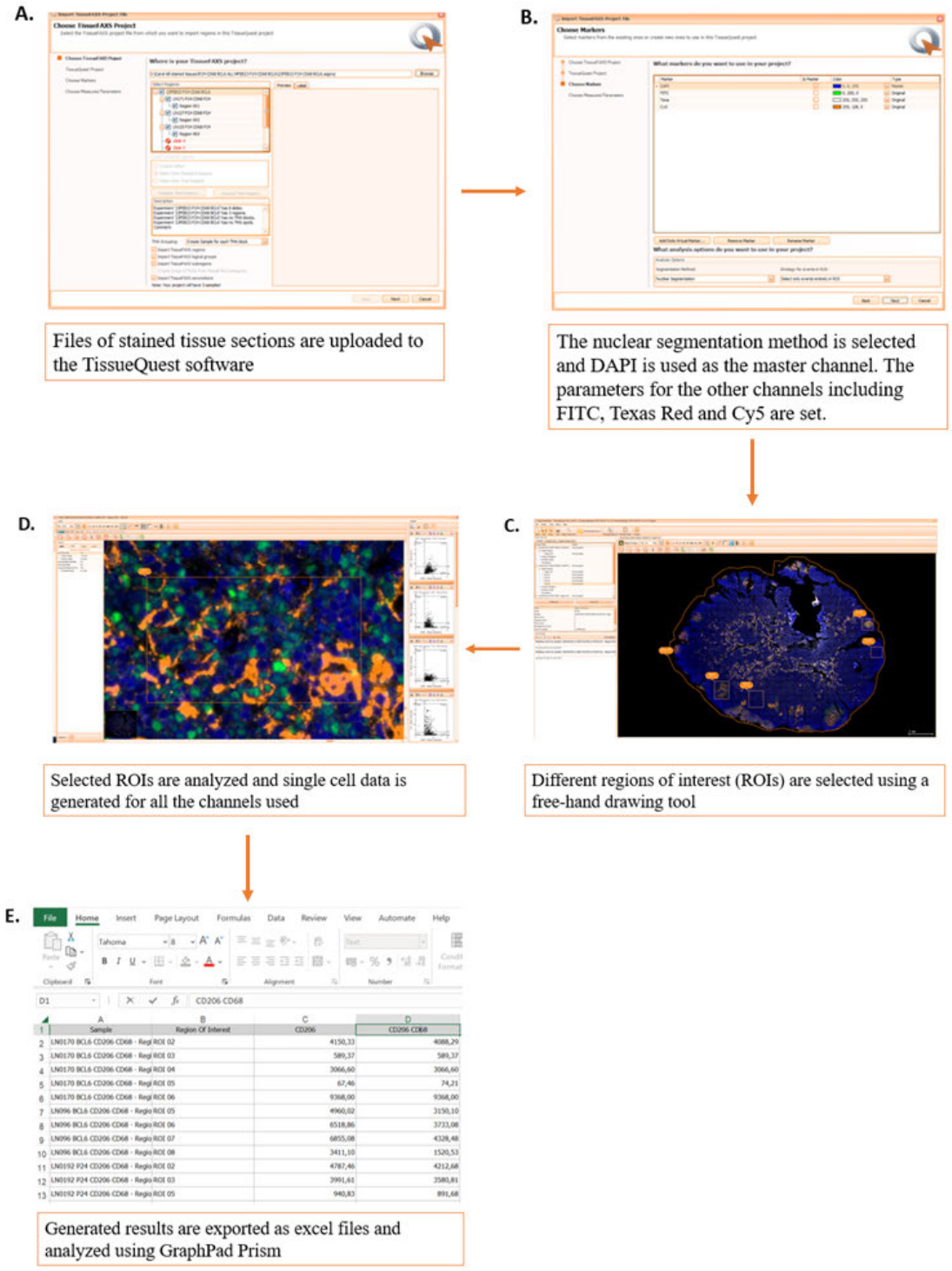
Supplementary Figure 4.4: Detection of HIV-1 RNA in lymph nodes of HIV positive LT individuals.

(A) Representative image of a whole HIV positive (LT) LN showing HIV-1 RNA (green), CD4 (red), and CD68 (white). Image shows detection of HIV-1 RNA in CD68 and CD4 co-expressing cells. RNAscope hybridization for HIV gag-pol RNA was detected using immunofluorescence microscopy. Single RNA transcripts are shown as punctate dots.



Supplementary Figure 4.5: Detection of HIV Gag p24 protein and HIV-1 RNA in tissues obtained from two LT donors.

Side-by-side comparison of samples stained for HIV Gag p24 (left) and HIV-1 RNA (right) using BCL6 (green), P24 (orange/ yellow), and HIV-1 RNA/GAG-POL (red).



Supplementary Figure 4.6: Image cytometry pipeline used for immunofluorescence microscopy image analysis using TissueQuest software (TissueGnostics, Vienna, Austria).

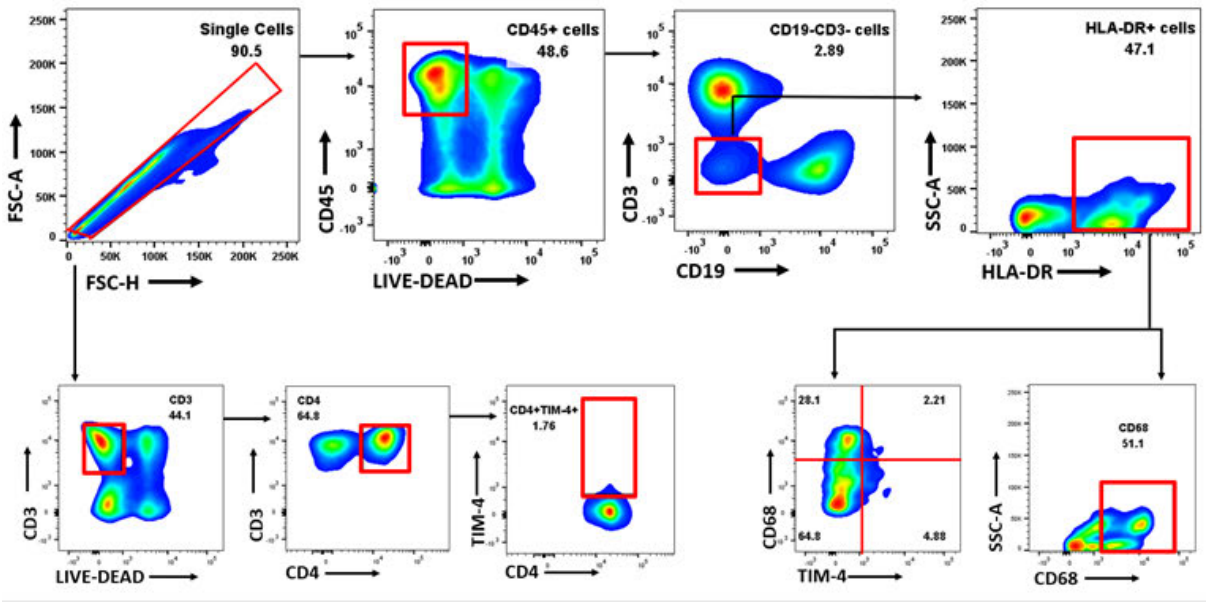
(A) Files used for analysis are uploaded in TissueQuest for analysis. (B) The nuclear segmentation method is selected. (C) The regions of interest are specified, and appropriate parameters are defined using 4' 6-diamidino-2-phenylindole (DAPI) as the master channel. (D) Single cell information for

Texas Red, fluorescein Isothiocyanate (FITC), cyanine 5 (Cy5) are displayed in scatter plots. (E) Summary results are exported in excel graphs.

Supplementary Table 4.1: Detailed patients' characteristics (Gag p24 detection and RNAscope)

Study group	*PID*	*Type of node*	*Age*	*Gender*	*HIV status*	*Treatment status*	*Absolute CD4 count (cells/ul)*	*Plasma viral load (copies/ml)*
Early treated	88	Inguinal	28	F	Pos	Yes	942	2055
Early treated	152	Inguinal	31	F	Pos	Yes	1168	<20
Early treated	171	Inguinal	25	F	Pos	Yes	1369	59
Early treated	172	Inguinal	27	F	Pos	Yes	624	370
Early treated	166	Inguinal	25	F	Pos	Yes	1800	614
Late treated	72	Inguinal	30	F	Pos	Yes	639	11000
Late treated	126	Inguinal	28	F	Pos	Yes	406	<20
Late treated	129	Inguinal	27	F	Pos	Yes	908	500
Late treated	133	Inguinal	27	M	Pos	Yes	834	7730
Late treated	184	Inguinal	33	F	Pos	Yes	555	6000
Late treated	186	Inguinal	26	F	Pos	Yes	554	3000
Late treated	190	Inguinal	24	F	Pos	Yes	225	<20
Late treated	191	Inguinal	23	F	Pos	Yes	-	<20
Late treated	192	Inguinal	31	F	Pos	Yes	847	<20
Late treated	194	Inguinal	24	F	Pos	Yes	854	<20
Late treated	215	Inguinal	-	F	Pos	Yes	751	<20

** Abbreviations: Patient identifier (PID), male (M), female (M), positive (Pos)*



Supplementary Figure 4.7: Flow cytometry gating strategy.

LN cells were identified as $CD45^{+}/CD3^{-}CD19^{-}/HLA\text{-}DR^{+}$ by flow cytometry. Populations were further gated to analyze the co-expression of markers CD68, CD206, CD11B, CD4 and TIM-4.

Supplementary Table 4.2: Detailed patients' characteristics (Flow cytometry)

Study group	*PID*	*Type of node*	*Age*	*Gender*	*HIV status*	*Treatment status*	*Absolute CD4 count (cells/ul)*	*Plasma viral load (copies/ml)*
HIV negative	106	Inguinal	29	F	Neg	N/A	928	N/A
HIV negative	110	Inguinal	27	F	Neg	N/A	659	N/A
HIV negative	111	Inguinal	29	F	Neg	N/A	667	N/A
HIV negative	113	Inguinal	29	F	Neg	N/A	704	N/A
HIV negative	167	Inguinal	27	F	Neg	N/A	-	N/A
HIV negative	168	Inguinal	25	F	Neg	N/A	-	N/A
Late treated	126	Inguinal	28	F	Pos	Yes	406	<20
Late treated	133	Inguinal	27	M	Pos	Yes	834	7730
Late treated	171	Inguinal	25	F	Pos	Yes	1369	59
Late treated	172	Inguinal	27	F	Pos	Yes	624	370
Late treated	186	Inguinal	26	F	Pos	Yes	554	3000
Late treated	190	Inguinal	24	F	Pos	Yes	225	<20
Late treated	202	Inguinal	24	F	Pos	Yes	883	<20
Late treated	203	Inguinal	29	M	Pos	Yes	-	<20

** Abbreviations: Patient identifier (PID), male (M), female (M), positive (Pos), negative (Neg)*

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CHAPTER 5: SYNTHESIS

5.1 GENERAL DISCUSSION

HIV and AIDS persist as a global health problem despite endless efforts to find a cure [1]. In 2001, HIV/AIDS was declared a global health crisis [2]. Thereafter, numerous efforts were made to mitigate severe disease outcomes as well as mobilize the resources required to develop a preventive vaccine or to cure people living with HIV. There is a broad consensus that scientific efforts should be directed towards the development of a safe, effective, and easily accessible HIV cure [3, 4]. Although combined antiretroviral therapy (cART) has significantly lowered AIDS-related mortalities, factors such as lack of access to drugs and drug resistance still complicate the effective use of ART. In addition, HIV continues to persist in various sanctuary sites resulting in the formation of long-lived HIV reservoirs [5-7]. It has been argued that macrophages harbor persistent HIV during cART [3]. However, conclusive evidence to support this notion has not yet materialized due to the lack of sensitive assays to quantify the macrophage reservoir as well as limited access to human tissue samples for researchers [8]. Sadly, people living with HIV (PLWH) have a higher risk of developing noncommunicable diseases such as cardiovascular disease and cancer [9, 10]. Recently, cumulative evidence has shown that PLWH have greater risk of contracting SARS-CoV-2 compared to uninfected individuals. In addition, global data has shown that PLWH have a 38% greater risk of developing severe COVID-19 compared to HIV negative individuals [11]. The inability to completely eradicate HIV, herein, highlights the need to gain more insight into HIV persistence and reservoir establishment.

The original aim of this study was to understand the HIV reservoir in lymph nodes. The COVID-19 lockdown in South Africa forced us to modify the original PhD proposal to include COVID-19 work for two major reasons. Firstly, my original study was suspended during the COVID-19 lockdown, which significantly delayed my original research work. Secondly, to better utilize the time during the lockdown we decided to use our immunology knowledge, skills, and tools, to contribute towards finding a solution to the COVID-19 pandemic while advancing my doctoral studies. Therefore, we changed the first part of my thesis to focus on investigating the impact of HIV infection on cellular immunity to SARS-CoV-2. After the lockdown, I resumed the original proposed work which was to investigate the role of macrophages in HIV reservoirs in lymph node tissues. It is important to note that both projects were executed successfully, and each study contributes towards partial fulfillment of the requirements for my dissertation.

We first investigated whether HIV infection has detrimental effects on SARS-CoV-2 T cell immunity and hampered cross-recognition of COVID-19 viral variants. We used peripheral blood samples from a newly established mechanisms cohort, which is an ongoing longitudinal study of COVID-19 patients. Samples from this cohort were used to gain insight into the impact of HIV infection on SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses during the first wave and second wave of the COVID-19 epidemic in South Africa. We showed that uncontrolled HIV infection is associated with reduced polyfunctionality and diminished cross-recognition of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses. Additionally, we showed that unsuppressed HIV infection impairs T-cell responses to SARS-CoV-2 infection. These findings may partly explain the high propensity for severe COVID-19 among PLWH and highlight their vulnerability to emerging SARS-CoV-2 variants of concern. The findings from this work were published in eLife journal in 2022 and are reported in chapter 2 of this thesis.

The second part of the study used excisional lymph node tissue samples and paired blood samples to interrogate the role on macrophages in HIV tissue reservoirs. The study participants enrolled in this study were drawn from a well-characterized cohort known as the Females Rising through Education, Support, and Health (FRESH). The FRESH cohort was established to detect, treat, and manage early HIV infection by early ART initiation and frequent HIV testing. Clinical characteristics of study participants are readily captured during the initial recruitment and treatment phase.

One of the major hurdles to the complete eradication of HIV is the establishment of persistent long-lived HIV-infected cells in various anatomic compartments. It is well established that HIV reservoirs persist in various $CD4^+$ T cell subsets [12, 13], but cumulative evidence suggests that tissue macrophages may be a minor but important component of the HIV reservoir, considering that it only takes one replicative competent virion to re-establish infection [14, 15]. Thus, understanding the contribution of tissue macrophages is vital for the complete elimination of latently infected cells in tissues. Moreover, identifying HIV reseirvoirs in tissues and the mechanisms involved in viral persistence will be essential for the development of effective HIV cure strategies. A growing body of literature has shown that macrophages are highly heterogenous cells and polarize to M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages in response to various stimuli [16, 17]. In addition, macrophage heterogeneity and polarization have been implicated in HIV persistence. However, the mechanisms pinning macrophages and their specific role in viral persistence remain unclear.

Therefore, we used tissue biopsies and lymph node (LN) mononuclear cells to perform a comprehensive characterization of LN macrophages during HIV-1 subtype C infection. We identified three main macrophage populations including $CD68^+$ (M1), $CD206^+$ (M2), and $CD68^+CD206^+$ macrophages in HIV negative and HIV positive LT individuals. $CD68^+$ macrophages were localized inside and outside

the GC while $CD206^+$ and $CD68^+CD206^+$ were localized in lymphatic vessels and outside the GCs. We also investigated the impact of HIV on macrophage frequency and observed a significantly higher macrophage frequency in HIV positive individuals compared to HIV negative individuals (Chapter 3). We expanded our research to further investigate the presence of HIV in $CD68^+$ macrophages. Imaging results showed the presence of HIV Gag p24 protein in HIV positive LT GCs ET GCs, with a higher frequency of HIV Gag p24 protein in LT GCs. The use of a combination of assays to study HIV infection in LN macrophages is vital for result validation. To further probe HIV infection in macrophages, we used RNAscope to show the presence of transcriptionally active HIV in LT GCs and we detected HIV-1 RNA in five of the six stained LN tissues (Chapter 4).

5.2 STUDY IMPLICATIONS AND FUTURE DIRECTIONS

Our COVID-19 studies highlight the importance of managing HIV infection and treatment as unsuppressed HIV may contribute to weaker immune responses to diseases such as COVID-19. Our studies mainly focused on investigating the impact of HIV infection on SARS-CoV-2-specific T-cell responses in the first wave and second wave of the COVID-19 epidemic in South Africa. Our results demonstrate that uncontrolled HIV infection is associated with low magnitude, reduced polyfunctionality, and diminished cross-recognition of SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses. Importantly, fully suppressed PLWH had comparable SARS-CoV-2-specific T-cell responses with HIV-seronegative individuals. These findings reveal the elevated risk of contracting SARS-CoV-2 among PLWH. Additionally, we also highlight the need to ensure uninterrupted access to cART for PLWH during the COVID-19 pandemic and beyond.

Although, we repeatedly showed robust in vitro T cell expansion following ex vivo peptide stimulation but limited expansion against mutant versions of the peptides, there is need to identify optimal peptides that were targeted by $CD8^+$ and $CD4^+$ T cells in the context of restricting MHC class I and II alleles. SARS-CoV-2 responses are generally very broad [18]; thus, it is not clear from these studies how the loss of T cell cross-recognition in Spike affects the overall protective immunity. Furthermore, investigating if the observed poor T cell cross-recognition between wave 1 and wave 2 is generalizable to the Delta and the Omicron variants is clearly warranted. Importantly, our data raise the question of whether CTL selection pressure plays a significant role in shaping emerging variants. This concept should be investigated using larger longitudinal studies with longer durations of follow-up. Previous work in this cohort examined the relationship between T cell and B cell responses and found a positive association between $CD8^+$ T cell frequency and several CD19 B cell subsets, which was attenuated in PLWH [19], suggesting that both arms of the immune system are impacted by HIV/SARS-CoV-2

coinfection. However, the current study did not examine this relationship at the antigen-specific level due to sample limitations. Future work is required to understand the relationship between T cells and humoral immunity and the impact of unsuppressed HIV infection on long-term protection.

The second part of this thesis reveals the potential contribution of human LN macrophages to HIV reservoirs in lymphoid tissues. Chapters 3 and 4 focused on the phenotypic and functional characterization of LN macrophages. Our major findings include firstly, that $CD68^+$ are distinguishable from $CD206^+$ macrophages by their location and function. Secondly, we discovered that HIV infection results in increased macrophage frequency despite plasma viral suppression. Other studies have shown an increase in $CD68^+$ macrophages in bone marrow during primary myelofibrosis [20]. However, the mechanisms underlying this increase are not known. Similarly, we did not conduct further research to probe the alteration of macrophage frequency in LN, hence the interplay between macrophage frequency and HIV infection is yet to be explored. Thirdly, having identified the various macrophage populations and their spatial localization in LN macrophages, we used immunofluorescence microscopy to identify HIV infected macrophages that were positive for HIV Gag p24 protein and HIV Gagpol RNA. Consistent with other studies in brain and urethral tissues, we detected transcriptionally active virus in LT individuals. Whether or not the transcriptionally active reservoirs can lead to productive infection remains to be explored. Lastly, we used TIM-4 to identify long-lived LN resident macrophages that have the greatest potential to contribute to HIV reservoirs in human lymph nodes. We postulate that targeting HIV-infected macrophages could be effective in reducing the levels of HIV trapped in microanatomical sites. Our studies highlight the role of LN macrophages in HIV persistence during cART.

5.3 STUDY LIMITATIONS

One of the major limitations of these studies was the inability to access peripheral blood mononuclear cells (PBMCS) and LN tissue biopsies during the COVID-19 pandemic. Consequently, our sample size was small. For instance, we could not quantify HIV-1 RNA in ET LN tissues and had fewer ET patient samples compared to LT samples. Comparing HIV-1 RNA levels in ET and LT individuals would have enlightened our understanding of the persistence of HIV in individuals initiating ART in less than 3 months of infection. Another notable limitation of this study was gender diversity. All the study participants recruited into the FRESH study are females. Therefore, we did not have the capacity to interrogate gender related differences in macrophage biology. Lastly, there are several other macrophage markers such as CD163, CD169 that have been described in literature, but we did not have the opportunity to interrogate their expression and distribution in LNs due to budget limitations. Future

studies should include these additional markers to allow for more nuanced characterization of macrophage subsets which will help to determine the biomarkers of macrophage reservoirs more precisely.

5.4 CONCLUDING REMARKS

An in-depth understanding of HIV pathogenesis and reservoir establishment has been hampered by the inability to access human blood and tissue samples. Here, we confirm that LN macrophages are heterogeneous. We detected $CD68^+$ (M1) and $CD206^+$ (M2) macrophages in LNs. Moreover, we show the presence of persistent HIV in ART-treated individuals. Understanding the functional roles of LN macrophages during HIV will inform the targeted elimination of HIV-infected macrophages. Follow-up studies will investigate the role of macrophages in the brain and gut using the markers identified in this study. Overall, an improved understanding of HIV persistence will require the use of a combination of sensitive assays to interrogate the macrophage reservoir.

Our study highlights the need to conduct further studies to more definitively determine the extent to which macrophages contribute to the persistence of the HIV reservoir in the setting of long-term effective virologic suppression. The lifespan of infected macrophages needs to be determined. There is need for more detailed characterization of the frequency and phenotype and spatial localization of latently infected macrophages. Future studies should also determine whether macrophages are an important source of cryptic viremia in sanctuary tissue sites such as lymphoid tissues, the brain, and other tissues. Moreover, there is need to determine the extent to which macrophage reservoirs contribute to rebound viremia following cART cessation. Answers to these outstanding questions will better inform research into HIV cure strategies.

Our data also reveals impaired SARS-CoV-2-specific T cell responses in individuals with unsuppressed HIV infection. Additionally, we observed poor cellular cross-recognition between variants and more pronounced cross recognition in individuals with unsupressed HIV. The muted responses in unsuppressed HIV infection may be attributable to low absolute CD4 count and immune activation. Importantly, we identified mutations in the Beta variant that could potentially reduce T cell recognition. Taken together, these data highlight the need to ensure uninterrupted access to ART for PLWH during the COVID-19 pandemic. Overall, our study findings demonstrate the persistence of HIV in human lymph nodes despite the early initiation of ART. We also highlight the need for monitored therapy to avoid treatment interruption as this may impact HIV pathogenesis resulting in viral rebound and may expose HIV-infected individuals to diseases such as COVID-19 and its variants.

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APPENDICES

APPENDIX A: ETHICAL APPROVAL

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

30 January 2023

Dr Alexander Sigal (38706)
Africa Health Research Institute (AHRI)

Dear Dr Sigal,

Protocol reference number: BREC/00001275/2020
Project title: Consequences of HIV and TB Co-Infection on COVID-19 Disease Dynamics, Severity and Immune Responses
Degree: Non-Degree

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**24 May 2023**
Expiration of Ethical Approval:	**23 May 2024**

I wish to advise you that your application for recertification received on 20 January 2023 for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 14 March 2023.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Figure A. 1: Ethical approval of the study

APPENDIX B: MANUSCRIPT 1

Manuscript 1: Tiza Ng'uni, **Caroline Chasara**, and Zaza M. Ndhlovu. **Major Scientific Hurdles in HIV Vaccine Development: Historical Perspective and Future Directions.** *Frontiers in Immunology*.

Authors' contributions: ZN formulated the idea and provided oversight in planning and organizing the literature search. T.N and I prepared the original manuscript draft with the supervision of ZN. Z.N, T.N, and I edited the manuscript. All authors approved of the manuscript before it was submitted and published in *Frontiers In Immunology*.

frontiers in Immunology

REVIEW
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Major Scientific Hurdles in HIV Vaccine Development: Historical Perspective and Future Directions

Tiza Ng'uni[1], *Caroline Chasara*[1] *and Zaza M. Ndhlovu*[1,2]*

[1] KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), 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, United States

Following the discovery of HIV as a causative agent of AIDS, the expectation was to rapidly develop a vaccine; but thirty years later, we still do not have a licensed vaccine. Progress has been hindered by the extensive genetic variability of HIV and our limited understanding of immune responses required to protect against HIV acquisition. Nonetheless, valuable knowledge accrued from numerous basic and translational science research studies and vaccine trials has provided insight into the structural biology of the virus, immunogen design and novel vaccine delivery systems that will likely constitute an effective vaccine. Furthermore, stakeholders now appreciate the daunting scientific challenges of developing an effective HIV vaccine, hence the increased advocacy for collaborative efforts among academic research scientists, governments, pharmaceutical industry, philanthropy, and regulatory entities. In this review, we highlight the history of HIV vaccine development efforts, highlighting major challenges and future directions.

Keywords: HIV, history of HIV-1 vaccines, efficacy trials, HIV prevention, HIV-1 vaccine design

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Edited by:
Marc H. V. Van Regenmortel, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Susan Zolla-Pazner, Icahn School of Medicine at Mount Sinai, United States
Stephanie Jost, Beth Israel Deaconess Medical Center and Harvard Medical School, United States

***Correspondence:**
Zaza M. Ndhlovu
zaza.ndhlovu@ahri.org

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INTRODUCTION

The HIV/AIDS epidemic remains a major global health challenge and continues to exert significant strain on healthcare resources in sub-Saharan Africa. According to the UNAIDS, globally, approximately 37.9 million people were living with HIV infection in 2018. In addition, there were 1.7 million new infections with approximately 770,000 AIDS-related deaths in the same year despite widespread rollout of antiretroviral therapy (ART) (1, 2). The global HIV incidence-to-prevalence ratio of 0.05 indicates that the number of HIV-infected people will continue to rise unless more effective preventive strategies are employed to reduce transmission (3). There is broad scientific consensus that the most effective approach to control and eventually end the HIV epidemic is to develop a preventive AIDS vaccine that is safe, effective, cost efficient, and easily accessible worldwide (4). Regrettably, despite over 30 years of rigorous HIV research and numerous vaccine trials, there is no licensed HIV vaccine currently on the market (5). The aim of this review is to discuss past and present approaches to vaccine development and clinical trials to date. The review also highlights current gaps in knowledge and proposes new directions and novel strategies toward developing an efficacious preventive HIV vaccine.

CHALLENGES IN HIV VACCINE DEVELOPMENT

The development of potent antiretroviral therapies, now delivered as a single pill once a day, has transformed HIV infection into a clinically manageable chronic disease. Globally, over 19 million people are now on life-long treatment, and test-and-treat strategies and oral pre-exposure prophylaxis (PrEP) could further reduce HIV transmissions. However, despite these remarkable advances, prolonged combined antiretroviral therapy (cART) mediated suppression of plasma viral loads to undetectable levels does not eradicate the virus, which often rapidly rebounds upon treatment interruption. In addition, while cART has decreased mortality and morbidity among people living with HIV (PLWH), long-term cART treatment is associated with increased occurrence of a range of serious non-AIDS events (SNAEs). These SNAEs include cardiovascular diseases, cancer, liver disease, long-term peripheral and central nervous system complications, renal and metabolic disorders, and osteoporosis (6). The many logistical limitations and cost challenges that come with providing life-long care to those living with HIV highlight the need for a preventive HIV vaccine (7). Desirable attributes of an HIV vaccine include elicitation of long-lasting all-round protection with a limited number of doses administered to the patient, the vaccine should also be affordable, easy to administer, and easy to store without the need of a cold chain. The HIV vaccine can either be preventive or therapeutic, which means it can either block HIV infection or could be used to treat HIV infected individuals (8).

Over the years, the greatest challenge in developing an effective HIV vaccine has been the high rate of mutation and recombination during viral replication (9). The enormous genetic diversity of HIV is mainly driven by the high rate of variability of the viral envelope (Env) glycoprotein, which ironically happens to be the main target of neutralizing antibodies (10). The HIV genome contains nine genes which encode 16 proteins including the major structural proteins Gag, Pol, and Env; accessory proteins Nef, Vif, Vpu, and Vpr; and regulatory proteins Tat and Rev. HIV diversity, which is mainly generated by the error prone viral reverse transcriptase, has various implications for disease progression and responses to ART (11). The high mutation rates of approximately 1–10 mutations per genome per replication cycle, extensive conformational adaptability, and massive glycan shielding of the Env enable the virus to evade the effects of neutralizing antibodies and other immune responses (12). Nonetheless, despite the high rate of variability, it has progressively been shown that polyvalent HIV vaccines can be developed and used to target conserved domains on the viral envelope (13). Most current efforts are aimed at inducing broadly neutralizing antibodies (bNAbs), which can neutralize the majority of HIV strains. The ability of bNAbs to neutralize a wide spectrum of HIV strains (broad cross-reactivity) is a major advantage (14). Moreover, the safety and remarkable antiviral activity of highly potent HIV specific bNAbs have been demonstrated in pre-clinical and clinical trials (15).

Beside high viral mutation and recombination rates, extraordinary worldwide genetic diversity is yet another hurdle to the development of a vaccine. HIV is composed of 4 groups: M (main), O (outlier), N (non-M/non-O), and P (pending). Group M is further subdivided into 9 subtypes/clades denoted by the letters A, B, C, D, F, G, H, J, and K. It has been shown that amino acid variations within subtypes can be as high as 30% with those between subtypes reaching as high as 42%. These amino acid variations are based on the subtypes and region of the genome being examined (16, 17). The difficulty of developing a universal vaccine is further compounded by the fact that 10–20% of HIV infected people in several parts of Africa, are infected with two or more viral variants (subtypes and recombinant forms) that circulate in these regions (18).

Other challenges that impact HIV vaccine development include an incomplete understanding of the correlates of immune protection, lack of appropriate animal models, and limited investments by the pharmaceutical industry (19, 20). In addition, most traditional immunogen delivery systems are unable to induce potent and long-lasting immunity against HIV and the traditional live attenuated or whole-inactivated virus methods, employed in the design of measles, mumps, and rubella vaccines, are not appropriate for HIV due to legitimate safety and regulatory concerns associated with the risk of permanent integration of proviral HIV DNA into the host genome (4, 21).

THE DEVELOPMENT OF EARLY HIV VACCINES

Following the isolation of HIV as a causative agent for AIDS in 1983–1984, numerous vaccine prototypes have to date failed to protect against HIV infection (22, 23). In 1984, it was speculated that an HIV vaccine would be developed and available for testing in approximately two years (24). In hindsight, it is clear that researchers underestimated the complexity of such a scientific undertaking. We now know that HIV is unlike any other viral disease for which effective vaccines were empirically developed (25, 26). Given the risk of irreversible HIV integration into the human genome, rational design of subunit vaccines is the only viable option. The introduction of recombinant DNA technologies in the mid-1980s presented the best approach to develop a safe and effective HIV vaccine. This idea was derived from the hepatitis B model for which the hepatitis B surface antigen was successfully cloned and expressed in yeast cells, thus allowing for a new recombinant hepatitis B vaccine to be manufactured and licensed in 1986 (24). Using the hepatitis B recombinant DNA model, HIV researchers developed a subunit vaccine based on genetically engineered antigens representing the outer envelope glycoproteins of HIV. The design was based on previous vaccine approaches that used virus subunits, synthetic peptides, and vaccinia-vector vaccines in animal models (27–29). Rapid developments in the molecular biology of HIV, such as the identification of the major viral structural proteins (30) and the cloning and sequencing of the HIV genome

(31), benefited initial vaccine development efforts. However, the genetic variability of HIV still remained the biggest obstacle to the success of these vaccine development efforts (32).

HIV VACCINES BASED ON THE INDUCTION OF NEUTRALIZING ANTIBODIES

Initially, scientists believed that neutralizing antibodies would be adequate to protect against HIV infection (33) and many of the HIV vaccines in this category were designed to primarily target the envelope glycoproteins, gp120 or gp160 (24). The first experimental immunization of humans against HIV/AIDS was done in Zaire (now the Democratic Republic of Congo) by Zagury and colleagues in 1986 (34). A small group of Zairians were vaccinated with a vaccinia vector (a recombinant HIV-vaccinia virus) expressing gp160, an envelope glycoprotein. The purpose of the study was to assess whether vaccination could induce neutralizing antibodies and cytotoxic T lymphocyte (CTL) responses in HIV negative individuals. Administration of the recombinant vaccinia virus vaccine by scarification elicited weak humoral and cellular immune responses that were boosted by four additional immunizations to achieve anamnestic humoral responses and cellular responses, which persisted for more than a year (35). Following this pioneering in human experimental HIV/AIDS vaccine trial, over 250 clinical trials have been carried out, with the majority being early-phase trials (phase 1 or 2) (36). Approximately 140 of these trials were conducted in the United States with several others being carried out in African countries and Thailand (24). Major vaccine trials have been completed and timelines are shown in **Table 1**.

VaxSyn and HIVAC-1e Vaccines

The first HIV vaccine trial carried out in the US investigated a recombinant envelope glycoprotein (rgpl60), VaxSyn, created in a baculovirus-insect cell system. This trial evaluated the safety and immunogenicity of a rgpl60 candidate vaccine in 72 healthy, HIV-negative adults. The vaccine recipients were randomly assigned to one of four groups to receive intramuscular injections of 40 or 80 μg of rgpl60, 10 μg of hepatitis B vaccine, or placebo in three doses (on days 0, 30, and 180) with an optional fourth dose on day 540. The placebo and hepatitis B vaccine groups served as control groups (37). Results showed that the vaccine was safe and well tolerated. It was observed that vaccines recipients receiving 40 or 80 μg of rgpl60 displayed mostly weak serum antibody responses to HIV envelope proteins. However, antibody titers noticeably increased after the third dose and declined over an 18-month period. The administration of a fourth dose resulted in homologous neutralizing activity and enhanced complement-mediated antibody-dependent activity in some vaccine recipients (37). Other studies showed that the administration of VaxSyn at a dose of 640 μg resulted in increased immunogenicity and higher rates of homologous neutralizing antibody responses, despite the titer being low (52) but failed to elicit sufficient protective neutralizing antibodies (37). After the VaxSyn trial, numerous envelope proteins were evaluated in 35 phase I trials, between 1988 and 2003. Collectively, these vaccine constructs induced binding and neutralizing antibodies that were remarkably durable and also primed $CD4^+$ T cell responses but no apparent CTL responses (24).

In 1988, a second recombinant vaccinia virus designed to express HIV gp160 (HIVAC-1e) entered phase 1 clinical trials in the US. The study enrolled 35 HIV-negative males (31 of whom had prior smallpox immunisations and 4 of whom were vaccinia naive). Study participants were randomly allocated to receive either standard New York strain vaccinia virus or HIVAC-1e. Results showed that vaccinia-naive subjects shed virus from the vaccination site for longer and at a higher titer than the vaccinia-primed individuals. It was also observed that *in-vitro* T-cell proliferative responses to one or more HIV antigen preparations developed in some vaccinia-primed subjects inoculated with HIVAC-1e. However, T-cell responses were short-lived, and no HIV-specific antibodies were detectable. Nonetheless, two vaccinia-naive subjects vaccinated with HIVAC-1e showed robust T-cell responses to homologous and heterologous whole virus stains and to the recombinant gp160 protein, which were detectable for over a year. HIV Env specific antibodies also developed in both subjects. Interestingly, despite HIVAC-1e inducing transient and robust T-cell responses, it failed to produce antibodies against HIV infection in other subjects (24, 38). This led researchers to posit that antibody responses could be improved by priming with a recombinant vaccinia vector expressing the HIV-1 envelope and later boosting with an envelope protein.

In 1991, a combined approach was used in a phase 1 vaccine trial conducted in the US. This trial primed with HIVAC-1e and later boosted with VaxSyn. The prime-boost approach, used in several clinical trials, significantly enhanced both humoral and cellular immune responses and induced neutralizing antibodies (24, 53, 54). Despite these promising results, the use of vaccinia virus vectors raised several concerns. For instance, there was notable decrease in the immunogenicity of the vector in individuals previously vaccinated against smallpox (55, 56). In addition, there were concerns that administering the replicating vaccinia to immunosuppressed individuals could result in severe disease (55, 56). These concerns led to the development of non-replicating poxvirus vectors in the early 1990s, based on two models, namely, a highly attenuated vaccinia virus (NYVAC) or an avian poxvirus, Canarypox (ALVAC), that is not able to replicate in mammalian cells (57, 58).

ALVAC-HIV Vector Vaccine

In 1993, an ALVAC-vector HIV vaccine, vCP125, expressing gp160 was tested alone or as a prime-boost combination with an adjuvanted gp160 subunit. The results revealed that the ALVAC-HIV vaccine significantly primed the neutralizing antibody response of the protein boost and induced CTL activity (59). Other ALVAC-vectors (vCP205, vCP300, vCP1433, vCP1452, and vCP1521) were developed not only to express the HIV envelope but also to express *gag* and other HIV genes to induce broader cell-mediated immune responses (60, 61). In 1999, the

TABLE 1 | Illustration of completed and documented HIV vaccine trials.

Vaccine Trial	Year	Site	Target group	Vaccine	Immune response	Result	Reference
VaxSyn	1987	Canada (Clade B)	72 adults	Recombinant envelope glycoprotein subunit (rgp160) of HIV	Neutralizing antibodies were detected	No vaccine efficacy	(37)
HIVAC-1e	1988	USA (Clade B)	35 male adults	Recombinant vaccinia virus designed to express HIV gp160	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(38)
Vax004	1998–2002	North America (Clade B)	5,417 MSM and 300 women	AIDSVAX B/B gp120 with alum	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(39, 40)
Vax003	1999–2003	Thailand (Clade B/E)	2,545 men and women IDUs	AIDSVAX B/E gp120 with alum	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(39, 40)
HVTN 505	2009–2013	United States (Clade B)	2,504 men or transgender women who have sex with men	Three vaccinations with DNA encoding HIV clade B *gag*, *pol* and *nef* as well as *env* from HIV clades A, B and C followed by an Ad5 vector-based vaccine encoding clade B *gag* and *pol* as well as *env* from clades A, B and C	Vaccine was unable to prevent infection or decrease viral load in vaccinated volunteers	No vaccine efficacy	(41, 42)
STEP/HVTN 502 trial	2004–2007	North America the Caribbean South America, and Australia (Clade B),,	3,000 MSM and heterosexual men and women	MRKAd5 HIV-1 *gag/pol/nef* trivalent vaccine	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(43, 44)
Phambili/ HVTN 503 trial	2003–2007	South Africa (Clade C)	801 adults	rAd5 (*gag/pol/nef*)	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(24)
RV144	2003–2009	Thailand (Clade B)	16,402 community-risk men and women	ALVAC-HIV (vCP1521) and AIDSVAX B/E vaccines	IgG antibody avidity for Env in vaccine recipients with low IgA	31.2% vaccine efficacy at 42 months	(45, 46)
HVTN 305	2012–2017	Thailand (Clade B/E)	162 women and men	ALVAC-HIV and AIDSVAX B/E		No vaccine efficacy	(47)
HVTN 306	2013–2020	Thailand (Clade B/E)	360 men and women aged 20–40 years	ALVAC-HIV and AIDSVAX B/E	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(48)
HVTN 097	2012–2013	South Africa (Clade B/E)	100 black Africans (men and women) aged 18–40 years	ALVAC-HIV (vCP1521) and AIDSVAX B/E	Induction of $CD4^+$ T cells directed to HIV-1 Env	No vaccine efficacy	(49)
HVTN 100	2015–2018	South Africa (Clade C)	252 men and women	ALVAC-HIV (vCP2438) and bivalent subtype C gp120/MF59	$CD4^+$ T-cell responses and gp120 binding antibody responses	No vaccine efficacy	(50)
HVTN 702	2016–2020	South Africa (702Clade C)	5,400 men and women	ALVAC-HIV (vCP2438) and bivalent subtype C gp120/MF59	Vaccine was unable to confer protection against HIV	No vaccine efficacy	(51)

MSM, men who have sex with men; IDUs, IV drug users.

second ALVAC-1 vector HIV vaccine was tested in Ugandan infants born to HIV infected mothers in a randomized placebo-controlled double-blind phase 1 trial. The vaccine construct expressed multiple genes such as *gp120*, the anchor region of *gp41*, *gag*, and *protease* (62–64). Even though the vaccine design was based on clade B genes, the researchers justified testing it in Uganda where clades A and D dominate based on the widespread cross-clade cellular immune responses observed in pre-clinical studies (63, 64). The results showed that the vaccine was safe in infants. Notably, vCP1521 was the prime employed in the Thai RV144 trial (discussed below) (45, 61).

VAX003 and VAX004 Efficacy Trials

1994 saw the emergence of two possible vaccine candidates (consisting of formulations of bivalent recombinant gp120 and alum) that were used in efficacy trials. These vaccine concepts were advanced to efficacy trials because they conferred protection to chimpanzees following HIV challenge and were safe and immunogenic in phase 1/2 clinical trials in humans (65, 66). The first two efficacy trials were carried out, from 1998 to 2003, by VaxGen in North America (VAX004; ClinicalTrials.gov Identifier: NCT00002441) and Thailand (VAX003; ClinicalTrials.gov Identifier: NCT00006327) (67–69). Based on the knowledge gained regarding genetic variability of HIV strains and the ability to use various co-receptors, the two initial candidate HIV vaccines were redesigned as bivalent gp120 vaccines (AIDSVAX B/B) for the North American trial and (AIDSVAX B/E) for the Thailand trial (the AIDSVAX B/E gp120 boost was also used in the RV144 trial) (45, 70). The two redesigned gp120 vaccines were derived from R5 and X4 strains (HIV strains using CCR5 and CXCR4 co-receptors, respectively) (71). The VAX004 efficacy trial recruited 5417 volunteers who were mainly men who have sex with men (MSM) in North America, and the VAX003 trial recruited 2545 volunteers comprising intravenous injection drug users in Bangkok, Thailand. Unfortunately, in 2003, data analysis revealed that the two vaccines did not prevent HIV acquisition and did not ameliorate disease (39, 40).

VACCINES DESIGNED TO STIMULATE T CELL IMMUNITY TO HIV

Multiple failures in antibody-based vaccines such as the VaxGen gp120 trials prompted the HIV vaccine field to begin pursuing T cell-based vaccines. There was a growing body of evidence demonstrating that $CD8^+$ T cell responses play a key role in controlling HIV infection. Animal studies showed that depletion of $CD8^+$ T cells in acute infection led to loss of virus control (72–75). Human studies also showed that the emergence of HIV-specific $CD8^+$ T cell responses coincided with the decline of viral load to a set point (76). Discovery of $CD8^+$ T cell driven viral mutations was another piece of evidence highlighting the importance of $CD8^+$ T cell responses in immune mediated control of HIV infection. Moreover, cumulative data showing $CD8^+$ T cell responses are largely responsible for spontaneous control of viremia for prolonged periods in the absence of medication, provided the impetus for the vaccine field to seriously pursue the development of T cell-based HIV vaccines (72, 77, 78). The candidate model vaccine vectors used for T cell-based vaccines were live recombinant viral vectors, mainly pox and adenovirus vectors (particularly the replication-defective adenovirus 5 [Ad5]), as well as DNA vaccines (79–81).

T Cell-Based Phase 2b Efficacy Trials

The first large T cell-based vaccine trial, the STEP trial also called HVTN 502 trial (ClinicalTrials.gov Identifier: NCT00095576), was a phase 2b multicentre, double-blind, randomized, placebo-controlled "test-of-concept" study that tested the efficacy of the MRKAd5 HIV-1*gag/pol/nef* vaccine. In December 2004, 3,000 participants were enrolled from Australia, Brazil, Canada, the Dominican Republic, Haiti, Jamaica, Peru, Puerto Rico, and the US where HIV subtype B is prevalent. The aim of the STEP trial was to determine whether the vaccine could prevent HIV infection, decrease the viral load in HIV-infected individuals, or both. Participants were randomly assigned to either vaccine or placebo groups, in a 1:1 ratio, to receive 3 injections of MRKAd5 HIV-1 *gag/pol/nef* vaccine or placebo (43, 44). The MRKAd5 HIV-1*gag/pol/nef* vaccine was also tested in a phase 2b trial (the Phambili/HVTN 503 trial; ClinicalTrials.gov Identifier: NCT00413725) in 801 adult South Africans. The goal of this trial was to evaluate the effectiveness of the vaccine in preventing infection in Southern Africa, where HIV subtype C is predominant. Unfortunately, vaccination and enrolment into both the STEP and Phambili trials were terminated in September 2007 following preliminary assessment that demonstrated no efficacy (24, 82). Furthermore, multivariate analysis of baseline risk factors revealed that the vaccination resulted in increased risk of HIV infection in some volunteers. In 2008, the final results of the trial were published, showing that cell-mediated immune responses elicited by this vaccine did not prevent HIV infection or blunt peak viral load (44). Additionally, there were slightly more HIV infections in the vaccine group compared to the placebo group in uncircumcised men with Ad5-neutralizing antibodies (43).

HVTN 505 (ClinicalTrials.gov Identifier: NCT00865566) was the next T cell-based phase 2b randomized, placebo-controlled, efficacy trial initiated in 2009 to test a potential DNA-primed vaccine in 2504 men or transgender women who have sex with men using a prime-boost regimen. The regimen consisted of three vaccinations with DNA encoding HIV clade B *gag*, *pol*, and *nef* as well as *env* from HIV clades A, B and C followed by an Ad5 vector-based vaccine encoding clade B *gag* and *pol* and *env* from clades A, B, and C (41, 42, 83). The experimental group received a DNA-primed vaccine injection on days 0, 28, and 56 followed by an Ad5 vector-based vaccine injection on day 168. The placebo group received placebo injections on days 0, 28, 56, and 168. Unfortunately, the trial was prematurely terminated after 47 months because interim analysis showed that the vaccine was not able to prevent infection or decrease viral load in vaccinated volunteers. Moreover, there was a slight increase in breakthrough infections in vaccine recipients compared to placebo controls (24).

The failure of conventional approaches to generate $CD8^+$ T cell-based vaccines prompted the scientific community to search for new approaches to HIV vaccine design. In 2008, NIAID encouraged researchers to go back to basics. A meeting convened by NIAID endorsed the expansion of research agenda to answer both basic science and novel vaccine design questions (24).

A VACCINE AGAINST HIV-1 IS POSSIBLE

The multiple setbacks in the HIV vaccine field led to substantial discussions regarding the optimal path toward a vaccine. The unexpected success of the RV144 trial (ClinicalTrials.gov Identifier: NCT00223080) showing modest but significant vaccine-induced protection (31.2% by 42 months) against HIV acquisition provided renewed hope that an HIV vaccine is possible. The RV144 was a randomized, double-blind phase 3 efficacy trial that utilized a recombinant canarypox vector vaccine, ALVAC-HIV (vCP1521), expressing Env (clade E), group-specific antigen (Gag) (clade B), and protease (Pro) (clade B), and an alum-adjuvanted AIDSVAX B/E and a bivalent HIV glycoprotein 120 (gp120) subunit vaccine (45, 46, 84). Vaccine recipients were given four priming injections of ALVAC-HIV (vCP1521) at months 0, 1, 3, and 6 with two booster injections of AIDSVAX B/E administered at months 3 and 6 (70). Immune correlates analyses revealed that the regimen induced HIV-specific humoral and cellular immune responses which resulted in reduced risk of HIV infection (inversely correlated with risk of HIV infection). Vaccine-induced responses included IgG antibodies binding to the HIV Env variable loops 1 and 2 (V1V2) and antibody-dependent cellular cytotoxicity (ADCC) in vaccine recipients with low IgA (70, 85–87). The magnitude and polyfunctionality of Env-specific $CD4^+$ T cells were also later shown to play a role in reducing the risk of HIV infection (70, 85–87).

Follow-Up Studies Based on the RV144 Trial

To date, only the RV144 trial has shown modest efficacy of 31.2% 42 months after the final vaccination. Rapid decline in protective antibody levels in most vaccine recipients led to the proposition that late boosts would induce durable protective immune response (88). Therefore, late boost studies (RV305 and RV306) were designed to assess immune responses generated in newly boosted vaccine recipients compared to RV144 vaccine recipients (47, 48).

RV305 Phase 2 Trial

The RV305 trial (ClinicalTrials.gov Identifier: NCT01435135) was a randomized, placebo-controlled, double-blind study conducted to investigate whether late boosts with ALVAC-HIV (vCP1521) or AIDSVAX B/E, administered either alone or in combination could enhance immune correlates of protection. The trial re-enrolled 162 healthy, HIV-negative Thai RV144 vaccine recipients to receive 2 additional boosts given 6–8 years after RV144 vaccination. Study participants were randomized into one of three groups to receive either vaccine or placebo. Group 1 received ALVAC-HIV and AIDSVAX B/E, group 2 received AIDSVAX B/E, and group 3 received ALVAC-HIV, or placebo, at weeks 0 and 24 (47). Results showed that plasma immunoglobulin G (IgG), IgA, and neutralizing antibody responses at week 2 were all significantly higher in groups 1 and 2 compared to the responses observed 2 weeks following the last RV144 vaccination. The boost also resulted in higher antibody titers against various Env antigens (such as gp120 and V1V2) which were above the levels observed at the peak RV144 vaccine time point in plasma and mucosal secretions (47, 89). While the antibody titers increased following the first boost, they did not increase following the second boost in RV305 vaccine recipients. Moreover, the administration of late boosts did not result in lasting antibody responses as they rapidly declined after boosting in all groups (47). Overall, it was concluded that administering late boosts to RV144 vaccine recipients (6–8 years following their last vaccination) was safe and well tolerated. In addition, it was observed that despite AIDSVAX B/E alone or in combination with ALVAC-HIV generating higher humoral and $CD4^+$ T cells responses in RV305 vaccine recipients, these responses were short-lived and subsequent boosts did not increase their magnitude (47).

RV306 Phase 2 Trial

The RV306 trial (ClinicalTrials.gov Identifier: NCT01435135) was a randomized, placebo-controlled, double-blind study conducted at three clinical sites in Thailand. This study was designed to determine whether boosting the RV144 regimen at either month 12, 15, or 18 following initial vaccination would improve the quality, magnitude or duration of humoral, cellular, and mucosal responses. In addition, it was designed to also establish the optimal boosting interval for further clinical development (48). The study enrolled 367 healthy, HIV-negative individuals and randomly allocated them to one of five groups to receive vaccine or placebo. All groups received the original RV144 vaccination regimen at months 0, 1, 3, and 6 as follows: ALVAC-HIV at months 0 and 1 followed by either ALVAC-HIV and AIDSVAX B/E or placebo at months 3 and 6. Group 1 received only the RV144 series and no additional boost, group 2 received additional ALVAC-HIV and AIDSVAX B/E or placebo at month 12, group 3 received AIDSVAX B/E alone or placebo at month 12, group 4a received ALVAC-HIV and AIDSVAX B/E or placebo at month 15, and group 4b received ALVAC-HIV and AIDSVAX B/E or placebo at month 18. No serious vaccine-related adverse events were reported across active groups. Furthermore, it was observed that groups with late boosts (groups 2, 3, 4a, and 4b) had increased peak plasma IgG-binding antibody levels against gp70 V1V2 relative to group 1 vaccine recipients with no late boost. It was also observed that boosting at month 12 (groups 2 and 3) did not increase gp120 responses compared with the peak responses after the RV144 priming regimen at month 6, but boosting at month 15 (group 4a) improved responses to gp120 A244gD–D11 and boosting at month 18 (group 4b) improved responses to both gp120 A244gD–D11 and gp120 MNgD–D11. In addition, boosting at

month 18 versus month 15 resulted in a significantly higher plasma IgG response to gp120 antigens but not gp70 V1V2 antigens. It was further observed that CD4 functionality and polyfunctionality scores following stimulation with HIV-1 Env peptides (92TH023) increased with delayed boosting. Additionally, the results showed that groups with late boosts had increased functionality and polyfunctionality scores relative to vaccine recipients with no late boost. Collectively, these results implied that additional boosting of the RV144 regimen with longer intervals between the initial vaccination and late boost could improve vaccine efficacy (48).

The promising results of RV144 clinical trials prompted the need to assess its efficacy against other clades (3, 90). RV144 originally designed to protect against HIV clade CRF01_AE BE infection in Thailand was modified to target HIV clade C. Therefore, HVTN designed and conducted a series of clinical trials including, HVTN 097, HVTN 100 and HVTN 702 (3).

HVTN 097 Phase 1b Vaccine Trial

The first vaccine concept tested by HVTN based on the RV144 concept was the HVTN 097 trial (ClinicalTrials.gov Identifier: NCT02109354), which was a randomized, controlled, double-blind phase 1b study done in South Africa (49). The trial was designed to assess the safety and immunogenicity of the vaccine regimen in healthy, HIV-uninfected South African adults. The regimen consisted of two prime doses of the experimental canarypox HIV vaccine ALVAC-HIV (vCP1521) followed by two booster shots of the AIDSVAX B/E. Study participants were randomized into three groups, in a 3:1:1 ratio, to receive the vaccine combined with tetanus and hepatitis B immunizations, the vaccine only or placebo. The tetanus and hepatitis B immunizations were included to assess possible cross-correlates of immune responses to HIV vaccine, however no significant differences in HIV immune responses were observed indicating that subsequent results were solely due to immune responses to HIV (49). The prime-boost vaccine regimen induced mostly Env-specific $CD4^+$ T cell responses at significantly higher levels compared to RV144 vaccine recipients (RV144 = 36.4%; HVTN 097 = 51.9%). IgG antibodies recognizing the V1V2 region and the IgG3 binding antibody responses to both gp120 and V1V2 antigens were also significantly higher among HVTN 097 vaccine recipients relative to RV144 recipients. ADCC antibody responses were also higher in HVTN 097 than in RV144, 72.6% (53 of 73) and 58.5% (114 of 195), respectively. These favourable results provided compelling rationale for conducting larger clinical trials in South Africa (49).

HVTN 100 Phase 1/2 Preventative Vaccine Trial

The HVTN 100 (ClinicalTrials.gov Identifier: NCT02404311) phase 1/2 randomized, controlled, double-blind study, was also conducted in South Africa to evaluate the safety, tolerability, and immunogenicity of the new modified vaccine regimen for subsequent efficacy testing. It consisted of an ALVAC-HIV (vCP2438) vector, expressing HIV Env gp120 (clade C ZM96), Env gp41, Gag, and Pro (all clade B) as well as a MF59-adjuvanted bivalent subtype C gp120 protein vaccine. The MF59 adjuvant was used to boost neutralizing antibodies and T cell responses (50). Enrolment took place from February to May 2015. Vaccine recipients received ALVAC-HIV (vCP2438) vector intramuscular (IM) injections at months 0 and 1 followed by co-administrations of ALVAC-HIV (vCP2438) and MF59-adjuvanted bivalent subtype C gp120 at months 3, 6, and 12 (50). The vaccine induced greater frequency of IgG3 responses to Env gp120, significantly higher $CD4^+$ T-cell responses and gp120 binding antibody responses compared to the RV144 regimen. Importantly, the IgG response exceeded the expected 63% threshold required for 50% vaccine efficacy that was calculated using a V1V2 correlate of protection model. Therefore, the HVTN 100 vaccine regime was advanced to a phase 2b/3 efficacy trial (HVTN 702) (50). It is important to note that despite the frequency of IgG3 and V1V2 antibody responses in HVTN 100 exceeding levels that were modeled to be required for protection, the correlate of reduced risk in RV144 was not response rate but rather the level of the antibodies (HIV-specific antibody responses resulting in reduced risk of HIV acquisition) (70, 85–87). Therefore, this criterion used for advancement to the phase 2b/3 HVTN 702 trial was not consistent with the findings of the RV144 trial.

HVTN 702 Phase 2b/3 Efficacy Trial

The HVTN 702 trial (ClinicalTrials.gov Identifier: NCT02968849) was a randomized, controlled, double-blind study conducted at 14 sites in South Africa from 2016 to 2020. The main objective was to assess the efficacy, safety, and tolerability of the ALVAC-HIV (vCP2438) plus bivalent Subtype C gp120/MF59 prime-boost vaccine regimen. The study enrolled 5407 HIV-uninfected sexually active individuals (both men and women), aged 18–35 years, and randomly allocated them to the vaccine or placebo arm. Vaccine recipients received an IM injection of ALVAC-HIV (vCP2438) at months 0 and 1 followed by co-administrations of ALVAC-HIV (vCP2438) + Bivalent Subtype C gp120/MF59 at months 3, 6, and 12 (51). The vaccine regimen employed in the HVTN 702 was modified to improve the efficacy and durability of the immune responses compared to RV144. Firstly, ALVAC-HIV vaccine construct contained clade C HIV genetic inserts to match those predominantly found in South Africa whereas the RV144 ALVAC contained clade B and E genetic inserts (those predominantly found in Thailand). Secondly, the boost in the HVTN 702 trial was a clade C genetically engineered HIV gp120 protein that was co-formulated with the MF59 adjuvant, whereas RV144 utilized the adjuvant alum. Thirdly, vaccine candidates in the HVTN 702 trial received five injections administered over a 12-month period (0, 1, 3, 6, and 12), whereas the RV144 vaccine candidates received four injections over a 6-month period (0, 1, 3, and 6). It was believed that a fifth dose at month 12 would potentially result in an extended protective effect (45, 51). Unfortunately, the trial was stopped on 23 January 2020 following an interim analysis by an independent data and safety monitoring board (DSMB). The DSMB analysed data from 2694 vaccine recipients and 2689 placebo recipients and

discovered that 129 HIV infections occurred among the vaccine recipients, and 123 HIV infections occurred among the placebo recipients (91, 92). These findings indicated that the vaccine was not effective in preventing HIV infection and the DSMB recommended discontinuation of further vaccinations but allowed follow-up to continue. This was a very disappointing and unexpected result given that prior studies showed the vaccine exhibited greater immunogenicity compared to RV144. The borderline statistical significance of the RV144 results of 31.2% protective effect has led others to question the veracity of the analyses (93). Clearly, more work is needed to understand the discrepancy between immunogenicity in the HVTN 100 study and the lack of efficacy in the HVTN 702 study; this will inevitably include revisiting the RV144 analyses.

CURRENT STATUS OF THE HIV VACCINE FIELD

During the past 30 years, only a few HIV vaccine regimens have been tested in phase 2b clinical trials (3, 24, 26, 36, 94). More recently, there has been strong advocacy for adaptive clinical trials aimed at accelerating vaccine development by rapid evaluation of vaccine candidates in small human studies and rapidly advancing promising candidates to efficacy trials (95–97). The new accelerated approach has resulted in more than 100 HIV vaccine concepts being clinically tested. Similar approaches have been adopted in the accelerated development of novel coronavirus disease 2019 (COVID-19), vaccines. Collaborative efforts such as the Pox-Protein Public Private Partnership (P5) which includes private industry, government agencies, the Bill and Melinda Gates foundation, and HVTN have contributed to accelerating vaccine trials through training and establishment of vaccine testing sites across the world. Additionally, partnerships between Janssen Pharmaceutical company, academic labs, and HVTN have championed the development and testing of mosaic-based vaccines. These private-public partnerships have led to the current rich pipeline of new vaccine concepts in preclinical trials and various stages of clinical trials. Although the vaccine field is trying to accelerate the extensive pipeline of vaccine concepts to efficacy trials, the decline of HIV incidence worldwide and the wider deployment of other HIV prevention tools such as Pre- exposure prophylaxis (PrEP) has complicated vaccine testing landscape by necessitating very large, more complex, and very expensive vaccine trial designs.

Passive Immunization Studies

For years, passive immunisation with protective antibodies has been used in the prevention and treatment of several bacterial and viral infections, subsequently influencing the current HIV vaccine field (98). The diverse mechanism of action of antibodies (through their interaction with the innate and adaptive arms of the immune system), coupled with their ability to bind and neutralize viruses, continues to make the antibody-based approach appealing to researchers (99, 100). The identification of various bNAbs with increased breadth and potency such as PG9, PG16, PGT121, PGT145, VRC01, VRC07, and 3BNC117 has provided an opportunity for their potential application in HIV vaccine research (101). Moreover, animal studies have demonstrated the protective and therapeutic properties of numerous bNAbs (102). Additionally, bNAbs have been shown to reduce viremia and delay viral rebound following ART interruption in HIV-infected individuals (103–105). However, it is unknown whether bNAbs are able to prevent HIV infection in humans (106). Hence, the need for the continued evaluation of the protective efficacy of passive immunization with bNAbs.

Early Passive Immunization Studies

The wealth of evidence from early passive immunization studies in animal models has revealed that the passive infusion of bNAbs resulted in protection from HIV infection (101). While several animal models have been used in passive immunisation studies, the most commonly used are Non-Human Primate (NHPs) and humanized mouse models (101). NHPs are typically infected with either simian immunodeficiency virus (SIV) or chimeric Simian/Human Immunodeficiency Virus (SHIVs), expressing the HIV Env in a SIV backbone (101). However, it has been shown that antibodies specific for the HIV Env protein are unable to neutralize SIV due to the difference in the HIV and SIV Env protein composition (102, 107). It is for this reason that SHIVs have been frequently used to infect NHPs, while humanized mouse models are directly infected with HIV in antibody protection studies (101, 102).

One of the earliest antibody protection studies in a SHIV challenge model, used polyclonal HIV IgG derived from HIV-infected chimpanzees. It was shown that the passive transfer of HIV IgG to pig-tailed macaques protected them from SHIV (based on the HIV DH12 strain) challenge (108). Another study showed that PGT121, a potent bNAb, protected monkeys from SHIV-SF162P3 challenge at serum concentrations that were lower than those previously observed (109). Several antibody protection studies evaluating the passive transfer of bNAbs in NHP models have demonstrated their ability to confer robust protection from HIV infection, even at low concentrations (97, 109–114). Furthermore, proof-of-principle studies of first-generation antibodies such as b12, that targets the CD4 binding site, in SHIV challenged monkeys have provided insight into the mechanism and durability of antibody protection (101, 115, 116). Similarly, antibody protection studies in mouse models have highlighted the protective efficacy of neutralizing antibodies (NAbs), such as b12 (117, 118) and BAT123 (119, 120). In addition, several studies have demonstrated that immunodeficient mice transplanted with human hematopoietic stem cells (hu-HSC) or bone marrow/liver/thymus (BLT) and passively immunized with bNAbs such as 2G12 (121), VRCO1 (122, 123), PG16 (124), and PG126 (125) were protected against HIV infection.

Overall, the passive infusion of SHIV challenged monkeys and HIV challenged humanized mice with bNAbs, particularly the potent second-generation antibodies, has provided evidence of

their ability to effectively protect against viral infection (101). While Fc receptor binding for antibody protection has proven to be important (126, 127), the specific mechanisms by which protection is rendered are not fully understood (101, 115). Nonetheless, phase 1 human studies previously conducted to evaluate the protective efficacy of bNAbs such as 3BNC117 and VRC01 have demonstrated short-term viral control (104, 128). The use of bNAb-based vaccines in human has generated tremendous interest and clinical trials are being conducted to investigate their ability to prevent HIV infection. For instance, antibody mediated prevention (AMP) studies are being conducted to test whether VRC01 can prevent HIV infection in men who have sex with men as well as heterosexual women (106).

Antibody Mediated Prevention Studies

Over the years, researchers have been studying and developing bNAbs as potential HIV vaccine candidates. Subsequently, the use of bNAb-based vaccines in human trials has generated tremendous interest and clinical trials such as the Antibody Mediated Prevention (AMP) studies (HVTN 703/HPTN 081 and HVTN 704/HPTN 085) are being conducted to test whether VRC01, a potent bNAb designed to target the $CD4^{+}$ binding site of the HIV-1 envelope glycoprotein, can prevent HIV infection in men who have sex with men as well as heterosexual women (106).

HVTN703/HPTN 081 Phase 2b Study

HVTN 703/HPTN 081 (ClinicalTrials.gov Identifier: NCT02568215) is a phase 2b randomized, controlled, double-blind study currently underway in sub-Saharan Africa. The study commenced in May 2016 and is expected to be completed by December 2020. This test-of-concept trial seeks to assess the safety, tolerability and efficacy of VRC01 in preventing HIV infection in healthy sexually active HIV-uninfected women. This AMP study has enrolled about 1900 HIV-uninfected sexually active women, aged 18-50 years, from several countries. Study participants, randomly allocated to one of three groups, in a 1:1:1 ratio, receive an intravenous (IV) infusion of 10 mg/kg VRC01 (low dose), 30 mg/kg VRC01 (high dose)m or placebo every 8 weeks (106, 129, 130).

HVTN704/HPTN 085 Phase 2b Study

The HVTN 704/HPTN 085 (ClinicalTrials.gov Identifier: NCT02716675) is another AMP study seeking to evaluate the safety, tolerability, and efficacy of VRC01 in preventing HIV-1 infection in healthy men and transgender (TG) men who have sex with men (MSM). The study commenced in March 2016 and the expected study completion date is February 2021. This study has enrolled 2701 HIV-uninfected men and transgender MSM in Brazil, Peru, Switzerland, and the United States (106, 130, 131). Participants in this study, like the HVTN 703/HPTN 081, were randomly allocated to one of three groups, in a 1:1:1 ratio, to receive a total of 10 IV infusion of 10 mg/kg VRC01 (low dose), 30 mg/kg VRC01 (high dose) or placebo every 8 weeks. The ultimate goal of the AMP trials is to identify and understand the characteristics of VRC01, such as optimal antibody concentration and effector functions, that correlate with protection against HIV infection (130).

CURRENT HIV VACCINE EFFICACY TRIALS

Currently, some of the ongoing phase 2b efficacy trials include HVTN 705/HPX2008 (Imbokodo study), HVTN 706/HPX3002 and PrepVacc. Janssen Pharmaceutical in collaboration with HVTN and academic labs are testing vaccine regimens that are designed to cover different types of HIV found across the world.

HVTN 705/HPX2008 (Imbokodo Study) Phase 2b Efficacy Trial

One such vaccine currently in efficacy trials is the HVTN 705/HPX2008 (Imbokodo study) (ClinicalTrials.gov Identifier: NCT03060629). This proof of concept study is a multicentre randomized, controlled, double-blind phase 2b/3 efficacy trial currently being conducted at 24 sites in 5 sub-Saharan African countries. This study commenced in November 2017 and is expected to be completed by May 2022. It aims to evaluate the efficacy, safety, and tolerability of a prime-boost regimen for the prevention of HIV infection. This study has enrolled 2600 healthy HIV-uninfected sexually active women, aged 18-35 years. Study participants, randomly assigned to either the experimental group or placebo group in a 1:1 ratio, received either the vaccine or placebo. The regimen consists of a tetravalent adenovirus vector vaccine, Ad26.Mos4.HIV (consisting of Ad26.Mos1.Gag-Pol, Ad26.Mos2.Gag-Pol, Ad26.Mos1.Env, and Ad26.Mos2S.Env clade C), and aluminium-phosphate adjuvanted clade C gp140 and Mosaic gp140 HIV protein vaccine. Vaccine recipients receive intramuscular (IM) injections of Ad26.Mos4.HIV at months 0 and 3 followed by IM injections of Ad26.Mos4.HIV and aluminum-phosphate adjuvanted clade C gp140 at months 6 and 12 whereas those in the placebo group will receive intramuscular injections of placebo. The primary endpoints will include; assessment of vaccine efficacy, number of participants with reactogenicity signs or symptoms, and adverse events (AEs). The secondary endpoints will include, immunogenicity, immune response biomarkers as correlates of risk of subsequent HIV acquisition, and genomic sequences of viral isolates from vaccine and placebo recipients (132, 133).

HVTN 706/HPX3002/Mosaico Phase 3 Efficacy Trial

Another mosaic-based vaccine concept currently in clinical trials is the HVTN 706/HPX3002 (ClinicalTrials.gov Identifier: NCT03964415), or Mosaico trail. This multicentre, randomized, controlled, double-blind phase 3 efficacy trial is currently underway in Europe, North America, and South America. It commenced in October 2019 and is expected to be completed by September 2023. This study seeks to assess the safety and efficacy of the Ad26.Mos4.HIV and adjuvanted clade C gp140 and Mosaic gp140 protein vaccine prime-boost vaccine regimen in healthy, HIV-uninfected MSM and transgender people. This study has enrolled approximately 3800 participants, aged 18-60 years, and randomly allocated to receive either the vaccine or placebo as outlined in the HVTN 705/HPX2008. The primary endpoint is to assess vaccine efficacy. Secondary endpoints are, to assess the

number of participants with solicited and unsolicited local and systemic adverse events (AEs), medically-attended adverse events (MAAEs) and SAEs, frequency and magnitude of HIV Env-specific humoral and cellular immune responses, antibody titers for Ad26, risky sexual behaviour, and Pre-Exposure Prophylaxis (PrEP) intake. Preliminary results reported at the International AIDS conference in Mexico city (IAS 2019), showed evidence of vaccine induced immune responses to different HIV strains circulating worldwide (133, 134).

PrepVacc Phase 2b Trial

Finally, the PrepVacc (ClinicalTrials.gov Identifier: NCT04066881) is another multicentre, randomised, controlled, double-blind phase 2b vaccine study currently underway in Mozambique, South Africa, Tanzania, and Uganda. The study seeks to evaluate the effectiveness/efficacy of a combination of two HIV vaccine regimens (DNA/AIDSVAX and DNA/CN54gp140+ MVA/CN54gp140) with PrEP (PrEPVacc). The study commenced in January 2020 and is projected to complete in March 2023. A total of 1668 healthy HIV-uninfected adults (18-40 years) are expected to be enrolled in the study and an equal number (278) will be randomised into one of six groups (i.e. Group A, B, C, D, E, and G) to receive either the vaccine regime or placebo with PrEP. The primary endpoints will include; evaluation of HIV infection in vaccine recipients and assessment of AEs associated with receiving either vaccine or PrEP regimes which may lead to the regimes being terminated. The ultimate goal is to determine if the vaccine leads to a decrease in HIV prevalence with adequate public health significance to justify implementation of the combination vaccine regimen (133, 135). A complete list of ongoing vaccine trials is illustrated in **Table 2**.

NEW HIV VACCINE CONCEPTS AND TECHNOLOGIES

Vaccines have been used for centuries to prevent and treat various diseases thereby saving millions of lives. Importantly, widespread vaccinations led to the successful eradication of smallpox and significant reduction in other infectious diseases such as polio and measles (136, 137). While modelling research show that a vaccine is essential to conclusively end the HIV epidemic (94), traditional vaccine formulations such as live attenuated and inactivated pathogens and subunit vaccines which offer robust protection against many deadly diseases, are not suitable for HIV vaccines (138). Moreover, cumulative evidence suggests that subunit vaccine designs do not elicit immune responses to levels required for protection, hence the need to utilize novel approaches such as targeted stimulation of broadly neutralizing antibody (bNAb) producing B cell precursors, novel viral vectors and combinatorial vaccine approaches.

Broadly Neutralizing Antibody (bNAbs) Vaccine Design

The most significant advance in the HIV vaccine field over the past decade has been the identification of bNAbs. Initially, the lack of evidence of antibody mediated suppression of HIV infection *in vivo* led to the belief that the human body was not capable of generating antibodies that can neutralize HIV. The discovery of human sera from HIV infected individuals that could neutralize a broad range of lab-adapted HIV-1 strains led to the rapid isolation and characterization of bNAbs (139–142). From then on, numerous bNAbs have been isolated, some of which can neutralize up to 99% of all known HIV-1 isolates (143). It is now well established that bNAbs responses can be generated during natural infection, but are quite rare and tend to occur much later in chronic infection (144, 145).

Isolation of bNAbs from HIV infected individuals motivated the design of immunogens capable of inducing bNAbs through vaccination. Over the years, bNAbs research has generated deeper insight into the virology and humoral immunity to HIV-1 infection. The wealth of knowledge gained has led to several HIV immunogen design approaches including germ-line targeting immunogens to molecular structural stabilization of envelope trimers such as eOD-GT8 (146, 147), the use of soluble trimers that mimic the native Env spike such as the BG505 SOSL664 (148), epitope targeted immunogen designs with minimal epitope fragments meant to focus the response on the right region of the Env spike, and minimize off target responses.

It has been shown that all bNAbs typically target the HIV envelope (Env) spike protein (149–151). However, HIV produces

TABLE 2 | Illustration of ongoing HIV vaccine trials.

Vaccine Trial	Year	Site	Target group	Vaccine	Immune response	Result	References
HVTN 703	2016-2020	Sub-Saharan Africa (Clade C)	1900 women	VRC01 broadly neutralizing monoclonal antibody	–	Pending	(106, 130)
HVTN 704	2016-2020	Brazil Peru Switzerland, United States (Clade B),,	2701 men and transgender persons	VRC01 broadly neutralizing monoclonal antibody	–	Pending	(106, 130)
HVTN 705	2017-2022	Sub-Saharan Africa (Clade C)	2600 women	Ad26.Mos4.HIV and adjuvanted clade C gp140 and Mosaic gp140 protein vaccine	–	Pending	(132, 133)
HVTN 706	2019-2023	Europe North America and South America (Clade C),,	3800 MSM and transgender persons	Ad26.Mos4.HIV and adjuvanted clade C gp140 and Mosaic gp140 protein vaccine	–	Pending	(133, 134)
PrepVacc	2020-2023	Mozambique South Africa, Tanzania, and Uganda (Clade C),	1668 Adults	DNA/AIDSVAX and DNA/CN54gp140 + MVA/CN54gp140) with PrEP.	–	Pending	(133, 135)

MSM, men who have sex with men; IDUs, intravenous drug users.

numerous non-functional Env proteins which divert antibody immune responses by displaying immunodominant epitopes, resulting in higher titers of non-neutralizing antibody responses (148, 152–154). Immunodominant epitopes tend to be easily accessible whereas most conserved epitopes (vulnerable sites) such as the CD4 binding site (CD4bs) have poor accessibility that limit bNAb recognition (154). One strategy used to increase the immunogenicity of immunodominant epitopes is epitope masking (155). Epitope masking is aimed at directing the immune response to sites of neutralization vulnerability by selectively allowing access to broadly neutralizing epitopes while masking immunodominant (non-neutralizing) regions (156). For instance, addition of glycans to mask immunodominant epitopes has been shown to reduce non-neutralizing antibody access (157, 158).

Another strategy to decrease the immunogenicity of non-neutralizing antibody (non-NAb) epitopes is the occlusion of immunodominant glycan holes. Serological studies conducted in an effort to get insight into the breadth of vaccine-elicited NAbs revealed that immunodominant strain-specific glycan holes on HIV Env contributed to the limited breadth of these monoclonal antibodies (mAbs) (157, 159–162). In addition, it has been shown that the addition of *N*-glycosylation sites to the V3 region or the glycan hole epitope at position 241/289 of the BG505 trimer suppressed the immunogenicity of its non-NAb epitopes while, in some instances, diverting the NAb responses to neoepitopes (163–165). Collectively, these novel strategies provide a platform for the optimization of the epitope targeted immunogen design approaches (97). Furthermore, advances in high throughput recombinant antibody technology has created the possibility of using bNAbs for prevention or treatment of HIV-1 infection as described above (166, 167).

While evidence has shown that cows immunized with BG505 SOSIP trimers rapidly developed broad and potent serum HIV specific NAb responses (168), large-scale bNAb research has not yet achieved the development of a vaccine that induces bNAb responses in other animal models or in humans (169, 170). Deep understanding of host and viral factors necessary and sufficient for the generation of bNAbs is imperative. The extensive Env diversity and the large glycan shield that cover the envelope trimer surface remain a major immunogen design challenge. Moreover, the extremely high somatic hypermutation required for bNAb function makes it very difficult to induce bNAbs by traditional vaccination protocols. To overcome the requirement for high mutation levels, novel approaches that involve priming the initial bNAb precursor B cells followed by sequential immunization aimed at driving the evolutionally intermediates are currently being evaluated in humans (171).

CMV-Based Vector Vaccines

Clearly, the limited immunogenicity of HIV is still a challenge. Most vaccines induce, weak, narrow, and short-lived immunity. Innovative approaches to overcome this limitation include the use of new viral vectors such as cytomegalovirus (CMV) or Ad26 viral vectors. CMV vectors have emerged as a type of virial vector with unique properties of inducing massive atypical immune responses that are capable of conferring sterilizing protection in animal studies (172). Special attributes of CMV vectored vaccines include the ability to maintain persistent immune stimulation and not being prone to attenuation by pre-existing immune responses. Also, CMV vector-induced HLA-E restricted $CD8^+$ T cell responses have the potential to provide vaccine efficacy in all individuals regardless of MHC-class I genotypes (173, 174).

CMV vectors represent a promising strategy in HIV vaccines because of the possibility of genetically reprogramming the vector to induce massive numbers of unusual $CD8^+$ T cell responses that can confer sterilizing immunity. The concept of genetic programming of the CMV vector to induce protective immune responses by vaccination was operationalized by Louis Picker and colleagues. In a series of studies, they used non-human primate models to demonstrate that CMV vectors can be genetically calibrated to induce MHC class II restricted as well as non-polymorphic MHC-E restricted $CD8^+$ T cell responses. Animals vaccinated with genetically engineered Rhesus CMV (RhCMV/) vectored SIV vaccines elicited very high frequencies of effector memory $CD8^+$ T cells that persisted in tissue sites and conferred stringent control of SIV/SHIV in 50% of the vaccinated animals without any apparent antibody responses (175–177).

Although CMV vectors show great promise, safety concerns regarding persistence and potential pathogenicity dampens enthusiasm to use such vaccines in humans. It is also not clear if humans can generate unconventional immune responses reported in animal studies. A study of HIV elite controllers showed that HLA-II restricted $CD8^+$ T cell responses exist in humans but are very rare (178), and highly unlikely to be elicited by vaccination. On the other hand, recent studies showed that HLA-E restricted responses are much more common in humans than previously appreciated, which opens up the possibility of inducing such responses by vaccination (179). To improve the safety of CMV vectors, the Picker group has managed to genetically modify CMV vectors to significantly reduce the capacity for the vector to widely disseminate while retaining its ability to superinfect, elicit, and maintain protective $CD8^+$ T cell responses (180). These safety improvements render support for testing CMV vectors in humans.

Combinatorial HIV Vaccine Design

This review has highlighted the inadequacy of standard vaccine approaches to elicit protective immunity against HIV. Therefore, it is imperative that future vaccines adopt multipronged approaches capable of eliciting more than one arm of the immune system. Examples of such approaches for HIV vaccines include the combination of HIV vaccines and non-vaccine approaches such as PrEP, microbicides or other non-vaccine HIV prevention methods. Non-human primate (NHP) studies have provided key proof-of-concept data (181, 182). The first NHP study of combined biomedical preventions (CBP), combined DNA prime recombinant adenovirus boost T cell-based vaccine with a vaginal microbicide gel (with a suboptimal concentration of an HIV-1 nucleocapsid zinc finger inhibitor) and administered the regime to rhesus macaques (183). CBP delayed simian-human immunodeficiency virus (SHIV)

infection in the break through animals and resulted in reduced viral load compared to individual treatments, highlighting strong synergy between the two approaches (183). Another study evaluated the combined effect of a tenofovir-containing vaginal gel co-administered with a protein-based HIV vaccine (containing proteins from clades B and C, adjuvanted with MF59) designed to induce T- and B-cell immunity. The vaccine alone did not offer protection against repeated $SHIV_{162P3}$ challenges, and 1% tenofovir alone showed an efficacy of 46%. But a combination of the vaccine with 1% tenofovir increased protective efficacy to 81% (182). Together, these studies demonstrate the potential of combinatorial approaches to HIV vaccine development and underscore the need for concerted efforts in pursuing such approaches.

CONCLUDING REMARKS

Although considerable progress has been made in understanding the structural and molecular biology of the virus, no HIV vaccine candidate has progressed to licensure, in spite of decades of very expensive research. Nonetheless, there is reason for cautious optimism that at least one of the many vaccine candidates, highlighted in this review, currently in pre-clinical or in efficacy trials will succeed. But, given the history of HIV vaccines, it is not far-fetched to think that success for the current vaccine candidates, if any, is most likely to be modest and will require several iterations to achieve significant protection. The glaring knowledge gap regarding the nature of immunity required for protection warrants basic science research. Given that an effective vaccine will need to stimulate more than one arm of the immune system, we recommend that combinatorial vaccine approaches capable of inducing innate and adaptive immune responses should actively be pursued. Finally, the huge cost of basic research, manufacture and regulatory processes required to take a vaccine candidate from the bench all the way to market, can only be achieved through collaborative efforts from all stake holders. Basic research scientists, vaccine trialists, governments and philanthropists, regulatory bodies, and pharmaceutical companies must continue to pull together to overcome this global challenge.

AUTHOR CONTRIBUTIONS

Conceptualization: ZN formulated the idea and provided oversight in planning and organizing the literature search. Writing (original draft preparation) was done by TNg'u and CC with the supervision of ZN. Writing (review and editing): TNg'u, CC, and ZN edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX C: MANUSCRIPT 2

Manuscript 2: Osmolar O. Baiyegunhi, Jaclyn Mann, Trevor Khaba, Thandeka Nkosi, Anele Mbatha, Funsho Ogunshola, **Caroline Chasara**, Nasreen Ismail, Thandekile Ngubane, Ismail Jajbhay, Johan Pansegrouw, Krista L. Dong, Bruce D. Walker, Thumbi Ndung'u & Zaza M. Ndhlovu: **CD8 lymphocytes mitigate HIV-1 persistence in lymph node follicular helper T cells during hyperacute-treated infection.** *Nature communications*.

Authors' contributions: ZMN, BDW, KD and TN initiated the study-cohorts. ZMN conceived the study. ZMN and OOB, designed the experiments. IJ and JP performed the lymph node biopsies. OOB performed digital droplet PCR, flow cytometry, viral RNA measurements and imaging experiments assisted by TK, ThaN, FO and I under the supervision of ZMN. OOB and ZMN analyzed the data. OOB, ZMN and JM wrote the manuscript. TN and BDW edited the manuscript. All authors approved of the manuscript before it was submitted and published in *Nature Communications*.

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CD8 lymphocytes mitigate HIV-1 persistence in lymph node follicular helper T cells during hyperacute-treated infection

Omolara O. Baiyegunhi[1,2], Jaclyn Mann[2], Trevor Khaba[2], Thandeka Nkosi[1], Anele Mbatha[2], Funsho Ogunshola[1,3], Caroline Chasara[1], Nasreen Ismail[2], Thandekile Ngubane[2], Ismail Jajbhay[4], Johan Pansegrouw[4], Krista L. Dong[3], Bruce D. Walker[1,2,3,5,6,7], Thumbi Ndung'u[1,2,3,8,9] & Zaza M. Ndhlovu[1,2,3]✉

HIV persistence in tissue sites despite ART is a major barrier to HIV cure. Detailed studies of HIV-infected cells and immune responses in native lymph node tissue environment is critical for gaining insight into immune mechanisms impacting HIV persistence and clearance in tissue sanctuary sites. We compared HIV persistence and HIV-specific T cell responses in lymph node biopsies obtained from 14 individuals who initiated therapy in Fiebig stages I/II, 5 persons treated in Fiebig stages III-V and 17 late treated individuals who initiated ART in Fiebig VI and beyond. Using multicolor immunofluorescence staining and in situ hybridization, we detect HIV RNA and/or protein in 12 of 14 Fiebig I/II treated persons on suppressive therapy for 1 to 55 months, and in late treated persons with persistent antigens. CXCR3$^+$ T follicular helper cells harbor the greatest amounts of *gag* mRNA transcripts. Notably, HIV-specific CD8$^+$ T cells responses are associated with lower HIV antigen burden, suggesting that these responses may contribute to HIV suppression in lymph nodes during therapy. These results reveal HIV persistence despite the initiation of ART in hyperacute infection and highlight the contribution of virus-specific responses to HIV suppression in tissue sanctuaries during suppressive ART.

[1] Africa Health Research Institute (AHRI), Durban, South Africa. [2] HIV Pathogenesis Programme, The Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa. [3] Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA, USA. [4] Prince Mshiyeni Memorial Hospital, Durban, South Africa. [5] Institute for Medical Sciences and Engineering and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA. [6] Howard Hughes Medical Institute, Chevy Chase, MD, USA. [7] Department of Immunology, Harvard Medical School, Boston, MA, USA. [8] Max Planck Institute for Infection Biology, Berlin, Germany. [9] Division of Infection and Immunity, University College London, London, UK. ✉email: zndhlovu@mgh.harvard.edu

Antiretroviral therapy (ART) does not eradicate HIV infection due in large part to early establishment and persistence of integrated proviruses in quiescent circulating and tissue reservoirs[1–3], which are resistant to drug or immune-mediated clearance. Additional mechanisms of persistence that involve ongoing replication have been suggested[4], including replication in germinal centers (GCs) within secondary lymphoid tissues, which have been described as sanctuary sites due to low $CD8^+$ T cell infiltration[5] and suboptimal penetration of antiretroviral drugs[6]. Ongoing virus replication, and to a lesser extent viral gene expression in the face of ART are contested concepts, with studies of individuals initiated on ART during chronic infection yielding conflicting results[6–12]. T follicular helper (Tfh) cells have been identified as a major source of persistent virus[13], but the precise subset of these cells enriched for HIV transcription on therapy within sanctuary sites is unknown. HIV persistence on ART is underscored by the nearly inevitable rebound of plasma viremia when treatment is interrupted, even after years of suppression, and is the major barrier to HIV cure[14].

It has previously been shown that very early initiation of ART can lead to prolonged remission when treatment is interrupted[15,16], but this is an infrequent occurrence. In most individuals, virus rebound occurs within weeks to months even in individuals initiated on ART during Fiebig stage I (hyperacute) HIV infection[14,17], despite rapid suppression of viremia and dramatically lower numbers of latently infected cells in peripheral blood (PB)[18–21]. Intriguingly, virus rebound kinetics following treatment interruption are heterogenous, sometimes taking a year or more[14,22]. The underlying immunological and virologic mechanisms responsible for the diverse viral rebound kinetics remain unknown. For instance, prolonged therapy in SIV infected macaques initiating therapy within 6 days of infection, prior to detectable plasma viremia, led to apparent elimination of infection after 600 days of suppressive ART in some animals[23]. However, such clearance of infection has not been observed in acute HIV infection, even in persons in whom therapy was initiated before detection of plasma viremia[20]. We previously showed that early treatment initiation enhances T cell functions in PB and limits viral diversity[24], but it is not clear if functional responses occur in tissues and whether such responses play a significant role in HIV suppression during ART. Limited access to tissue samples from persons initiating therapy before peak viremia has impeded a better understanding of the impact of early therapy on the lymphoid reservoir.

Here we analyzed 64 excisional lymph node (LN) biopsies and paired PB samples obtained from a well pedigreed cohort of individuals, where some initiated ART during hyperacute HIV infection. We investigated the impact of blunting peak viremia on the microanatomical location, cellular source and role of T cell responses on HIV persistence in LNs. Study participants were drawn from a unique hyperacute HIV infection cohort termed Females Rising through Education, Support and Health (FRESH). FRESH is a prospective study of uninfected 18–23-year-old women at high risk of HIV infection established at the epicenter of the HIV epidemic in South Africa, where yearly incidence rates approach 10%. The participants were offered PrEP when it became available in South Africa. Uptake was very high (90%) but retention was poor similar to other PrEP programs in the region. Despite vigorous prevention efforts, twice weekly monitoring for viral RNA has identified and treated (Tx) persons at the onset of plasma viremia, allowing for immediate institution of ART in many cases resulting in peak plasma viral loads that are sometimes <1000 RNA copies/ml and the preservation of $CD4^+$ T cell numbers[18]. Our results show that despite ART-induced blunting of peak viremia[18] and augmentation of functional HIV-specific T cell responses[24], HIV Gag p24 protein and viral RNA can persist in the LNs of Fiebig I/II Tx donors even after 4.5 years of fully suppressive ART, and these viral antigens are enriched in LN $CXCR3^+$Tfh cells. We also show that superior functioning T cell responses were associated with lower HIV antigen persistence in the LNs.

Results

Hyperacute HIV infection as a model to interrogate antigen persistence in lymph nodes. To determine the impact of immediate initiation of ART in hyperacute HIV infection (before peak viremia) on HIV clearance from sanctuary sites, we studied 14 women aged 18–26 who initiated ART during hyperacute HIV infection (Fiebig I/II Tx) and achieved full suppression of plasma viremia within a median of 15 days (range, 6–33). LNs were obtained by excisional biopsy after treatment for a median of 370 days (range, 19–1647). All remained fully suppressed except for one donor who had a transient viral load blip prior to LN excision. Five additional individuals identified in Fiebig stages III–V of infection and started on ART 1 day after diagnosis were also included. Three additional control groups were included: 13 HIV negative (HIVneg) donors; 17 individuals who initiated treatment in Fiebig VI and beyond (late Tx); and 15 untreated individuals whose duration on infection is unknown (unTx). Detailed characteristics of the cohorts are in Table 1. In total, 95% of the study participants were females.

Long-term persistence of HIV Gag p24 antigen in germinal centers (GCs) of individuals initiating antiretroviral therapy during hyperacute HIV-infection. To investigate HIV persistence in LNs of individuals initiating ART in Fiebig stages I/II, we measured HIV Gag p24 antigen in excisional LN biopsies by multicolor immunofluorescence (IF) staining of formalin-fixed paraffin-embedded LNs and imaging of tissue sections (Supplementary Fig. 1). The transcription factor BCL-6 was used to identify active GCs[25] (Supplementary Fig. 1a–d) and images were quantified for Gag p24 content using the algorithm for area measurements in TissueQuest (TissueGnostics)[26]. Figure 1a shows a representative image of HIV Gag p24 LN staining for a participant who was diagnosed in Fiebig stage I, initiated ART within 48 h and achieved persistent plasma viremia suppression within 33 days. The LN sample shown was obtained after 479 days of uninterrupted ART treatment with undetectable viremia, and depicts HIV Gag p24 antigen within a GC, which

Table 1 Characteristics of study participants at the time of lymph node excision.

Characteristic	Fiebig stage I/II treated	Fiebig stage III–V treated	Late treated[a]	Untreated	HIV negative
No. of participants (% female)	14 (100%)	5 (100%)	17 (94%)	15 (80%)	13 (100%)
Median (IQR) age of participants (years)	22 (20–24)	22 (19–24)	24 (22–27)	24 (24–29)	22 (21–23)
Median (IQR) CD4 Count (no. of cells/μl)	911 (717–1120)	942 (696–1086)	589 (454–792)	595 (401–720)	976 (792–1180)
Median (IQR) plasma viral load copies/ml	<20 (<20–<20)	<20 (<20–<20)	<20 (<20–<20)	11,000 (1900–22,000)	NA
Median (IQR) days on treatment	370 (31–550)	120 (46–677)	571 (100–762)	NA	NA

NA not applicable, *IQR* interquartile range.
[a]Donors whose Fiebig stage of infection was either Fiebig VI or unknown are defined as late treated.

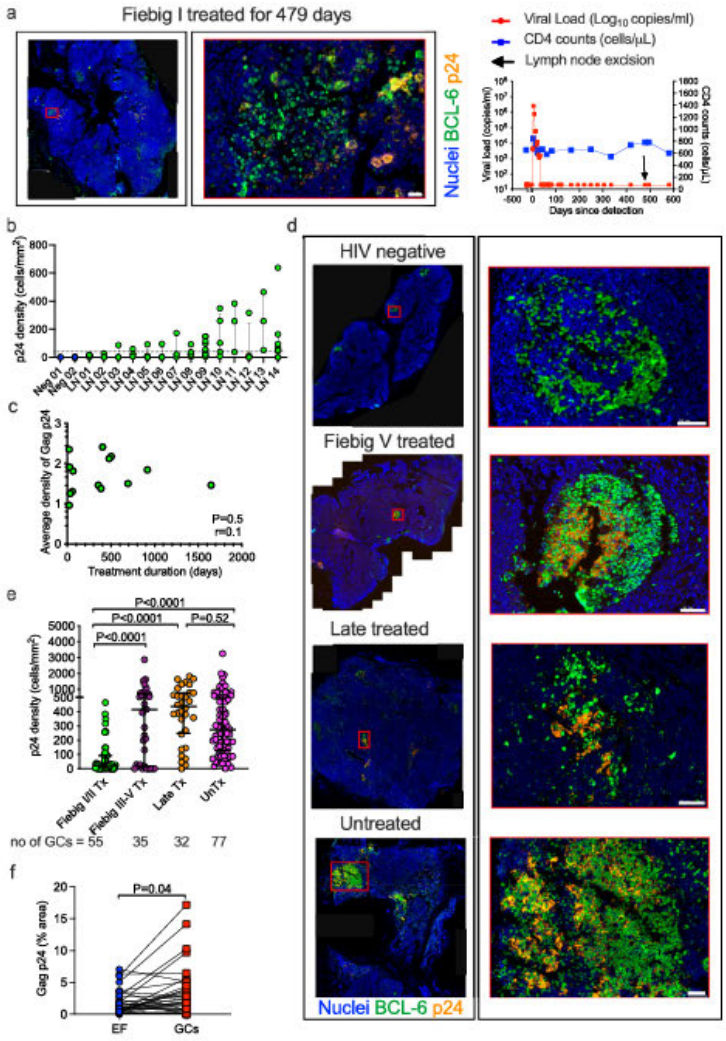


Fig. 1 HIV Gag p24 persists in germinal centers (GCs) despite early ART initiated in Fiebig stages I/II. a IF images of Gag p24 antigen (yellow) and BCL-6 (green) staining in lymph node (LN) sections and a scatter plot showing CD4+ T cell counts, plasma viral loads and the time of LN excision for a Fiebig I treated (Tx) participant. Nuclei are counterstained with DAPI (blue) and scale bar is 20 μm. **b** Gag p24 density per GC computed from TissueQuest (TissueGnostics, Vienna) analysis of IF LN sections (HIV negative, $n = 2$; Fiebig I/II Tx, $n = 14$), and **c** correlation analyses of average density of Gag p24 per donor and the treatment duration prior to LN excision. **d** Representative IF images of Gag p24 antigen (yellow) and BCL-6 (green) staining (scale bar is 50 μm) and **e** aggregate data from TissueQuest (TissueGnostics, Vienna) analysis of IF LN sections (Fiebig I/II Tx, $n = 14$; Fiebig III–V Tx, $n = 3$; late Tx, $n = 8$; and untreated (unTx), $n = 13$). Each dot represents the density of Gag p24 per GC and the total number of GCs analyzed per group is displayed. **f** Comparison of the total percentage area staining for Gag p24 within GCs and outside the GCs (EF) for all donors ($n = 36$). Three independent experiments were conducted with similar results. Error bars represent interquartile range. Data are presented as median ± interquartile range. All statistical tests are two-sided. Two-tailed p values from Mann–Whitney U test (**f**) or adjusted p values from Dunn's multiple comparison's test (**e**) are shown. Spearman rho (r) values and p values are reported for correlation analyses (**c**). Dotted line denotes threshold of detection (**b**). Source data are provided as a source data file.

was present in 5 of the 7 GCs examined in this LN (Supplementary Fig. 1e).

Gag p24 staining and imaging were conducted on a total of 14 LNs from fully suppressed Fiebig I/II Tx donors obtained at a median of 370 days (range, 19–1647 days) post-ART initiation. Twelve of 14 (86%) of these Fiebig I/II Tx donors had detectable HIV Gag p24 in at least one GC, and overall 42 of 55 GCs evaluated (76%) were positive for Gag p24. In two Fiebig I/II Tx donors there was no detectable HIV Gag p24 despite examining more than more than four GCs (Fig. 1b). In those with detectable HIV Gag p24, quantitative image analysis revealed no correlation between the amount of HIV Gag p24 present in the LN tissue section and treatment duration prior to LN excision (Fig. 1c). Notably, regardless of treatment duration, Fiebig I/II Tx donors had significantly less detectable HIV Gag p24 compared to Fiebig III–V Tx ($p < 0.0001$), late Tx ($p < 0.0001$) and unTx ($p < 0.0001$) donors, though there was considerable overlap (Fig. 1d, e and Supplementary Fig. 1f, g). This result was also consistent in a subset of donors Tx beyond 1 year (Supplementary Fig. 1h). Quantitative image analysis of all treated LNs revealed a trend of greater area percent of Gag p24 staining in some GCs compared to extrafollicular areas of the tissue ($p = 0.04$, Fig. 1f and Supplementary Fig. 1i). Together, these data demonstrate that early ART initiation in Fiebig stage I/II limits the magnitude of HIV Gag p24 antigen in LNs, but that Gag p24 can persist predominantly in follicular areas even after 4.5 years of fully suppressive treatment.

Lymph nodes of Fiebig I/II treated individuals harbor HIV-1 RNA. To determine if viral RNA transcription was occurring, which is required to produce infectious virions, we used an in situ hybridization (ISH) assay called RNAscope[27] to probe for HIV-1 *gag-pol* RNA within LN sections. 12 Fiebig I/II Tx, 4 Fiebig III–V Tx, 2 late Tx, 4 unTx and 3 HIVneg LN samples were analyzed based on sample availability. Viral RNA was detected as punctate dots in LNs from all HIV-infected persons and there were no signals in the HIVneg controls (Fig. 2a, b and Supplementary Fig. 2). Productively infected viral RNA$^+$ cells were identified as a dense spherical signal, whereas follicular dendritic cell (FDC)-bound virus particles were defined by a diffuse lattice-like pattern consistent with previous reports[28]. Combined RNAscope® ISH *gag-pol* staining with IF staining for CD4$^+$ T cells confirmed viral RNA (green) within CD4$^+$ T cells (red, Fig. 2c). RNAscope staining was quantified using Fiji[29]. Ten of 12 Fiebig I/II Tx donors had detectable but significantly lower amounts of HIV RNA compared to late Tx ($p = 0.04$) and unTx ($p = 0.001$) donors (Fig. 2d). However, there was no difference in RNA density between Fiebig I/II Tx and Fiebig III–V Tx donors. Notably, there was a positive correlation between Gag p24 density measured by IF and *gag-pol* RNA measured by in situ hybridization in Fiebig I/II Tx donors ($p = 0.002$; $r = 0.8$, Fig. 2e). The results are consistent with the persistence of viral RNA despite very early ART initiation in hyperacute infection and durable plasma virus suppression.

Discordant HIV-1 RNA loads in plasma and lymph nodes. To better define active virus transcription within LN mononuclear (LNMCs) cells and to determine the viral loads in the LNs of aviremic individuals initiated on treatment either very early or later in infection. We measured cell-associated viral loads in LNMCs using a commercial viral load assay Cobas® Ampliprep HIV-1 test. We found a hierarchy of LNMC viral loads with the values lowest in patients that initiated therapy in Fiebig stages I/II (Fig. 2f). Interestingly, neither the peak plasma viral load (Fig. 2g), treatment duration before LN excision (Fig. 2h), nor the time to suppression (Fig. 2i) impacted viral RNA persistence in the LN. Overall, quantifiable amounts HIV RNA persists in the LNs of most Fiebig I/II Tx individuals and the magnitude of LN viral loads was not dependent on the duration of treatment.

Expansion of GCTfh cells in early ART-treated individuals. Identifying the cellular phenotypes of persistent HIV-1 protein and transcripts during therapy will be critical for future anti-HIV interventions. While follicular T helper (Tfh) cells are a key component of the adaptive immune response to HIV-1 infection and provide cognate help to B cells[30,31] and CD8$^+$ T cells[32,33], these cells also serve as a major HIV reservoir[13,34]. Moreover, HIV antigen can be trapped in the follicular dendritic lymphoreticular network within LNs and persist for years[35], thus we interrogated persistence within these cell subsets.

Given that Tfh are major targets of HIV infection, we first sought to determine the extent to which early ART mitigates HIV-induced Tfh expansion. We defined GCTfh as CD4$^+$CD45RA$^-$CXCR5^{hi}PD-1^{hi} and nonGCTfh cells as CD4$^+$CD45RA$^-$CXCR5$^+$PD-1$^+$ in LNMCs (Fig. 3a and Supplementary Fig. 3a) consistent with previous Tfh studies[36]. HIV negatives ($n = 9$) had very low frequencies of GCTfh cells (median 1.3%, IQR; 0.6% to 1.5%) of antigen experienced (CD45RA$^-$) CD4$^+$ T cells whereas nonGCTfh cells were 11% (IQR; 8.5% to 13%, Fig. 3b). HIV infection resulted in significant expansion of GCTfh (Fig. 3c). Treatment initiation impacted the extent of GCTfh expansion. Immediate therapy was associated with significant diminution of GCTfh expansion (Fiebig I/II Tx vs. unTx $p = 0.002$), which was comparable among all treatment groups ($p =$ ns, Fig. 3c). Notably, HIV-induced Tfh expansion was restricted to GCTfh, as no significant expansion of nonGCTfh cells were observed between the groups (Fig. 3d). To verify these observations, we quantified the area of GCs and area densities of GCTfh in situ using FFPE LNs (Fig. 3e–g). Consistent with flow cytometry data GCs and GCTfh cell densities were expanded in HIV infection and significantly greater in unTx infection compared to Fiebig I/II Tx and HIV negative controls (Fig. 3f, g). The numbers of GCs per patient (Supplementary Fig. 3b, c, e) and the average area of GCs (Supplementary Fig. 3d, f) correlated with HIV p24 and RNA measurements in tissue (Supplementary Fig. 3c–f). Furthermore, comparisons of cell phenotype markers of activation or inflammation (Supplementary Fig. g–j) as well as the CD4$^+$/PD1$^+$ ratio of cells within GCs (Fig. S3k), among the treatment groups were inconclusive. Together, these data show that early treatment initiated in Fiebig I/II mitigates HIV-induced GCTfh expansion. Reduced HIV targets in GCs might partly explain reduced HIV persistence in LN of individuals who initiate therapy early.

To gain more insight on cellular targets of HIV infection in LNs, we investigated if there was a particular subset of GCTfh that was selectively expanded. We quantified previously described[37,38] GCTfh subsets namely; GCTfh1 defined as CXCR3$^+$CCR6$^-$, GCTfh2 as CXCR3$^-$CCR6$^-$, double positive (dp)GCTfh as CXCR3$^+$CCR6$^+$ and R6$^+$GCTfh defined as CXCR3$^-$CCR6$^+$ (Fig. 3h) among our study groups (Fig. 3i) and determined their relationship with HIV Gag p24 densities. While subsets had varying frequencies (Fig. 3i), within the Fiebig I/II and III–V Tx donors, there was a trend of higher frequency of GCTfh1 being associated with greater degrees of Gag p24 positivity ($p = 0.08$; $r = 0.5$) (Fig. 3j), whereas R6$^+$GCTfh displayed a weak negative association ($p = 0.08$; $r = 0.5$) (Fig. 3k). Overall, these results show that while early treatment mitigates GCTfh responses, subset distribution of Tfh cells might impact virus persistence in early treated LNs.

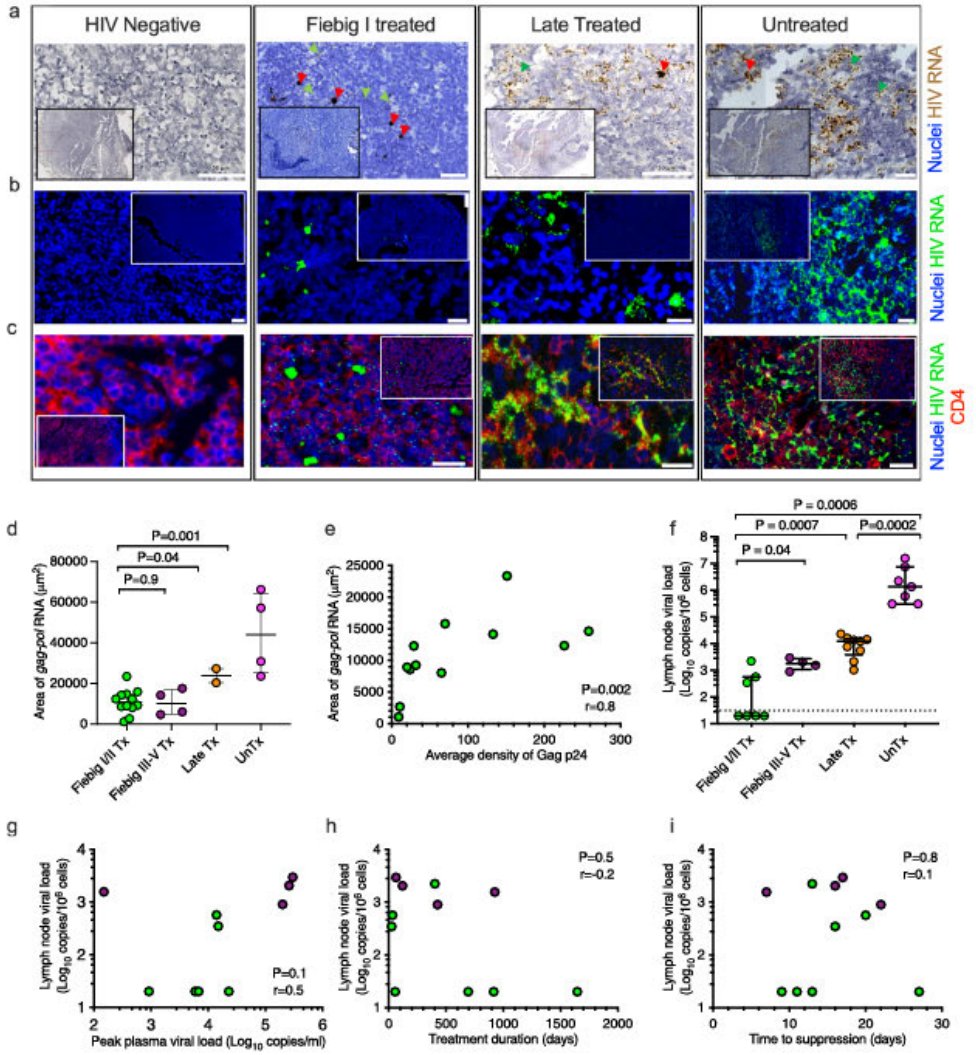


Fig. 2 HIV-RNA persistence in lymph nodes of Fiebig I/II treated individuals. HIV-RNA detection in lymph nodes (LN) of Fiebig I/II treated individuals using RNAscope (**a**–**e**) and Cobas® AmpliPrep HIV-1 test (**f**–**i**). **a** RNAscope hybridization for HIV *gag-pol* RNA was detected using 3, 3′-diaminobenzidene (DAB, brown) or **b**, **c** fluorescent Opal polymers (green). Representative images for HIV negative, Fiebig I/II treated (Tx), late Tx and untreated (unTx) HIV-infected LN sections are shown. Single RNA transcripts are seen as punctate dots; clusters of transcripts are also observed. Red arrowheads identify HIV RNA$^+$ cells and green arrowheads identify virions on follicular dendritic cells. **c** Images showing multiplexed RNAscope *gag-pol* hybridization (green) coupled with IF staining for CD4$^+$ cells (red). Three independent experiments were conducted with similar results. Scale bars are 50 µm (**a**) or 20 µm (**b**, **c**). **d** RNA signals quantified in micrographs using Fiji [(ImageJ software, Fiebig I/II Tx, $n = 12$; Fiebig III–V Tx, $n = 4$; late Tx, $n = 2$; and unTx, $n = 4$). Five fields of view are analyzed per sample and averaged. **e** A correlation analysis of area staining of *gag-pol* RNA and Gag p24 density for Fiebig I/II Tx LNs. **f** Viral RNA loads are quantified in lymph node mononuclear cells (LNMCs) (Fiebig I/II Tx, $n = 7$; Fiebig III–V Tx, $n = 4$; late Tx, $n = 9$; and unTx, $n = 7$). Viral loads below the limits of detection of the assay are assigned a value of 20. Correlation analysis of LNMCs' viral loads with **g** peak plasma viral loads, **h** treatment duration, and **i** time to suppression for Fiebig I/II Tx, $n = 7$; and Fiebig III–V, $n = 4$; donors. All statistical tests are two-sided and p values are from the Mann–Whitney U test (**d**, **f**). Spearman rho (r) values and p values are reported for correlation analyses (**e**, **g**–**i**). Dotted line denotes threshold of viral load detection. Error bars represent interquartile range (**d**, **f**). Data are presented as median and interquartile range (**d**, **f**). Source data are provided as a source data file.

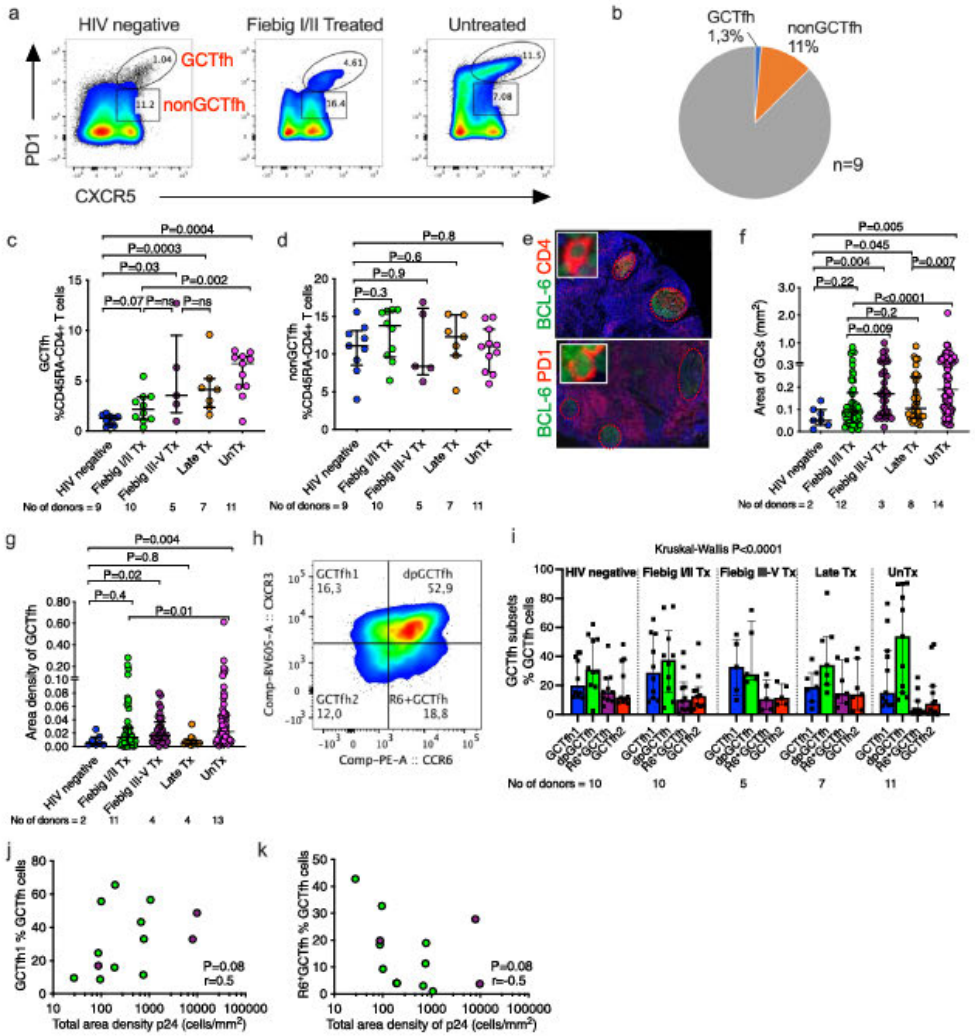


Fig. 3 Expansion of Germinal center T follicular helper (GCTfh) cells during HIV-1 infection. a Representative flow cytometry plots, **b** pie chart and **c** summary plots comparing the proportions of GCTfh (CXCR5^{hi}PD-1^{hi}), and **d** nonGCTfh (PD-1^{+}CXCR5^{+}) cells in HIV negative (HIVneg), Fiebig I/II treated (Tx), Fiebig III–V Tx, Late Tx and untreated HIV-infected (unTx) groups. **e** Representative images showing lymph node sections stained with BCL-6 (green) to define germinal centers and CD4 (red), or PD-1 (red) to localize GCTfh cells. Three independent experiments were conducted with similar results. Aggregate results for the (**f**) area of GCs and (**g**) area density of GCTfh cells computed using TissueQuest (TissueGnostics, Vienna) software. **h** Representative flow cytometry plot showing gating for Tfh subsets and **i** aggregate data across the groups. **j** Correlation analysis of Gag p24 density measured using image cytometry with GCTfh1 (CXCR3^{+}CCR6^{-}) and **k** R6^{+}GCTfh (CXCR3^{-}CCR6^{+}) subsets distribution measured using flow cytometry ($n = 13$). All statistical tests are two-sided and p values are from the Mann–Whitney U test (**c**, **d**, **f**, **g**, **i**). Spearman rho (r) and p values are reported for correlation analysis (**j**, **k**). The number of donor samples analyzed in each group is indicated. Error bars represent interquartile range. Data are presented as median and interquartile range (**c**, **d**, **f**, **g**, **i**). Source data are provided as a source data file.

Lastly, given the notion that HIV-specific CD4^{+} T cells might be more susceptible to infection and contribute to viral persistence[39], we used class II tetramers to characterize HIV-specific Tfh responses in LN tissues. DRB1*11:01 and DRB1*13:01 class II tetramers previously described[38,40] were used to identify HIV-specific GCTfh and nonGCTfh cells in 3 Fiebig I/II Tx, 2 late Tx and 4 unTx donors within our cohort expressing the class II DRB1*11:01 and DRB1*13:01 alleles (Supplementary Fig. 3l, m). These results demonstrate that HIV-specific Tfh responses are induced during early Tx HIV infection.

However, we did not have sufficient tetramer$^+$ data to determine if antigen specific CD4$^+$ T cells are preferentially infected.

CXCR3$^+$ Tfh cells harbor a greater burden of persistent HIV RNA in lymph nodes obtained from treated individuals with sustained plasma viral suppression. Since image analysis for Gag p24 indicated that most of the HIV antigen was confined within discrete regions of GCs, and flow data showed differential correlation between levels of Gag p24 and GCTfh subsets, we next stained for other markers shown to be highly expressed on human Tfh[38]. We also used FDC markers to identify residual Gag p24 that has been reported to persist on FDCs[35]. IF imaging of serial sections stained with different combinations of antibodies and detected with Opal fluorophores revealed that Gag p24 co-localized with several phenotypic markers (Fig. 4a and Supplementary Fig. 4), including PD1 (Fig. 4a, i), CD4 (ii), CXCR3 (iii, iv), CCR6 (iv) and FDC (v). To more definitively identify the Tfh subset that habored the most HIV infection burden, we quantified HIV RNA in LNMCs isolated from LN tissue of 3 Fiebig I/II Tx, 2 Fiebig III–V Tx and 3 late Tx donors and FACS-sorted into the 4 different Tfh subsets (Fig. 4b). HIV mRNA was detectable using digital droplet PCR in all the subsets (Fig. 4c). Importantly, when we analyzed the cells based on expression of chemokine receptors, CXCR3 and CCR6, we found that CXCR3$^+$ Tfh subsets harbored significantly greater amounts of HIV RNA than other subsets ($p = 0.003$, Fig. 4c).

To further interrogate preferential infection of CXCR3$^+$ Tfh cells, we used a broadly neutralizing antibody called 3BNC117 to stain HIV-infected cells expressing the HIV envelope protein (gp120), while simultaneously staining for CXCR3. 3BNC117 targets the CD4 binding site on the surface of HIV-1 Envelope (Env) glycoprotein[41]. Assay validation showed clear 3BNC117 staining of LNMCs that were infected with NL4-3 in vitro for 7 days compared to uninfected control (Fig. 4d). Further validation showed ex vivo staining of LNMCs of a viremic donor with no staining observed for two HIV negative donors (Fig. 4e). Having validated the assay, we performed ex vivo staining of seven paired LNMC and PBMC samples obtained from seven Fiebig I/II treated donors. Representative flow plots for one donor and aggregate data for seven donors showed detection of HIV-1 Env (3BNC117) positive LNMCs at significantly greater frequency compared to paired PBMC samples ($p = 0.03$, Fig. 4f). To confirm detection of low frequency HIV-1 positive cells ex vivo, we intracellularly stained aliquots of the same samples with anti-Gag p24 antibody. Similarly, Gag p24$^+$CD4$^+$ T cells were readily detectable in LNMCs compared to PBMCs ($p = 0.01$, Supplementary Fig. 5a). We phenotyped infected cells by dual staining of 3BNC117 and CXCR3 and observed a trend toward more Env$^+$CD4$^+$ T cells co-expressing CXCR3 ($p = 0.06$, Fig. 4g) than those not expressing CXCR3. Together, these data suggest that CD4$^+$CXCR3$^+$ expressing Tfh cells may be preferentially infected in vivo compared to other subsets.

Impact of HIV-specific CD4$^+$ and CD8$^+$ T cell responses on HIV persistence in the lymph node during ART. We previously showed that immediate ART initiation augments HIV-specific T cell function in PB[24]. To investigate the effects of early ART on LN responses, we begun by investigating if there were compartmental differences in the frequency of HIV-specific responses between LN and PB. We used intracellular cytokine staining (ICS) to measure the proportions of HIV-specific CD4$^+$ and CD8$^+$ T cells in LNs and paired blood samples using 14 fully suppressed Fiebig I/II Tx donors on uninterrupted therapy for greater than a year. Representative flow plots for one donor and aggregate data show significantly higher frequency of PB Gag-specific CD8$^+$ T cells ($p = 0.05$) compared to LN responses (Fig. 5a). HIV-specific CD4$^+$ T cell frequencies also trended toward greater frequencies in PB relative to LN ($p = 0.06$; Fig. 5b). Next, we investigated whether HIV-specific CD8$^+$ T cell responses limit HIV persistence in the LN, and found a negative correlation between the frequency of HIV-specific LN CD8$^+$ T cell responses and HIV Gag p24 density ($p = 0.02$, $r = -0.7$; Fig. 5c). There was no correlation observed between peripheral CD8$^+$ T cell responses and the amount of persistent Gag p24 antigen in the LN, suggesting the peripheral responses may not accurately depict HIV persistence in LNs. Notably, there was no correlation between LN or peripheral CD4$^+$ T cell responses and persistent HIV Gag p24 in the LN (Fig. 5d).

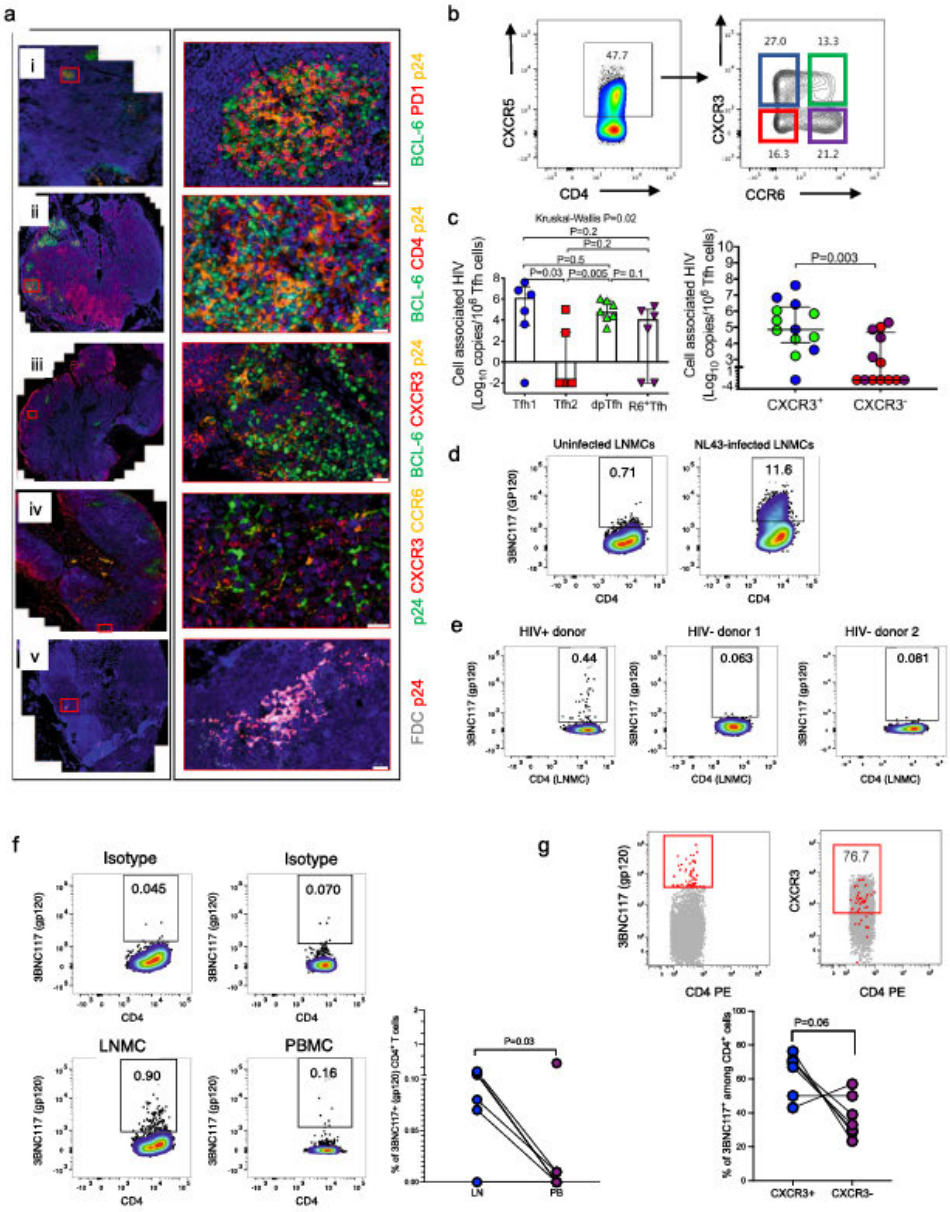
Considering that proliferative CD8$^+$ T cell responses are often associated with protection[42,43], we next measured virus-specific responses by carboxyfluorescein succinimidyl ester (CFSE) dilution. Representative flow plots for a donor with low and a donor with high Gag p24 density are shown (Fig. 5e). Aggregate data show proliferative Gag-specific CD8$^+$ and CD4$^+$ T cell responses negatively correlated with HIV Gag p24 burden (both CD8 and CD4, $p = 0.04$, $r = -0.7$; Fig. 5f). Together, these data show an association between maintenance of functional cellular responses and reduced HIV viral antigens in LNs.

Given most of the residual virus was concentrated within GCs, we next assessed the capacity of HIV-specific T cell responses to traffic into the GCs by enumerating the frequencies of CXCR5$^+$ HIV-specific responses in LN, which denote capacity to migrate into GCs. Representative flow plot and aggregate data show lower CXCR5$^+$ HIV-specific (gamma secreting) CD8$^+$ T cells compared to CXCR5$^-$ CD8$^+$ T cells among Fiebig I/II and III–V treated donors ($p = 0.001$), suggesting reduced capacity of CD8$^+$ T cells to migrate into GCs (Fig. 5g). These data are consistent with our recent publication where we showed by IF imaging that CD8$^+$ T cells are largely excluded from GCs[44]. These data partly explain the observed greater HIV antigen burden in GCs relative to extrafollicular areas in some donors. Additionally, there was no correlation between plasma CXCL-13 (part of the CXCR5-CXCL-19 axis crucial for recruitment of immune cells into GCs) and density of HIV antigens in LNs (Supplementary Fig. 5b). Together, these data show that reduced functional HIV-specific CD8$^+$ T cell responses within GCs might contribute to HIV persistence in this tissue microenvironment.

Discussion

Comprehensive understanding of ART-mediated HIV suppression in tissue sanctuary sites is critical to the design, optimization, and evaluation of curative strategies. Moreover, a therapeutic vaccine for HIV-1 infection would need to induce robust anti-HIV immune responses in ART suppressed individuals to mediate post-treatment viral control. Here, we used a very well characterized cohort of persons with hyperacute HIV infection to conduct a comprehensive analysis of HIV persistence in LNs following ART initiation in Fiebig stage I/II and to elucidate Tfh cell responses which are critical for robust B cell and CD8$^+$ T cell functions.

Most donors exhibited persistent HIV antigens in LN despite prompt blunting of initial peak viremia and sustained plasma viral suppression for as long as 55 months, suggesting that early therapy initiation may not fully eradicate persistent virus in lymphoid tissue sites. Immediate therapy reduced GCTfh expansion which is typically associated with dysregulation of B cell responses due to excessive GC reactions in unTx HIV infection[36,45]. Moreover, mitigated GCTfh responses decreased the number of cellular targets of HIV infection. Importantly, the association between functional



immune responses and reduced viral burden in LNs indicates that T cell responses contribute toward elimination of infected cells during therapy. Combined, these data highlight the need to prioritize elimination of active HIV persistence in LNs as a critical step to achieving a cure or prolonged HIV remission off therapy.

Our unique ability to obtain excisional LN biopsies in the FRESH cohort allowed for characterization of sites of virus persistence within the LN architecture in persons in whom peak viremia is blunted. The topological analysis of persistent HIV antigens within intact LN tissues identified greater HIV protein antigen burden within B cell follicles in some patients. Notably,

Fig. 4 CXCR3$^+$ Tfh cells contribute to HIV persistence in treated hyperacute HIV-1 infection. Representative IF images characterizing HIV Gag p24$^+$ cells in the germinal centers (BCL-6$^+$, green). **a** The co-localization of Gag p24 antigens (yellow color in (i–iii), green in (iv), red in (v)) with cells expressing (i) PD1$^+$ in red, (ii) CD4$^+$ in red, (iii) CXCR3$^+$ in red, and (iv) CCR6$^+$; yellow color, surface markers and (v) follicular dendritic cells (FDC, gray color) are assessed by immunofluorescence microscopy. Green, red, and yellow signals are from Opal fluorophores 520, 570 and 690 (PerkinElmer) and nuclei are counterstained with DAPI (blue). **b** Representative flow cytometry plots showing gating for FACs-sorted Tfh subsets. **c** HIV mRNA quantified in FACs-sorted Tfh subsets (from $n = 8$ donors) using digital droplet PCR. Absolute numbers of quantified HIV transcripts are equated to absolute cell numbers determined using the expression of β2M. Amounts of HIV mRNA within CXCR3$^+$ and CXCR3$^-$ subsets are also compared. **d** In vitro NL4-3 infected and uninfected LNMCs surface stained with 3BNC117 monoclonal antibody. **e** Flow plots of showing ex vivo 3BNC117 LNMC staining for one HIV positive and two HIV negative donors. **f** Representative flow plot and aggregate data show proportion of 3BNC117$^+$ CD4$^+$ T cells in LNMC and paired PBMCs for seven donors. **g** Representative flow plot and aggregate data show proportion of 3BNC117$^+$ CD4$^+$ T cells that either co-express or do not express CXCR3. Statistical differences are calculated using Mann–Whitney *U* (**c**, **f**, **g**) and Kruskal–Wallis (**c**) tests and all statistical tests are two-sided. Error bars represent interquartile range (**c**). Data are presented as median and interquartile range (**c**). Source data are provided as a source data file.

onset and duration on therapy did not significantly affect the amount of detectable Gag p24 protein, consistent with the notion of rapid HIV reservoir establishment followed by very slow decay rate[46]. Moreover, there are variable decay dynamics between active and latent HIV reservoirs. Active HIV reservoirs which are majorly responsible for low-level viremia during ART have been implicated in higher virological failure rates, persistent immune activation and inflammation[47]. Thus, the need for identifying all sources of persistent virus during ART. Importantly, our data suggest that LN GCs may be major sites of HIV persistence, with the potential to be a source of rebound viremia upon treatment interruption.

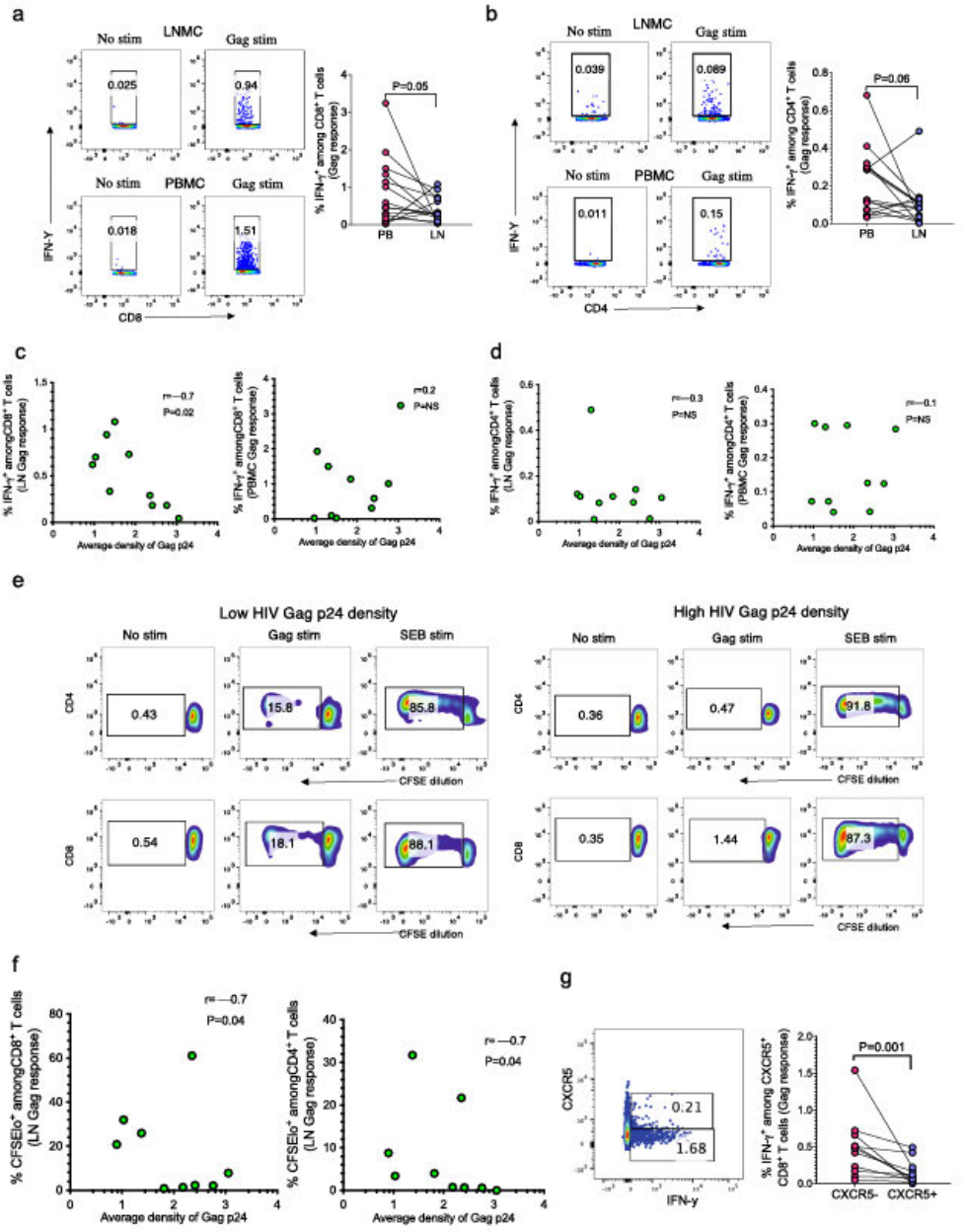
Using a highly specific ISH assay, RNAscope[48,49], which, in addition to HIV antigen detection provided further evidence in support of persistent HIV transcription in LNs in the face of ART in 86% of very early treated donors. We identified densely spherical signals by RNAscope staining in some early treated individuals suggestive of productively infected viral RNA$^+$ cells, consistent with a previous report in which active HIV RNA transcription was detected in the LNs of patients who initiated therapy in the chronic phase of illness[13]. Moreover, our data reveal heterogeneity in the amount of persistent HIV transcripts in very early treated aviremic individuals despite similar levels of peak viremia and rapid plasma viral suppression kinetics following ART initiation. Viral antigen persistence and ongoing transcription could indicate that HIV continues to cause immune damage in anatomical sites despite full suppression in PB, but it may also suggest that even in early treated individuals, priming, stimulation and harnessing of HIV-specific immunity for curative strategies will not be insurmountable because functional HIV-specific immunity is preserved.

Identifying cellular reservoirs of HIV in tissues has been a major area of research (reviewed in ref. [50]). These studies describe GCTfh subset compositions anatomically and phenotypically during HIV infection and their contributions to persistent virus. The observed positive correlation between the proportions of GCTfh1 cells (which are CXCR3$^+$CCR6$^-$GCTfh) and detection of greater amounts of HIV RNA relative to CXCR3$^+$ Tfh cells, indicates that CXCR3 may yet be another phenotypic marker of Tfh cells that have greater HIV transcription activity on ART. These data are consistent with a study that reported greater amounts of SIV DNA in CXCR3$^+$ GCTfh compared to CXCR3$^-$ GCTfh in macaques[51]. It is reasonable to attribute increased HIV burden in the CXCR3$^+$ Tfh subset to CXCR3 being used as an alternative co-receptor for HIV entry, which has previously been reported[52]. However, confirmatory work is needed. In any case, we have identified a marker for HIV-infected cells during ART that could be targeted for elimination as part of an HIV eradication strategy. Whether or not the CXCR3$^+$ Tfh population identified in this study represents the same population as the PD-1$^+$ subset that was recently implicated in HIV persistence is intriguing and warrants further investigation[13,53].

Low CD8$^+$ T cell density in GCs is thought to be a major reason for persistently high HIV antigen burden in this anatomical niche[54,55]. Interestingly, we detected significantly greater proliferative responses in individuals with little to no detectable HIV antigens compared to those with greater LN HIV antigen burden. It is difficult to determine if the low antigen environment leads to the development of better CD8$^+$ T cell function or whether superior CD8$^+$ T cell functionality results in lower antigen burden in LN tissue. However, we did not have longitudinal data to unpack this conundrum. Nevertheless, these findings are consistent with our recent study showing that very early ART is associated with functionally superior cellular responses[24]. Indeed, other studies have demonstrated the cytolytic activity of CXCR5$^+$ CD8$^+$ T cells against LCMV and HIV-infected cells[56,57]. Together, these data suggest that HIV-specific T cell responses contribute to HIV suppression in LN during therapy[58]. However, longitudinal studies using serial biopsies from the same donor are needed to confirm these findings.

A notable limitation of the study is that we could only obtain one LN sample per study participant, thus we could not conduct intra-individual longitudinal HIV decay kinetics, or longitudinally define the immune responses in tissues associated with control. This limitation was partly overcome by sampling LNs from many participants over a very wide time range. Serial LN studies have been reported in the absence of complications[59]. Alternatively, future studies could attempt serial fine needle aspirates (FNA), which is a quick procedure, less invasive and more amendable to serial sampling[60]. This approach results in loss of spatial information. Moreover, cell yields from FNAs may be limiting for some of the studies described here, nevertheless, cell subset distribution in FNAs is representative of whole LNs and will be advantageous for the characterization of tissue cell subsets, HIV reservoir quantification among other LN tissue studies[60]. Additional limitations of our study are the lack of gender and age diversity within the study population. Although we studied mainly young women, this is the group at disproportionate risk of infection and women are underrepresented in most studies to date[61]. Higher immune activation[62] among other sex-linked immunological factors in HIV infection may prevent the generalization of our results to HIV-infected males. In addition, well described structural changes to the LN with age including, collagen deposition and fibrosis[63,64], might dampen immune responses during early ART. These issues should be addressed in future studies. As with many immunology studies, sample sizes and quantities are a limiting factor. Specifically, due to the subtle differences between the early Fiebig stages, much larger samples sizes in the groups might be useful to tease out subtle but clinically relevant differences.

In conclusion, our results demonstrate HIV persistence in LNs despite prompt and durable ART-mediated plasma viral suppression. HIV structural proteins and HIV RNA persist in LNs of in 12 of 14 individuals, albeit at lower levels compared to



treatment in chronic infection. Given sample limitations, one cannot rule out persistent infection in the other two individuals. Among those with detectable infection, GCs serve as the primary anatomical site of HIV persistence and the major cellular source is CXCR3 expressing Tfh cells. Together, our results emphasize the importance of very early initiation of ART in Fiebig stage I/II to reduce the amounts of persistent virus in the LN and highlight the need for interventions to completely eradicate residual viremia in immune privileged and anatomically compartmentalized tissue sites.

Fig. 5 HIV-specific CD8$^+$ T cell responses limit the amount of persistent HIV antigens in lymph nodes during ART. a, **b** Intracellular cytokine staining was conducted after stimulating PBMCs and lymph node mononuclear cells (LNMCs) with HIV-Gag. **a** Representative flow cytometry plots and aggregate data of 14 donors showing IFN-γ$^+$ CD8$^+$ T cells, and **b** IFN-γ$^+$ CD4$^+$ T cells after stimulation with HIV-1 clade C Gag peptide pools. Correlation analysis of average Gag p24 density; measured from image analysis, with the frequency of **c** IFN-γ$^+$CD8$^+$ T cells and **d** IFN-γ$^+$CD4$^+$ T cells in LNMCs and PBMCs. **e** Representative flow cytometry plots of CFSE-labeled CD4$^+$ and CD8$^+$ T cells after 7-days of stimulation of LNMCs with HIV-1 clade C Gag peptide pools. **f** Aggregate data correlating CFSEloCD8$^+$ and CFSEloCD4$^+$ T cell responses and Gag p24 density. **g** Representative flow plot and aggregate data showing frequency of HIV Gag-specific CXCR5$^+$ CD8$^+$ T cells. All statistical tests are two-sided and *p* values are from the Mann–Whitney *U* test (**a**, **b**, **g**). Spearman rho (*r*) and *p* values are reported for correlation analysis (**c**, **d**, **f**). Source data are provided as a source data file.

Methods

Study approval. All study participants provided written informed consent prior to inclusion in the study. Ethical approval for the study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (protocol number BF298/14) and the Institutional Review Board of Massachusetts General Hospital (protocol number 2015-P001018).

Study population, samples, and performance site. Study participants were drawn from the HIV Pathogenesis Programme (HPP) lymph node study (LNS) cohort, Durban, South Africa. Recruitment into the HPP LNS cohort were from the FRESH cohort described in ref. [16] ($n = 41$) and another primary HIV infection cohort in Durban, South Africa where participant's time of infection is less defined ($n = 35$). Axillary, cervical or inguinal LNs were surgically excised at Prince Mshiyeni Hospital in Umlazi, and 120 ml paired PB was also obtained from each participant. Viral load measurements were performed by HIV-1 RNA testing using the NucliSens EasyQ v2.0 assay (BioMérieux Clinical Diagnostics, Marcy-l'Étoile, France), through a certified commercial laboratory. CD4$^+$ T cell counts were enumerated by Tru-Count technology and analyzed on a FACSCalibur flow cytometer (Becton Dickinson (BD) New Jersey, USA). Sample processing and laboratory studies were performed at the Africa Health Research Institute in Durban, South Africa.

Lymph node and blood sample processing. Excised LNs were sectioned into two, one section was fixed in 10% formal-saline (Sigma-Aldrich, St. Louis, Missouri, USA) for IF microscopy studies, while the second section was macerated to release LNMCs according to the method of Schacker et al.[65]. The cells were passed through a mesh screen and harvested by centrifugation (625 × *g*, 6 min, room temperature (RT)].

Peripheral blood mononuclear cells (PBMCs) were isolated from patient's blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in liquid nitrogen[66].

Viral RNA quantification in lymph node mononuclear cells (LNMCs). Cryopreserved LNMCs (10 million cells) were lysed, and viral RNA was quantified using the Cobas® AmpliPrep HIV-1 test (Roche, Mannheim, Germany) at an accredited clinical laboratory using standardized protocols.

Immunofluorescence (IF) microscopy. IF microscopy staining was performed on 4 μM sections of formalin-fixed paraffin-embedded (FFPE) LNs using the Opal 4-color fluorescent IHC kit (PerkinElmer, Waltham, MA, USA). Sections were deparaffinized using xylene (Honeywell research chemicals) and rehydrated, prior to antigen retrieval using AR6 buffer (20 min, 100 °C, (PerkinElmer)). Next, two blocking steps (2 × 10 min, RT) were performed with the Dako peroxidase-blocking reagent (Agilent Technologies, Glostrup, Denmark) and Bloxall block (Vector Laboratories, Burlingame, CA, USA). The slides were washed with 0.05% Tween 20 in Tris-buffered saline (TBS-T) for 5 min, sequentially probed with the primary antibody (30 min, RT), and Opal polymer HRP (20 min, RT (PerkinElmer)) and detected using the Opal polymer 520 (10 min, RT). This protocol was repeated for the second and third antibodies with Opal polymers 570 and 690 respectively, followed by counterstaining with spectral DAPI (PerkinElmer) to make a total of four different fluorochromes. Primary antibodies used in these combinations include anti-human BCL-6 ((clone PG-B6p) Dako/Agilent Technologies), CCR6 ((R6H1) Thermo Fisher Scientific, Waltham MA, USA), CD4 ((clone 4B12) Dako/Agilent Technologies), CXCR3 ((clone 6H1L8) Thermo Fisher Scientific), FDC ((cloneCNA.42), Dako/Agilent Technologies) p24 ((clone Kal-1), Dako/Agilent Technologies), and PD-1 (clone NAT105) Abcam, Cambridge, MA, USA). Slides were mounted with Dako fluorescence mounting medium (Agilent Technologies) and imaged with the Axio Observer, ×20 objective lenses, a Hamamatsu C13440-20C camera and TissueFAXS imaging software (TissueGnostics, Vienna, Austria).

RNAscope® in situ hybridization (ISH). RNAscope® ISH was conducted using the RNAscope® 2.5 HD assay kit (Advanced Cell Diagnostics (ACD), Newark, CA, USA, Cat No: 322300) and the RNAscope® multiplex fluorescent kit v2.0 (ACD, Cat No: 323100) as per manufacturer's instructions. Briefly, pre-treated samples were hybridized with the clade C HIV-1 *gag-pol* probe (Cat No: 317691) at 40 °C for 16 h. Next, the samples were incubated with signal amplification probes and horseradish peroxidase conjugated secondary antibodies. The signal was detected with either diaminobenzidine for the RNAscope® 2.5 HD assay (ACD) or with Opal fluorophores (PerkinElmer) for the multiplex fluorescent assay. Slides were imaged with Axio Observer and TissueFAXS imaging software (TissueGnostics).

Quantitative image analysis. Quantitative image analysis of Gag p24 in IF images of whole tissue section scans was conducted with TissueQuest software (TissueGnostics). Two independent experiments of total area measurements and nuclear segmentation analyses were performed on each whole tissue scan. The numerical data generated from the analyses are displayed in scattergrams. Grey-scale images were analyzed and each channel was processed separately by the software using DAPI as a master marker. In cases where images were stained with another nuclear marker such as BCL-6, then the FITC channel was used as a virtual channel for nuclei identification. Negative control slides were used to set the threshold values in the scattergrams and to distinguish specific staining signals from non-specific or background fluorescence signals. Although HIV Gag p24 staining was generally intense, there was no notable spillover of the signal to other channels (Supplementary Fig. 1a, b). Also, p24 co-staining was only observed with FDCs and CD4 markers but not CD8 cells (Supplementary Fig. 1c, d).

Analysis of *gag-pol* RNA signals was done using Fiji, an open-source software based on ImageJ (ImageJ version: 2.0.0-rc-69/1.52p) which is optimized for biological image analysis[29]. Briefly, images were segmented using the color segmentation plugin with the algorithm for Hidden Markov Model. Thresholding was applied to the segmented image and the total area of brown RNA signals was measured and recorded. Five images were analyzed per sample and averaged. Pixel measurements were converted to μm using the scale bar.

Flow cytometry analysis. Freshly isolated or frozen LNMCs and PBMCs were characterized using flow cytometry analysis with standardized protocols[67]. Cells were stained with LIVE/DEAD Fixable Blue dead cell stain kit (Thermo Fisher Scientific), CD3 Brilliant Violet (BV) 711 (BioLegend, San Diego, CA, USA), CD8 BV786 (BD Biosciences, San Jose, CA), CD4 BV650 (BD Biosciences) CXCR5 Alexa Fluor (AF) 488 (BD Biosciences), PD-1 BV421 (BioLegend), CCR6 Phycoerythrin ((PE) BioLegend), CXCR3 BV605 (BioLegend) and CD45RA PE-Cyanine (Cy)−7 (BioLegend), for 30 min at RT.

For ICS, PBMCs or LNMCs were either left unstimulated or stimulated with HIV clade C overlapping peptide (OLP) pools spanning Gag, Nef, or Env proteins or Staphylococcal enterotoxin B (SEB, 0.5 μg/ml) in the presence of GolgiStop and GolgiPlug protein transport inhibitors (BD Biosciences) for 16 h at 37 °C, prior to surface staining with the panel of antibodies comprising LIVE/DEAD fixable Aqua dead cell stain (Thermo Fisher Scientific), CD3 BV711, CD4 BV650 and CD8 BV786. After fixation and permeabilization with the BD Cytofix/Cytoperm kit (BD Biosciences), cells were again stained using TNF-α A700 (BD Biosciences) and IFN-γ PE-Cy7 (BioLegend) antibodies.

T cell proliferation was measured by labeling LNMCs with CFSE, stimulating cells with HIV clade C OLP pools for 7 days and staining with CD3 BV711, CD4 BV650 and CD8 BV786. Stained cells were acquired using an LSRFortessa (BD Biosciences) with FACSDiva™ software or sorted using the FACS Aria Fusion (BD Biosciences). Data were analyzed using the FlowJo version 10.0.8 (Flowjo, LLC, Ashland, Oregon).

HIV-infected cells were identified by surface staining with biotinylated 3BNC117 antibody followed by streptavidin PE (Thermo Fisher Scientific) and/or intracellular staining with HIV Gag p24 RD1 ((clone KC57), Beckman Coulter, Indianapolis, USA) after fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences).

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

Digital droplet PCR. Total RNA was extracted from FACS-sorted LNMC Tfh subsets using Qiagen RNeasy kit (Qiagen) after lysing cells with QIAzol lysis reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions, and used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was used as a template for HIV *gag* mRNA quantification by TaqMan digital droplet PCR assay using custom probes (Assay ID: APCE4R6, Thermo Fisher Scientific) in a two-step digital droplet PCR reaction. PCR thermal cycling was conducted following optimized cycling conditions: an initial denaturation at 95 °C for 10 min, 40 cycles of 30 s at 94 °C, 1 min at 60 °C, followed by a final incubation at 98 °C for 10 min and holding at 4 °C until reading time. After PCR amplification, droplets were measured in the QX200 ddPCR Droplet Reader (Bio-Rad), and target gene copy number was analyzed using QuantaSoft analysis software (Bio-Rad) and recorded as mRNA copies/20 µl. Absolute *gag* mRNA counts were normalized to the expression of the housekeeping gene β2M.

Statistical analyses. All statistical analyses were conducted with GraphPad Prism version 7.0 for macOs (GraphPad Software, San Diego, California, USA) and p values were considered significant if <0.05. Specifically, the Mann–Whitney U and Kruskal–Wallis H tests were used for group comparisons. Additional post hoc analyses were performed using the Dunn's multiple comparisons test. Correlations between variables were defined by the Spearman's rank correlation test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data are provided with this paper.

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Author contributions

Z.M.N., B.D.W., K.L.D. and T.N. initiated the study cohorts. Z.M.N. conceived the study. Z.M.N. and O.O.B. designed the experiments. ThaNg recruited the study participants, I.J. and J.P. performed the lymph node biopsies. N.I., ThaNk and A.M. processed the samples. O.O.B. performed all the experiments with the assistance of T.K., ThaNk, F.O., A.M., and C.C. under the supervision of Z.M.N. O.O.B. and Z.M.N. analyzed the data. O.O.B., Z.M.N. and J.M. wrote the manuscript. T.N. and B.D.W. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Zaza M. Ndhlovu.

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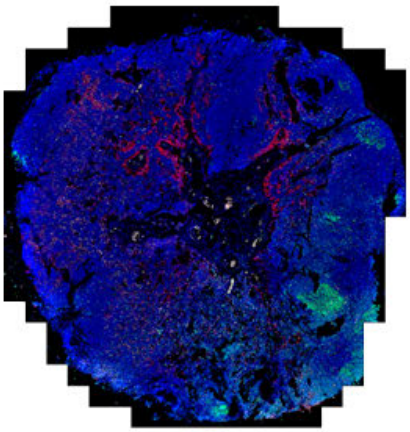
APPENDIX D: WHOLE LYMPH NODE TISSUES USED TO PHENOTYPE AND SPATIALLY LOCALIZE MACROPHAGES



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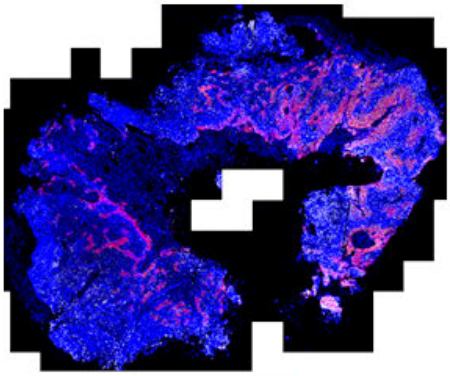

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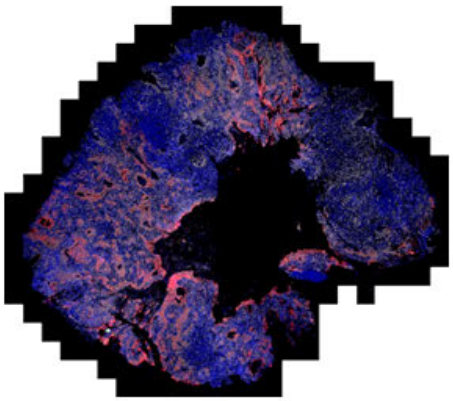

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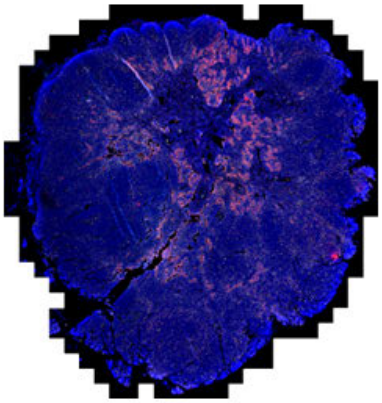

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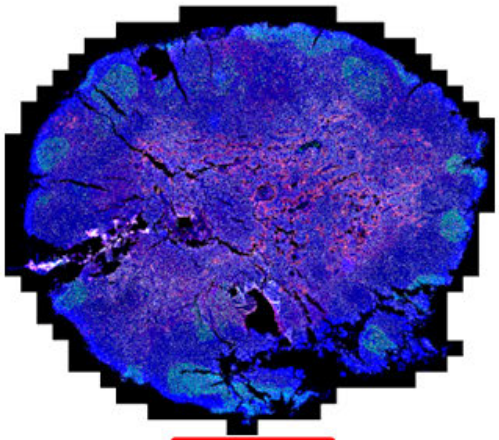

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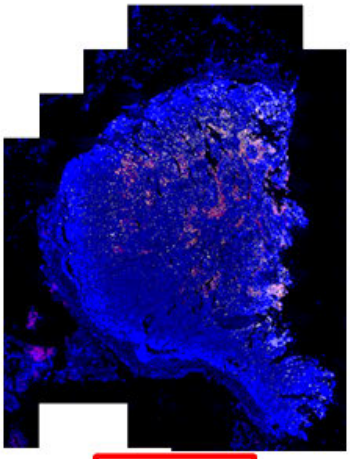

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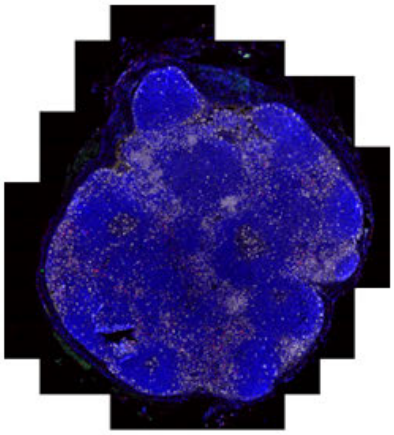

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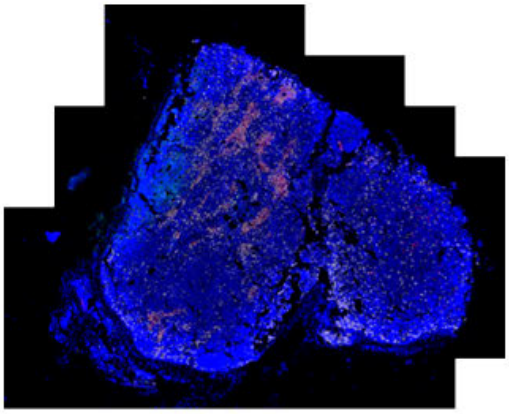

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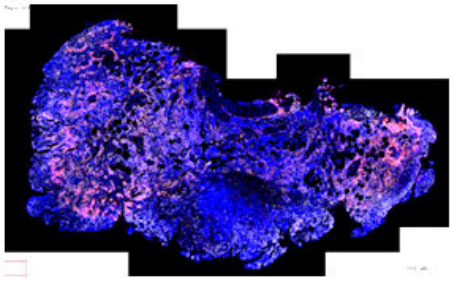

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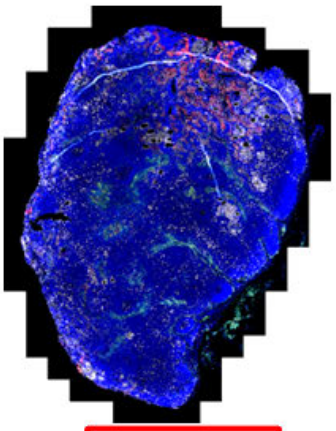

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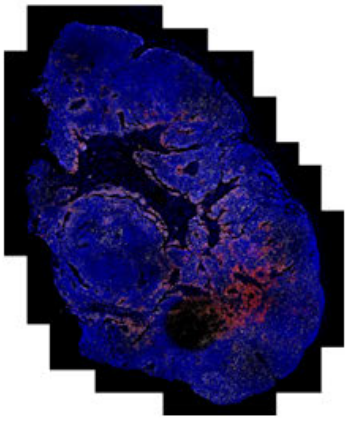
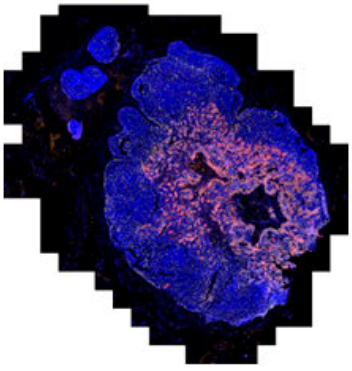


PID 63



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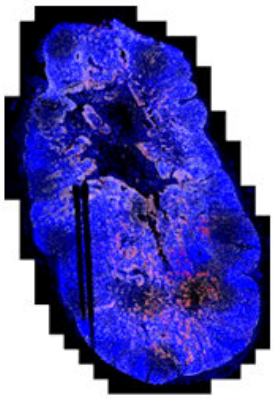

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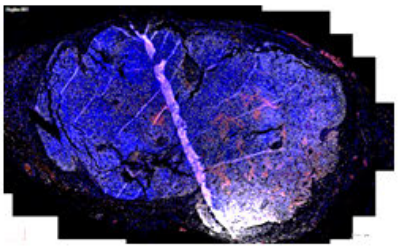

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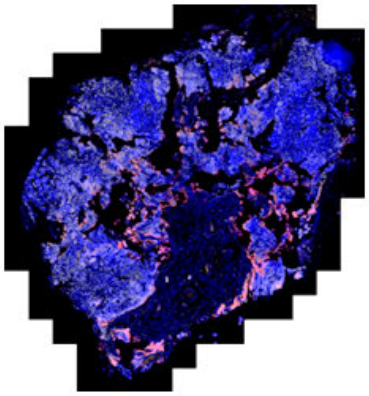

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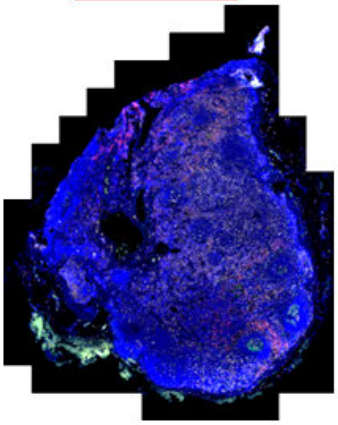

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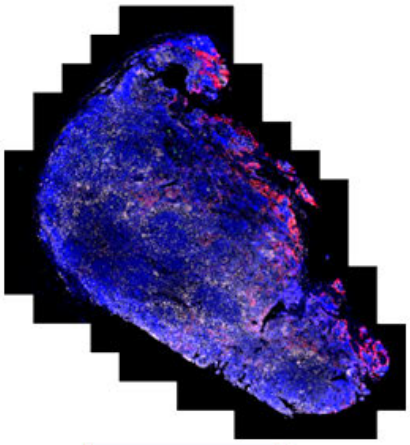
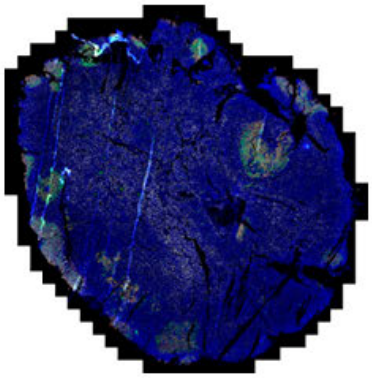


PID 95069

APPENDIX E: WHOLE LYMPH NODE TISSUES USED TO QUANTIFY HIV GAG P24 PROTEIN AND HIV-1 RNA



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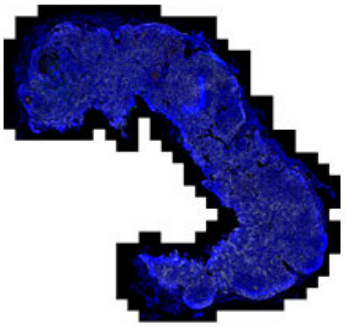

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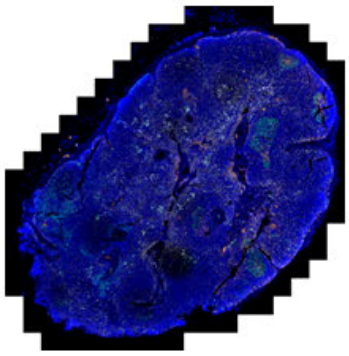
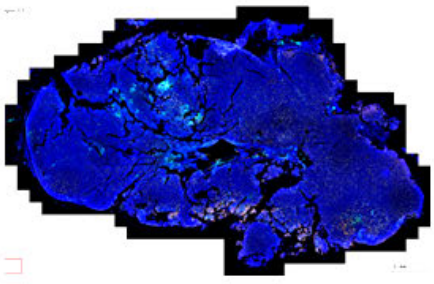


PID 190

PID 191



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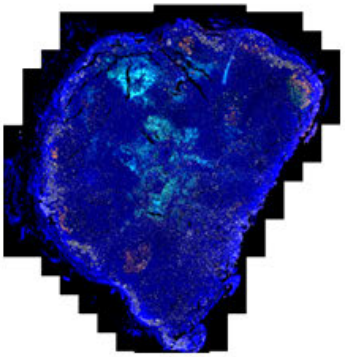

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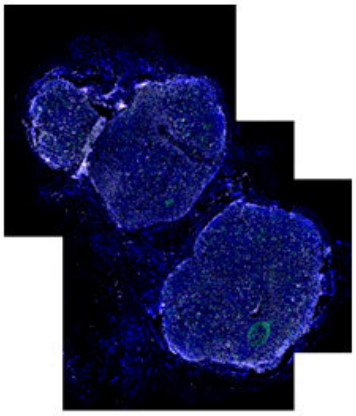

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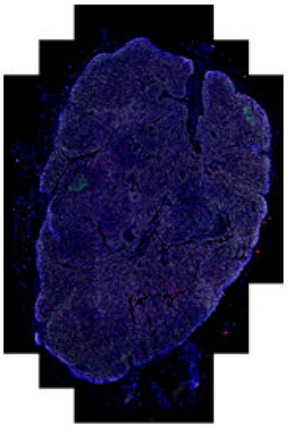

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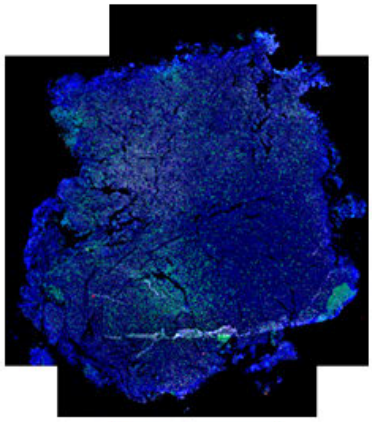

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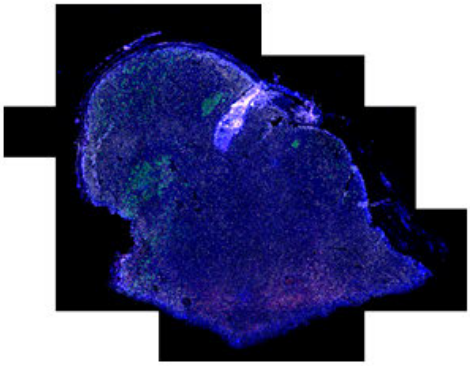

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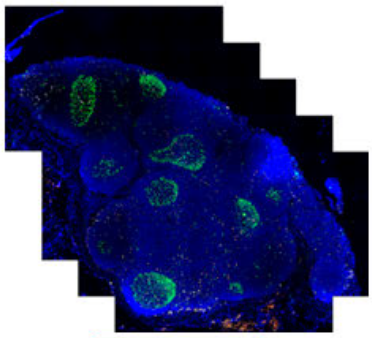


PID 192

APPENDIX F: TRAINING CERTIFICATES

Certificate of Completion

THIS ACKNOWLEDGES THAT

Caroline Chasara

has successfully completed the

IMAGING AFRICA MICROSCOPY WORKSHOP

held at the University of Cape Town, South Africa

21-26 October 2022

[redacted]

TENG-LEONG CHEW, WORKSHOP CO-DIRECTOR

[redacted]

MICHAEL REICHE, WORKSHOP CO-DIRECTOR

Certificate

This is to confirm that

Caroline Chasara

Passed, presented a poster, and completed 40 hours

IUIS-FAIS-SANTHE-ISZ IMMUNO-ZAMBIA Course

„Advanced Immunology of the Big Four: COVID-19, HIV, TB, Malaria"

December 4 – 9, 2022

[redacted]

Dieter Kabelitz
University of Kiel, Germany
IUIS Education Committee
Chair

Zaza Ndhlovu
Africa Health Research Institute
Immunological Society of Zambia
Chair

Clive Gray
Stellenbosch University, South Africa
IUIS Education Committee
Vice-Chair & Immunopaedia

Rosana Pelayo
Mexican Institute for Social Security, Mexico
IUIS Education Committee
Vice-Chair

William Kilembe
Center for Family Health
Research in Zambia,
ISZ –Secretary General

Michelle Letarte
University of Toronto, Canada
IUIS Education Committee
Past-Chair

Certificate

This is to confirm that

Caroline Chasara

Participated in the grant writing exercise and completed 15 hours

IUIS-FAIS-SANTHE-ISZ IMMUNO-ZAMBIA Course

„Advanced Immunology of the Big Four: COVID-19, HIV, TB, Malaria"

December 4 – 9, 2022

[redacted]

Dieter Kabelitz
University of Kiel, Germany
IUIS Education Committee
Chair

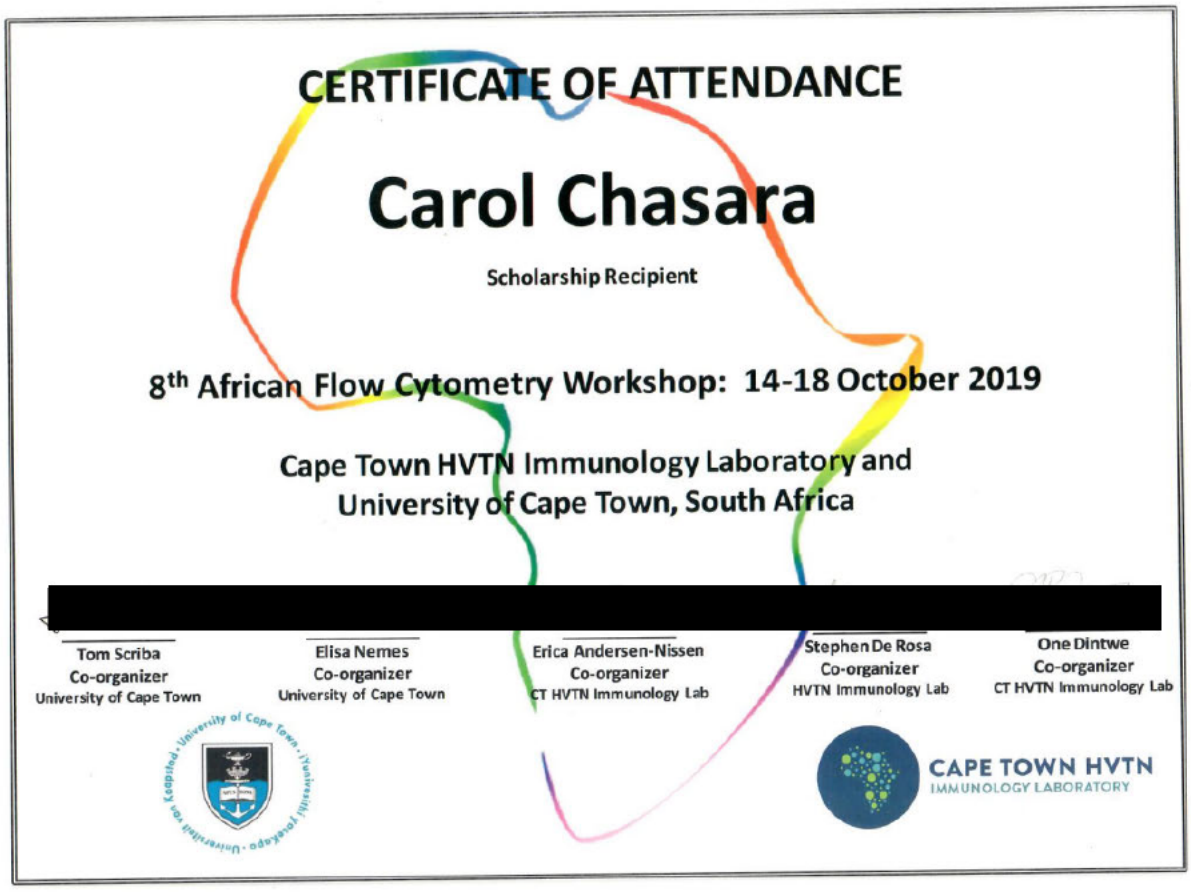
Zaza Ndhlovu
Africa Health Research Institute
Immunological Society of Zambia
Chair

Clive Gray
Stellenbosch University, South Africa
IUIS Education Committee
Vice-Chair & Immunopaedia

Rosana Pelayo
Mexican Institute for Social Security, Mexico
IUIS Education Committee
Vice-Chair

William Kilembe
Center for Family Health
Research in Zambia,
ISZ –Secretary General

Michelle Letarte
University of Toronto, Canada
IUIS Education Committee
Past-Chair



CERTIFICATE OF ATTENDANCE

Carol Chasara

Scholarship Recipient

8th African Flow Cytometry Workshop: 14-18 October 2019

Cape Town HVTN Immunology Laboratory and
University of Cape Town, South Africa

[redacted]

Tom Scriba
Co-organizer
University of Cape Town

Elisa Nemes
Co-organizer
University of Cape Town

Erica Andersen-Nissen
Co-organizer
CT HVTN Immunology Lab

Stephen De Rosa
Co-organizer
HVTN Immunology Lab

One Dintwe
Co-organizer
CT HVTN Immunology Lab

CAPE TOWN HVTN
IMMUNOLOGY LABORATORY