Restoration of cellular immunity in HIV-infected individuals on antiretroviral therapy

by

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Declaration

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List of abbreviations

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cy7
APOBEC3G	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
ASCs	Antigen secreting cells
ART	Antiretroviral therapy
ARVs	Antiretroviral drugs
AZT	Zidovudine
BAFF	B cell activating factor
BCN	Broadly cross neutralising
BFA	Brefeldin-A
BLyS	B lymphocyte stimulator
BST-2	Bone marrow stromal cell antigen-2
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CFP-10	Culture filtrate protein
CLRs	C-type lectin receptors
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CpG	Cytosine-phosphate-guanine
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) ligand
CXCR4	Chemokine (C-X-C motif) receptor
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ddI-EC	Didanosine-enteric coating
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
d4T	Stavudine
EBV	Epstein-Barr virus
ECD	Electron coupled dye
ED	Early differentiated
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
Env	Envelope glycoprotein
FACS	Fluorescence-activated cell sorter
FCS	Foetal Calf Serum
FITC	Fluorescein isothyocyanate
FMO	Fluorescent Minus One
Foxp3	Forkhead box p3
FSC	Forward Scatter
FTC	Emtricitabine

GALT	gut-associated lymphoid tissue
Gag	Group specific antigen
gp	glycoprotein
h	hours
HAART	Highly active antiretroviral treatment
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV/HIV-1	Human Immunodeficiency type 1
HLA	Human leukocyte antigen
HPV	Human Papillomavirus
HSV	Herpes Simplex Virus
hsCRP	High sensitive C-reactive protein
ICS	Intracellular cytokine staining
IRF	Interferon response factor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INI	Integrase inhibitor
Int	Intermediate
IP-10	IFN-γ inducible protein 10
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
LAG-3	Lymphocyte activation gene-3 protein
LBP	LPS-binding protein
LD	Late differentiated
LDL	Lower detection limit
LPS	Lipopolysaccharide
LTNPs	Long-term non progressors
MHC	Major histocompatibility complex
Mtb	Mycobacterium tuberculosis
min	minutes
MIP	Macrophage inflammatory protein
ml	millilitre
mm	millimetre
mRNA	Messenger ribonucleic acid
nAb	Neutralising antibodies
Nef	Negative regulatory factor
NF-κB	Nuclear factor kappa B
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOD	Nucleotide binding oligomerisation domain
NRTI	Nucleoside reverse transcriptase inhibitor
NSAID	Non-steroidal anti-inflammatory drug
NtRTI	Nucleotide reverse transcriptase inhibitor
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cy5

PE-Cy5.5	Phycoerythrin-Cy5.5
PE-Cy7	Phycoerythrin-Cy7
PD-1	Programmed death-1
PI	Protease inhibitor
Pol	Polymerase protein
PPD	Purified protein derivative
PRRs	Pathogen recognition receptors
Qdot	Quantum dot
RANTES	Regulated on activation, normal T expressed and secreted
Rev	Regulator of expression of virion
RIG	Retinoic acid-inducible gene
RLRs	RIG-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute cell culture medium
RT	Room temperature
SEB	Staphylococcal enterotoxin B
SERINC	Serine incorporator
SIV	Simian Immunodeficiency Virus
ss	Single-stranded
SSC	Side scatter
Tat	Trans-activator of transcription
TB	Tuberculosis
T_{CM}	T cell central memory
TCR	T cell receptor
TD	Terminally differentiated
TDF	Tenofovir
T_{EM}	T cell effector memory
Tfh	T follicular helper cells
TGF	Transforming growth factor
Th	T helper
Tim-3	T cell immunoglobulin mucin-3
TLR	Toll-like receptors
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cells
T_{SCM}	Stem memory T cells
T_{TM}	T cell transitional memory
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	Viral infectivity factor
Vivid	Violet-fluorescent reactive dye
Vpr	Viral protein R
Vpu	Viral protein unique
WHO	World Health Organisation
3TC	Lamivudine
α	alpha
β	beta
γ	gamma
μg	microgram
μl	microlitre

%	percent
°C	Degrees Celsius
Δ	Delta
x g	times gravity (centrifugation speed)

Abstract

During the course of HIV pathogenesis, the virus induces multiple defects in immune cells, altering their functional ability to efficiently control HIV itself and other infections. Whilst the widespread implementation of antiretroviral therapy (ART) has led to reduced morbidity and mortality in most HIV-infected individuals having access to treatment, we still do not know whether full restoration of immune function occurs. The aim of this study was to assess the extent to which ART restores both phenotypic and functional T and B cell immunity.

HIV-infected women were studied before and 1 year after ART initiation. In Chapter 2, the effect of ART on T cell activation and differentiation profiles was evaluated in HIV-infected individuals (n=28; pre- and post-ART), and compared to HIV-uninfected age- and sex-matched controls (n=23). In Chapter 3, the restoration of co-pathogen specific CD4+ T cells was determined by comparing their cytokine secretion ability and memory differentiation profiles in response to *Mycobacterium tuberculosis* and cytomegalovirus in HIV-infected (n=15; pre- and post-ART), compared to uninfected (n=9) individuals. Finally, Chapter 4 examined changes in B cell activation and memory profiles in HIV-infected persons (n=19; pre- and post-ART), and compared profiles to HIV-uninfected individuals (n=19). Multiparameter flow cytometry was performed to address the study objectives.

T cell activation, as measured by CD38 and HLA-DR expression, was significantly reduced one year after ART for both CD4+ and CD8+ T cells, but normalisation to levels in HIV-uninfected individuals did not occur, despite suppression of viral load. In addition, skewed CD4+ and CD8+ T cell memory profiles were not completely restored. Furthermore, no change in the cytokine production capacity and memory profile of pathogen-specific CD4+ T cells was found before and after ART, but pathogen-specific CD4+ T cells exhibiting a late differentiated profile (CD27-CD45RO+) had a lower ability to replenish ($p=0.02$; $r=-0.5$) compared to cells with an early differentiated profile (CD27+CD45RO+; $p=0.04$; $r=0.45$) prior to ART. Similar to T cells, activated B cells (CD40+CD86+) were only partially normalised post-ART, and remained significantly higher than B cells of HIV-uninfected individuals. The frequency of all B cell memory subsets were comparable between HIV-treated and uninfected individuals, with the exception of plasmablasts, whose frequency was still

significantly higher than in HIV-uninfected subjects.

In summary, these results demonstrate that HIV-infected women on suppressive ART show a substantial but only partial normalisation of T cell and B cell memory subsets, and lower levels of T cell and B cell activation. In addition, restoration of co-pathogen specific memory CD4+ T cells upon treatment was dependent on their memory profile before ART. Understanding the impact of HIV on T and B cell dysfunction and restoration upon ART may provide important insights into the mechanisms of HIV pathogenesis in the era of ART.

CHAPTER 1

Literature review

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1.1 Introduction

1.1.1 Epidemic burden of HIV

In 1983, the human immunodeficiency virus (HIV) was discovered (Barre-Sinoussi *et al*., 1983), and three decades later it remains a huge public health threat globally. The number of people living with HIV worldwide rose from 33.3 million to 36.7 million in the period from 2010 to 2015 (**Figure 1.1**; UNAIDS, 2016a). This is the result of both new infections, of which there were roughly 2.1 million in the last year, as well as more people with HIV surviving longer than before. In fact, for the past 5 years, the number of newly HIV-infected people per year remained stable, but death rates were reduced by roughly one third, attributed to an increase in antiretroviral therapy (ART) coverage. Even though Sub-Saharan Africa accounted for a 13% and 38% decrease in HIV incidence and death cases respectively, it remains the region of the world most impacted by the HIV epidemic. More than half (~52%) of all adults and children living with the virus reside in Sub-Saharan Africa, where 46% of all new HIV infections occurred (UNAIDS, 2016a). In South Africa, the number of people living with HIV rose from 4.72 million in 2002 to 7.03 million in 2016 making it currently the country with the highest HIV prevalence of 18.9% in the world (Statistic South Africa, 2016). Women aged 15 years and more are the most affected in the country with a prevalence of 22.3%. However, ART is available at no cost via the public health system and 3.4 million South Africans are accessing treatment as of March 2016, making South Africa the largest ART programme compared to any country in the world (https://africacheck.org/reports/yes-south-africa-has-the-worlds-largest antiretroviral-therapy-programme/). This has resulted in a 10 years increase in life expectancy since 2004.

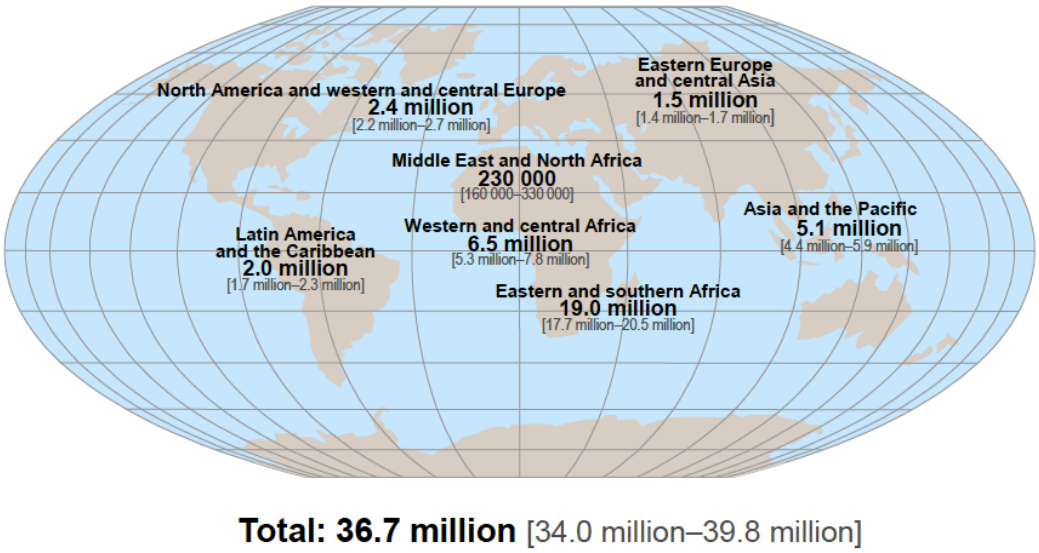

Figure 1. 1. A total estimate of the number of people infected with HIV in 2015 (taken from UNAIDS, 2016a).

1.1.2 HIV structure and life cycle

HIV-1 is classified within the family Retroviridae, belonging to the *Lentivirus* genus. (Fanales-Belasio *et al*., 2010). HIV has a genome consisting of two copies of single-stranded RNA (ssRNA) encoding nine genes (Frankel & Young, 1998). The virus is composed of three main proteins that are important for the effective synthesis of new virions: the enzyme Pol, and the structural proteins, Gag and Env. The Pol protein codes for three enzymes: a reverse transcriptase, which transcribes HIV RNA into DNA for incorporation into the host cell, an integrase and a protease which allow the integration of the viral DNA into the host chromosome, and the cleavage of Gag and Gag-Pol polyproteins for packaging, respectively (Yang *et al*., 2012). The Gag and Env proteins form the nucleocapsid and envelope glycoproteins, respectively (Frankel & Young, 1998). HIV also encodes regulatory (Tat, Rev) and accessory (Vif, Vpr, Vpu, Nef) proteins that control the transcription and translation of the viral RNA as well as its transport from the nucleus to the cytoplasm (Lever, 2005). Despite the fact that the HIV accessory proteins are dispensable for viral infection *in vitro*, these proteins contribute to pathogenesis *in vivo* by escaping the host antiviral responses (Collins & Collins, 2014). For example, Vif counteracts the function of the host restriction factor apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) by preventing the deamination of cysteine residues in the viral

genome, enabling viral replication (Simon *et al*., 2015). Vpu neutralises the bone marrow stromal cell antigen-2 (BST-2) restriction factor, allowing infected cells to release viral particles at budding (van Damme *et al*., 2008; McNatt *et al*., 2013). The Nef protein downregulates CD4 and major histocompatibility complex (MHC) class I expression, and can also antagonise the function of the host cell factor serine incorporator (SERINC), enhancing viral infectivity (Fackler, 2015).

HIV uses specific receptors to infect different immune cell types. To enter and replicate in CD4+ T cells, the major target of HIV, the virus uses the CD4 receptor and the chemokine receptors CXCR4 or CCR5 (Berger *et al*., 1999). The replication cycle of HIV consists of several distinct steps; first, the virus binds to CD4 via its gp120 glycoprotein (Myszka *et al*., 2000), inducing a conformational change that allows the virus to further attach to CCR5 or CXCR4 (Wu, 2010). This interaction results in viral and host cell membrane fusion through gp41, enabling entry of the viral RNA into the host cell (**Figure 1.2**; Melikyan, 2008). To prevent viral sensing by cytoplasmic restriction factors, nucleotides can be recruited via pores within the capsid for the synthesis of DNA by reverse transcriptase enzyme (Jacques et al., 2016). The newly formed DNA is then transported to the nucleus for integration into the host chromosome (Fanales-Belasio *et al*., 2010). Once integrated, the provirus uses the host cell RNA polymerase II to start transcribing more copies of the multiply spliced messenger (mRNA) transcripts that are initially translated into the regulatory proteins, Tat and Rev (Scherer *et al*., 2007). Both proteins migrate back to the nucleus, where Tat enhances viral transcription and Rev directs the transport of RNA transcripts to the cytoplasm via the nuclear pores (Lever, 2005). Finally, these RNA molecules are translated into enzymatic and structural proteins, and transported to the cell surface for packaging into viral particles. A capsid structure forms and immature virions bud from the cell to form infectious particles (Lever, 2005).

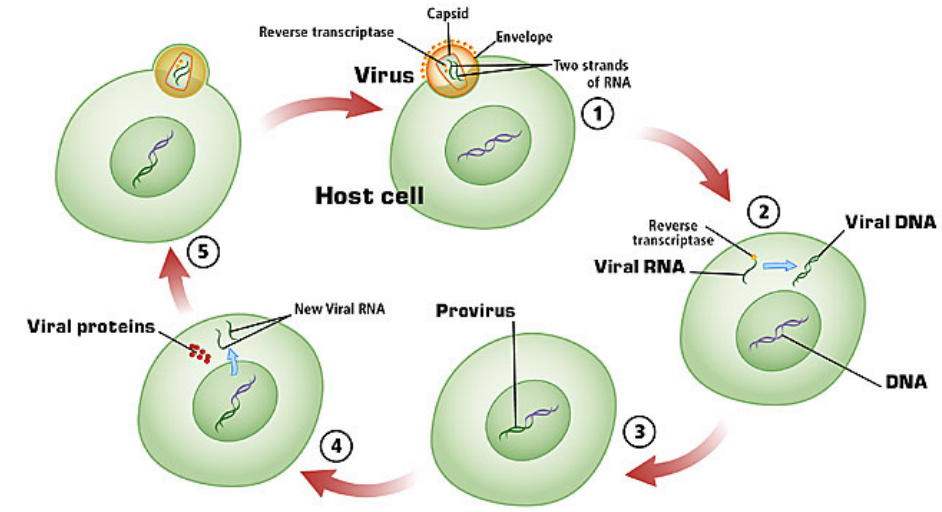

Figure 1. 2. Replication steps of HIV. 1) Delivery of the capsid containing HIV RNA and reverse transcriptase into the cytoplasm upon membrane fusion. 2) Reverse transcriptase converts the viral RNA into DNA. 3) Viral DNA known as provirus is integrated into the host genome. 4) The transcription of the integrated provirus into mRNA is followed by the translation and packaging of viral proteins into new virions. 5) Immature virions bud out from the cell membrane, acquiring the ability to produce mature particles (Taken from https://www.learner.org/courses/biology/archive/images/1922.html).

1.2 Pathogenesis of HIV infection

This section describes the mechanisms associated with the loss of CD4+ T cells during the course of HIV infection, the consequences of this depletion and how the immune system attempts to control the virus.

1.2.1 Loss of CD4+ T cells

HIV infection is marked, in most infected individuals, by a gradual decrease of CD4+ T cells resulting in AIDS if left untreated. Early in infection, a significant loss in memory CD4+ T cells occurs predominantly in the gastrointestinal mucosa, which has been demonstrated in both SIV-infected rhesus macaques and HIV-infected individuals (Mattapallil *et al*., 1998; Veazey *et al*., 1998; Mehandru *et al*. 2004). In this tissue, HIV mainly targets memory CD4+ T cells with an activated phenotype and expressing the HIV co-receptor CCR5, amplifying viral replication for further systemic spread (Veazey *et al*., 2000). Unlike the peripheral blood and lymph nodes,

the gut-associated lymphoid tissue (GALT) is rich in memory CCR5+CD4+ T cells, comprising more than 60% of these cells, hence their profound depletion in this compartment (Li *et al*., 2005; Mattapallil *et al*., 2005). Brenchley and colleagues reported that this loss in memory CCR5+CD4+ T cells does not only occur during the early stages of HIV infection, but continues during chronic infection (Brenchley *et al*., 2004).

To date, we do not have a complete understanding of how HIV causes CD4+ T cell depletion. Direct and indirect mechanisms have been proposed to contribute to the loss of CD4+ T cells. Direct killing of HIV-infected CD4+ T cells may occur either by virus-induced cytolysis or HIV-specific CD8+ T cells targeting HIV-infected CD4+ T cells for destruction (Appay *et al*., 2002b; Mattapallil *et al*., 2005). Direct killing of virally infected CD4+ T cells alone cannot account for the loss of these cells, since only a small proportion of CD4+ T cells (0.001-1%) are infected during chronic HIV infection (Douek *et al*., 2002). Thus, indirect mechanisms are thought to play a major role in CD4+ T cell loss. Apoptosis of bystander cells has been reported in lymphoid tissues of HIV-infected individuals (Finkel *et al*., 1995). More recently, it has been proposed that the majority (>95%) of CD4+ T cells die due to abortive HIV infection, when non-permissive, resting cells undergo caspase-1-mediated pyroptosis, whilst only a small proportion of productively infected CD4+ T cells die by caspase-3-mediated apoptosis (Doitsh *et al*., 2010; 2014). With regard to indirect ways by which CD4 count declines, one such mechanism is the inability of the host immune system to replenish the CD4+ T cells killed by HIV (Ho *et al*., 1995; Wei *et al*., 1995). Okoye and colleagues have also reported a progressive loss of central memory (CM) CD4+ T cells, which then fail to renew the pool of effector memory (EM) CD4+ T cells (Okoye *et al*., 2007). Furthermore, thymic dysfunction as a result of HIV infection impedes the *de novo* synthesis of naive CD4+ T cells (Douek *et al*., 1998). Another major contributor to CD4+ T cell loss appears to be systemic immune hyperactivation (Hazenberg *et al*., 2000), a concept that is dealt with in detail in Section 1.3.

Taken together, these studies demonstrate that upon HIV infection, different mechanisms are initiated to drive the loss of CD4+ T cells observed during the acute and chronic stages of HIV infection. This renders the immune system vulnerable to

opportunistic infections, leading to disease progression and AIDS in the absence of treatment.

1.2.2 Opportunistic infections

Clinically, disease progression in HIV-infected persons is associated with the severity of CD4 loss and the development of opportunistic infections (Egger *et al*., 2002; Sterne *et al*., 2009). These opportunistic diseases are caused by different viral, bacterial or fungal pathogens, such as Cytomegalovirus (CMV), *Mycobacterium tuberculosis* (Mtb) or *Candida*, among others. In the cohort of African women studied in this thesis, during acute HIV infection, some of the women had vaginal discharge and genital tract disease of unknown aetiology, as well as upper respiratory tract infections (Mlisana *et al*., 2014). Furthermore, skin rashes and fungal infections were found in these women over the full course of infection (approximately 4 years), while lower respiratory tract infections, shingles and tuberculosis (TB) emerged at later stages of infection. Similar opportunistic infections were reported in HIV-infected individuals from Uganda (Rubaihayo *et al*., 2016). However, it is worth mentioning that the development of opportunistic infections is not always associated with low CD4 counts, since both TB and *Candida* infection may occur at any CD4 count (Saharia & Koup, 2013). Altogether, these data show that HIV infection is associated with a range of co-infections, further overwhelming the immune system of the HIV-infected individual. Therefore, it is of crucial importance to clearly define the relationship between HIV and these pathogens in HIV-infected patients, and whether adjunctive therapy (in addition to ART) may be of benefit against other infections, as discussed in Sections 1.4 and 1.5.

1.2.3 Host immune responses to HIV

During the interaction between HIV and the host immune system, the innate arm directs the adaptive arm of the immune system to initiate specific immune responses in an attempt to control and clear the virus. These events are summarised in **Figure 1.3** below.

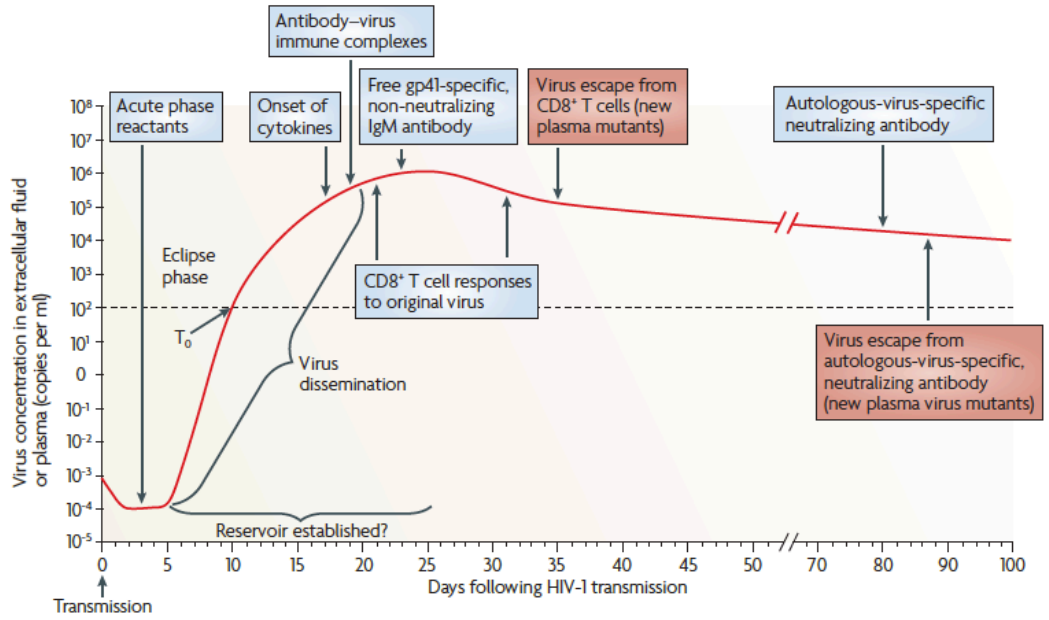

Figure 1. 3. The early wave of innate and adaptive immune responses following HIV infection. During the eclipse phase, elevated levels of plasma acute phase reactants are initially detected when viral replication occurs mostly in mucosal tissues and draining lymph nodes. An increase in cytokine levels in the plasma is observed when plasma viral load is first detected (T_0). A few days later, the first antibody-virus immune complexes emerge with the exponential increase in plasma viral load. After the initial rise in viral load, HIV-specific CD8+ T cell responses develop with a subsequent appearance of circulating gp41-specific non-neutralising IgM antibodies. Within 10 days, the virus can evolve to escape recognition from CD8+ T cells. In addition, viral reservoirs are likely established during this time. Approximately 80 days after infection, the detection of neutralising antibodies against the infecting virus occurs; CD8+ and neutralising antibody responses coincide with the decrease in plasma viraemia before viral set point is established. Shortly thereafter, viruses able to escape antibody neutralisation can arise in plasma (taken from McMichael *et al*., 2010).

Upon pathogen encounter, the immune system resorts to innate immunity, known as the first line of defence against invading pathogens. The innate immune system is composed of a range of cell types, including phagocytes (macrophages, monocytes and dendritic cells; DCs), professional antigen presenting cells (DCs), as well as cytolytic cells (neutrophils and natural killer; NK cells; Carrington & Alter, 2012). Using pathogen recognition receptors (PRRs), innate cells can recognise diverse pathogens by sensing molecular structures (pattern associated molecular patterns; PAMPs) commonly found as part of their cell walls, or involving recognition of their nucleic acid (Thompson *et al*., 2011). Innate cells possess a range of surface and intracellular PRRs (toll-like receptors, TLRs; C-type lectin receptors, CLRs; the retinoic acid-inducible gene (RIG)-like receptors, RLRs and the nucleotide binding

oligomerisation domain (NOD)-like receptors, NLRs), whose triggering results in the secretion of proinflammatory cytokines and chemokines for the recruitment and further activation of immune cells, as well as for the inhibition of viral replication (Mogensen *et al*., 2010; Carrington & Alter, 2012; Altfeld & Gale, 2015). One study showed that TLR triggering on DCs induced the production of type I interferons, hence decreasing the ability of DCs to spread HIV to CD4+ T cells (Thibault *et al*., 2009). However, HIV can affect the function of innate cells. For instance, a reduction in the number of circulating DC subsets, namely plasmacytoid DC (pDC) and myeloid DC (mDC) was observed during acute and chronic HIV infection (Barron *et al*., 2003; Donaghy *et al*., 2003). The decline in pDC numbers was further associated with decreased IFN-α secretion (Feldman *et al*., 2001), possibly due to the suppression of TLR signalling (Martinelli *et al*., 2007). Despite the negative effect of HIV on innate immune cells, these cells remain indispensable as they shape adaptive immune responses (Carrington & Alter, 2012). In line with this, a direct association between NK cell activity and HIV viral load was reported in the absence of HIV-specific CD8+ T cell responses in very early acute HIV infection, prior to seroconversion (Alter *et al*., 2007).

HIV-specific T cells have been associated with the control of HIV infection. For CD8+ T cells, it has been shown that during primary HIV infection the reduction in viral load coincides with the emergence of CD8+ T cells specific for HIV (Koup *et al*., 1994). This observation was also observed in SIV-infected rhesus macaques during the primary stage of infection (Matano *et al*., 1998). In these animals, depletion of CD8+ T cells resulted in failure to control SIV infection, demonstrated by an increase in viral load, which was suppressed upon re-appearance of SIV-specific CD8+ T cells (Schmitz *et al*., 1999). In addition, *in vivo* depletion of CD8+ T cells in SIV-infected rhesus macaques at 70 days post infection resulted in increased viral load that was more obvious in SIV-controllers compared to progressors (Chowdhury *et al*., 2015). To control HIV infection, CD8+ T cells can eliminate infected cells via non-lytic (production of HIV suppressive factors known as RANTES, MIP-1α and MIP-1β) and lytic (release of cytotoxic granules or triggering of the Fas-Fas ligand pathway) mechanisms (Cocchi *et al*., 1995; Gulzar & Copeland, 2004).

During HIV infection, intensive research has been conducted on the quantity and quality of HIV-specific T cell responses to define what aspects of the immune response are associated with the control of HIV. Some studies have looked at the quantity by measuring the magnitude and breadth of T cell responses in HIV-infected individuals (Addo *et al*., 2003; Masemola *et al*., 2004; Gray *et al*., 2009). Using the IFN-γ ELISpot assay, these studies measured HIV-specific T cell responses across the full HIV genome and found that plasma viral load was not associated with the magnitude or breadth of HIV-specific CD8+ T cells, suggesting that the overall quantity of HIV-specific T cell responses is not related to viral control when using IFN-γ as a readout. However, others have indicated that CD8+ T cells targeting specific HIV proteins associate with HIV pathogenesis, where high breadth of Gag-specific responses correlates with low viral load while the breadth of Env-specific CD8+ T cell responses were related to high viral load (Kiepiela *et al*., 2007; Rolland *et al*., 2008). Other studies have rather focused on the quality of HIV-specific T cell responses, assessing the polyfunctional potential (i.e. the capacity of cells to simultaneously produce multiple cytokines), the proliferative and cytotoxic capacities or memory differentiation profile of these cells (Migueles *et al*., 2002; Betts *et al*., 2006; Addo *et al*., 2007; Almeida *et al*., 2007; Day *et al*., 2007; Burgers *et al*., 2009; Kunwar *et al*., 2013). In one study, Long-term non progressors (LTNPs) were characterised by increased frequencies of polyfunctional CD8+ T cells (co-expressing CD107a, IFN-γ, TNF-α, MIP-1β and IL-2) when compared to individuals with chronic HIV infection (Betts *et al*., 2006). In LTNPs, HIV-specific CD8+ T cells also showed increased proliferating potential compared to progressors (Migueles *et al*., 2002). Furthermore, in LTNPs, *in vitro* activated HIV-specific CD8+ T cells exhibited enhanced capacity to lyse HIV-infected CD4+T cells, as measured by higher levels of perforin and granzyme B than progressors (Migueles *et al*., 2008; Hersperger *et al*., 2011). It is important to point out that the majority of these studies were focusing on the functional features of HIV-specific CD8+ T cells from LTNPs, a sub-group of individuals representing less than 5% of HIV-infected individuals (Kumar, 2013) and are not hence representative of the total HIV population. Riou and co-workers then reported in acutely HIV-infected individuals an association between lower viral set point and elevated frequencies of Gag-specific CD8+ T cell responses with polyfunctional ability (expression of IFN-γ, TNF-α, MIP-1β and CD107a; Riou *et al*., 2014). Collectively, these studies indicate that the quality of HIV-specific T cell responses

may play a pivotal role in the control of viral replication, showing that CD8+ T cells endowed with polyfunctional capacity, high proliferative potential and/or enhanced cytotoxic function favour HIV control.

CD4+ T cells are also essential for the control of HIV infection, as they provide help to DCs in order to trigger HIV-specific CD8+ T cell responses (Ostrowski *et al*., 2000; Huang *et al*., 2008). In addition, the capacity of HIV-specific CD4+ T cells to acquire cytolytic function (expression of granzyme and perforin) has been reported in HIV-infected individuals (Appay, 2004; Zheng *et al*., 2009). Zheng and colleagues demonstrated the killing of HIV-infected macrophages, and activated CD4+ T cells expressing HLA-DR by Nef-specific CD4+ T cells (Zheng *et al*., 2009). Furthermore, elevated levels of HIV-specific CD4+ T cells expressing granzyme A were associated with a lower viral load set point during acute infection (Soghoian *et al*., 2012). From these reports, it appears that CD4+ T cells can thus contribute to control of HIV either via cytotoxic function or their capacity to induce CD8+ T cell responses, or indeed to provide help to B cells for the initiation of antibody responses (Sant & McMichael, 2012).

HIV infection can elicit antibody-mediated immune responses that can be both non-neutralising and neutralising. The initial B cell response to HIV is detected as antibody complexes 8 days after the appearance of plasma virus, while free antibody specific to HIV envelope gp41 emerged around 13 days later (Tomaras *et al*., 2008). On the other hand, the detection of envelope gp120-specific antibodies only occurred 2 weeks after the appearance of envelope gp41 antibodies. Although these antibodies are able to bind virions, they are non-neutralising, and do not appear to inhibit viral replication (Tomaras *et al*., 2008). The generation of neutralising antibodies (nAb) against autologous viruses occurs months after HIV infection but whilst these antibodies can neutralise autologous virus, HIV escape renders them unable to neutralise heterologous viruses, which evolve rapidly in response to selective antibody pressure (Moog *et al*., 1997; Richman *et al*., 2003; Wei *et al*., 2003; Gray *et al*., 2007). However, roughly 20% of HIV-infected individuals can induce broadly cross neutralising (BCN) antibodies against heterologous viruses years after infection (Gray *et al*., 2009; Simek *et al*., 2009; Gray *et al*., 2011). Although still incompletely understood, some factors could explain why some individuals are able to develop BCN antibodies.

Neutralisation breadth was related to the length of infection and levels of virus, as well as to continuous viral escape from broadly neutralising antibodies uncovering novel and various target sites (Gray *et al*., 2011; Wibmer *et al*., 2013). Due to the difficulties encountered in inducing these antibodies through vaccination, the infusion of broadly neutralising antibodies to chronically SHIV-infected rhesus macaques and humans led to a robust decrease in plasma viral load (Barouch *et al*., 2013; Caskey *et al*., 2015; Lynch *et al*., 2015) , and a large clinical trial is currently underway in South Africa to test this concept in humans (Rubens *et al*., 2015).

Despite the induction of the host immune response, the control of HIV is only partial and the majority of HIV patients develop AIDS without therapy. This is in part due to viral escape, where HIV mutates in the face of selection pressure and avoids recognition by HIV-specific CD8+ T cells and antibodies; this occurs during both acute and chronic infections (Borrow *et al*., 1997; Cao *et al*., 2003). Some regions of HIV are conserved and required for replicating efficiently (viral 'fitness'), and viruses that are selected for mutations in these regions may display fitness impairment, leading to partial immune control and lower viral replication (Lewis *et al*., 2012). It is well known that CD4+ T cells are the principal targets of HIV, and there is some evidence that HIV preferentially infects HIV-specific CD4+ T cells (Douek *et al*., 2002), resulting in CD4+ T cells providing suboptimal or no help to CD8+ T cells and B cells for effective responses (Kuerten *et al*., 2008; Boswell *et al*., 2014). In addition, T cell and B cells can lose their function as they become "exhausted" and non-functional due to the persistence of viral replication (Day *et al*., 2006; Moir *et al*., 2008).

1.3 Systemic immune hyperactivation during HIV infection

This section introduces the concept of immune activation during HIV infection and its effects on immune cells, with an emphasis on T cells and B cells, the focus of this thesis. The potential mechanisms causing this aberrant immune activation and its consequences in the absence of treatment are also discussed.

Now recognised as a main contributor to HIV pathogenesis, immune activation is associated with a number of cellular events including activation, proliferation and apoptosis, that affect both innate and adaptive immune cells (d'Ettorre *et al*., 2011). The activation of NK cells, DCs, monocytes and macrophages has been previously

reported in the context of HIV infection (Brown *et al*., 2008; Burdo *et al*., 2011; Yonkers *et al*., 2011; Hearps *et al*., 2012; Campillo-Gimenez *et al*., 2014). Elevated proportions of inflammatory monocytes with an activated phenotype (CD11b+CD62L-) were found in the blood of HIV-infected compared to HIV-uninfected patients (Hearps *et al*., 2012). In addition, DCs from HIV-infected individuals displayed increased levels of activation and maturation markers (CD83 and CD86) compared to HIV-uninfected controls (Yonkers *et al*., 2011). These activated cells in turn secrete high levels of proinflammatory cytokines (such as IFN-α, IL-1β, IL-6, IL-8 and TNF-α) and chemokines (MIP-1α, MIP-1β, RANTES) to further drive immune activation (Appay & Kelleher, 2016). Immune cells from the adaptive arm, namely T cells and B cells are also activated during HIV infection (Appay & Sauce, 2008).

1.3.1 T cell activation

Previous analyses of chronically HIV-infected individuals have reported higher levels of activation markers (CD38, HLA-DR or co-expression of both markers) on CD4+ and CD8+ T cells in blood (Liu *et al*., 1997; Giorgi *et al*., 1993; 1999; 2002; Hazenberg *et al*. 2003). These studies found T cell activation to predict CD4+ T cell loss and disease progression more strongly even than plasma viral load. Others further showed that T cell activation before or at the set point (when activation reaches a plateau, in most cases 6 months post-acute infection) predicted CD4+ T cell loss independently of the degree of viral replication (Deeks *et al*., 2004; van Ansten *et al*., 2004; Hunt *et al*., 2008).

During HIV infection, T cell activation is also associated with markers of inflammation and coagulation. In acutely HIV-infected individuals, a substantial increase in plasma cytokine levels was found to be associated with high viral load (Stacey *et al*., 2009). The levels of certain cytokines (IL-1α and IL-7) were predictive of CD4+ T cell loss, while others were associated with T cell activation set point (TGF-β1 and IL-8) and rapid disease progression (IP-10; Roberts *et al*., 2011; Liovat *et al*., 2012). In addition, faster disease progression was associated with elevated levels of IL-6 in the plasma of chronically HIV-infected subjects compared to uninfected controls (Eller *et al*., 2011). Equally, there are increased levels of coagulation markers

during untreated HIV infection (Funderburg *et al*.; 2010). Before the start of antiretroviral therapy, elevated levels of D-dimers were found to be associated with higher risk of AIDS and death in individuals with advanced HIV disease (Boulware *et al*., 2011). Overall, high levels of these inflammatory (IL-6 and hsCRP) and coagulation (D-dimers) biomarkers are predictive of cardiovascular disease and mortality in HIV patients (Baker & Duprez, 2010).

1.3.2 B cell activation

Apart from T cells, B cells are also highly activated in HIV infection. Studies on B cells, primarily in the peripheral blood, have reported higher expression of activation markers (CD70, CD71, CD80 and CD86) in individuals with chronic HIV infection (Martínez-Maza *et al*., 1987; Malaspina *et al*., 2003; De Milito *et al*., 2004). In addition, chronically HIV-infected individuals showed high levels of proliferating B cells (Kovacs *et al*., 2001). B cell hyperactivity is also manifested by a polyclonal B cell activation and hypergammaglobulinaemia (Lane *et al*., 1983; Morris *et al*., 1998; Levesque *et al*., 2009). Morris and colleagues found elevated frequencies of B cells spontaneously producing immunoglobulin (Ig) in the serum of chronically HIV-infected individuals (Morris *et al*., 1998). Moreover, elevated levels of soluble markers of B cell activation (such CXCL13) and B cell-stimulatory cytokines (IL-10 and IL-6) have been found in HIV-infected persons (Martínez-Maza & Breen, 2002; Widney *et al*., 2005).

Altogether, these studies underline the importance of immune activation, described here by the activation of T cells and B cells, to drive disease progression. A range of mechanisms can contribute to this hyperimmune activation.

1.3.3 Causes of immune activation

The mechanisms contributing to immune activation can be both direct and indirect. HIV-derived antigens can activate the immune system directly. The interaction between HIV gp120 and CD4 receptors enhanced the activity of the protein tyrosine kinase $p56^{lck}$, resulting in CD4+ T cell activation (Hivroz *et al*., 1993). In addition, Zimmermann and co-workers recently showed that the activation of CD4+ T cells depends on the alignment of gp120–CD4 receptor binding with respect to T cell

receptor (TCR) engagement site, where proximal CD4-TCR signalling in response to gp120 favoured complete CD4+ T cell activation (Zimmermann *et al*., 2015). The HIV Nef protein can contribute to immune activation by downmodulating the TCR-CD3 complex from infected T cells (Schindler *et al*., 2006). Even though B cells are not directly infected by HIV, the binding of HIV gp120 to the CD21 receptor on B cells can result in polyclonal B cell activation and transfer of viral particles to activated T cells (Moir *et al*., 2000). Furthermore, B cells were found to become activated (expressing the activation marker Fas) following exposure to HIV Tat protein (Huang *et al*., 1997). B cell activation was linked to the direct triggering of the spleen tyrosine kinase (SYK) and c-Jun N-terminal kinase (JNK) signalling pathways by HIV (Perisé-Barrios *et al*., 2014).

The activation of innate cells via direct interaction either with HIV proteins or TLR ligands within the viral genomic RNA can also lead to immune activation. Uridine-rich sequences within HIV ssRNA mediated the activation of pDC, monocytes and NK cells through TLR 7/8 ligation (Alter *et al*., 2007; Meier *et al*., 2008). The activation of pDC to produce IFN-α occurred in response to HIV RNA upon CD4-mediated endocytosis and endosomal acidification followed by sensing primarily through TLR7 (Beignon *et al*., 2005). Furthermore, IFN-α production was mediated upon activation of the IRF7 pathway in early endosomes, where HIV localises depending on the interaction between HIV envelope protein and the CD4 receptor on pDC (O'Brien *et al*., 2011; Reszka-Blanco *et al*., 2015; O'Brien *et al*., 2016). Moreover, it has been recently proposed that the accumulation of HIV DNA in the cytoplasm of abortively infected cells can induce the release of the proinflammatory cytokine IL-1β upon caspase-1 activation (Doitsh *et al*., 2010). In summary, these studies show that viral components can directly induce immune activation.

The process of immune activation proves to be more complex than being mediated by HIV components alone in this direct manner, and there are also indirect contributors to hyperimmune activation. During HIV infection, the epithelial barrier of the gastrointestinal tract is disrupted due to a massive loss in CD4+ T cell helper (Th) subsets (such as Th17 cells) that are key players in gut homeostasis and in the defence against invading microbes at mucosal sites (Brenchley *et al*., 2004; 2007; 2008). As a result, HIV-infected individuals display an increased permeability of the gut

epithelium (Mudd & Brenchley, 2016). Consequently, the process of microbial translocation occurs, allowing bacterial components (such as LPS and bacterial DNA) to access the systemic circulation (Brenchley *et al*., 2007; Jiang *et al*., 2009). Brenchley and colleagues showed that individuals with chronic HIV infection had high levels of plasma LPS, which associated directly with the extent of CD8+ T cell and pDC activation. The first report to link the gut microbiome to systemic immune activation demonstrated a significant correlation between the proportions of Bacteroidales (through 16S rDNA quantification) and the levels of activated CD8+ T cells in chronically HIV-infected subjects (Ellis *et al*., 2011). Furthermore, changes in the gut microbial environment of chronically HIV-infected persons resulted in elevated Prevotella bacteria, which strongly associated with microbial translocation (measured by LPS and sCD14 plasma levels) and T cell activation (CD38 and HLA-DR expression; Dillon *et al*., 2014). Different classes of bacteria can induce the release of proinflammatory cytokines (IL-1β, IFN-γ and TNF-α) further promoting immune activation (Dinh *et al*., 2015).

Besides microbial translocation, co-infections with other pathogens may also play a role in chronic immune activation (Haas *et al*., 2011). Elevated frequencies of CD8+ T cells expressing CD38 and HLA-DR were reported in women co-infected with HIV and HCV relative to those infected with HIV alone (Kovacs *et al*., 2008). This CD8+ T cell activation was substantially higher in viraemic than non-viraemic women (HIV+HCV+ and HIV+HCV-, respectively). In addition, frequencies of activated B cells (expression of CD69 and CD86) correlated with EBV DNA load, which associated with higher levels of IL-6 and TNF-α (Petrara *et al*., 2012). Furthermore, increased expression of CD38 on CD8+ T cells specific for CMV and EBV was found in acutely HIV-infected individuals and correlated strongly with HIV viral load (Doisne *et al*., 2004).

Overall, these findings demonstrate that multiple processes occur and function together to drive immune activation and HIV disease progression, leading to serious consequences for the immune system.

1.3.4 Consequences of immune activation

Hyperimmune activation of the immune system can fuel further HIV replication. During HIV infection, the release of proinflammatory cytokines such as TNF-α, IFN-α and IL-1β can contribute to T cell activation, which directly results in elevated levels of NF-κB, inducing the transcription of HIV provirus, and providing more viruses for the infection of new target cells (Kawakami *et al*., 1988; Decrion *et al*., 2005; Doitsh *et al*., 2014). In addition, these cytokines may promote the translocation of bacterial products into the systemic circulation by altering epithelial barrier function, thereby further driving immune activation and HIV replication (Stockmann *et al*., 2000; Brenchley *et al*., 2007; Nazli *et al*., 2010). Even under antiretroviral therapy, where HIV replication is suppressed, immune activation may persist and there are consequences associated with this (Lederman *et al*., 2011; Sandler *et al*., 2011), as discussed in Section 1.5. Generalised immune activation in HIV-infected individuals may also lead to secondary lymphoid tissues fibrosis, primarily in the lymph nodes, as demonstrated by collagen deposition in paracortical T cell zones (Schacker *et al*., 2002). In addition, within the first 80 days of infection, nearly 50% of germinal centers were shown to be lost in secondary lymphoid tissues in the terminal ileum Peyer's Patches in HIV-infected individuals (Levesque *et al*., 2009). These data suggest that changes in lymph node architecture affecting both the paracortical T cell zone and B cell follicles (germinal centers), where the generation of T cell and B cell responses to antigens occurs, respectively, may impede the initiation of these immune responses (Estes, 2013). Furthermore, a reduction in the number of hematopoietic progenitor cells with functional alterations were demonstrated in HIV-infected individuals, as well as decreased thymopoiesis possibly due to thymic involution or HIV infection of the thymus (Stanley *et al*., 1993; Douek *et al*., 1998; Kalayjian *et al*., 2003; Sauce *et al*., 2011). Consequently, this alters the ability of the immune system to produce new lymphocytes to replenish those destroyed or rendered dysfunctional. Therefore, HIV-infected patients are unable to compensate for the immune activation-induced increased cell turnover (proliferation and/or apoptosis) and differentiation from naive to antigen-experienced cells displaying an exhausted phenotype, possessing a reduced ability to proliferate and exert effector functions (Appay & Sauce, 2008; Moir & Fauci, 2009). Collectively, these reports suggest that continuous viral replication and immune

activation can induce defects in the phenotype and function of immune cells, discussed in the next Section.

1.4 HIV-induced phenotypic and functional defects in lymphocytes

This section focuses on defects in T and B lymphocytes and their subsequent impairment to effectively coordinate immune responses.

1.4.1 T cell defects

Chronic immune activation can drive the expansion of memory T cells causing an imbalance in T cell subset distribution (Papagno *et al*., 2004). T cells can be grouped into naive, central memory, transitional memory (TM), effector memory and terminal effector cells, according to their differentiation phenotype and function (Paiardini & Müller-Trutwin, 2013). Upon antigen encounter and activation, naive T cells undergo clonal expansion, and become long-lived memory or effector T cells that migrate to sites of infection, with cytotoxic and cytokine secretion abilities (Pennock *et al*., 2013). Effector T cells are generally short-lived and the majority of these cells disappear once an infection has resolved, with the residual cells becoming memory T cells (Broere *et al*., 2011). Memory T cells are generally long-lived cells and the hallmark of re-encountering antigen is a rapid and more potent secondary response (Jameson & Masopust, 2009). In turn, based on the expression of the chemokine receptor CCR7 required for T cell homing to lymphoid tissues, memory T cells have been classified as CCR7+ central memory T cells (T_{CM}), self-renewing cells located in lymphoid tissues or CCR7- effector memory T cells (T_{EM}) with the ability to migrate to peripheral tissues (Lanzavecchia & Sallusto, 2005).

Chronic HIV infection drives the exhaustion of CD8+ T cells, limiting cell proliferative and polyfunctional abilities. T cell exhaustion is often characterised by increased expression of various inhibitory receptors including programmed cell death protein-1 (PD-1), the T cell immunoglobulin mucin-3 (Tim-3), lymphocyte activation gene-3 protein (LAG-3) and CD160 (Trautmann *et al*., 2006; Jones *et al*., 2008; Blackburn *et al*., 2009; Peretz *et al*., 2012). In chronically HIV-infected individuals, the expression of Tim-3 was upregulated on HIV-specific CD8+ T cells and Tim-3+CD8+ T cells were unable to secrete cytokine (IFN-γ) or undergo proliferation (as

measured by Ki67 expression) following stimulation with antigens likely due to impaired triggering of Stat5, Erk1/2, and p38 pathways (Jones *et al*., 2008). Furthermore, compared to both HIV-uninfected subjects and elite controllers, individuals with chronic HIV infection had higher expression of HIV-specific CD8+ T cells co-expressing CD160+PD-1+ and these cells had lower ability to perform effector functions (CD107a, IFN-γ and TNF-α) in response to TCR stimulation relative to HIV-specific CD8+ T cells expressing only CD160+ or PD-1+ (Peretz *et al*., 2012). More recently, Pombo and colleagues demonstrated that even in elite controllers HIV-specific CD8+ T cells can exhibit an exhausted phenotype (PD-1+CD160+2B4+) and the frequency of these cells were comparable to those found in chronic progressors (Pombo *et al*., 2015). However, unlike progressors, elite controllers appear to maintain a subset of HIV-specific CD8+ T cells that, despite co-expressing CD160 and 2B4, are endowed with cytolytic activity (perforin expression) possibly playing a role in the control of HIV (Pombo *et al*., 2015).

Studies of HIV-infected persons have demonstrated a loss of naive CD4+ and CD8+ T cells that is likely as a result of thymic dysfunction or increased differentiation of these cells into memory and effector cells due to chronic immune stimulation (Rabin *et al*., 1995; Roederer *et al*., 1995; Douek *et al*., 1998; Sallusto *et al*., 1999; Li *et al*., 2011). In the blood of HIV-infected persons, T_{CM} and T_{TM} CD4+ T cells were identified as the main reservoirs of HIV (Chomont *et al*., 2009). In SIV-infected rhesus macaques, the progressive decline of T_{CM} CD4+ T cells sets the tempo of disease progression to AIDS (Okoye *et al*., 2007). The maintenance of T_{CM} CD4+ T cells has been associated with lower viraemia and prolonged survival (Letvin *et al*., 2006; Potter *et al*., 2007). For instance, Potter and colleagues have shown that the frequencies of T_{CM} CD4+ T cells were significantly lower in viraemic individuals compared to elite controllers who had similar percentages of T_{CM} CD4+ T cells as uninfected controls. Moreover, in response to p24 Gag, T_{CM} CD4+ T cells in elite controllers had a greater ability to secrete cytokines (IFN-γ or IL-2 or both; but largely IL-2) than cells from viraemic patients (Potter *et al*., 2007). The depleted memory T cells during the course of HIV infection may also include memory cells for a range of opportunistic pathogens, impairing T cell responses against these co-pathogens (Saharia & Koup, 2013).

Specialised CD4+ Th subsets, based on their cytokine and transcriptional profiles (namely Th1, Th2, Th17, Th22, regulatory T cells (Treg) and follicular helper T cells (Tfh) cells; Paiardini & Müller-Trutwin, 2013) can be affected differentially during HIV infection. The loss of Th17 and Th22 subsets has been demonstrated in HIV-infected individuals (El Hed *et al*., 2010; Kim *et al*., 2012; Alvarez *et al*., 2013; Page *et al*., 2014). Alvarez and co-workers reported a selective depletion of Th17 cells, as shown by a decrease in the frequency of CD4+ T cells producing IL-17 from the blood of chronically HIV-infected subjects in comparison to uninfected controls (Alvarez *et al*., 2013). In addition, *in vitro* co-culture of sorted CCR6+CD4+ T cells in the presence of polyclonal stimulus and cytokines (IL-1β and IL-23) showed a preferential infection and depletion of Th17 cells by CCR5 or CXCR4-tropic HIV isolates (Alvarez *et al*., 2013). A lower frequency of peripheral Th22 cells in HIV-infected compared to HIV-uninfected individuals has also been demonstrated (Page *et al*., 2014), and this study also showed that a lower frequency of Th22 cells was associated with higher CD8+ T cell activation in HIV-infection.

In contrast to the loss of Th17 and Th22 cells, there is expansion of Treg and Tfh during HIV infection. Treg can suppress HIV-specific T cell responses, thereby contributing to HIV-induced immune dysfunction (Aandahl *et al*., 2004; Kinter *et al*., 2007). Increased frequencies and numbers of CD4+ Treg (CD25+FoxP3+) were found in mucosal tissues of chronically HIV-infected subjects with respect to uninfected controls, while in the blood the frequencies of CD4+ Treg were marginally elevated in viraemic patients (Epple *et al*., 2006). Other studies of HIV and SIV infection have reported the accumulation of CD4+ Tfh cells in secondary lymphoid tissues (Hong *et al*., 2012; Lindqvist *et al*., 2012; Petrovas *et al*., 2012). For example, Petrovas and colleagues have shown that the majority of rhesus macaques with chronic SIV infection had higher frequencies of CD4+ Tfh cells in their lymph nodes than acutely SIV-infected or uninfected control animals (Petrovas *et al*., 2012). The expansion of CD4+ Tfh cells did not appear to be because these cells were resistant to SIV infection, but rather was associated with SIV-induced immune activation and increased IL-6 levels in the plasma of chronically SIV-infected animals. In line with this, another study group demonstrated increased frequencies of SIV-infected CD4+ Tfh cells (by SIV DNA quantification) in lymph nodes of rhesus macaques with chronic infection compared to sooty mangabeys, natural primate hosts of SIV (Brenchley *et al*., 2012).

More recently, it has been shown in chronically HIV-infected individuals that CD4+ Tfh cells were enriched with HIV-specific CD4+ T cells following stimulation of lymph node mononuclear cells with Gag, Pol and Env peptide pools (Perreau *et al*., 2013). In addition, plasma viral load positively associated with the frequencies of Tfh cells that had the highest quantity of HIV DNA and supported productive HIV infection in *in vitro* culture (Perreau *et al*., 2013). However, how HIV infection affects the function of Tfh remains unclear. Expansion of CD4+ Tfh cells was associated with elevated germinal center B cells and increased levels of IgG and IgA antibodies in response to SIV antigens in the plasma of chronically SIV-infected animals, underlining the key role of Tfh in the stimulation of B cell responses (Petrovas *et al*., 2012; Perreau *et al*., 2013). Furthermore, the accumulation of Tfh in individuals with chronic HIV infection was associated with skewed B cell differentiation (Lindqvist *et al*., 2012).

1.4.2 B cell defects

Another hallmark of HIV infection is the occurrence of severe B cell defects that are associated with HIV-induced immune activation (Moir & Fauci, 2009). HIV induces phenotypic alterations in peripheral blood by provoking a skewing of B cell subpopulations (Moir & Fauci, 2013). Immature transitional B cells, having newly emigrated from bone marrow, and mature naive B cells, represent antigen-inexperienced B cells (Sims *et al*., 2005; Bemark, 2015). Memory B cells, that have responded to antigen and undergone somatic hypermutation upon antigen encounter, are characterised by their capacity to rapidly respond and produce high affinity antibodies when they are re-exposed to the same antigen (Kaminski *et al*., 2012; Bemark, 2015). Previous studies have identified classical memory B cells based on the expression of three markers, namely IgD, IgM and CD27 (Black *et al*., 1978; Klein *et al*., 1998). These memory B cells consist of roughly 60% unswitched B cells (IgM+IgD+ and IgM+IgD-), and 40% class-switched (IgM-IgD-), expressing other Ig isotypes such as IgG, IgA and IgE, and <1% of IgD+IgM- B cells (Yoshida *et al*., 2010; Kaminski *et al*., 2012). More recently, with the use of other markers such as CD21 and CD10, classical CD27+ memory B cells may additionally be grouped into activated and resting, memory B cells (Ho *et al*., 2006; Moir *et al*., 2008). However, a low proportion (1-4%) of memory B cells lacking the expression of CD27 has been

described in the blood of HIV-infected persons and represent tissue-like memory B cells (Fecteau *et al*., 2006; Moir *et al*., 2008; 2010). Terminally differentiated antibody producing B cells encompass plasmablasts (partially differentiated and proliferating) and plasma cells (fully differentiated and rare in peripheral blood; Montezuma-Rusca *et al*., 2015).

HIV infection results in the skewing of B cell subpopulations, creating an imbalance of some subsets. A number of studies have shown a substantial decrease in the frequency of naive B cells, while the frequency of immature transitional B cells increased in the blood of HIV-infected individuals relative to HIV-uninfected individuals (Malaspina *et al*., 2006; Moir *et al*., 2008; Pensieroso *et al*., 2013a). In addition, one study reported the depletion of CD27+ memory B cells in the blood of HIV-infected persons (De Milito *et al*., 2001). Furthermore, Moir and colleagues reported elevated frequencies of activated memory B cells and plasmablasts in individuals with chronic HIV infection, while resting memory B cell frequencies were reduced compared to uninfected controls (Moir *et al*., 2010). The loss of resting memory B cells can translate into a decrease in T cell-dependent or –independent antigen-specific memory B cell responses (Titanji *et al*., 2006; Hart *et al*., 2007; Moir *et al*., 2010), as well as compromised antibody responses to vaccination (Malaspina *et al*., 2005; Ho *et al*., 2011). For instance, there were lower tetanus toxoid and pneumococcal antibody (Ab) levels in HIV-infected than uninfected controls, and tetanus toxoid IgG and pneumococcal IgM levels positively associated with frequencies of switched memory and IgM memory B cells, respectively, in HIV-infected persons (Hart *et al*., 2007). In addition, two months after a single dose of the trivalent inactivated influenza vaccine, HIV-uninfected subjects showed substantially higher influenza-specific memory B cell responses relative to HIV patients (Malaspina *et al*., 2005). This could be explained by the lack of CD4+ T cell help due to B cells expressing low levels of CD25, impeding their ability to respond to activated CD4+ T cells or due to increased ligation of PD-1 on Tfh cells resulting in inadequate help to B cells, hence affecting the generation of B cell responses (Moir *et al*., 2003; Cubas *et al*., 2013). As opposed to resting memory B cells, higher frequency of tissue-like memory B cells were observed in HIV-infected than uninfected individuals (Moir *et al*., 2008). In this study, the frequency of antigen secreting cells (ASCs, measured by B cell ELISpot) against HIV gp120 were enhanced in these tissue-like memory B cells

after polyclonal stimulation. In contrast, a more recent study using gp140 probes showed that HIV-specific B cell responses were enriched not only in tissue-like memory B cells but also in activated memory and resting memory B cells, the latter two making up most (activated memory, 50.9% and resting memory, 31.2% vs tissue-like memory, 16.1%) of the total HIV-specific responses (Kardava *et al*., 2014). Differences in the techniques used may explain these contrasting findings. Unlike with the use of HIV envelope probes, the ELISpot assay requires *in vitro* culture, and it is possible that memory B cells may have not survived due to the susceptibility of these cells, primarily activated memory B cells to apoptosis in HIV infection (Kardava *et al*., 2014).

Overall, HIV infection dysregulates the differentiation pattern of lymphocytes and preferentially depletes some subsets, crippling their capacity to induce effective immune responses. However, whether HIV-induced dysregulation of these subsets are completely corrected following antiretroviral therapy remain to be clarified, and is still the subject of intensive research.

1.5 Antiretroviral therapy (ART) for HIV

The widespread availability of ART for HIV has had a major impact on the pandemic. This section summarises current ART guidelines for when to start ART and the benefits of early ART, elaborating on its effect on the immune system as well as transmission, and highlighting factors that influence CD4+ T cell recovery and whether phenotypic and functional alterations caused by HIV are reversed upon ART. In addition, the consequences and limitations of ART are discussed, along with new strategies proposed to improve or complement the efficacy of ART.

1.5.1 Current guidelines for ART initiation

According to the recent WHO guidelines on HIV treatment, HIV-infected persons should initiate ART irrespective of their CD4 count or WHO clinical stage, with priority being given to patients with CD4 counts ≤350 cells/ml and displaying serious or progressive HIV disease (WHO, 2015). This recommendation was proposed based on a systematic review of collective data from a range of studies demonstrating that HIV patients who commence ART early (at least at a CD4 counts of 500 cells/mm^3)

have a lower risk of developing AIDS and non-AIDS-related diseases and better immune recovery compared to those initiating ART late (WHO, 2015). A large randomised controlled trial conducted in 2076 HIV-infected Africans with baseline CD4 counts of 500 cells/mm^3 or more showed that patients who started ART immediately had a reduced risk of AIDS and non-AIDS-related diseases, and death in comparison to individuals who were in a deferred ART group, who received ART when they met the WHO criteria for starting ART at CD4 counts less than 200 or 350 or 500 cells/mm^3 (TEMPRANO ANRS12136 Study Group, 2015). Further evidence supporting early HIV treatment has also come from two published clinical trials, one of them being the Strategic Timing of Antiretroviral Treatment (START) trial with a similar design to the TEMPRANO study. The START trial determined the occurrence of severe AIDS and non-AIDS-related diseases or mortality in HIV patients (with CD4 counts ≥500 cells/mm^3) who were taking ART either immediately or later (when CD4 counts fell below 350 cells/mm^3). Compared to the 96 events reported in deferred ART arm, only 42 events occurred in the immediate therapy group, representing roughly a 56% decrease in serious AIDS- and non-AIDS-defining events (INSIGHT START Study Group *et al*., 2015). In addition, the HIV Prevention Trials Network (HPTN) 052 study randomised HIV serodiscordant couples with baseline CD4 counts ranging between 350 and 550 cells/mm^3 to receive early therapy or late therapy (when CD4 counts dropped below 250 cells/mm^3). A staggering 93% reduction in the overall risk of HIV transmission was reported in HIV-infected individuals receiving early ART (Cohen *et al*., 2016). Taken together, the outcomes of these studies underline the enormous benefits of early ART. However, the implementation of these new guidelines involve the scaling-up and sustainability of national ART programmes for maximum and life-long coverage of all HIV-infected individuals, which is a major challenge in resource-limited regions, particularly in Sub-Saharan African, where most HIV-infected persons reside. Interestingly, Kuznik and colleagues showed that early initiation of ART (CD4 counts >500 cells/ml) in HIV-infected persons from a range of countries (South Africa, Nigeria, Uganda and India) was cost-effective relative to a deferred ART group (Kuznik *et al*., 2016). Given these benefits to individual health, reducing transmission and cost-effectiveness, WHO issued guidelines recommending initiating ART irrespective of CD4 count (UNAIDS, 2016b). In response to this, South Africa modified its policy and began offering ART to all

HIV-infected persons in September 2016, regardless of their CD4 counts (Department of Health, 2016).

1.5.2 Classes of drugs, toxicity and treatment switch

The use of combination ART for the treatment of HIV infection has proven to be of great benefit, leading to sustained decreases in HIV viral load within 6 months, and these levels remain stable and below the limit of detection within a year of treatment, resulting in immune reconstitution (Autran *et al*., 1997; Lederman *et al*., 1998). The antiretroviral (ARVs) drugs currently available target distinct stages of the HIV life cycle and may be divided into different classes based on their mode of action (Arts & Hazuda, 2012). These include: 1) nucleoside reverse transcriptase inhibitors (NRTIs), 2) nucleotide reverse transcriptase inhibitors (NtRTIs), 3) non-nucleoside reverse transcriptase inhibitors (NNRTIs), 4) integrase inhibitors (INIs), 5) protease inhibitors (PIs) and 6) entry inhibitors. Briefly, NRTIs, NtRTIs and NNRTIs inhibit the function of HIV reverse transcriptase by preventing the copying of viral RNA into DNA. While INIs inhibit the integration of HIV DNA into the host chromosome, PIs prevent processing of viral proteins and packaging into mature forms. Entry inhibitors block the binding of HIV gp120 to CD4 or CCR5 on target cells (Arts & Hazuda, 2012). The recommended combination of ARVs consists of a backbone of two drugs from the nucleoside/nucleotide family and a third drug from any other class of ARVs, including NNRTIs, INIs, PIs and entry inhibitors (Akanbi *et al*., 2012). In South Africa, the preferred first-line regimen given to HIV-infected individuals consists of two NRTIs (tenofovir, TDF and emtricitabine, FTC or lamivudine, 3TC) and one NNRTI (efavirenz, EFV; Meintjes *et al*., 2014). Unlike other classes of drugs like INIs and PIs, NNRTIs can be used over the long-term as they are well-tolerated and more affordable (Meintjes *et al*., 2014). However, HIV-infected individuals may be required to switch regimens due to virological failure caused by the accumulation of resistance mutations to certain drugs, or drug toxicity. For instance, TDF, 3TC and EFV are associated with renal failure, anaemia and neuropsychiatric side effects, respectively (Meintjes *et al*., 2014).

1.5.3 Factors associated with CD4 restoration and immune recovery

ART is without a doubt highly effective, as it has reduced the emergence of opportunistic infections in HIV patients resulting in clear improvements in morbidity and mortality (Lawn & Wood, 2011). However, one early study demonstrated that roughly 60% and 16% of HIV-infected individuals on long-term ART failed to reconstitute their CD4 counts to levels above 500 and 200 cells/mm^3, respectively, even though viral load was maintained at undetectable levels (Kaufmann *et al*., 2003). These so-called 'immunological non-responders' spend a greater length of time at low CD4 counts and are thus more susceptible to AIDS and non-AIDS related illnesses (like opportunistic infections, renal and cardiovascular events) as well as death relative to immunological responders (Tan *et al*., 2008; Lapadula *et al*., 2013). Several factors have been identified that influence the extent of CD4+ T cell recovery upon ART, the strongest being baseline CD4 count prior to the start of ART (Corbeau & Reynes, 2011). Other factors, summarised in **Table 1.1**, include age, co-infections, time since HIV infection and cellular senescence.

Table 1. 1. Parameters influencing CD4+ T cell restoration upon ART

Factors	Main results	References
Baseline CD4 counts prior to ART	Lower CD4 counts at start of ART were associated with CD4 counts <500cells/mm^3 at 5 years of ART	(Kaufmann *et al*., 2005)
Age	Younger age independently predicted higher gain in CD4 counts at 4 years of ART	(Kaufmann *et al*., 2002)
Co-active infections	After 1 year of ART, HCV seropositivity was associated with lower CD4+ T cell recovery	(Greub *et al*., 2000)
Timing of ART	Early ART start (within 1 year) normalised CD4 counts better than late ART start (after 1 year)	(Okulicz *et al*., 2015)
Senescence	Highest frequency of CD27+CD8+ T cells was associated with high CD4+ T cell recovery at 1 to 2 years of ART	(Seu *et al*., 2013)

Another important parameter influencing CD4+ T cell reconstitution is prolonged immune activation during ART (Anthony *et al*., 2003; Hunt *et al*., 2003), discussed in further detail below.

1.5.4 ART and normalisation of immune activation

Despite a substantial decrease in the levels of immune activation in chronically HIV-infected individuals on ART, abnormal immune activation, measured by cell surface or soluble markers, can persist and has been linked to lower CD4+ T cell reconstitution and increased mortality (Hunt *et al*., 2011; Hunt, 2012). A study assessing T cell activation levels before and 1 year after ART showed that increased CD8+ T cell activation before ART could predict slower CD4+ T cell recovery, while continued CD8+ T cell activation predicted higher risk of mortality in HIV patients on ART (Hunt *et al*., 2011). In addition, elevated levels of microbial products such as LPS or markers of monocyte/macrophage activation (*e.g.* sCD14), the proinflammatory cytokine IL-6 and cycling T cells have been associated with poor CD4+ T cell reconstitution (Lederman *et al*., 2011). It is also unclear whether the levels of immune activation measured upon ART decrease to the extent of those found in HIV-uninfected individuals. Whole genome transcriptional profiling of CD4+ T cells showed increased gene transcripts associated with immune activation (cell cycling, cytokines and chemokines) in ART-treated patients with mean CD4 counts of 789/μl and undetectable viral load compared to HIV-uninfected controls (Vigneault *et al*., 2011). In addition, the increased percentages of activated T cells and NK cells (as measured by HLA-DR expression) as well as plasma levels of monocyte/macrophage (sCD163) and B cell (IgG, IgA and IgM) activation markers in HIV-infected individuals did not return to normal after 8 years of ART compared to an HIV-uninfected control group (Psomas *et al*., 2016). A further study assessed the effect of ART on the serum levels of immune activation, examining B cell activation and markers of inflammation in HIV-infected individuals on therapy (Regidor *et al*., 2011). A substantial decrease in serum levels of sCD27, sCD30, CXCL13, IgG and IgA was observed in HIV-infected subjects on ART for 2 to 3 years, but this did not reach levels found in HIV-uninfected controls. On the contrary, compared to HIV-uninfected controls, Wada and co-workers showed normalisation of seven biomarkers of immune activation and inflammation, including three (IL-2, IFN-γ, sIL-2Ra) that were associated with T cell activation following one year of ART, although sCD14 and TNF-α, related to monocyte/macrophage activation, did not normalise (Wada *et al*., 2015). It is worth noting that the timing of ART commencement can determine the levels of immune activation in HIV-infected individuals on treatment (Burdo *et al*.,

2011; Chevalier *et al*., 2013; Jain *et al*., 2013). Lower CD4+ and CD8+ T cell activation (measured by CD38 and HLA-DR expression) and a reduced HIV reservoir were reported in HIV patients who initiated ART within 6 months of infection compared to HIV patients who started ART late (a minimum of 2 years after infection) (Jain *et al*., 2013). Similarly, the start of ART during acute HIV infection resulted in a decrease in monocyte activation (sCD163) levels to comparable levels in HIV-uninfected controls, which was not the case when ART was initiated during chronic HIV infection (Burdo *et al*., 2011). Overall, the results indicate that immune activation is not completely normalised upon ART and may influence the extent of immune recovery. Consequently, sustained immune activation is associated with non AIDS-related events (such as cardiovascular disease) which are currently the main cause of morbidity and mortality in ART-treated patients (Kuller *et al*., 2008; Sandler *et al*., 2011; Duprez *et al*., 2012). For example, after 6 months of ART, lower levels of sCD14, IL-6 and kynurenine-tryptophan ratio were found to predict a lower common carotid intima media thickness (a marker for cardiovascular events) in a longitudinal study of HIV patients on ART for a median of 7 years (Siedner *et al*., 2016).

1.5.5 ART and normalisation of lymphocyte differentiation

In addition to sustained immune activation, Lederman and colleagues showed decreased T_{CM} and T_{EM} CD4+ T cell subsets in patients with immunologic failure despite suppressive ART (Lederman *et al*., 2011). There is evidence for full or partial normalisation of previously skewed memory subsets in HIV-infected individuals on ART. In HIV-infected individuals who initiated ART at CD4 counts >350 cells/mm^3, the number of naive and memory CD4+ T cells regularised by 1 year of ART with respect to uninfected controls (Robbins *et al*., 2009). On the contrary, another study showed partial restoration of naive and memory T cell counts after 3 years ART (Valdez *et al*., 2002). Although long-term ART (8 years) normalised the quantity of T_{EM} and effector CD4+ T cells, the number of naive and T_{CM} CD4+ T cells did not return to levels found in uninfected subjects (Rallon *et al*., 2013). A recent cross-sectional study of HIV-infected persons who started ART either early or late in infection reported that the frequencies of terminally differentiated CD4+ T cells remained elevated after a year in both treated groups and did not reach levels found in uninfected controls, suggesting that even early ART may not restore some HIV-

induced T cell perturbations (Amu *et al*., 2016). The incomplete normalisation of stem memory T cells (T_{SCM}), known to differentiate into other memory subsets like T_{CM} and T_{TM}, has been shown in SIV-infected rhesus macaques on ART and may explain irreversible normalisation of these subpopulations of T cells (Cartwright *et al*., 2016). With regards to B cells, it has been previously demonstrated that ART did not improve the percentages of classical memory (CD27+) B cells lost in HIV patients, as normalisation did not occur compared to HIV-uninfected controls (De Milito *et al*., 2001; Chong *et al*., 2004). Other studies have investigated the effect of ART on the subset distribution of B cells (Moir *et al*., 2008; 2010; Rethi *et al*., 2013; Amu *et al*., 2014; Rainwater-Lovett *et al*., 2014). One year of ART resulted in an increase in the frequency of naive and resting memory B cells with a concomitant decrease in immature transitional, activated memory, tissue-like memory B cells and plasmablasts in chronically HIV-infected persons although only naive B cells and plasmablasts were normalised relative to uninfected controls (Moir *et al*., 2010). ART during the early stages of HIV infection corrected the frequency of resting memory B cells, improving the memory B cell responses to influenza vaccine (Moir *et al*., 2010). In addition, while the frequency of activated and tissue-like memory B cells improved upon prolonged ART, those of resting memory B cells remained reduced in HIV-infected individuals (Amu *et al*., 2014). Therefore, except for resting memory B cells, normalisation of activated and tissue-like memory B cells were reported in virally-suppressed ART-treated patients (Pensieroso *et al*., 2013b). However, another study indicated that there is correction of resting memory B cells counts in younger HIV-infected subjects on ART for approximately 4 years (van Epps *et al*., 2014). Equally, Rainwater-Lovett and colleagues reported that following 1 year ART, the frequency of resting memory B cells were comparable between HIV-infected children at the age of 2-5 years old and uninfected controls (Rainwater-Lovett *et al*., 2014). In sum, despite somewhat contrasting data, ART can partially correct the skewed differentiation profiles of both T cells and B cells.

1.5.6 Restoration of antigen-specific responses

Numerous studies have assessed whether antigen-specific immunity is reconstituted during ART (Keane *et al*., 2004; Migueles *et al*., 2009; Moir *et al*., 2010; Cellerai *et al*., 2011; Hsu *et al*., 2013; Jambo *et al*., 2014). In a group of HIV-infected individuals

on ART for a median of 5 years, CD8+ T cell proliferation (CFSE labelling) and polyfunctional (simultaneous production of multiple cytokines) abilities in response to autologous HIV-infected CD4+ T cell targets were not restored compared to LTNPs matched for viral load (Migueles *et al*., 2009). In contrast, Cellerai and co-workers reported that HIV seroconverters on ART for at least 4 years restored T cell functionality (as measured by IFN-γ, IL-2 and TNF-α) in response to Gag relative to LTNPs (Cellerai *et al*., 2011). Others have investigated the reconstitution of other pathogen-specific T cell responses in ART-treated HIV-infected individuals. A slow rise in T cell IFN-γ responses to *Candida* antigens peaked 5 years after ART in the majority of HIV patients, but remained lower than responses found in HIV-uninfected controls (Burgess *et al*., 2006). For CMV, in one study elevated levels of CMV-specific T cell responses were found in the HIV treated group on long-term ART compared to the untreated group, while another study showed an early increase in the frequency of CMV-specific CD4+ T cells after 1 month ART, with a decline at 12 and 24 months ART relative to baseline levels (Naeger *et al*., 2010; Hsu *et al*., 2013). Likewise, for Mtb, while one study demonstrated no change in CD4+ T cell polyfunctional ability (IFN-γ, IL-17 and TNF-α) in response to Mtb purified protein derivative (PPD) post-ART, another study rather reported higher polyfunctional responses (IFN-γ, IL-2 and TNF-α) to PPD in these subjects post- compared to pre-ART (Sutherland *et al*., 2010; Jambo *et al*., 2014). With regards to B cells, using gp140 probes, lower frequencies of HIV-specific B cell responses were reported in HIV-infected subjects on ART for roughly 5 years compared to untreated elite controllers (Buckner *et al*., 2016). In addition, memory B cell responses to influenza hemagglutinin were similar between elite controllers and ART-treated individuals, whereas memory B cell responses to tetanus toxin C fragment were substantially reduced in HIV patients on ART compared to elite controllers. Overall, these conflicting studies highlight the need for additional research to clarify the recovery of antigen-specific immunity upon effective ART, a subject dealt with, at least for functional T cell responses, in Chapter 3 of this thesis.

1.5.7 Paradoxical ART and Immune reconstitution inflammatory syndrome (IRIS)

IRIS is an inflammatory response resulting in the deterioration of clinical status in a subgroup of patients early (within 6 months) after the start of ART (Wilkinson *et al*.,

2015). Globally, an estimate of 10-27% of HIV-infected individuals on ART develop IRIS (Haddow *et al*., 2012). The disease can manifest itself as paradoxical IRIS, namely when a pre-existing opportunistic infection initially responding to treatment worsens after ART; or unmasking IRIS, when a previously undetected infection appears after ART with excessive inflammatory responses (Murdoch *et al*., 2008; Meintjes *et al*., 2008). A number of risk factors have been associated with the development of IRIS, including low CD4 counts and high HIV RNA levels at the start of ART, a rapid decrease in viral load with an increase in CD4 counts post-therapy, as well as the presence of opportunistic infections closer to the time of ART initiation (Grant *et al*., 2010; Müller *et al*., 2010; Wilkinson *et al*., 2015). The most common pathogens associated with IRIS are Mtb, CMV, *Cryptococcus neoformans* and Hepatitis C and B viruses (Sharma & Soneja, 2011). A higher risk of paradoxical TB-IRIS has been associated with lower pre-ART levels of IL-6 and LPS-binding protein (LBP) and higher levels of CRP (Haddow *et al*., 2011; Goovaerts *et al*., 2013). Anti-inflammatory compounds like corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) have been used for the treatment of IRIS. A randomised placebo-controlled trial of 4 weeks course of prednisone was conducted in patients with paradoxical TB-IRIS (Meintjes *et al*., 2010). A decreased hospitalisation rate and therapeutic procedures were observed following prednisone treatment as a result of the latter likely inducing a substantial decrease in serum levels of IP-10, IFN-γ, TNF-α, IL-6, IL-10 and IL-12p40 (Meintjes *et al*., 2012). NSAIDs known to block the generation of prostaglandin mediators of inflammation are used as a first-line treatment for mild IRIS as opposed to corticosteroids that are required in severe cases of IRIS (Meintjes *et al*., 2012). Overall, in an attempt to restore functional immunity and improve the health of HIV-infected subjects, ART can lead to IRIS whose treatment with corticosteroids is associated with serious side effects in already immunocompromised individuals (Barber *et al*., 2012; Wilkinson *et al*., 2015). Thus, other therapies may be required for better management of IRIS.

1.5.8 Adjunct therapies

ART has succeeded in controlling HIV replication and improving immune function, but levels of immune activation do not reach baseline levels observed in HIV-uninfected individuals, and are associated with non-AIDS related morbidity and

mortality in ART-treated patients (Deeks & Phillips, 2009; Paiardini & Müller-Trutwin, 2013). Thus, the use of other drugs in combination with ART may be necessary to reduce immune activation in HIV-infected individuals. In line with this, due to their capacity to suppress cellular activation by interfering with the TLR signalling pathway within endosomes, two antimalarial drugs namely, chloroquine and hydroxychloriquine have been tested in HIV infection (Kyburz *et al*., 2006; Murray *et al*., 2010). In the absence of ART, chronically HIV-infected individuals on chloroquine therapy showed a decrease in the frequency of activated CD38+HLA-DR+CD8+ T cells as well as proliferating T cells (Murray *et al*., 2010). In addition, a reduced frequency of CD4+Ki67+ T cells, plasma LPS and IL-6 levels were reported in ART-treated immunologic non-responders during hydroxychloroquine treatment (Piconi *et al*., 2011). In contrast, Paton and co-workers showed in a randomised double-blind placebo-controlled trial that the administration of hydroxycloroquine in HIV-infected subjects not receiving ART did not induce a decrease in CD8+ T cell activation (measuring CD38 and HLA-DR expression), but induced a reduction in CD4+ T cell counts with a concomitant increase in plasma viral load (Paton *et al*., 2012). The use of cyclosporin A (an immunosuppressive cyclic oligopeptide known to prevent T cell activation and proliferation) has been reported, and while one study showed an increase in CD4+ T cell counts in HIV patients taking ART and cyclosporin A, other studies demonstrated no immunologic benefit or change in CD4+ T cell counts and proviral DNA levels (Rizzardi *et al*., 2002; Lederman *et al*., 2006; Markowitz *et al*., 2010). Other compounds such as valganciclovir and adalimumab (an anti-TNF monoclonal antibody), aimed at reducing immune activation by treating co-infections (like CMV) or blocking proinflammatory responses, respectively (Hunt, Martin, *et al*., 2011; Tabb *et al*., 2013). For instance, acutely SIV-infected rhesus macaques treated with adalimumab showed a decrease in expression levels of TNF genes and preserved CD4+ T cells but no change in T cell activation and plasma viral load compared to untreated animals (Tabb *et al*., 2013). Furthermore, homeostatic cytokines such as IL-7 has been used in conjunction with ART in HIV-infected patients, and data showed an increase in naive and T_{CM} CD4+ T cells, suggesting an improvement in T cell homeostasis (Levy *et al*., 2012). Moreover, the administration of IL-21 to ART-treated SIV-infected pigtailed rhesus macaques supplemented with probiotics demonstrated an increase in the frequency and function of Th17 cells and a decrease in microbial translocation (measured by sCD14 and LBP levels) when

compared to control animals on ART only (Ortiz *et al*., 2016). Altogether, these reports highlight the benefits of these compounds but nevertheless with limited success, implying that more effective strategies need to be developed to reduce immune activation in HIV infection while having a positive effect in decreasing viral load and increasing CD4+ T cell counts.

1.5.9 Limitations of ART

Effective ART clearly suppresses HIV replication in the majority of HIV-infected individuals, but despite this, there are some limitations to it. Access to ART is limited in developing countries due to the high cost or lower coverage (due to poor health infrastructure) and in countries such as South Africa where there is ready access to therapy, life-long adherence and the monitoring and toxicities associated with treatment represent substantial cost and healthcare challenges (Ford *et al*., 2011; Sahay *et al*., 2011). In addition, even when viral replication is controlled, there remains an increased risk of HIV-associated illnesses such as cancer, cardiovascular diseases and other end-organ diseases likely due to sustained immune activation and inflammation as a result of ongoing or residual viral replication (Hunt, 2012). Therefore, there is a pressing need to eradicate the virus to completely cure HIV infection, something that current ART is not able to achieve. However, the establishment of viral reservoirs in latently infected memory CD4+ T cells in HIV-infected individuals occurs early in infection and is a major obstacle to curing HIV, because these latently infected cells are invisible to clearance by the immune system and not susceptible to ARVs, representing a potential source of viral replication when treatment is discontinued (Chun *et al*., 1997; Finzi *et al*., 1997). HIV latency is generated when HIV-infected activated CD4+ T cells survive cellular infection to return to a resting memory state, or upon interaction of mDC or CCR7 ligands (CCL19 and CCL21) with resting CD4+ T cells (Saleh *et al*., 2007; Siliciano & Greene, 2011; Evans *et al*., 2013). Strategies for HIV cure can be grouped into functional cure or remission, a state of long-term control of viral replication without ART, and sterilising cure, representing complete purging of the virus (Katlama *et al*., 2013). A number of studies have proven that the commencement of ART during acute infection could restrict seeding of HIV reservoirs and possibly control viral replication when ART is interrupted (Strain *et al*., 2005; Archin *et al*., 2012; Josefsson *et al*., 2013; Buzon et al., 2014). One key example is the

case of the HIV-infected Mississippi baby who initiated ART within 30 hours of life for 18 months, after which viral load was suppressed and HIV reservoirs remained undetectable for more than 2 years after discontinuation of ART (Persaud *et al*., 2013). In addition, in 14 adult patients from the VISCONTI cohort who started ART within 2 months of HIV infection for roughly 3 years, viral load was effectively controlled for more than 7 years after ART was interrupted (Sáez-Cirión *et al*., 2013). A French teenager was also able to suppress viraemia for 12 years after stopping ART (Hayden, 2015). Together, these results suggest that there is a possibility to maintain HIV replication at very low levels in the absence of ART for a long period of time. However, this does not mean that the virus has been completely eradicated in these individuals. To date, only one individual, known as the "Berlin patient", has been scientifically verified to be cured from HIV. This HIV-infected individual, who developed acute myeloid leukaemia, showed no viral rebound after receiving a stem cell transplant from a homozygous donor with a 32 base-pair deletion in CCR5 (CCR5Δ32) following cessation of ART (Hütter *et al*., 2009; Allers *et al*., 2011). Currently novel therapeutic approaches are being tested, including gene therapy approaches and latency reversing agents to purge viral reservoirs (Katlama *et al*., 2013). Interestingly, some of these approaches involve the use of activation of the immune system, for example by the use of TLR7 agonists. In summary, ARTs are currently limited, but therapies are being pursued that have the potential for curing HIV.

1.6 Aims and objectives of the thesis

The overall aim of this thesis was to investigate the impact of antiretroviral therapy for HIV on the phenotype and function of T and B cells. Levels of residual immune activation, differentiation profiles of T and B cells, and functional T cell responses specific for co-pathogens (Mtb and CMV) were evaluated. This was studied in the peripheral blood of a cohort of chronically HIV-infected South African women after short-term ART, where samples pre-ART and 1 year post-ART were examined, and compared to HIV-uninfected individuals from the same community.

Hypothesis:

Effective antiretroviral therapy normalises the phenotype of T and B cells, and decreases immune activation in both T and B cell subsets. The ability of T cells to produce cytokines in response to specific co-pathogens is restored following short-term ART.

Objective 1: To determine the activation and memory phenotypes of CD4+ and CD8+ T cells during chronic HIV infection and after antiretroviral therapy

Rationale: Despite the improvement in CD4+ T cell counts upon suppressive ART, hyperimmune activation may persist and influence CD4+ T cell recovery. The extent, to which specific memory T cell subsets are normalised on ART, and the relationship with ongoing immune activation is not fully defined. Therefore, we aimed to assess T cell differentiation, activation and proliferation profiles in HIV-infected individuals before and after ART initiation. These phenotypic profiles were compared to those in age- and sex-matched HIV-uninfected controls. Our findings give insight into the reconstitution of particular T cell memory subsets and the extent to which T cell activation and proliferation levels decline after 1 year of ART.

This aspect of the study is presented in Chapter 2.

<u>Objective 2:</u> To assess whether functional T cell immunity to co-pathogens is recovered after successful antiretroviral therapy

Rationale: HIV infection not only causes the loss of CD4+ T cells, but also compromises their function by modifying their phenotype and altering their ability to secrete cytokines in response to HIV itself or other pathogens. Even though effective ART improves CD4+ T cell counts, its effect on the functional restoration of specific immunity to common co-pathogens (in this case Mtb and CMV), and the factors associated with this recovery have not been fully characterised. Here, we aimed to compare the memory differentiation profile and cytokine production ability of Mtb and CMV-specific CD4+ T cells pre- and post-ART in HIV-infected individuals and healthy uninfected individuals. Our data give important insights into the restoration of antigen-specific T cell responses when HIV replication is controlled by effective ART.

This aspect of the study is presented in Chapter 3 and has been published as:

Catherine Riou*, **<u>Ramla F. Tanko</u>***, Andreia P. Soares, Lindi Masson, Lise Werner, Nigel J. Garrett, Natasha Samsunder, Quarraisha Abdool Karim, Salim S. Abdool Karim, Wendy A. Burgers. **Restoration of CD4+ responses to co-pathogens in HIV-infected individuals on antiretroviral therapy is dependent on T cell memory phenotype.** *Journal of Immunology* 2015; 195:2273-2281. **contributed equally to this work. [See Appendix]*

<u>Objective 3:</u> To identify changes in B cell activation and differentiation profiles in HIV-infected persons on suppressive antiretroviral therapy

Rationale: In addition to T cells, HIV infection severely impacts B cells, resulting in skewing of B cell subsets, and impaired B cell and antibody responses. However, the extent to which HIV-induced B cell defects are corrected upon ART remains unclear. Thus, we aimed to examine the level of B cell activation, proliferation, and memory subset distribution prior to initiation of therapy and after suppressive therapy. HIV-infected individuals were compared to healthy uninfected subjects. The relationship between 'deactivation' of B cells and T cells (Objective 1) was also determined. Our results provide novel information on the recovery of B cell memory subsets, the degree to which B cell activation and proliferation declines upon ART, as well as the relationship between immune activation in the B cell and T cell compartments.

This aspect of the study is presented in Chapter 4 and has been published as:

Ramla F. Tanko, Andreia P. Soares, Tracey L. Müller, Nigel J. Garrett, Natasha Samsunder, Quarraisha Abdool Karim, Salim S. Abdool Karim, Catherine Riou, Wendy A. Burgers. **Effect of antiretroviral therapy on the memory and activation profiles of B cells in HIV-infected African women.** *Journal of Immunology* 2017; 198: 1220-1228. *[See Appendix]*

CHAPTER 2

Impact of antiretroviral therapy on T cell activation and differentiation during chronic HIV infection.

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2.1 Introduction

Chronic HIV infection is characterised by the gradual decline in CD4+ T cells that are essential in co-ordinating adaptive immune responses. In the absence of effective antiretroviral treatment (ART), this loss in CD4 cells as a consequence of sustained viral replication is accompanied by an increase in CD8 counts and a reduced CD4 to CD8 ratio (Frazer *et al*., 1986). A hallmark of HIV infection is chronic immune activation, which has a deleterious effect and a fundamental role in HIV pathogenesis, by significantly contributing to CD4+ T cell loss and driving disease progression (Hazenberg. *et al.*, 2003). The state of immune activation results in increased activation, proliferation, depletion and exhaustion of T cells (Hellerstein & McCune, 1997; Mohri *et al*., 2001; Hazenberg *et al*., 2003; Papagno *et al*., 2004). In addition, increased immune activation can cause alterations in T cell phenotype by inducing a decrease in naive T cells and a parallel accumulation of more differentiated effector cells (Sallusto *et al*., 1999; Papagno *et al*., 2004; Appay & Sauce, 2008). This skewed differentiation profile may reflect thymic dysfunction, preventing the renewal of naive T cells, or antigen-driven differentiation of naive T cells, increasing the proportion of effector memory T cells (Douek *et al*., 1998; Li *et al*., 2011; Sauce *et al*., 2011). Overall, this results in the loss of memory resources for opportunistic and pathogenic infectious agents.

Numerous drivers of immune hyperactivation during HIV infection have been described. These include 1) the interaction of HIV itself either via the envelope glycoprotein gp120 or single-stranded HIV RNA motifs that act as ligands for toll-like receptors (TLR7/8) on innate cells (Heil *et al*., 2004; Meier *et al*., 2007; Del Cornò *et al*., 2016), 2) the process of microbial translocation, where bacterial components enter the circulation as a result of leaky epithelium within the gastrointestinal tract and activate a range of additional TLRs (Brenchley *et al*., 2007; Zevin *et al*., 2016); 3) the subsequent release of proinflammatory cytokines by innate cells such as dendritic cells, macrophages or monocytes as a result of TLR stimulation (Haas *et al*., 2011); 4) the highly proinflammatory process of pyroptosis of CD4+ T cells after abortive infection with HIV (Doitsh *et al*., 2014); and 5) reactivation of co-infecting, chronic viruses like hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and herpes simplex virus (HSV) (Doisne *et al*., 2004; Sheth *et al*., 2008). All together,

these mechanisms contribute to the overactivation of the immune system, promoting disease progression.

With the introduction of ART, the majority of HIV-infected individuals fully suppress viral load, display a progressive increase in CD4 counts, and reduced opportunistic infections, resulting in a substantial improvement in their health (Guihot *et al*., 2011; Lawn & Wood, 2011; Williams *et al*., 2011; Delaugerre *et al*., 2015). However, up to one quarter of individuals fail to reconstitute their CD4 counts substantially, as a result of delayed initiation of treatment and ongoing immune activation, and hence are at high risk of AIDS related morbidities (Corbeau & Reynes, 2011) as well as non-AIDS related illnesses, such as certain types of cancer and cardiovascular disease (Hunt, 2012). Nevertheless, HIV-induced T cell phenotypic defects can be partially corrected upon ART. HIV-infected individuals on ART showed an overall decline in CD4+ and CD8+ T cell activation (commonly measured by CD38 and HLA-DR expression) and proliferation (measured by Ki67 expression), but it still appears to remain higher than in HIV-uninfected individuals (Hazenberg *et al*., 2000; Anthony *et al*., 2003; Hunt *et al*., 2003; Almeida *et al*., 2007; Glencross *et al*., 2008; Hunt *et al*., 2011). Naive and memory CD4+ T cells are also partially normalised in treated-HIV patients (Lepej *et al*., 2006; Rallon *et al*., 2013; Emu *et al*., 2014).

Despite the irrefutable clinical and immunologic benefits of ART, HIV-infected individuals on suppressive treatment may still display immune defects. In fact, we still do not completely understand the extent to which specific T cell memory subsets are recovered upon ART, their relationship with ongoing immune activation, and whether the recovery profile is similar between the CD4 and CD8 compartments. African cohorts, in particular, are less well-studied than Caucasian cohorts. With this in mind, the aim of this study was to determine the effect of ART on the activation, proliferation and memory differentiation profiles of CD4+ and CD8+ T cells in chronically HIV-infected South African women, before and 12 months after the initiation of ART. These profiles were compared to age- and sex-matched HIV-uninfected controls from the same community.

2.2 Material and Methods

2.2.1 Study participants

Twenty-eight women were recruited from the Centre of AIDS Programme of Research in South Africa (CAPRISA) 002 HIV acute infection cohort in KwaZulu-Natal, as described previously (van Loggerenberg *et al*., 2008; Mlisana *et al*., 2014). The study participants were infected for a median of 4.2 years [Interquartile range (IQR): 2.7-5.6 years]. To estimate the time of HIV infection, a prospective RNA positive/antibody negative result or the midpoint between the last antibody negative test and the first antibody positive enzyme-linked immunosorbent assay test were used. ART was offered according to the South African National HIV treatment guidelines (at a CD4 count of <200 cells/mm^3 prior to October 2012; <350 cells/mm^3 until March 2015). The majority of the participants (25/28) were taking standard first-line therapy, namely TDF/3TC/EFV (n=14) or TDF/FTC/EFV (n=4) or ddI-EC/3TC/EFV (n=3) or TDF/3TC/NVP (n=1) or AZT/3TC/NVP (n=1) or d4T/3TC/EFV (n=1) or d4T/3TC/NVP (n=1), while only one individual was on a second-line regimen, consisting of AZT/3TC/Lpvr/r (as indicated in **Appendix Table 1)**. Two participants switched drug regimens during the study period, namely CAP200 (ddI-EC /3TC/ EFV to TDF /3TC/EFV at month 11) and CAP255 (d4T/3TC/EFV to AZT/3TC/EFV at month 10). Peripheral blood samples were taken at two time-points pre- and post-ART initiation. An additional 23 HIV-uninfected women who were matched for age, gender and ethnicity were provided from the CAPRISA 004 1% Tenofovir microbicide gel trial (Abdool Karim *et al*., 2010). These women were either in the pre-intervention or in the placebo phase of the trial. No participants had active TB over the study period or any immune reconstitution inflammatory syndrome following ART. Ethical approval for the study was obtained from the Research Ethics Committees at the University of KwaZulu-Natal and University of Cape Town. All participants provided written informed consent prior to participating in the study.

2.2.2 Determination of HIV plasma viral load, CD4 and CD8 counts

Plasma HIV viral load and CD4 count were assessed at each study visit. During the study period, the viral load PCR assay switched from Roche AMPLICOR HIV-1 Monitor test version 1.5 (lower detection limit (LDL) of 400 RNA copies/ml) to Roche

Taqman version 1.0 in June 2010 (LDL 40 RNA copies/ml), and then to Roche Taqman version 2.0 in January 2012 (LDL 20 RNA copies/ml). The FACSCalibur TruCOUNT method (BD Biosciences) was used to measure absolute blood CD4+ and CD8+ T cell counts.

2.2.3 Sample processing

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Pharmacia) density gradient centrifugation and cryopreserved in freezing media consisting of heat-inactivated fetal calf serum (FCS; Invitrogen) containing 10% dimethylsulfoxide (DMSO; Sigma-Aldrich). Cells were stored in liquid nitrogen until use. Cryopreserved PBMC were thawed and rested in R10 (RPMI 1640 plus 10% heat-inactivated FCS and 50 U/ml of penicillin-streptomycin) at 37°C with 5% CO_2 for 3 hours before staining.

2.2.4 Monoclonal antibodies and staining

All the antibodies used in this study are listed in **Table 2.1**, together with the commercial sources of these antibodies, their corresponding clones and optimal titers, determined by titrations for a 50 μl staining volume. Briefly, PBMC were stained with a viability dye (Vivid), then labeled with antibodies against surface markers, fixed, permeabilised and subsequently stained intracellularly for Ki67. Cells were then resuspended in 1X CellFix (BD Biosciences) and kept at 4^0C until acquisition. Samples were acquired within 24 hours on a BD Fortessa using FACSDiva software and analysed using FlowJo (version 9.9.3; TreeStar).

Table 2. 1. List of antibodies for flow cytometry

	Markers	Fluorochromes	Clone	Manufacturer	Titer (µl)
T cell panel					
Exclusion	CD14	Pacific Blue[a]	Tük4	Invitrogen	1.25
Exclusion	CD19	Pacific Blue[a]	SJ25-CI	Invitrogen	1.25
Lineage	CD3	PE-Cy7	Sk7	BD Biosciences	0.63
Lineage	CD4	PE-Cy5.5	S3.5	Invitrogen	0.3
Lineage	CD8	Qdot-705	3B5	Invitrogen	0.1
Memory	CD27	PE-Cy5	1A4CD27	Beckman Coulter	2.5
Memory	CD45RO	ECD	UCHL1	Beckman Coulter	0.6
Activation	HLA-DR	APC-Cy7	L243	BD Biosciences	2.5
Activation	CD38	APC	HIT2	BD Biosciences	10
Proliferation	Ki67	FITC	7B11	Invitrogen	0.25

[a]The viability dye Vivid (Invitrogen) was also detected in the Pacific Blue channel

2.2.5 Gating strategy

The gating strategy used to identify T cells and memory subsets is shown in **Figure 2.1**. A time gate was first applied to eliminate any potential shifts in fluorescence intensity, followed by singlets (to exclude doublets), live cells, lymphocytes and CD3+ cells. CD14 and CD19 markers were used to exclude monocytes and B cells, respectively, concomitant with exclusion of dead cells. CD3+ T cells were further discriminated using CD4 and CD8 markers. Gates were set based on FMO controls.

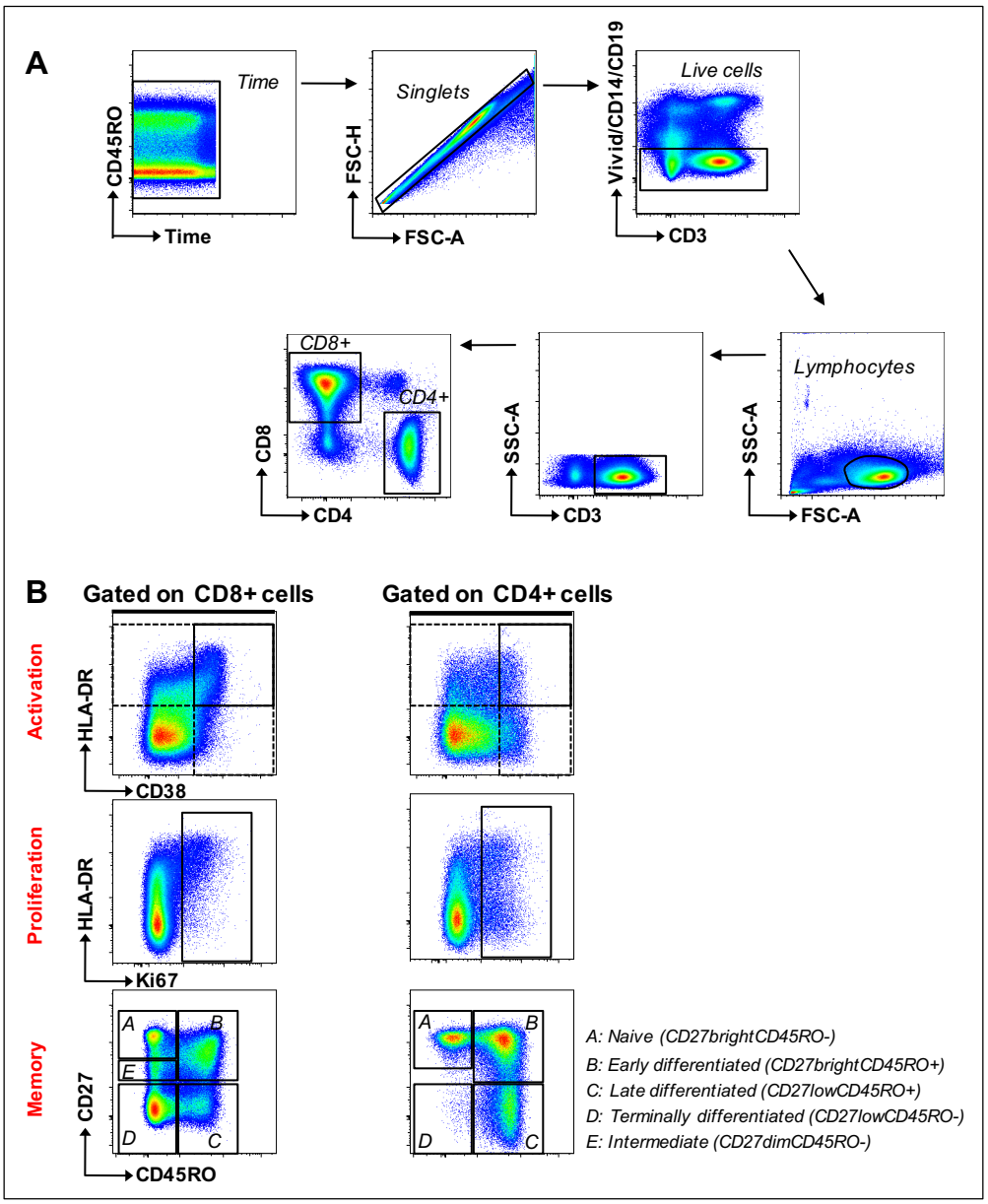

Figure 2. 1. Gating strategy. PBMC were stained directly *ex vivo*. Gating is shown for one HIV-infected individual before ART initiation. **(A)** Identification of T cells and subsets. A time gate to ensure no shift in fluorescence intensity over time was applied, followed by a singlet gate for exclusion of doublets. A dump channel comprising CD14 and CD19 markers as well as the viability dye Vivid were used to exclude monocytes, B cells and dead cells, respectively. The CD3 marker identified T cells, while CD4 and CD8 markers specifically stained for their corresponding CD4+ and CD8+ T cell subsets. **(B)** Gating to identify T cell activation (CD38 and HLA-DR), proliferation (Ki67) and differentiation (CD27 and CD45RO). Four distinct T cell subsets were delineated: naive (A. CD27brightCD45RO-), early (B. CD27brightCD45RO+), late (C. CD27lowCD45RO+), and terminally (CD27lowCD45RO-) differentiated cells. An additional subset, namely intermediate (E. CD27dimCD45RO-) was unique to CD8+ T cells.

2.2.6 Statistical analyses

Statistical analyses were performed using Prism (GraphPad version 5.0). The Mann-Whitney U test and the Wilcoxon Signed Rank test were used for unpaired and paired samples, respectively. Correlations between the different groups were determined by the non-parametric Spearman Rank correlation test. A p-value of <0.05 was considered to be statistically significant.

2.3 Results

2.3.1 Changes in HIV plasma viral load and T cell counts after ART initiation

Twenty-eight HIV-infected women were studied prior to and one year after the initiation of ART in this study. We first examined how the study participants responded to ART treatment, by analysing clinical parameters associated with immune recovery. Pre-ART samples were obtained at a median of 1.9 months (IQR: 0.4-2.7) before ART initiation (**Appendix Table 1**). The median plasma viral load was 38,513 HIV RNA copies/ml (IQR: 8,205-76,793), while the median CD4 and CD8 counts were 279 cells/mm^3 (IQR: 228-330) and 1,018 cells/mm^3 (IQR: 642-1,395), respectively. Prior to ART, viral load inversely correlated with CD4 counts, as expected ($r=-0.52$, $p=0.005$; data not shown). After a median of 12.2 months of ART (IQR: 11.3-12.9), plasma viral load was significantly reduced ($p<0.0001$; **Figure 2.2A**), and was below the detection limit of the assay used (i.e <20 copies/ml, <40 copies/ml or <400 copies/ml) in the majority of participants (21/28, **Appendix Table 1**). The remaining 7 participants still had detectable but low levels of virus in their plasma (median: 64 copies/ml). The decrease in plasma viraemia was accompanied by a significant increase ($p<0.0001$; **Figure 2.2B**) in absolute CD4 counts, which occurred in 26/28 individuals. The median increase in the absolute number of circulating CD4 cells post-ART was 249 cells/mm^3. In contrast to the increase in absolute CD4 counts, no significant change was observed in the absolute CD8 counts pre- and post-ART ($p=0.19$, **Figure 2.2C**). However, it is worth noticing that in the majority of subjects (71.4%, 20/28), the absolute CD8 counts decreased (median of 122 cells/mm^3; **Figure 2.2C**) following ART. These changes in both CD4 and CD8 counts led to a significant increase in the CD4:CD8 ratio ($p<0.0001$; **Figure 2.2D**), with a median fold change of 2.5 after 1 year of ART. Of note, 2 participants, CAP248

and CAP262 did not reconstitute their CD4 counts. CAP248 was one of the participants with low but detectable viral load (121 RNA copies/ml; **Appendix Table 1**) after 1 year of ART; and CAP262 exhibited viral suppression, low CD8 count and her CD4:CD8 ratio doubled after treatment. However, it is worth noting that both participants at later time points (>1 year) recovered their CD4 counts upon ART (data not shown).

Overall, these results demonstrate that in all participants, 12 months of ART suppressed HIV replication to a large degree, and led to a significant improvement in absolute CD4 counts in most of the treated patients.

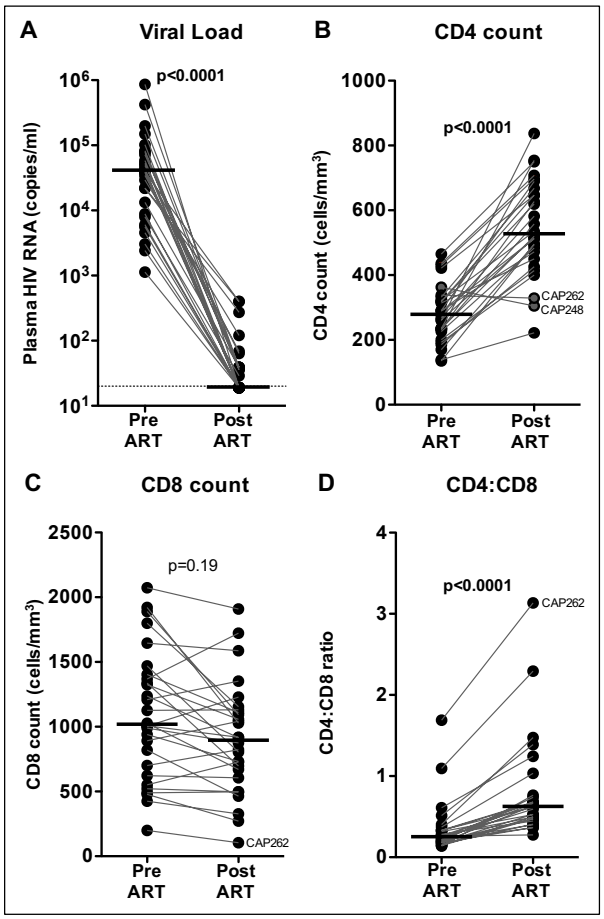

Figure 2. 2. Clinical parameters before and after ART. (A) Plasma viral load, **(B)** absolute CD4 count, **(C)** absolute CD8 count, **(D)** CD4/CD8 ratio before (pre-ART) and after 12 months of ART (post-ART) in HIV-infected individuals (n=28). The horizontal dotted line indicates the detection limit of the assay. The two grey dots depict two individuals who did not reconstitute their CD4 counts after ART. Horizontal solid lines indicate the median. Statistical significance was calculated using a t-test.

2.3.2 Substantial decrease in activation and proliferation of CD4+ and CD8+ T cells after ART

Excessive immune activation, a major contributor to CD4+ T cell loss and HIV pathogenesis, is characterized by increased activation and proliferation of immune cells, including T cells (Mohri *et al*., 2001; Hazenberg *et al*., 2003). ART has been highly successful in reducing T cell activation and proliferation, but normalisation is often incomplete and is likely dependent on the timing and duration of ART, and whether ART can reverse the immune damage caused by HIV. Thus, to evaluate the extent to which ART impacts T cell hyperactivation in our cohort of African women, we first measured the expression of activation markers CD38 and HLA-DR pre-and post-ART and compared them to profiles obtained in HIV-uninfected controls. **Figure 2.3A** shows representative flow cytometry plots of CD38 and HLA-DR expression on CD4+ and CD8+ T cells from one HIV-uninfected and one HIV-infected individual before and after ART. As expected, the frequency of activated CD4+ T cells were significantly higher in HIV-infected individuals prior to ART compared to HIV-uninfected controls (CD38: medians 8.5% vs 1.6%; HLA-DR: median 7.4% vs 1.5% and CD38/HLA-DR co-expression: 1.7% vs 0.1%; $p<0.0001$; **Figure 2.3B**). After ART, the frequency of activated CD4+ T cells decreased significantly (CD38: median 5.5%; HLA-DR: median 3.3%; and CD38/HLA-DR: median 0.3%) but still remained significantly higher than HIV-uninfected subjects ($p<0.0001$ for CD38 and CD38/HLA-DR, and $p=0.0006$ for HLA-DR; **Figure 2.3B**). Similar to CD4+ T cells, HIV also induced elevated expression of CD38 and HLA-DR on CD8+ T cells, as expected (Hunt *et al*., 2008). The frequency of activation markers expressed on CD8+ T cells was higher than on CD4+ T cells (CD38: medians 14.4% vs 8.5%; HLA-DR: medians 23.7% vs 7.4% and CD38/HLA-DR: medians 9.2% vs 1.7%), and while ART also induced a significant reduction in CD8+ activation, the levels observed after 12 months of treatment remained significantly higher than HIV-uninfected individuals (**Figure 2.3C)**. Of note, one individual, CAP200, had an increase in CD38 expression on both CD4+ and CD8+ T cells post-ART, while the expression of HLA-DR alone or co-expressed with CD38 was reduced on CD4+ T cells and remained unchanged on CD8+ T cells (**Figure 2.3B**). This patient switched her drug regimen at 11 months (**Appendix Table 1**).

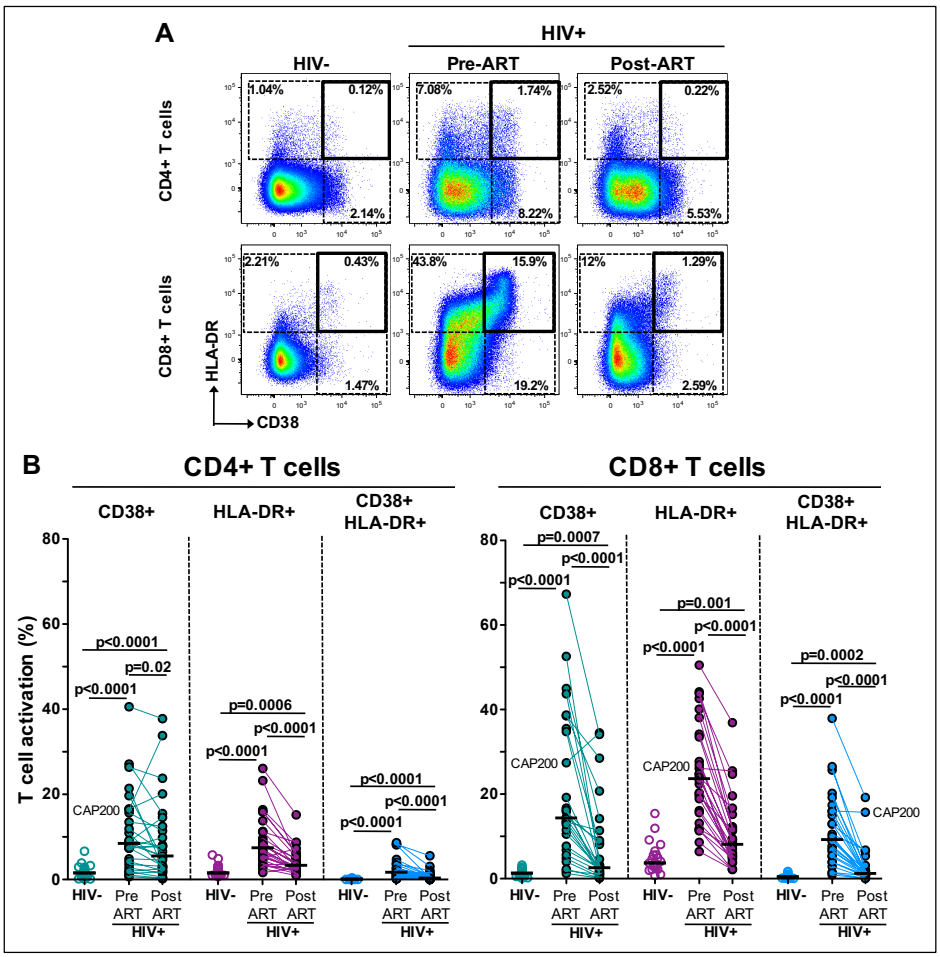

Figure 2. 3. Effect of ART on CD4+ and CD8+ T cell activation levels. (A) Representative flow plots of CD38 and HLA-DR expression on T cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. **(B)** Frequencies of CD38+ (teal), HLA-DR+ (plum) and CD38+HLA-DR+ (blue) CD4+ and CD8+ T cells in 23 HIV-uninfected (open circles) and 28 HIV-infected (closed circles) individuals pre- and post-ART. Horizontal lines represent the median. Statistical significance was calculated using the Mann-Whitney U test and Wilcoxon Signed Rank for unmatched and matched samples, respectively.

Next, the effect of ART on T cell proliferation was determined in 18 participants for whom Ki67 staining was available. **Figure 2.4A** shows representative flow cytometry plots of CD4+ and CD8+ T cell proliferation measured by Ki67 expression from one HIV-uninfected and one HIV-infected subject before and after ART. The frequency of Ki67+ T cells was significantly higher in HIV-infected subjects compared to uninfected controls (CD4: median 2.9% vs 0.42% and CD8: 2.1% vs 0.38%; $p<0.0001$; **Figure 2.4B**). Even though ART led to a significant reduction in the frequency of Ki67+ T cells (medians of 1% and 0.6% for CD4+ and CD8+ T cells with corresponding p-values, $p=0.0003$ and $p=0.0002$, respectively), they were still significantly elevated compared to the uninfected control group ($p<0.0001$ for CD4+ and $p<0.02$ for CD8+ T cells). Thus, with the exception of one individual who had a

slight increase in Ki67 expression in her CD4 compartment (CAP353), the remaining ART-treated patients (n=17) displayed a reduced frequency of proliferating T cells.

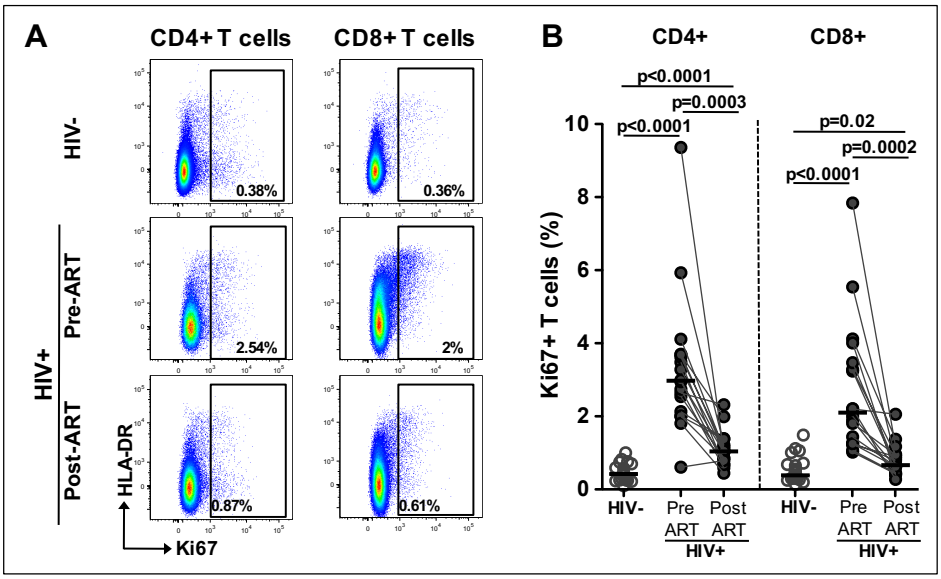

Figure 2. 4. Effect of ART on T cell proliferation. (A) Representative flow plots of Ki67 expression in CD4+ and CD8+ T cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. Numbers represent the frequencies of proliferating (Ki67+) T cells. **(B)** Frequencies of Ki67+ T cells in HIV-uninfected (n=23; open circles) and HIV-infected pre- and post-ART (n=18; closed circles) individuals. Horizontal lines represent the median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively.

Taken together, these data show that both CD4+ and CD8+ T cells are highly activated and have an increased turnover during HIV infection. ART reduces activation and proliferation of both CD4+ and CD8+ T cells, but this reduction does not lead to normalisation to similar levels in HIV-uninfected individuals.

2.3.3 Relationship between CD4+ and CD8+ T cell activation before and after ART

We next investigated the association between CD4+ and CD4+ T cell activation in 28 HIV-infected individuals pre- and post-ART initiation. A significant positive correlation was found between the frequency of CD4+ and CD8+ T cells expressing CD38 ($p<0.0001$, $r=0.89$; **Figure 2.5A**), HLA-DR ($p=0.0002$, $r=0.65$; **Figure 2.5B**) or co-expressing CD38 and HLA-DR ($p<0.0001$, $r=0.79$; **Figure 2.5C**) pre-ART. These positive correlations were also observed after 1 year of ART (CD38: $p<0.0001$, $r=0.91$; **Figure 2.5D**; HLA-DR: $p<0.0001$, $r=0.85$; **Figure 2.5E**; and CD38/HLA-DR: $p<0.0001$, $r=0.82$; **Figure 2.5F**). Moreover, there was also a positive correlation

between the frequencies of proliferating CD4+ and CD8+ T cells pre- and post-ART, (n=18, p=0.0005, r=0.74 and p=0.02, r=0.56, respectively; data not shown).

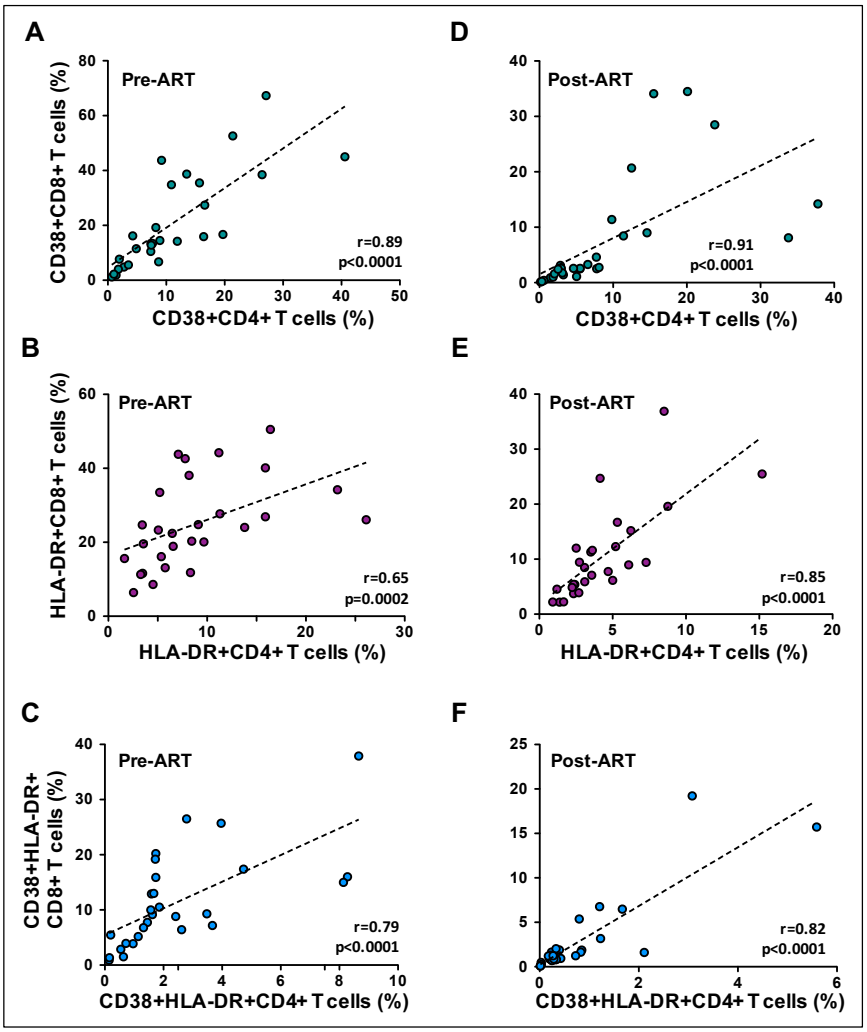

Figure 2. 5. Association between CD4+ and CD8+ T cell activation before and after ART. Correlation of the frequency of **(A)** CD38+ (teal), **(B)** HLA-DR+ (plum) and **(C)** CD38+HLA-DR+ (blue) expressing CD4+ T cells and the frequency of CD38+, HLA-DR+, CD38+HLA-DR+ on CD8+ T cells (n=28), respectively, in HIV-infected individuals prior to ART initiation. Correlation of the frequency of **(D)** CD38+, **(E)** HLA-DR+ and **(F)** CD38+HLA-DR+ expressing CD4+ T cells and the frequency of CD38+, HLA-DR+, CD38+HLA-DR+ expressing CD8+ T cells (n=28), respectively, post-ART. Statistical significance was calculated using a non-parametric Spearman Rank test. The dashed line represents a linear regression fit.

Overall, these findings reveal that the activation and proliferation levels in CD4+ T cells reflect those in CD8+ T cells both in ART-naive and in ART-treated HIV-infected women, where residual activation and proliferation remain in both T cell compartments. These data indicate that the 'de-activation' rate of CD4+ and CD8+ T cells after ART is similar, or alternatively that the factors driving or maintaining

activation of T cells post-ART continue to affect both T cell subsets as they did prior to ART.

2.3.4 Differentiation profiles of CD4+ and CD8+ T cells prior to and after ART

HIV infection results in the skewing of T cell subsets from naive to more differentiated (effector and memory) phenotypes, and memory CD4+ T cells are preferentially targeted by the virus (Roederer *et al*., 1995; Brenchley *et al*., 2004). However, even though the imbalance in T cell subset distribution can be corrected upon ART (Hellerstein *et al*., 2003), the extent to which the recovery of the different memory subsets occur is still not fully characterised. Therefore, we investigated the impact of ART on T cell differentiation in our 28 HIV-treated participants in comparison to 23 HIV-uninfected controls. **Figure 2.6A** shows representative flow cytometric plots of CD27 and CD45RO expression on CD4+ T cells from one HIV-uninfected and one HIV-infected individual (pre- and post-ART). Based on these two markers, four separate CD4+ T cell subsets were identified, namely naive (CD27brightCD45RO-), early differentiated (ED; CD27brightCD45RO+, comprising central and transitional memory subsets), late differentiated (LD; CD27lowCD45RO+ corresponding to effector memory cells) and terminally differentiated cells (TD; CD27lowCD45RO-, also known as effector cells). Compared to HIV-uninfected controls, HIV-infected participants displayed significantly lower frequencies of naive and TD CD4+ T cells (Naive: medians 48% vs 38%, $p=0.02$ and TD: medians 4% vs 1%, $p<0.0001$; **Figure 2.6B**), and higher frequencies of ED and LD CD4+ T cells (ED: medians 33% vs 43%, $p=0.002$ and LD: medians 10% vs 16%, $p=0.05$; **Figure 2.6B**), although the latter was not statistically different. ART led to a substantial increase in naive CD4+ T cells and a concomitant decrease in LD (Naive: medians 38% vs 44%, $p=0.006$ and LD: medians 16% vs 11%, $p=0.0003$) with respect to HIV-untreated subjects. The median frequencies of ED and TD CD4+ T cell subsets were comparable pre- and post-ART. While ART fully normalised the frequency of naive and LD subsets, the ED and TD CD4+ subsets did not return to normal levels observed in HIV-uninfected controls ($p=0.20$, $p=0.44$, $p=0.004$ and $p<0.0001$, respectively). These changes in the distribution of CD4+ T cell memory subsets between HIV-uninfected, HIV-untreated and HIV-treated participants are further illustrated in **Figure 2.6C.**

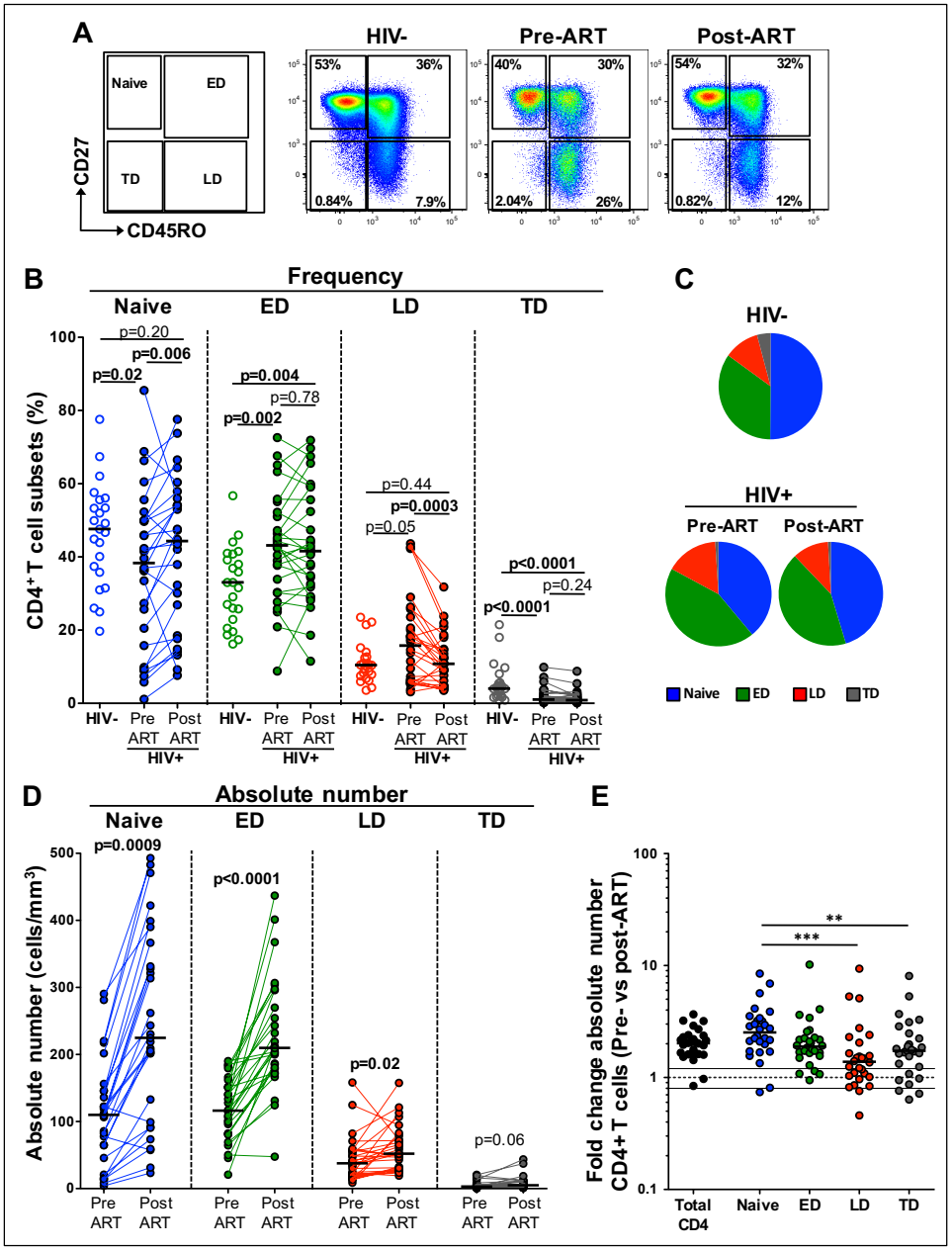

Figure 2. 6. Memory profiles of CD4+ T cells before and after ART. (A) Representative flow plots of total CD4 subset distribution in one HIV-uninfected and one HIV-infected individual pre- and post-ART. Naive: CD27brightCD45RO-, blue; Early Differentiated (ED: CD27brightCD45RO+, green); Late Differentiated (LD: CD27lowCD45RO+, red) and Terminally Differentiated (TD: CD27lowCD45RO-, grey). The frequencies of each subset are indicated. Frequency **(B)** and absolute number **(D)** of CD4+ T cell subsets in HIV-uninfected (n=23; open circles) and HIV-infected individuals pre-and post-ART initiation (n=28; closed circles). Horizontal bars represent the median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively. **(C)** Pie charts showing the overall distribution of CD4+ T cell subsets in HIV-uninfected (n=23) and HIV-infected participants (n=28; pre- and post-ART). Each slice of the pie represents the median proportion of each CD4+ T cell subset, as indicated by the color at the bottom of the graph. **(E)** Fold change in the total, naive, ED, LD and TD absolute CD4+ T cell count over 12 months of ART. The vertical dotted line indicates no change from the time point prior to ART. The solid lines at 0.8 and 1.2 represent 20% change above which a change was considered significant. Statistical comparisons were calculated using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

To take into account variation in absolute CD4 counts pre- and post-ART, the changes in CD4+ T cell memory subsets upon treatment were also assessed in absolute number. We found a significant increase in the number of naive and ED CD4+ T cell subsets (Naive: p=0.0009, ED: p<0.0001; **Figure 2.6D**) after ART. Relatively small overall changes were observed in the median absolute number of LD and TD after ART (LD: p=0.02 and TD: p=0.06; **Figure 2.6D)**. Moreover, to evaluate the dynamics of CD4+ T cell memory subset reconstitution upon treatment, we examined the fold change in absolute number of CD4+ T cell subsets pre- and post-ART. All four subsets expanded following 1 year of ART, with naive CD4+ T cells exhibiting the largest expansion (median: 2.5), which was higher than the increase in LD and TD CD4+ subsets (medians: 1.4, p<0.001 and 1.7, p<0.01 respectively; **Figure 2.6E**).

For CD8+ T cells, a similar analysis was performed. An additional CD8+ subset, namely intermediate cells (CD27dimCD45RO-) was identified, as shown in the representative flow cytometric plots from one HIV-uninfected and one HIV-infected individual (pre- and post-ART; **Figure 2.7A**). As previously described, this subset is distinct from effector cells and is characterised by CD57 and CD127 expression, and appears to be a differentiation stage between central memory and effector memory cells (Burgers *et al*., 2009). As for CD4+ T cells, HIV infection led to a significantly lower frequency of naive CD8+ T cells, and there was a concomitant increase in ED and LD CD8+ T cell subsets when compared to HIV-uninfected controls (Naive: medians 18% vs 48%, p<0.0001; ED: medians 24% vs 6%, p<0.0001; and LD: medians 8% vs 3%, p=0.002; **Figure 2.7B**). In contrast to CD4+ T cells, although there was a trend towards a greater proportion of TD CD8+ T cells, the frequencies did not differ significantly between HIV-uninfected and HIV-infected individuals (medians: 24% vs 33%, respectively; p=0.13). There was also no significant difference in the frequency of Inter CD8+ T cells between the HIV-infected and the HIV-uninfected groups (medians: 7% vs 9%, respectively; p=0.07). Following suppressive therapy, there was a significant increase in naive CD8+ T cell frequency, with a simultaneous decrease in ED and Inter CD8+ T cell frequencies relative to HIV-untreated subjects (Naive: medians 31% vs 18%, p<0.0001; ED: medians 15% vs 24%, p<0.0001and Inter: medians 5% vs 7%, p=0.0005; **Figure 2.7B**). No substantial difference in the frequencies of LD and TD CD8+ T cell subsets was found between pre- and post-ART time points (LD: medians 8% vs 8%, p=0.19 and TD: medians 33% vs 29%, p=0.89;).

However, ART-induced changes in CD8+ T cell subset frequencies was partial, as only naive cells significantly increased but still remained lower compared to HIV-uninfected subjects (p=0.01). These were compensated by decreases in ED, Inter and LD subsets post-ART. (**Figure 2.7B & C**).

While the median absolute CD8 count did not differ pre- and post-treatment, substantial individual variation was observed amongst participants. Thus, to take this into account, changes in the absolute number of each CD8+ memory subsets were assessed, and we observed, although not significant as for CD4+ T cells, a trend towards an increase in the number of naive CD8+ T cells (p=0.08) after treatment, but in slightly fewer individuals (23/28) compared to 26/28 in CD4+ T cells (**Figure 2.7D**). The absolute number of ED CD8+ T cells and inter subset, which constitutes the smallest proportion of the CD8+ T cell population significantly decreased in the majority of the patients post-ART (ED: 24/28, p<0.0001 and inter: 24/28, p=0.02). In contrast, the absolute number of LD and TD remained similar pre- and post-ART (LD: 24/28, p=0.28 and TD: 21/28, p=0.28). Lastly, to further assess the effect of ART, we measured the fold change in absolute number of CD8+ T cell subsets pre- and post-ART (**Figure 2.7E**). Only naive CD8+ T cells expanded with a median fold change of 1.4, while the other four subsets contracted (median fold changes: 0.5, 0.6, 0.7 and 0.8 for ED, Inter, LD and TD; respectively). The contraction in the ED subset was the highest, and differed significantly from the decrease in the TD subset upon ART (p<0.05).

Overall, our results reveal that HIV skews memory T cell profiles and promotes excessive differentiation by decreasing the frequency of peripheral naive T cells, while augmenting the memory subsets (ED and LD cells). Nevertheless, one year of ART was not sufficient for complete normalisation of CD4+ and CD8+ T cell subsets, despite suppression of viral load. It will be important to determine whether HIV-induced defects persist despite successful ART, or if merely a longer period on ART may be necessary for full normalisation of T cell subsets.

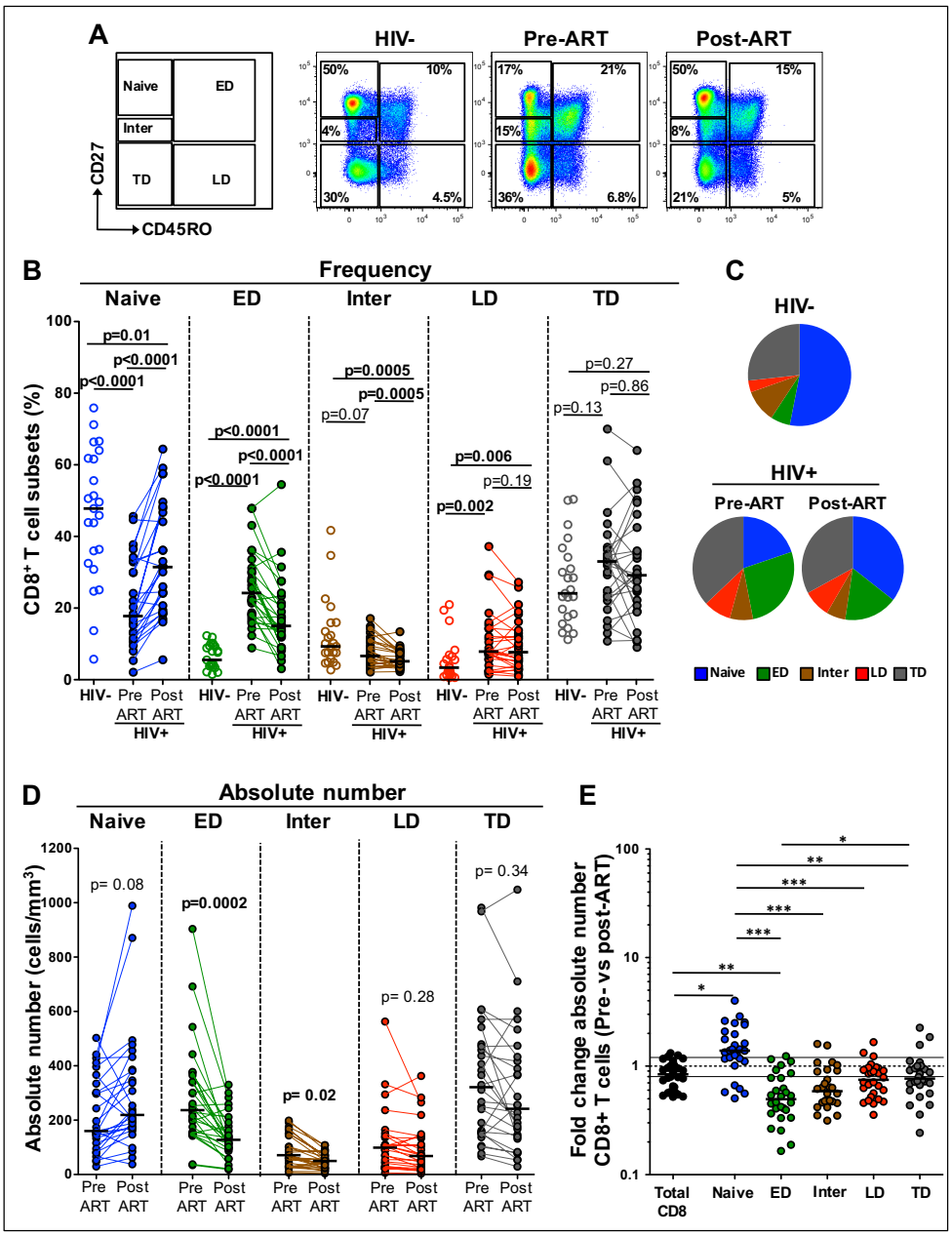

Figure 2. 7. Memory profiles of CD8+ T cells before and after ART. (A) Representative flow plots of total CD8 subset distribution in one HIV-uninfected and one HIV-infected individual pre- and post-ART. Naive: CD27brightCD45RO-, blue; Early Differentiated (ED: CD27brightCD45RO+, green); Intermediate (Inter: CD27dimCD45RO-, brown), Late Differentiated (LD: CD27lowCD45RO+, red) and Terminally Differentiated (TD: CD27lowCD45RO-, grey). The frequencies of each subset are indicated. Frequency **(B)** and absolute number **(D)** of CD8+ T cell subsets in HIV-uninfected (n=23; open circles) and HIV-infected individuals pre-and post-ART initiation (n=28; closed circles). Horizontal bars represent the median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively. **(C)** Pie charts showing the overall distribution of CD8+ T cell subsets in HIV-uninfected (n=23) and HIV-infected participants (n=28; pre- and post-ART). Each slice of the pie represents the median proportion of each CD8+ T cell subset, as indicated by the color at the bottom of the graph. **(E)** Fold change in the total, naive, ED, Inter, LD and TD absolute CD8+ T cell count over 12 months of ART. The vertical dotted line indicates no change from the time point prior to ART. The solid lines at 0.8 and 1.2 represent 20% change above which a change was considered significant. Statistical comparisons were calculated using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.3.5 Activation levels within CD4+ and CD8+ T cell subsets before and after ART

Finally, in order to define whether HIV-induced hyperactivation affects all memory T cell subsets to the same extent, we determined HLA-DR expression on each T cell memory subsets within CD4+ and CD8+ T cells (n=28; pre- and post-ART), and compared them to profiles in HIV-uninfected controls (n=23). During HIV infection, for both CD4+ and CD8+ T cells, all antigen-experienced subsets (ED, Inter, LD and TD) became activated (3-6 fold greater than the same subsets in HIV-uninfected subjects), as shown by a significant upregulation of HLA-DR expression (CD4: $p<0.0001$ for ED, LD and TD; and CD8: ED, Inter, LD, TD: $p<0.0001$; **Figures 2.8A & B**). With respect to naive cells, naive CD8+ T cells demonstrated~11 fold higher levels of activation than the HIV-uninfected group, while naive CD4+ T cells showed little or no activation. Following 1 year of ART, HLA-DR expression on all subsets was markedly reduced for both CD4+ (decrease of ~2 fold for ED and LD, ~1 fold for TD) and CD8+ T cells (decrease of ~3 fold for ED, ~2 fold for Inter, LD and TD). The degree of deactivation was comparable across all CD4+ and CD8+ memory subsets. Naive CD8+ T cells also deactivated upon ART (decrease of ~4 fold). With regards to CD4+ T cells, none of the subsets normalised their activation levels post-ART to HIV-uninfected levels, whereas in three subsets of CD8+ T cells (ED, LD and TD), activation reached similar levels to those in HIV-uninfected individuals ($p=0.1$ for ED and LD; $p=0.07$ for TD). The reduction in HLA-DR expression post-therapy is further demonstrated in **Figures 2.8C & D** showing the fold change in activation, where overall all subsets contracted (CD4 median fold change decrease: Naive 0.48, ED 0.45, LD 0.60 and TD 0.75; CD8 median fold changes: Naive 0.2, ED and Inter 0.4, LD and TD 0.5). The decrease in HLA-DR expression was more pronounced for CD8+ T cells than CD4+ T cells. Taken together, these data suggest that HIV induced activation of all T cells, irrespective of their differentiation stage, and these defects within each subset appeared to be almost fully normalised in the CD8 compartment (ED, LD and TD activation profiles are comparable to HIV-uninfected controls), while the restoration was only partial in the CD4 compartment (where activation profiles on all memory subsets remained elevated compared to HIV-uninfected controls) after 1 year of suppressive therapy. Of note, similar profiles were observed using as the readout for activation the marker CD38 (alone or in combination with HLA-DR), as well as using the proliferation marker Ki67 (**Appendix Figure 1**).

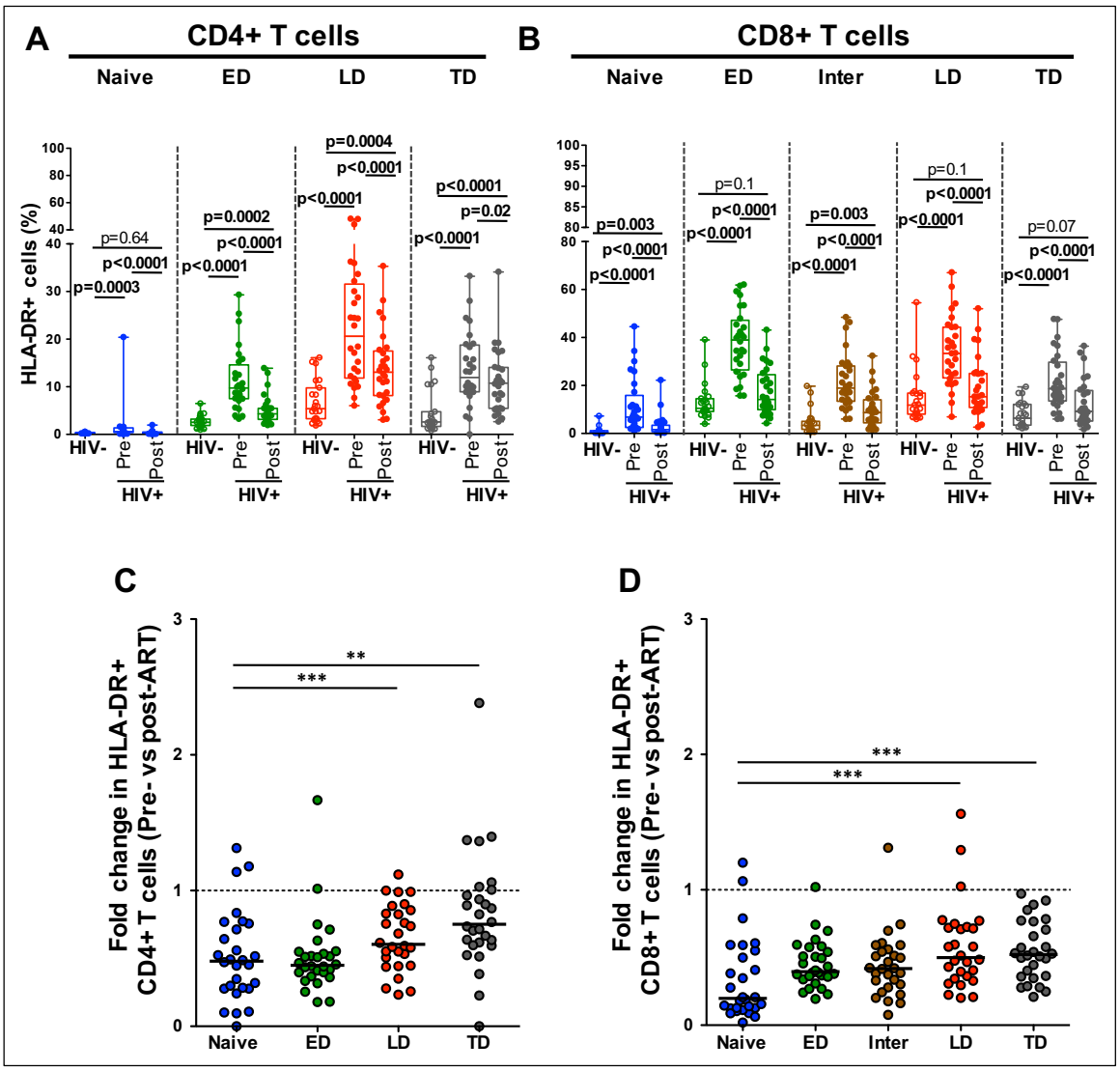

Figure 2. 8. Activation profiles of CD4+ and CD8+ T cell subsets before and after ART. Frequencies of **(A)** HLA-DR+CD4+ T cells and **(B)** HLA-DR+CD8+ T cells, in HIV-uninfected (n=23; open circles) and HIV-infected (pre- and post-ART; n=28; closed circles) individuals. Blue, green, brown, red and grey represent the Naive, ED, Inter, LD and TD subsets. Data are shown as box and whisker (interquartile range) plots. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank test for unpaired and paired samples, respectively. Fold change in HLA-DR frequencies of **(C)** Naive, ED, LD and TD CD4+ T cells and **(D)** Naive, ED, Inter, LD and TD CD8+ T over 12 months of ART. The vertical dotted line indicates no change from the time point prior to ART. Statistical comparisons were calculated using a one-way ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001.

2.4 Discussion

HIV directly impacts the immune system by inducing a profound loss in CD4+ T cells and a state of generalised immune activation, thereby promoting disease progression and development of AIDS. Although successful ART improves CD4+ T cell numbers, excessive immune activation may persist and influence CD4+ T cell reconstitution and the health of the individual. The degree to which specific memory T cell subsets are normalised on ART, and the association with sustained immune activation, is not fully defined. Therefore, this Chapter focused on the longitudinal assessment of the impact of short-term ART on the activation, proliferation and memory differentiation profiles of CD4+ and CD8+ T cells in HIV-infected African women (n=28). These profiles were compared to age- and sex-matched HIV-uninfected controls from the same community (n=23). Our results showed high levels of T cell activation and proliferation, as well as perturbations in the differentiation profiles of both CD4+ and CD8+ T cell subsets upon HIV infection, as expected. Whilst there was a decrease in activation and skewing of T cell memory subsets, one year of ART was insufficient to completely normalise these immune defects when compared to HIV-uninfected individuals. We found a positive correlation between the levels of activation of CD4+ and CD8+ T cell subsets before and after ART initiation, suggesting that similar factors may affect both subsets. Lastly, hyperactivation as a result of HIV infection was comparable among memory subpopulations within CD4+ and CD8+ T cells, and while ART almost completely normalised activation levels of subpopulations within the CD8 compartment, this was not the case for CD4+ T cells.

CD4+ T cell count, plasma viral load and CD4:CD8 ratio are key clinical parameters to predict disease progression to AIDS and to evaluate how well HIV patients on ART respond to treatment (Taylor *et al*., 1989). In this study, we confirmed findings from other studies that one year of ART induces substantial gains in CD4+ T cell counts and effectively suppresses viral load in most HIV-treated individuals (Deeks *et al*., 2000; DeHovitz *et al*., 2000; Kaufmann *et al*., 2000; Hunt *et al*., 2003). Regardless of the drug regimen, the gain in peripheral CD4+ T cells takes place in more than one step (Corbeau & Reynes, 2011). During the first few weeks of therapy, the prompt initial increase may be explained by the release of trapped memory CD4+ T cells from lymphoid tissues to the bloodstream as a result of reduced immune activation and low

expression of adhesion molecules (VCAM-1 and ICAM-1) on the surface of CD4+ T cells (Autran *et al*., 1997; Bucy *et al*., 1999). A recent study has shown that the memory CD4+ T cells released into the blood are predominantly CCR4-expressing and have a Th17-like functionality (Mahnke *et al*., 2016). The second boost in CD4 counts after several months of therapy is somewhat due to new synthesis of naive CD4+ T cells by the thymus, or prolonged CD4+ T cell half life (Bucy *et al*., 1999; Steffens *et al*., 2001; Kovacs *et al*., 2001; Mohri *et al*., 2001). However, it is notable that for two participants (CAP248 and 262) no improvement in CD4 count was observed after one year of ART, but CD4 recovery occurred at a later stage (> 1 year ART; data not shown). Failure or delay to recover CD4+ T cells may be due to multiple reasons, including CD4 count and level of T cell activation at the time of ART initiation (Kaufmann *et al*., 2005; Goicoechea *et al*., 2006; Le *et al*., 2013), length of ART, age (Kaufmann *et al*., 2002; Allers *et al*., 2014) and co-infections (Almeida *et al*., 2007; Kaufmann *et al*., 2011).

Chronic HIV infection is marked by a state of generalised immune activation, manifested by an increase in T cell activation, and an accelerated and constant T cell turnover (proliferation and death), which results in the exhaustion of the immune system, inhibiting its capacity to replenish the T cell pool (Hazenberg *et al*., 2000; McCune, 2001). As previously reported (Liu *et al*., 1997; Mohri *et al*., 2001; Hazenberg *et al*., 2003), our data showed increased CD4+ and CD8+ T cell activation and proliferation in chronically HIV-infected women. Interestingly, we found a positive association between the frequency of activated CD4+ and activated CD8+ T cells, as well as the frequency of proliferating CD4+ and proliferating CD8+ T cells pre-ART. Despite this association, there were differences in the activation and proliferation of CD4+ compared to CD8+ T cells. Our observation that CD8+ T cells were highly activated but there were lower frequencies of proliferation cells compared to CD4+ T cells concurs with what has been reported previously (Funderburg *et al*., 2008). The difference in CD4+ and CD8+ T cell activation could be as a result of the distribution of memory subsets within the compartments, where central and effector memory CD4+ T cells entered the cell cycle (Ki67 expression) compared to naive CD4+ T cells, whilst effector memory CD8+ T cells were found to express the activation marker CD69 (Funderburg *et al*., 2008), or alternatively, due to differential expression of cytokine receptors between CD4+ and CD8+ T cells (Boyman, 2010).

Catalfamo and colleagues reported differences in homeostatic responses between CD4+ and CD8+ T cells; CD4+ T cells proliferate in response to CD4+ T cell loss and viral load, as opposed to CD8+ T cells, whose proliferation is driven largely by viral load (Catalfamo *et al*., 2008). In our study, we also evaluated whether ART normalised the levels of T cell activation and proliferation in HIV-treated individuals to that seen in uninfected controls. Our results show a substantial global decrease in the frequency of activated (CD38 and/or HLA-DR) and proliferating (Ki67) CD4+ and CD8+ T cells, however these parameters remained significantly higher than in HIV-uninfected individuals. These findings are consistent with previous reports, suggesting only a partial reduction in immune activation (Mohri *et al*., 2001; Valdez *et al*., 2002;. Hunt *et al*., 2003; Lim *et al*., 2007). Upon ART, failure to eradicate viral reservoirs and the irreversible damage of the gut epithelium, both established early in HIV infection, are a potential source of virus and bacterial components that could account for this sustained immune activation despite effective ART (Finzi *et al*., 1997; Brenchley *et al*., 2007). Consequently, HIV-infected people on ART become at high risk of non-AIDS related diseases and mortality. In a number of studies, elevated levels of inflammation (as measured by IL-6 and C-reactive protein; CRP) and coagulation (D-dimers) markers were found to independently predict increased mortality and death associated with cardiovascular diseases among treated HIV-infected individuals (Kuller *et al*., 2008; Duprez *et al*. 2012). In addition, the risk of cardiovascular disease has been associated with monocyte activation during HIV infection (Funderburg *et al*., 2012).

Based on their differentiation status, T cell subsets can be classified into multiple subgroups namely, naive, central memory, transitional memory, effector memory and terminal effector cells (Paiardini & Müller-Trutwin, 2013). During HIV infection, naive T cells are depleted as a result of reduced thymic output, or elevated differentiation of these cells into memory and effector cells due to constant antigen exposure and increased immune activation (Nokta *et al*., 2001). In this study, HIV-infected women showed skewed T cell differentiation profiles, with a decreased naive cell frequency and increased memory subset frequency (ED and LD cells), which normalised only partially to levels observed in HIV-uninfected individuals. These data are in accordance with previous reports (Robbins *et al*., 2009; Rallon *et al*., 2013; Serrano-Villar *et al*., 2014) In addition, there was HIV-induced activation of both

naive and memory T cells, as previously reported (Ssewanyana *et al*., 2009). Our data also showed that "de-activation" of all memory T cell subsets occurred upon ART, with a more pronounced effect on the CD8 compartment. The imbalance in CD4+ T cell homeostasis as a result of continuous destruction of memory CD4+ T cells could explain the incomplete recovery of these cells upon ART (Ssewanyana *et al*., 2009).

Our study had some limitations. We confined ourselves to HIV-uninfected controls matched for age, sex and HIV risk. Using pre-infection samples from the same participants who went onto treatment after being infected with HIV would have provided a more accurate view of normalisation of T cell activation and differentiation profiles upon ART, since there is substantial variation in these profiles in a population. It could also be beneficial to include an additional low-risk HIV-uninfected control group. Furthermore, memory subsets were identified based on two markers, CD27 and CD45RO that cannot discriminate between central memory and transitional memory cells found within the ED (CD27brightCD45RO+) subset. Thus, additional markers such as CCR7 should be included in future studies to determine the effect of ART on these subsets. Moreover, our cohort consisted of women only, and it is worth noting that male sex was associated with lower CD4 gains after treatment independent of starting CD4 count (Gandhi *et al*., 2006). In addition, thymic output has been described to be reduced in men compared to women, thus testing whether our findings are applicable in a cohort of men is warranted (Pido-Lopez *et al*. 2001; Hunt *et al*. 2003). Lastly, it is important to note that recovery of T cell phenotype from peripheral blood does not necessarily mean reconstitution at other immunological sites of importance in HIV pathogenesis, such as the gastrointestinal tract, where HIV causes significant damages. Previous reports have shown that compared to peripheral blood, CD4+ T cells are substantially more depleted in the gastrointestinal tract of HIV-infected persons, and that this loss in gut CD4+ T cells is only partially restored upon ART (Brenchley *et al*., 2004; Guadalupe *et al*., 2003). Thus, to address reconstitution of T cells in the gut, studies investigating T cell recovery in gut biopsies could be performed. Another important point is that despite the apparent overall normalisation of T cell memory profiles upon ART, certain memory T cells specific for opportunistic pathogens depleted during HIV infection may not be restored upon ART, raising the issue of the 'quality' and not just quantity of immune recovery (Geldmacher *et al*., 2010; Pauza *et al*., 2011; Saharia & Koup, 2013), a subject that is dealt with in Chapter

3 (Riou, Tanko *et al*., 2015). Additionally, the effect of ART on the recovery of immune cells other than T cells should be investigated for a better assessment of functional immune recovery. In this regard, Chapter 4 of this thesis covering our recently published study (Tanko *et al*., 2017) evaluated the influence of ART on B cells.

In summary, our study has demonstrated hyperactivation and skewing of T cell memory phenotypes in African women with chronic HIV infection. Administration of ART during chronic HIV infection led to successful vial load suppression and CD4+ T cell increases. However, T cell activation and proliferation levels remained higher than HIV-uninfected subjects and the frequency of T cell memory subsets did not completely normalise, implying partial recovery following short-term (1 year) ART. In addition, whilst activation levels were nearly completely regularized within memory CD8+ T cells, they were only partially restored within memory CD4+ T cells, implying differential dynamics of deactivation between the two compartments. A follow-up study will be required to determine whether longer-term ART may enable full immune normalisation of T cell phenotypes and deactivation in our cohort.

CHAPTER 3

Restoration of CD4+ responses to co-pathogens in HIV-infected individuals on antiretroviral therapy is dependent on T cell memory phenotype.

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Contribution: Ramla Tanko performed the majority of the experiments, analysed the data and co-wrote the paper with her supervisors Catherine Riou and Wendy Burgers.

3.1 Abstract

Antiretroviral therapy (ART) induces rapid suppression of viral replication and a progressive replenishment of CD4+ T cells in HIV-infected individuals. However, the effect of ART on restoring pre-existing memory CD4+ T cells specific for common co-pathogens is still unclear. To better understand the dynamics of antigen-specific CD4+ T cells during ART, we assessed the frequency, functional capacity and memory profile of CD4+ T cells specific for *Mycobacterium tuberculosis* (Mtb) and cytomegalovirus (CMV) in 15 HIV-infected individuals before and one year after ART initiation.

After ART initiation, the frequency of Mtb-specific CD4+ T cells showed little change whereas CMV-specific CD4+ T cells were significantly lower (p=0.003). There was no difference in the polyfunctional or memory profile of antigen-specific CD4+ T cells before and after ART. The replenishment of antigen-specific CD4+ T cells correlated with the memory differentiation profile of these cells prior to ART. Pathogen-specific CD4+ T cells exhibiting a late differentiated profile (CD45RO+CD27-) had a lower

capacity to replenish (p=0.019, r=-0.5) compared to cells with an early-differentiated profile (CD45RO+CD27+; p=0.04, r=0.45).

In conclusion, restoration of co-pathogen-specific memory CD4+ T cells during treated HIV infection is related to their memory phenotype, where early differentiated cells (such as most Mtb-specific cells) have a higher replenishment capacity compared to late differentiated cells (such as most CMV-specific cells). These data identify an important, hitherto unrecognised, factor that may limit restoration of co-pathogen immunity in HIV-infected individuals on ART.

3.2 Introduction

The hallmarks of untreated HIV infection are a progressive loss of CD4+ T cells, sustained cellular activation and chronic inflammation (Moir *et al.*, 2011; Okoye & Picker, 2013; Doitsh *et al.*, 2014). In addition to the numerical depletion of CD4+ T cells, HIV can also alter the functional capacity of these cells, impairing their proliferative potential, altering their cytokine secretion profiles and changing their phenotypic characteristics in response to HIV antigens as well as various co-pathogens (Shearer & Clerici, 1991; Luciano *et al.*, 2007; Morou *et al.*, 2014; Boswell *et al.*, 2014). Both of these quantitative and qualitative alterations can lead to increased susceptibility to opportunistic infections, including tuberculosis (TB), Candidiasis and Human Papilloma Virus (HPV) infection (Saharia & Koup, 2013). Indeed, HIV is the best-recognised risk factor for TB disease even before profound CD4+ T cell deficiency (Sonnenberg *et al.*, 2005; Geldmacher *et al.*, 2012). The introduction of antiretroviral therapy (ART) has drastically decreased morbidity and mortality in HIV-infected individuals (Williams *et al.*, 2011), inducing a rapid reduction of plasma viral load and a progressive repletion of CD4+ T cells (Guihot *et al.*, 2011). Although the clinical benefit of ART is undeniable, the extent to which ART can fully "normalise" functional immunity remains unclear (Corbeau & Reynes, 2011). HIV-infected individuals on ART exhibit a differential degree of recovery of co-pathogen-specific CD4+ T cell responses, depending on the pathogen they target (Schluger *et al.*, 2002; Keane *et al.*, 2004; Burgess *et al.*, 2006; Wilkinson *et al.*, 2009; Sutherland *et al.*, 2010; Hsu *et al.*, 2013; Jambo *et al.*, 2014). For example, it has been shown that the restoration of CMV-specific CD4+ T cells occurs early after ART (Hsu *et al.*, 2013),

but appears to be short-lived (Keane *et al.*, 2004). Conversely, *Candida*-specific CD4 responses recover slowly (Burgess *et al.*, 2006). Contrasting data exist on the degree of recovery of *Mycobacterium tuberculosis* (Mtb)-specific CD4+ T cell responses upon ART. Jambo *et al.* showed in a cross-sectional study that the frequency and polyfunctional profile of Mtb-specific CD4+ T cell responses were similar in ART-naive or treated individuals (Jambo *et al.*, 2014), while Sutherland *et al.* reported that ART increases the polyfunctional capacity of these cells (Sutherland *et al.*, 2010). Other studies described only a partial reconstitution of Mtb-specific CD4+ T cell responses after ART (Schluger *et al.*, 2002; Wilkinson *et al.*, 2009).

It is of particular importance to define the factors that associate with successful pathogen-specific CD4+ T cell recovery upon ART, as limited "normalisation" of functional CD4+ T cell responses could account for sustained incidence of opportunistic infections. Several parameters influence the degree and dynamics of recovery of the overall CD4+ T cell compartment in response to ART, such as age, CD4 count at the time of treatment initiation, and timing of ART initiation after HIV infection (Kaufmann *et al.*, 2005; Aiuti & Mezzaroma, 2006; Le *et al.*, 2013). However, it is still unclear why CD4+ T cells of different pathogen specificities have different profiles of restoration, and the mechanisms mediating this variable recovery of memory CD4+ T cells to co-pathogens are still incompletely understood. Thus, to better understand the effect of successful ART on the dynamics of recovery of co-pathogen-specific CD4+ T cells, we compared the magnitude, functional capacity and memory differentiation profiles of Mtb- and CMV-specific CD4+ T cells before and one year after ART initiation in a cohort of HIV-infected individuals, and HIV-uninfected controls.

3.3 Materials and Methods

3.3.1 Study participants

Blood samples were collected from 15 women participating in the CAPRISA 002 study, a cohort study following HIV-infected women from HIV seroconversion until five years on treatment. The cohort is situated in KwaZulu-Natal, South Africa, and has been previously described (van Loggerenberg *et al.*, 2008; Mlisana *et al.*, 2014). Participants were selected based on sample availability. Blood samples from 9 HIV-

uninfected participants were provided from the CAPRISA 004 vaginal microbicide (1% tenofovir) gel trial (Abdool Karim *et al.*, 2010). An additional 14 HIV-uninfected participants from CAPRISA 004 were studied for immune activation. HIV-uninfected participants were from the same community as the HIV-infected individuals and age-matched; they were either in the pre-intervention phase or in the placebo arm of the trial. For HIV-infected individuals, the time of infection was estimated either as the same date as a prospective RNA positive/antibody negative measurement or taken as the midpoint between the last antibody negative test and first antibody positive enzyme-linked immunosorbent assay test. Participants in the cohort were offered ART according to South African national HIV treatment guidelines (at a CD4 count of <200 cells/mm^3 prior to October 2012; <350 cells/mm^3 until the present). Eight of the 15 participants were taking standard first-line therapy (TDF/3TC/EFV), 3 were on ddI-EC/3TC/EFV, and 1 each on d4T/3TC/EFV, TDF/3TC/NVP, d4T/3TC/NVP and AZT/3TC/LPV/r. Two participants switched drug regimens while on study, namely CAP200 (EFV/3TC/ddI-EC to EFV/3TC/TDF at month 11), and CAP255 (EFV/3TC/d4T to EFV/3TC/AZT at month 10). No participants had active TB during the study period, or exhibited any immune reconstitution disease upon HIV treatment. Ethical approval for the study was obtained from the University of KwaZulu-Natal and University of Cape Town Research Ethics Committees. All participants provided written informed consent to participate in the study.

3.3.2 Determination of plasma viral load and CD4 counts

Plasma HIV viral loads and CD4 counts were quantified at each study visit. Over the course of the study, the viral load PCR assay switched from Roche AMPLICOR HIV-1 monitor test version 1.5 [lower detection limit (LDL) of 400 RNA copies/ml], to Roche Taqman version 1.0 on June 1, 2010 (LDL 40 RNA copies/ml), and then to Roche Taqman version 2.0 on January 9, 2012 (LDL 20 RNA copies/ml). Absolute blood CD4 and CD8 T cell counts were measured using the FACSCalibur TruCOUNT method (BD Biosciences) and expressed as cells/mm^3. Plasma samples matching the visits where PBMC were tested for antibodies to CMV and CMV DNA. Seropositivity was determined using the Cobas CMV IgG Assay (Roche), and CMV viral load was detected and quantified using a CMV R-gene PCR kit (Argene), with a detection limit of 30 copies/ml.

3.3.3 Cell preparation

PBMC were isolated by standard Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia) and cryopreserved in 90% heat-activated foetal calf serum (FCS, Invitrogen) plus 10% DMSO and stored in liquid nitrogen until needed. Cryopreserved PBMC were thawed and rested in R10 [RPMI 1640 containing 10% heat-inactivated FCS (Sigma) and 50 U/ml of penicillin-streptomycin] at 37°C and 5% CO_2 for 8 hours prior to antigen stimulation.

3.3.4 Antigens and cell stimulation

Following resting, PBMC were stimulated using Mtb Purified Protein Derivative (PPD, 10 μg/ml; Statens Serum Institute), a pool of CMV peptides consisting of 138 peptides (15mers overlapping by 11 amino acids) covering the entire HCMV pp65 protein (2 μg/ml; NIH AIDS Reagent Program) and Staphylococcal Enterotoxin B (SEB, 2.5 μg/ml; Sigma), used as a positive control. Of note, we found no major differences in the memory profile, cytokine secretion potential and polyfunctional capacity between peptide and protein-stimulated PBMC (Bunjun *et al.*, manuscript in preparation). Stimulations were performed in the presence of co-stimulatory antibodies, anti-CD28 and anti-CD49d (both at 1 μg/ml; BD Biosciences) at 37°C for 16 hours. Brefeldin A (BFA; Sigma) was added after 1 hour at a concentration of 10 μg/ml. Surface and intracellular cytokine staining was performed at the end of the incubation period. Sufficient cells were available for assessing CMV and SEB responses in only 12/15 HIV-infected participants.

3.3.5 Surface phenotypic and intracellular cytokine staining and flow cytometry

The following antibodies were used for surface and intracellular staining: CD3-APC-H7 (SK7; BD Biosciences), CD4-PE-Cy5.5 (S3.5; Invitrogen), CD8-Qdot 705 (3B5; Invitrogen), CD27-PE-Cy5 (1A4CD27; R&D Systems), CD45RO-ECD (UCHL1; R&D Systems), IFN-γ-Alexafluor 700 (B27; BD Biosciences), IL-2-APC (MQ1-17H12; BD Biosciences), TNF-α-PE-Cy7 (MAb11; eBiosciences), CD14-PacBlue (Tük4; Invitrogen), CD19-PacBlue (SJ25-CI; Invitrogen) and a violet amine viability dye ("Vivid"; Molecular Probes). All antibodies were titrated to obtain optimal concentrations prior to use. After stimulation, PBMC were first stained with Vivid for

20 min at room temperature. Cells were then surface stained with CD4, CD8, CD45RO, CD27, CD14 and CD19 antibodies. Cells were fixed and permeabilised using Cytofix/Cytoperm buffer (BD Biosciences) and stained intracellularly with CD3, IL-2, TNF-α and IFN-γ**.** Cells were then re-suspended in 1X CellFix (BD Biosciences). Samples were acquired on a BD Fortessa using FACSDiva software and analysed using FlowJo (version 9.8.2; TreeStar). The gating strategy is presented in **Figure 3.1**. A positive cytokine response was defined as at least twice the background (in the presence of co-stimulatory antibodies and no antigen). For phenotyping the memory profile of cells producing cytokines, a cut-off of 40 events was used. Polyfunctionality of antigen-specific cells was analysed using Pestle (version 1.7) and Spice (version 5.35) software (Roederer *et al.*, 2011). Immune activation was measured by surface staining of unstimulated cells using the CD3, CD4, CD8, CD14 and CD19 antibodies listed above, as well as CD38-APC (HIT2) and HLA-DR-APC-Cy7 (L243) (both BD Biosciences), along with the inclusion of Vivid.

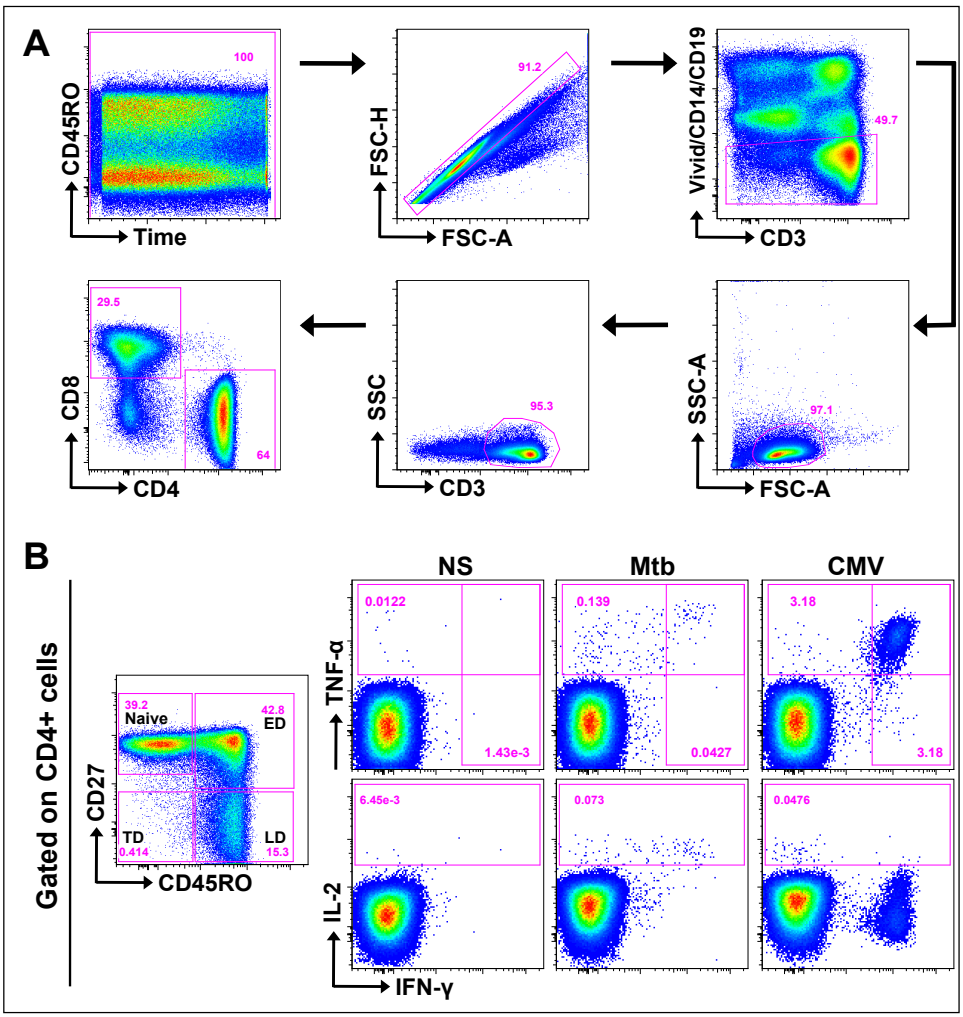

Figure 3. 1. Gating strategy. (A) Singlets were identified in forward scatter plots. Dead cells, B cells and monocytes were excluded by Vivid, CD19 and CD14 labelling, respectively. **(B)** Memory subsets were discriminated based on the expression of CD27 and CD45RO, allowing for the discrimination of four subpopulations: Naive: CD45RO-CD27+; ED: CD45RO+CD27+; LD: CD45RO+CD27- and TD: CD45RO-CD27-. Gates applied for the identification of cytokine positive cells were set according to the unstimulated samples (NS).

3.3.6 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 5.0; San Diego). Non-parametric statistical tests were used for all comparisons. The Mann-Whitney U test and the Wilcoxon Signed Rank test were used for unmatched and paired samples, respectively, and the Kruskal-Wallis ANOVA using Dunn's test for multiple comparisons. Correlations were performed using the Spearman Rank test. A p-value of <0.05 was considered statistically significant.

3.4 Results

3.4.1 Effect of ART on the restoration of co-pathogen-specific CD4+ T cells

In order to better understand the replenishment dynamics of CD4+ T cells specific for co-pathogens in HIV-infected individuals on treatment, we studied 15 HIV-infected individuals before and after ART initiation. The cohort consisted of women infected with HIV-1 for a median of 4.9 years (IQR: 3.8-5.8 years) at the time of treatment initiation (**Table 3.1**). Pre-ART samples were obtained at a median of 1.7 months (IQR: 0.8-2.5) prior to starting ART. The median plasma viral load was 21,955 HIV RNA copies/ml (IQR: 4,743-52,800), and the median CD4 count was 289 cells/mm^3 (IQR: 193-322).

Table 3. 1. Characteristics of study participants

		PRE-ART				POST-ART			
PID	**Pre-ART duration of infection (Years)**	**Viral Load (RNA copies/ml)**	**CD4 count (cells/mm^3)**	**CD4:CD8 ratio**	**Months Pre ART initation**	**Viral Load (RNA copies/ml)**	**CD4 count (cells/mm^3)**	**CD4:CD8 ratio**	**Months post ART initation**
CAP200	3.8	423,000	135	0.24	2.5	<20	496	0.68	13.5
CAP206	5.2	198,277	289	0.15	0.2	<40	512	0.50	11.0
CAP222	6.1	3,049	319	0.61	3.2	<20	619	1.24	11.3
CAP237	5.2	4,743	308	0.57	0.0	<20	528	1.05	12.0
CAP244	7.3	21,955	318	0.32	0.0	273	647	0.53	12.3
CAP248	6.6	29,258	363	0.26	2.8	121	305	0.27	6.7
CAP255	3.7	8,830	226	0.18	3.8	<20	484	0.53	18.1
CAP257	4.8	52,800	170	0.19	2.0	<20	494	0.47	12.0
CAP262	5.8	1,130	338	1.69	1.7	<20	329	3.13	12.1
CAP267	5.4	41,338	269	0.27	1.3	<20	347	0.35	8.7
CAP268	4.2	4,537	186	0.18	0.9	<20	426	0.63	12.0
CAP276	1.4	22,000	232	0.37	2.3	<400	627	1.03	20.2
CAP277	4.9	7,997	322	0.22	1.0	29	535	0.66	10.0
CAP279	2.7	58,100	193	0.40	0.8	<20	400	1.48	12.0
CAP316	4.1	5,612	436	0.33	2.4	<20	708	0.67	11.8
Median	4.9	21,955	289	0.27	1.7	121	496	0.66	12
Range	1.4 - 7.3	1,130 - 423,000	135 - 436	0.15 - 1.69	0 - 3.8	<20 - 273	305 - 758	0.27 - 3.13	6.7 - 20.2

After a median of 12 months of treatment (IQR: 11-12.3 months), all individuals exhibited some degree of viral suppression ($p<0.0001$, **Figure 3.2A**), which was below the limit of detection of the assay in 12/15 individuals and <300 RNA copies/ml in the remainder. An increase in absolute CD4 count occurred in 13/15 individuals over the period measured ($p<0.0001$, **Figure 3.2B**). The median fold change in absolute CD4 count was 1.9 (IQR: 1.6-2.3), and when adjusted for length of treatment, the replenishment rate of CD4+ T cells was 20 cells/mm^3/month (IQR: 14-26, data not shown); this was accompanied by a concomitant doubling of the CD4/CD8 ratio (0.27 vs. 0.66; $p=0.0007$, **Figure 3.2C**). As previously reported (Kaufmann *et al.*, 2005;

Aiuti & Mezzaroma, 2006; Le *et al.*, 2013), the degree of CD4 reconstitution was inversely related to absolute CD4 count at ART initiation ($p<0.0001$, $r=-0.85$) and the duration of HIV infection ($p=0.016$, $r=-0.61$, data not shown). We also measured immune activation before and after ART using the markers CD38 and HLA-DR. There was a significant reduction in CD4+ and CD8+ T cells co-expressing CD38+ and HLA-DR+ ($p=0.0002$ and $p=0.0001$, **Figure 3.2D**), as well as HLA-DR+ alone ($p=0.0001$ for both subsets). Residual levels of immune activation after one year of ART remained significantly greater than healthy, HIV-uninfected individuals for both markers (**Figure 3.2D)**. We found no association between the degree of CD4 reconstitution and CD4+ T cell activation before or after ART (data not shown).

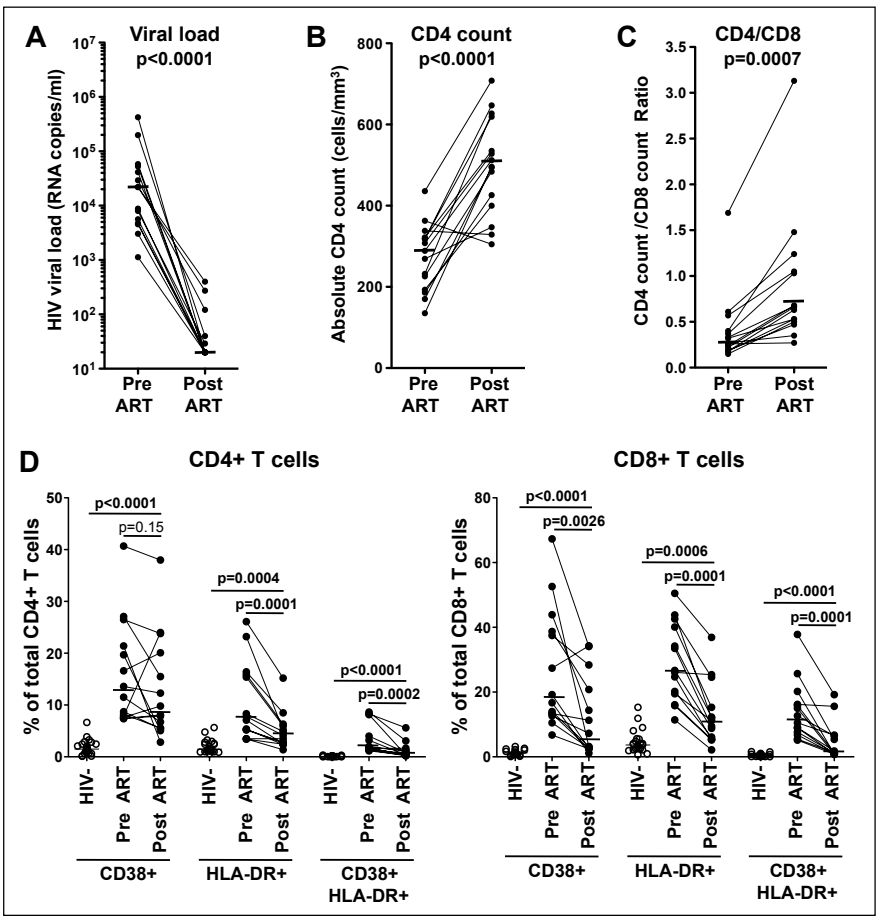

Figure 3. 2. Effect of ART on clinical and activation parameters. Plasma viral load (**A**), absolute CD4 count (**B**) and CD4/CD8 ratio (**C**) before (Pre-ART) and 12 months after ART initiation (Post-ART) in 15 HIV-infected individuals. (**D**) T cell activation (CD38, HLA-DR) is shown for CD4+ and CD8+ T cells. Closed circles depict HIV-infected individuals (n=14) and open circles depict HIV-uninfected individuals (n=23). Statistical analysis was performed using non-parametric statistical tests (Mann-Whitney and Wilcoxon matched pair tests for unmatched and matched samples, respectively). A p-value <0.05 was considered significant. Effective ART of 1-year duration results in an overall decline in T cell activation, although this still remained higher than in HIV-uninfected individuals.

To determine whether the dynamics of co-pathogen-specific CD4+ T cells mirror the reconstitution of the total CD4+ T cell population, we measured the magnitude of Mtb- and CMV-specific CD4+ T cell responses, as well as SEB-reactive CD4+ cells, before and after ART initiation. **Figure 3.3A** shows representative flow cytometry plots of cytokine production (IFN-γ, IL-2 and TNF-α) by CD4+ T cells in response to Mtb, CMV and SEB stimulation in one HIV-infected patient (CAP267). At baseline (before ART initiation), 11/15 individuals tested had a detectable Mtb response, with a median frequency of 0.08% of total CD4+ T cells (range, 0-0.9%; **Figure 3.3B**). CMV responses were detected in 10/12 individuals tested and the median frequency was 0.35% (range: 0-4.1%). SEB responses were detectable in 12/12 participants with with a median frequency of 16% (range: 8.6-32.5%). The frequency of Mtb-specific and SEB-responding CD4+ T cells were comparable pre- and post-ART, whilst the frequency of CMV-specific CD4+ T cells was significantly lower after treatment (median 0.35% vs 0.14%, p=0.003, **Figure 3.3C**). To take into account variation in absolute CD4 counts across the cohort, the absolute number of antigen-specific CD4+ T cells was calculated. The number of Mtb-specific and SEB-responding CD4+ T cells increased significantly after ART (p=0.033 and p=0.016, respectively), whilst the number of CMV-specific CD4+ T cells remained unchanged upon treatment (**Figure 3.3C**), consistent with the decreased frequency in the context of increases in absolute CD4 numbers. Overall, these data reveal that the dynamics of reconstitution of antigen-specific CD4+ T cells vary according to their memory phenotype. As for chronic infections, pathogen-specific memory T cells (such as EBV and HCV) are maintained with a distinct degree of memory differentiation (Appay *et al.*, 2002a). It is thus plausible that the reconstitution potential of cells will vary based on their antigen-specificity.

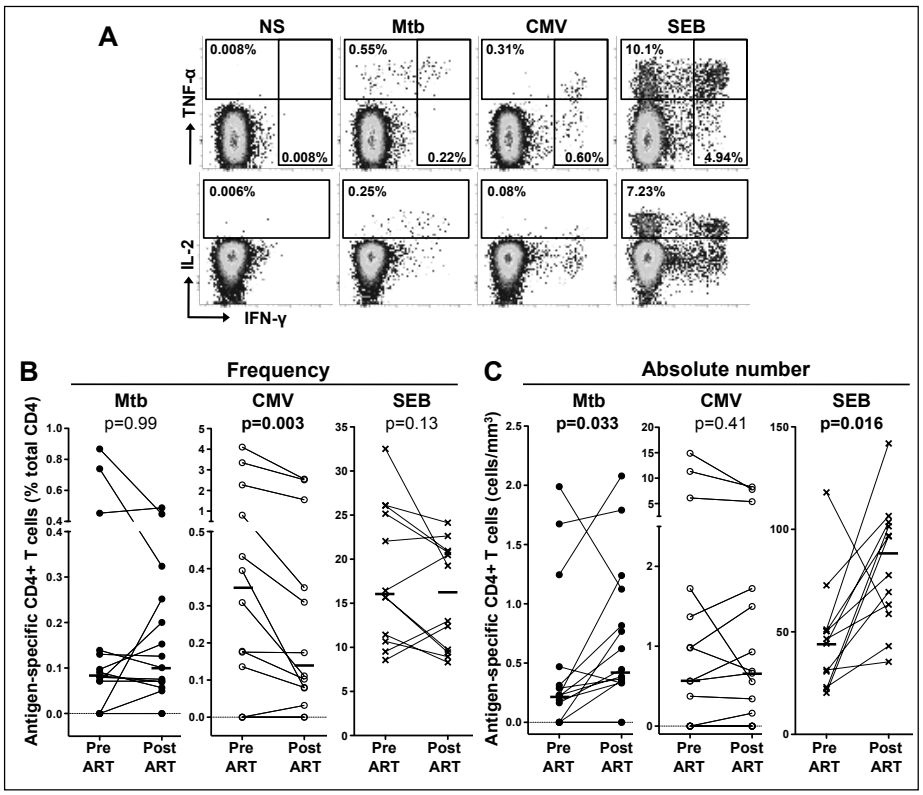

Figure 3. 3. Effect of ART on Mtb-, CMV- and SEB-specific CD4+ T cell responses. (A) Representative flow cytometry plots of the expression of TNF-α, IFN-γ and IL-2 from CD4+ T cells after stimulation with Mtb and CMV antigens and SEB, in one study participant. NS corresponds to unstimulated PBMC. The frequency of cytokine-producing cells expressed as a percentage of the total CD4+ T cell population are indicated. Frequency **(B)** and absolute number **(C)** of Mtb-, CMV- and SEB-specific CD4+ T cell responses before and after ART. Horizontal bars represent the median. Statistical comparisons were performed using a Wilcoxon matched pairs test.

3.4.2 Relationship between the dynamics of restoration of total and co-pathogen-specific CD4+ T cells

To further investigate the restoration dynamics of co-pathogen-specific CD4+ T cells, we examined the relationship between the fold change in the absolute number of antigen-specific CD4+ T cells pre- and post-ART (in individuals with detectable antigen responses at both time points, n=11 for Mtb, n=10 for CMV and n=12 for SEB), and the absolute total CD4 count variation. **Figure 3.4A** shows that whilst the magnitude of cell replenishment of Mtb-specific and SEB-responding CD4+ T cells was comparable to that of total CD4+ T cells (approximately a two-fold increase over the 12 months of ART), the extent of restoration of CMV-specific CD4+ T cells was significantly lower (p=0.018, p=0.013 and p=0.0008, to Mtb, SEB and total CD4+ cells, respectively). Consistent with these observations, the degree of restoration of

Mtb- and SEB-responding CD4+ T cells corresponded closely with the overall CD4 recovery, as measured in fold-change of the absolute CD4 count, with a slope close to 1 (**Figure 3.4B**). In contrast, for CMV-specific CD4+ T cell responses the slope was 0.37, indicating a reduced replenishment rate of these cells compared to the total CD4 compartment (**Figure 3.4B**). Of note, whilst all study participants were CMV seropositive, none had detectable CMV viral replication at either visit, pre- or post-ART, indicating that a difference in CMV antigen load at the two visits was not a contributing factor to the reduced replenishment capacity of CMV-specific T cells.

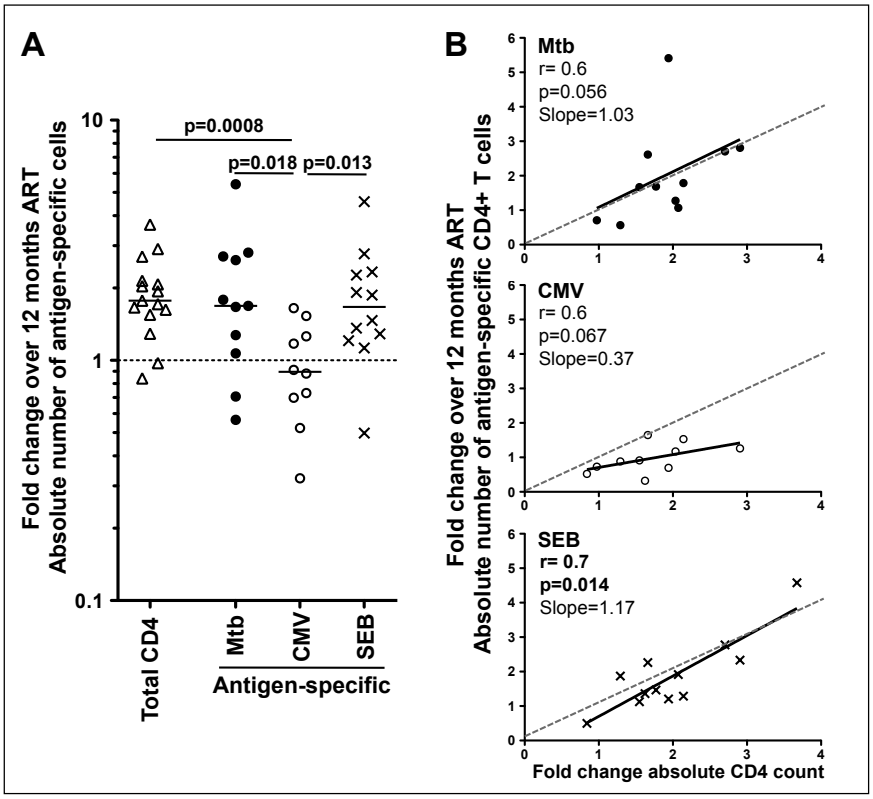

Figure 3. 4. Restoration dynamics of Mtb-, CMV- and SEB-specific CD4+ T cells after ART. (A) Fold change in the total, Mtb-, CMV- and SEB-specific absolute CD4+ T cell count over 12 months of ART. The horizontal dotted line indicates no change from the baseline pre-ART time point. Statistical comparisons were performed using a non-parametric Mann-Whitney test. **(B)** Association between the fold change of the absolute number of antigen-specific CD4+ T cells and the fold change in the absolute CD4 count pre- and post-ART. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation. The solid line represents a linear regression fit, and the dashed line depicts the ideal slope of 1.

3.4.3 Effect of ART on the functional capacity of co-pathogen-specific CD4+ T cells

ART has been shown to gradually restore the polyfunctional capacity of HIV-specific T cells (Rehr *et al.*, 2008; Mahnke *et al.*, 2012), but the effect of ART on the functionality of CD4+ T cells specific for other pathogens is unclear. Thus, we evaluated the cytokine secretion profiles of Mtb-, CMV- and SEB-reactive CD4+ T cells before and after ART initiation in detail (**Figure 3.5**). We included 9 HIV-uninfected individuals in this analysis for comparison. Our first finding was that the cytokine production profile of CD4+ T cells varied significantly according to their specificity ($p=0.002$, **Figure 3.5A**), with Mtb-specific responses consisting of a greater proportion of polyfunctional cells producing all three cytokines, in addition to a predominant TNF-α monofunctional subset (~40% of the response), whilst CMV-specific CD4+ responses exhibited predominantly a IFN-γ+TNF-α+ phenotype (~60%; **Figure 3.5B**). ART did not significantly alter the cytokine secretion profile of CD4+ T cells from baseline, regardless of their specificity for Mtb, CMV or SEB (**Figure 3.5A&B**). Moreover, the polyfunctional characteristics of Mtb-specific and SEB-responsive CD4+ T cell responses were comparable to those observed in HIV-uninfected individuals. In contrast, CMV responses were significantly skewed in HIV-infected individuals compared to HIV-uninfected subjects ($p=0.016$ and $p=0.045$ for pre- and post-ART, **Figure 3.5A**), with a lower proportion of cells co-producing IFN-γ, IL-2 and TNF-α, and a higher proportion of IFN-γ monofunctional cells (**Figure 3.5B**). These data demonstrate that, unlike for Mtb-specific CD4 responses, HIV infection impaired the functional potential of CMV-specific CD4 responses towards a less polyfunctional profile, and one year of ART did not restore the CMV functional profile.

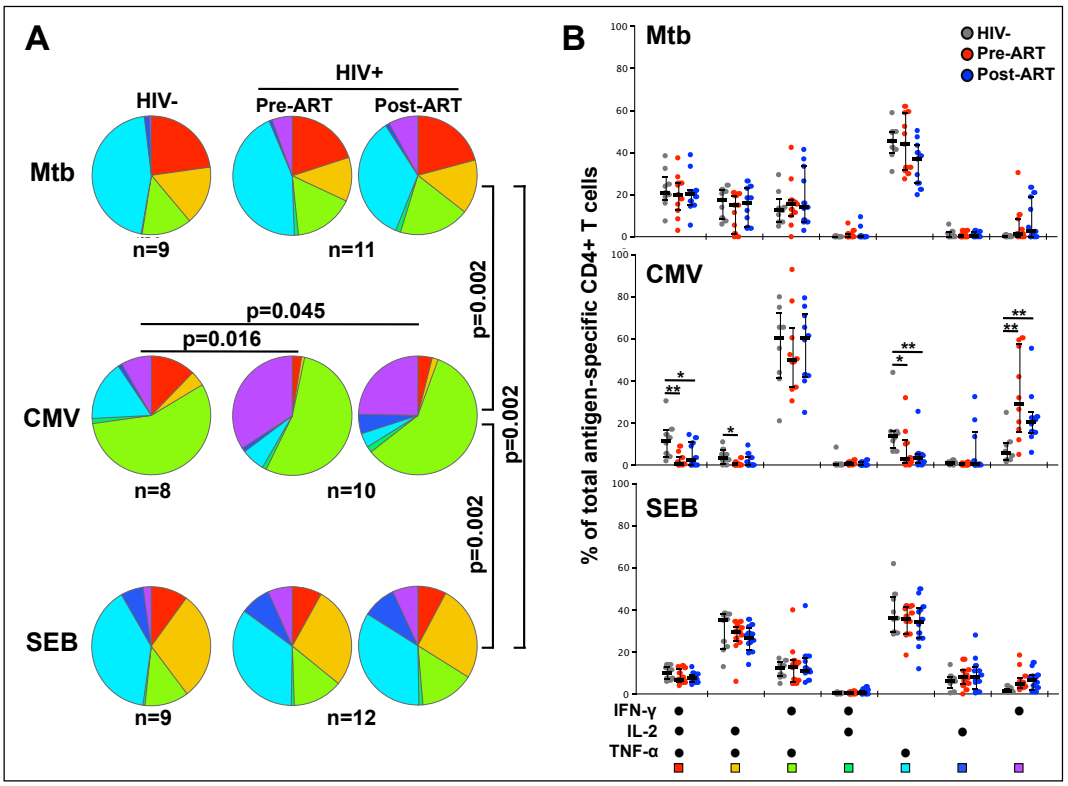

Figure 3. 5. Polyfunctional capacity of Mtb-, CMV- and SEB-specific CD4+ T cells in HIV-infected and uninfected individuals pre- and post-ART. Pie charts **(A)** and graphs **(B)** representing the cytokine secretion ability of Mtb-, CMV- and SEB-specific CD4+ T cell responses in HIV-uninfected individuals (n=9) and HIV-infected individuals pre-and post-ART initiation. Each section of the pie chart represents a specific combination of cytokines, as indicated by the color at the bottom of the graph. Horizontal bars depict the median with interquartile range indicated. Statistical comparisons were performed using a Wilcoxon rank-sum test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4.4 Impact of the memory phenotype of pathogen-specific CD4+ T cells on their replenishment potential upon ART

Depending on their memory differentiation profile, CD4+ T cells are endowed with distinct survival and proliferation capacities (Sallusto *et al.*, 1999, 2004). Thus, to investigate whether the cell maturation phenotype could impact the replenishment capacity of co-pathogen-specific CD4+ T cells, we first compared the differentiation profiles of Mtb- and CMV-specific CD4+ T cells at baseline, prior to ART initiation. **Figure 3.6A** shows a representative contour plot of distinct memory subsets in the CD4 compartment pre-ART in one HIV-infected individual. Based on the expression of CD45RO and CD27, we were able to discriminate four different memory subpopulations (Riou *et al.*, 2012), namely: Naive (CD45RO-CD27+), Early Differentiated (ED: CD45RO+CD27+, encompassing central and transitional memory

subsets), Late Differentiated (LD: CD45RO+CD27-, encompassing effector memory cells) and Terminally Differentiated (TD: CD45RO-CD27-, or effector cells). In HIV-infected subjects, although Mtb- and CMV-specific CD4+ T cells were represented in both ED and LD subsets, they comprised significantly distinct profiles (**Figure 3.6B**, left panel). Mtb-specific cells exhibited primarily an early-differentiated profile [median 51%, interquartile range (IQR) 42-62], whereas CMV-specific cells demonstrated a substantial enrichment of the late differentiated phenotype (median 73%, IQR 48-86), as previously described (Casazza *et al.*, 2006). Of note, ART did not markedly alter the overall memory differentiation profile of Mtb-specific or CMV-specific CD4+ T cells (**Figure 3.7**). In addition, HIV-uninfected individuals exhibited similarly distinctive memory CD4 differentiation profiles for Mtb and CMV (**Figure 3.6B**, right panel).

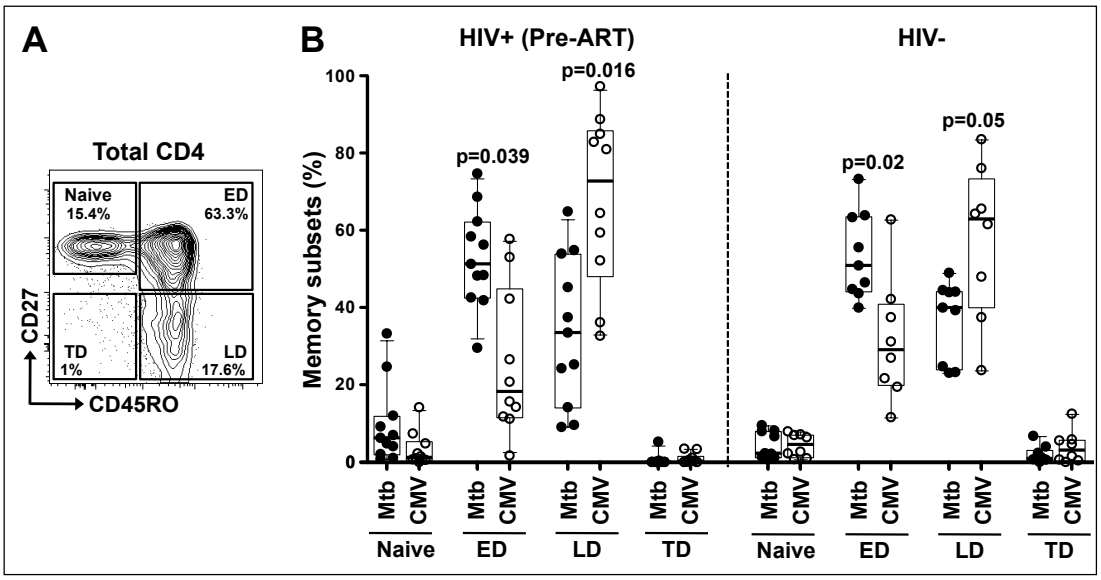

Figure 3. 6. Memory profiles of Mtb- and CMV-specific CD4+ T cells in HIV-infected and uninfected individuals. (A) Representative example of total CD4 memory subset distribution in one HIV-infected individual. Naive: CD45RO-CD27+, Early Differentiated (ED: CD45RO+CD27+), Late Differentiated (LD: CD45RO+CD27-) and Terminally Differentiated (TD: CD45RO-CD27-). The frequencies of each subset are indicated. **(B)** Memory profile of Mtb- and CMV-specific CD4+ T cells in HIV-infected individuals pre-ART (left panel) and HIV-uninfected individuals (right panel). Results are shown as box and whisker (10-90 percentile) plots. Each dot depicts an individual and the horizontal bar is the median. Statistical comparisons were performed using a one-way ANOVA non-parametric Kruskal-Wallis test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

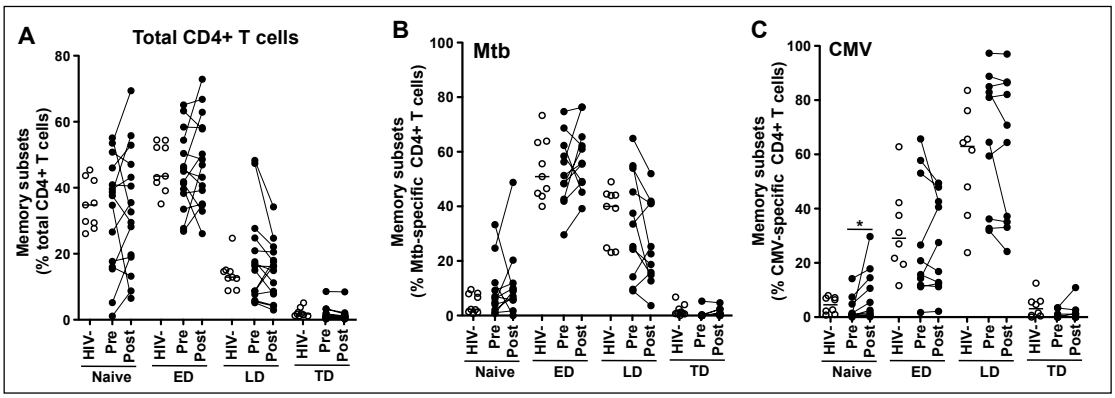

Figure 3. 7. Comparison of the memory profile of Mtb- and CMV-specific CD4+ T cells pre- and post-ART. Memory profile of total CD4+ T cells **(A)**, Mtb-specific CD4+ T cells **(B)** and CMV-specific CD4+ T cells **(C)** pre- and post-ART initiation. For comparison the memory profile of total and antigen-specific CD4 responses in HIV-uninfected individuals (n=9) is presented on each graph with open circles. Each symbol represents one individual. Statistical comparisons were performed using a Wilcoxon matched pairs test for comparison of pre- and post-ART time points and the Mann Whitney test for comparisons with HIV-uninfected individuals. * $p < 0.05$.

We next investigated the relationship between the memory phenotype of pathogen-specific CD4+ T cells at the time of ART initiation and the capacity of these cells to be maintained and/or replenished upon treatment. **Figure 3.8A** shows that the fold change in the absolute number of pathogen-specific CD4+ T cells over 12 months on ART inversely correlated with the proportion of cells exhibiting a late differentiated phenotype (p=0.019, r=-0.5). Consistent with this, a positive correlation was observed between the fold change in the absolute number of pathogen-specific CD4+ T cells and the proportion of these cells exhibiting an early-differentiated phenotype (p=0.04, r=0.45). Of note, the proportion of late-differentiated Mtb-specific CD4+ T cell responses (in the absence of CMV responses) also correlated inversely with the fold change in the absolute number of Mtb-specific cells (r=-0.62, p=0.038, data not shown), and so too when 'naïve-like' Mtb-specific cells were grouped together with ED cells, we observed a trend towards a positive association with the fold change in the absolute number of Mtb-specific cells (r=0.54, p=0.05, data not shown). This relationship between the memory profile and the ART-induced repletion potential of pathogen-specific CD4+ T cells is further illustrated in **Figure 3.8B**, showing specific examples from two participants. In CAP257, where the pre-ART CMV-specific CD4 response showed an enrichment in cells with a late differentiated phenotype (82%), their ability to expand over 12 months on ART was considerably lower than Mtb-

specific CD4 responses, characterised by an early differentiated phenotype (77.4%) (Fold change 1.2 for the CMV response vs. 2.8 for the Mtb response). In CAP244, where the memory profile of both Mtb and CMV-specific responses were similar (~60% of late differentiated cells), the fold change in the absolute number of CD4+ T cells to both pathogens was comparable.

Overall, these data reveal that the ability of co-pathogen-specific memory CD4+ T cells to expand upon ART appears to be related to their memory phenotype, where early differentiated cells have a higher replenishment capacity compared to late differentiated cells.

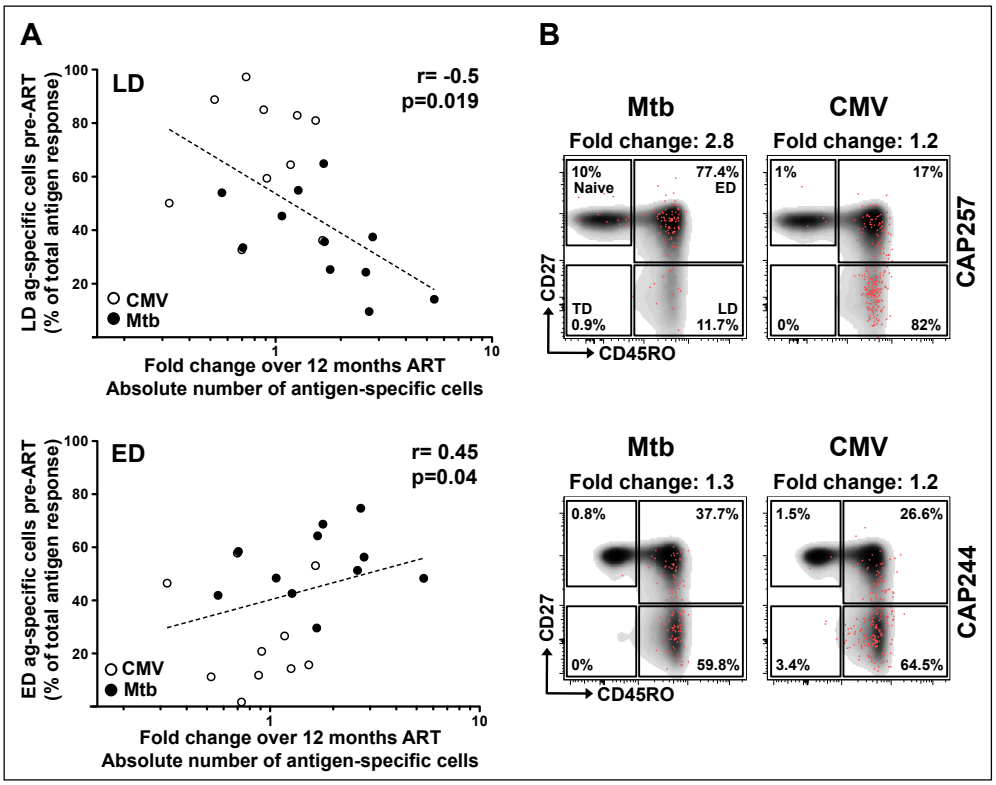

Figure 3. 8. Relationship between the memory profile of Mtb- and CMV-specific CD4+ T cells and the dynamics of restoration of these cells. (A) Association between the proportion of antigen-specific CD4+ T cells exhibiting a late differentiated (LD; top panel) or early differentiated (ED; bottom panel) profile with the fold change in antigen-specific CD4+ T cell absolute count after ART. Statistical associations were performed by a two-tailed non-parametric Spearman rank correlation. **(B)** Representative examples of the memory profile of Mtb- or CMV-specific CD4+ T cells pre-ART in two individuals. The frequencies of each subset are indicated. For each example, the fold change in antigen-specific absolute CD4+ T cell count pre-and post-ART is indicated at the top of each plot.

3.5 Discussion

The introduction of ART has changed the clinical pattern of HIV infection significantly, with considerable reductions in morbidity and mortality. Whilst it is clearly established that ART leads to a progressive replenishment of the CD4+ T cell compartment in the majority of cases, the extent to which pre-existing co-pathogen-specific CD4+ T cells are restored, and the causes of variable restoration, are poorly defined. In this study, we investigated the dynamics of restoration of memory CD4+ T cells specific for a bacterial co-pathogen (Mtb) and a viral pathogen (CMV) in 15 HIV-infected individuals in response to ART. Our main finding was that the extent of reconstitution of pathogen-specific cells was related to their memory differentiation profile at the time of ART initiation, where cells exhibiting an early differentiation memory profile (including central memory and transitional memory cells) had a higher replenishment potential compared to late differentiated (effector memory) T cells.

Multiple mechanisms contribute to the increase in CD4+ T cells in blood in response to antiretroviral treatment for HIV (Wilson & Sereti, 2013). Over the first few months of ART, there is a redistribution of CD4+ T cells from the lymph nodes to the blood (Pakker *et al.*, 1998; Bucy *et al.*, 1999), leading to a rapid initial rise in CD4+ counts. Moreover, homeostatic cell proliferation (Marchetti *et al.*, 2006), decreased cell death (Piconi *et al.*, 2010) and increased thymic output (Delgado *et al.*, 2002; Dion *et al.*, 2007) also play a role in the replenishment of CD4+ T cells. While the CD4 absolute cell count at the time of treatment initiation is one of the main factors dictating the level to which CD4+ T cells are restored (Kaufmann *et al.*, 2005; Le *et al.*, 2013), other parameters such as the activation level of T cells at the time of treatment (Goicoechea *et al.*, 2006), age (Kaufmann *et al.*, 2002; Allers *et al.*, 2014) or active co-infections (Almeida *et al.*, 2007; Kaufmann *et al.*, 2011) also influence the degree of reconstitution of the CD4 compartment. Thus, CD4+ T cell recovery upon ART appears to be dependent on both the extent of immune damage at the time of treatment initiation and the regenerative capacities of these cells.

We demonstrate here that despite successful viral suppression and robust CD4 gains in the majority of participants, the dynamics of Mtb- and CMV-specific CD4+ T cell recovery were distinct. This shows that not all T cell subpopulations have the same

potential to be replenished. Several factors and/or cellular attributes could account for these differences. Firstly, the regenerative capacities of pathogen-specific CD4+ T cells could be determined by their intrinsic ability to survive or proliferate, and it has been clearly established that CD4+ T cells exhibiting an early differentiated memory profile have an enhanced survival and proliferative potential compared to cells with a late differentiated phenotype (Sallusto *et al.*, 1999, 2004). Our data are in accordance with these observations, showing that the extent of replenishment of pathogen-specific CD4+ T cells correlates with their memory status, with superior recovery observed for antigen-specific CD4+ T cells exhibiting an early memory phenotype. Mechanistically, cell responsiveness to common γ-chain cytokines may account for the distinct renewal potential of these different cell subsets (Ahlers & Belyakov, 2010, Hodge *et al.*, 2011, Prezzemolo *et al.*, 2014). In our study, the replenishment potential of Mtb- or CMV-specific CD4+ T cells did not associate with their ability to produce IL-2 (data not shown), however we have observed that responsiveness to IL-7 distinguishes ED and LD CD4+ subsets, with ED memory T cells being more responsive to exogenous IL-7 and IL-2 (as measured by Stat5 phosphorylation), compared with LD memory CD4+ T cells (C. Riou, unpublished observation). Further analysis comparing the level of expression of cytokine receptors and cytokine-induced proliferation potential between Mtb- and CMV-specific CD4+ T cells would be needed to define the role of these signaling pathways in the restoration of immune memory cells upon ART.

Although Mtb responses displayed primarily an early-differentiated phenotype, there were substantial inter-individual differences, ranging from 30% to 75% of the response. The CD4+ memory profile is dictated by numerous factors, including continual antigen exposure in high burden settings (Schuetz *et al.*, 2012), previous TB, TB treatment and cure (Tapaninen *et al.*, 2010; Adekambi *et al.*, 2012; Marin *et al.*, 2012). A recent study carried out in a similar geographic setting as the present study noted dynamic changes in the T cell response to Mtb in the context of latent TB infection and HIV co-infection (Mitchell *et al.*, 2012). It is worth noting that we also detected antigen-specific cells with a 'naïve-like' phenotype, previously described for Mtb and other infections (Caccamo *et al.*, 2006; Marin *et al.*, 2012; Chatterjee *et al.*, 2015). Although making up only a minor proportion of the total Mtb- and CMV-specific response, these cells expanded post ART in the majority of individuals compared to other memory subsets that were characterised by more variable expansion and

contraction. For CMV, this expansion led to a significantly greater frequency of this subset after ART. These cells may fall within the recently described memory stem cell subset, comprising memory cells endowed with a greater ability to proliferate and persist long-term compared to central memory (Gattinoni *et al.*, 2011; Lugli *et al.*, 2013). Thus, the composition of the memory pool at the time of ART initiation may influence the restoration of antigen-specific CD4+ T cells.

To determine whether short-term ART affected not just quantitative but also qualitative aspects of co-pathogen immunity, we assessed the ability of cells to simultaneously secrete multiple cytokines, which for some infections, such as HIV and leishmaniasis, are a correlate of immune control or protection (Betts *et al.*, 2006; Darrah *et al.*, 2007). Although the significance of polyfunctional cells in TB is unclear (Prezzemolo *et al.*, 2014), we demonstrated that over 50% of Mtb-specific CD4+ cells produced three or two cytokines simultaneously, a profile that did not significantly change after ART; moreover, this was similar to the profile from HIV-uninfected individuals. Our data are in apparent contrast to previous findings (Sutherland *et al.*, 2010) showing that ART leads to an improved polyfunctional profile in Mtb-specific CD4+ T cell after short-term treatment, but this may be due to cohort differences, as the participants in that study were characterised by more advanced immunosuppression and higher viral loads than in the present study. Since it has been reported that the impairment of the functional capacity of Mtb-specific CD4+ T cells is related to HIV disease status (Day *et al.*, 2008), it is likely that differences in the severity of HIV pathology at the time of treatment initiation between the two studied groups could account for these discrepancies. The polyfunctional profile we observed for CMV-specific CD4+ cells is consistent with that previously reported (Casazza *et al.*, 2006), with simultaneous IFN-γ and TNF-α, and IFN-γ alone dominating the response. The CMV profile also did not change after ART, and in this case was less polyfunctional compared to HIV-infected individuals. Our study only examined paired samples from 12 months after ART, and it is possible that prolonged ART may restore CMV functional profiles (Rehr *et al.*, 2008).

It is important to note that although we demonstrate an expansion of Mtb-specific CD4+ T cells after ART, restoration of the magnitude and measures of functionality of the Mtb-specific CD4+ Th1 response determined by the production of three

cytokines is a coarse measure of functional immunity to TB, and may not reflect protective immunity. Several additional CD4+ T cell subsets and cytokines contribute to TB immunity (Scriba *et al.*, 2008; McBride *et al.*, 2013; Wozniak *et al.*, 2010; Slight *et al.*, 2013), and we have found an even greater diversity of CD4+ T cell subsets specific for Mtb (Riou *et al.*, 2016). An additional issue is that we do not know the extent of pathogen-specific CD4+ recovery in tissues upon ART, which appears defective for TB in the lungs even after prolonged viral suppression (Jambo *et al.*, 2014). These considerations may account for the persistently greater susceptibility of successfully treated HIV-infected individuals to TB (Girardi *et al.*, 2005).

Our study had several limitations. We were constrained by the availability of paired samples prior to and after ART, limiting us to studying 15 individuals in detail. Testing the generalisability of our findings in larger cohorts is warranted. Also, we focused our study on short-term restoration of pathogen-specific immunity (after 1 year of ART), and although study participants exhibited suppressed HIV replication, decreased immune activation and improved CD4 counts, studying responses after longer-term therapy may provide further important insights and more definitive conclusions on the restoration of pathogen-specific immunity. Furthermore, the participants we studied had nadir CD4 counts of >135 cells/mm^3 (median 289 cells/mm^3), and we did not have access to participants commencing treatment at lower CD4 counts (e.g. <50 cells/mm^3). These highly immunocompromised patients would be the most relevant group to study with respect to susceptibility to CMV disease. In this context, although CMV-specific cells replenished more poorly than Mtb-specific cells after ART, they were present at a high frequency prior to ART initiation, that may well have been sufficient to provide protective immunity to CMV. While we did not observe a correlation between nadir CD4 counts and the degree of antigen replenishment likely due to a small sample size analysed, it is still plausible that the extent of CD4 depletion at the time of ART initiation could impact the degree and dynamics of antigen-specific CD4+ T cell recovery. Moreover, we delineated memory subsets based on two markers, CD27 and CD45RO. These markers cannot differentiate between central memory and transitional memory cells within the CD27+CD45RO+ ED subset, and inclusion of a marker such as CCR7 would afford an additional degree of distinction that may reveal further differences in the ability of these memory subsets to expand after ART, and should be included in future studies of reconstitution of

pathogen-specific immunity. Lastly, it would be of great interest to concomitantly evaluate the degree of replenishment of HIV-specific T cells. However, due to sample availability, we were not able to investigate the effect of ART on HIV-specific T cell responses. It has been shown that ART can induce an initial increase in HIV-specific T cell responses, but following complete suppression of HIV viral load there is a decline in HIV-specific T cell responses due to insufficient HIV antigen levels (Macatangay & Rinaldo, 2015). Further research addressing these important limitations is needed to confirm and extend our findings.

Understanding the host and cellular factors that contribute to successful immune restoration on effective antiretroviral treatment for HIV, including both the overall CD4+ T cell compartment and co-pathogen-specific CD4 immunity, is of crucial importance, as only partial recovery of these cells could result in a persistently heightened risk of particular opportunistic infections. In this report, we showed that the renewal potential of pre-existing pathogen-specific CD4+ T cells was related to their memory differentiation profile. It will be important to determine whether this principle holds true in a wider context for other co-pathogens. Incomplete restoration of functional immunity and the persistence of elevated susceptibility to particular co-infections have implications for targeted interventions in the treated HIV-infected population. Overall, our findings underscore the complexity of immune reconstitution on ART, and the importance of preserving functional immunity with early ART.

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Contribution: Conceived and designed the experiments: C.R., N.J.G., L.W., Q.A.K., S.A.K. and W.A.B. Performed the experiments: R.F.T., A.P.S. and L.M. Analysed the

data: C.R. and R.F.T. Contributed reagents/materials/analysis tools: N.J.G., N.S., Q.A.K. and S.A.K. Wrote the paper: C.R., R.F.T. and W.A.B. All authors approved the final manuscript.

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CHAPTER 4

Effect of antiretroviral therapy on the memory and activation profiles of B cells in HIV-infected African Women.

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Contribution: Ramla Tanko performed the majority of the experiments, analysed the data and wrote the paper.

4.1 Abstract

HIV infection induces a wide range of effects in B cells, including skewed memory cell differentiation, compromised B cell function and hypergammaglobulinaemia. However, data on the extent to which these B cell abnormalities can be reversed by antiretroviral therapy (ART) are limited.

To investigate the effect of ART on B cells, the activation (CD86) and differentiation (IgD, CD27 and CD38) profiles of B cells were measured longitudinally in 19 HIV-infected individuals before (median, 2 months) and after ART initiation (median, 12 months) and compared to 19 age-matched HIV-uninfected individuals, using flow cytometry.

Twelve months of ART restored the typical distribution of B cell subsets, increasing the proportion of naive B cells (CD27-IgD+CD38-) and concomitantly decreasing the immature transitional (CD27-IgD+CD38+), unswitched memory

(CD27+IgD+CD38-), switched memory (CD27+IgD-CD38- or CD27-IgD-CD38-) and plasmablast (CD27+IgD-CD38^{high}) subsets. However, B cell activation was only partially normalised post-ART, with the frequency of activated B cells (CD86+CD40+) reduced compared to pre-ART levels (p=0.0001), but remaining significantly higher compared to HIV-uninfected individuals (p=0.0001). Interestingly, unlike for T cell activation profiles, the extent of B cell activation prior to ART did not correlate with HIV plasma viral load, but positively associated with plasma sCD14 levels (p=0.01, r=0.58).

Overall, ART partially normalises the skewed B cell profiles induced by HIV, with some activation persisting. Understanding the effect of HIV on B cell dysfunction and restoration following ART may provide important insights into mechanisms of HIV pathogenesis.

4.2 Introduction

Systemic immune hyperactivation is a hallmark of HIV infection, affecting a range of immune cells, including both T cells and B cells (Appay & Sauce, 2008). Multiple B cell defects have been reported in HIV-infected individuals, including alteration in the distribution of B cell memory subsets, with the accumulation of differentiated B cells (Moir *et al*., 2001, 2003, 2008; Malaspina *et al*., 2006), excessive B cell activation (Malaspina *et al*., 2003; De Milito *et al*., 2004;) and increased cell turnover (Kovacs *et al*., 2001). These B cell perturbations lead to functional abnormalities, as demonstrated by hypergammaglobulinemia, decreased B cell responsiveness to both T cell-dependent and T cell-independent antigens (Malaspina *et al*., 2005; Titanji *et al*., 2006; Hart *et al*., 2007) and compromised responses to vaccination (French *et al*., 1998; Malaspina *et al*., 2005; Pasricha *et al*., 2006).

The specific mechanisms contributing to B cell abnormalities are only partially known, and multiple factors may account for their dysfunction. HIV-driven alteration of the cytokine and chemokine environment has been described as a source of B cell dysfunction (Müller *et al*., 1998; Malaspina *et al*., 2006; Swingler *et al*., 2008; Lantto *et al*., 2015) and it has also been proposed that specific HIV proteins may have a direct effect on B cells (Schnittman *et al*., 1986; Swingler *et al*., 2003). Several studies, performed mostly in cross-sectional Caucasian cohorts, have investigated the effect of

antiretroviral therapy (ART) on B cells, reporting that suppressive ART can partially or completely normalise B cell phenotypic defects, as shown by the replenishment of naive B cells (D'Orsogna *et al*., 2007; Moir *et al*., 2008b, 2010; Amu *et al*., 2014), contraction of activated B cells (Moir *et al*., 2010; Pensieroso *et al*., 2013; Rethi *et al*., 2013; Amu *et al*., 2014; Abudulai *et al*., 2016) and increase in B cell survival potential (van Grevenynghe *et al*., 2011). It is still uncertain, however, whether normalisation of B cell memory subsets results in improved B cell immune responses to antigens, including influenza, measles, pneumococcus and hepatitis B (Kroon *et al*., 1998; Malaspina *et al*., 2003; Titanji *et al*., 2006; Hart *et al*., 2007; Whitaker *et al*., 2012).

There is a paucity of published studies on female and African populations with regard to B cell activation and restoration of B cell immunity following successful treatment of HIV (Béniguel *et al*., 2004; Longwe *et al*., 2010). There are cogent reasons to believe there may be differences in Africans compared to Caucasian cohorts. African cohorts have demonstrated higher baseline levels of T cell activation, significantly different T cell memory differentiation profiles (Eggena *et al*., 2005; Roetynck *et al*., 2013), and consistently weaker cellular and humoral reactivity to some vaccines (Monath *et al*., 2002; Muyanja *et al*., 2014). A variety of factors may influence immune activation and therefore normalisation of immune profiles after ART, such as genetic, gender and environmental differences, the latter including higher antigenic exposure, diet and gut microbiota. Furthermore, a variety of sex-specific differences in the response to infections have been described. Women have higher levels of immune activation and faster progression of HIV disease than men with the same viral load (Meier *et al*., 2009). These effects have been attributed to oestrogen receptor signalling and/or differences in expression of key X-chromosome-expressed immune regulators, such as toll-like receptors and CD40L (Hagen & Altfeld, 2016). Additional factors such as HIV strains, treatment regimens and delayed access to HIV treatment could result in distinct outcomes with respect to immunity after ART.

Thus, in this study, to define the extent to which ART restores B cell phenotype, we measured the memory differentiation and activation profiles of B cells longitudinally in chronically HIV-infected African women before and 12 months after ART initiation, and compared these profiles to age- and sex-matched HIV-uninfected individuals.

4.3 Materials and Methods

4.3.1 Description of study participants

Study participants consisted of 19 women from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 002 HIV acute infection cohort in KwaZulu-Natal, previously described (van Loggerenberg *et al*., 2008; Mlisana *et al*., 2014). Peripheral blood samples were obtained at two time-points, during chronic infection pre-ART initiation, and post-ART initiation. With respect to ART regimens, 15 of the 19 participants were taking current standard first-line therapy (TDF/3TC/EFV or TDF/FTC/EFV), and 1 each were taking D4T/3TC/EFV, D4T/3TC/NVP, AZT/3TC/NVP and AZT/3TC/LPV/r. One participant (CAP255) switched ART regimens during the study period (D4T/3TC/EFV to AZT/3TC/EFV at month 10). No participants had active TB during the study period, or exhibited any immune reconstitution inflammatory syndrome upon HIV treatment. An additional 19 HIV-uninfected women who were matched for age and ethnicity were studied, from the CAPRISA 004 1% tenofovir microbicide gel trial (Abdool Karim *et al*., 2010). These women were either in the pre-intervention or in the placebo arms of the trial. For HIV-infected individuals, either a prospective RNA positive/antibody negative reading or the midpoint between the last antibody negative test and the first antibody positive enzyme-linked immunosorbent assay test were used to determine the time after infection. HAART was given according to the South African national HIV treatment guidelines (at a CD4 count of <200 cells/mm^3 prior to October 2012; <350 cells/mm^3 until March 2015). Ethical approval for the study was obtained from the Research Ethics Committees at the University of KwaZulu-Natal and University of Cape Town. All participants provided written informed consent prior to participating in the study.

4.3.2 Measurements of HIV plasma viral load and CD4 counts

Plasma HIV viral load and CD4 count were assessed at each study visit. Over the course of the study, the viral load PCR assay switched from Roche AMPLICOR HIV-1 monitor test version 1.5 (lower detection limit (LDL) of 400 RNA copies/ml) to Roche Taqman version 1.0 in June 2010 (LDL 40 RNA copies/ml), and then to Roche Taqman version 2.0 in January 2012 (LDL 20 RNA copies/ml). The FACSCalibur

TruCOUNT method (BD Biosciences) was used to measure blood absolute CD4+ and CD8+ T cell counts.

4.3.3 Sample processing

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Pharmacia) density gradient centrifugation and cryopreserved in freezing media [heat-inactivated foetal calf serum (FCS; Invitrogen) containing 10% dimethylsulfoxide (Sigma-Aldrich)]. Cells were stored in liquid nitrogen until use. Cryopreserved PBMC were thawed and rested in R10 (RPMI 1640 plus 10% heat-inactivated FCS and 50 U/ml of penicillin-streptomycin) at 37°C with 5% CO_2 for 3 hours before staining.

4.3.4 Antibodies, Surface and Intracellular staining (ICS)

The following antibodies were used in three different staining panels: CD19 ECD (J3-119), CD27 PE-Cy5 (1A4CD27; both Beckman Coulter), IgD APC-Cy7 (IA6-2), CD10 BV605 (HI10a), CD21 PE-Cy7 (Bu32), CD40 PerCP-Cy5.5 (5C3; all Biolegend), CD38 APC (HIT2), CD86 PE (IT2.2), CD3 PE-Cy7 (SK7), HLA-DR APC-Cy7 (L243; all BD Biosciences), CD4 PE-Cy5.5 (S3.5), CD8 Qdot-705 (3B5), CD19 Pacific Blue (SJ25-CI), CD14 Pacific Blue (Tük4), CD3 Pacific Blue (UCHT1), Ki67 FITC (7B11; all Invitrogen) and a violet viability reactive dye ("Vivid"; Molecular Probes). All antibodies were titrated prior to use to obtain optimal titres for staining. Briefly, PBMC were stained with Vivid, then labelled with antibodies against surface markers, fixed, permeabilised and subsequently stained intracellularly with Ki67. Cells were then re-suspended in 1X CellFix (BD Biosciences) and kept at 4^0C until acquisition. Samples were acquired on a BD Fortessa using FACSDiva software and analysed using FlowJo (version 9.9.3; TreeStar). The gating strategy to identify B cell subsets is shown in **Figure 4.1**.

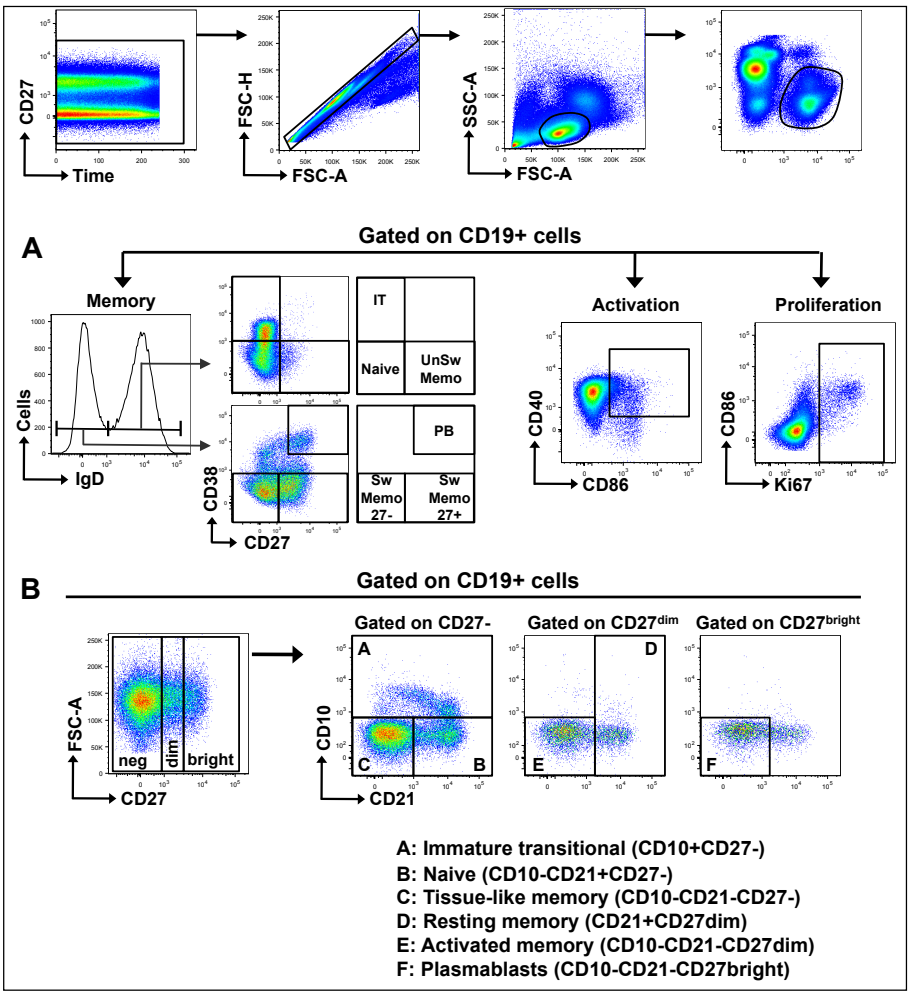


Figure 4. 1. Gating strategies. A time gate was included to ensure no shift in fluorescence intensity over time, followed by a singlet gate to exclude doublets from the analysis. CD3 and CD14 markers were used to exclude T cells and monocytes, respectively, which were on the same channel as the viability dye Vivid, used to exclude dead cells. The core marker CD19 was used to identify B cells. **(A)** Identification of B cell memory subsets based on IgD, CD27 and CD38 expression. **(B)** Alternative gating strategy based on CD27, CD10 and CD21 expression to delineate memory B cell subsets.

4.3.5 Enzyme-linked Immunosorbent assays (ELISA)

Plasma samples were used to measure soluble CD14 (sCD14, a marker of monocyte/macrophage activation) from the same women pre- and post-ART. One participant was not included in this analysis due to the sample being unavailable. Plasma (n=18) was tested in duplicate using commercially available ELISA kits according to the manufacturers' protocol (R&D Systems). In addition, 30 plasma samples from HIV-uninfected women from the same cohort were included. Samples were diluted 1:200, data were collected using a SpectraMax Plus reader (Molecular Devices) and point-to-point curve fits were used to calculate sCD14 concentrations

from the standard curves. Data were analysed using SoftMax Pro software (Version 3.2.1; Molecular Devices).

4.3.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 5.0). The Mann-Whitney U test and the Wilcoxon Signed Rank test were used for unmatched and paired samples, respectively. Correlations between the different groups were determined by the non-parametric Spearman Rank test. A p-value of <0.05 was considered statistically significant.

4.4 Results

4.4.1 Effect of antiretroviral therapy (ART) on the memory differentiation and activation of B cells

To define the extent to which ART normalises HIV-induced alteration of B cells, we first determined the distribution of B cell subsets in 19 HIV-infected individuals before and one year after ART initiation, and compared it to 19 HIV-uninfected matched controls. Pre-ART samples were obtained during chronic infection at a median of 4.9 years [Interquartile range (IQR): 2.8-5.8 years] after the estimated date of HIV infection, and a median of 1.5 months (IQR: 0.05-2.7) prior to starting ART. The median plasma viral load at this time was 34,325 HIV RNA copies/ml (IQR: 7,997-78,573), and the median CD4 count was 314 cells/mm^3 (IQR: 260-369) (**Table 4.1**). After a median of 12 months of treatment (IQR: 12-13 months), all individuals exhibited viral suppression ($p=0.0001$), the majority to <40 copies/ml, and an increase in both absolute CD4 count and CD4/CD8 ratio (both $p=0.0001$) (**Table 4.1** and data not shown).

Table 4. 1. Clinical parameters of study participants.

	HIV-infected (pre-ART)	HIV-infected (post-ART)	*P*-value
Sample size	**19**	**19 (paired)**	
CD4 count (cells/mm^3) *	314 [260-369]	629 [514-696]	p=0.0001
CD4/CD8 ratio *	0.23 [0.18-0.33]	0.61 [0.48-0.74]	p=0.0001
Viral load (RNA copies/ml) *	34,325 [7,997-78,573]	<20 [<20-39]	p=0.0001
Duration of ART (months) *	NA	12 [12-13]	

* Median and Interquartile range, NA: Not Applicable

Figure 4.2A shows representative flow plots of each B cell subset from one HIV-uninfected and one HIV-infected individual pre- and post-ART. Based on the expression of CD27, IgD and CD38, six B cell subsets were identified, namely immature transitional B cells (CD27-IgD+CD38+), naive B cells (CD27-IgD+CD38-), unswitched memory (CD27+IgD+CD38-), CD27+ switched memory B cells (IgD-CD38-), CD27- switched memory B cells (IgD-CD38-) and plasmablasts (CD27+IgD-CD38^{high}). There was a marked skewing of B cell subsets in chronic HIV infection prior to ART (**Figure 4.2B**). When compared to HIV-uninfected participants, HIV-infected individuals were characterised by significantly higher frequencies of immature transitional B cells, unswitched memory B cells and plasmablasts (median: 9.3% [IQR: 6.3-13.3] vs 14.8% [IQR: 10.6-20]; 1.5% [IQR: 1.2-1.7] vs 2.3% [IQR: 2-5.8] and 0.44% [IQR: 0.3-1] vs 2.1% [IQR: 1.4-4], respectively), and significantly lower proportions of naive B cells (median: 65.8% [IQR: 60-72.1] vs 46.9% IQR: 39.7-55.7) (**Figure 4.2B**). Additionally, the frequencies of switched memory B cells, expressing CD27 or not were comparable in HIV-uninfected and ART naive HIV-infected individuals (~8% and ~12%, respectively). It is worth mentioning that the frequency of un-switched memory B cells in our healthy study population was about 7 times lower when compared to a previous study reporting that the proportion of unswitched memory was comparable to switched memory (representing about 15% of total B cells) in a predominantly male cohort from London (Hart *et al.*, 2007). This could be explained by major differences in the study cohort such as sex, geographical location and low versus high disease burden country. Following 12 months of ART, the frequency of immature transitional, naive and unswitched memory B cells were normalised, returning to levels comparable to those observed in HIV-uninfected individuals (**Figure 4.2B**). The frequency of plasmablasts was also reduced in response to ART (p=0.004), but remained significantly elevated compared to the HIV-

uninfected group. To further define the restoration dynamics of B cell subsets upon ART, we compared the fold change in their frequencies pre- and post-ART. After one year of ART, only naive B cells displayed an overall expansion (median 1.3 fold [IQR: 1.2-1.5]); amongst the subsets decreasing upon ART, plasmablasts exhibited the largest contraction, with a median fold change of 0.38 [IQR: 0.22-0.72]. The contraction of other B cells subsets was more moderate with a median fold change of 0.51 [IQR: 0.39-0.92] for unswitched memory B cells, 0.65 [IQR: 0.37-0.77] for immature transitional B cells, 0.79 [IQR: 0.5-1] for CD27+ switched memory B cells and 0.87 [IQR: 0.69-1] CD27- switched memory B cells (**Figure 4.2C**). Although ART had a predominant effect on reducing the frequency of plasmablasts (~60% reduction), this was not sufficient for their normalisation with respect to HIV-uninfected individuals.

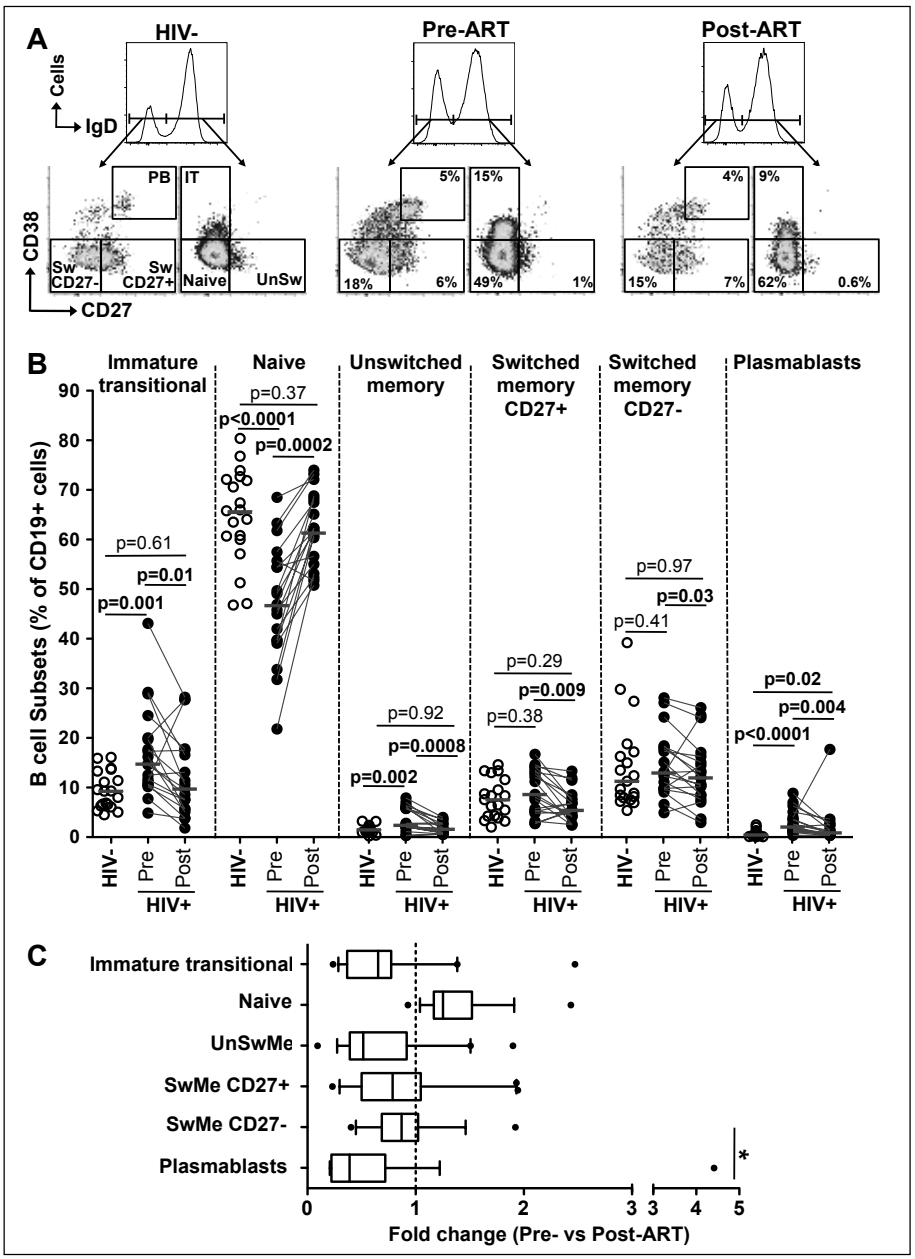
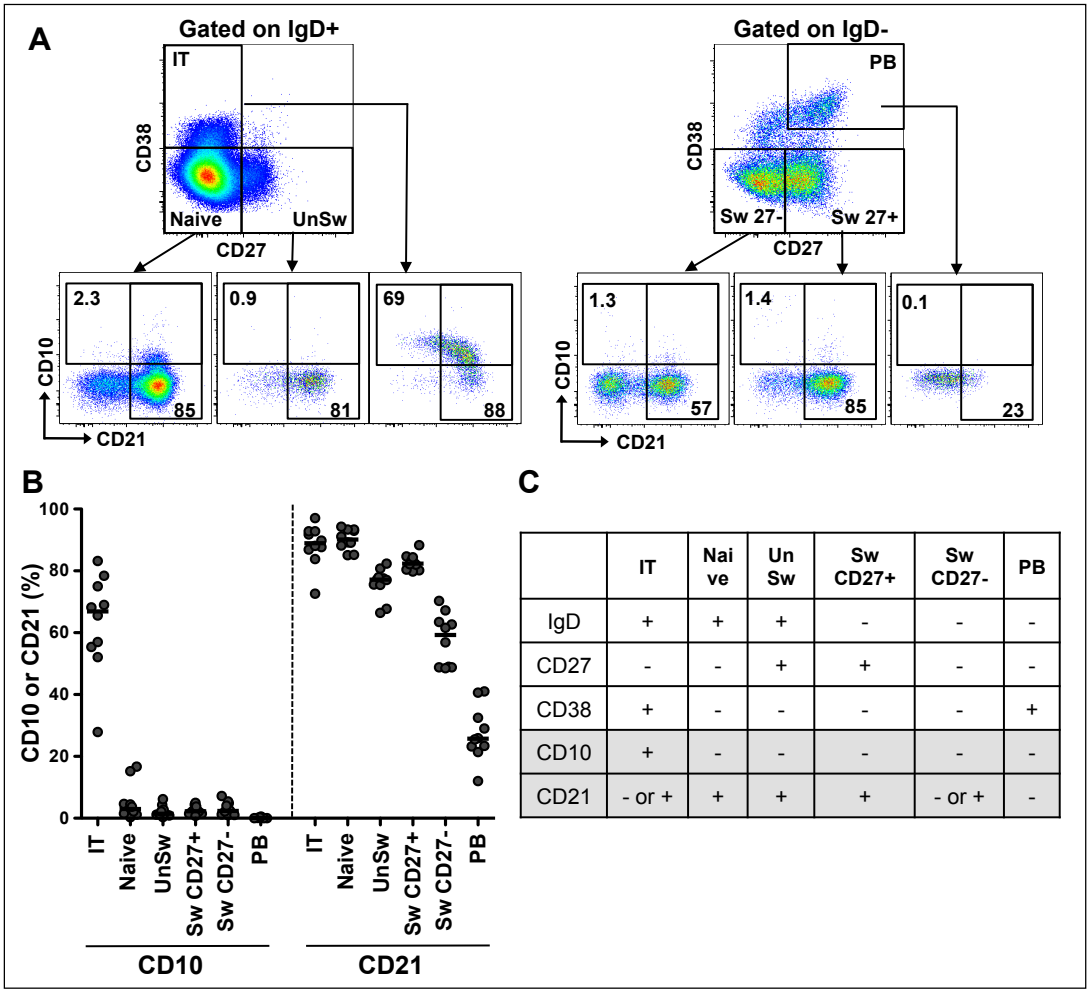


Figure 4. 2. Effect of ART on the memory differentiation profile of B cells. (A) Representative flow plots of CD27, IgD and CD38 expression in B cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. Numbers represent the frequencies of each B cell subset, namely immature transitional (CD27-IgD+CD38+), naive (CD27-IgD+CD38-), Unswitched memory (CD27+IgD+CD38-), switched CD27+ memory (IgD-CD38-), switched CD27- memory (IgD-CD38-) and plasmablasts (CD27+IgD-CD38high). **(B)** Frequencies of B cell subsets in HIV-uninfected (n=19; open circles) and HIV-infected pre- and post ART (n=19; closed circles) individuals. Horizontal lines indicate the median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively. **(C)** Fold change in the frequency of B cell subsets over 12 months of ART. Data are shown as box and whisker (interquartile range) plots. The vertical dotted line indicates no change from the time point prior to ART. Statistical comparisons were calculated using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Alternative B cell markers such as CD27, CD10 and CD21 have been used to delineate B cell memory subsets (Moir & Fauci, 2009), allowing for the identification of three additional B cell memory subsets, namely tissue-like memory (CD10-CD21-CD27-), resting memory (CD21+CD27dim) and activated memory (CD10-CD21-CD27dim) B cells (**Figure 4.1B**). The expression of CD10 and CD21 on B cell subsets is shown in **Figure 4.3**. Thus, to validate our data, we defined the evolution of B cell memory subpopulations pre- and post-ART using this set of markers in 10 of the HIV-infected individuals from our study (**Figure 4.4**). ART led to a significant reduction in the frequencies of tissue-like memory, activated memory B cells and plasmablasts, and an increase in the frequencies of naive and resting memory B cells. These observations confirm the data described above, and are in line with published data (Moir *et al*., 2010). Of note, using this alternative gating strategy, we found no significant difference in the frequencies of immature transitional B cells between the two time-points. This may be due to the small sample size (a subset of n=10) used for this set of markers, since further analysis of the same individuals using IgD, CD38 and CD27 also showed no significant difference in immature transitional B cells (data not shown).



	IT	Naive	Un Sw	Sw CD27+	Sw CD27-	PB
IgD	+	+	+	-	-	-
CD27	-	-	+	+	-	-
CD38	+	-	-	-	-	+
CD10	+	-	-	-	-	-
CD21	- or +	+	+	+	- or +	-

Figure 4. 3. Expression of CD10 and CD21 in B cell subsets delineated using IgD, CD27 and CD38 expression. (A) Representative flow plots from one HIV-infected individual after ART. **(B)** Frequencies of CD10 and CD21 in each B cell subsets in HIV-infected individuals post-ART (n=10). The majority (~70%) of immature transitional (IT) B cells express CD10, while all other B cell subsets were predominantly negative for this marker. CD10 expression on IT B cells may be an underestimation due to non-optimal CD10 separation. **(C)** Summary of the phenotype of B cells subsets. Although the majority of IT B cells express CD21, T1 IT B cells are CD21lo.

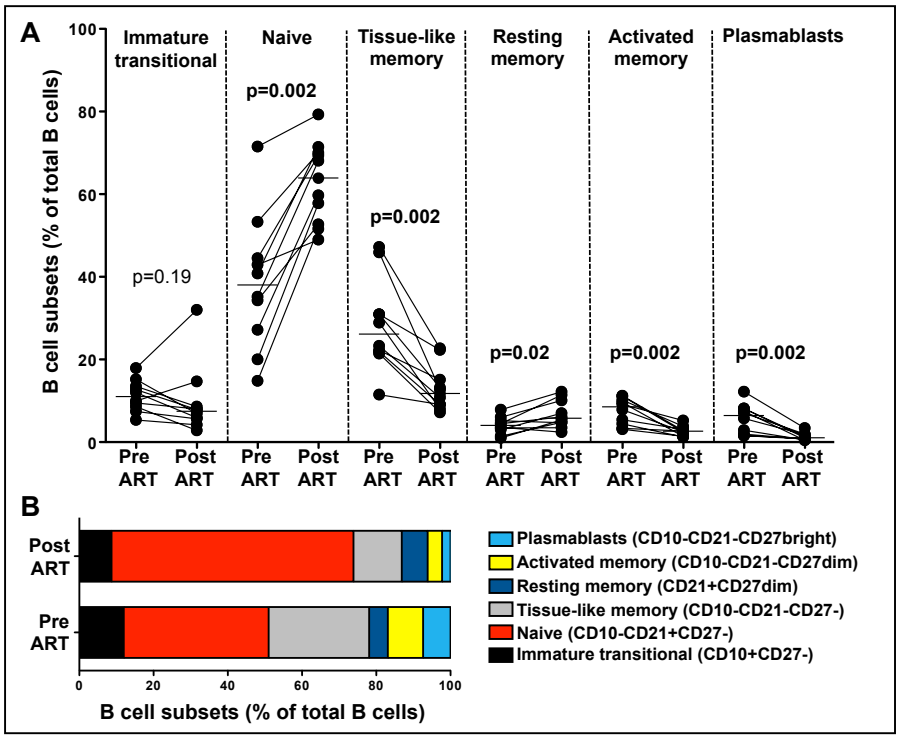


Figure 4. 4. Evolution of B cell subsets defined based on CD27, CD10 and CD21 expression pre- and post-ART. (A) Frequencies of B cell subpopulations before and after ART in 10 HIV-infected participants. Horizontal lines indicate the median. Statistical significance was calculated using Wilcoxon paired test. **(B)** Stacked bar graph showing the median frequency of each B cell subset before and after ART. Each colour identifies a B cell subpopulation, as indicated in the adjacent key. The gating strategy is shown in **Figure 4.1.**

The effect of ART on B cell activation and proliferation were also evaluated. **Figure 4.5A** shows representative flow plots of B cell activation, measured as the frequency of B cells co-expressing CD86 and CD40, and proliferation (Ki67 expression) from one HIV-uninfected and one HIV-infected individual pre- and post-ART. The frequency of activated B cells (CD86+CD40+) was significantly higher in HIV-infected subjects prior to ART compared to HIV-uninfected participants (median: 14% [IQR: 9.2-15.6] vs 1.7% [IQR: 1.3-2.3]; **Figure 4.5B**). In response to ART, the frequency of activated B cells was substantially reduced but remained significantly higher than HIV-uninfected participants (**Figure 4.5B)**. It is important to mention that during HIV infection, activated B cells were mostly confined to switched memory cells (i.e. IgD- B cells), with a median of 25% (IQR: 19.6-32) of switched cells co-expressing CD40 and CD86. On the contrary, the activation level of unswitched B cells (i.e. IgD+ B cells) was only marginal, with less that 5% of these cells positive for CD40 and CD86 (median: 4.5% [IQR: 2.4-5]; data not shown).

Similar to activated B cells, there were significantly more Ki67+ B cells in ART-naive HIV-infected individuals compared to HIV-uninfected controls (median: 5% [IQR: 2.9-7.2] vs 1.3% [IQR: 0.9-1.6]); and while ART led to a significant decline in proliferating B cells, they remained elevated compared to the HIV-uninfected group (p=0.001, **Figure 4.5C**). Of note, the frequencies of proliferating (Ki67+) and activated (CD86+CD40+) B cells were positively associated with the frequency of plasmablasts following ART (p=0.004, r=0.62 and p=0.003, r=0.63, respectively, data not shown), suggesting that residual B cell activation and proliferation upon ART may be explained by the persistence of an elevated frequency of plasmablasts, consistent with our findings presented in **Figure 4.2B**.

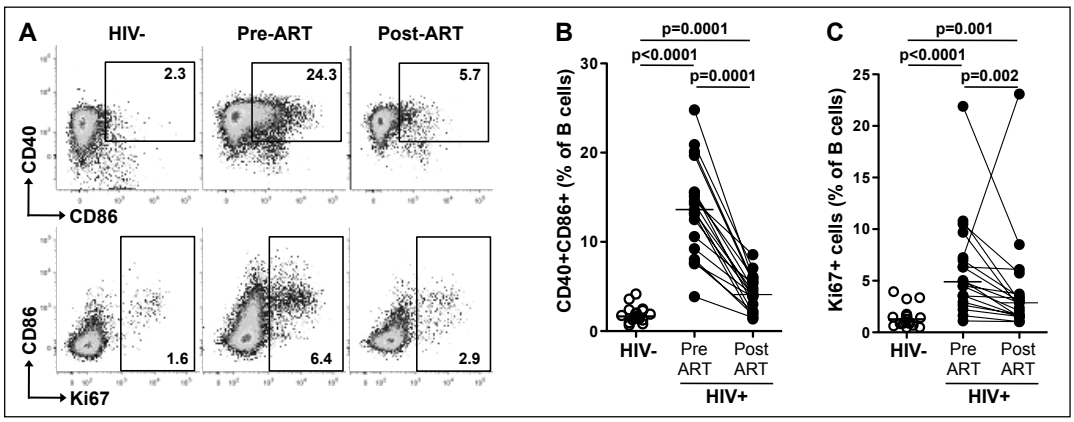


Figure 4. 5. Effect of ART on B cell activation and proliferation. (A) Representative flow plots of CD40, CD86 and Ki67 expression in B cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. Numbers represent the frequencies of activated (CD86+CD40+) and proliferating (Ki67+) B cells. **(B)** Frequencies of CD86+CD40+ B cells in HIV-uninfected (n=19; open circles) and HIV-infected pre- and post-ART (n=19; closed circles) individuals. **(C)** Frequencies of proliferating B cells as measured by Ki67 expression. Horizontal lines represent the median. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively.

Overall, our data show that HIV infection skews the B cell memory differentiation profile and causes abnormal B cell activation and proliferation. Defects in B cell memory phenotype were largely normalised within one year of ART, while B cell activation and proliferative capacities were only partially reduced and longer periods of suppressive therapy may be required for normalisation.

4.4.2 B cell defects during HIV infection do not directly associate with HIV viral load

It has been clearly established that hyperimmune activation of T cells during HIV infection is related to viral replication (Deeks *et al*., 2004) and is partially normalised upon ART (Hunt *et al*., 2003). This was confirmed in our experimental setting, where we had T cell activation data for 18 of the participants in the current study, as well as 14 additional subjects from the same cohort. The proportion of activated CD4+ T cells (measured by the expression of HLA-DR) was positively associated with plasma viral load (p=0.001, r=0.54; **Figure 4.6A**) in HIV-infected subjects pre-ART. While 12 months of ART led to a substantial reduction in the frequency of HLA-DR expression by T cells, T cell activation levels remained significantly higher than HIV-uninfected subjects (**Figure 4.7)**. Thus, in order to determine whether HIV load was also the driving factor for B cell activation, we examined the relationship between B cell phenotype and HIV plasma viral load before ART initiation. As previously stated, switched memory B cells (i.e IgD- B cells) are highly activated during HIV infection, thus we focused our analyses on this particular subset. Surprisingly, unlike for T cells, we found no association between the frequency of activated (CD86+CD40+) switched memory B cells and HIV viral load (p=0.71, r=0.39; **Figure 4.6B**). Moreover, none of the B cell memory subset frequencies were found to be associated with viral load (data not shown). Finally, the extent of CD4+ or CD8+ T cell activation did not correlate with the level of switched B cell activation (p=0.78, r=0.07; **Figure 4.6C** and data not shown). Together, these results show that HIV differentially affects the T cell and B cell compartments, where B cell activation does not mirror T cell activation and is not directly associated with the levels of HIV. This suggests that additional factors besides HIV replication may contribute to B cell activation.

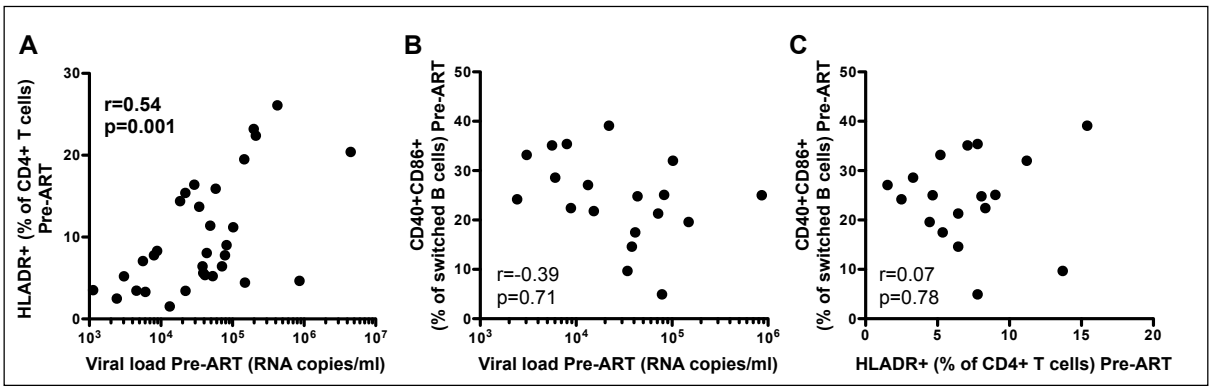


Figure 4. 6. Relationship between CD4+ T cell and B cell activation and plasma viral load pre-ART. Correlation of **(A)** the frequency of HLA-DR expressing CD4+ T cells (n=32) and **(B)** the frequency of CD40+CD86+ switched memory B cells (n=18) and plasma viral load in HIV-infected individuals prior to ART initiation. **(C)** Correlation between B cell activation (CD86+CD40+) and CD4+ T cell activation (HLA-DR+) in 18 HIV-infected participants before ART. Statistical significance was calculated using a non-parametric Spearman Rank test.

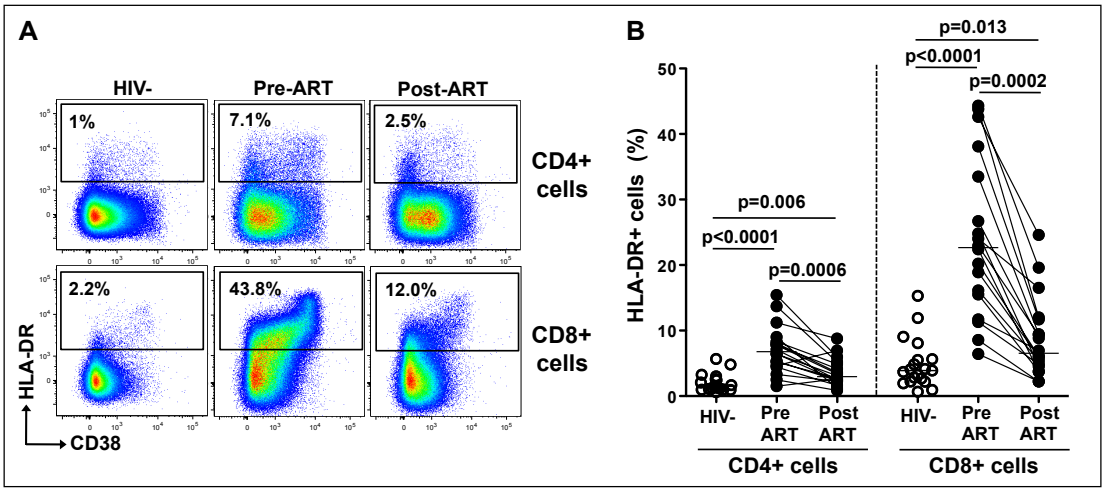


Figure 4. 7. Effect of ART on CD4+ and CD8+ T cell activation levels measured by HLA-DR expression. (A) Representative flow plots of HLA-DR expression on T cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. **(B)** Frequencies of HLA-DR+ CD4+ and CD8+ T cells in 19 HIV-uninfected (open circles) and 18 HIV-infected (closed circles) individuals pre- and post-ART. Horizontal lines represent the median. Statistical significance was calculated using Mann-Whitney U test and Wilcoxon Signed Rank for unmatched and matched samples, respectively.

4.4.3 Additional contributors of B cell defects during HIV infection

The translocation of bacterial products, resulting from HIV-induced damage to the gastrointestinal tract, is an important contributor of systemic immune activation (Brenchley *et al*., 2007). Therefore, we investigated the relationship between the activation and skewed differentiation profile of B cells and the level of plasma sCD14,

a marker of monocyte/macrophage activation, that may be an indicator, in the absence of other infections, of microbial translocation. Prior to ART, plasma sCD14 levels associated negatively with the frequency of naive B cells (p=0.036, r=-0.50; data not shown) and positively with the frequency of immature transitional B cells (p=0.003, r=0.75; **Figure 4.8A**) and the frequency of activated (CD86+CD40+) switched memory B cells (p=0.01, r=0.58; **Figure 4.8B**). Moreover, a positive correlation was also observed between sCD14 levels and the frequency of activated HLA-DR+CD4+ T cells (p=0.045, r=0.49; **Figure 4.8C**). It is worth noting that the levels of plasma sCD14 were not significantly changed after 12 months of ART (**Figure 4.9**), and there were no associations with residual B cell activation post-ART (data not shown).

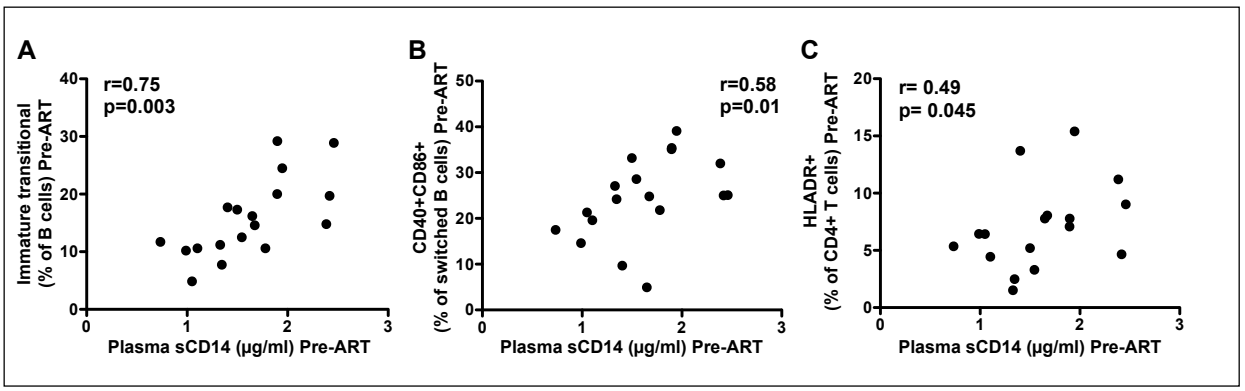


Figure 4. 8. Relationship plasma sCD14 levels and B cell phenotype and CD4+ T cell activation pre-ART. Correlation of **(A)** the frequency of immature transitional B cells, **(B)** the frequency of CD86+CD40+ switched memory B cells and **(C)** and the frequency of HLA-DR+ CD4+ T cells with plasma sCD14 levels in HIV-infected individuals (n=18 and n=17, respectively). Statistical significance was calculated using a non-parametric Spearman Rank test.

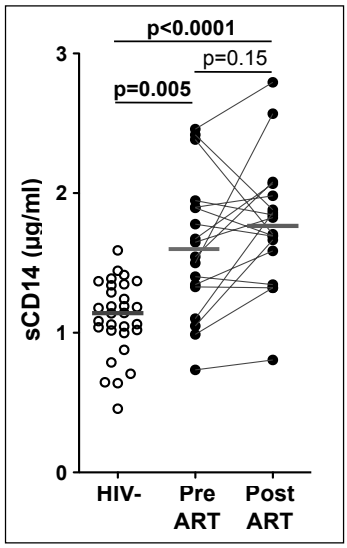


Figure 4. 9. Effect of ART on plasma levels of sCD14. Soluble CD14 was measured in 18 HIV-infected women and 30 HIV-uninfected women from the same community by ELISA (R&D Systems). Horizontal black lines represent the median. Statistical significance was calculated using a non-parametric Wilcoxon matched test (pre- and post-ART) and the Mann-Whitney test for comparisons with HIV-uninfected individuals.

Overall, these data suggest a possible impact of microbial translocation on B cell dysfunction, as suggested by sCD14 levels correlating with HIV-induced B cell skewing and hyperactivation in untreated chronic HIV infection. However, their influence may be relatively minor, since there are consistently elevated levels of sCD14 after ART, when B cell defects are largely reversed. It is clear that a variety of factors may directly or indirectly influence B cell defects during HIV infection.

4.5 Discussion

Despite the fact that HIV does not directly infect B cells, the virus causes several B cell abnormalities that contribute to HIV pathogenesis (Moir & Fauci, 2013). Whilst these B cell defects may be reverted upon antiretroviral therapy (ART), this effect has mostly been studied in cross-sectional Caucasian cohorts. Thus, in this study, we assessed longitudinally the impact of ART on the memory and activation profile of B cells in South African adult women (n=19); and compared these profiles to age-matched HIV-uninfected women from the same community (n=19). Our data show that 1) HIV-induced alterations of B cells did not associate with viral burden but rather correlated with plasma sCD14 concentration and 2) 12 months of ART largely normalised the distribution of B cell memory subsets whilst activation of B cells was only partially reduced.

HIV induces multiple defects in B cells, altering their memory and activation profiles and function (Moir *et al*., 2003; Ho *et al*., 2006; Malaspina *et al*., 2006; Moir & Fauci, 2009). In our experimental setting, we showed that, in HIV-infected African women, B cells were skewed towards a more differentiated memory phenotype (with the accumulation of plasmablasts and the reduction of naive B cells) and were hyperactivated (as measured by CD86 expression) when compared to HIV-uninfected persons. These observations are in accordance with previous studies performed primarily in male Caucasian populations (Malaspina *et al*., 2003; Moir *et al*., 2003, 2008b, 2010; Titanji *et al*., 2005; Ho *et al*., 2006), highlighting that similar abnormalities occur in African women. To date, the drivers of B cell alterations during HIV infection have not been fully elucidated. Even though HIV does not directly target B cells for infection, the interaction between HIV and B cells has been previously described, where HIV glycoprotein gp120 can bind to B cell receptors (such as CD21,

DC-SIGN and variable heavy chain 3 (VH3) immunoglobulin (Ig), resulting in polyclonal B cell activation and Ig production (Moir *et al*., 2000). Furthermore, HIV Nef can interact directly with B cells, resulting in suppression of Ig class switching (Qiao *et al*., 2006; Xu *et al*., 2009). How each of these mechanisms contribute to B cell dysfunction is unknown (Moir & Fauci, 2009).

It has also been proposed that excessive B cell activation may be driven by pro-inflammatory cytokines, such as IFN-α, TNF-α, IL-6 and IL-10, molecules commonly up-regulated during HIV-induced systemic inflammation (Ruffin *et al*., 2012). HIV infection also leads to increased production of B cell-specific growth factors such as BLyS/BAFF from macrophages, dendritic cells and granulocytes (Fontaine *et al*., 2011; Poudrier *et al*., 2015; Gomez *et al*., 2016;), promoting B cell dysregulation right from the early stages of infection. Additionally, HIV pathogenesis has also been linked to extensive damage to the gastrointestinal tract, leading to the release of bacterial products into the circulation (Brenchley *et al*., 2007). These products may induce aberrant B cell activation via TLR signalling (Jiang, 2012), by both direct and indirect means. Stimulation of monocytes by bacterial LPS leads to the release of cytokines that contribute to the inflammatory environment. Additionally, human B cells express a range of TLRs, including high levels of TLR9 (Hornung *et al*., 2002), and abundant bacterial DNA as a result of HIV-induced microbial translocation (Jiang *et al*., 2009) may stimulate B cells directly. In fact, TLR9 stimulation of human B cells promotes their activation, proliferation and the generation of plasmablasts (He *et al*., 2004; Jiang *et al*., 2007). The potential role of microbial products as direct factors fuelling B cell hyperactivation is further inferred by the fact that in other chronic diseases leading to gut damage (such as inflammatory bowel disease), B cells exhibit aberrant activation profiles (Noronha *et al*., 2009).

We found an association pre-ART of B cell activation and differentiation profile with the concentration of plasma sCD14, secreted upon monocyte/macrophage activation by LPS, which may be a surrogate marker of microbial translocation. Interestingly, whilst there was a relative normalisation of B cells post-ART, plasma sCD14 concentration did not decrease overall upon treatment. This phenomenon has been observed in several studies, where sCD14 levels remain elevated even after several years on ART (d'Ettorre *et al*., 2014; Hattab *et al*., 2014; Rudy *et al*., 2015), and

unreversed damage to the gastrointestinal tract has been proposed to account for this. These data confirm that in African women, inflammation persists after ART even while there is a substantial decrease in cellular activation. The absence of any decrease in sCD14 after ART, despite a substantial normalisation of B cells profiles, suggests that sCD14 plays only a partial role in contributing to B cell activation. Alternatively, we could speculate that microbial translocation and viral-induced factors might act synergistically to drive B cell activation and skewing, and ART uncouples this effect by suppressing viral replication even in the face of ongoing inflammation. Whilst we did not find an association of HIV viral load with B cell dysfunction pre-ART, this relationship may not be linear, with a multitude of viral-induced factors influencing B cell activation and differentiation directly and indirectly (Haas *et al*., 2011). Overall, this suggests that a combination of inflammatory mediators such as cytokines, B cell growth factors and microbial products may directly and indirectly stimulate B cell differentiation and proliferation, favouring their functional impairment and exhaustion.

Several studies have shown that ART leads to a near normalisation of peripheral B cell memory subsets (D'Orsogna *et al*., 2007; Moir *et al*., 2008b; Amu *et al*., 2014, 2016) and the extent of restoration was linked to the timing of ART initiation (Pensieroso *et al*., 2009; Moir *et al*., 2010;), the duration of treatment or related to the age of the patients (Amu *et al*., 2014; Van Epps *et al*., 2014). Very limited data are available on the effect of ART on B cells in African cohorts. To our knowledge, only one cross-sectional study has investigated how ART influences the restoration of B cell numbers in an African adult population (Longwe *et al*., 2010). It is of importance to define, in countries disproportionately affected by the HIV pandemic and where access to ART is limited and often started during advanced lymphopenia, whether HIV treatment can restore B cell profiles to similar levels observed in HIV-uninfected individuals (of note, the CD4 count pre-treatment in the participants included in this study was <350 cells/mm^3). Our data showed that while 12 months of ART largely normalised the distribution of memory subsets, B cell activation remained significantly elevated compared to HIV-uninfected individuals. The persistence of these cycling, activated B cells composed mainly of plasmablasts could reflect residual systemic inflammation, despite viral suppression. Indeed, it has been shown that even upon successful ART, inflammation, microbial translocation and germinal center destruction still persist (Brenchley *et al*., 2007; Levesque *et al*., 2009; Klatt *et al*., 2013; Ghislain *et al*., 2015).

These observations are in line with results reported in Caucasian cohorts (Moir *et al*., 2008b, 2010; Fogli *et al*., 2012), showing that the normalisation of B cell subsets can be achieved even when treatment is initiated at a late stage of chronic HIV infection.

The limitations of this study include a cohort exclusively composed of women; further studies could test the generalisability of our findings in men. Additionally, we only analysed B cells from peripheral blood and this may not be representative of B cell profile within the tissues; it is plausible that B cell residing in germinal center are differentially affected during HIV infection and that the extent and/or kinetics of their restoration during treatment may differ. For future studies, use of additional phenotypic markers such as CD10 and CD24 could improve the definition of B cell subsets, in particular immature transitional B cells. Furthermore, we did not examine the relationship of relative normalisation of B cell activation and differentiation with restoration of functional B cell immunity, which may not always return (Wheatley *et al*., 2016). Further studies of humoral immunity after ART, by investigation of both memory B cell responses and long-lived plasma cells to a variety of vaccines and infections, are warranted in larger African cohorts. This is particularly important if functional B cell immunity is not fully restored and revaccination to certain pathogens may be required. In this respect, early ART initiation, compared to those treated during chronic HIV infection, has demonstrated a benefit for restoration of B cell functionality (Moir *et al*., 2010).

In conclusion, our study has shown that the B cell compartment is highly disturbed in chronically HIV-infected African women, an understudied group disproportionately affected by the HIV pandemic, as it is for Caucasian cohorts. Differences in methodology limit our ability to directly compare our results in African women to published studies in western cohorts, but we observed a substantial reduction in B cell activation and a relative normalisation of skewed B cell subsets as observed in Caucasian cohorts, despite the commencement of ART in chronic HIV infection. Identifying the drivers of B cell activation and defective differentiation may provide a better understanding of the mechanisms associated with B cell dysfunction. This can potentially lead to the development of intervention strategies to improve B cell function during HIV infection, for both HIV-specific humoral immunity and responses to vaccines targeting other pathogens.

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Contribution: Conceived and designed the experiments: A.P.S., N.J.G., Q.A.K., S.A.K. C.R and W.A.B. Performed the experiments: R.F.T., T.L.M. and A.P.S. Analysed the data R.F.T, C.R. and W.B. Contributed reagents/materials/analysis tools: N.S., N.J.G., Q.A.K. and S.A.K. Wrote the paper: R.F.T., C.R. and W.A.B. All authors approved the final manuscript.

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CHAPTER 5

Discussion

Untreated HIV infection is associated with a profound loss in CD4+ T cells and a state of generalised immune activation (Okoye & Picker, 2013; Doitsh *et al.,* 2014). Excessive activation of the immune system contributes to HIV pathogenesis and disease progression by causing several abnormalities in T cell and B cells, affecting both their phenotype and function, and hence impairing the generation of effective T cell and B cell responses both to HIV itself and other pathogens (Moir & Fauci, 2009; Saharia & Koup, 2013; Morou *et al*., 2014). The era of widespread ART has led to a significant decrease in morbidity and mortality, as well as reduced opportunistic and other infections in HIV-infected individuals (Guihot *et al*., 2011; Williams *et al*., 2011). However, despite inhibition of viral replication and improvement in CD4+ T cell numbers in the majority of treated patients, excessive immune activation may persist and influence CD4+ T cell reconstitution (Corbeau & Reynes, 2011). In addition, the extent to which functional immunity is fully restored after ART is not completely defined. For example, whether memory T cell and B cell subsets are fully normalised on ART, and the association with sustained immune activation, is poorly characterised. Furthermore, it is not known whether functional T cell immunity to specific co-pathogens (*Mtb* and CMV) are restored upon ART, and what factors are associated with this recovery. The overall focus of this thesis was on the assessment of the influence of short-term (1 year) ART on the phenotype (activation, proliferation and memory differentiation) of T cells and B cells as well as the functional restoration (cytokine production) of T cell responses to co-pathogens in HIV-infected African women. Studying the changes in HIV-induced phenotypic and functional defects after therapy may reveal how these features may influence immunity after treatment, CD4+ reconstitution and continued susceptibility to infections.

The cohort used for these experiments consisted of study participants in the CAPRISA acute infection study from the Durban and Vulindlela areas of Kwazulu-Natal, a province in South Africa where HIV incidence is the highest, particularly in women

(Huerga *et al*., 2016). The HIV incidence is a staggering estimate between 7 to 10 per 100 person-years in women aged between 18 and 35 years (Abdool Karim *et al*., 2012; Nel *et al*., 2012) The CAPRISA acute infection study comprised a large cohort of HIV-infected women recruited early in HIV infection to study the natural history of HIV infection in Southern Africa (van Loggerenberg *et al*., 2008); all women in the cohort were infected with subtype C (Williamson *et al*., 2003). Participants were followed for up to 4 years and roughly half of the women were found to be rapid progressors (CD4 count <350 cells/μl within 2 years of infection; Mlisana *et al*., 2014). This is a well-established cohort and comprehensive studies on these women have been previously conducted to characterise adaptive and innate immunity, host genetics and viral factors (Abrahams *et al*., 2009; Burgers *et al*., 2009; Gray *et al*., 2009; Mlotshwa *et al*., 2010; Chopera *et al*., 2011; Gray *et al*., 2011; Naranbhai *et al*., 2012; Riou *et al*., 2014; Bandawe *et al*., 2015). With regards to T cell immunity, one study showed that the memory phenotype of HIV-specific CD8+ T cells at 6-9 months after infection correlated with viral set point at 12 months (more differentiated HIV-specific CD8+ T responses correlated with a higher viral load set point; Burgers *et al*. 2009). In terms of B cell responses to HIV, broadly neutralising antibodies were identified in certain individuals in the CAPRISA 002 cohort (Gray *et al*., 2011). These and other extensive studies performed in the CAPRISA 002 cohort have underlined important parameters of the immune response to HIV that are associated with disease progression in a cohort of African HIV-infected women. In relation to these past findings, our study further characterised these women as they initiated ART to determine the degree to which functional immune recovery occurred, and what factors influenced this restoration. Limited data are available on the effect of ART on T cells and B cells in African cohorts, which are less well-studied than Caucasian cohorts. Thus, it was of particular importance to assess whether ART could normalise HIV-induced cellular defects, particularly in individuals from countries extremely affected by the HIV pandemic, and where access to ART is often initiated during advanced lymphopenia (of note, the CD4 count pre-ART for the participants in our study was <350 cells/mm^3).

During HIV infection, the process of immune activation induces the activation, proliferation and differentiation of T cells, creating more CD4+ target cells for HIV and fuelling continued HIV replication (Paiardini & Müller-Trutwin, 2013). The attempt to constantly replenish the pool of T cells to balance their loss results in the

exhaustion and dysfunction of the immune system (McCune, 2001). Chapter 2 investigated the effect of HIV on the phenotype of T cells during chronic HIV infection. Increased T cell activation, proliferation and differentiation was observed in HIV-infected individuals compared to uninfected individuals. In addition to T cell defects, HIV causes alterations in the phenotype of B cells, inducing B cell dysfunction (Moir *et al*., 2003; Malaspina *et al*., 2006; Moir & Fauci, 2009). Chapter 4 determined the effect of HIV on B cells, and as for T cells, data indicated higher B cell activation, proliferation and differentiation in the blood of HIV-infected women compared to healthy controls. To date, the mechanisms and drivers of T cell and B cell abnormalities during HIV infection are not fully understood. A positive association was found between plasma viral load and T cell activation but not B cell activation, implying that HIV replication levels have a more direct effect on activating T cells. An indirect mechanism mediating immune activation involves severe damage to the gastrointestinal mucosal surfaces during HIV infection, resulting in microbial translocation, where bacterial components (such as LPS) released into the bloodstream mediate immune activation (Brenchley *et al*., 2007). Human T cells and B cells express no or low levels of TLR4 (Hornung *et al*., 2002) and the fact that we found an association between the levels of both activated T and B cells and the concentration of plasma sCD14 imply an indirect activation of both subsets, likely through monocyte/macrophage activation by LPS, as previously described (Brenchley *et al*., 2007; Marchetti *et al*., 2008; Jiang, 2012). All together, our results indicate that in the absence of treatment, individuals with chronic HIV infection in our cohort displayed substantial perturbations in both T cell and B cell subsets, likely affecting the function of these cells.

HIV infection does not only result in a global depletion in CD4+ T cells, but memory CD4+ T cells specific for the virus and other pathogens (such as Mtb and CMV) are also lost, impairing the generation and preservation of T cell responses (Douek *et al*., 2002; Geldmacher & Koup, 2012). In this study, Chapter 3 investigated whether pre-existing memory CD4+ T cells specific for common co-pathogens, Mtb and CMV, are recovered upon suppression of HIV by treatment, by assessing their frequency, functional capacity and memory profile before and after ART. We found no change in the frequency of Mtb-specific CD4+ T cells and a substantial decrease in the frequency of CMV-specific CD4+ T cells post-ART, consistent with previous reports (Hsu *et al*.,

2013; Jambo *et al*., 2014). In addition, the polyfunctional profile of both co-pathogens did not differ before and after ART. Our data for Mtb are in contrast with Sutherland and colleagues, who reported that short-term ART increases the polyfunctional capacity of Mtb-specific CD4+ T cells (Sutherland *et al.*, 2010). This may be explained by differences in the cohort, as the subjects in their study were characterised by more advanced immunosuppression and higher viral loads compared to participants in our cohort. Most importantly, our data showed for the first time that the degree of replenishment of co-pathogen specific CD4+ T cells is associated with their memory status pre-ART, with greater recovery for cells exhibiting an early differentiated memory profile (such as most Mtb-specific CD4+ T cells). This suggests that the renewal potential of T cells depends on the state in which they are found prior to ART, and the targeted pathogen.

Multiple factors have been associated with the extent of CD4+ T cell recovery in treated individuals, including the absolute CD4 count at ART initiation and T cell activation levels at the time of treatment (Kaufmann *et al.*, 2005; Goicoechea *et al*., 2006; Le *et al*., 2013). Our sample size was too small to identify whether these factors influenced the reconstitution of T cells in treated patients in this cohort. Nonetheless, in Chapter 2, we showed that short-term ART substantially but only partially normalised T cell activation levels and differentiation profiles compared to uninfected controls. The substantial decrease in activation was not just limited to T cells, as a significant decrease in B cell activation and a relative normalisation of skewed B cell subsets were observed in the same ART-treated individuals (Chapter 4). Together, these data indicate that 1 year ART partially normalised both compartments (T cells and B cells). However, the fact that ART was initiated during the chronic stage of HIV infection may explain in part why complete normalisation did not occur in these individuals. In line with this, several studies have reported that the degree of restoration of T cell and B cell phenotypes is dependent on the timing of ART initiation, where a more complete normalisation of T cell and B cell subsets to levels found in healthy individuals occurs when ART is initiated during primary HIV infection (Kaufmann *et al*., 2000; Moir *et al*., 2010; Allers *et al*., 2014). In addition, HIV-infected individuals who initiated ART early (<6 months after infection) exhibited reduced T cell activation compared to those who started ART late (≥2 years after infection) (Jain *et al*., 2013). Together, these reports imply that alterations of the

immune system are less pronounced in early stages of HIV infection, hence leading to a better immune recovery. However, it is important to note that despite early administration of ART, levels of T cell activation are still not fully normalised (Kaufmann *et al*., 2000; Jain *et al*., 2013).

High levels of immune activation on successful ART have been previously reported in HIV-individuals and are associated with cardiovascular disease and mortality (Küller *et al.*, 2008; Hunt *et al*., 2011a; Kaplan *et al*., 2011; Sandler *et al*., 2011). To date, the mechanisms potentially contributing to this sustained immune activation despite effective therapy have not been fully elucidated. Residual viral replication may be responsible for prolonged immune activation during ART (Corbeau & Reynes, 2011). Viral reservoirs established early in HIV infection may persist during antiretroviral treatment either because the drugs are unable to reach these sites or due to the maintenance of long-lived latently infected cells that allow replication during natural homeostatic processes of cell turnover (Chun *et al*., 1997; Finzi *et al*., 1997; Siliciano *et al*., 2003; Chomont *et al*., 2009). In addition, the breach in mucosal surfaces in the gastrointestinal tract (gut damage) as a result of HIV infection may be irreversible in many patients even with early initiation of ART, suggesting ongoing bacterial translocation driving residual immune activation (Brenchley *et al*., 2007; Jenabian *et al*., 2015). Together, these factors promote HIV persistence and impede HIV cure. The size of the latent viral reservoir has been associated with residual immune activation and HIV replication during ART, and HIV individuals who initiated ART early (<6 months after infection) were found to have lower T cell activation levels and a smaller HIV reservoir (Jain *et al*., 2013; Cockerham *et al*., 2014; Ruggiero *et al*., 2015). Interestingly, some "shock and kill" strategies to purge HIV reservoirs are testing TLR agonists (the very molecules that contribute to immune activation during infection) to reactivate virus from latently infected cells. However, this method may also contribute to systemic immune activation via TLR signaling further sustaining viral replication, and would have to be administered in conjunction with ART to prevent the seeding of new reservoirs (Battistini & Sgarbanti, 2014). Alternative therapeutic approaches are required for restoring gut damage and controlling immune activation, thereby possibly decreasing the size of the HIV reservoir (Sandler & Douek, 2012). The most recent WHO guidelines recommend ART be initiated regardless of CD4 counts (WHO, 2015), as has now been

implemented in South Africa. Earlier treatment may improve the likelihood of full immune recovery over time, before irreversible damage to the immune system is established.

Limitations and future studies

There were a number of limitations to these studies to be highlighted, that can be addressed in follow-up studies. Our cohort consisted exclusively of women and previously Meier and co-workers showed that women have elevated levels of immune activation and faster disease progression rate compared to men with the same viral load (Meier *et al*., 2009). This may imply a differential response to ART with respect to the resolution of immune activation between the sexes. Additional studies are therefore needed to make our findings generalisable to men. Our study also only focused on the analysis of T and B cell subsets from the peripheral blood, and this may not be representative of their profile within tissues. It has been previously reported that during the course of HIV infection, the majority of CD4+ T cells are lost in secondary lymphoid tissues such as the gastrointestinal tract (Brenchley *et al*., 2004), and the extent to which these are repopulated and deactivated is not fully known. In addition, structural changes occur in primary lymphoid tissues such as lymph nodes, including collagen deposition and formation of hyperplastic germinal centers, where there is increased B cell expansion and influx of Tfh cells that provide critical B cell help (Hong *et al*., 2016). However, during HIV infection, both abnormal T cell activation and disturbances in memory B cells are associated with altered B cell responses (Cagigi *et al*., 2010; Cubas *et al*., 2013). Therefore, it may be necessary to evaluate the restoration of T and B cell subsets in lymphoid compartments and evaluate B cell memory and humoral immunity after ART. This may have implications for revaccination to certain pathogens if functional B cell immunity is still absent after therapy. Lastly, our study focused on short-term (1 year) treatment of HIV and normalisation of T and B cell phenotypes and co-pathogen-specific T cell immunity. Thus, studying the effect of longer-term therapy on phenotypic and functional recovery of T and B cells may be more conclusive as to whether complete immune recovery can occur, particularly in persons that were treated relatively late in HIV infection.

In summary, this study has shown abnormalities in T and B cells in a cohort of chronically HIV-infected African women, as observed previously for Caucasian cohorts. ART was administered in chronic HIV infection, which still led to effective and rapid viral load suppression and an increase in CD4+ T cell numbers. However, we observed only a partial recovery of HIV-induced T cell and B cell perturbations (activation and differentiation profiles) following short-term ART relative to healthy individuals. In addition, these studies showed that the renewal potential of pre-existing pathogen specific CD4+ T cells is related to their memory profile before ART, identifying an important factor that may limit restoration of functional immunity in HIV-treated individuals. Overall, our data has added to the body of work previously done in the CAPRISA 002 cohort by showing that in a cohort of South African women 1 year after ART, the participants in this cohort were able to partially revert HIV-induced cellular defects at least in peripheral blood. Understanding the cellular factors that contribute to successful immune recovery upon effective HIV treatment and identifying the drivers of continued immune activation and defective differentiation is of pivotal importance. This may give insights into adjunct therapies to administer in combination with ART to fully restore immune function in HIV-infected individuals.

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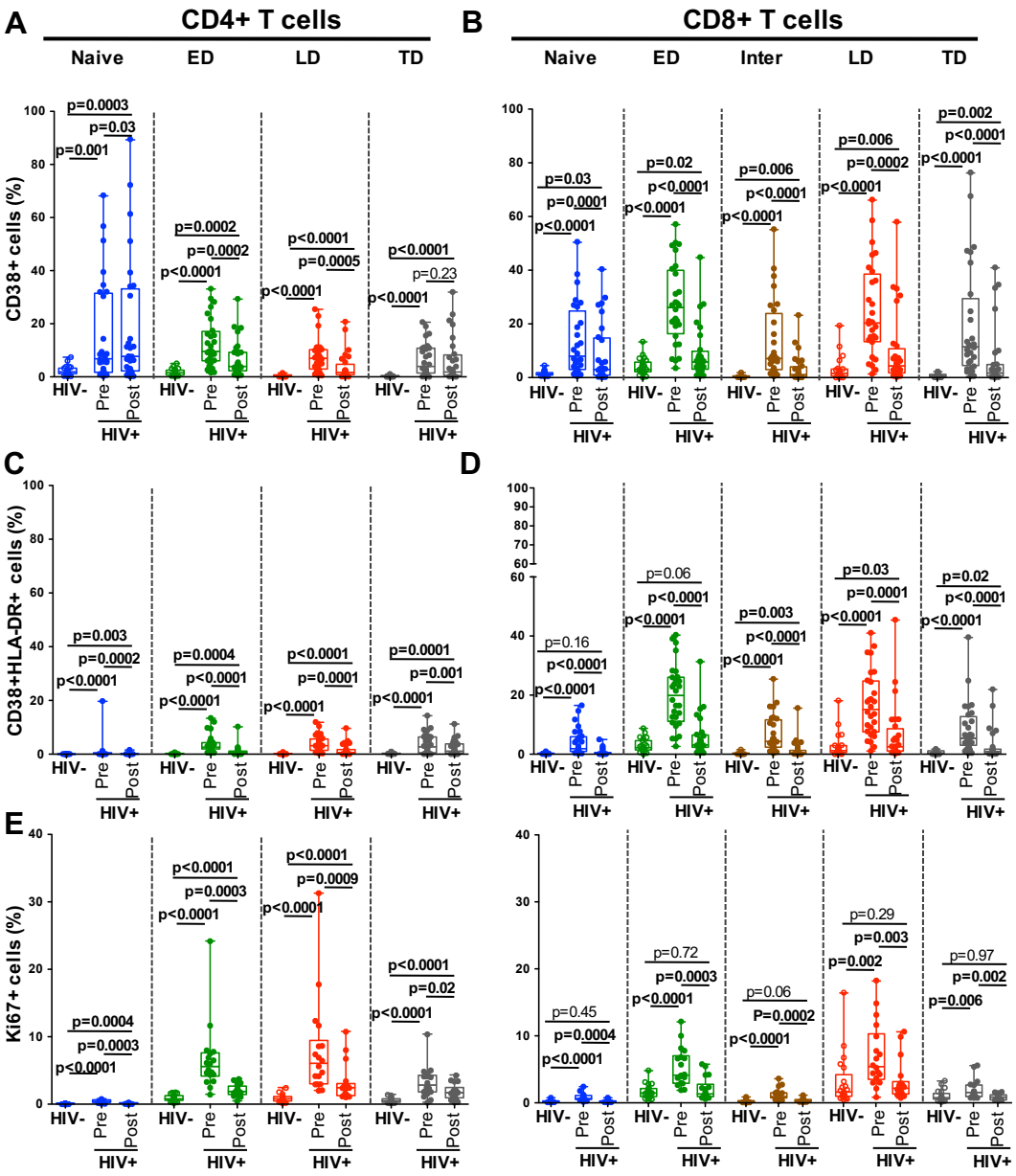
Appendix

Table A.1. Clinical characteristics of study participants

Sex: 100% female
Median age for HIV-uninfected individuals: 31(IQR: 27-34)

	Pre-ART					Post-ART					
Participant Identification	Years of infection prior ART	Viral load (RNA copies/ml)	CD4 count (Cells/mm^3)	CD8 count (Cells/mm^3)	Months before ART initiation	Viral load (RNA copies/ml)	CD4 count (Cells/mm^3)	CD8 count (Cells/mm^3)	Months after ART initiation	Drug regimens	Age at sampling (years)
CAP177	5.6	82,556	201	1,126	1.9	36	414	1,131	12.4	TDF/3TC/EFV	43
CAP200	3.8	423,000	135	552	2.5	<40	496	727	13.5	ddI-EC/3TC/EFV TDF/3TC/EFV	37
CAP206	5.2	198,277	289	1,921	0.2	<40	512	1,028	11	TDF/3TC/EFV	42
CAP221	3.6	48,800	233	700	0.0	64	558	823	12.4	ddI-EC/3TC/EFV	30
CAP222	6.1	3,049	319	522	3.2	<20	619	498	11.3	TDF/3TC/EFV	27
CAP228	7.3	102,110	260	1,890	0.1	<20	837	1,093	12.2	TDF/3TC/EFV	46
CAP244	7.3	21,955	318	1,004	0.0	273	647	1229	12.3	TDF/3TC/EFV	31
CAP248	6.6	29,258	363	1,403	2.8	121	305	1,110	6.7	TDF/3TC/EFV	29
CAP255	3.7	8,830	226	1,237	3.8	<20	484	916	18.1	d4T/3TC/EFV AZT/3TC/EFV	32
CAP257	4.8	52,800	170	892	2.0	<20	494	1,042	12.0	TDF/3TC/NVP	34
CAP261	8.2	34,325	430	2,073	1,5	69	696	1,909	10.9	TDF/FTC/EFV	25
CAP262	5.8	1,130	338	200	1.7	<20	329	105	12.1	TDF/3TC/EFV	29
CAP264	3.5	39,000	139	490	1.9	<400	222	498	12.3	d4T/3TC/EFV	27
CAP267	5.4	41,338	269	1,008	1.3	<20	582	919	16.5	d4T/3TC/NVP	32
CAP268	4.2	4,537	186	1,027	4.4	<20	426	671	12.0	TDF/3TC/EFV	26
CAP276	1.4	22,000	232	623	2.3	<400	627	606	20.2	ddI-EC/3TC/EFV	28
CAP277	4.9	7,997	322	1,470	1.0	29	535	805	10.0	TDF/3TC/EFV	42
CAP279	2.7	58,100	193	478	0.8	<20	400	271	12.0	ddI-EC/3TC/EFV	34
CAP280	5.5	78,573	236	990	3,9	<20	520	875	12,2	TDF/3TC/EFV	33
CAP299	4.8	6,047	465	425	3,8	<20	750	327	11,7	TDF/FTC/EFV	31
CAP316	4.1	5,612	436	1,325	2.4	<20	708	1,058	11.8	AZT/3TC/Lpvr/r	30
CAP319	1.9	71,454	262	1,338	2,1	<40	514	697	13,1	TDF/3TC/EFV	24
CAP326	2.7	2,423	332	1,210	2,8	<20	668	1,352	16,9	TDF/3TC/EFV	26
CAP349	4.1	148,898	291	1,369	0.0	36	689	1,723	10,9	TDF/FTC/EFV	33
CAP351	2.8	38,026	314	940	1,3	<20	450	732	12,9	TDF/3TC/EFV	36
CAP353	0.8	13,300	422	820	2,2	<40	646	465	12,8	AZT/3TC/NVP	24
CAP381	2.6	860,702	236	1,646	0.0	<20	754	1,587	11,4	TDF/3TC/EFV	27
CAP384	2.0	43,598	289	1,799	0.0	<20	472	1,155	11,2	TDF/FTC/EFV	27
Median	4.2	38,513	279	1,018	1.9	<20	528	896	12.2		31
IQR	2.7-5.6	8,205-76,793	228-330	642-1,395	0.4-2.7	<20-40	456-663	622-1126	11.3-12.9		27-34



Appendix Figure 1. Activation and proliferation profiles of T cell subsets before and after ART. Frequencies of subset distribution **(A)** CD38+CD4+, **(B)** CD38+CD8+, **(C)** CD38+HLA-DR+CD4+, **(D)** CD38+HLA-DR+CD8+, **(E)** Ki67+CD4+**, (F)** Ki67+CD8+ T cells in HIV-uninfected (n=23; open circles) and HIV-infected (n=28; Closed circles). Blueberry, clover, mocha, maraschino and iron represent the Naive, ED, Inter, LD and TD subsets. Data are shown as box and whisker (interquartile range) plots. Statistical significance was calculated using a Mann-Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively.

Publications

Restoration of CD4$^+$ Responses to Copathogens in HIV-Infected Individuals on Antiretroviral Therapy Is Dependent on T Cell Memory Phenotype

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Antiretroviral therapy (ART) induces rapid suppression of viral replication and a progressive replenishment of CD4$^+$ T cells in HIV-infected individuals. However, the effect of ART on restoring pre-existing memory CD4$^+$ T cells specific for common copathogens is still unclear. To better understand the dynamics of Ag-specific CD4$^+$ T cells during ART, we assessed the frequency, functional capacity, and memory profile of CD4$^+$ T cells specific for *Mycobacterium tuberculosis* and CMV in 15 HIV-infected individuals before and 1 y after ART initiation. After ART initiation, the frequency of *M. tuberculosis*–specific CD4$^+$ T cells showed little change, whereas CMV-specific CD4$^+$ T cells were significantly lower ($p = 0.003$). There was no difference in the polyfunctional or memory profile of Ag-specific CD4$^+$ T cells before and after ART. The replenishment of Ag-specific CD4$^+$ T cells correlated with the memory differentiation profile of these cells prior to ART. Pathogen-specific CD4$^+$ T cells exhibiting a late differentiated profile (CD45RO$^+$CD27$^-$) had a lower capacity to replenish ($p = 0.019$; $r = -0.5$) compared with cells with an early differentiated profile (CD45RO$^+$CD27$^+$; $p = 0.04$; $r = 0.45$). In conclusion, restoration of copathogen-specific memory CD4$^+$ T cells during treated HIV infection is related to their memory phenotype, in which early differentiated cells (such as most *M. tuberculosis*–specific cells) have a higher replenishment capacity compared with late differentiated cells (such as most CMV-specific cells). These data identify an important, hitherto unrecognized, factor that may limit restoration of copathogen immunity in HIV-infected individuals on ART. ***The Journal of Immunology*****, 2015, 195: 2273–2281.**

The hallmarks of untreated HIV infection are a progressive loss of CD4$^+$ T cells, sustained cellular activation, and chronic inflammation (1–3). In addition to the numerical depletion of CD4$^+$ T cells, HIV can also alter the functional capacity of these cells, impairing their proliferative potential, altering their cytokine secretion profiles, and changing their phenotypic characteristics in response to HIV Ags as well as various copathogens (4–7). Both of these quantitative and qualitative alterations can lead to increased susceptibility to opportunistic infections, including tuberculosis (TB), Candidiasis, and human papilloma virus infection (8). Indeed, HIV is the best-recognized risk factor for TB disease even before profound CD4$^+$ T cell deficiency (9, 10). The introduction of antiretroviral therapy (ART) has drastically decreased morbidity and mortality in HIV-infected individuals (11), inducing a rapid reduction of plasma viral load and a progressive repletion of CD4$^+$ T cells (12). Although the clinical benefit of ART is undeniable, the extent to which ART can fully normalize functional immunity remains unclear (13). HIV-infected individuals on ART exhibit a differential degree of recovery of copathogen-specific CD4$^+$ T cell responses, depending on the pathogen they target (14–20). For example, it has been shown that the restoration of CMV-specific CD4$^+$ T cells occurs early after ART (19), but appears to be short-lived (15). Conversely, *Candida*-specific CD4 responses recover slowly (16). Contrasting data exist on the degree of recovery of *Mycobacterium tuberculosis*–specific CD4$^+$ T cell responses upon ART. Jambo et al. (20) showed in a cross-sectional study that the frequency and polyfunctional profile of *M. tuberculosis*–specific CD4$^+$ T cell responses were similar in ART-naive or treated individuals, whereas Sutherland et al. (18) reported that ART increases the polyfunctional capacity of these cells. Other studies described only a partial reconstitution of *M. tuberculosis*–specific CD4$^+$ T cell responses after ART (14, 17).

It is of particular importance to define the factors that associate with successful pathogen-specific CD4$^+$ T cell recovery upon ART, as limited "normalization" of functional CD4$^+$ T cell responses could account for sustained incidence of opportunistic infections.

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Abbreviations used in this article: ART, antiretroviral therapy; ED, early differentiated; IQR, interquartile range; LD, late differentiated; LDL, lower detection limit; SEB, staphylococcal enterotoxin B; TB, tuberculosis.

Several parameters influence the degree and dynamics of recovery of the overall $CD4^+$ T cell compartment in response to ART, such as age, CD4 count at the time of treatment initiation, and timing of ART initiation after HIV infection (21–23). However, it is still unclear why $CD4^+$ T cells of different pathogen specificities have different profiles of restoration, and the mechanisms mediating this variable recovery of memory $CD4^+$ T cells to copathogens are still incompletely understood. Thus, to better understand the effect of successful ART on the dynamics of recovery of copathogen-specific $CD4^+$ T cells, we compared the magnitude, functional capacity, and memory differentiation profiles of *M. tuberculosis*– and CMV-specific $CD4^+$ T cells before and 1 y after ART initiation in a cohort of HIV-infected individuals and HIV-uninfected controls.

Materials and Methods

Study participants

Blood samples were collected from 15 women participating in the CAPRISA 002 study, a cohort study following HIV-infected women from HIV seroconversion until 5 y on treatment. The cohort is situated in KwaZulu-Natal, South Africa, and has been previously described (24, 25). Participants were selected based on sample availability. Blood samples from nine HIV-uninfected participants were provided from the CAPRISA 004 vaginal microbicide (1% tenofovir) gel trial (26). An additional 14 HIV-uninfected participants from CAPRISA 004 were studied for immune activation. HIV-uninfected participants were from the same community as the HIV-infected individuals and age-matched; they were either in the preintervention phase or the placebo arm of the trial. For HIV-infected individuals, the time of infection was estimated either as the same date as a prospective RNA-positive/Ab-negative measurement or taken as the midpoint between the last Ab negative test and first Ab-positive ELISA test. Participants in the cohort were offered ART according to South African national HIV treatment guidelines (at a CD4 count of <200 cells/mm^3 prior to October 2012; <350 cells/mm^3 until the present). Eight of the 15 participants were taking standard first-line therapy (TDF/3TC/EFV), 3 were on ddI-EC/3TC/EFV, and 1 each on d4T/3TC/EFV, TDF/3TC/NVP, d4T/3TC/NVP, and AZT/3TC/LPV/r. Two participants switched drug regimens while on study, namely CAP200 (EFV/3TC/ddI-EC to EFV/3TC/TDF at month 11) and CAP255 (EFV/3TC/d4T to EFV/3TC/AZT at month 10). No participants had active TB during the study period or exhibited any immune reconstitution disease upon HIV treatment. Ethical approval for the study was obtained from the University of KwaZulu-Natal and University of Cape Town Research Ethics Committees. All participants provided written informed consent to participate in the study.

Determination of plasma viral load and CD4 counts

Plasma HIV viral loads and CD4 counts were quantified at each study visit. Over the course of the study, the viral load PCR assay switched from Roche AMPLICOR HIV-1 monitor test version 1.5 (lower detection limit [LDL] of 400 RNA copies/ml), to Roche TaqMan version 1.0 on June 1, 2010 (LDL 40 RNA copies/ml), and then to Roche TaqMan version 2.0 on January 9, 2012 (LDL 20 RNA copies/ml). Absolute blood CD4 and CD8 T cell counts were measured using the FACSCalibur TruCOUNT method (BD Biosciences) and expressed as cells/mm^3. Plasma samples matching the visits where PBMC were studied were tested for Abs to CMV and CMV DNA. Seropositivity was determined using the Cobas CMV IgG Assay (Roche), and CMV viral load was detected and quantified using a CMV R-gene PCR kit (Argene), with a detection limit of 30 copies/ml.

Cell preparation

PBMC were isolated by standard Ficoll–Hypaque density gradient centrifugation (Amersham Pharmacia) and cryopreserved in 90% heat-inactivated FCS (Invitrogen) plus 10% DMSO and stored in liquid nitrogen until needed. Cryopreserved PBMC were thawed and rested in R10 (RPMI 1640 containing 10% heat-inactivated FCS [Sigma-Aldrich] and 50 U/ml of penicillin-streptomycin) at 37°C and 5% CO_2 for 8 h prior to Ag stimulation.

Ags and cell stimulation

Following resting, PBMC were stimulated using *M. tuberculosis* Purified Protein Derivative (10 μg/ml; Statens Serum Institute), a pool of CMV peptides consisting of 138 peptides (15-mers overlapping by 11 aa) covering the entire HCMV pp65 protein (2 μg/ml; National Institutes of Health AIDS Reagent Program) and staphylococcal enterotoxin B (SEB; 2.5 μg/ml; Sigma-Aldrich), used as a positive control. Of note, we found no major differences in the memory profile, cytokine secretion potential, and polyfunctional capacity between peptide and protein-stimulated PBMC (R. Bunjun, C. Riou, A.P. Soares, N. Thawer, T.L. Muller, A. Kiravu, Z. Ginbot, W. Hanekom, G. Walzl, R.J. Wilkinson, and W.A. Burgers, manuscript in preparation). Stimulations were performed in the presence of costimulatory Abs anti-CD28 and anti-CD49d (both at 1 μg/ml; BD Biosciences) at 37°C for 16 h. Brefeldin A (Sigma-Aldrich) was added after 1 h at a concentration of 10 μg/ml. Surface and intracellular cytokine staining was performed at the end of the incubation period. Sufficient cells were available for assessing CMV and SEB responses in only 12 out of 15 HIV-infected participants.

Surface phenotypic and intracellular cytokine staining and flow cytometry

The following Abs were used for surface and intracellular staining: CD3–allophycocyanin-H7 (SK7; BD Biosciences), CD4–PE-Cy5.5 (S3.5; Invitrogen), CD8-Qdot 705 (3B5; Invitrogen), CD27–PE-Cy5 (1A4CD27; R&D Systems), CD45RO-ECD (UCHL1; R&D Systems), IFN-γ–Alexa Fluor 700 (B27; BD Biosciences), IL-2–APC (MQ1-17H12; BD Biosciences), TNF-α–PE-Cy7 (MAb11; eBioscience), CD14–Pacific Blue (Tük4; Invitrogen), CD19–Pacific Blue (SJ25-CI; Invitrogen), and a violet amine viability dye ("Vivid"; Molecular Probes). All Abs were titrated to obtain optimal concentrations prior to use. After stimulation, PBMC were first stained with Vivid for 20 min at room temperature. Cells were then surface stained with CD4, CD8, CD45RO, CD27, CD14, and CD19 Abs. Cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and stained intracellularly with CD3, IL-2, TNF-α, and IFN-γ. Cells were then resuspended in 1× CellFix (BD Biosciences). Samples were acquired on a BD Fortessa using FACSDiva software and analyzed using FlowJo (version 9.8.2; Tree Star). The gating strategy is presented in Supplemental Fig. 1. A positive cytokine response was defined as at least twice the background (in the presence of costimulatory Abs and no Ag). For phenotyping the memory profile of cells producing cytokines, a cutoff of 40 events was used. Polyfunctionality of Ag-specific cells was analyzed using Pestle (version 1.7) and Spice (version 5.35) software (27). Immune activation was measured by surface staining of unstimulated cells using the CD3, CD4, CD8, CD14, and CD19 Abs listed above, as well as CD38-APC (HIT2) and HLA-DR–allophycocyanin-Cy7 (L243) (both BD Biosciences), along with the inclusion of Vivid.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5.0; San Diego, CA). Nonparametric statistical tests were used for all comparisons. The Mann–Whitney *U* test and Wilcoxon signed-rank test were used for unmatched and paired samples, respectively, and the Kruskal–Wallis ANOVA using Dunn's test for multiple comparisons. Correlations were performed using the Spearman rank test. A p value <0.05 was considered statistically significant.

Results

Effect of ART on the restoration of copathogen-specific $CD4^+$ T cells

In order to better understand the replenishment dynamics of $CD4^+$ T cells specific for copathogens in HIV-infected individuals on treatment, we studied 15 HIV-infected individuals before and after ART initiation. The cohort consisted of women infected with HIV-1 for a median of 4.9 y (interquartile range [IQR]: 3.8–5.8 y) at the time of treatment initiation (Table I). Pre-ART samples were obtained at a median of 1.7 mo (IQR: 0.8–2.5) prior to starting ART. The median plasma viral load was 21,955 HIV RNA copies/ml (IQR: 4,743–52,800), and the median CD4 count was 289 cells/mm^3 (IQR: 193–322). After a median of 12 mo of treatment (IQR: 11–12.3 mo), all individuals exhibited some degree of viral suppression ($p < 0.0001$, Supplemental Fig. 2A), which was below the limit of detection of the assay in 12 out of 15 individuals and <400 RNA copies/ml in the remainder. An increase in absolute CD4 count occurred in 13 out of 15 individuals over the period measured ($p < 0.0001$; Supplemental Fig. 2B). The median fold change in absolute CD4 count was 1.9 (IQR: 1.6–2.3), and when

adjusted for length of treatment, the replenishment rate of $CD4^+$ T cells was 20 cells/mm^3/month (IQR: 14–26, data not shown); this was accompanied by a concomitant doubling of the CD4/CD8 ratio (0.27 versus 0.66; $p = 0.0007$; Supplemental Fig. 2C). As previously reported (21–23), the degree of CD4 reconstitution was inversely related to absolute CD4 count at ART initiation ($p < 0.0001$; $r = -0.85$) and the duration of HIV infection ($p = 0.016$; $r = -0.61$, data not shown). We also measured immune activation before and after ART using the markers CD38 and HLA-DR. There was a significant reduction in $CD4^+$ and $CD8^+$ T cells coexpressing $CD38^+$ and $HLA\text{-}DR^+$ ($p = 0.0002$ and $p = 0.0001$; Supplemental Fig. 2D), as well as $HLA\text{-}DR^+$ alone ($p = 0.0001$ for both subsets). Residual levels of immune activation after 1 y of ART remained significantly greater than healthy, HIV-uninfected individuals for both markers (Supplemental Fig. 2D). We found no association between the degree of CD4 reconstitution and $CD4^+$ T cell activation before or after ART (data not shown).

To determine whether the dynamics of copathogen-specific $CD4^+$ T cells mirror the reconstitution of the total $CD4^+$ T cell population, we measured the magnitude of *M. tuberculosis*– and CMV-specific $CD4^+$ T cell responses, as well as SEB-reactive $CD4^+$ cells, before and after ART initiation. Fig. 1A shows representative flow cytometry plots of cytokine production (IFN-γ, IL-2, and TNF-α) by $CD4^+$ T cells in response to *M. tuberculosis*, CMV, and SEB stimulation in one HIV-infected patient (CAP267). At baseline (before ART initiation), 11 out of 15 individuals tested had a detectable *M. tuberculosis* response, with a median frequency of 0.08% of total $CD4^+$ T cells (range, 0–0.9%; Fig. 1B). CMV responses were detected in 10 out of 12 individuals tested, and the median frequency was 0.35% (range: 0–4.1%). SEB responses were detectable in 12 out of 12 participants with a median frequency of 16% (range: 8.6–32.5%). The frequency of *M. tuberculosis*–specific and SEB-responding $CD4^+$ T cells were comparable pre- and post-ART, whereas the frequency of CMV-specific $CD4^+$ T cells was significantly lower after treatment (median 0.35 versus 0.14%, $p = 0.003$; Fig. 1B). To take into account variation in absolute CD4 counts across the cohort, the absolute number of Ag-specific $CD4^+$ T cells was calculated. The number of *M. tuberculosis*–specific and SEB-responding $CD4^+$ T cells increased significantly after ART ($p = 0.033$ and $p = 0.016$, respectively), whereas the number of CMV-specific $CD4^+$ T cells remained unchanged upon treatment (Fig. 1C), consistent with the decreased frequency in the context of increases in absolute CD4 numbers. Overall, these data reveal that the dynamics of reconstitution of Ag-specific $CD4^+$ T cells vary according to their pathogen specificity.

Relationship between the dynamics of restoration of total and copathogen-specific $CD4^+$ T cells

To further investigate the restoration dynamics of copathogen-specific $CD4^+$ T cells, we examined the relationship between the fold change in the absolute number of Ag-specific $CD4^+$ T cells pre- and post-ART (in individuals with detectable Ag responses at both time points, $n = 11$ for *M. tuberculosis*, $n = 10$ for CMV, and $n = 12$ for SEB), and the absolute total CD4 count variation. Fig. 2A shows that although the magnitude of cell replenishment of *M. tuberculosis*–specific and SEB-responding $CD4^+$ T cells was comparable to that of total $CD4^+$ T cells (an ∼2-fold increase over the 12 mo of ART), the extent of restoration of CMV-specific $CD4^+$ T cells was significantly lower ($p = 0.018$, $p = 0.013$, and $p = 0.0008$, to *M. tuberculosis*, SEB, and total $CD4^+$ cells, respectively). Consistent with these observations, the degree of restoration of *M. tuberculosis*– and SEB-responding $CD4^+$ T cells corresponded closely with the overall CD4 recov-

Table I. Characteristics of study participants

Participant Identification	Pre-ART Duration of Infection (y)	Pre-ART				Post-ART			
		Viral Load (RNA Copies/ml)	CD4 Count (Cells/mm^3)	CD4/CD8 Ratio	Months before ART Initiation	Viral Load (RNA Copies/ml)	CD4 Count (Cells/mm^3)	CD4/CD8 Ratio	Months after ART Initiation
CAP200	3.8	423,000	135	0.24	2.5	<20	496	0.68	13.5
CAP206	5.2	198,277	289	0.15	0.2	<40	512	0.50	11.0
CAP222	6.1	3,049	319	0.61	3.2	<20	619	1.24	11.3
CAP237	5.2	4,743	308	0.57	0.0	<20	528	1.05	12.0
CAP244	7.3	21,955	318	0.32	0.0	273	647	0.53	12.3
CAP248	6.6	29,258	363	0.26	2.8	121	305	0.27	6.7
CAP255	3.7	8,830	226	0.18	3.8	<20	484	0.53	18.1
CAP257	4.8	52,800	170	0.19	2.0	<20	494	0.47	12.0
CAP262	5.8	1,130	338	1.69	1.7	<20	329	3.13	12.1
CAP267	5.4	41,338	269	0.27	1.3	<20	347	0.35	8.7
CAP268	4.2	4,537	186	0.18	0.9	<20	426	0.63	12.0
CAP276	1.4	22,000	232	0.37	2.3	<400	627	1.03	20.2
CAP277	4.9	7,997	322	0.22	1.0	29	535	0.66	10.0
CAP279	2.7	58,100	193	0.40	0.8	<20	400	1.48	12.0
CAP316	4.1	5,612	436	0.33	2.4	<20	708	0.67	11.8
Median	4.9	21,955	289	0.27	1.7	<20	496	0.66	12
IQR	3.8–5.8	4,743–52,800	193–322	0.95–0.40	0.8–2.5	<20–40	400–619	0.5–1.05	11–12.3

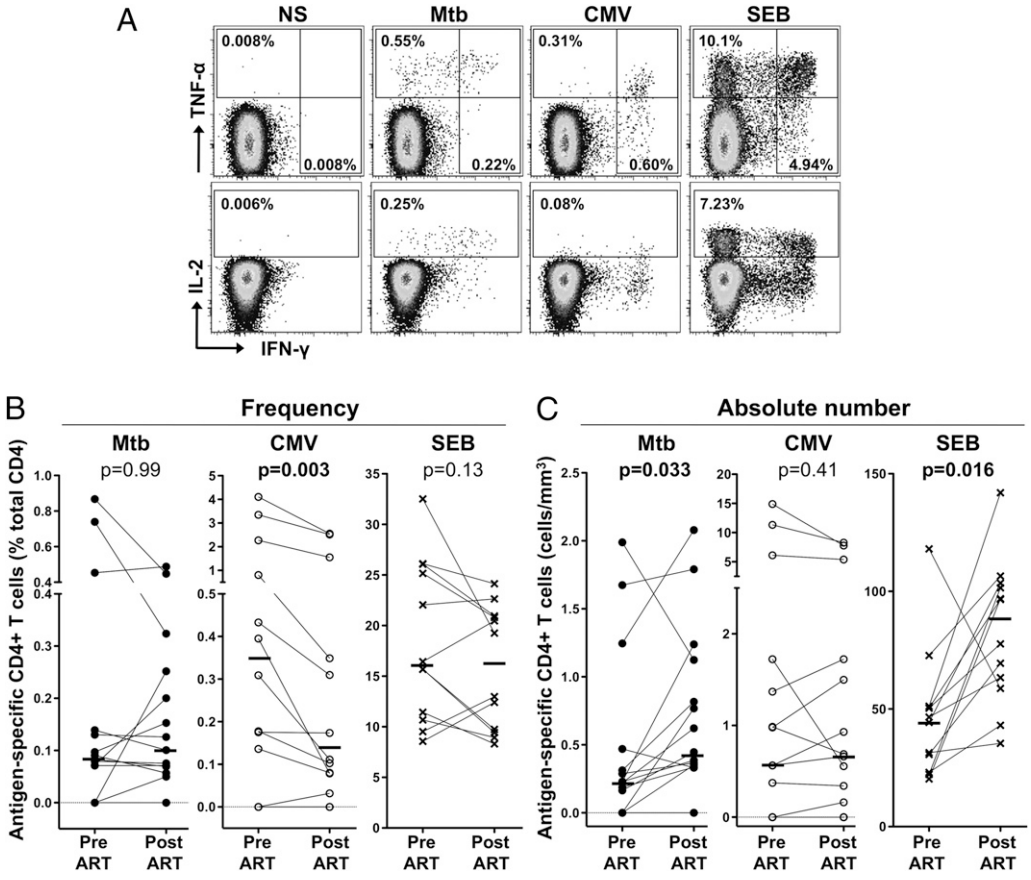

FIGURE 1. Effect of ART on *M. tuberculosis* (Mtb)-, CMV-, and SEB-specific $CD4^+$ T cell responses. (**A**) Representative flow cytometry plots of the expression of TNF-α, IFN-γ, and IL-2 from $CD4^+$ T cells after stimulation with *M. tuberculosis* (Mtb) and CMV Ags and SEB, in one study participant. NS corresponds to unstimulated PBMC. The frequency of cytokine-producing cells expressed as a percentage of the total $CD4^+$ T cell population are indicated. Frequency (**B**) and absolute number (**C**) of *M. tuberculosis*–, CMV-, and SEB-specific $CD4^+$ T cell responses before and after ART. Horizontal bars represent the median. Statistical comparisons were performed using a Wilcoxon matched-pairs test.

ery, as measured in fold-change of the absolute CD4 count, with a slope close to 1 (Fig. 2B). In contrast, for CMV-specific $CD4^+$ T cell responses the slope was 0.37, indicating a reduced replenishment rate of these cells compared with the total CD4 compartment (Fig. 2B). Of note, although all study participants were CMV seropositive, none had detectable CMV viral replication at either visit, pre- or post-ART, indicating that a difference in CMV Ag load at the two visits was not a contributing factor to the reduced replenishment capacity of CMV-specific T cells.

Effect of ART on the functional capacity of copathogen-specific $CD4^+$ T cells

ART has been shown to gradually restore the polyfunctional capacity of HIV-specific T cells (28, 29), but the effect of ART on the functionality of $CD4^+$ T cells specific for other pathogens is unclear. Thus, we evaluated the cytokine secretion profiles of *M. tuberculosis*–, CMV-, and SEB-reactive $CD4^+$ T cells before and after ART initiation in detail (Fig. 3). We included nine HIV-uninfected individuals in this analysis for comparison. Our first finding was that the cytokine production profile of $CD4^+$ T cells varied significantly according to their specificity ($p = 0.002$; Fig. 3A), with *M. tuberculosis*–specific responses consisting of a greater proportion of polyfunctional cells producing all three cytokines, in addition to a predominant TNF-α monofunctional subset (~40% of the response), whereas CMV-specific $CD4^+$ responses exhibited predominantly a IFN-γ^+TNF-α^+ phenotype (~60%; Fig. 3B). ART did not significantly alter the cytokine secretion profile of $CD4^+$ T cells from baseline, regardless of their specificity for *M. tuberculosis*, CMV, or SEB (Fig. 3A, 3B). Moreover, the polyfunctional characteristics of *M. tuberculosis*–specific and SEB-responsive $CD4^+$ T cell responses were comparable to those observed in HIV-uninfected individuals. In contrast, CMV responses were significantly skewed in HIV-infected individuals compared with HIV-uninfected subjects ($p = 0.016$ and $p = 0.045$ for pre- and post-ART; Fig. 3A), with a lower proportion of cells coproducing IFN-γ, IL-2, and TNF-α, and a higher proportion of IFN-γ monofunctional cells (Fig. 3B). These data demonstrate that, unlike for *M. tuberculosis*–specific CD4 responses, HIV infection impaired the functional potential of CMV-specific CD4 responses toward a less polyfunctional profile, and 1 y of ART did not restore the CMV functional profile.

Impact of the memory phenotype of pathogen-specific $CD4^+$ T cells on their replenishment potential upon ART

Depending on their memory differentiation profile, $CD4^+$ T cells are endowed with distinct survival and proliferation capacities (30, 31). Thus, to investigate whether the cell maturation phenotype could impact the replenishment capacity of copathogen-specific $CD4^+$ T cells, we first compared the differentiation profiles of *M. tuberculosis*– and CMV-specific $CD4^+$ T cells at baseline, prior to ART initiation. Fig. 4A shows a representative contour plot of distinct memory subsets in the CD4 compartment pre-ART in one HIV-infected individual. Based on the expression of CD45RO and CD27, we were able to discriminate four different memory subpopulations (32), namely: naive ($CD45RO^-CD27^+$), early differentiated (ED; $CD45RO^+CD27^+$, encompassing central and transitional

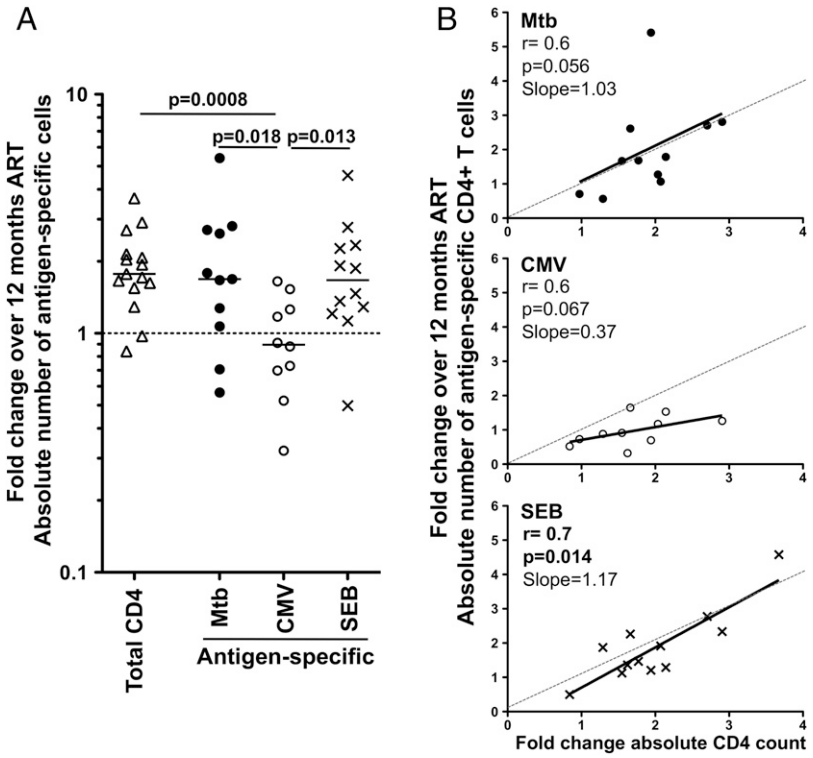

FIGURE 2. Restoration dynamics of *M. tuberculosis*– (Mtb), CMV-, and SEB-specific $CD4^+$ T cells after ART. (**A**) Fold change in the total, *M. tuberculosis*–, CMV-, and SEB-specific absolute $CD4^+$ T cell count over 12 mo of ART. The horizontal dotted line indicates no change from the baseline pre-ART time point. Statistical comparisons were performed using a nonparametric Mann–Whitney *U* test. (**B**) Association between the fold change of the absolute number of Ag-specific $CD4^+$ T cells and the fold change in the absolute CD4 count pre- and post-ART. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation. The solid line represents a linear regression fit, and the dashed line depicts the ideal slope of 1.

memory subsets), late differentiated (LD; $CD45RO^+CD27^-$, encompassing effector memory cells), and terminally differentiated ($CD45RO^-CD27^-$, or effector cells). In HIV-infected subjects, although *M. tuberculosis*– and CMV-specific $CD4^+$ T cells were represented in both ED and LD subsets, they comprised significantly distinct profiles (Fig. 4B, *left panel*). *M. tuberculosis*–specific cells exhibited primarily an ED profile (median 51%, interquartile range [IQR] 42–62), whereas CMV-specific cells demonstrated a substantial enrichment of the LD phenotype (median 73%, IQR 48–86), as previously described (33). Of note, ART did not markedly alter the overall memory differentiation profile of *M. tuberculosis*–specific or CMV-specific $CD4^+$ T cells (Supplemental Fig. 3). In addition, HIV-uninfected individuals exhibited similarly distinctive memory CD4 differentiation profiles for *M. tuberculosis* and CMV (Fig. 4B, *right panel*).

We next investigated the relationship between the memory phenotype of pathogen-specific $CD4^+$ T cells at the time of ART initiation and the capacity of these cells to be maintained and/or replenished upon treatment. Fig. 5A shows that the fold change in the absolute number of pathogen-specific $CD4^+$ T cells over 12 mo on ART inversely correlated with the proportion of cells exhibiting a late differentiated phenotype ($p = 0.019$; $r = -0.5$). Consistent with this, a positive correlation was observed between the fold change in the absolute number of pathogen-specific $CD4^+$ T cells and the proportion of these cells exhibiting an ED phenotype ($p = 0.04$; $r = 0.45$). Of note, the proportion of LD

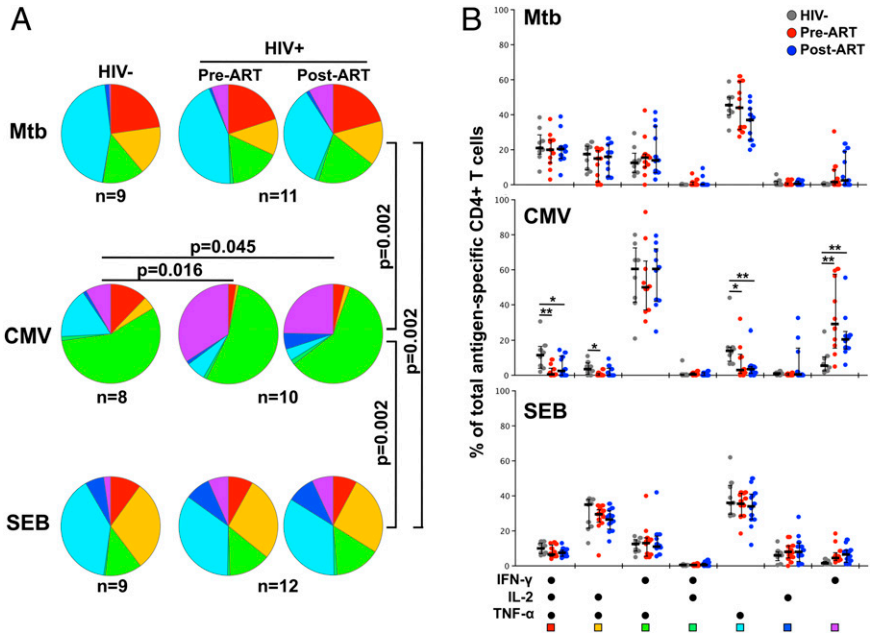

FIGURE 3. Polyfunctional capacity of *M. tuberculosis*– (Mtb), CMV-, and SEB-specific $CD4^+$ T cells in HIV-infected and uninfected individuals pre- and post-ART. Pie charts (**A**) and graphs (**B**) representing the cytokine secretion ability of *M. tuberculosis*–, CMV-, and SEB-specific $CD4^+$ T cell responses in HIV-uninfected individuals ($n = 9$) and HIV-infected individuals pre- and post-ART initiation. Each section of the pie chart represents a specific combination of cytokines, as indicated by the color at the bottom of the graph. Horizontal bars depict the median with interquartile range indicated. Statistical comparisons were performed using a Wilcoxon rank-sum test. $*p < 0.05$, $**p < 0.01$.

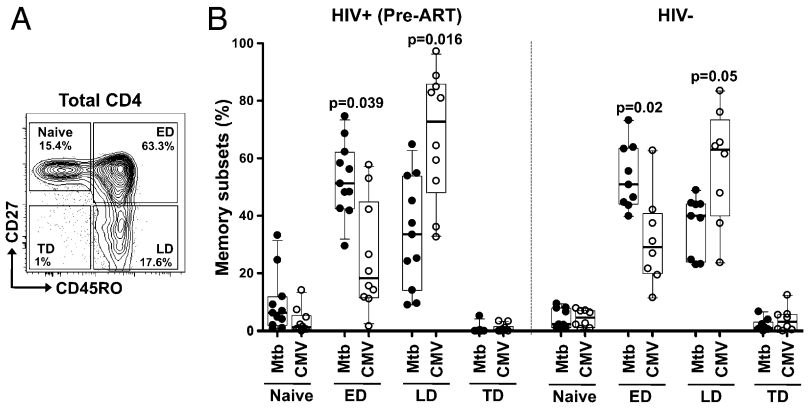

FIGURE 4. Memory profiles of *M. tuberculosis*– (Mtb) and CMV-specific CD4$^+$ T cells in HIV-infected and uninfected individuals. (**A**) Representative example of total CD4 memory subset distribution in one HIV-infected individual. Naive (CD45RO$^-$CD27$^+$), ED (CD45RO$^+$CD27$^+$), LD (CD45RO$^+$CD27$^-$), and TD (CD45RO$^-$CD27$^-$). The frequencies of each subset are indicated. (**B**) Memory profile of *M. tuberculosis*– and CMV-specific CD4$^+$ T cells in HIV-infected individuals pre-ART (*left panel*) and HIV-uninfected individuals (*right panel*). Results are shown as box and whisker (10–90 percentile) plots. Each dot depicts an individual, and the horizontal bar is the median. Statistical comparisons were performed using a one-way ANOVA nonparametric Kruskal–Wallis test.

M. tuberculosis–specific CD4$^+$ T cell responses (in the absence of CMV responses) also correlated inversely with the fold change in the absolute number of *M. tuberculosis*–specific cells ($r = -0.62$; $p = 0.038$; data not shown), and so too when naive-like *M. tuberculosis*–specific cells were grouped together with ED cells, we observed a trend toward a positive association with the fold change in the absolute number of *M. tuberculosis*–specific cells ($r = 0.54$; $p = 0.05$; data not shown). This relationship between the memory profile and the ART-induced repletion potential of pathogen-specific CD4$^+$ T cells is further illustrated in Fig. 5B, showing specific examples from two participants. In CAP257, in which the pre-ART CMV-specific CD4 response showed an enrichment in cells with an LD phenotype (82%), their ability to expand over 12 mo on ART was considerably lower than *M. tuberculosis*–specific CD4 responses, characterized by an ED phenotype (77.4%) (fold change 1.2 for the CMV response versus 2.8 for the *M. tuberculosis* response). In CAP244, in which the memory profile of both *M. tuberculosis* and CMV-specific responses were similar (~60% of LD cells), the fold change in the absolute number of CD4$^+$ T cells to both pathogens was comparable.

Overall, these data reveal that the ability of copathogen-specific memory CD4$^+$ T cells to expand upon ART appears to be related to their memory phenotype, in which ED cells have a higher replenishment capacity compared with LD cells.

Discussion

The introduction of ART has changed the clinical pattern of HIV infection significantly, with considerable reductions in morbidity and mortality. Although it is clearly established that ART leads to a progressive replenishment of the CD4$^+$ T cell compartment in the majority of cases, the extent to which pre-existing copathogen-specific CD4$^+$ T cells are restored, and the causes of variable restoration, are poorly defined. In this study, we investigated the

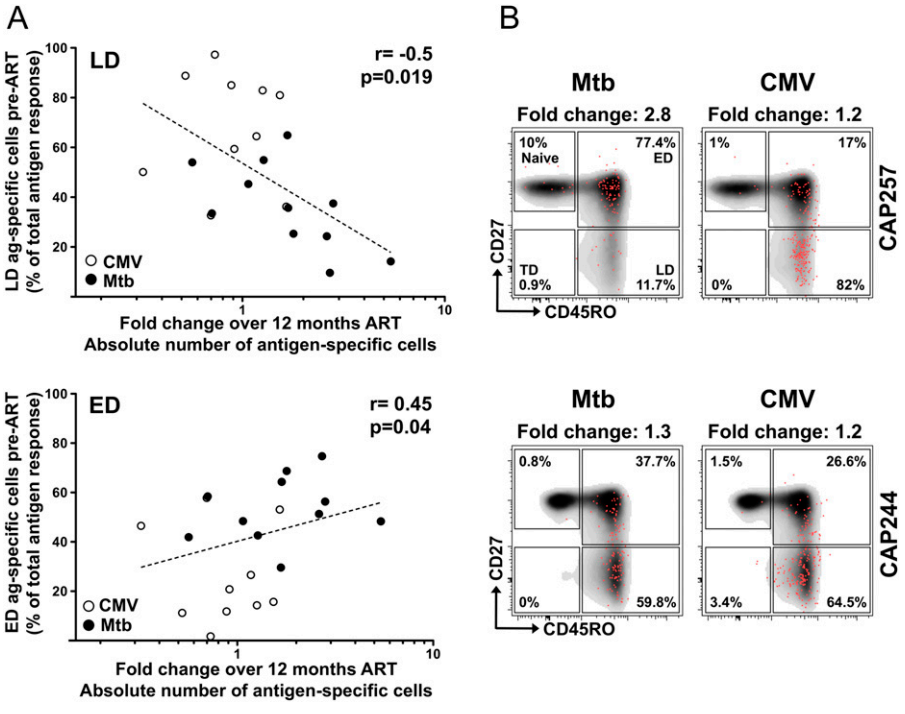

FIGURE 5. Relationship between the memory profile of *M. tuberculosis*– (Mtb) and CMV-specific CD4$^+$ T cells and the dynamics of restoration of these cells. (**A**) Association between the proportion of Ag-specific CD4$^+$ T cells exhibiting an LD (*top panel*) or ED (*bottom panel*) profile with the fold change in Ag-specific CD4$^+$ T cell absolute count after ART. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation. (**B**) Representative examples of the memory profile of *M. tuberculosis*– or CMV-specific CD4$^+$ T cells pre-ART in two individuals. The frequencies of each subset are indicated. For each example, the fold change in Ag-specific absolute CD4$^+$ T cell count pre- and post-ART is indicated at the top of each plot.

dynamics of restoration of memory CD4$^+$ T cells specific for a bacterial copathogen (*M. tuberculosis*) and a viral pathogen (CMV) in 15 HIV-infected individuals in response to ART. Our main finding was that the extent of reconstitution of pathogen-specific cells was related to their memory differentiation profile at the time of ART initiation, in which cells exhibiting an ED memory profile (including central memory and transitional memory cells) had a higher replenishment potential compared with LD (effector memory) T cells.

Multiple mechanisms contribute to the increase in CD4$^+$ T cells in blood in response to antiretroviral treatment for HIV (34). Over the first few months of ART, there is a redistribution of CD4$^+$ T cells from the lymph nodes to the blood (35, 36), leading to a rapid initial rise in CD4$^+$ counts. Moreover, homeostatic cell proliferation (37), decreased cell death (38) and increased thymic output (39, 40) also play a role in the replenishment of CD4$^+$ T cells. Although the CD4 absolute cell count at the time of treatment initiation is one of the main factors dictating the level to which CD4$^+$ T cells are restored (21, 23), other parameters such as the activation level of T cells at the time of treatment (41), age (42, 43), or active coinfections (44, 45) also influence the degree of reconstitution of the CD4 compartment. Thus, CD4$^+$ T cell recovery upon ART appears to be dependent on both the extent of immune damage at the time of treatment initiation and the regenerative capacities of these cells.

We demonstrate in this study that despite successful viral suppression and robust CD4 gains in the majority of participants, the dynamics of *M. tuberculosis*– and CMV-specific CD4$^+$ T cell recovery were distinct. This shows that not all T cell subpopulations have the same potential to be replenished. Several factors and/or cellular attributes could account for these differences. First, the regenerative capacities of pathogen-specific CD4$^+$ T cells could be determined by their intrinsic ability to survive or proliferate, and it has been clearly established that CD4$^+$ T cells exhibiting an ED memory profile have an enhanced survival and proliferative potential compared with cells with an LD phenotype (30, 31). Our data are in accordance with these observations, showing that the extent of replenishment of pathogen-specific CD4$^+$ T cells correlates with their memory status, with superior recovery observed for Ag-specific CD4$^+$ T cells exhibiting an early memory phenotype. Mechanistically, cell responsiveness to common γ-chain cytokines may account for the distinct renewal potential of these different cell subsets (46–48). In our study, the replenishment potential of *M. tuberculosis*– or CMV-specific CD4$^+$ T cells did not associate with their ability to produce IL-2 (data not shown); however, we have observed that responsiveness to IL-7 distinguishes ED and LD CD4$^+$ subsets, with ED memory T cells being more responsive to exogenous IL-7 and IL-2 (as measured by Stat5 phosphorylation), compared with LD memory CD4$^+$ T cells (C. Riou, unpublished observations). Further analysis comparing the level of expression of cytokine receptors and cytokine-induced proliferation potential between *M. tuberculosis*– and CMV-specific CD4$^+$ T cells would be needed to define the role of these signaling pathways in the restoration of immune memory cells upon ART.

Although *M. tuberculosis* responses displayed primarily an ED phenotype, there were substantial interindividual differences, ranging from 30–75% of the response. The CD4$^+$ memory profile is dictated by numerous factors, including continual Ag exposure in high burden settings (49), previous TB, TB treatment, and cure (50–52). A recent study carried out in a similar geographic setting as the current study noted dynamic changes in the T cell response to *M. tuberculosis* in the context of latent TB infection and HIV coinfection (53). It is worth noting that we also detected Ag-specific cells with a naive-like phenotype, previously described for *M. tuberculosis* and other infections (51, 54, 55). Although making up only a minor proportion of the total *M. tuberculosis*– and CMV-specific response, these cells expanded post-ART in the majority of individuals compared with other memory subsets, that were characterized by more variable expansion and contraction. For CMV, this expansion led to a significantly greater frequency of this subset after ART. These cells may fall within the recently described memory stem cell subset, comprising memory cells endowed with a greater ability to proliferate and persist long-term compared with central memory (56, 57). Thus, the composition of the memory pool at the time of ART initiation may influence the restoration of Ag-specific CD4$^+$ T cells.

To determine whether short-term ART affected not just quantitative but also qualitative aspects of copathogen immunity, we assessed the ability of cells to simultaneously secrete multiple cytokines, which for some infections, such as HIV and leishmaniasis, are a correlate of immune control or protection (58, 59). Although the significance of polyfunctional cells in TB is unclear (48), we demonstrated that >50% of *M. tuberculosis*–specific CD4$^+$ cells produced three or two cytokines simultaneously, a profile that did not significantly change after ART; moreover, this was similar to the profile from HIV-uninfected individuals. Our data are in apparent contrast to previous findings (18) showing that ART leads to an improved polyfunctional profile in *M. tuberculosis*–specific CD4$^+$ T cell after short-term treatment, but this may be due to cohort differences, as the participants in that study were characterized by more advanced immunosuppression and higher viral loads than in the current study. Because it has been reported that the impairment of the functional capacity of *M. tuberculosis*–specific CD4$^+$ T cells is related to HIV disease status (60), it is likely that differences in the severity of HIV pathology at the time of treatment initiation between the two studied groups could account for these discrepancies. The polyfunctional profile we observed for CMV-specific CD4$^+$ cells is consistent with that previously reported (33), with simultaneous IFN-γ and TNF-α, and IFN-γ alone dominating the response. The CMV profile also did not change after ART and in this case was less polyfunctional compared with HIV-infected individuals. Our study only examined paired samples from 12 mo after ART, and it is possible that prolonged ART may restore CMV functional profiles (29).

It is important to note that although we demonstrate an expansion of *M. tuberculosis*–specific CD4$^+$ T cells after ART, restoration of the magnitude and measures of functionality of the *M. tuberculosis*–specific CD4$^+$ Th1 response determined by the production of three cytokines is a coarse measure of functional immunity to TB and may not reflect protective immunity. Several additional CD4$^+$ T cell subsets and cytokines contribute to TB immunity (61–64), and we have found an even greater diversity of CD4$^+$ T cell subsets specific for *M. tuberculosis* (C. Riou, N. Strickland, A.P. Soares, B. Corleis, D.S. Kwon, E.J. Wherry, R.J. Wilkinson, and W.A. Burgers, submitted for publication). An additional issue is that we do not know the extent of pathogen-specific CD4$^+$ recovery in tissues upon ART, which appears defective for TB in the lungs even after prolonged viral suppression (20). These considerations may account for the persistently greater susceptibility of successfully treated HIV-infected individuals to TB (65).

Our study had several limitations. We were constrained by the availability of paired samples prior to and after ART, limiting us to studying 15 individuals in detail. Testing the generalizability of our findings in larger cohorts is warranted. Also, we focused our study on short-term restoration of pathogen-specific immunity (after 1 y of ART), and although study participants exhibited suppressed HIV replication, decreased immune activation, and improved CD4 counts, studying responses after longer-term therapy may provide further important insights and more definitive conclusions on the

restoration of pathogen-specific immunity. Furthermore, the participants we studied had nadir CD4 counts of >135 cells/mm^3 (median 289 cells/mm^3), and we did not have access to participants commencing treatment at lower CD4 counts (e.g., <50 cells/mm^3). These highly immunocompromised patients would be the most relevant group to study with respect to susceptibility to CMV disease. In this context, although CMV-specific cells replenished more poorly than *M. tuberculosis*–specific cells after ART, they were present at a high frequency prior to ART initiation, which may well have been sufficient to provide protective immunity to CMV. Moreover, the extent of CD4 depletion at the time of ART initiation could impact the degree and dynamics of Ag-specific CD4$^+$ T cell recovery. Lastly, we delineated memory subsets based on two markers, CD27 and CD45RO. These markers cannot differentiate between central memory and transitional memory cells within the CD27$^+$CD45RO$^+$ ED subset, and inclusion of a marker such as CCR7 would afford an additional degree of distinction that may reveal further differences in the ability of these memory subsets to expand after ART and should be included in future studies of reconstitution of pathogen-specific immunity. Further research addressing these important limitations is needed to confirm and extend our findings.

Understanding the host and cellular factors that contribute to successful immune restoration on effective antiretroviral treatment for HIV, including both the overall CD4$^+$ T cell compartment and copathogen-specific CD4 immunity, is of crucial importance, as only partial recovery of these cells could result in a persistently heightened risk of particular opportunistic infections. In this report, we showed that the renewal potential of pre-existing pathogen-specific CD4$^+$ T cells was related to their memory differentiation profile. It will be important to determine whether this principle holds true in a wider context for other copathogens. Incomplete restoration of functional immunity and the persistence of elevated susceptibility to particular coinfections have implications for targeted interventions in the treated HIV-infected population. Overall, our findings underscore the complexity of immune reconstitution on ART and the importance of preserving functional immunity with early ART (66).

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Disclosures

The authors have no financial conflicts of interest.

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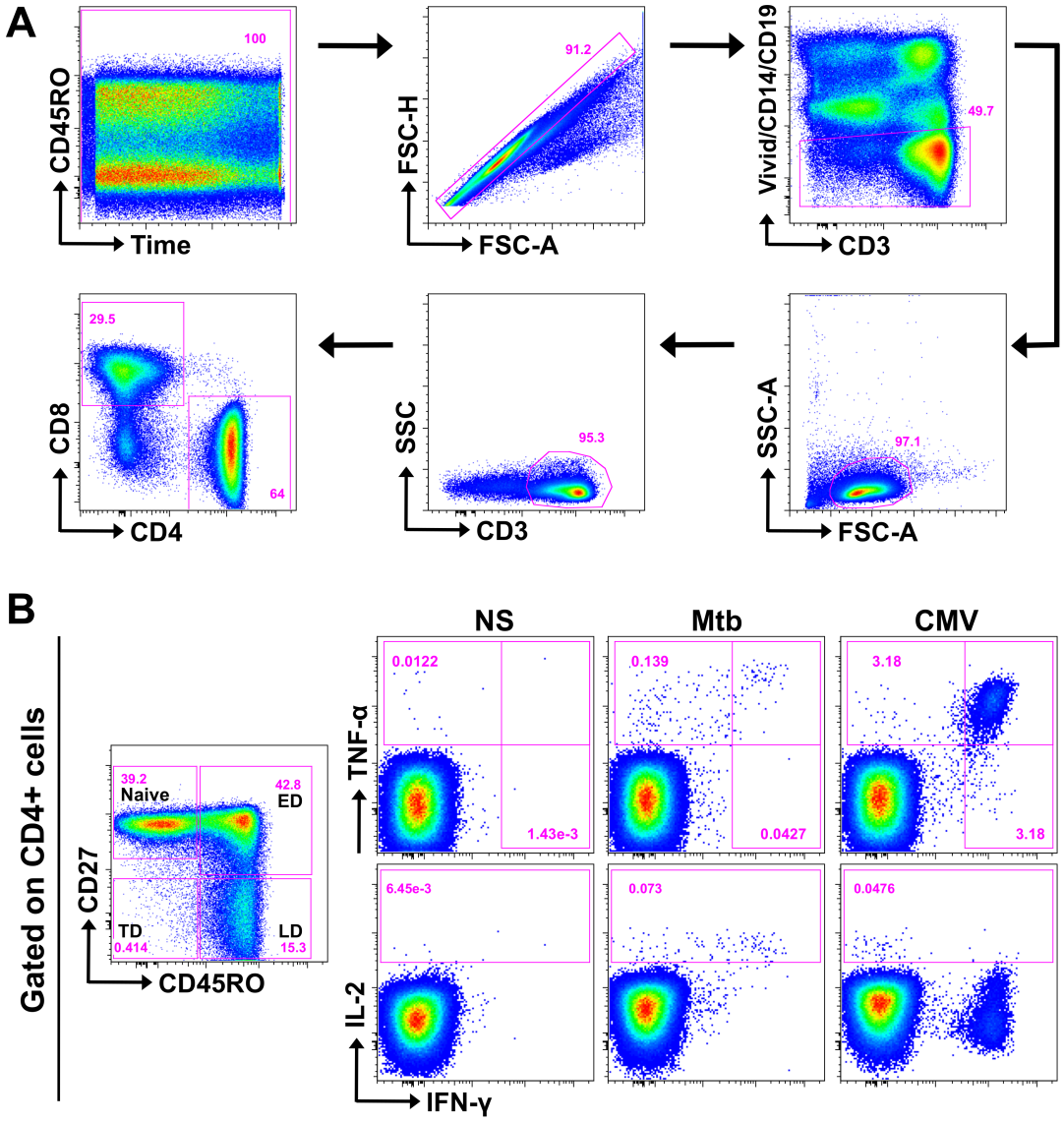
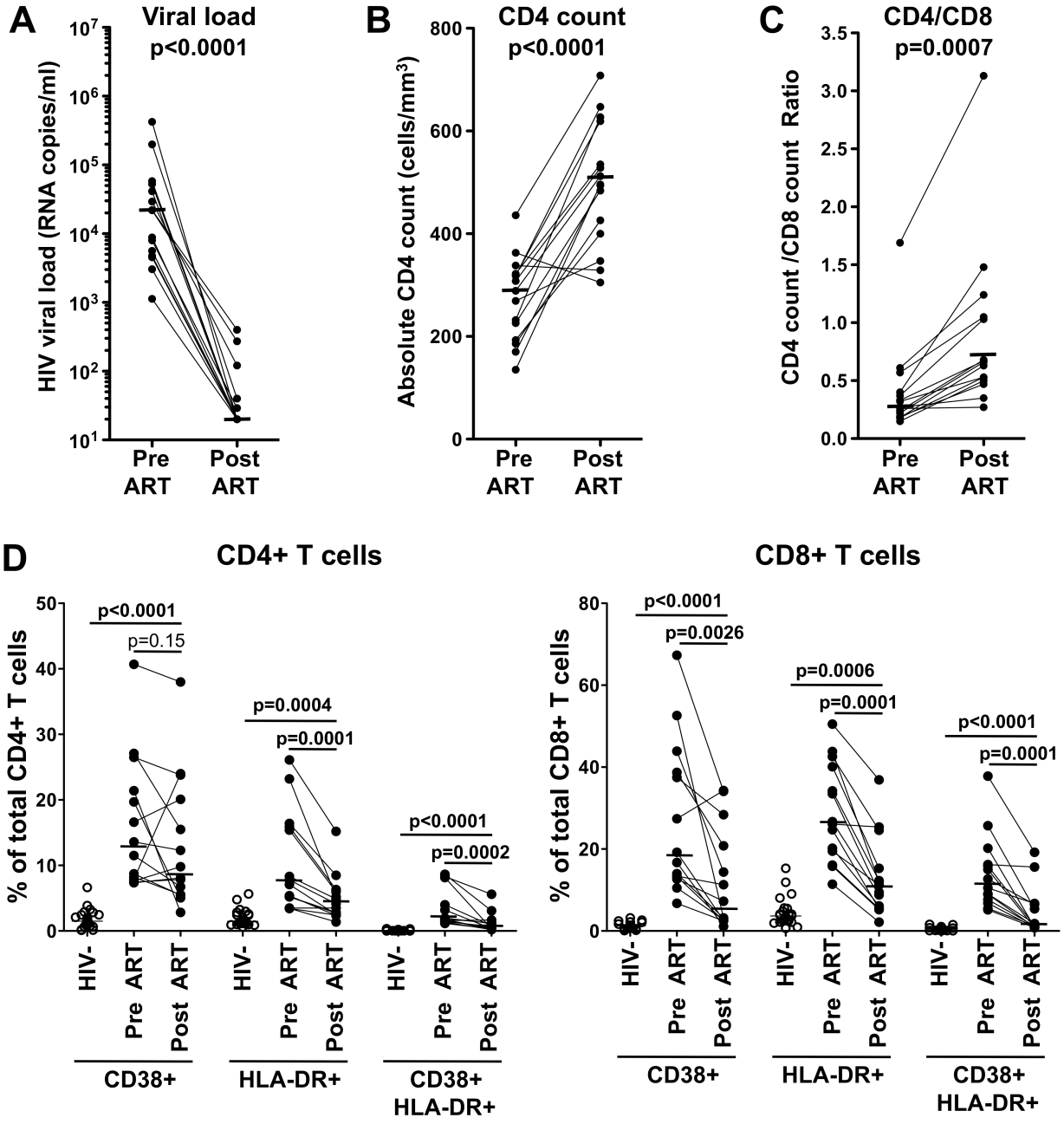


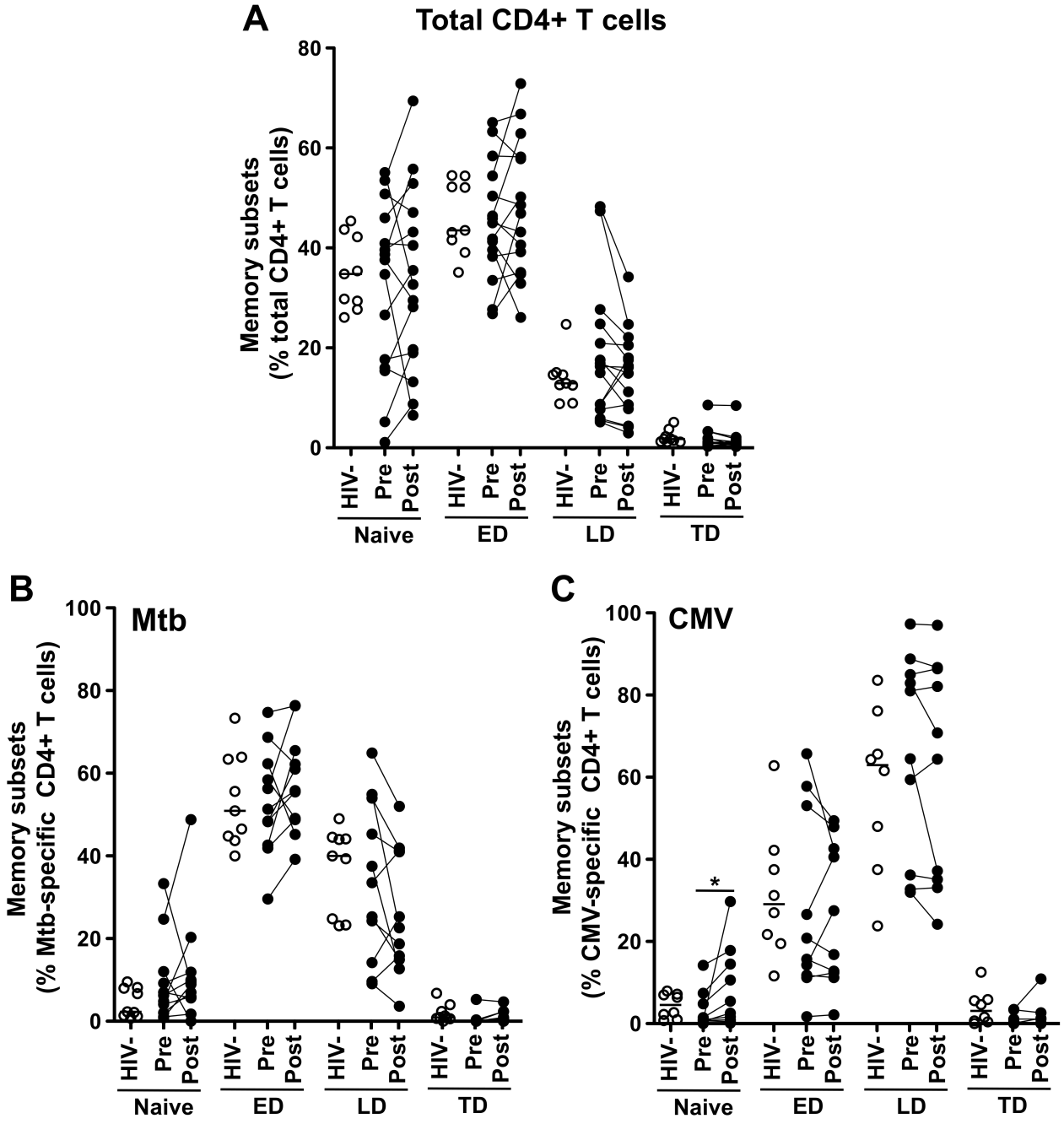
Supplementary Figure S1: Gating strategy. Singlets were identified in forward scatter plots. Dead cells, B cells and monocytes were excluded by Vivid, CD19 and CD14 labeling, respectively. Memory subsets were discriminated based on the expression of CD27 and CD45RO, allowing for the discrimination of four subpopulations (Naïve: CD45RO-CD27+; Early-differentiated (ED): CD45RO+CD27+, Late differentiated (LD): CD45RO+CD27-, and Terminally-differentiated (TD): CD45RO-CD27-). Gates applied for the identification of cytokine positive cells were set according to the unstimulated samples (NS).

Supplementary Figure S2



Supplementary Figure S2. Effect of ART on clinical and activation parameters. Plasma viral load (**A**), absolute CD4 count (**B**) and CD4/CD8 ratio (**C**) before (Pre-ART) and 12 months after ART initiation (Post-ART) in 15 HIV-infected individuals. **(D)** T cell activation (CD38, HLA-DR) is shown for CD4+ and CD8+ T cells. Closed circles depict HIV-infected individuals (n=14) and open circles depict HIV-uninfected individuals (n=23). Statistical analysis was performed using non-parametric statistical tests (Mann-Whitney and Wilcoxon matched pair tests for unmatched and matched samples, respectively). A p-value <0.05 was considered significant. Effective ART of 1 year duration results in an overall decline in T cell activation, although this still remained higher than in HIV-uninfected individuals.

Supplementary Figure S3



Supplementary Figure S3: Comparison of the memory profile of Mtb- and CMV-specific CD4+ T cells pre- and post-ART. Memory profile of total CD4+ T cells (**A**), Mtb-specific CD4+ T cells (**B**) and CMV-specific CD4+ T cells (**C**) pre- and post-ART initiation. For comparison the memory profile of total and antigen-specific CD4 responses in HIV-uninfected individuals (n=9) is presented on each graph with open circles. Each symbol represents one individual. Statistical comparisons were performed using a Wilcoxon matched pairs test for comparison of pre- and post-ART time points and the Mann Whitney test for comparisons with HIV-uninfected individuals. * $p < 0.05$.

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Effect of Antiretroviral Therapy on the Memory and Activation Profiles of B Cells in HIV-Infected African Women

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Human immunodeficiency virus infection induces a wide range of effects in B cells, including skewed memory cell differentiation, compromised B cell function, and hypergammaglobulinemia. However, data on the extent to which these B cell abnormalities can be reversed by antiretroviral therapy (ART) are limited. To investigate the effect of ART on B cells, the activation (CD86) and differentiation (IgD, CD27, and CD38) profiles of B cells were measured longitudinally in 19 HIV-infected individuals before (median, 2 mo) and after ART initiation (median, 12 mo) and compared with 19 age-matched HIV-uninfected individuals using flow cytometry. Twelve months of ART restored the typical distribution of B cell subsets, increasing the proportion of naive B cells ($CD27^-IgD^+CD38^-$) and concomitantly decreasing the immature transitional ($CD27^-IgD^+CD38^+$), unswitched memory ($CD27^+IgD^+CD38^-$), switched memory ($CD27^+IgD^-CD38^-$ or $CD27^-IgD^-CD38^-$), and plasmablast ($CD27^+IgD^-CD38^{high}$) subsets. However, B cell activation was only partially normalized post-ART, with the frequency of activated B cells ($CD86^+CD40^+$) reduced compared with pre-ART levels ($p = 0.0001$), but remaining significantly higher compared with HIV-uninfected individuals ($p = 0.0001$). Interestingly, unlike for T cell activation profiles, the extent of B cell activation prior to ART did not correlate with HIV plasma viral load, but positively associated with plasma sCD14 levels ($p = 0.01$, $r = 0.58$). Overall, ART partially normalizes the skewed B cell profiles induced by HIV, with some activation persisting. Understanding the effects of HIV on B cell dysfunction and restoration following ART may provide important insights into the mechanisms of HIV pathogenesis. ***The Journal of Immunology*****, 2017, 198: 000–000.**

Systemic immune hyperactivation is a hallmark of HIV infection, affecting a range of immune cells, including both T cells and B cells (1). Multiple B cell defects have been reported in HIV-infected individuals, including alteration in the distribution of B cell memory subsets, with the accumulation of differentiated B cells (2–5), excessive B cell activation (6, 7), and increased cell turnover (8). These B cell perturbations lead to functional abnormalities, as demonstrated by hypergammaglobulinemia, decreased B cell responsiveness to both T cell-dependent and T cell-independent Ags (9–11), and compromised responses to vaccination (9, 12, 13).

The specific mechanisms contributing to B cell abnormalities are only partially known, and multiple factors may account for their dysfunction. HIV-driven alteration of the cytokine and chemokine environment has been described as a source of B cell dysfunction (5, 14–16); and it has also been proposed that specific HIV proteins may have a direct effect on B cells (17, 18). Several studies, performed mostly in cross-sectional Caucasian cohorts, have investigated the effect of antiretroviral therapy (ART) on B cells, reporting that suppressive ART can partially or completely normalize B cell phenotypic defects, as shown by the replenishment of naive B cells (19–22), contraction of activated B cells (21–25), and increase in B cell survival potential (26). It is still uncertain, however, whether normalization of B cell memory subsets results in improved B cell immune responses to Ags, including influenza, measles, pneumococcus, and hepatitis B (6, 10, 11, 27, 28).

There is a paucity of published studies on female and African populations with regard to B cell activation and restoration of B cell immunity following successful treatment of HIV (29, 30). There are cogent reasons to believe there may be differences in African compared with Caucasian cohorts. African cohorts have demonstrated higher baseline levels of T cell activation, significantly different T cell memory differentiation profiles (31, 32), and consistently weaker cellular and humoral reactivity to some vaccines (33, 34). A variety of factors may influence immune activation and therefore normalization of immune profiles after ART,

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The online version of this article contains supplemental material.

Abbreviations used in this article: ART, antiretroviral therapy; CAPRISA, Centre for the AIDS Program of Research in South Africa; IQR, interquartile range; LDL, lower detection limit.

such as genetic, gender, and environmental differences, the latter including higher antigenic exposure, diet, and gut microbiota. Furthermore, a variety of sex-specific differences in the response to infections have been described. Women have higher levels of immune activation and faster progression of HIV disease than men with the same viral load (35). These effects have been attributed to estrogen receptor signaling and/or differences in expression of key X-chromosome-expressed immune regulators, such as TLR and CD40L (36). Additional factors such as HIV strains, treatment regimens, and delayed access to HIV treatment could result in distinct outcomes with respect to immunity after ART.

Thus, in this study, to define the extent to which ART restores the B cell phenotype, we measured the memory differentiation and activation profiles of B cells longitudinally in chronically HIV-infected African women before and 12 mo after ART initiation, and compared these profiles to age- and sex-matched HIV-uninfected individuals.

Materials and Methods

Description of study participants

Study participants consisted of 19 women from the Centre for the AIDS Program of Research in South Africa (CAPRISA) 002 HIV acute infection cohort in KwaZulu-Natal, as previously described (37, 38). Peripheral blood samples were obtained at two time points, during chronic infection pre-ART initiation, and post-ART initiation. With respect to ART regimens, 15 of the 19 participants were taking current standard first-line therapy (TDF/3TC/EFV or TDF/FTC/EFV), and four took one of D4T/3TC/EFV, D4T/3TC/NVP, AZT/3TC/NVP, or AZT/3TC/LPV/r. One participant (CAP255) switched ART regimens during the study period (D4T/3TC/EFV to AZT/3TC/EFV at month 10). No participants had active tuberculosis during the study period, or exhibited any immune reconstitution inflammatory syndrome upon HIV treatment. An additional 19 HIV-uninfected women who were matched for age and ethnicity were studied, from the CAPRISA 004 1% tenofovir microbicide gel trial (39). These women were either in the preintervention or placebo arm of the trial. For HIV-infected individuals, either a prospective RNA positive/Ab negative reading or the midpoint between the last Ab negative test and the first Ab positive ELISA test were used to determine the time postinfection. Highly active ART was given according to the South African national HIV treatment guidelines (at a CD4 count of $<$200 cells/mm^3 prior to October 2012; $<$350 cells/mm^3 until March 2015). Ethical approval for the study was obtained from the Research Ethics Committees at the University of KwaZulu-Natal and the University of Cape Town. All participants provided written informed consent prior to participating in the study.

Measurements of HIV plasma viral load and CD4 counts

Plasma HIV viral load and CD4 count were assessed at each study visit. Over the course of the study, the viral load PCR assay switched from Roche AMPLICOR HIV-1 monitor test version 1.5 (lower detection limit [LDL] of 400 RNA copies/ml) to Roche Taqman version 1.0 in June 2010 (LDL 40 RNA copies/ml), and then to Roche Taqman version 2.0 in January 2012 (LDL 20 RNA copies/ml). The FACSCalibur TruCOUNT method (BD Biosciences) was used to measure blood absolute CD4$^+$ and CD8$^+$ T cell counts.

Sample processing

PBMC were isolated by Ficoll-Hypaque (Amersham Pharmacia) density gradient centrifugation and cryopreserved in freezing media (heat-inactivated FCS; Invitrogen) containing 10% DMSO (Sigma-Aldrich). Cells were stored in liquid nitrogen until use. Cryopreserved PBMC were thawed and rested in R10 (RPMI 1640 plus 10% heat-inactivated FCS and 50 U/ml of penicillin-streptomycin) at 37°C with 5% CO_2 for 3 h before staining.

Abs, surface, and intracellular staining

The following Abs were used in three different staining panels: CD19 ECD (J3-119), CD27 PE-Cy5 (1A4CD27; both Beckman Coulter), IgD APC-Cy7 (IA6-2), CD10 BV605 (HI10a), CD21 PE-Cy7 (Bu32), CD40 PerCP-Cy5.5 (5C3; all BioLegend), CD38 APC (HIT2), CD86 PE (IT2.2), CD3 PE-Cy7 (SK7), HLA-DR APC-Cy7 (L243; all BD Biosciences), CD4 PE-Cy5.5 (S3.5), CD8 Qdot-705 (3B5), CD19 Pacific Blue (SJ25-CI), CD14 Pacific Blue (Tük4), CD3 Pacific Blue (UCHT1), Ki67 FITC (7B11; all Invitrogen), and a violet viability reactive dye (Vivid; Molecular Probes). All Abs were titrated prior to use to obtain optimal titers for staining. Briefly, PBMC were stained with Vivid, then labeled with Abs against surface markers, fixed, permeabilized, and subsequently stained intracellularly with Ki67. Cells were then resuspended in 1× CellFix (BD Biosciences) and kept at 4°C until acquisition. Samples were acquired on a BD Fortessa using FACSDiva software and analyzed using FlowJo (version 9.9.3; TreeStar). The gating strategy to identify B cell subsets is shown in Supplemental Fig. 1.

ELISAs

Plasma samples were used to measure soluble CD14 (sCD14; a marker of monocyte/macrophage activation) from the same women pre- and post-ART. One participant was not included in this analysis due to the sample being unavailable. Plasma (n = 18) was tested in duplicate using commercially available ELISA kits according to the manufacturer's protocol (R&D Systems). In addition, 30 plasma samples from HIV-uninfected women from the same cohort were included. Samples were diluted 1:200, data were collected using a SpectraMax Plus reader (Molecular Devices), and point-to-point curve fits were used to calculate sCD14 concentrations from the standard curves. Data were analyzed using SoftMax Pro software (Version 3.2.1; Molecular Devices).

Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 5.0). The Mann–Whitney U test and the Wilcoxon Signed Rank test were used for unmatched and paired samples, respectively. Correlations between the different groups were determined by the non-parametric Spearman Rank test. A p value $<$0.05 was considered statistically significant.

Results

Effect of ART on the memory differentiation and activation of B cells

To define the extent to which ART normalizes HIV-induced alteration of B cells, we first determined the distribution of B cell subsets in 19 HIV-infected individuals before and 1 y after ART initiation, and compared it to 19 HIV-uninfected matched controls. Pre-ART samples were obtained during chronic infection at a median of 4.9 y (interquartile range [IQR]: 2.8–5.8 y) after the estimated date of HIV infection, and a median of 1.5 mo (IQR: 0.05–2.7) prior to starting ART. The median plasma viral load at this time was 34,325 HIV RNA copies/ml (IQR: 7,997–78,573), and the median CD4 count was 314 cells/mm^3 (IQR: 260–369) (Table I). After a median of 12 mo of treatment (IQR: 12–13 mo), all individuals exhibited viral suppression (p = 0.0001), the majority to $<$40 copies/ml, and an increase in both absolute CD4 count and CD4/CD8 ratio (both p = 0.0001) (Table I, data not shown).

Fig. 1A shows representative flow plots of each B cell subset from one HIV-uninfected and one HIV-infected individual pre- and post-ART. Based on the expression of CD27, IgD, and CD38, six B cell subsets were identified, namely immature transitional B cells (CD27$^-$IgD$^+$CD38$^+$), naive B cells (CD27$^-$IgD$^+$CD38$^-$), unswitched memory (CD27$^+$IgD$^+$CD38$^-$), CD27$^+$ switched memory B cells (IgD$^-$CD38$^-$), CD27$^-$ switched memory B cells (IgD$^-$CD38$^-$), and plasmablasts (CD27$^+$IgD$^-$CD38^{high}). There was a marked skewing of B cell subsets in chronic HIV infection prior to ART (Fig. 1B). When compared with HIV-uninfected participants, HIV-infected individuals were characterized by significantly higher frequencies of immature transitional B cells, unswitched memory B cells, and plasmablasts (median: 9.3% [IQR: 6.3–13.3] versus 14.8% [IQR: 10.6–20]; 1.5% [IQR: 1.2–1.7] versus 2.3% [IQR: 2–5.8] and 0.44% [IQR: 0.3–1] versus 2.1% [IQR: 1.4–4], respectively), and significantly lower proportions of naive B cells (median: 65.8% [IQR: 60–72.1] versus 46.9% [IQR: 39.7–55.7]) (Fig. 1B). Additionally, the frequencies of switched memory B cells expressing CD27 or not were comparable in HIV-uninfected and ART naive HIV-infected individuals

Table I. Clinical parameters of study participants

	HIV-Infected (Pre-ART)	HIV-Infected (Post-ART)	*p* Value
Sample size	19	19 (paired)	
CD4 count (cells/mm^3)[a]	314 (260–369)	629 (514–696)	$p = 0.0001$
CD4/CD8 ratio[a]	0.23 (0.18–0.33)	0.61 (0.48–0.74)	$p = 0.0001$
Viral load (RNA copies/ml)[a]	34,325 (7,997–78,573)	<20 (<20–39)	$p = 0.0001$
Duration of ART (months)[a]	NA	12 (12–13)	

[a]Median and interquartile range.
NA, not applicable.

(~8 and ~12%, respectively). It is worth mentioning that the frequency of unswitched memory B cells in our healthy study population was ~7 times lower when compared with a previous study reporting that the proportion of unswitched memory was comparable to switched memory (representing ~15% of total B cells) in a predominantly male cohort from London (11). Following 12 mo of ART, the frequency of immature transitional, naive and unswitched memory B cells were normalized, returning to levels comparable to those observed in HIV-uninfected individuals (Fig. 1B). The frequency of plasmablasts was also reduced in response to ART ($p = 0.004$), but remained significantly elevated compared with the HIV-uninfected group. To further define the restoration dynamics of B cell subsets upon ART, we compared the fold change in their frequencies pre- and post-ART. After 1 y of ART, only naive B cells displayed an overall expansion (median 1.3 fold [IQR: 1.2–1.5]); among the subsets decreasing upon ART, plasmablasts exhibited the largest contraction, with a median fold change of 0.38 (IQR: 0.22–0.72). The contraction of other B cells subsets was more moderate with a median fold change of 0.51 (IQR: 0.39–0.92) for unswitched memory B cells, 0.65 (IQR: 0.37–0.77) for immature transitional B cells, 0.79 (IQR: 0.5–1) for CD27$^+$ switched memory B cells and 0.87 (IQR: 0.69–1) CD27$^-$ switched memory B cells (Fig. 1C). Although ART had a predominant effect on reducing the frequency of plasmablasts (~60% reduction), this was not sufficient for their normalization with respect to HIV-uninfected individuals.

Alternative B cell markers such as CD27, CD10, and CD21 have been used to delineate B cell memory subsets (40), allowing for the identification of three additional B cell memory subsets, namely tissue-like memory (CD10$^-$CD21$^-$CD27$^-$), resting memory (CD21$^+$CD27dim), and activated memory (CD10$^-$CD21$^-$ CD27dim) B cells (Supplemental Fig. 1B). The expression of CD10 and CD21 on B cell subsets is shown in Supplemental Fig. 2. Thus, to validate our data, we defined the evolution of B cell memory subpopulations pre- and post-ART using this set of markers in 10 of the HIV-infected individuals from our study (Supplemental Fig. 3). ART led to a significant reduction in the frequencies of tissue-like memory, activated memory B cells, and plasmablasts, and an increase in the frequencies of naive and resting memory B cells. These observations confirm the data described above, and are in line with published data (21). Of note, using this alternative gating strategy, we found no significant difference in the frequencies of immature transitional B cells between the two time points. This may be due to the small sample size (a subset of $n = 10$) used for this set of markers, because further analysis of the same individuals using IgD, CD38, and CD27 also showed no significant difference in immature transitional B cells (data not shown).

The effects of ART on B cell activation and proliferation were also evaluated. Fig. 2A shows representative flow plots of B cell activation, measured as the frequency of B cells coexpressing CD86 and CD40, and proliferation (Ki67 expression) from one HIV-uninfected and one HIV-infected individual pre- and post-ART. The frequency of activated B cells (CD86$^+$CD40$^+$) was significantly higher in HIV-infected subjects prior to ART compared with HIV-uninfected participants (median: 14% [IQR: 9.2–15.6] versus 1.7% [IQR: 1.3–2.3]; Fig. 2B). In response to ART, the frequency of activated B cells was substantially reduced but remained significantly higher than in HIV-uninfected participants (Fig. 2B). It is important to mention that during HIV infection, activated B cells were mostly confined to switched memory cells (i.e., IgD$^-$ B cells), with a median of 25% (IQR: 19.6–32) of switched cells coexpressing CD40 and CD86. On the contrary, the activation level of unswitched B cells (i.e., IgD$^+$ B cells) was only marginal, with less than 5% of these cells positive for CD40 and CD86 (median: 4.5% [IQR: 2.4–5]; data not shown).

Similar to activated B cells, there were significantly more Ki67$^+$ B cells in ART-naive HIV-infected individuals compared with HIV-uninfected controls (median: 5% [IQR: 2.9–7.2] versus 1.3% [IQR: 0.9–1.6]) and although ART led to a significant decline in proliferating B cells, they remained elevated compared with the HIV-uninfected group ($p = 0.001$, Fig. 2C). Of note, the frequencies of proliferating (Ki67$^+$) and activated (CD86$^+$CD40$^+$) B cells were positively associated with the frequency of plasmablasts following ART ($p = 0.004$, $r = 0.62$ and $p = 0.003$, $r = 0.63$, respectively, data not shown), suggesting that residual B cell activation and proliferation upon ART may be explained by the persistence of an elevated frequency of plasmablasts, consistent with our findings presented in Fig. 1B.

Overall, our data show that HIV infection skews the B cell memory differentiation profile and causes abnormal B cell activation and proliferation. Defects in B cell memory phenotype were largely normalized within 1 y of ART, whereas B cell activation and proliferative capacities were only partially reduced and longer periods of suppressive therapy may be required for normalization.

B cell defects during HIV infection do not directly associate with HIV viral load

It has been clearly established that hyper-immune activation of T cells during HIV infection is related to viral replication (41) and is partially normalized upon ART (42). This was confirmed in our experimental setting, where we had T cell activation data for 18 of the participants in the current study, as well as 14 additional subjects from the same cohort. The proportion of activated CD4$^+$ T cells (measured by the expression of HLA-DR) was positively associated with plasma viral load ($p = 0.001$, $r = 0.54$; Fig. 3A) in HIV-infected subjects pre-ART. Although 12 mo of ART led to a substantial reduction in the frequency of HLA-DR expression by T cells, T cell activation levels remained significantly higher than in HIV-uninfected subjects (Supplemental Fig. 4). Thus, to determine whether HIV load was also the driving factor for B cell activation, we examined the relationship between B cell phenotype and HIV plasma viral load before ART initiation. As previously

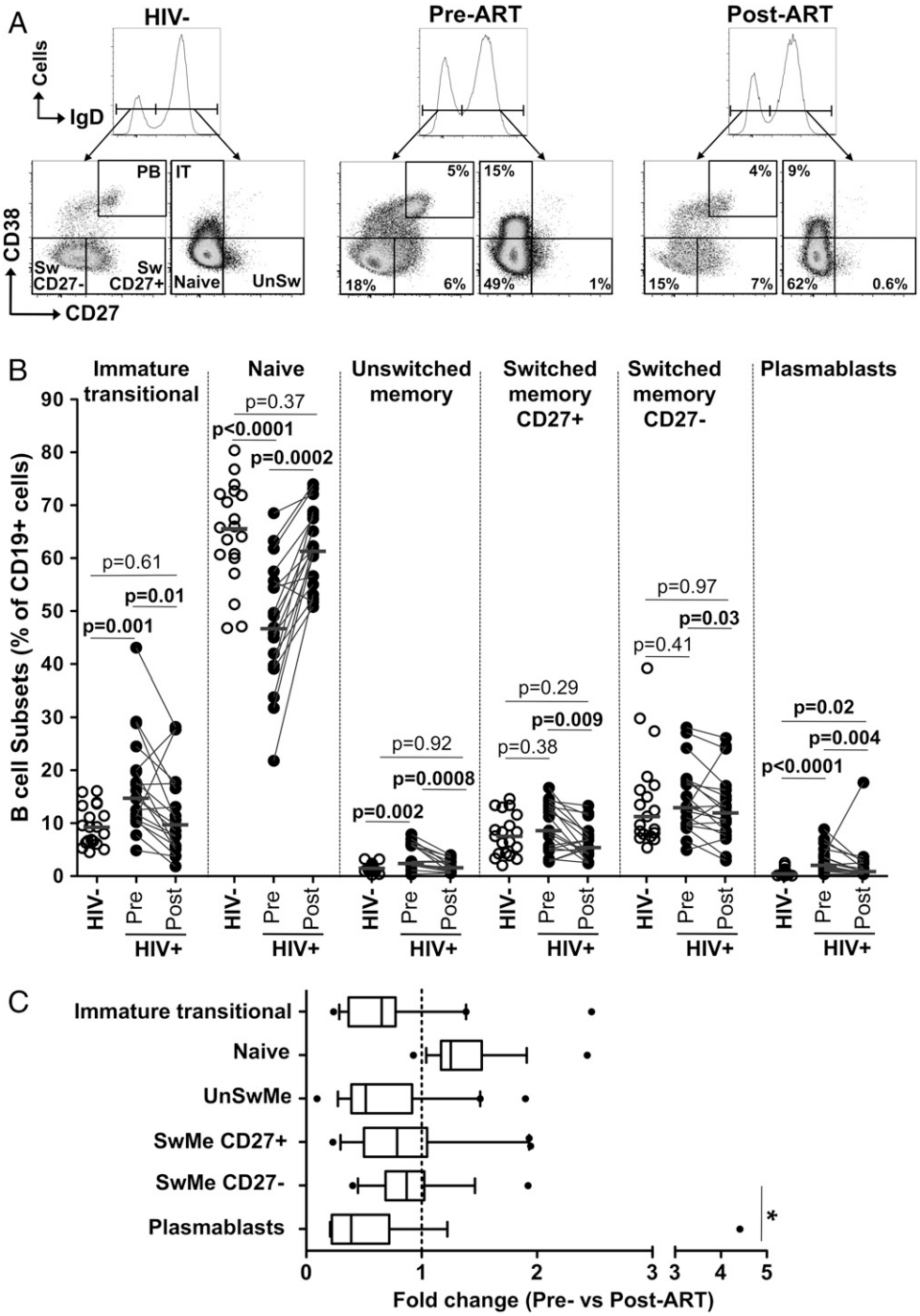

FIGURE 1. Effect of ART on the memory differentiation profile of B cells. (**A**) Representative flow plots of CD27, IgD, and CD38 expression in B cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. Numbers represent the frequencies of each B cell subset, namely immature transitional ($CD27^-IgD^+CD38^+$), naive ($CD27^-IgD^+CD38^-$), Unswitched memory ($CD27^+IgD^+CD38^-$), switched $CD27^+$ memory (IgD^-CD38^-), switched $CD27^-$ memory (IgD^-CD38^-), and plasmablasts ($CD27^+IgD^-CD38^{high}$). (**B**) Frequencies of B cell subsets in HIV-uninfected (n = 19; open circles) and HIV-infected pre- and post ART (n = 19; closed circles) individuals. Horizontal lines indicate the median. Statistical significance was calculated using a Mann–Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively. (**C**) Fold change in the frequency of B cell subsets over 12 mo of ART. Data are shown as box and whisker (interquartile range) plots. The vertical dotted line indicates no change from the time point prior to ART. Statistical comparisons were calculated using a one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

stated, switched memory B cells (i.e., IgD^- B cells) are highly activated during HIV infection, thus we focused our analyses on this particular subset. Surprisingly, unlike for T cells, we found no association between the frequency of activated ($CD86^+CD40^+$) switched memory B cells and HIV viral load (p = 0.71, r = 0.39; Fig. 3B). Moreover, none of the B cell memory subset frequencies were found to be associated with viral load (data not shown). Finally, the extent of $CD4^+$ or $CD8^+$ T cell activation did not correlate with the level of switched B cell activation (p = 0.78, r = 0.07; Fig. 3C, data not shown). Together, these results show that HIV differentially affects the T cell and B cell compartments, where B cell activation does not mirror T cell activation and is not directly associated with the levels of HIV. This suggests that additional factors besides HIV replication may contribute to B cell activation.

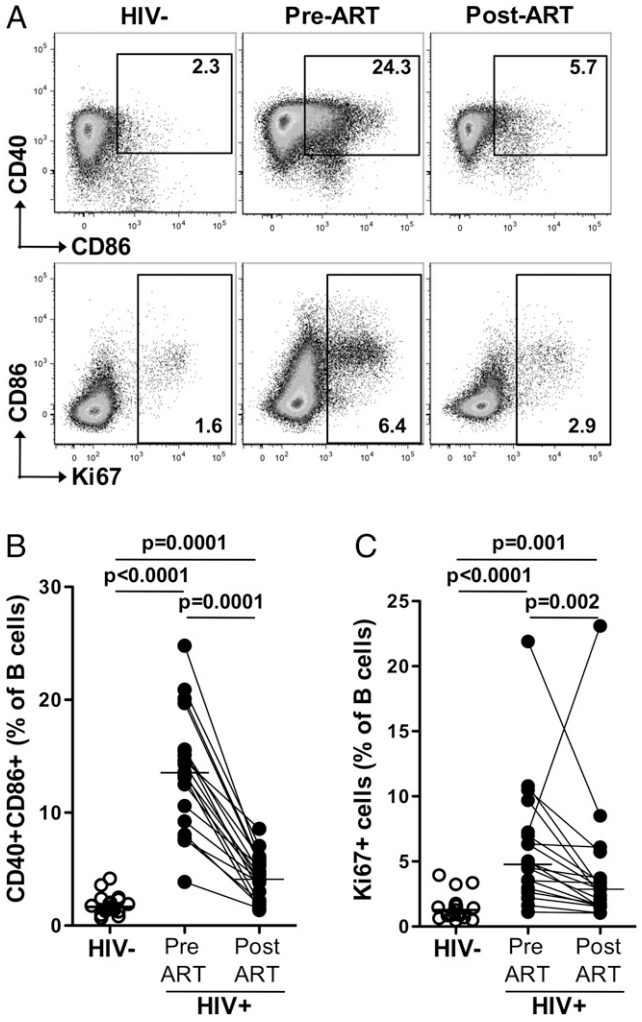

FIGURE 2. Effect of ART on B cell activation and proliferation. (**A**) Representative flow plots of CD40, CD86, and Ki67 expression in B cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. Numbers represent the frequencies of activated ($CD86^+CD40^+$) and proliferating ($Ki67^+$) B cells. (**B**) Frequencies of $CD86^+CD40^+$ B cells in HIV-uninfected (n = 19; open circles) and HIV-infected pre- and post-ART (n = 19; closed circles) individuals. (**C**) Frequencies of proliferating B cells as measured by Ki67 expression. Horizontal lines represent the median. Statistical significance was calculated using a Mann–Whitney U test and Wilcoxon Signed Rank for unpaired and paired samples, respectively.

Additional contributors of B cell defects during HIV infection

The translocation of bacterial products resulting from HIV-induced damage to the gastrointestinal tract is an important contributor of systemic immune activation (43). Therefore, we investigated the relationship between the activation and skewed differentiation profile of B cells and the level of plasma sCD14, a marker of monocyte/macrophage activation, which may be an indicator, in the absence of other infections, of microbial translocation. Prior to ART, plasma sCD14 levels associated negatively with the frequency of naive B cells (p = 0.036, r = −0.50; data not shown) and positively with the frequency of immature transitional B cells (p = 0.003, r = 0.75; Fig. 4A), and the frequency of activated ($CD86^+CD40^+$) switched memory B cells (p = 0.01, r = 0.58; Fig. 4B). Moreover, a positive correlation was also observed between sCD14 levels and the frequency of activated HLA-DR^+ $CD4^+$ T cells (p = 0.045, r = 0.49; Fig. 4C). It is worth noting that the levels of plasma sCD14 were not significantly changed after 12 mo of ART (Supplemental Fig. 5), and there were no associations with residual B cell activation post-ART (data not shown).

Overall, these data suggest a possible impact of microbial translocation on B cell dysfunction, as suggested by sCD14 levels correlating with HIV-induced B cell skewing and hyperactivation in untreated chronic HIV infection. However, their influence may be relatively minor, because there are consistently elevated levels of sCD14 after ART, when B cell defects are largely reversed. It is clear that a variety of factors may directly or indirectly influence B cell defects during HIV infection.

Discussion

Despite the fact that HIV does not directly infect B cells, the virus causes several B cell abnormalities that contribute to HIV pathogenesis (44). Although these B cell defects may be reverted upon ART, this effect has mostly been studied in cross-sectional Caucasian cohorts. Thus, in this study we assessed longitudinally the impact of ART on the memory and activation profile of B cells in South African adult women (n = 19); and compared these profiles to age-matched HIV-uninfected women from the same community (n = 19). Our data show that: 1) HIV-induced alterations of B cells did not associate with viral burden but rather correlated with plasma sCD14 concentration; and 2) 12 mo of ART largely normalized the distribution of B cell memory subsets whereas activation of B cells was only partially reduced.

HIV induces multiple defects in B cells, altering their memory and activation profiles and function (3, 5, 40, 45). In our experimental setting, we showed that in HIV-infected African women B cells were skewed toward a more differentiated memory phenotype (with the accumulation of plasmablasts and the reduction of naive B cells) and were hyperactivated (as measured by CD86 expression) when compared with HIV-uninfected persons. These observations are in accordance with previous studies performed primarily in male Caucasian populations (3, 20, 21, 6, 45, 46), highlighting that similar abnormalities occur in African women. To date, the drivers of B cell alterations during HIV infection have not been fully elucidated. Even though HIV does not directly target B cells for infection, the interaction between HIV and B cells has been previously described, where HIV gp120 can bind to B cell receptors such as CD21, DC-SIGN, and variable H chain 3 (VH3) Ig, resulting in polyclonal B cell activation and Ig production (47). Furthermore, HIV Nef can interact directly with B cells, resulting in suppression of Ig class switching (48, 49). How each of these mechanisms contributes to B cell dysfunction is unknown (40).

It has also been proposed that excessive B cell activation may be driven by proinflammatory cytokines, such as IFN-α, TNF-α, IL-6, and IL-10, molecules commonly up-regulated during HIV-induced systemic inflammation (50). HIV infection also leads to increased production of B cell-specific growth factors such as BLyS/BAFF from macrophages, dendritic cells, and granulocytes (51–53), promoting B cell dysregulation right from the early stages of infection. Additionally, HIV pathogenesis has also been linked to extensive damage to the gastrointestinal tract, leading to the release of bacterial products into the circulation (43). These products may induce aberrant B cell activation via TLR signaling (54), by both direct and indirect means. Stimulation of monocytes by bacterial LPS leads to the release of cytokines that contribute to the inflammatory environment. Additionally, human B cells express a range of TLRs, including high levels of TLR9 (55), and abundant bacterial DNA as a result of HIV-induced microbial translocation (56) may stimulate B cells directly. In fact, TLR9 stimulation of human B cells promotes their activation, proliferation, and the generation of

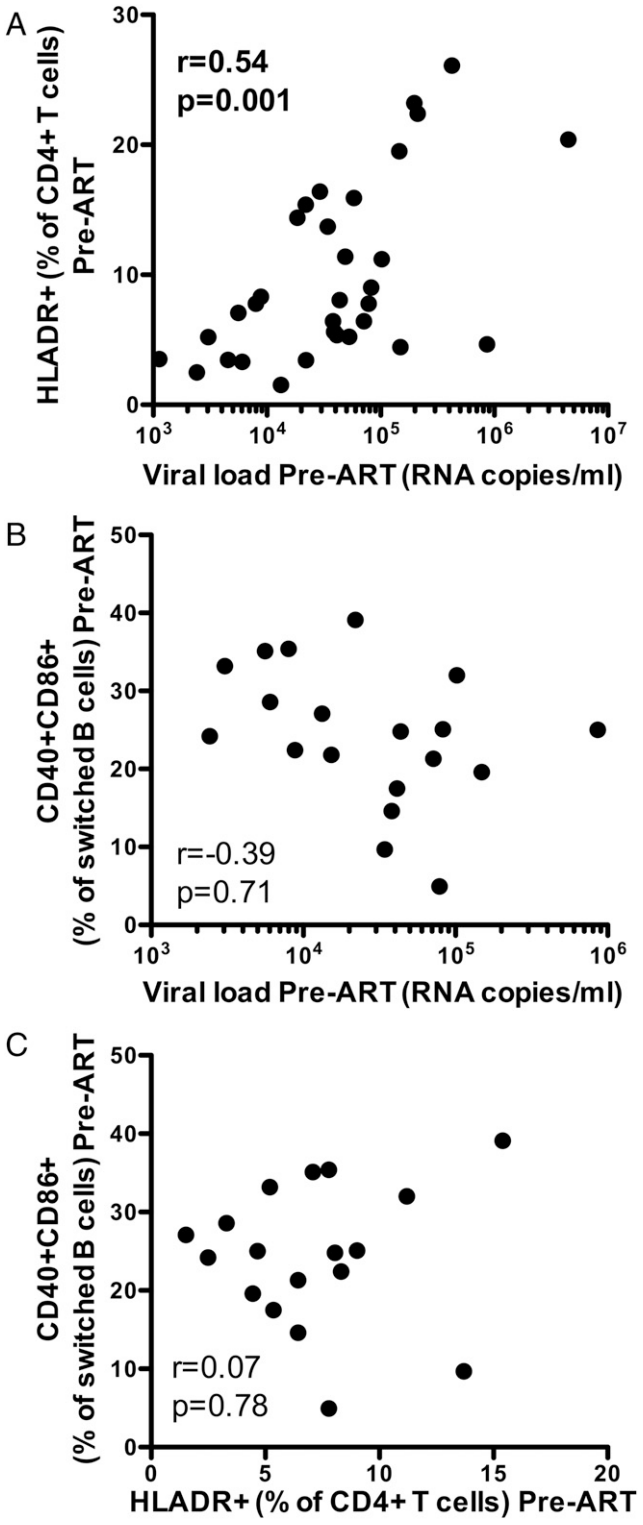

FIGURE 3. Relationship between $CD4^+$ T cell and B cell activation and plasma viral load pre-ART. Correlation of (**A**) the frequency of HLA-DR expressing $CD4^+$ T cells (n = 32) and (**B**) the frequency of $CD40^+CD86^+$ switched memory B cells (n = 19) and plasma viral load in HIV-infected individuals prior to ART initiation. (**C**) Correlation between B cell activation ($CD86^+CD40^+$) and $CD4^+$ T cell activation ($HLA\text{-}DR^+$) in 18 HIV-infected participants before ART. Statistical significance was calculated using a non-parametric Spearman Rank test.

plasmablasts (57, 58). The potential role of microbial products as direct factors fueling B cell hyperactivation is further inferred by the fact that in other chronic diseases leading to gut damage (such as inflammatory bowel disease), B cells exhibit aberrant activation profiles (59).

We found an association pre-ART of B cell activation and differentiation profile with the concentration of plasma sCD14, secreted upon monocyte/macrophage activation by LPS, which may be a surrogate marker of microbial translocation. Interestingly, although there was a relative normalization of B cells post-ART, plasma sCD14 concentration did not decrease overall upon treatment. This phenomenon has been observed in several studies, where sCD14 levels remain elevated even after several years on ART (60–62), and unreversed damage to the gastrointestinal tract has been proposed to account for this. These data confirm that in African women, inflammation persists after ART even when there is a substantial decrease in cellular activation. The absence of any decrease in sCD14 after ART, despite a substantial normalization of B cell profiles, suggests that sCD14 plays only a partial role in contributing to B cell activation. Alternatively, we could speculate that microbial translocation and viral-induced factors might act synergistically to drive B cell activation and skewing, and ART uncouples this effect by suppressing viral replication even in the face of ongoing inflammation. Although we did not find an association of HIV viral load with B cell dysfunction pre-ART, this relationship may not be linear, with a multitude of viral-induced factors influencing B cell activation and differentiation directly and indirectly (63). Overall, this suggests that a combination of inflammatory mediators such as cytokines, B cell growth factors, and microbial products may directly and indirectly stimulate B cell differentiation and proliferation, favoring their functional impairment and exhaustion.

Several studies have shown that ART leads to a near normalization of peripheral B cell memory subsets (19, 20, 22, 64); and the extent of restoration was linked to the timing of ART initiation (21, 65), the duration of treatment, or the age of the patients (22, 66). Very limited data are available on the effects of ART on B cells in African cohorts. To our knowledge, only one cross-sectional study has investigated how ART influences the restoration of B cell numbers in an African adult population (30). In countries disproportionately affected by the HIV pandemic and where access to ART is limited and often started during advanced lymphopenia, it is of importance to define whether HIV treatment can restore B cell profiles to similar levels observed in HIV-uninfected individuals (of note, the CD4 count pretreatment in the participants included in this study was $<$350 cells/mm^3). Our data showed that although 12 mo of ART largely normalized the distribution of memory subsets, B cell activation remained significantly elevated compared with HIV-uninfected individuals. The persistence of these cycling, activated B cells composed mainly of plasmablasts could reflect residual systemic inflammation, despite viral suppression. Indeed, it has been shown that even upon successful ART, inflammation, microbial translocation, and germinal center destruction still persist (43, 67–69). These observations are in line with results reported in Caucasian cohorts (20, 21, 70), showing that the normalization of B cell subsets can be achieved even when treatment is initiated at a late stage of chronic HIV infection.

The limitations of this study include a cohort exclusively composed of women; further studies could test the generalizability of our findings in men. Additionally, we only analyzed B cells from peripheral blood and this may not be representative of B cell profiles within the tissues; it is plausible that B cells residing in the germinal center are differentially affected during HIV infection and that the extent and/or kinetics of their restoration during treatment may differ. For future studies, use of additional phenotypic markers such as CD10 and CD24 could improve the

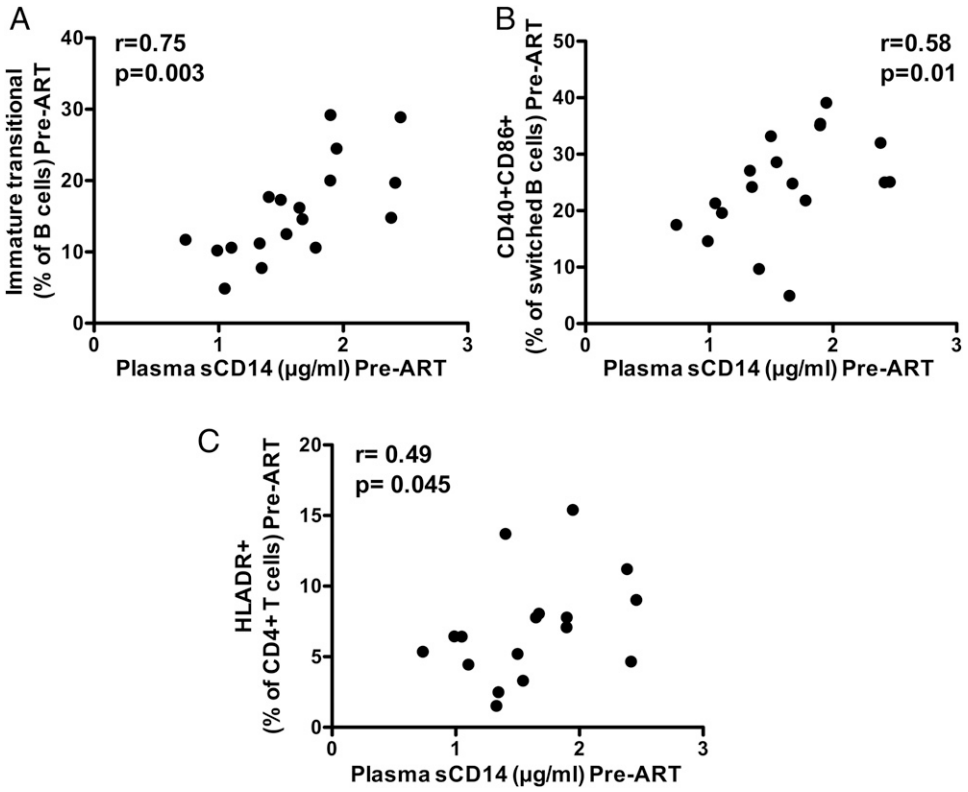

FIGURE 4. Relationship between plasma sCD14 levels and B cell phenotype and $CD4^+$ T cell activation pre-ART. Correlation of (**A**) the frequency of immature transitional B cells, (**B**) the frequency of $CD86^+$ $CD40^+$ switched memory B cells, and (**C**) and the frequency of HLA-DR^+ $CD4^+$ T cells with plasma sCD14 levels in HIV-infected individuals (n = 18 and n = 17, respectively). Statistical significance was calculated using a non-parametric Spearman Rank test.

definition of B cell subsets, in particular immature transitional B cells. Furthermore, we did not examine the relationship of relative normalization of B cell activation and differentiation with restoration of functional B cell immunity, which may not always return (71). Further studies of humoral immunity after ART are warranted in larger African cohorts, by investigation of both memory B cell responses and long-lived plasma cells to a variety of vaccines and infections. This is particularly important if functional B cell immunity is not fully restored and revaccination to certain pathogens may be required. In this respect, early ART initiation, compared with those treated during chronic HIV infection, has demonstrated a benefit for restoration of B cell functionality (21).

In conclusion, our study has shown that the B cell compartment is highly disturbed in chronically HIV-infected African women, an understudied group disproportionately affected by the HIV pandemic, as it is for Caucasian cohorts. Differences in methodology limit our ability to directly compare our results in African women to published studies in western cohorts, but we observed a substantial reduction in B cell activation and a relative normalization of skewed B cell subsets as observed in Caucasian cohorts, despite the commencement of ART in chronic HIV infection. Identifying the drivers of B cell activation and defective differentiation may provide a better understanding of the mechanisms associated with B cell dysfunction. This can potentially lead to the development of intervention strategies to improve B cell function during HIV infection, for both HIV-specific humoral immunity and responses to vaccines targeting other pathogens.

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Disclosures

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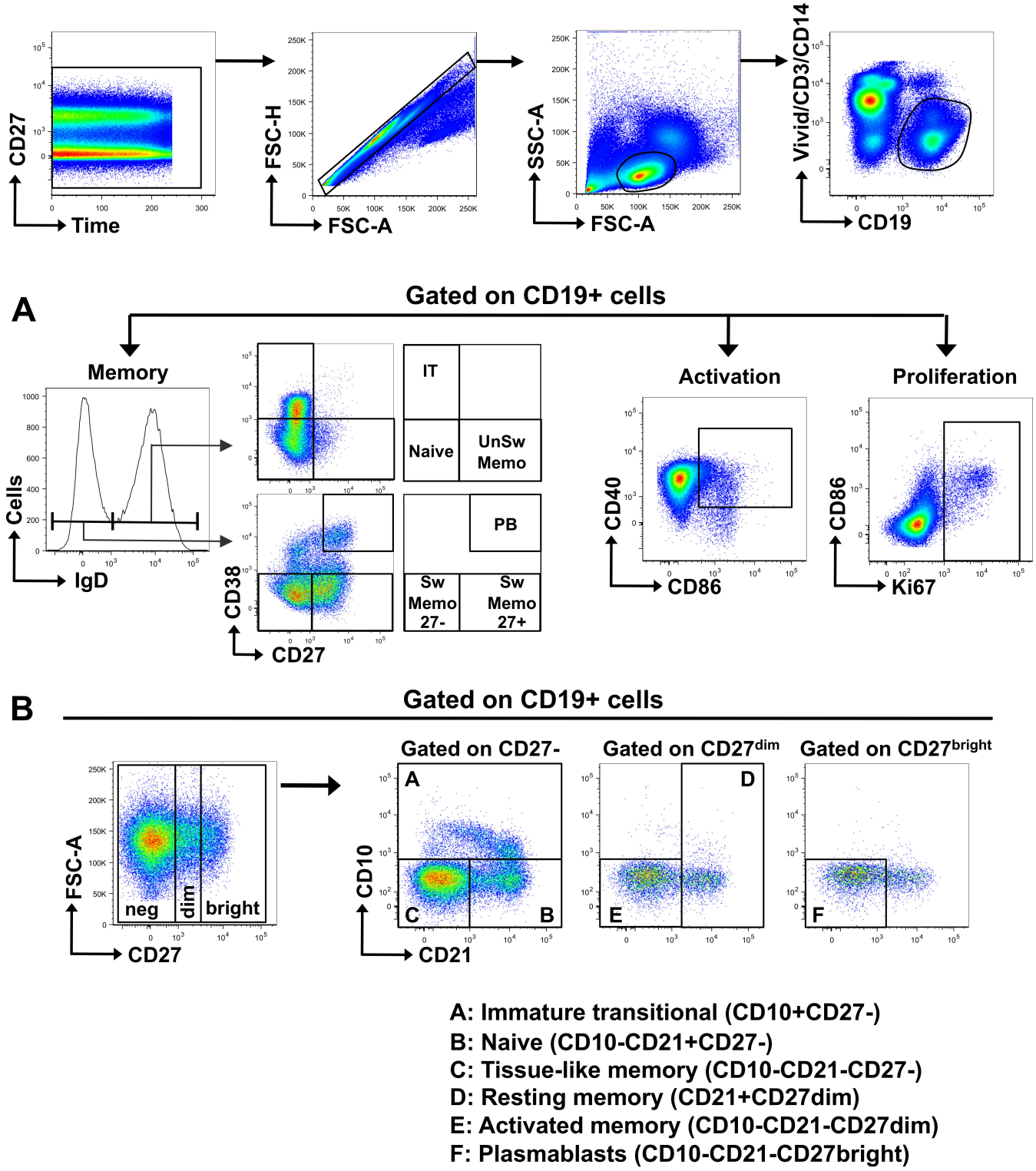
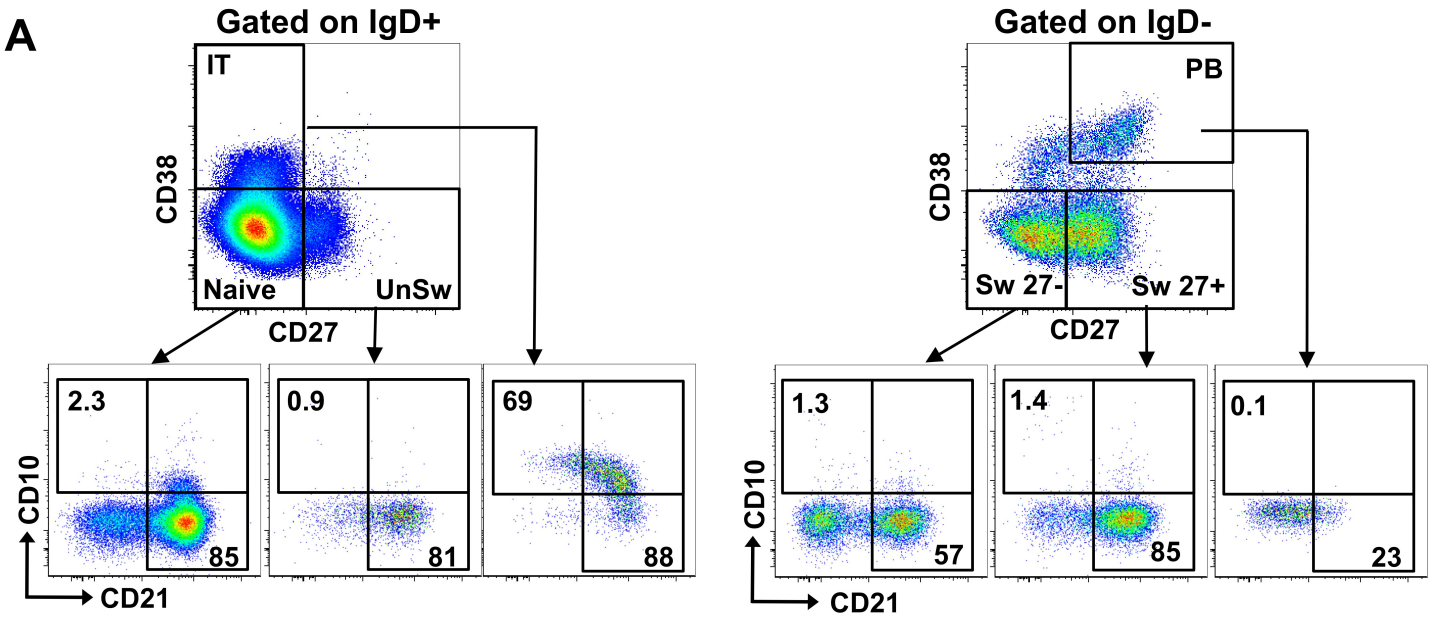
Supplemental Figure 1

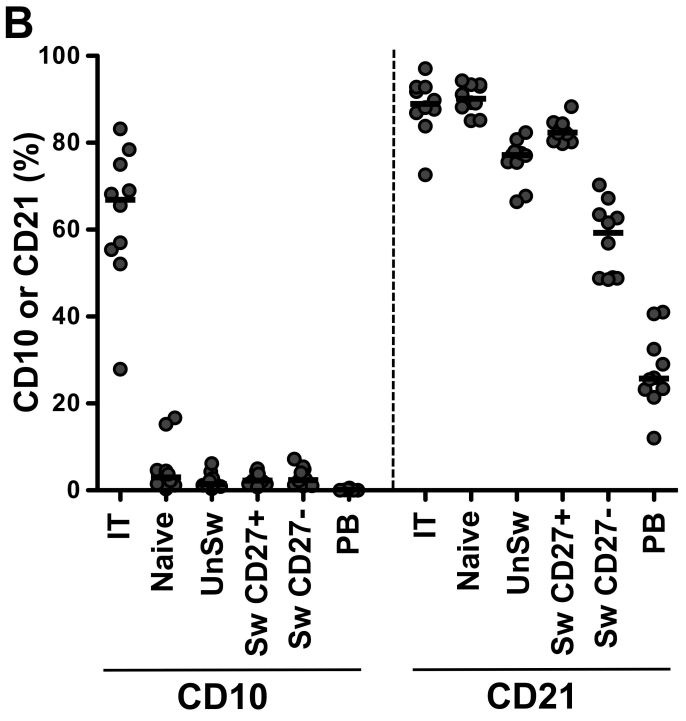


A: Immature transitional (CD10+CD27-)
B: Naive (CD10-CD21+CD27-)
C: Tissue-like memory (CD10-CD21-CD27-)
D: Resting memory (CD21+CD27dim)
E: Activated memory (CD10-CD21-CD27dim)
F: Plasmablasts (CD10-CD21-CD27bright)

Figure S1. Gating strategies. A time gate was included to ensure no shift in fluorescence intensity over time, followed by a singlet gate to exclude doublets from the analysis. CD3 and CD14 markers were used to exclude T cells and monocytes, respectively, which were on the same channel as the viability dye Vivid, used to exclude dead cells. The core marker CD19 was used to identify B cells. **(A)** Identification of B cell memory subsets based on IgD, CD27 and CD38 expression. **(B)** Alternative gating strategy based on CD27, CD10 and CD21 expression to delineate memory B cell subsets.

Supplemental Figure 2





C

	IT	Naive	UnSw	Sw CD27+	Sw CD27-	PB
IgD	+	+	+	-	-	-
CD27	-	-	+	+	-	-
CD38	+	-	-	-	-	+
CD10	+	-	-	-	-	-
CD21	- or +	+	+	+	- or +	-

Figure S2: Expression of CD10 and CD21 in B cell subsets delineated using IgD, CD27 and CD38 expression. (A) Representative flow plots from one HIV-infected individual after ART. **(B)** Frequencies of CD10 and CD21 in each B cell subsets in HIV-infected individuals post-ART (n=10). The majority (~70%) of immature transitional B cells express CD10, while all other B cell subsets were predominantly negative for this marker. CD10 expression on IT B cells may be an underestimation due to non-optimal CD10 separation. **(C)** Summary of the phenotype of B cells subsets. Although the majority of IT B cells express CD21, T1 IT B cells are CD21lo.

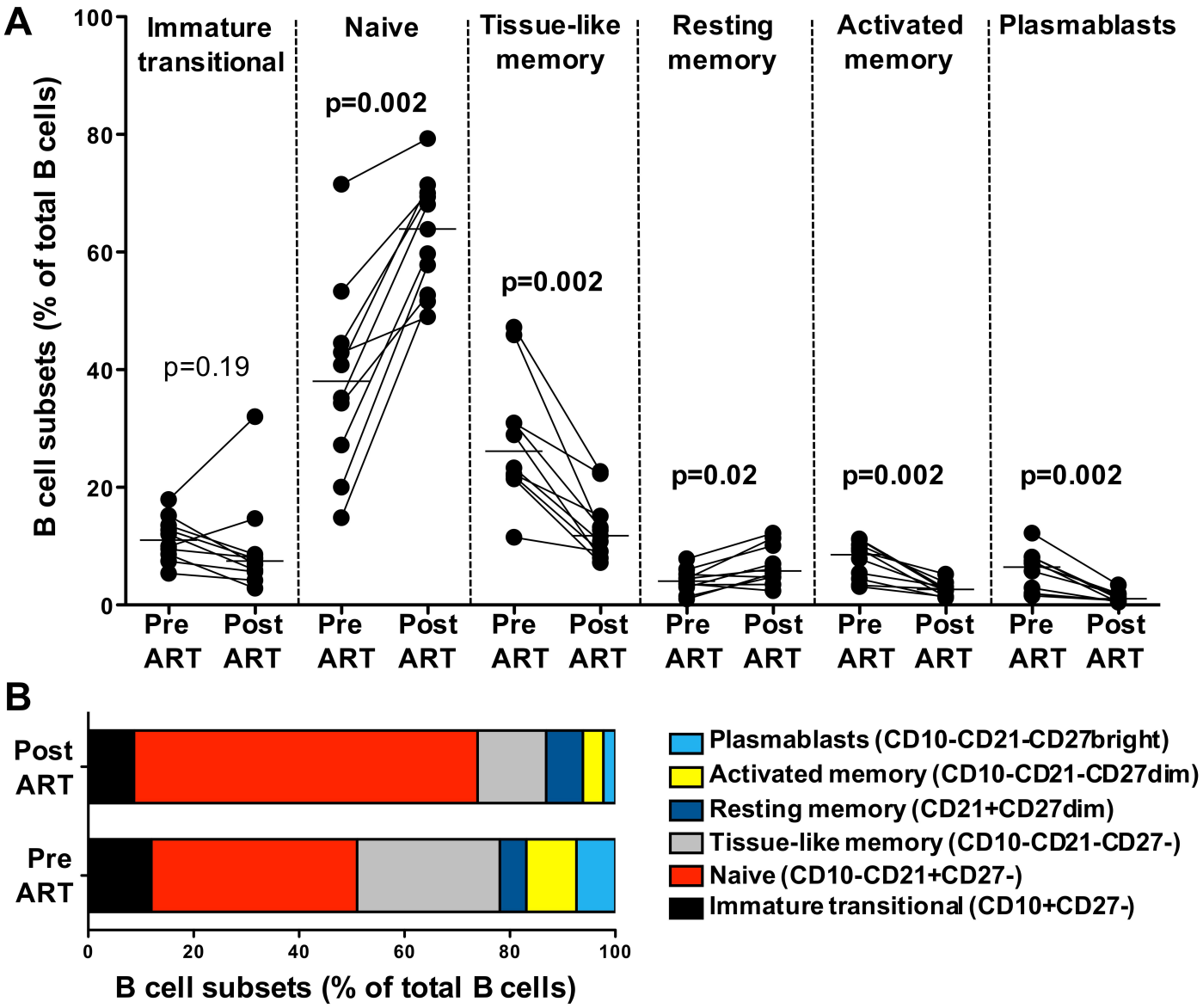


Figure S3. Evolution of B cell subsets defined based on CD27, CD10 and CD21 expression pre- and post-ART. (A) Frequencies of B cell subpopulations before and after ART in 10 HIV-infected participants. Horizontal lines indicate the median. Statistical significance was calculated using Wilcoxon paired test. (**B**) Stacked bar graph showing the median frequency of each B cell subset before and after ART. Each color identifies a B cell subpopulation, as indicated in the adjacent key. The gating strategy is shown in Supplemental Figure S1.

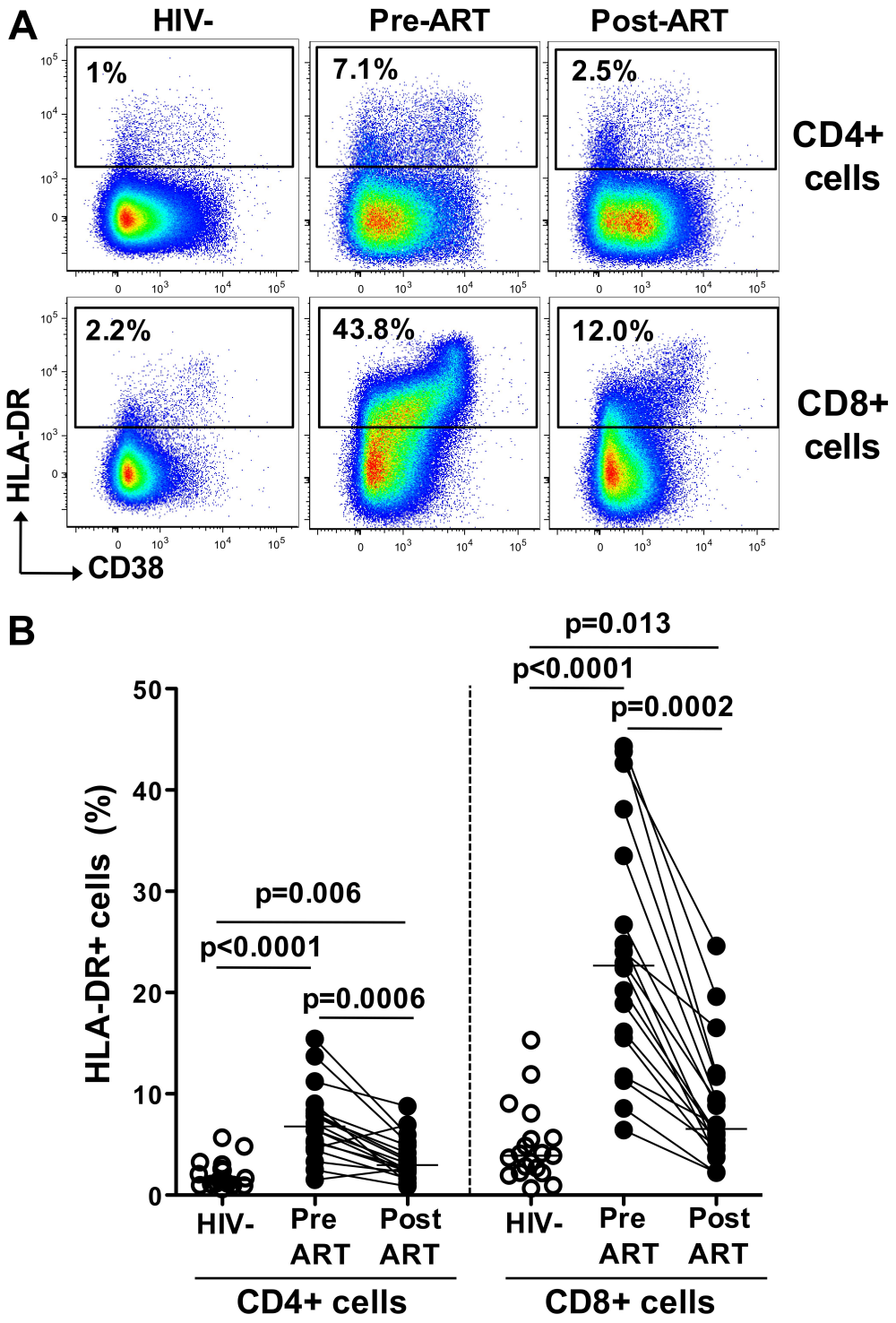


Figure S4. Effect of ART on CD4+ and CD8+ T cell activation levels measured by HLA-DR expression. (A) Representative flow plots of HLA-DR expression on T cells from one HIV-uninfected and one HIV-infected (pre- and post-ART) individual. **(B)** Frequencies of HLA-DR+ CD4+ and CD8+ T cells in 19 HIV-uninfected (open circles) and 18 HIV-infected (closed circles) individuals pre- and post-ART. Horizontal lines represent the median. Statistical significance was calculated using Mann-Whitney U test and Wilcoxon Signed Rank for unmatched and matched samples, respectively.

Supplemental Figure 5

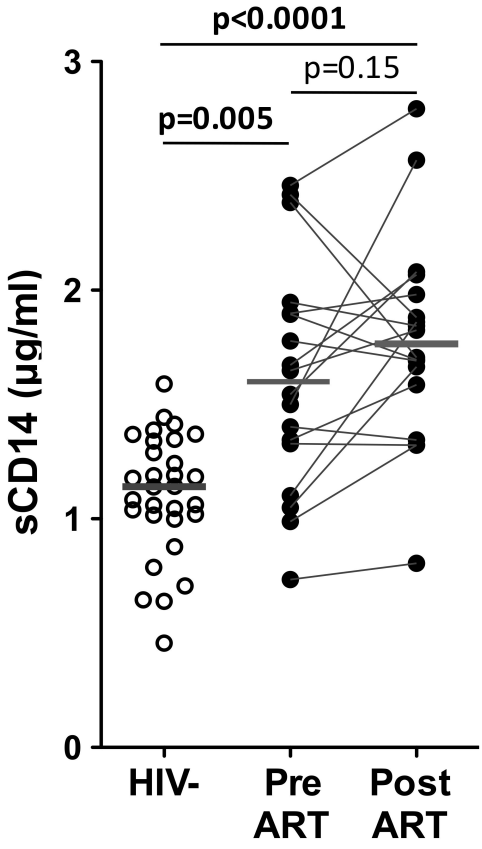


Figure S5. Effect of ART on plasma levels of sCD14. Soluble CD14 was measured in 18 HIV-infected women and 30 HIV-uninfected women from the same community by ELISA (R&D Systems). Horizontal black lines represent the median. Statistical significance was calculated using a non-parametric Wilcoxon matched test (pre- and post-ART) and the Mann-Whitney test for comparisons with HIV-uninfected individuals.