Characteristics and Clinical Relevance of Lost T-Cell Homeostasis and T-cell Dysregulation In HIV-1 Infected Patients Receiving Antiretroviral Therapy.

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Abstract

In the early years of the HIV epidemic, HIV infection was considered a terminal disease characterized by the onset of opportunistic infections and malignancies known as AIDS-defining illnesses (ADIs). The earliest surrogate laboratory marker for this syndrome was a decline in the CD4:CD8 ratio. Upon the identification of HIV as the causative agent of the disease, studies of viral dynamics led to a better understanding of HIV pathogenesis. Results from these studies led to the establishment of the CD4+ T-cell count and HIV viral load as the preferred biomarkers for the monitoring and management of HIV related clinical outcomes. Over the last 2 decades, the advent of effective antiretroviral therapy (ART) has transformed this disease from an acute and lethal condition to a manageable chronic infection. Although ADIs are no longer a major threat to successfully treated HIV patients, an emerging set of non-AIDS illnesses (NADIs) have been identified. These comorbidities, which are usually found in aging individuals, are postulated to result from persistent immune dysfunction that is generally associated with chronic inflammation and immunological aging; a process often referred to as inflammaging. This new clinical picture has prompted research on the long-term residual immune changes stemming from prior chronic HIV infection in the context of successful antiretroviral therapy. The work presented in this thesis provides a foundation for the rational monitoring of treated HIV patients, using a composite measurement of clinically available immune markers. Whereas other studies have historically stressed the depletion of CD4+ T-cell counts as the hallmark of HIV-mediated immunologic dysfunction; the findings described herein indicate that the immuno-pathogenic impact of HIV infection is multifaceted and only partially reversible with long-term suppressive treatment. This thesis also provides a basis for further understanding the role of inflammaging in the context of successfully treated HIV infection. Our findings may prove relevant to the development of new HIV treatment strategies in the era of effective ART.

Résumé

Au commencement de l'épidémie du VIH, l'infection par le VIH était perçue comme une maladie mortelle caractérisée par l'émergence d'infections et de tumeurs opportunistes liées au SIDA; la phase terminale de la maladie. La dérégulation du rapport CD4:CD8, fût l'un des premiers marqueurs associés à l'immuno-pathologie causée par le VIH. Les analyses de recherche sur les dynamiques de réplication virale ont contribué aux connaissances accrues de la pathogénèse du VIH. Suite aux résultats de ces analyses, le taux de cellules T CD4 et la charge virale du VIH sont devenus les marqueurs de choix pour le suivi et le traitement des conditions cliniques liées à l'infection par le VIH. Au cours des 20 dernières années, l'avènement de la trithérapie a révolutionné la vie des personnes infectées par le VIH en transformant l'infection par le VIH en une maladie chronique. Cependant, bien que les maladies liées au SIDA ne représentent plus un problème majeur pour les individus traités pour le VIH, certaines de maladies non-liées au SIDA sont désormais une cause majeure de mortalité chez les individus infectés par le virus. Ces maladies sont généralement observées chez les personnes âgées, et semblent être associées à des dérèglements immunitaires qui caractérisent un état d'inflammation chronique et de sénescence immunitaire appelé « inflammaging ». Cette nouvelle présentation clinique de l'infection par le VIH a influencée de nouvelles recherches sur les conséquences de la chronicité de la maladie sur le système immunitaire. La présente thèse propose le choix rationnel d'un groupe de marqueurs immunologiques qui permettraient une évaluation intégrale du système immunitaire des patients traités pour le VIH. Bien que la baisse du taux de cellules T CD4 soit généralement considérée comme le principal dérèglement immunitaire chez les patients infectés par le VIH, les résultats présentés dans cette thèse indiquent que l'infection par le VIH affecte d'autres paramètres immunitaires, qui ne sont pas complètements rétablis malgré un traitement efficace. Cette thèse contribue également à une meilleure compréhension de la sénescence immunitaire dans un

contexte d'infection par le VIH sous contrôle thérapeutique. Nos résultats pourraient être utiles pour le développement de nouvelles stratégies de traitement du VIH à l'ère des traitements antirétroviraux efficaces.

Preface

The thesis is manuscript-based, composed of a literature review and a manuscript Chapter, and prepared in accordance to the guidelines outlined by the department of Graduate and Postdoctoral Studies. Below is a list of the manuscripts included in this thesis as well as the author contribution:

Chapter 2: Delay in cART Initiation Results in Persistent Immune Dysregulation and Poor Recovery of T-cell Phenotype Despite a Decade of Successful HIV Suppression

Patricia Ndumbi, Julian Falutz, Nitika Pant Pai, and Christos M. Tsoukas.

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Contributions of authors: Ndumbi P. and Tsoukas C.M. designed the research; Ndumbi P. performed the analyses and prepared the manuscript; Pai NP. provided assistance with the statistical methods used and Falutz J. critically reviewed the final version of the manuscript.

Chapter 3: Characteristics and determinants of T-cell phenotype normalization in HIV-1-infected individuals receiving long-term antiretroviral therapy.

Patricia Ndumbi, Jennifer Gillis, Janet Raboud, Curtis Cooper, Robert S Hogg, Julio SG Montaner, Ann N Burchell, Mona R Loutfy, Nima Machouf, Marina B Klein, Chris Tsoukas and The Canadian Observational Cohort (CANOC) collaboration.

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Contributions of authors: Ndumbi P. and Tsoukas C.M. designed the research; Ndumbi P. prepared the manuscript; Ndumbi P., Raboud J. and Gillis. J. interpreted the data; Raboud J. and Gillis. J. provided assistance with the statistical methods used; Klein M., Cooper C., Hogg R.S., Loutfy M.R., Machouf N., Montaner J. and Burchell A.N. critically reviewed the final version of the manuscript.

<u>Chapter 4:</u> Clinical impact of altered T-cell homeostasis in treated HIV patients enrolled in a large Canadian Observational Cohort.

Patricia Ndumbi, Jennifer Gillis, Janet Raboud, Marina Klein, Curtis Cooper, Robert S Hogg, Mona R Loutfy, Nima Machouf, Ann Burchell, Julio SG Montaner, Chris Tsoukas and The Canadian Observational Cohort (CANOC) collaboration

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<u>Chapter 5:</u> Comprehensive Evaluation of the Immune Risk Phenotype in Successfully Treated HIV-infected Individuals

Patricia Ndumbi, Louise Gilbert and Christos M. Tsoukas

Under review at PLoS One.

Contributions of authors: Ndumbi P. and Tsoukas C.M. designed the research ; Ndumbi P. prepared the manuscript, performed the laboratory assays and analysed the data; Gilbert L. provided assistance with the laboratory assays.

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List of abbreviations

ADIs - AIDS-defining illnesses

AICD - activation induced cell death

AIDS - acquired immune deficiency syndrome

APCs - antigen presenting cells

ART - antiretroviral therapy

ARV - AIDS-associated retrovirus

CDC - centers for disease control and prevention

CMI - cell-mediated immunity

CMV - cytomegalovirus

CTLs - cytotoxic T-lymphocytes

CVD - cardiovascular disease

FADD - fas-associated death domain

FasL - fas-ligand

GRID - gay-related immune deficiency

HAART - highly active antiretroviral treatment

HC - homeostatic cycling

HIV - human immunodeficiency virus

HL - hayflick limit

HPE - homeostatic peripheral expansion

HsCRP - high-sensitivity C-reactive protein

HTLV-III - human T-lymphotrophic virus type III

IDDM - type-1 insulin-dependent diabetes mellitus

IFN-γ - interferon gamma

IN - integrase

IRP - immune risk phenotype

KLRG1 - killer cell lectin-like receptor G1

KS - kaposi sarcoma

LAV - lymphadenopathy-associated virus

MACS - multicenter AIDS cohort study

NADIs - non-AIDS defining illnesses

NK - natural killer

NNRTI - non-nucleoside reverse transcriptase inhibitors

NRTI - nucleoside reverse-transcriptase inhibitors

PCP - pneumocystis jirovecii pneumonia

PD-1 - programmed death-1

PI - protease inhibitors

PR - proteases

pre-T-cells - precursor T-cells

RA - rheumatoid arthritis

RT - reverse transcriptase

SIV - simian immunodeficiency virus

SLE - systemic lupus erythematous

spMHC - self-peptide/MHC

TCR - T-cell receptor

T_{CM} - central memory T-cells

T_{EM} - effector memory T-cells

T_{FH} - follicular helper T-cells

Tim-3 - T-cell immunoglobulin and mucin domain-containing molecule 3

TLRs - toll-like receptors

TNF-α - tumor necrosis factor alfa

TRECs - TCR rearrangement excision circles

Tregs - Regulatory T-cells

T_{RM} - tissue-resident memory T-cells

Chapter 1:

Introduction & Thesis Objectives

1.1 Introduction to HIV/AIDS

Over the last 3 decades, there have been significant achievements in the treatment and management of HIV/AIDS, the most important of which being the development of effective antiretroviral therapy (ART). HIV-infected patients who have access to ART and remain adherent to it are able to control viral replication, reduce virus transmission, improve immune function and avoid AIDS-related syndromes. This progress has transformed HIV disease from a dreadful terminal condition characterized by opportunistic infections and malignancies known as AIDS-defining illnesses (ADIs) to a manageable chronic infection. Although ADIs are no longer a major threat among successfully treated HIV patients, an emerging set of non-AIDS comorbidities have been identified. These comorbidities that are usually found in aging individuals, appear to be the result of a complex interplay of the viral induced immune deficiency, chronic inflammation and treatment side effects. Despite effective ART, HIV infected individuals are still at higher risk for poor clinical outcomes compared to healthy uninfected individuals and current research efforts now focus on understanding this important residual pathophysiology.

1.1.1 Acquired Immune Deficiency Syndrome

The first official description of a new acquired immune deficiency syndrome (AIDS) took place in 1981, when the Centers for Disease Control and Prevention (CDC) reported unusual cases of PneumoCystis Jirovecii Pneumonia (PCP) and of Kaposi Sarcoma (KS) among young homosexual men living in New York and Los Angeles [1,2]. During this period opportunistic infections such as PCP, a fungal infection of the lungs, and Kaposi Sarcoma, were both rare in the United States of America and had only occurred in severely immuno-compromised individuals. The CDC report therefore concluded that the affected men experienced “a cellular-immune dysfunction related to a common exposure that predisposed individuals to opportunistic infections” [2]. Since no

causative agent was identified at the time, the diagnosis of AIDS in suspected cases was solely based on clinical presentation and laboratory abnormalities. These abnormalities included the presence of immune dysregulation, as noted by an inversion of the CD4:CD8 T-cell ratio. Furthermore, those with AIDS also experienced severe lymphopenia, dysfunctional cellular immunity characterized by reduced lymphocyte proliferation and abnormally high levels of activation and inflammation markers [3,4]. Initial epidemiologic findings lead to the inaccurate assumption that this condition was restricted to homosexual men. As a result, the term gay-related immune deficiency (GRID) was coined [5,6]. It quickly became apparent that the disease also affected intravenous drug users, Haitians, haemophiliacs, heterosexual women and babies born from affected mothers [7-11]. Thus, in 1982, the term Acquired Immunodeficiency Syndrome (AIDS) was proposed by the CDC **[12]**.

The frequent occurrence of immune abnormalities in asymptomatic individuals belonging to these at risk groups, as well as the demonstration that transmission occurred in clusters, suggested that an infectious agent transmittable through blood and sexual contact[1,4,10,12-14]. Certain herpes viruses were originally thought to be the causative agent of AIDS [15,16] since years prior to the development of AIDS, patients commonly had a history of mononucleosis-like symptoms such as lymphadenopathy, fever and fatigue. Of more obvious interest was cytomegalovirus (CMV), because it was found in very high prevalence among these patients [2,17,18]. Furthermore, high titers of antibodies against CMV were observed and CMV end-organ diseases such as retinitis and colitis were common and often led to death [1,19]. Finally, CMV had previously been shown to cause CD4:CD8 T-cell dysregulation, to have transient immunosuppressive properties and to be associated with Kaposi Sarcoma [20-22]. However, subsequent studies showed that the causative role of CMV in Kaposi Sarcoma was implausible [23]. Furthermore since this virus was ubiquitous in the general population, and particularly among homosexual men, the sudden appearance of the

AIDS syndrome was perplexing. Failure to associate AIDS at that time with any known etiologic agent lead scientists to hypothesize the existence of a newly recognized pathogen; the most probable being a retrovirus. Two groups in the USA and one in France described the AIDS-associated retrovirus (ARV), the human T-lymphotrophic virus type III (HTLV-III) and lymphadenopathy-associated virus (LAV) [24-26]. Ultimately these three virions were found to be the same, and a common more appropriate descriptive terminology: Human Immunodeficiency Virus (HIV) was introduced.

1.1.2 Human Immunodeficiency Virus

In 1983 Luc Montagnier and Francoise Barre-Sinoussi, researchers at the Institut Pasteur in Paris, were able to isolate this new retrovirus from blood samples of symptomatic haemophiliacs [24]. A year later, Dr. Robert Gallo provided the first empirical evidence that the same virus was the causative agent of AIDS [26-28]. The biological features of HIV have been extensively reviewed by Dr. Jay Levy [25]. This retrovirus is classified within the lentivirus genus. As a prototypic retrovirus, HIV converts its RNA genome into DNA by a process called reverse transcription. In addition to its genomic material, the HIV virion carries enzymes that are critical for its development such as reverse transcriptase (RT), proteases (PR) and integrase (IN). Based on studies conducted shortly after discovery of the virus, it became evident that it had a particular tropism for CD4+ T-cells [29].

1.1.3 HIV pathogenesis

The primary mode of HIV transmission is through sexual contact [30]. The virus generally gains entry inside the host by crossing gastrointestinal or genital mucosae. Following an initial local replication within target cells of these mucosal surfaces, HIV is disseminated throughout the lymphatic tissues [31]. The virus then infects its target cells by attaching to a CD4 molecule and a chemokine receptor (CCR5 or CXCR4) on the cell surface [32].

The natural course of HIV infection can be divided into 3 main phases: acute infection, chronic infection and AIDS. Acute infection occurs shortly after HIV transmission and can last up to up to three months. During the initial weeks of acute infection, Clinical features associated with HIV patients include mononucleosis-like symptoms such as myalgia, pharyngitis, fever, rash, and swollen lymph nodes [33]. This phase is characterized by the presence of high levels of infectious virus in the peripheral blood [34,35]. In response to this high HIV viremia, CD8+ T-cells undergo a significant expansion in the peripheral circulation. These cells have the capacity to suppress viral replication via cytotoxic or non-cytotoxic anti-HIV responses [36-38]. The expansion of CD8+ T-cells creates an imbalance in the CD4:CD8 T-cell ratio. The inversion of this ratio is the first of many immune abnormalities that are established during acute infection, and that are ultimately associated with progressive immune dysfunction [39]. Moreover, a massive depletion of CCR5-expressing memory CD4+ T-cells occurs in the gut associated lymphoid tissue (GALT) [40]. CD4+CCR5+ T-cells represent prime targets for viral infection and replication, mainly owing to their activated phenotype [41]. These GALT-associated T-cells also constitute the majority of the total body CD4+ T-cell population [42]. Therefore, very early in the infection, HIV imposes a significant strain on the immune system's ability to maintain CD4+ T-cell homeostasis in the body [43].

Antibodies to HIV antigens such as Gag, Env and p24 are only formed and detected approximately 12 weeks post-infection [44,45]. Together with CD8+ T-cells, HIV-specific neutralizing antibodies contribute to the cell-mediated and humoral responses against the virus [46,47]. The emergence of HIV-specific immune responses coincides with an important decline in HIV viral load, which marks the beginning of the chronic phase of the infection [48]. Although the duration of this phase can vary greatly between individuals it often progresses fairly slowly, lasting approximately 10 years in untreated patients [49,50]. Chronic HIV infection is often described as a period of clinical latency due to asymptomatic state of the patients. However, the term "latency" can be misleading since a persistent and gradual deterioration of the immune system occurs. This is manifested by a persistently low CD4:CD8 ratio and a slow but steady depletion of CD4+ T-cell cells [4]. Other key immune features of chronic HIV infection include: increased T-cell apoptosis, loss of naïve CD4+ T-cells, altered bone marrow infrastructure and impaired thymic function [51-55]. These perturbations are believed to be in part, the result of chronic immune activation, which is essentially a generalized state of continuous activation of cells of the immune system. Immune activation becomes apparent as the expression of markers of cellular activation (CD38, CD69 and HLA-DR), proliferation (Ki67) inflammation (IL-6, D-dimer, hsCRP and sCD14) and apoptosis (CD95, Annexin V, 7A6) increase on the surface of T-cells and in the plasma [56-58]. The state of generalized activation that characterizes chronic HIV infection ultimately leads to the destruction of lymphoid tissue architecture, which in turn has deleterious effects on the maintenance of lymphocyte homeostasis [55,59].

In the absence of efficacious treatment, the inevitable outcome of this progressive immune dysregulation is a severe deterioration of immune function and a rebound of the HIV viral load. This last phase of the disease, also known as AIDS, is characterized by very high HIV viremia,

severely low CD4+ T-cell counts (below 200 cells/mm3), altered cytokine production and poor proliferative response to neoantigens [60-63]. It is at this stage that patients become susceptible to opportunistic infections and AIDS-defining diseases such as KS and PCP.

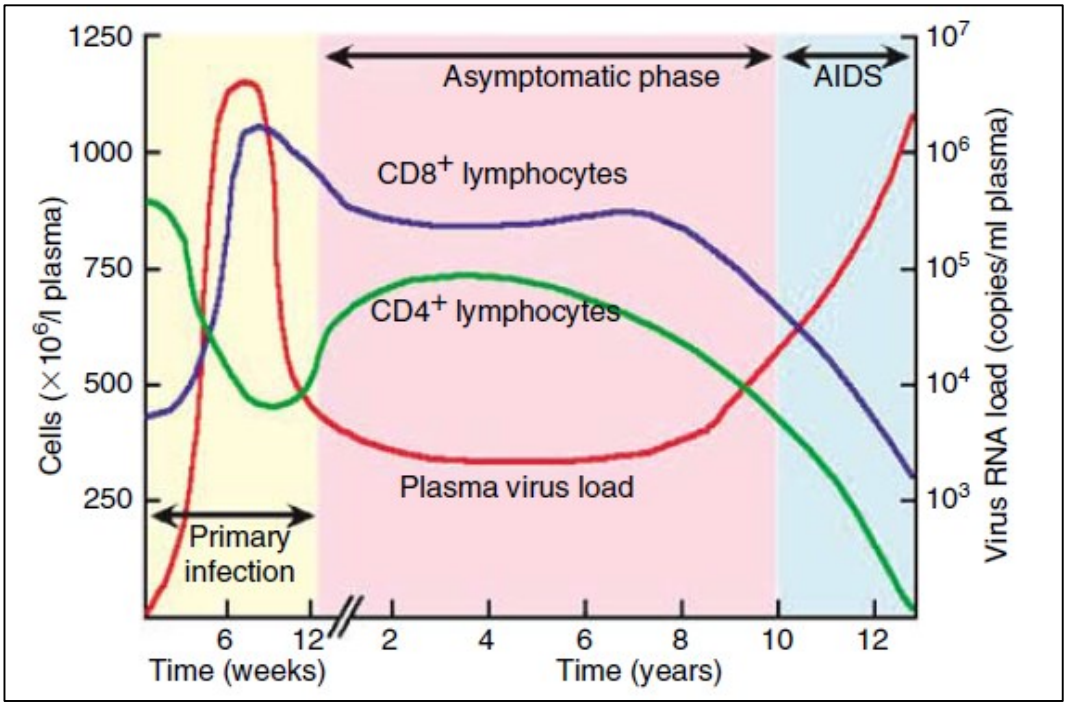

Figure 1 [64]. **Schematic of typical course of HIV-1 infection showing changes in CD4 and CD8 T-cell counts in peripheral blood and pVL** (Reprinted with permission from Macmillan Publishers Ltd: Immunol Cell Biol ; 85(1):6-15, copyright 2006).

1.1.4 Highly Active Antiretroviral Therapy

In 1987, the first successful anti-HIV drug called AZT (zidovudine) was approved in the USA. This drug could reduce viral replication by inhibiting HIV reverse transcriptase. The remarkable benefits of AZT included a marked decrease in opportunistic infections and an increase in immune function among treated patients [65]. Unfortunately, owing to the hyper-mutability of the virus, drug resistant HIV variants rapidly emerged and the efficacy of AZT was short-lived [66]. Consequent attempts to control viral replication with dual nucleoside therapy also failed.

The grim outlook of the first 15 years of the AIDS epidemic took a pivotal turn with the advent of Highly Active Anti-Retroviral Treatment (HAART) in 1996. HAART is incontestably the most significant advance in the clinical management of HIV infection. It was first introduced as the concurrent use of combinations of 3 anti-HIVdrugs belonging to at least two classes. These included nucleoside reverse-transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI). In 2007 two more classes of drugs became widely available for the treatment of HIV, entry inhibitors and integrase inhibitors. All drugs were designed to target specific steps in the infection and replication cycle of the virus [67].

In the years that followed its approval, HAART has contributed to a substantial reduction in AIDS-associated morbidity and mortality; thus converting a nearly universally fatal disease into a manageable chronic disease [68]. However, as HIV infected individuals live longer, we are now witnessing a change in their mortality and morbidity profile. While the extensive use of antiretroviral drugs has dramatically decreased the occurrence of AIDS defining illnesses (ADIs), the proportion of non-AIDS defining illnesses (NADIs) (prominently including hepatic, cardiovascular and renal diseases as well as non-AIDS malignancies) has significantly increased

among successfully treated HIV+ patients [69-71]. In the general population, some of these clinical disorders are observed in the uninfected elderly and are typically associated with chronic inflammation. Thus, despite successful drug-mediated viral suppression, HIV patients remain at higher risk for age-associated comorbidities compared to uninfected individuals.

Cumulative drug toxicity may account for some of these complications. For instance, Tenofovir, has been associated with nephrotoxicity [72], Abacavir possibly with myocardial infarction and d4T with lipodistrophy [73,74]. Similarly, certain commonly used PIs have been associated with an increased risk of hypercholesterolemia and cardiovascular disease (CVD) [75,76]. However, the latest generation of drugs is generally associated with less toxicity, limited side effects and higher antiviral potency [77]. Furthermore, current treatment guidelines recommend regimens not only based on their antiviral efficacy, but also on their long-term toxicity [78]. Finally, findings from the SMART study have demonstrated that the intermittent use of ART through guided treatment interruption is associated with higher levels of activation markers and an increased risk of CVD, when compared to continuous use of ART [79,80]. Antiretroviral therapy (ART) - associated toxicity may therefore only partially explain the continuously increasing prevalence of NADIs. A rapidly growing body of evidence suggest that most successfully treated HIV individuals exhibit signs of persistent inflammation and immune dysfunction [81-83]. This indicates that, although ART can partially restore the immune system, irreversible immune dysregulation lingers over the course of the disease [84]. These immune abnormalities have been shown to be strong predictors of non-AIDS morbidity and mortality [80,85]. The rising burden of NADIs during chronic HIV disease highlights the importance of elucidating the root causes of persistent immune dysregulation under ART, how they cause morbidity and whether they are reversible.

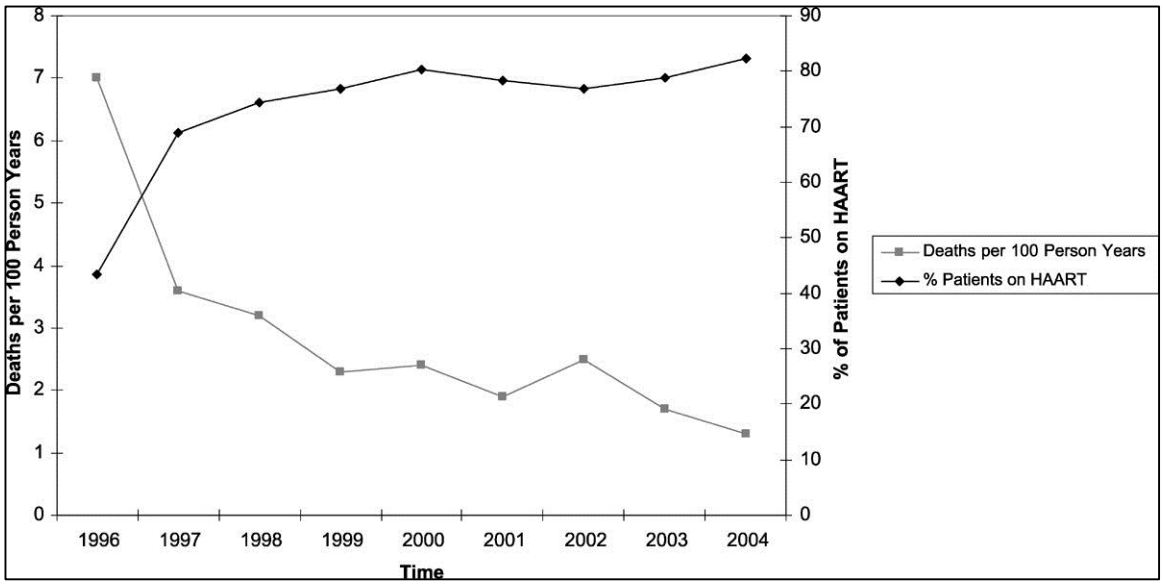

Figure 2 [70]: **Mortality and HAART use over time** (Reprinted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: J Acquir Immune Defic Syndr; 43(1): 27-34, copyright 2006)

1.1.5 Evolution of therapeutic approaches

During the early years of the epidemic, although it was generally accepted that patients with severely low CD4+ T-cell counts should be treated, no consensus existed as to the adequate timing of the treatment. In 1995, David Ho and colleagues provided seminal data on the dynamics and pathogenesis of HIV infection, which has since influenced the therapeutic approach to HIV disease. They demonstrated that ART-mediated suppression of viral replication (measured by plasma levels of HIV RNA) was associated with a concomitant increase in CD4+ T-cell counts [86]. Based on this novel understanding of HIV pathophysiology, a "hit hard, hit early" approach to the disease was recommended. This notion involved an aggressive treatment of the virus with ART at the earliest stage of the infection [87]. However, issues relating to adverse side effects, drug resistance and drug toxicity made this approach unpopular.

Today, due to the availability of a new generation of more potent and better-tolerated drugs, there is a broad consensus that therapy should be initiated as early as possible. Many guidelines now suggest that treatment should be started when CD4 levels fall below 500 cells/μL [88,89]. Evidence from a large non-randomized study by the NA-ACCORD has highlighted the positive effect of early treatment initiation. In this study, it was observed that patients initiating ART with CD4+ T-cell counts below 350 cells/μL had a 69% increase in the mortality risk, when compared to those initiating treatment at CD4 levels below 500 cells/mL [90]. Although these findings suggested a plausible clinical benefit from early treatment initiation, they were subjected to limitations intrinsic to the retrospective design of the study. These limitations included the potential occurrence of unmeasured confounders, which may have influenced the analysis. Recent data from the first randomized controlled trial assessing the potential benefits of early treatment initiation indicate that early ART initiation is associated with a reduced incidence of both AIDS and non-AIDS related events [91]. From an immune point of view, early ART initiation has also been associated with benefits such as improved functional immune responses [92,93]. Furthermore, several studies have reported better proliferative capacity and higher frequency of cytokine-secreting CD4+ T-cells in individuals receiving treatment during acute infection [94,95]. Collectively, these findings suggest that earlier ART initiation may be associated with better clinical and immune outcomes.

1.1.6 The end of AIDS

The use of biological markers ("biomarkers") is instrumental for the diagnosis, staging and prognosis of diseases. Ideally, a good biomarker should predict relevant clinical outcomes while clarifying pathogenic mechanisms [96]. In the early years of the epidemic, a better understanding of HIV viral dynamics and pathogenesis led to the establishment of the CD4+ T-cell count and HIV viral load as the preferred biomarkers of HIV disease clinical outcomes [63,97]. Early studies demonstrated a clear correlation between higher levels of HIV RNA and faster decline of CD4+ T-cell counts, ultimately leading to more rapid disease progression [98]. Conversely, CD4+ T-cell counts were associated with higher risk for AIDS-related diseases and shorter survival [99]. Initially, the key focus of HIV clinical management was to prevent AIDS-related morbidity and mortality; which had been associated with severe immune deficiency [100,101]. However, in the current era of long-term suppressive ART, AIDS-defining illnesses no longer represent the primary clinical challenge [70]. As previously mentioned, non-AIDS events such as cardiovascular, renal and hepatic diseases; are now the most clinically-relevant outcomes in successfully treated HIV patients. Of importance, these emerging clinical end-points are also associated with chronic inflammation and immune dysregulation [85,102].

Current HIV care therefore requires a new understanding and evaluation of biomarkers in the context of long-term viral suppression. In the past couple of years, a growing focus has been placed on exploring novel biomarkers of immune reconstitution and health status during treated HIV disease. Among these markers, a series of immune mediators that reflect senescence and chronic activation of the immune system have been identified. For instance, in a recent study higher frequencies of activated ($CD38^{+}HLA\text{-}DR^{+}$) and senescent ($CD28^{-}CD57^{+}$) T-cells were associated with an increased risk of subclinical carotid artery disease among treated HIV-positive women

[103]. Furthermore, persistently high levels of inflammatory cytokines such as IL-6 have also been linked to all-cause mortality among HIV patients receiving suppressive ART [104]. Overall these findings highlight the need to better characterize the pathogenesis of treated HIV disease.

1.2 Homeostatic Regulation of T-cells

Much that is understood regarding the impact of HIV infection on the integrity of the immune system is derived from the analysis of peripheral blood lymphocytes. Although it is generally associated with an immune deficiency disease, a closer look at HIV immune pathogenesis shows that it is first and foremost an immune activation and dysregulation disease [4,105]. Immune activation and dysregulation always antecede the clinical presence of immune deficiency. The virus affects both the innate and adaptive arms of the immune system and T-cells are particularly affected. Prior to discussing the impact of HIV infection on T-cell dynamics (section 1.3), an overview of the mechanisms of T-cell production, survival and homeostatic regulation will be provided.

1.2.1 Biology of T-cells

The immune system is a very complex network of cells and organs that protects the body from a variety of infections, tumors and toxic molecules. In vertebrates, the immune system consists of two arms: the innate immunity and the adaptive immunity. Although the two arms of the immune system carry out distinct functions, a constant interaction exists between innate and adaptive cells during the course of an immune response [106,107]. Cells of the innate immune system primarily consist of granulocytes (mast cells, eosinophils, basophils), phagocytes (neutrophils, macrophages, and dendritic cells) and natural killer (NK) cells. The principal mediators of the

adaptive immunity include B-lymphocytes, which confer a humoral or antibody-mediated immunity, and T-lymphocytes, which confer a cell-mediated immunity (CMI) [108]. CMI plays an important role in the clearance of tumour cells and intracellular pathogens such as viruses, fungi, protozoa and mycobacteria [109-111]. Here we explore the characteristics, role and function of T-lymphocytes as the effectors of antigen-specific CMI.

1.2.2 T-cell production

Primary lymphoid organs such as the thymus and the bone marrow play an important role in the generation and development of T-cells. Precursors T-cells (pre-T-cells) originate from haematopoietic stem cells in the bone marrow and eventually migrate to the thymus where they undergo maturation [112]. It is within the thymus that T-cell receptor (TCR) gene rearrangement take place. In addition to assuring a normal T-cell development, TCR rearrangement plays an important role in the generation of the T cell repertoire diversity.

Early thymic immigrants lack both the CD4 and CD8 co-receptors and are therefore called double-negative (CD4-CD8-) T-cells. Double negative cells can differentiate into either γδ or αβ lineages. However, the majority of pre-T-cells (>95%) adopt an αβ phenotype [113]. Later in the course of their maturation αβ pre-T-cells simultaneously acquire CD4 and CD8 markers, thus becoming double-positive (CD4+CD8+) pre-T-cells. At this stage of their thymic development, pre-T-cells are subjected to two rigorous selection processes: positive selection and negative selection. These two mechanisms require an interaction between the TCR and a self-peptide/MHC (spMHC) complex expressed in the thymus [114]. During positive selection, pre-T-cells that adequately recognize and bind the spMHC complex on thymic stromal cells mature into a single-positive stage where they express either CD4 or CD8 on their surface. The differentiation of double positive pre-T-cells into either CD4 or CD8 single positive pre-T-cells is dependent on the type of MHC

molecule recognized by the TCR. Pre-T-cells that recognize MHC-I molecules will cease to express the CD4 co-receptor and acquire a CD8 phenotype, while those that recognize MHC-II molecules will lose their CD8 expression and acquire a CD4 phenotype [115,116].

Following positive selection, survivor pre-T-cells migrate to the thymic medulla where they interact with spMHC complex on antigen presenting cells (APCs) such as dendritic cells, B-cells and macrophages. T-cell precursors that bind to the spMHC complex with too high of an affinity are eliminated via programmed cell death (apoptosis). This process is called negative selection [117]. Thus, by eliminating self-reactive pre-T-cells from the repertoire, thymic education ensures the generation of T-cells that can distinguish "non-self" (or external) antigens from antigens that originate within the body [118]. Failure to induce self-tolerance can ultimately lead to the emergence of autoimmune diseases [119]. While the majority of pre-T-cells are eliminated during thymic education, a select pool of survivors differentiates into two functionally and phenotypically distinct subsets that are exported to the peripheral blood and lymphoid organs: helper or CD4+ T-cells and cytotoxic or CD8+ T-cells. Maintaining a balance between these two T-cell subsets is essential for an optimal performance of the immune system.

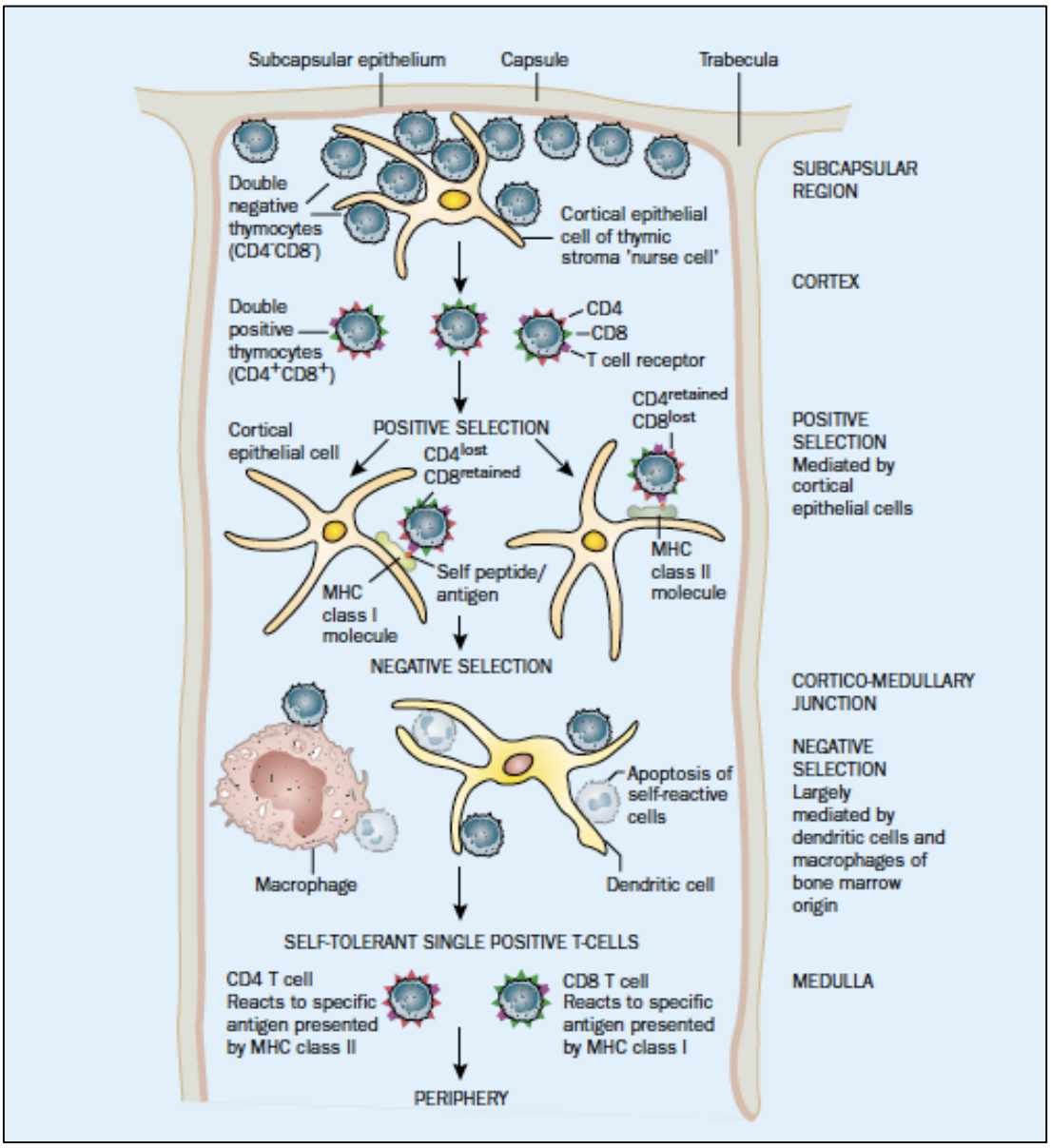

Figure 3 [108]: **T-cell development in the thymus** (Reprinted with permission from Elsevier: Lancet ; 357(9270): 1777-89, copyright 2001).

1.2.3 Structure of the TCR

The T-cell receptor (TCR) is a multimeric signaling molecule that is found on the surface of all T-cells. Its main function is to recognize antigen:MHC complexes. Its structure consists of a heterodimer composed of two different protein chains. 95% of T-cells have TCRs consisting of an αβ heterodimer, while 5% have TCRs consisting of a γδ heterodimer. The TCR is associated with 2 main accessory molecules which play an important role in signal transduction during T-cell activation: a CD3 complex and a ς chain. Together with the TCR, these molecules form the TCR complex [120]. In addition to these accessory molecules, the TCR requires the presence of co-receptors such as CD4 and CD8 in order to efficiently bind to MHC-II and MHC-I molecules, respectively [121]. Upon engagement of the TCR complex and its co-receptor (either CD4 or CD8) with the appropriate antigen:MHC complex, an elaborate series of signals is propagated from the receptor to the inside of the T-cell, which ultimately leads to the transcription of genes involved in T-cell proliferation and differentiation [122-125].

1.2.4 T-cell interaction with APC

By definition, an APC is a cell type capable of processing antigens and displaying them on their surface via an MHC molecule for subsequent recognition by T-cells. As previously mentioned, T-cells can recognize antigen presented in the context of MHC-I (CD8+ T-cells) or MHC-II (CD4+ T-cells). There are two classes of APCs: non-professional and professional. While non-professional APCs only express MHC-I, professional APCs display both MHC-I and MHC-II on their surface. These cells can therefore present antigens to both CD8+ T-cells and CD4+ T-cells, respectively. In addition to constitutively expressing MHC-II, these cells also provide important co-stimulatory molecules that promote the activation, clonal expansion and differentiation of these

cells [126,127]. For this reason, the term APC generally refers to professional APCs. There are three types of APCs: dendritic cells, macrophages and B-cells [128-130]. Dendritic cells are the predominant type of APCs. These cells use pattern recognition receptors such as the toll-like receptors (TLRs), to scan tissues and blood for pathogens like viruses and bacteria [131]. Upon encounter and ingestion of a foreign antigen, immature dendritic cells become activated and begin to express co-stimulatory molecules that are essential for T-cell activation. These cells also up-regulate the surface expression of CCR7, a protein that allows them to migrate to secondary lymphoid tissues (eg: lymph node, spleen and mucosa associated lymphoid tissue), where they undergo maturation and become potent T-cell activators [132].

1.2.5 T-cell co-stimulation

The TCR signalling process described in section 1.2.3 is not by itself sufficient for the induction of an effective immune response. T-cells require a second signal to be productively activated [126]. This co-stimulatory signal is MHC independent and is mediated by molecules from the CD28 receptor family (CD28 and ICOS) and the tumor necrosis factor receptor family (CD27, OX-40, 4-1BB and CD40L) [133]. The best characterized of these co-stimulatory molecules is the cell surface protein CD28. The primary role of T-cells dual stimulation through the TCR and CD28 is to promote T-cell proliferation, differentiation and cytokine production [134]. The ligands of CD28 are the B7.1 (CD80) and B7.2 (CD86) molecules, which are expressed on the surface of activated APCs such as dendritic cells [135]. Signal transduction through the TCR complex, in the absence of CD28:B7 co-stimulation, can result in anergy or in cell death [136]. In addition to playing a critical role in T-cell activation and survival, CD28 expression can also be a useful marker to identify terminally differentiated T-cell subpopulations. We will discuss the importance of this marker in section 1.4.

1.2.6 Phenotype and function attributes of T-cell subpopulation

T-cells are very phenotypically and functionally heterogeneous, it is therefore important to characterize them. The classification of T-cell subpopulations can be very complex, and requires the identification of various surface and intracellular markers. In this section, we will provide an overview of the phenotypic and functional characteristics of specific T-cell subpopulations as well as their role during cell-mediated immune responses.

CD4 and CD8 phenotypes. T-cells are primarily distinguished based on their surface expression of CD4 and CD8 molecules. The main function of CD4+ T-cells, also called T helper (Th) cells, is to orchestrate immune responses by secreting chemical mediators called cytokines. These cytokines activate other immune cells such as CD8+ T-cells, B-lymphocytes and macrophages, thus allowing them to mount potent immune responses against pathogens [137,138]. CD4+ T-cells can be further subdivided based on the pattern of their cytokine production. The two major subtypes of helper CD4+ T-cells include: Th1 and Th2 cells. The roles of these subtypes tend to be mutually exclusive, as one often dominates the other during specific immune responses. Th1 responses are usually prominent against intracellular pathogens. They involve the secretion of pro-inflammatory cytokines such as interferon gamma (IFN-γ) by CD4+ T-cells. This cytokine can activate macrophages and induce cytotoxic NK cells. Antigen-specific cytotoxic CD8+ T-cells and opsonizing antibodies such as IgG are a key feature of Th1 responses. Conversely, Th2 responses generally occur during immune responses against extra-cellular bacteria, parasites or allergens. These responses are characterized by the secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines, which play an important role in neutralizing antibody production, eosinophil activation and macrophage inhibition [138].

In addition to Th1 and Th2, CD4+T-cells can differentiate into other functionally distinct subtypes such as Tregs, Th17 and T_{FH} cells. Regulatory T-cells or Tregs contribute to self-tolerance and immune homeostasis by actively suppressing the activation of the immune system and preventing the pathological self-reactivity that characterizes autoimmune diseases. Immunosuppressive cytokines such as TGF-β and Interleukin 10 (IL-10) have been associated with Tregs function [139]. Th17 are implicated in mucosal anti-microbial immunity. They also play an important role in inflammation and tissue injury and have been associated with autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. Their main effector cytokines include IL-17a, IL-21, and IL-22 [140,141]. Follicular helper T-cells or T_{FH} are effector CD4+ T-cells that are found within B-cell follicles of secondary lymphoid organs. These cells can be identified by their constitutive expression of the B-cell follicle homing receptor CXCR5. T_{FH} cells facilitate the somatic hypermutation and isotype switching within the germinal centers via the expression of CD40 ligand and the secretion of IL-21 and IL-4 [142].

Aside from their helper function, CD4+ T-cells can demonstrate cytotoxic potential against tumours and virally infected cells [143,144]. These "cytotoxic" CD4+ T-cells can kill their target cells via the Fas-FasL pathway or by the release of lytic granules such as perforins and granzymes. Both of these pathways result in the recruitment and activation of caspases, a group of proteolytic enzymes that are essential for the initiation of apoptotic processes in target cells [145,146]. Thus, although CD4+ T-cells have important helper functions, they can also have direct cytotoxic effects during cell mediated immune responses.

CD8+ T-cells, also known as cytotoxic T-lymphocytes (CTLs), have the ability to directly kill tumour cells, virus-infected cells and some parasites via both the Fas-FasL and perforin/granzyme

pathways [147,148]. In addition to their cytotoxic functions, CD8+ T-cells have been shown to mediate non-cytotoxic anti-viral responses via the release of soluble factors such as RANTES, MIP-1 alpha and MIP-1 beta [149-151]. Furthermore, CTLs have the ability to secrete large amount of pro-inflammatory cytokines such IFN-γ and tumor necrosis factor-α (TNF-α). In addition to having direct anti-microbial and antitumor properties, these cytokines can also recruit and activate effector cells such as macrophages [152-154].

Naïve, Effector and Memory phenotypes. In addition to their co-receptor expression, T-cells can be further categorized on the basis of their differentiation following antigen exposure. As such, within the peripheral T-cell pool, CD4+ and CD8+ T-cells can be further divided into three main phenotypically diverse subsets: naïve, effector and memory T-cells. Naïve T-cells are mature T-cells that have just exited the thymus but have not yet encountered a foreign antigen. These cells typically express CCR7, which allows them to recirculate through secondary lymphoid tissues where the majority of them (>95%) reside [155]. Other markers that are typically expressed on naïve T-cells include: CD45RA, CD27 and CD28 [156]. Upon encounter with a cognate antigen via APC presentation in lymphoid tissues, naïve T-cells undergo rapid rounds of proliferation and differentiate into short-lived effector T-cells. As a result of their differentiation into effector cells, T-cells cease to express CCR7 since they no longer need to recirculate through lymphoid tissues in search of a foreign antigen. These activated T-cells develop highly specialized functions such as: target cell killing, pro-inflammatory cytokines secretion, macrophage activation, stimulation of antibody-producing B-cells, and therefore very efficient at eradicating pathogens [157]. However, following antigen clearance, effector T-cells are subjected to activation induced cell death (AICD). AICD is a type of post-infection apoptosis that occurs as a result of repeated rounds of TCR stimulation. This process results in the contraction of the immune response via the removal of

effector clones that are no longer useful. This is an important step in the maintenance of T-cell homeostasis [158-160]. In fact, impairments in the AICD mechanism have been associated with the development of autoimmune diseases and lymphomas [161]. Less than 10% of effector T-cells survive the AICD process and differentiate into long-lived memory T-cells [158].

Memory T-cells are antigen-experienced T-cells with the capacity to expand rapidly upon secondary challenge with the same pathogen. These cells can greatly improve immune efficacy by mounting a faster and stronger immune response against recall antigens [162,163]. There are two major types of memory T-cells: central memory T-cells (T_{CM}) and effector memory T-cells (T_{EM}). These cells are characterized on the basis of their phenotype, homing capacity and effector functions. Most memory T-cells cease to express CD45RA, and acquire a CD45RO phenotype [164]. T_{CM} express CCR7, which allows them to migrate to secondary lymphoid organs. These cells have a high proliferative capacity, but little effector functions. However, upon antigenic stimulation they readily differentiate into effector cells. Conversely, T_{EM} do not express CCR7 and therefore predominant in peripheral tissues. These cells have a low proliferative capacity, but display immediate effector functions upon antigenic stimulation, which allows for rapid protection from recurring infections [165,166]. More recently, a third type of memory T-cells called tissue-resident memory T-cells (T_{RM}) has been identified. These cells have a phenotype identical to that of T_{EM}, but mostly reside in epithelial tissues where they mediate strong host responses against microbes. They have also been shown to produce a wide range of cytokines and to have cytotoxic properties [167].

In summary, T-cells are comprised of very complex and heterogeneous subpopulations; and the differentiation of naïve T-cells into effector and memory T-cells constitute the quintessential basis of T-cell mediated immunity.

1.2.7 T-cell homeostasis in clinical settings

The term "homeostasis" was initially coined by the American physiologist Walter Canon in 1929. It describes the maintenance of an internal biologic equilibrium in the face of perturbations [168]. Homeostatic regulation occurs in all living organisms and is critical in keeping a variety of biotic parameters within a normal physiologic range. Many diseases result from homeostatic imbalance. For instance excess secretion of thyroid hormones in the blood leads to hyperthyroidism, a hypermetabolic state [169]. Likewise, lymphocyte homeostasis is essential for the maintenance of a healthy and stable immune system. Lymphocyte imbalance can be used as a powerful diagnostic tool for the detection of various diseases. Lymphocytosis, an abnormal increase in circulating lymphocytes counts, is usually associated with viral infections and some types of malignancies such as lymphoma and leukaemia [170,171]. On the other hand, lymphopenia, which is characterized by abnormally low levels of lymphocytes, generally reflects a state of immune deficiency indicative of a primary genetic disorder or a secondary cause resulting from pathogens such as HIV, mycobacterium tuberculosis or corona virus [172-174].

T-cell homeostasis is a mechanism through which the size and diversity of the peripheral T-cell pool is maintained. This process is crucial for the maintenance of the functional integrity of the immune system. T-cells should represent 65-85% of peripheral blood lymphocytes, and should have a CD4:CD8 T-cell ratio of 2:1 (normal range: 1.2 – 3.3). The inability to maintain these parameters indicates a loss of T-cell homeostasis and an immune dysregulation respectively.

Defects in T-cell homeostasis have been associated with autoimmune diseases such as rheumatoid arthritis (RA), type-1 insulin-dependent diabetes mellitus (IDDM) and systemic lupus erythematous (SLE) [175-178]. For instance, pre-diabetic individuals are characterized by an increased prevalence of activated CD8+ T-cells and a reduced frequency of total T-cells (CD3+) [175,179]. Similarly, immune dysregulation plays a role in the development of age-associated comorbidities such as cardiovascular and renal diseases [180,181].

1.2.8 Homeostatic control of T-cells

T-cell homeostasis is achieved through various mechanisms that regulate the production, differentiation and destruction of T-cells. These processes involve cellular interactions between T-cells and APCs, access to specific cytokines and apoptotic pathways. Here we provide an overview of the homeostatic mechanisms that regulate T-cell numbers and diversity.

Thymopoesis. Thymopoesis is the process occurring in the thymus that is responsible for the generation and differentiation of T-cells. Seminal studies by Miller et al. have demonstrated that the thymus plays an important role in T-cell homeostasis [182,183]. In the circulation, recent thymic emigrants are distinguishable from other T-cells through the high concentration of TCR rearrangement excision circles (TRECs) that are found intracellularly. TRECs are episomal DNA fragments that are generated during the TCR gene rearrangement, Since TRECs do not replicate and are passed on to a single daughter-cell following mitosis, the frequency of TREC-positive cells is a useful marker for the enumeration of newly produced naïve T-cells in the body [184]. As we age, the thymus involutes and leads to a rapid decline of intra-thymic T-cell production. This is reflected by a reduction in the level of TREC-positive T-cells [185]. In a study by Murray et al., reduced proportions of TREC-positive naïve T-cells were associated with chronological aging and T-cell activation [186]. Therefore, early in life, the thymus plays a critical role in the replenishment

of the naïve T-cell pool. Age-associated thymic involution can disrupt the homeostatic regulation of the naïve compartment. Naïve T-cell homeostasis is critical for the maintenance of the T-cell repertoire diversity and for immune reconstitution following T-cell depletion. Disruptions within the naïve T-cell compartment have been associated clinical disorders such as RA, SLE and HIV [187-189].

Homeostatic cycling. Homeostatic cycling (HC) is the basal level of T-cell division that occurs in the periphery in order to preserve T-cell numbers and diversity in non-lymphopenic hosts [190]. As discussed in the previous paragraph, although thymopoesis is important for the generation of new T-cells, it is not sufficient for the maintenance of the peripheral T-cell pool. Previous findings have shown that after the third decade of age, the majority (>90%) of naïve T-cells present in the periphery are TREC-negative. This suggests that these cells have undergone post-thymic cellular division [186]. Thus, following thymic involution, T-cell survival and maintenance in the periphery is mostly dependent on HC [191]. This process is regulated by interactions of T-cells with cytokines and spMHC identical to those encountered during intra-thymic positive selection [192]. HC of naïve T-cells requires interaction with spMHC and IL-7, a gamma chain (γ_c) cytokine constitutively produced by dendritic cells and stromal cells in the lymphoid tissues. Without both of these signals, the life span of these cells is significantly reduced [193-195]. Therefore, naive T-cell homeostasis drastically shifts from a thymus-dependant mechanism during youth, to a peripheral expansion mechanism in adulthood. HC is also essential for the long-term maintenance of memory T-cell homeostasis. However, unlike naïve T-cells, memory CD4+ and CD8+ T-cells are not equivalently regulated. The survival of memory CD8+ T-cells requires access to IL-15 (another γ_c cytokine), but is independent of spMHC interaction [158,196]. Although the mechanisms involved in memory CD4+ T-cells survival have not been completely elucidated, the

HC of these cells appears to principally involve interaction with IL-7. TCR signalling may contribute but is not required for the turnover of this population [193]. The pro-survival properties of IL-7 and IL-15 stem from their ability to up-regulate levels of anti-apoptotic factors such as Bcl-2 and Bcl-xL, which play an important role in the control of the cell cycle and the regulation of apoptosis [197,198].

Homeostatic peripheral expansion. Although T-cell homeostasis is strictly regulated under healthy conditions, dramatic changes occur during lymphopenic settings. For instance, prolonged T-cell subset imbalance and dysfunction is often observed in patients who have received cytotoxic chemotherapy [199,200]. In lymphopenic hosts, the size and diversity of the T-cell repertoire is maintained via a process termed homeostatic peripheral expansion (HPE). HPE is essentially an exaggerated form of homeostatic cycling that occurs following severe T-cell depletion. Similarly to HC, HPE of naïve T-cells relies on dual access to spMHC and IL-7, which are largely available in lymphopenic settings [201]. Therefore, while HC of naïve T-cells occurs at a very slow rate, HPE results in excessive and rapid rounds of cellular division. This lymphopenia-induced proliferation causes a phenotypic and functional conversion of naïve T-cells into memory cells. Therefore, in lymphopenic hosts, the replenishment of the memory T-cell pool mainly depends on HPE of naïve T-cells [202]. In the periphery, naïve T-cells can interact with either high-affinity foreign antigens or low-affinity self-antigens. Thus, depending on the antigenic milieu, HPE can generate a memory T-cell repertoire that is skewed toward a specific antigen or that recognizes self-antigens. Consequently, as we age, each lymphopenic incident causes a gradual transition of polyclonal naive T-cells into clonally expanded memory T-cells [203,204]. This phenomenon illustrates how chronic viral infections such as CMV and HIV can progressively jeopardize the homeostatic equilibrium of the immune system by depleting the naïve compartment and reducing

repertoire diversity. Interestingly, studies have shown that during HPE some memory T-cells may repopulate the CD45RA+ T-cell compartment by re-expressing this marker [205]. Although these T-cells re-acquire a CD45RA phenotype, they have been shown to be terminally differentiated effectors with a low proliferative capacity, high pro-inflammatory functions and cytotoxic properties [206,207]. These terminally differentiated effector memory T-cells called "TEMRA", are also resistant to apoptosis and gradually accumulate in the periphery over time. This can ultimately lead to a shrinking of the immune space and repertoire [208]. Furthermore, the accumulation of TEMRA cells has been associated with poor clinical outcomes such as graft rejection [209].

Activation Induced Cell Death. The maintenance of T-cell homeostasis is achieved not only through cellular proliferation but also through cellular destruction. As previously mentioned, the peripheral T-cell pool greatly expands during the course of an immune response [157]. However, following antigen clearance, it is important for the immune system to re-establish its equilibrium. AICD is a very important mechanism that preserves the balance of the T-cell repertoire via the clonal deletion of activated T-cells that are no longer useful. This mechanism relies on the interaction between Fas-ligand (FasL) and a Fas molecule on activated T-cells [210,211]. The binding of FasL triggers the trimerization of the Fas receptor, which enables the recruitment of the Fas-associated death domain (FADD). FADD then binds and activates caspase-8, which in turn initiates a series of downstream events that result in cellular apoptosis [212,213]. AICD is critical to for the removal of highly activated or self-reactive T-cells. Defects in this mechanism can therefore lead to clinical disorders such as inflammatory and autoimmune diseases [214,215].

In conclusion, the maintenance of T-cell homeostasis is of key importance for the long-term integrity of the immune system. In healthy individuals immune equilibrium is preserved through clonal deletion and access to survival signals (spMHC and cytokines). However, repeated or persistent lymphopenic events can lead to the gradual destabilization and erosion of the T-cell compartment. This will progressively impair the immune system's capacity to maintain long-term homeostasis. Similarly, failure to control the expansion of activated T-cell clones can have nefarious effects on immune health and long-term survival.

1.3 HIV-induced altered T-cell phenotype

As highlighted in the previous section, phenotypic analysis of patients' lymphocytes can be very useful in determining the level of immune dysfunction. Currently, the clinical evaluation of immune recovery during treated HIV-infection is largely based on CD4+ T-cell counts. However, monitoring this immune parameter alone, only offers a partial assessment of the true status of the immune system. Indeed, other T-cell parameters are altered during HIV disease and it is unclear whether they can be normalized with effective ART. Here we review the different ways in which HIV-infection contributes to altered T-cell homeostasis and profound immune dysregulation.

1.3.1 CD4+ T-cell depletion

CD4+ T-cell depletion is the hallmark of HIV-associated immune deficiency. However, the precise mechanisms leading to the loss of CD4+ T-cells during HIV disease are still the subject of vigorous scientific investigation. Over the years various models have been proposed including both direct and indirect cytopathic effects of the virus.

The tap and drain model. The first theory on HIV pathogenesis originated in the mid-1990s, and was proposed by David Ho and colleagues. To explain the progressive depletion of CD4+ T cells, they suggested a "tap and drain" model, according to which the destruction of CD4+ T-cells by the HIV virus (the drain), triggers a homeostatic proliferative response (the tap) that attempts to replenish the depleted T-cell compartment. However, over time, the continual *de novo* infection and destruction of CD4+ T-cells creates a state of high turnover that ultimately leads to immune exhaustion. This model was supported by experimental data showing that HIV treatment with the protease inhibitor Ritonavir was associated with a rapid drop in the viral load and subsequent increase in CD4+ T-cell counts [86,216]. However this view has been challenged by a number of observations. First of all, during early HIV infection, the fraction of dying CD4+ T-cells was shown to be higher than the fraction of productively infected CD4+ T-cells [217,218] Secondly, several studies had shown that in the CD8+ T-cell compartment, the level of proliferative exhaustion was at least as high as in the CD4+ T-cell subset [219-221]. Collectively, these findings suggested that HIV-infection of target cells is most likely not the main cause of CD4+ T-cell depletion.

The immune activation model. HIV disease is characterized by a state of generalized chronic immune activation**.** This is exemplified by the up-regulation of T-cell activation markers and inflammatory cytokine release during the course of the disease [222,223]. According to the immune activation model, chronic activation of the immune system may contribute to progressive CD4+ T-cell destruction by providing new target cells for HIV replication and, by causing apoptosis of uninfected "bystander" T-cells via AICD [57,224]**.** This process can also lead to the depletion of the naïve T-cell repertoire, through the relentless activation of these cells and their differentiation into memory cells [225,226]. Finally, the pro-inflammatory environment associated with immune activation can cause severe architectural and functional damage to important

lymphoid organs such as the bone marrow, thymus and lymph node [227]. Seminal work by Haase and colleagues, have demonstrated that immune activation can induce fibrosis of lymphoid tissues via the production and deposition of collagen around high endothelial venules [227]. These organs play a crucial role in the generation, development and homeostasis of CD4+ T-cells. One of the most compelling findings for the role of chronic immune activation in disease progression originates from work on sooty mangabeys, which are the natural host of the simian immunodeficiency virus (SIV). Seminal data by Silvestri and colleagues showed that, despite elevated plasma viral loads, natural SIV-infection was characterized by the absence of immunopathology, the maintenance of the peripheral CD4+ T-cell pool and non-progression to AIDS. These positive outcomes appeared to be the result of attenuated levels of immune activation during the chronic phase of SIV disease [228]. The limited immune activation observed in SIV-infected sooty mangabeys is markedly different from the generalized immune activation that characterizes pathogenic HIV and SIV infections. This was supported by other studies that demonstrated that HIV- infected individuals with decreased immune activation were also able to maintain CD4+ T cell levels in the face of high viremia [225,229]. Therefore, while HIV may initiate the early events leading to CD4+ T-cell destruction, virus-induced chronic immune activation appears to be substantially responsible for exacerbating and fuelling the ultimate depletion of this subset.

The lymphocyte redistribution model. T-cells continuously recirculate through lymphoid tissues via the blood and lymphatics. This recirculation is mediated by the interaction between T-cells and adhesion molecules on high endothelial cells in venules [230]. Pro-inflammatory cytokines such as IFN-γ and TNF-α, have been shown to affect lymphocyte migration by increasing the expression of adhesion molecules [231-233]. Thus, according to the redistribution model, HIV-

induced immune activation causes the up-regulation of adhesion molecules such as ICAM-1 and VCAM-1, which enables the sequestration of circulating T-cells within lymphoid tissues [234]. This theory was supported by data showing that decreased expression of adhesion molecules, following ART-mediated viral suppression, was associated with a redistribution of sequestered T-cells from lymphoid tissues to the peripheral circulation [235]. There are however some pitfalls in this model. First of all, although post-ART changes in peripheral cellularity were inversely correlated with changes in lymph node cellularity; the fluctuations in cell numbers were not mirrored between the two compartments. Furthermore, despite viral suppression and reduced immune activation, not all study participants exhibited an inverse correlation between peripheral and tissue cellularity. Finally, AIDS associated CD4+ T-cell depletion has previously been demonstrated in both the periphery and the lymph nodes [236]**.**

The blind T-cell homeostasis model. The blind T-cell homeostasis (BTH) model was first proposed by Adleman and Wofsy in 1993. This model suggests the existence of a homeostatic mechanism that regulates T-cell numbers in a subset independent manner [236]. The total number of (CD3+) T-cells approximates the sum of CD4+ and CD8+ T-cells. The only other cell type that expresses the CD3 marker are the NK-T-cells, which only accounts for a very small proportion of the CD3+ population [237]. According to the BTH model, the immune system "experiences" the loss of both CD4+ and CD8+ T-cells as a total reduction of CD3+ T-cells, rather than distinguishing between the two subsets. As a result of this reduction in total T-cell numbers, CD4+ and CD8+ T-cell production is increased in order to maintain a constant level of circulating T-cells. This implies that the immunological space created after a lymphopenic event can be replenished with either CD4+ or CD8+ T-cells. Therefore, in clinical contexts such as HIV infection, the preferential and continuous destruction of CD4+ T-cells ultimately leads to a gradual expansion of CD8+ T-cells

that attempt to fill the immunologic space. This hypothesis is supported by the observation that total T-cell numbers do not decline in CD4-depleted and CD4-knockout mice despite the absence of CD4+ T-cells [236,238,239]. The data from these findings all demonstrate the immutability of T-cell counts despite changes within the T-cell subsets. It should be noted that one study by Vezys et al. demonstrated that the introduction of new memory CD8+ T-cells following a prime-boost immunization technique, did not significantly impact on the size of the CD4+ T-cell compartment [240].

Resolving the conundrum. All the mechanisms that have been proposed to explain HIV-associated CD4+ T-cell depletion are based on strong supporting experimental observation. Clearly, the models presented above should not be considered as being mutually exclusive. CD4+ T-cells may be lost as a result of direct viral-mediated killing and impaired regenerative capacity, especially during acute HIV infection. However, during chronic infection, viral-induced immune activation and AICD are the major contributors to the destruction of infected and uninfected CD4+ T-cells. Furthermore, ongoing inflammation may cause the redistribution and confinement these cells to lymphoid tissues. Chronic inflammation may also impair CD4+ T-cell homeostasis by affecting the integrity of lymphoid tissues. Finally, homeostatic responses to specific CD4+ T-cell destruction may lead to the gradual replacement of these cells by expanded CD8+ T-cell clones. The cause of CD4+ T-cell depletion in HIV disease is therefore multifactorial and far more complex than the viral-induced CD4+ T-cell destruction proposed by the initial “tap and drain” model.

1.3.2 CD8+ T-cell differentiation and dysfunction

In addition to CD4+ T-cell depletion, chronic HIV-infection induces complex phenotypic and functional remodelling of the CD8+ T-cell subset. These changes can persist despite ART-mediated

viral suppression. The key characteristic of this remodelling is the loss of CD28 co-stimulatory molecule expression on the surface of most of these cells [241]. Empirical data has demonstrated that CD8+CD28- T-cells represent a subset of cells that have reached replicative senescence [219]. In vitro analysis has indicated that the transcription of the CD28 gene has been permanently silenced in these cells [242]. Key features of these senescent cells include the inability to proliferate or perform cytotoxic effector functions in response to antigenic stimulation [243]. However these cells have the capacity to produce high amounts of inflammatory cytokines such as IL-6 and TNF-α [244]. Another feature of these cells is their capacity to resist apoptosis, which enables them to accumulate in the periphery and evade homeostatic control [245]. In non-HIV settings, the accumulation of these cells has been associated with clinical disorders such as RA, and anklyosing spondylitis [246,247].

In HIV infection, the clinical significance of the predominance of these cells has been the subject of extensive investigation: is their expansion a consequence of chronic antigenic stimulation or a homeostatic mechanism aiming to replace depleted CD4+ T-cells? It appears that CD28+ and CD28- cytotoxic T-cells play different roles in HIV pathogenesis. The presence of CD8+CD28+ T-cells has previously been associated with anti-HIV activity and asymptomatic clinical status [241,248,249]. On the other hand, empirical data has indicated a direct correlation between CD8+CD28- T-cell expansion and HIV disease progression; which may be explained by the reduced effector functions of these cells [243,250]. Furthermore, the expansion of HIV-specific CD8+CD28- T-cells has been associated with a gradual decline in both CD4+ T-cell counts and CD4:CD8 T-cell ratio [251-253]. The phenotypic and functional heterogeneity of the HIV-specific CD8+ T-cell pool may therefore explain why high levels of viremia often persist in the face of large numbers of CD8+ T-cells [254]. Taken as a whole these experiments suggest that while

CD8+CD28+ T-cells provide an effective immune response against HIV infection, CD8+CD28- effectors contribute to the maintenance of T-cell homeostasis by replenishing the immunological gap created by CD4+ T-cell depletion [252]. These cells do not only play an important role in T-cell homeostasis; they also indirectly contribute to HIV pathogenesis by replacing functionally competent CD8+CD28+ T-cells and by releasing high levels of inflammatory mediators that have pathogenic effects of uninfected CD4+ T-cells [244,252].

Multiple studies have indicated that CD8+CD28- T-cells can represent more than 60 percent of circulating CD8+ T-cells in HIV patients, whereas they only constitute a third of this proportion in sero-negative individuals [250,255]. Interestingly, tetramer analyses of late differentiated CD8+CD28- T-cells in HIV patients have revealed that the majority of these cells specifically recognize CMV. These CMV-specific senescent T-cells can account for up to 50% of the total CD8+ T-cell compartment of HIV patients [243]. Viral co-infections such as CMV may contribute to the expansion of a CD8+ T-cell pool that is oligoclonal. This can ultimately result in the skewing and shrinking of the immune repertoire. The CD8+CD28- T-cell population can be subdivided based on the expression of two surface markers: CD27 and CD57. When used in conjunction with the loss of CD28, the expression of CD57 and the loss of CD27 are believed to represent the furthermost stage of replicative senescence [256,257]. These terminally differentiated effector memory cells are part of the TEMRA subset mentioned in section 1.2.8.

1.3.3 CD4:CD8 T-cell ratio dysregulation

Prior to the establishment of CD4+ T-cells as the primary target of the HIV virus, the CD4:CD8 T-cell ratio was the preferred biomarker for determining HIV disease diagnosis and progression. The expansion of CD8+ T-cells leading to an inversion of the CD4:CD8 T-cell ratio is indeed the first step in the development of a series of HIV-mediate immune abnormalities [4]. The dysregulation

of the CD4:CD8 T-cell ratio is a common feature of viral infections, whereby antigen-specific CD8+ T-cells expand significantly in an attempt to clear the infection. However, following viral clearance these cells normally return to their physiologic level [258]. A distinctive characteristic of treated chronic HIV infection is the persistence of high CD8+ T-cell levels despite viral suppression. This eventually leads to a profound and prolonged dysregulation of the CD4:CD8 T-cell ratio [181].

Various theories have attempted to elucidate the persistently low CD4:CD8 T-cell ratio in the context of treated HIV infection. Studies have found that patients who achieve undetectable levels of HIV replication (plasma HIV RNA below 50 copies/ml) can exhibit minimal levels of residual viremia that are only detectable by ultra-sensitive assays [259]. It was suggested that this very low viremia was sufficient for the maintenance of high frequencies of CD8+ T-cells. Residual viremia has been shown to contribute to persistent immune activation via chronic antigen presentation; however, it is unlikely that it would account for the large proportion of CD8+ T-cell observed in these individuals. Indeed, data from successfully treated individuals have indicated that HIV-specific CD8+ T-cells only account for a very small proportion of the CD8+ T-cell repertoire [260,261]. Independent of HIV, other viral infections such as CMV have been shown to contribute to high levels of CD8+ T-cells [262,263]. This virus can generate up to a 60% increase in the frequency of memory CD8+ T-cells [264]. Interestingly, a study by Naeger and colleagues, demonstrated high CMV-specific CD8+ T-cell responses in treated HIV-infected individuals [265]. Thus, the persistent CD8+ T-cell lymphocytosis observed in successfully treated HIV-infected patients might be explained by the presence of a subclinical CMV co- infection.

The clinical relevance of this immune dysregulation is underscored by several observations. First, in the context of untreated HIV, the CD4:CD8 T-cell ratio has been associated with disease progression [266]. Among treated HIV+ individuals this marker was linked to ongoing immune dysfunction and increased risk of non-AIDS morbidity and mortality [102]. In non-HIV settings, inverted CD4:CD8 T-cell ratios were also associated with poor clinical outcomes. [267].

1.3.4 Impaired T-cell homeostasis

In the early 1990's, studies monitoring the effect of T-cell subset depletion in mice, demonstrated that CD4-deficient mice exhibited abnormally high levels of CD8+ T-cells, while maintaining stable numbers of total T-cells (CD3+) [239,268]. Conversely, CD8-deficient mice were also able to maintain constant levels of CD3+ T-cells, in the face subset alterations [269,270]. In 1993, it was proposed that the maintenance of CD3+T-cell counts previously observed in CD4-deficient and CD8-deficient mice, suggested the occurrence of a homeostatic process that is independent of changes within individual T-cell subsets. The term blind T-cell homeostasis was coined to describe this phenomenon. The BTH theory was supported by findings from studies on mice treated with anti-CD4 antibodies. 16 weeks following the treatment of these mice, a normalization of the peripheral T-cell count was observed. However, an inverted CD4:CD8 T-cell ratio, driven by a persistent CD4+ T-cell lymphopenia and CD8+ T-cell lymphocytosis, was also detected. This process was shown to occur in both the peripheral blood and the spleen. [236].

In 1995, Margolick and colleagues provided empirical data demonstrating the role of this mechanism in the pathogenesis of HIV. In their study, they followed 321 HIV-1 positive homosexual men, enrolled in the Multicenter AIDS Cohort Study (MACS), over a period of 4 years prior to seroconversion and 5 years post seroconversion. Their results showed an initial drop in CD3+ T-cell levels shortly after seroconversion. However, this parameter was quickly

normalized and remained stable during the subsequent 2 years, despite a marked decrease in CD4+ T-cells. The stabilization of total T-cell numbers was attributed to a concomitant CD8+ T-cell lymphocytosis [271]. Subsequent data demonstrated that the maintenance of the CD3+ T-cell numbers was associated with good clinical prognosis. Conversely, the decline of total T-cell numbers, characterized by the loss of both CD4+ and CD8+ T-cells, was indicative of the exhaustion of T-cell regenerative processes and predicted the onset of AIDS [174]. Later studies have found CD3+ T-cell counts to be a better marker of HIV related mortality and morbidity, than CD4+ T-cell counts and plasma viral load levels [272]. These findings highlight the potential role of total T-cell numbers as an independent marker of HIV disease progression.

BTH has also been observed in non-HIV settings. For instance, individuals with bare lymphocyte syndrome (who are unable to express class I and/or class II MHC molecules) can maintain normal CD3+ T-cell numbers despite the absence of either one of the T-cell subsets [273]. Similarly, in bone marrow transplant recipients, the total T-cell count rapidly returns to normal, despite persistent alterations in both CD4+ and CD8+ T-cell counts [200,274]. Therefore, following severe lymphopenic events, BTH can indirectly result in long-term immune imbalance. In HIV-uninfected populations, failure to maintain BTH has been associated with deleterious clinical outcomes such as SLE, RA, Crohn's disease and active pulmonary tuberculosis [275-277]. Overall, these studies underscore the critical role of BTH in HIV pathogenesis, disease progression and clinical outcomes. .

1.3.5 Altered T-cell Phenotype

Collectively, all the data presented above suggest that HIV induces changes to the immune system that are not fully captured by monitoring CD4+ T-cell counts. These changes occur sequentially and each reflects a specific stage of the disease. The first step in this series of events is the

expansion of CD8+ T-cells in response to HIV viremia. This leads to an immediate inversion of the CD4:CD8 T-cell ratio that will persist throughout the course of the disease. Multiple factors may contribute to the persistent dysregulation of the ratio. First, the progressive depletion of CD4+ T-cells as a result of ongoing virus-mediated destruction and activation induced cell death. Second, the accumulation of apoptosis-resistant CD8+CD28- T-cells that fill the immune space previously occupied by CD4+T-cells. Furthermore, viral co-infections such as CMV may exacerbate CD4:CD8 T-cell dysregulation by fuelling the expansion of terminally differentiated CD8+ T-cells. Ultimately, in absence of efficacious treatment, the exhaustion of T-cell regenerative processes may result in the loss of T-cell homeostasis. This last phase is characterized by the depletion of both CD4+ and CD8+ T-cells.

1.4 HIV-infection and immune senescence

The dramatic improvement in the life expectancy of HIV-infected individuals receiving effective ART, has allowed scientists to investigate the long-term effect of chronic HIV infection on the immune system. The results of these investigations have shown that independent of CD4+ T-cell counts; long-term HIV-infection ultimately induces a state of pre-mature immune senescence [278]. Immune senescence can be defined as the progressive remodelling of the immune system that occurs with age. This remodelling reflects the cumulative exposure of the aging host to antigenic stressors such as chronic or latent pathogens. This process eventually leads to the dysregulation and deterioration of the immune system and is associated with poor clinical outcomes [279,280]. In this section we highlight the phenotypic and clinical features of immune senescence and its role in HIV pathogenesis.

1.4.1 Markers of immune senescence

Remodelling of the T-cell repertoire. The hallmark feature of immune senescence is the oligoclonal expansion of terminally differentiated CD8+CD28- T-cells [281]. Interestingly, a large proportion of these terminally differentiated clones tend to be specific for CMV; which appears thus to be a key driver of immunological aging [264,282]. The accumulation of CMV-specific CD8+ T-cells contributes to the inversion of the CD4:CD8 T-cell ratio, which has previously been associated with T-cell senescence and dysfunction [283]. Immune senescence is also marked by a reduction of the T-cell repertoire diversity mainly via the depletion of peripheral naïve T-cells, especially in the CD8 compartment [284]. These cells could be lost as a result of both age-associated thymic involution and their progressive conversion into a memory phenotype following repeated antigenic stimulation [285].

Replicative senescence and immune exhaustion. Additional features of immune senescence include the up-regulation of the CD57 and killer cell lectin-like receptor G1 (KLRG1) molecules on the surface of T-cells. These glycoproteins are putative markers of replicative senescence. They are generally expressed on NK cells and T-lineage lymphocytes, where they have been reported to identify late differentiated effectors with reduced proliferative capacity and a high susceptibility to apoptosis [286,287]. The acquisition of the inhibitory receptors programmed death-1 (PD-1) and T-cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) is also an important marker of immunological aging. The expression of both of these molecules is highly correlated with T-cell exhaustion, decreased proliferation and impaired cytokine secretion [288,289].

Inflammaging. Chronic inflammation and immune activation are key signatures of an aging immune system. Indeed, immune senescence has been associated with the establishment of a pro-inflammatory milieu as noted by the increased production of inflammatory mediators such as TNF-

α, IL-6 and CRP [290-292]. Persistent immune activation resulting from exposure to chronic antigens (eg: HIV and CMV) or from microbial translocation following damage to the intestinal mucosa, is believed to be a key driver of this age-related systemic inflammation, also referred to as "inflammaging" [293-295].

***Telomere attrition*.** Telomeres are tandem sequences of $(TTAGGG)_n$ nucleotide repeats that protect the distal ends of chromosomes from degradation; their integrity is essential for optimal cellular proliferation [296,297]. Following multiple rounds of cellular division, telomeres become gradually shorter until they reach a critical point called the Hayflick limit (HL). The HL consists of a cellular mechanism that inhibits proliferative potential via cell cycle arrest [298]. Although this process is important for tumor control, it can have a negative impact on the function of the immune system. Indeed, shortened telomeres have been shown to be a defining feature of terminally differentiated and exhausted T-cells [219,299]. Thus, telomere attrition represents the cellular basis of replicative senescence.

1.4.2 Immune Risk Phenotype

Despite efficacious treatment, HIV persists within the host and continues to challenge the immune system over time. As a result, chronically infected HIV individuals develop a pattern of immune abnormalities comparable to those observed in uninfected individuals who are decades older. These changes include the inversion of the CD4:CD8 T-cell ratio, the expansion of late differentiated T-cell populations, the depletion of naïve T-cells and the persistence of a pro-inflammatory milieu [181,278,300,301]. These immune alterations resemble the immune risk phenotype (IRP) previously described in very elderly healthy individuals followed in the Swedish OCTO longitudinal study [302,303] The IRP occurs in 15-20% of octagerians (>85 years old) and

consists of a combination of phenotypic and serologic markers associated with immune senescence. This cluster of markers include: a low CD4:CD8 T-cell ratio, an expansion of CD8+CD28- T-cells and the presence of CMV seropositivity. Collectively these immune-related defects have been associated with an increased risk of mortality and morbidity among octagerians [304]. It thus appears that both HIV disease and natural aging involve the inflation of dysfunctional CD8+ T cells with features of replicative senescence and a dominant specificity to CMV antigens.

CMV is a ubiquitous Herpes virus whose sero-prevalence increases with age [305]. In immune-competent hosts, CMV infections are usually asymptomatic and result in life-long latency [306,307]. Empirical data from a multitude of studies suggest that latent CMV infection induces dramatic changes to immune repertoire diversity and plays a direct role in the emergence of immunosenescent T-cells [264,282,283]. Chronic CMV infection has also been shown to progressively drive the replicative senescence of circulating T-cells by contributing to the shortening of their telomeres [308]. Interestingly, CMV prevalence is particularly high among those infected with HIV and studies have shown that CMV co-infection can exacerbate the senescent phenotype associated with chronic HIV disease [309]. As HIV-infected individuals grow older, the multiple unresolved immune alterations that persist during the course of the disease may act synergistically with age-associated immune abnormalities to generate or accelerate negative long-term clinical outcomes. Considering the similarities between HIV-related and age-related immune changes, understanding the interplay between HIV infection and biological aging is of critical importance.

1.4.3 Clinical outcomes associated with immune senescence

In the uninfected elderly, immune senescence has been linked to poor clinical outcomes. Studies

have reported an association between chronic inflammation and the development of age-related pathologies [310,311]. Similarly, inflammatory mediators such as IL-6 and TNF-α have been correlated to all-cause mortality [292,312]. Furthermore, the oligoclonal expansion of late-differentiated effectors and the depletion of circulating naïve T-cells have been shown to contribute to a reduction of the T-cell repertoire diversity [282]. This immunological remodelling is postulated to cause disruptions in immune-surveillance, leading to an increased susceptibility to neo-pathogens and reduced response to vaccination [313]. Finally, telomere shortening has previously been linked to increased risk of cardiovascular disease, diabetes and cancer [314,315]. Collectively, these studies suggest that time-dependent remodelling of the immune system may significantly contribute to comorbidities associated with aging.

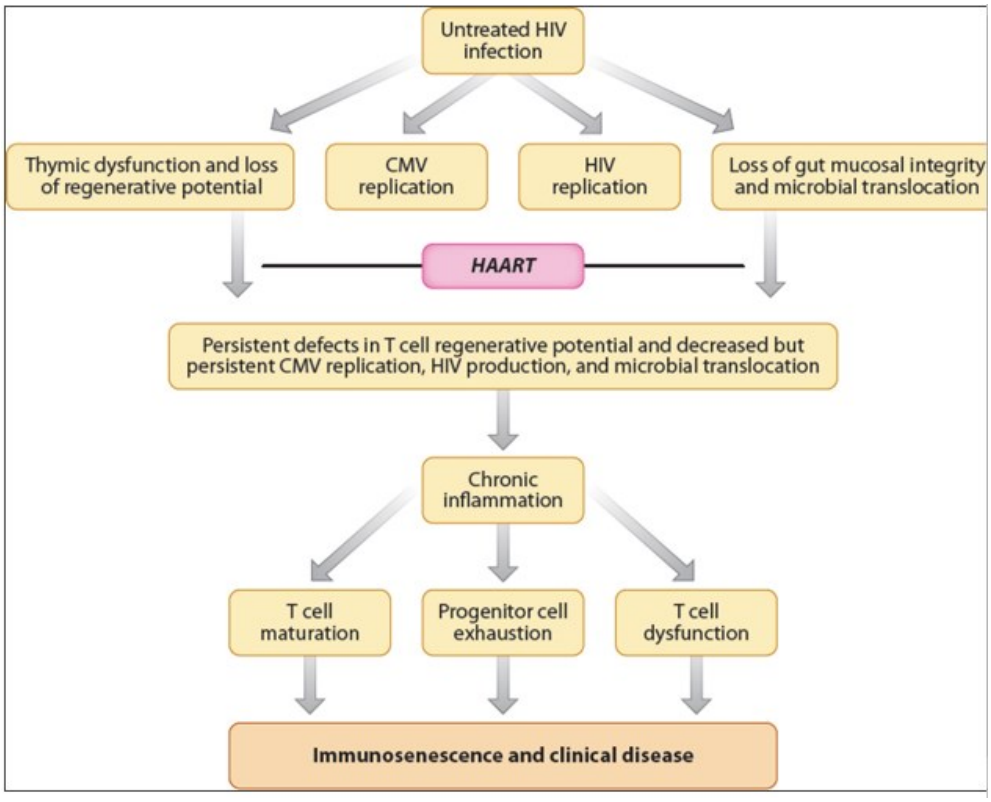

Figure 4 [278]: **HIV Infection, Inflammation and Immunosenescence** (Reprinted with permission from Annual Reviews: Annu Rev Med; 62:141-55, copyright 2011)

In recent years, immune senescence has gained significant interest in the field of HIV. A variety of studies have reported that, among treated HIV individuals, immune senescence is a major contributor of increased risk of age-related clinical manifestations such as cardiovascular, renal, and metabolic diseases [79,102,316]. This suggests that a detailed examination of markers of immunological aging may be useful in the clinical care of HIV-infected individuals.

1.5 Thesis rationale

HIV disease is characterized by a spectrum of immune changes that take place during acute infection and persist throughout the chronic phase of the disease. The virus engages all components of host defence, ultimately leading to a severe and fatal immune deficiency. A unique property of this retrovirus is its lymphocytotrophic propensity. Although all lymphocytes are impacted throughout the infection, and the hallmark of the disease is the profound CD4+ T-cell cytopenia and chronic immune activation. HIV however also induces substantial alterations to T-cell subsets that lead to: 1) severe immune dysregulation (CD4:CD8 T-cell ratio inversion), 2) immune deficiency (secondary to CD4+ T-cell depletion) and 3) loss of T-cell homeostasis (impaired CD3+ T-cell homeostasis). These phenotypic changes occur progressively over time and reflect the gradual deterioration of the immune system.. Although the CD4+ T-cell count is the preferred immune surrogate marker for monitoring HIV disease progression, during therapy, CD4+ T-cell restoration alone may not accurately reflect complete immune recovery as T-cell dysregulation persists and T-cell homeostasis is often lost. A dysregulated and chronically activated phenotype has been associated with the recent emergence of non-AIDS related comorbidities including cardiovascular, renal, and liver diseases. There is thus a crucial need not only to develop novel and comprehensive markers of long-term immune recovery in successfully treated HIV+ patients but

also to identify immune markers that are prognostic for age-related comorbidities. Monitoring CD3+ T-cell levels and the CD4:CD8 T-cell ratio, in addition to the CD4+ T-cell count, may be essential for a more complete evaluation of immune recovery.

The beneficial effects of ART stem from its ability to efficiently control viral replication and restore CD4+ T-cell counts. However, it is still unclear to what extent long term effective ART can restore other immune parameters such as CD3+ T-cell levels and the CD4:CD8 T-cell ratio; or whether these parameters are associated with poor clinical outcomes in those successfully treated for HIV. Furthermore, most treatment outcome studies have a relatively short follow-up duration, which may not be sufficient to capture slow longitudinal changes in immune parameters. The work described in this thesis will provide evidence supporting the idea that despite very long-term ART-mediated viral suppression HIV-induced altered T-cell phenotype (TCP) is not recovered in the majority of patients, especially those who initiated treatment at advanced stages of their disease. The thesis also provides a comprehensive evaluation of the IRP as a composite marker of immune senescence in the context of HIV and cardiovascular disease.

1.6 Thesis objectives

The availability of more effective and better-tolerated HIV drug regimens has shifted the prognosis of HIV-positive individuals from a few years to several decades. This dramatic improvement in life expectancy allows for the evaluation of very long-term immune outcomes in those living with the virus. This thesis will present original work that contributes to the general knowledge of long-term immune recovery in the context of successfully treated HIV infection. To address immune recovery and generate a greater understanding about the of long-term effects of chronic HIV disease on the immune system, this thesis will evaluate (I) whether a decade of effective ART can

reverse HIV-induced altered TCP (II) the characteristics and determinants of ART-mediated TCP normalization (III) the clinical impact of altered T-cell homeostasis on the morbidity and mortality profile of successfully treated HIV patients (IV) the phenotypic and functional immune characteristics associated with an immune risk phenotype in the context of treated HIV infection.

Bridge from chapter 1 to chapter 2

HIV induces T-cell phenotypic dysfunction that includes CD4+ T-cell depletion, CD4:CD8 T-cell dysregulation and altered CD3+ T-cell homeostasis. Although effective ART can suppress viral suppression and promote CD4+ T-cell reconstitution, little is known about the evolution of other HIV-induced phenotypic alterations subsequent to long-term effective therapy. The pilot study presented in this chapter was designed to explore patterns of complete T-cell phenotypic recovery in a cohort of patients receiving suppressive ART for more than a decade.

Chapter 2:

Delay in cART Initiation Results in Persistent Immune Dysregulation and Poor Recovery of T-cell Phenotype Despite a Decade of Successful HIV Suppression

Delay in cART Initiation Results in Persistent Immune Dysregulation and Poor Recovery of T-cell Phenotype Despite a Decade of Successful HIV Suppression

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The data summarized in this paper were presented in part at the 14th International Workshop on HIV Observational Databases - IWHOD *(Abstract #**14_172**) and at the 18th* International AIDS Conference *(Abstract #**15953**)*

2.1 Abstract

Background: Successful combination antiretroviral therapy (cART) increases levels of CD4+ T-cells, however this increase may not accurately reflect long-term immune recovery since T-cell dysregulation and loss of T-cell homeostasis often persist. We therefore assessed the impact of a decade of effective cART on immune regulation, T-cell homeostasis, and overall T-cell phenotype.

Methods: We conducted a retrospective study of 288 HIV+ cART-naïve patients initiating therapy. We identified 86 individuals who received cART for at least a decade, of which 44 consistently maintained undetectable plasma HIV-RNA levels throughout therapy. At baseline, participants were classified into three groups according to pre-treatment CD4+ T-cell counts: Group I (CD4 <200 cells/mm^3); Group II (CD4: 200-350 cells/mm^3); Group III (CD4 >350 cells/mm^3). Outcomes of interest were: (1) CD4+ T-cell count restoration (CD4 >532 cells/mm^3); (2) normalization of CD4:CD8 T-cell ratio (1.2-3.3); (3) maintenance of CD3+ T-cell homeostasis (CD3: 65%-85% of peripheral lymphocytes); (4) normalization of the complete T-cell phenotype (TCP).

Results: Despite a decade of sustained successful cART, complete T-cell phenotype normalization only occurred in 16% of patients, most of whom had initiated therapy at high CD4+ T-cell counts (>350 cells/mm^3). The TCP parameter that was the least restored among patients was the CD4:CD8 T-cell ratio.

Conclusions: Failure to normalize the complete T-cell phenotype was most apparent in patients who initiated cART with a CD4+ T-cell count <200 cells/mm^3. The impact of this impaired T-cell phenotype on life-long immune function and potential comorbidities remains to be elucidated.

2.2 Introduction

The immune system of healthy individuals is characterized by the maintenance of homeostasis via a balanced T-cell phenotype (TCP) [204]. Although the hallmark of HIV infection is progressive CD4+ T-cell depletion, other impairments in immune phenotype such as loss of T-cell homeostasis and severe T-cell subset dysregulation also occur.

T-cell subset dysregulation was the earliest noted surrogate marker of AIDS in the early 1980s [4,317]. It is characterized by a low CD4:CD8 T-cell ratio (<1.2) resulting from the depletion of CD4+ T-cells and the concomitant expansion of the CD8+ population of T-cells in the peripheral blood. Studies have shown that in HIV seropositive individuals receiving long-term combination antiretroviral therapy (cART), CD4:CD8 T-cell ratio dysregulation is correlated with higher risk of developing coronary artery disease [318]. In non-HIV settings, low CD4:CD8 T-cell ratios are also associated with poor clinical outcomes in patients with common variable immune deficiency (CVID) and in healthy individuals over the age of 60 [319,320].

T-cell homeostasis was first described in 1993 by Adleman and Wofsy as the normal physiologic state by which the immune system maintains a constant number of circulating CD3+ T-cells, irrespective of changes within the CD4+ and CD8+ T-cell compartments [236]. Loss of T-cell homeostasis often occurs in HIV-infected individuals, and is manifested by a failure to maintain normal levels of circulating CD3+ T-cells [321]. Data from 372 seroconverters enrolled in the Multicenter AIDS Cohort Study (MACS) showed that in the absence of adequate treatment, the loss of T-cell homeostasis in HIV infection could predict impending AIDS and death [322]. We recently showed for the first time that impairment in T-cell homeostasis is also associated with morbidity and mortality among HIV-positive patients who receive effective combination

antiretroviral therapy (cART) [323]. In non-HIV clinical contexts, altered T-cell homeostasis has also been linked with deleterious clinical disorders such as rheumatoid arthritis, Crohn's disease and systemic lupus erythematosus [275,276]. The triad of low CD4+ T-cell count, dysregulated CD4:CD8 T-cell ratio, and loss of T-cell homeostasis characterizes the abnormal T-cell phenotype induced by HIV infection [4,324].

The goals of effective cART are to suppress HIV viral replication and restore immune competence. Successful cART usually results in increased CD4+ T-cell counts, yet the increase may not reflect a complete immune recovery since ratio dysregulation and altered T-cell homeostasis often persist [324-327]. Failure to recover a balanced T-cell phenotype may put HIV-infected patients at risk for non-viral morbidities despite cART-mediated viral control [318,323,328,329]. However, there is a scarcity of information on the effect of long-term suppressive cART on the normalization of the TCP. It is currently unknown whether this parameter can be completely restored in successfully treated individuals.

A major impediment to the evaluation of true immune recovery among HIV-positive patients is the limited number of surrogate markers available for clinical use. Measuring the total number of circulating CD4+ T-cells often necessitates the simultaneous determination of CD8+ and CD3+ T-cell numbers. Therefore, monitoring CD3+ T-cell levels and CD4:CD8 T-cell ratio, in addition to CD4+ T-cell counts, may provide further insight into immune restoration. Due to the availability of more effective and better-tolerated antiretrovirals (ARVs), HIV-positive patients are now able to achieve sustained viral suppression over longer periods of time, thus allowing for the evaluation of very long-term immune recovery. In this pilot study, we assessed the extent of T-cell phenotype recovery among a group of treated HIV-positive males who achieved and maintained viral suppression for at least a decade.

2.3 Methods

Ethics Statement

Institutional approval for this study was obtained from the Research Ethics Board (REB) of the Montreal General Hospital. The REB approves the anonymous use of data retrospectively abstracted from clinical care databases without requiring patient consent. Patients sign a general waiver upon opening a medical chart.

Cohort description

We conducted a retrospective cohort analysis of a treatment-naïve group of 288 HIV-positive individuals followed at the Montreal General Hospital. Patients initiated cART as of 01-Jan-1995. Combination ART was defined as a combination of at least three anti-HIV drugs: either two nucleoside reverse transcriptase inhibitors (NRTIs) in combination with a non-NRTI (NNRTI) or a protease inhibitor (PI), two PIs in combination with at least one NRTI; or a combination of one PI, one NNRTI, and at least one NRTI. All patients had regular follow-up at three month intervals. At each follow-up, clinical, virologic, and immune evaluations were performed including longitudinal documentation of CD4:CD8 T-cell ratios as well as CD4+, CD8+, and CD3+ T-cell counts and percentages. Each study participant had a minimum follow-up of ten years from the time of cART initiation.

Participant selection

Inclusion criteria:

- Adults (>18 years of age) and of male gender
- Date of cART initiation
- Documented therapy with cART for a minimum of ten years
- Complete adherence with cART and trimestrial clinic visits
- Confirmation of viral suppression at each visit (<50 copies/mL)

- Clinical and immune phenotype data available during the entire follow-up period

Exclusion criteria:

- Participants with incomplete baseline and follow-up data
- Participants with clinical loss to follow-up exceeding twelve months
- Participants with more than two consecutive viral load blips throughout their follow-up

Outcomes

The primary outcome of interest was the restoration of a balanced T-cell phenotype defined as meeting all six of the following criteria: CD3+ T-cell percent: 65%-85%; CD4:CD8 T-cell ratio 1.2-3.3; CD4+ T-cell count: 532-1170 cells/mm^3; CD4+ T-cell percent: 39%-55%; CD8+ T-cell count 236-651 cells/mm^3; CD8+ T-cell percent 18%-31%. These ranges represent the mean ±2 standard deviations and were derived from 124 healthy age-matched controls recruited at the Montreal General Hospital. These controls were all HIV-negative, had normal physical examinations, and were also screened for the presence of primary immune deficiencies and autoimmune diseases.

The primary outcome was chosen based on the known alterations in T-cell phenotype induced by HIV-1 infection that include low CD4+ T-cells, loss of CD3+ T-cell homeostasis, and a low CD4:CD8 T-cell ratio. Using our established normal ranges, the cutoffs for these parameters were as follows: low CD4+ T-cells (<532 cells/mm^3); loss of T-cell homeostasis (low <65% or high >85%) CD3+ T-cell percentages; low CD4:CD8 T-cell ratio (<1.2). Since the sum of the CD4+ and CD8+ T-cell percentages proportionally define the T-cell compartment of circulating lymphocytes and are also used to generate the CD4:CD8 T-cell ratio, we also assessed the percentages of these subsets. The subset percentages are also not affected by fluctuations in lymphocyte numbers and their use ensured a thorough evaluation of the TCP.

Statistical Analysis

Patients were assigned based on their baseline pre-treatment CD4+ T-cell counts as follows: Group I–CD4+ T-cell count <200 cells/mm^3; Group II–CD4+ T-cell count = 200-350 cells/mm^3; Group III–CD4+ T-cell count >350 cells/mm^3. Patient characteristics were compared according to the baseline CD4+ T-cell group, and differences were assessed using the Kruskal-Wallis test for continuous variables and Fisher's Exact test for categorical variables. Linear mixed effects models to account for correlation between repeated measurements within each individual, assuming an autoregressive of order 1 covariance structure, were used to estimate the annual rate of change in CD4+ T-cell count, CD4:CD8 ratio, and CD3+ T-cell percentage. An analysis of the residuals on each model suggested the need for a transformation of the outcome data in order to meet the assumptions. Thus in the final model, rates of change in CD4+ T-cell counts were estimated using square-root transformed absolute CD4+ T-cell values, which provides variance stabilization for repeated measures and has been previously used for CD4+ T-cell trajectory analysis [330,331]. For the same reasons, the rates of change in CD4:CD8 T-cell ratios were estimated using natural logarithm transformed data. No transformation was required for the CD3+ T-cell percentages. The final model was adjusted for baseline age, CMV, and baseline HIV viral load (log10). All statistical hypothesis tests were two-sided and performed at the 0.05 level of significance. All analyses were done using the SAS software, version 9.2 (SAS Institute Inc. Cary, NC).

2.4 Results

Characteristics of the study population

From the original cART-naïve group of 288 HIV+ patients, we identified 86 males (29.9%) who remained consistently on cART for at least 10 years without any treatment interruptions. Among these 86 males, 44 (51.2%) met the inclusion criteria. These individuals achieved and maintained plasma HIV-RNA under the level of detection (<50 copies/ml) throughout their entire treatment time. The number of participants per group was 19, 14, and 11 for Group I, Group II, and Group III, respectively. The median time from HIV diagnosis to treatment initiation was six years (IQR: 2-10) and there was no significant difference between the groups. All patients were initiated on a 2 NRTI (3TC, AZT, or d4T) plus 1 PI (indinivir, saquinavir, or ritonavir) regimen, except for one patient in Group I who was on a 2 NRTI plus 1 NNRTI (nevirapine) regimen.

At baseline, the mean age of the patients was 42 years (IQR: 38.5-49.5), all patients were male, 57% were men who have sex with men (MSM), 75% were CMV seropositive, and 20% presented with an AIDS defining illness (ADI). The mean baseline HIV-RNA was 4.5 $\log_{10}$ copies/ml (IQR: 3.8-4.9), and the median duration of HIV treatment with cART was 14 years (IQR: 13-14). Baseline characteristics are summarized in Table 1.

Normalization of the T-cell phenotype and clinical outcomes

CD4+ T-cell count

At baseline, only 2 of the 44 patients had a normal CD4+ T-cell count. These patients belonged to Group III. After a decade of suppressive cART, only 50% of Group I patients normalized their CD4+ T-cell count, while 86% of Group II, and 100% of Group III patients recovered this parameter (Table 2). Linear regression analysis showed that the rate of change in

square root CD4+ T-cell counts was significantly higher in Group I (2.19/year, 95% CI = 1.47-2.92) compared to Group II (1.39/year, 95% CI = 0.64-2.16) and Group III (1.08/year, 95% CI = 0.41 to 1.75) within the first 5 years of treatment. In the subsequent 5 years, the annual change in square root CD4+ T-cell counts had decreased across the three groups (Group I: 0.64/year, 95% CI = 0.23-1.05; Group II: 0.57/year, 95% CI = 0.13-1.01, and Group III: 0.77/year, 95% CI = 0.38-1.15). However, there were no significant differences across the groups (Table 3).

CD4:CD8 T-cell ratio

At treatment initiation, the CD4:CD8 T-cell ratio was dysregulated in all patients except for two individuals in Group III. By year 10, the proportion of patients with normalized CD4:CD8 T-cell ratio was highest in Group III (73%) and lowest in Group I (11%). Less than half of Group II (43%) normalized this parameter (Table 2). Linear regression analysis showed that similarly to CD4+ T-cell counts, the annual increase in CD4:CD8 T-cell ratio within the first five years of treatment was highest in Group I (0.27/year, 95% CI = 0.17-0.37) compared to Group II (0.13/year, 95% CI = 0.03-0.23) and Group III (0.11/year, 95% CI = 0.02-0.21). However, no difference in the annual rate of change was observed during the last five years across the groups: Group I: 0.09/year, 95% CI = 0.04-0.14; Group II: 0.08/year, 95% CI = 0.02-0.14; Group III: 0.04/year, 95% CI = -0.01-0.09 (Table 3). A descriptive representation of the CD4+ and CD8+ T-cell percentage trajectories showed that the CD4:CD8 T-cell ratio imbalance was only successfully reversed in Group III (Figure 1).

CD3+ T-cell homeostasis

Despite differences in T-cell subset balance, the proportion of patients with normal T-cell homeostasis at baseline and at follow-up was similar across the groups (Table 2). Linear regression

analysis showed that there was no significant difference in the annual rate of change in CD3+ T-cell percentage across the three groups during the first half (Group I: 0.90/year, 95% CI = -1.08 to 2.88; Group II: -0.5/year, 95% CI = -2.62 to 1.62; Group III: -0.02/year, 95% CI = -1.86 to 1.82), and the last half of the follow-up (Group I: -0.23/year, 95% CI = -1.32 to 0.86; Group II: -0.1/year, 95% CI = -1.26 to 1.06; Group III: -0.02/year, 95% CI = -1.03 to 0.99) (Table 3).

Complete T-cell Phenotype

Only 16% of all patients had a completely normalized TCP by the end of the follow-up period. Comparing individual groups, we observed that 45% of individuals in Group III achieved TCP normalization while only 7% of Group II and 5% of Group I normalized (Table 2). Overall, among the 44 patients, we found that the TCP parameter with the lowest normalization rate was the CD4:CD8 T-cell ratio with only 36% of all patients normalizing this parameter after a decade of suppressive therapy, 50% of those restoring this parameter belonged to Group III.

Clinical Outcomes

Throughout the course of the follow-up, 11 patients (25%) developed a cardiovascular event (CVE), as defined by the occurrence of an acute coronary syndrome (myocardial infarction, diagnosed unstable angina, or stroke). All of these individuals were co-infected with CMV. Overall, 32% of Group I and 36% of Group II developed a CVE. However, no CVE was observed among Group III patients (Table 1). After ten years of follow-up, only three deaths occurred within the sample: 2 (11%) in Group I and 1 (7%) in Group II. There were no reported deaths among Group III patients.

2.5 Discussion

In the very first description of AIDS in 1981, the authors described a new acquired severe immune deficiency that was characterized by a loss of CD4+ T-cells (Leu 3+), a concomitant increase in CD8+ T-cells (Leu 2+), resulting in a low CD4:CD8 T-cell (Leu3+/Leu2+) ratio, and a severe loss of CD3+ T-cell (Leu 1+) homeostasis [4]. In this study, we describe results obtained from a cohort of 44 well-defined HIV-positive patients with long-term complete viral suppression following cART initiation. We detected a distinct subset of individuals, primarily among patients who initiated antiretroviral therapy at very low CD4+ T-cell counts, who failed to normalize key T-cell phenotype parameters.

The CD4+ T-cell count is currently the preferred marker for monitoring HIV progression, as low levels of CD4+ T-cells are associated with high risk of co-morbidities and mortality in HIV patients [332,333]. Our findings show that despite the fact that three quarters of the population achieved a normal CD4+ T-cell count by the end of the follow-up, only about a third of the patients had a normal CD4:CD8 T-cell ratio at year ten. This suggests that recovery of CD4+ T-cell numbers does not always reflect the normalization of other T-cell phenotype parameters. Interestingly, our longitudinal analysis showed that Group I exhibited the greatest fold-increase in CD4+ T-cell counts within the first five years of treatment. However, this recovery rate slowed down in the second half of the follow-up, resulting in subnormal levels of CD4+ T-cells at the end of the follow-up despite continuous viral suppression. Our findings are in line with previous studies showing that HIV-positive patients initiating therapy at advanced stages of the disease experience important increases in CD4+ T-cell counts during the early phase of therapy, but exhibit substantially reduced annual increases during the rest of the follow-up [331,334-336]. Several studies have suggested that the initial spike observed within circulating CD4+ T-cells during early treatment results from

a redistribution of memory T-cells from lymphoid tissues to the periphery, rather than from de novo synthesis [337]. T-cell regenerative processes might thus be severely or even irreversibly impaired in patients who initiate therapy at advanced stages of the infection.

Among the 44 patients, we found that the parameter that was the least conserved was the CD4:CD8 T-cell ratio. Failure to restore a normal CD4:CD8 T-cell ratio has been linked with an increased risk of non-AIDS related events, such as cardiovascular disease [318]. Furthermore, a low CD4:CD8 T-cell ratio is an important component of the immune risk phenotype (IRP), which is associated with increased morbidity and mortality in seronegative individuals over the age of 60 [338,339]. The inability to restore the ratio could similarly put HIV-positive patients at risk for non-AIDS mortality and comorbidities. Interestingly, a recent study by the Canadian Observational Cohort Collaboration (CANOC) has shown that T-cell ratio normalization might be associated with decreased risk of AIDS defining illnesses and mortality among treated patients [340]. Despite these findings, there is still a paucity of data available on long-term CD4:CD8 T-cell ratio recovery in patients receiving efficacious HIV therapy. Our study shows that among successfully treated HIV patients, T-cell ratio normalization is most frequent in those who initiate therapy at CD4+ T-cell counts above 350 cells/mm^3. Despite over a decade of HIV suppression, more than half of Group II and most of Group I maintained persistently low ratios. A descriptive representation of CD4+ and CD8+ T-cell subset trajectories show that the ratio normalization observed in Group III is the result of both an increase in CD4+ T-cell levels and a concomitant decrease in CD8+ T-cell levels (Figure 1). This is a clear reversal of the post HIV seroconversion T-cell subset trend previously reported in cART-naive HIV-patients [322]. CD8+ T-cell percentages remained abnormally high in Groups I and II throughout the follow-up. Since all patients maintained HIV suppression, it is unlikely that HIV replication would account for the disproportionate CD8+ T-cell expansion

observed in these two groups. It is possible that co-morbid chronic viral infections, such as cytomegalovirus (CMV) infection, contribute to CD8+ T-cell lymphocytosis [341]. CMV, a ubiquitous herpes virus, establishes a lifelong latent infection in humans [342,343]. Naeger et al found that CMV-specific CD8+ T-cell responses are high in successfully treated HIV-positive patients [265]. Interestingly, CMV prevalence was highest among Group I and Group II patients. The persistent CD8+ lymphocytosis and low CD4:CD8 T-cell ratio observed in these groups might thus be partially explained by a subclinical CMV infection. Future studies are required to investigate the nature of the CMV immune response in such individuals.

We also noted that a quarter of our patient population developed cardiovascular events (CVE) throughout the follow-up period, however none of these patients belonged to Group III. The occurrence of CVE among Group I and II patients is particularly interesting considering that these two groups had the highest prevalence of CMV seropositivity. In fact, all of the eleven patients who experienced a cardiovascular event were co-infected with CMV. Although the clinical outcomes associated with acute CMV infection have been the subject of considerable investigation, the long-term impact of chronic asymptomatic CMV infection still needs to be elucidated. Considerable evidence seems to indicate an association between CMV seropositivity and an increased risk of cardiovascular morbidity via the induction of a pro-inflammatory response [344,345].

Numerous reports of untreated individuals revealed that failure of T-cell homeostasis is an important landmark in HIV disease progression [321,322,346,347]. We recently showed for the first time that even in those receiving potent cART, lost T-cell homeostasis was associated with morbidity and mortality [323]. However, to our knowledge, studies investigating long-term

changes in circulating CD3+ T-cells in those with well-controlled HIV replication remain scarce. Our findings indicate that despite a high rate of CD4:CD8 T-cell dysregulation, most patients maintained normal CD3+ T-cell levels at the end of the follow-up. Therefore, CD3+ T-cell percentages remained relatively constant within each group despite drastic shifts in the CD4+ and CD8+ T-cell subsets as shown in Figure 1. These results are in accordance with previous findings in AIDS free HIV-infected individuals, where little variations were observed in CD3+ T-cell levels regardless of substantial changes in the CD4+ and CD8+ compartments [322]. This is in agreement with the concept of blind T-cell homeostasis, and indicates the existence of a physiologic mechanism that strives to maintain constant T-cell numbers in the face of intra-subset fluctuations. This phenomenon has also been observed in HIV-negative individuals, where CD3+ T-cell percentages were shown to be stable despite low (<1) or high (>1) CD4:CD8 T-cell ratios [338]. Although some studies have measured T-cell homeostasis in terms of CD3+ T-cell counts, in this study it was defined in terms of CD3+ T-cell percentages as we consider this parameter to be a more reliable marker of the proportional stability of the circulating T-cell pool. Indeed, T-cell percentages vary less than absolute counts since they are not affected by lymphocyte fluctuations [348-350].

Finally, the complete TCP was only restored in a small number of patients (16 %), most of which had initiated treatment at high baseline CD4+ T-cell counts (>350 cells/mm^3). Whether the other patients will eventually normalize TCP with longer treatment time remains unknown. It is unlikely that the type of treatment regimen used had a significant impact on the outcomes, since all but one patient were on a PI-based regimen. The clinical importance of a multiparametric assessment of T-cell recovery was also underlined in a study by Torti et al. [351]. The T-cell markers evaluated in that study included CD4+ T-cell counts, CD4+ T-cell percentages, and

CD4:CD8 T-cell ratios. We feel that the inclusion of the CD3+ T-cell percentage in our analysis provides a more comprehensive measure of multiparametric T-cell recovery as it takes into consideration both CD4+ and CD8+ T-cell levels. Furthermore, our study had a follow-up period of 10 years (vs. a median of 4 years in Torti et al.), which to our knowledge, is the longest evaluation of a multiparametric T-cell phenotype recovery in HIV patients with consistently sustained viral suppression to levels below 50 copies/mL.

The current pilot study has a few limitations. First, we restricted our analysis to males because of the limited number of females being followed in our clinic, thus optimizing the homogeneity of the study population. Our results may therefore not apply to HIV-positive women. Secondly, the sample size of our study population was limited by the small size of our cohort and the rigorousness of our selection criteria. Studies using larger cohorts are therefore needed to validate these findings. It was previously demonstrated that early cART initiation can improve immunological outcomes presumably through a reduction of T-cell activation while on-therapy [352]. However, we could not assess the level of immune activation in each group, since inflammation markers are not usually collected from our patients during routine clinical visits. Finally, due to the small proportion of patients who achieved TCP normalization, the impact of the TCP on clinical outcomes could not be evaluated.

Treatment for HIV is life-long. Therefore, long-term and comprehensive evaluation of immune recovery is important. Historically, T-cell phenotypic recovery in HIV-infected patients was assessed by monitoring CD4+ T-cell counts. The originality of our work stems from the fact that this is the first study looking at an aggregate of specific T-cell phenotypic markers (CD4+ T-cell counts, CD4:CD8 T-cell ratio, and CD3+ T-cell percentages) in order to investigate immune

recovery from an allostatic perspective. The rationale for the choice of these parameters is based on their individual association with poor clinical outcomes in both HIV and non-HIV settings. Although the sample size was small, an important strength of our study lies in the long duration of the follow-up period and requirement that all subjects maintained viral load levels below 50 copies of HIV-1 RNA at all time points. These study criteria allowed us to investigate long-term patterns of immune recovery in the context of highly effective viral suppression.

In this pilot study, the analysis was specifically focused on patients with prolonged and optimal viral suppression, as this represents the ideal clinical context for long-term immune recovery. Our findings show that normalization of the T-cell phenotype is best achieved when cART is initiated at baseline CD4+ T-cell counts >350 cells/mm^3. Furthermore, despite increases in CD4+ T-cell counts, very few patients who initiate therapy at advanced stages of HIV infection recover a normal T-cell phenotype, even after a decade of therapy. Overall, our findings indicate that HIV infection causes profound TCP alterations that are not completely reversed with treatment. Larger cohort studies will be important to determine the long-term clinical impact of this persistent dysregulation, in order to assess whether an altered TCP is a risk profile associated with higher morbidity and mortality among HIV-infected patients.

2.6 Acknowledgement

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2.7 Figure legend

Figure 1. Longitudinal evaluation of the CD4:CD8 T-cell ratio from the time of cART initiation

Mean values of circulating CD4+ and CD8+ T-cell percentages as a function of years since cART initiation in Groups I, II, and III. Vertical bars indicate the 95% confidence intervals. The horizontal dashed lines represent upper and lower limits of the normal reference range for CD4+ T-cell percentages, and the horizontal dotted lines represent upper and lower limits of the normal reference range for CD8+ T-cell percentages.

2.8 Tables and Figures

Table 1. Demographics and clinical characteristics for 44 HIV-positive patients on combination antiretroviral therapy

Characteristics	All Patients (n=44)	Group I (n=19)	Group II (n=14)	Group III (n=11)	P-value
Age	42 (39-50)	44 (35-58)	44(38-54)	42(35-44)	0.46
HIV risk factor					
Hemophiliac/blood	13	7	4	2	
MSM	25	10	8	7	
Heterosexual	6	2	2	2	
Caucasian	42 (95%)	17 (89%)	14 (100%)	11 (100%)	0.14
Baseline VL (log_{10} copies/mL)	4.5 (3.8-4.9)	4.8 (4.6-5.1)	3.8 (3.2-4.9)	4.1 (3.3-4.4)	0.14
Nadir CD4 counts (cells/mL)	130 (30-268)	30 (20 -70)	178 (77-293)	308 (231-350)	0.0001
ADI at baseline	9 (20%)	6 (32%)	2 (14%)	1 (9%)	0.12
HCV co-infection	13 (30%)	6 (32%)	4 (29%)	3 (27%)	0.79
CMV co-infection	33 (75%)	16 (84%)	11 (79%)	6 (55%)	0.08
Cardiovascular Events	11 (25%)	6 (32%)	5 (36%)	0 (0%)	0.08

Values are number and percentage, or median and interquartile range. Group I: CD4+ T-cell count <200 cells/mm^3; Group II: CD4+ T-cell count = 200-350 cells/mm^3; Group III: CD4+ T-cell count >350 cells/mm^3. MSM, men who have sex with men; VL, viral load; ARV, antiretroviral; ADI, AIDS defining illness; HCV, hepatitis C virus; CMV, cytomegalovirus.

Table 2. Proportion of patients with normal T-cell parameters at baseline and year 10

	Baseline				
T-cell parameters	**All (n=44)**	**Group I (n=19)**	**Group II (n=14)**	**Group III (n=11)**	**P-value**
CD3+ T-cell percent	33 (75)	13 (68)	10 (71)	10 (91)	0.37
CD4+ T-cell count	2 (5)	0	0	2 (18)	0.06
CD4:CD8 T-cell ratio	2 (5)	0	0	2 (18)	0.06
TCP	1 (2)	0	0	1 (10)	0.25
	Year 10				
T-cell parameters	**All (n=44)**	**Group I (n=19)**	**Group II (n=14)**	**Group III (n=11)**	**P-value**
CD3+ T-cell percent	35 (80)	14 (73)	11 (78)	10 (91)	0.57
CD4+ T-cell count	33 (75)	10 (52)	12 (86)	11 (100)	0.007
CD4:CD8 T-cell ratio	16 (36)	2 (11)	6 (43)	8 (73)	0.002
TCP	7 (16)	1 (5)	1 (7)	5 (45)	0.02

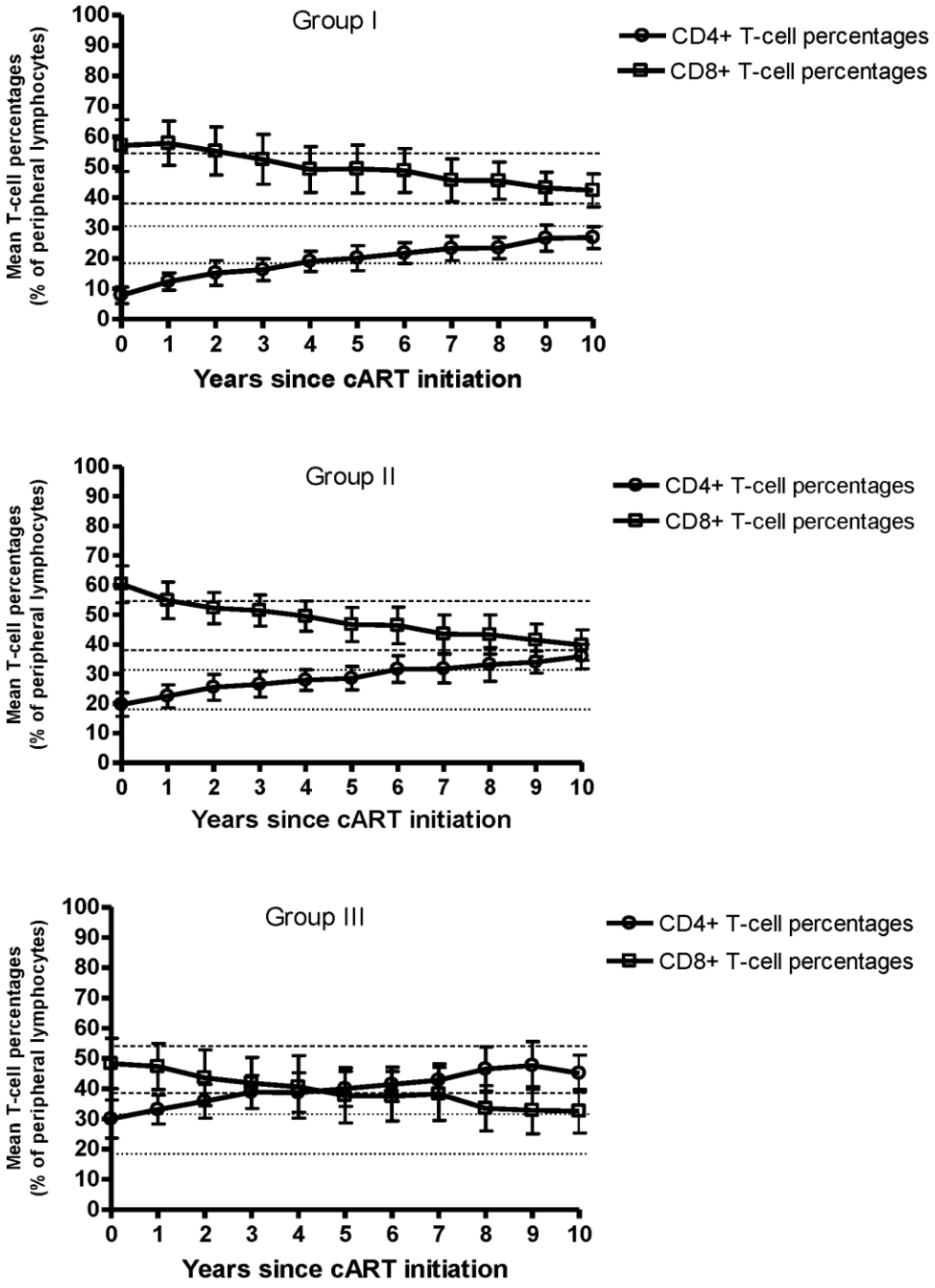
Data are presented as n (%)

Table 3. Estimated differences in slope of T-cell parameters between groups based on multivariate linear mixed effect models

Years 0-5	Parameters	Groups	Slope Difference	P-value
	CD4	Group 1 - 3	1.12	0.003
		Group 2 - 3	0.32	0.41
		Group 2 - 1	-0.8	0.02
	CD4:CD8	Group 1 - 3	0.16	0.002
		Group 2 - 3	0.02	0.74
		Group 2 - 1	-0.14	0.004
	CD3	Group 1 - 3	0.92	0.37
		Group 2 - 3	-0.48	0.66
		Group 2 - 1	-1.4	0.14
Years 6-10	**Parameters**	**Groups**	**Slope Difference**	**P-value**
	CD4	Group 1 - 3	-0.13	0.75
		Group 2 - 3	-0.2	0.65
		Group 2 - 1	-0.07	0.85
	CD4:CD8	Group 1 - 3	0.05	0.19
		Group 2 - 3	0.04	0.32
		Group 2 - 1	-0.01	0.79
	CD3	Group 1 - 3	-0.22	0.77
		Group 2 - 3	-0.09	0.91
		Group 2 - 1	0.13	0.85

Data represented are square root CD4 counts, geometric mean CD4:CD8 ratio, and mean CD3+ T-cell percent

Figure 1. Longitudinal evaluation of the CD4:CD8 T-cell ratio from the time of cART initiation



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Bridge from chapter 2 to chapter 3

The preliminary data presented in chapter 2 indicated that long-term suppressive ART was associated with partial T-cell phenotype (TCP) recovery in the majority of our study population. Complete TCP normalization occurred pre-dominantly in those patients who initiated therapy at high CD4+ T-cell counts. The findings of this pilot study suggested that early treatment initiation may be necessary to ensure full TCP recovery. The cohort study presented in chapter 3 aimed to validate our preliminary findings in a larger cohort of treated HIV+ patients. This study also provided a comprehensive evaluation of the characteristics and determinants of TCP normalization.

Chapter 3:

Characteristics and determinants of T-cell phenotype normalization in HIV-1 infected individuals receiving long-term antiretroviral therapy

Characteristics and determinants of T-cell phenotype normalization in HIV-1 infected individuals receiving long-term antiretroviral therapy

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3.1 Abstract

Objectives: Although combination antiretroviral therapy (cART) can restore CD4 T-cell numbers in HIV infection, alterations in T-cell regulation and homeostasis persist. We assessed the incidence and predictors of reversing these alterations with cART.

Methods: ART-naïve adults (n=4459) followed within the Canadian Observational Cohort and exhibiting an abnormal T-cell phenotype (TCP) prior to cART initiation were studied. Abnormal TCP was defined as having either (1) low CD4 T-cells (<532 cells/mm^3), (2) lost T-cell homeostasis (CD3 < 65% or > 85%) or (3) CD4:CD8 ratio dysregulation (ratio <1.2). To thoroughly evaluate the TCP, CD4 and CD8 T-cell percentages and absolute counts, were also analyzed for a median duration of 3.14 years (IQR=1.48-5.47). Predictors of TCP normalization were assessed using adjusted Cox proportional hazards models.

Results: At baseline: 96% had CD4 depletion, 32% lost homeostasis and 99% exhibited ratio dysregulation. With treatment, a third normalized CD4 T-cell counts, but only 85 (2%) individuals normalized their TCP. In a multivariable model adjusted for age, measurement frequency, and baseline regimen; higher baseline CD4 T-cell counts and time-dependent viral suppression independently predicted TCP normalization [HR for baseline CD4 =1.42 (1.31-1.54) per 100-cell increase; p ≤.0001, HR for time-dependent suppressed VL= 3.69 (1.58-8.61); p-value≤0.01].

Conclusions: Despite effective cART, complete TCP recovery occurred in very few individuals and was associated with baseline CD4 T-cell count and viral load suppression. HIV alterations of the T-cell phenotype are incompletely reversed by long-term antiretroviral therapy.

Key words: HIV, T-cell homeostasis, Immune dysregulation, Immune recovery, ART, CANOC

3.2 Introduction

Homeostatic regulation occurs in all living organisms and is critical in keeping many biologic parameters within a physiologic range [353,354]. The immune system of healthy individuals is characterized by the maintenance of T-cell homeostasis and a balanced T-cell phenotype. This is achieved through complex and tightly regulated processes such as: thymic output, access to cytokines, naïve T-cell differentiation into memory cells, and antigen-independent peripheral T-cell proliferation [355-357]. Although progressive CD4 T-cell depletion is the hallmark of HIV disease, impaired T-cell homeostasis and profound immune dysregulation are often observed during the course of the infection.

In the early 1980s, prior to the identification of the human immunodeficiency virus (HIV), the earliest diagnostic marker of AIDS was a reversion of the CD4:CD8 ratio [358,359]. The imbalance of these T-cell subsets defines HIV-mediated immune dysregulation. It begins in early disease preceding the progressive loss of CD4 T-cells and deteriorates during untreated infection. Furthermore, altered T-cell homeostasis occurs mostly in the late stage of HIV disease and is manifested by a failure to maintain physiologically normal levels of T-cells [321]. All T-cells have a CD3 phenotype and characteristically express either CD4 or CD8 cell surface molecules. T-cell homeostasis was first described in 1993, as the normal physiologic state by which the human body maintains a constant number of circulating (CD3+) T-cells irrespective of fluctuations within the CD4 and CD8 T-cell compartments [236]. Maintaining the size and diversity of the peripheral T-cell pool is of crucial importance for a healthy and balanced immune system [275,360]. Low CD4 T-cell counts, low CD4:CD8 ratio and loss of T-cell homeostasis are part of a continuum of immune abnormalities that occur during progressive HIV infection [324].

The use of potent combination antiretroviral therapy (cART) has resulted in sustainable

reductions of HIV viral load to undetectable levels and to improved CD4 T-cell counts [325]. However, cART-mediated CD4 T-cell restoration may not accurately reflect complete recovery of immune phenotype since T-cell dysregulation and altered T-cell homeostasis may persist.

In non-HIV immune based disease states such as rheumatoid arthritis, Crohn's disease, systemic lupus erythematous (SLE), and Sjogren's syndrome; impaired T-cell homeostasis has been linked to several deleterious clinical outcomes [176,275]. Burn injury is associated with altered T-cell homeostasis and subsequent decreased resistance to infections[361]. Patients with active pulmonary tuberculosis also have loss of T-cell homeostasis and a decreased CD4:CD8 T-cell ratio[277]. Individuals with common variable immune deficiency (CVID) have persistently low CD4:CD8 T-cell ratios that are associated with poor clinical outcomes[319].

Most notably, immune dysregulation has been associated with accelerated immune senescence (the gradual decline in immune function occurring with age) and with increased risks of non-AIDS related co-morbidities such as coronary artery diseases, diabetes, and liver impairment [362]. A low CD4:CD8 T-cell ratio is an important component of the immune risk phenotype (IRP); it has been found to be associated with increased morbidity and mortality in sero-negative individuals over the age of 60 [329,338]. In HIV-infected individuals, the potential risks for co-morbidities from failure to restore T-cell homeostasis and to normalize the CD4:CD8 T-cell ratio are currently unknown.

Despite previous findings that altered T-cell homeostasis has predictive value in determining impending AIDS and that CD4:CD8 ratio dysregulation correlates with higher risks of developing coronary disease, few, if any, studies have assessed the effect of long-term successful cART on altered T-cell homeostasis and T-cell ratio dysregulation [321,363,364]. Thus, the degree to which these HIV-mediated immune alterations can be reversed by effective cART remains to be

elucidated. We, therefore, assessed the incidence and predictors of complete T-cell phenotype normalization in antiretroviral-naïve HIV-positive patients initiating cART.

3.3 Methods

Cohort Description

The Canadian Observational Cohort (CANOC) collaboration is an observational cohort study of antiretroviral-naïve HIV-positive patients initiating cART on or after January 1, 2000 [365]. CANOC participants represent nearly a quarter of Canadians currently on cART after this date. This collaboration is open to all Canadian HIV treatment cohorts with more than 100 eligible patients and currently includes 8 participating cohorts across Canada. Eligibility criteria for inclusion into CANOC include: documented HIV infection, residence in Canada, aged 18 years and older, initiation of a first antiretroviral regimen comprised of at least 3 individual agents, and at least 1 measurement of HIV-1 RNA viral load and CD4+ T-cell count within 6 months of initiating cART. Patient selection and data extraction are performed locally at the data centers of the participating cohort studies. Non-nominal data from each cohort on a predefined set of demographic, laboratory, and clinical variables are then pooled at the Project Data Centre in Vancouver, British Columbia. The last date of follow-up in the cohort for the current analysis was August 22, 2010. All participating cohorts have received approval from their institutional ethics boards to contribute non-nominal patient specific data.

Ethical Consideration

The human subjects activities of CANOC were approved by the Simon Fraser University Research Ethics Board, the University of British Columbia Research Ethics Board and the following local institutional review boards of the participating cohorts: Providence Health Care Research Institute Office of Research Services, The Ottawa Hospital Research Ethics Board, University Health Network Research Ethics Board, Véritas IRB, Biomedical C (BMC) Research Ethics Board of the McGill University Heath Centre, University of Toronto HIV Research Ethics Board (HIV REB), and Women's College Hospital Research Ethics Board. Local cohorts have obtained written consent except the following: HOMER (IRB approves the retrospective use of anonymous administrative data without requiring consent; an information sheet for participants is provided in lieu of a consent form); Ottawa Hospital Cohort (IRB approves the anonymous use of data retrospectively abstracted from clinical care databases without requiring consent); UHN (REB, MLMC and the MUHC IRBs approve the anonymous use of data retrospectively abstracted from clinical care databases without requiring consent); MUHC and EARTH patients sign a general waiver on opening a medical chart at the hospital but no specific study related consent).

Eligibility

Participants included in the current analysis had an altered T-cell phenotype at baseline. Participants were eligible for analysis if a) they came from sites able to provide electronic data on all of the following immunological markers: CD4 and CD8 T-cell counts and percentages, CD3 T-cell percentages and CD4:CD8 T-cell ratios, b) had at least 1 record with all 6 markers within 2 years prior to starting cART and c) had a at least two follow-up records with all 6 markers greater than 30 days apart following the initiation of treatment. Note that inclusion criteria for enrollment in CANOC require that each individual have CD4 T-cell counts within 6 months of starting cART.

Therefore, all 4459 individuals included in the study had CD4 measures within 6 months of treatment initiation; of those, 4359 (97.8%) had all three baseline T-cell measures (CD4, CD8 and CD3) within 6 months of starting therapy. Only 100 (2.2%) individuals had complete TCP values between 6 months and 2 years. Thus our 2 year pre-cART window does not introduce any significant bias in terms of baseline evaluation. The data for all patients meeting eligibility criteria at participating sites were included in CANOC.

Primary Outcome

HIV-induced altered T-cell phenotype involves one of: (1) low CD4 T-cells (<532 cells/mm^3), (2) lost T-cell homeostasis (low (<65%) or high (>85%) CD3 T-cell percent) or (3) ratio dysregulation (CD4:CD8 ratio <1.2). For patients without CD3 T-cell percent measurements, CD3 T-cell percent was calculated as the sum of CD4 and CD8 T-cell percentages. Considering that CD4 and CD8 T-cell percentages and absolute counts are the main components of total T-cell levels and CD4:CD8 T-cell ratio, we also assessed each of these markers in order to ensure a thorough evaluation of the TCP.

The primary outcome of interest was the achievement of a healthy T-cell phenotype on at least 2 sequential visits at least 30 days apart. T-cell phenotype recovery was defined as meeting all 6 of the following criteria: CD3 T-cell percent: 65-85%, CD4/CD8 ratio: 1.2-3.3, CD4 T-cell counts: 532-1170 cells/mm^3, CD4 T-cell percent: 39-55%, CD8 T-cell counts: 236-651 cells/mm^3 and CD8 T-cell percent: 18-31%. These values were derived from 124 healthy controls (62 males and 62 females) recruited at the Montreal General Hospital. These controls had a median age of 39 (IQR=32-47), were all HIV-negative and received thorough physical examinations. Furthermore, these individuals were also screened for the presence of primary immune deficiencies

and auto-immune diseases.

Patients who failed to maintain at least one of these parameters within its physiological range were considered to have an altered TCP.

Statistical Methods

Demographic and clinical characteristics at baseline are summarized using medians and interquartile ranges (IQR) for continuous variables and frequencies and proportions for categorical variables. Baseline values were defined as the closest values within 2 years of initiating cART. Duration of the follow-up period was measured from the time of cART initiation. Time to normalization of T-cell phenotype was assessed using the Kaplan-Meier survival method. Predictors of T-cell phenotype normalization were assessed using univariate and multivariable Cox proportional hazards models. The assumption of proportional hazards was checked and met for each covariate. Based on previous studies analyzing the effect of HIV infection on the immune system, socio-demographic and clinical covariates potentially associated with immune dysregulation were considered for inclusion in the analyses [366,367]. Since there were few events, we were conservative with regard to the number of variables included in the model. Due to the great deal of missing data for variables such as ethnicity, HIV risk factors and HCV co-infection, and since these factors were neither associated with the primary outcome in an adjusted model nor changed the inference of the other covariates under consideration, we did not include these variables in the final multivariable model.

All analyses were performed using SAS software version 9.3 (SAS Institute, Cary, North Carolina, USA).

3.4 Results

Among the 6673 initially ART-naïve HIV-positive individuals followed within CANOC, 2214 were excluded from the analysis. Of these, 1071 came from 2 sites that didn't have electronic records of T-cell percentages available. Information on excluded participants and reason of exclusion has been detailed in **Figure 1**. 4459 patients met the inclusion criteria for this study. These individuals were studied for a median duration of 3.14 years (IQR=1.48-5.47 years), with the median year of cART initiation in 2005 (IQR=2002-2007)**.** Most individuals were on an NNRTI-based or PI-based regimen at the time of treatment initiation.

The demographic and clinical baseline characteristics of the study population are summarized in **Table 1**. At baseline 96% of the patients had CD4 T-cell counts <532 cells/mm^3, 32% had CD3 percentages outside the homeostatic range (65-85% of circulating lymphocytes) and 99% had a CD4:CD8 ratio <1.2. Among individuals with low CD4 T-cells, 53% had CD4 T-cell counts <200 cells/mm^3 which connotes severe HIV disease [368]. Six hundred and five individuals exhibited AIDS defining illnesses at baseline.

Following treatment initiation, 68% achieved normal T-cell homeostasis, 6.6% a balanced CD4:CD8 ratio and 30% a normal CD4 T-cell count throughout the course of their follow-up. Of the individuals that did not normalize their CD4:CD8 ratio (93%) during the study period, 96% had elevated CD8 T-cell percentages and 68% had elevated CD8 T-cell counts.

Only 85 (2%) individuals reached the primary endpoint of normalizing all the components of the T-cell phenotype during the follow-up period. The probability of normalizing the complete T-cell phenotype after 5 years of treatment was 0.08 (0.03-0.14) percent for people who initiated therapy with CD4 T-cell counts within the normal range and 0.008 (0.003-0.012) percent for those who initiated therapy at CD4 T-cell counts<200 cells/mm^3.

Figure 2 shows Kaplan-Meier (KM) curves comparing the time to T-cell phenotype normalization according to the degree of immune alteration at baseline. Panels A, B and C display the KM curves for time to TCP normalization by baseline CD4 T-cell counts, baseline CD4:CD8 ratio and baseline CD3 percentages, respectively. In all three panels, time to TCP normalization was shorter among individuals who maintained their immune parameters within physiological ranges at baseline. Note that for the purpose of the survival analysis, the CD4 T-cell categories: 532-1170 cells/μL and >1170 cells/μL were collapsed due to the very small number of patients with baseline CD4 T-cells above 1170 cells/μL (n=8).

Table 2 shows the hazard ratios of T-cell phenotype normalization associated with covariates of interest from univariate proportional hazards models. Participants with a high CD8 percentage at baseline and those that were intravenous drug users (IDU) were less likely to normalize. Conversely, participants with a high CD4:CD8 ratio at baseline and those with sustained viral suppression were more likely to normalize their T-cell phenotype. In a multivariable proportional hazards model adjusted for age, rate of measurement of immune markers and baseline regimen; higher baseline CD4 T-cell counts and HIV viral load suppression were associated with increased likelihood of T-cell phenotype normalization (HR = 1.42 per 100 cell increase in baseline CD4 T-cell count, 95% CI= [1.31, 1.54], $p \leq .0001$ and HR = 3.69, 95% CI= [1.58, 8.61], $p \leq 0.01$, respectively) (**Table 3**).

3.5 Discussion

We studied the predictors of complete T-cell phenotype normalization in a well-described, large cohort of treatment naïve HIV seropositive patients with an altered immune phenotype who were initiating cART. At baseline, although almost all the patients exhibited CD4 T-cell depletion and CD4:CD8 ratio dysregulation, only a third of the population had lost their CD3 T-cell homeostasis. Despite a trend of increasing CD4 T-cell counts, only one third of our sample normalized their CD4 T-cell counts during the study period and very few (2%) normalized their complete T-cell phenotype. The overall probability of normalizing the complete T-cell phenotype 5 years after treatment initiation was greater in those with baseline CD4 T-cell counts within the normal range compared to those with baseline CD4 T-cell counts<200 cells/mm^3 (0.08 vs. 0.007). We found that a high CD4 T-cell count at baseline was associated with an increased hazard of normalizing the complete T-cell phenotype; and that individuals who were unable to suppress their viral load over time were less likely to have normal TCP.

Since a change in the marker will be documented sooner on average for a patient monitored closely than for a patient followed infrequently, the rate of measurement of a marker is associated with the chance of observing a change in that marker. Our previous paper on rates of viral load measurement documented associations of rates of measurement with characteristics such as geographic region, HIV risk factor and age [27]. Failure to adjust for rates of measurement of CD4 T-cell counts may result in spurious associations of normalization of phenotype with covariates associated with more frequent measurement. Our multivariable analysis shows that after adjusting for the frequency of laboratory measurements, CD4 T-cell counts and HIV viral load suppression still have a statistically significant effect on normalization. . We detected no statistically significant associations of age and type of baseline regimen with the likelihood of normalizing the complete

T-cell phenotype.

Despite the fact that a third of the study population restored their CD4 T-cell counts and that 68% achieved T-cell homeostasis, only 7% of the patients were able to normalize their CD4:CD8 ratio. This was due to a disproportionate contribution of the CD8 T-cell compartment to the overall ratio. Indeed, the majority of patients with dysregulated ratio had abnormally high levels of circulating CD8 T-cells. This phenomenon was reflected in the univariate analysis where high percentages of CD8 T-cells were associated with a decreased likelihood of normalizing.

Although immune recovery has been extensively studied in numerous cohorts, research has focused almost exclusively on CD4 T-cell recovery [369,370]. There is a paucity of data available on long-term CD4:CD8 ratio recovery and, to our knowledge, no studies on T-cell homeostasis restoration in treated HIV+ patients. The findings from our study are in line with previous studies showing that the mechanism of T-cell homeostasis is a "blind" process that occurs irrespective of changes in the CD4 and CD8 compartment [371,372]. In HIV disease, the failure of T-cell homeostasis has been shown to coincide with the onset of clinically defined AIDS.

While previous studies have measured T-cell homeostasis in terms of CD3 T-cell counts, in this study we chose to look at T-cell homeostasis in terms of CD3 T-cell percentages as we consider percentage to be a more reliable marker with less between measurement variations. [349,373]. Furthermore, while CD3 T-cell counts reflects the total lymphocyte count in the blood, CD3 T-cell percentage avoids the impact of lymphocyte fluctuations and is a better measure of proportional stability of circulating T-cells [348,349,374].

In a study by Margolick et al., a group of 372 seroconverters enrolled in the MACS cohort were followed for 8 years post-seroconversion. The study data showed that individuals who did not develop clinical AIDS were able to maintain homeostatic levels of T-cells for several years,

despite significant declines in their CD4 T-cell counts. However, among those who developed AIDS, a loss of T-cell homeostasis (characterized by a substantial decline in the total level of circulating CD3 T-cells) was observed approximately 18 months prior to the onset of AIDS [375]. Using artificial intelligence tools, we previously published on the utility of CD3 T-cell percentages in predicting mortality and morbidity in HIV+ treated individuals [272]. These findings highlight the potential role of blind T-cell homeostasis as an independent marker of HIV disease progression and AIDS onset. Studies of HIV-negative individuals have emphasized the importance of T-cell homeostasis in maintaining the integrity of the immune system. Disruption of T-cell homeostasis has been associated with autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) [176,276,376]. There is a paucity of data linking morbidity as an outcome of long-term disrupted T-cell homeostasis in those with HIV disease. We have recently reported data from CANOC, showing for the first time that loss of T-cell homeostasis is associated with poor clinical outcomes in those treated for HIV-infection [377].

It is unclear to what extent HIV accounts for persistent immune dysregulation following successful therapy. Since HIV primarily leads to CD4 T-cell depletion it was not surprising that successful ART significantly increased CD4 T-cell counts in our cohort of patients. Although the CD8 T-cell compartment rapidly expands early in HIV infection other co morbid viral infections may contribute and sustain this expansion. These viral co-infections include cytomegalovirus (CMV) and Epstein-Barr virus (EBV), which are known to sustain the expansion of CD8 T-cells during their chronic infection phase [341,378]. A recent study by Naeger et al., showed that CMV-specific CD8 T-cell response is high in successfully treated HIV-infected individuals [265]. Thus, the persistent CD8 T-cell lymphocytosis and the low CD4:CD8 ratio observed in long-term treated and virologically suppressed HIV-infected patients might be partially explained by a subclinical

CMV infection. In HIV seronegative individuals, CMV infection is also associated with immune senescence [379,380]. An important characteristic of immune senescence is the large expansion of CD8+CD28- T-cells. The frequency of these cells increases with progressive HIV disease and can account for up to 50% of the CD8 T-cell compartment, thus contributing to the dysregulation of the CD4:CD8 ratio [381]. Geriatric longitudinal studies found a CD4:CD8 T-cell ratio of < 1 and CMV-seropositivity to be part of an "immune risk phenotype" (IRP) that is associated with higher mortality and morbidity rates [283]. Elderly individuals with an IRP have increased susceptibility to infections, reactivation of latent pathogens, and decreased responses to vaccination [382], reflecting an age-related loss of T-cell responsiveness.

Due to the strict definition of the outcome variables, a significant number of patients were excluded from the analysis because of missing data on one or more of the 6 required immunological markers at baseline and/or at follow-up. Finally, because thereis a fair amount of heterogeneity in T-cell reference values across various studies and populations, it is possible that our definition of TCP may not apply to patients outside of our study demographics.

In conclusion, disruptions in T-cell homeostasis resulting from infections or medical interventions are generally expected to be transient events [383]. However, our data shows a striking lack of recovery of the T-cell phenotype despite successful treatment. It remains to be determined if HIV infection results in irreversible phenotypic as well as functional changes within the T-cell compartment or whether co-morbid chronic viral infections such as CMV prevent the normalization of T-cell homeostatic and regulatory processes. For those with HIV, the clinical importance of maintaining a normal phenotype as it relates to long-term morbidities remains to be elucidated. The data presented in this study suggest that the concept of immune reconstitution should not be restricted to CD4 T-cell counts as HIV-infection is associated with other changes

within the T-cell compartment that are not immediately restored by antiretroviral therapy. These residual immune abnormalities in many patients resemble the Immune Risk Phenotype of the elderly and might thus be associated with deleterious clinical outcomes in the long term. The data presented in this study reflect the need for more in depth research into this complex area of long-term management of HIV infection.

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The CANOC Collaboration includes:** ***Investigators*:* Gloria Aykroyd (Ontario HIV Treatment Network, OHTN), Louise Balfour (University of Ottawa, OHTN Cohort Study, OCS Co-Investigator), Ahmed Bayoumi (University of Toronto, OCS Co-Investigator), Ann Burchell (Ontario HIV Treatment Network), John Cairney (University of Toronto, OCS Co-Investigator), Liviana Calzavara (University of Toronto, OCS Co-Investigator), Angela Cescon (British Columbia Centre for Excellence in HIV/AIDS), Curtis Cooper (University of Ottawa, OCS Co-Investigator), Kevin Gough (University of Toronto, OCS Co-Investigator), Silvia Guillemi (British Columbia Centre for Excellence in HIV/AIDS, University of British Columbia), P. Richard Harrigan (British Columbia Centre for Excellence in HIV/AIDS, University of British Columbia), Marianne Harris (British Columbia Centre for Excellence in HIV/AIDS), George Hatzakis (McGill University), Robert Hogg (British Columbia Centre for Excellence in HIV/AIDS, Simon Fraser University), Sean Hosein (CATIE), Don Kilby (University of Ottawa, Ontario HIV Treatment Network), Marina Klein (Montreal Chest Institute Immunodeficiency Service Cohort, McGill University), Richard Lalonde (The Montreal Chest Institute Immunodeficiency Service

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3.7 Conflict of interest and Source of funding

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3.8 Figure legend

Figure 1. Flow chart of inclusion criteria

Flow chart detailing information on excluded participants and reason for exclusion. Among the 6673 initially ART-naïve HIV-positive individuals followed within CANOC, 4459 patients met the inclusion criteria for this study.

Figure 2. Time to T-cell phenotype normalization

Kaplan-Meier survival curves for time to T-cell phenotype normalization by degree of immune alteration. CD4 T-cell counts, CD4:CD8 ratio and CD3 T-cell percentages were stratified according to levels of dysregulation versus normal physiological range. Panel A: Kaplan-Meier plot of time to T-cell Immunophenotype profile normalization by baseline CD4 T-cell count (the CD4 T-cell count group 532-1170 cells/mm^3 includes 8 individual with a CD4 T-cell count >1170 cells/mm^3); panel B: Kaplan-Meier plot of time to T-cell Immunophenotype Profile Normalization by CD4:CD8 ratio (the CD4:CD8 ratio group 1.2-3.3 includes 1 individual with a CD4:CD8 ratio >3.3); panel C: Kaplan-Meier plot of time to T-cell Immunophenotype Profile Normalization by CD3 percent.

3.9 Tables and Figures

Table 1. Demographic and Clinical Baseline Characteristics for 4459 HIV+ Patients on Combination Antiretroviral Therapy

Variable	Included (N=4459)
Province	
British Columbia	2066 (46%)
Ontario	1727 (39%)
Quebec	666 (15%)
Age at first ARV Treatment (years)	40.0 (34.0-46.7)
Sex	
Male	3668 (82%)
Female	790 (18%)
Ethnicity	
Caucasian	1368 (31%)
Black	231 (5%)
Aboriginal	208 (5%)
Mixed	136 (3%)
Other	224 (5%)
Unknown/ Missing	2292 (51%)
HIV Risk Factor	
Men who have Sex with Men	1501 (34%)
Intravenous Drug Users	935 (21%)
From Endemic Country	365 (8%)
Unknown/ Missing	1471 (33%)

Variable	Included (N=4459)
Year of first ARV treatment	2005 (2002-2007)
Baseline Regimen	
NNRTI-based	2022 (45%)
Boosted PI-based	2006 (45%)
Single PI-based	352 (8%)
NRTI only	79 (2%)
Baseline CD4 (cells/mm^3)	190 (105-280)
<200 cells/mm^3	2275 (51%)
200-349 cells/mm^3	1505 (34%)
350-531 cells/mm^3	492 (11%)
532-1170 cells/mm^3 (within normal range)	179 (4%)
>1170 cells/mm^3	8 (0%)
Baseline CD8 (cells/mm^3)	764 (500-1130)
<236 cells/mm^3	255 (6%)
236-651 cells/mm^3 (within normal range)	1504 (34%)
652-1200 cells/mm^3	1746 (39%)
>1200 cells/mm^3	954 (21%)
Baseline CD3% (percentage)	76% (69-83%)
<50%	167 (4%)
50-64%	549 (12%)
65-85% (within normal range)	3040 (68%)
>85%	703 (16%)
Baseline CD4:CD8 ratio	0.22 (0.14-0.36)

Variable	Included (N=4459)
<0.5	3874 (87%)
0.5-1.1	552 (12%)
1.2-3.3 (within normal range)	32 (1%)
>3.3	1 (0%)
Baseline Viral Load (log 10 copies/mL)	4.9 (4.4-5.0)
Presence of AIDS defining illness at Baseline	605 (14%)
Hepatitis B virus Co-infection	
Yes	250 (6%)
No	1233 (28%)
Unknown/ Missing	2976 (66%)
Hepatitis C virus Co-infection	
Yes	965 (22%)
No	2413 (54%)
Unknown/ Missing	1081 (24%)

Variables are described in terms of frequency and percentage, or median and interquartile range. NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; ARV, antiretroviral; AIDS, acquired immunodeficiency syndrome.

Table 2. Univariate Analysis of Time to Immunophenotype Normalization

Variable	N	Hazard Ratio	95% Confidence Interval	P-value
Demographic Parameters				
Age (Continuous per 10 years)	4459	1.26	(1.02,1.56)	0.03
Sex	4458			
Female		1	--	--
Male		1.59	(0.82,3.07)	0.17
Province	4459			
British Columbia		1	--	--
Ontario		1.37	(0.85,2.20)	0.19
Quebec		1.40	(0.76,2.60)	0.28
Ethnicity	2167			
Caucasian		1	--	--
Black		0.19	(0.03,1.37)	0.10
Aboriginal		0.67	(0.21,2.19)	0.51
Mixed		0.56	(0.13,2.31)	0.42
Other		0.63	(0.19,2.05)	0.44
Risk Factors	2998			
Men who have Sex with Men		1.41	(0.84,2.36)	0.19
Injection Drug Users		0.53	(0.28,1.02)	0.06
From Endemic Country		0.80	(0.34,1.86)	0.60
Baseline Regimen	4459			
NNRTI-based		1	--	--

Variable	N	Hazard Ratio	95% Confidence Interval	P-value
NRTI only		0.48	(0.07, 3.54)	0.48
Single PI-Based		1.08	(0.52, 2.25)	0.83
Boosted PI-based		1.33	(0.84, 2.10)	0.23
Rate of measurement	4459			
<3 measures per year		1.26	(0.60,2.64)	0.55
3-4 measures per year		1	--	--
4-6 measures per year		2.26	(1.21,4.22)	0.01
>6 measures per year		2.50	(1.21,5.18)	0.01
Year of First ARV (continuous per year)	4459	1.01	(0.91,1.12)	0.80
Hepatitis C Co-infected	3378	0.78	(0.44,1.38)	0.39
Hepatitis B Co-infected	1483	0.38	(0.12,1.23)	0.11
AIDS at baseline	4258	0.76	(0.39,1.47)	0.42
Baseline Immunological Parameters				
Baseline CD4 count	4459			
<200 cells/mm^3		0.11	(0.05,0.25)	<.0001
200-349 cells/mm^3		0.40	(0.19,0.81)	0.01
350-531 cells/mm^3		1.14	(0.56,2.33)	0.72
532-1170 cells/mm^3 (within normal range)		1	--	--
Continuous (per 100 cell increase)	4459	1.36	(1.27,1.47)	<.0001
Baseline CD4%	4459			
<14%		0.02	(0.01,0.04)	<.0001
14-24%		0.11	(0.05,0.22)	<.0001

Variable	N	Hazard Ratio	95% Confidence Interval	P-value
25-38%		0.38	(0.18,0.78)	<.01
39-55% (within normal range)		1	--	--
Continuous (per 0.1 unit increase)	4459	3.24	(2.69,3.89)	<.0001
Baseline CD8 count	4459			
<236 cells/mm^3		1.14	(0.51,2.55)	0.76
236-651 cells/mm^3 (within normal range)		1	--	--
652-1200 cells/mm^3		0.82	(0.51,1.31)	0.40
>1200 cells/mm^3		0.40	(0.19,0.84)	0.02
Continuous (per 100 cell increase)	4459	0.95	(0.90,0.99)	0.02
Baseline CD8%	4459			
18-31% (within normal range)		1	--	--
32-60%		0.40	(0.18,0.87)	0.02
>60%		0.07	(0.03,0.17)	<.0001
Continuous (per 0.1 unit increase)	4459	0.56	(0.49,0.65)	<.0001
Baseline CD3%	4459			
<50%		0.26	(0.04,1.85)	0.18
50-64%		0.55	(0.25,1.21)	0.14
65-85% (within normal range)		1	--	--
>85%		0.74	(0.39,1.41)	0.36
Continuous (per 0.1 unit increase)	4459	1.11	(0.92,1.35)	0.28
Baseline Ratio	4459			
<0.5		0.03	(0.01,0.05)	<.0001
0.5-1.1		0.14	(0.07,0.29)	<.0001

Variable	N	Hazard Ratio	95% Confidence Interval	P-value
1.2-3.3 (within normal range)		1	--	--
Continuous (per 0.1 unit increase)	4459	1.20	(1.17,1.24)	<.0001
Baseline lymphocyte count (Continuous per 100 cell increase)	4459	1.00	(0.97,1.03)	0.99
Suppressed VL at Baseline	4459	1.14	(0.36,3.61)	0.82
Viral suppression (time-dependent)	4459	2.90	(1.44,5.84)	<.01
Viral Load Continuous (time-dependent)	4459	0.65	(0.47,0.92)	0.01

NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; ARV, antiretroviral; AIDS, acquired immunodeficiency syndrome; VL, viral load.

Table 3. Multivariable Proportional Hazards models of Time to Normalization of Immunophenotype profile

Parameter	Hazard Ratio	95% Confidence Interval	P-value
Age (continuous per 10 years)	1.14	(0.92, 1.40)	0.23
Rate of measure			
<4 measures per year	1	--	
4-6 measures per year	2.23	(1.36, 3.68)	0.002
>6 measures per year	2.64	(1.38, 5.05)	0.003
Baseline Regimen			
NNRTI-based	1	--	--
NRTI only	0.41	(0.06, 3.01)	0.38
Single PI-Based	0.89	(0.42, 1.88)	0.75
Boosted PI-based	1.28	(0.80, 2.04)	0.30
Baseline CD4 count (continuous per 100 cells)	1.44	(1.33, 1.55)	<0.0001
Suppressed Viral Load (<50 copies/mL) (time-dependent)	2.84	(1.43, 5.63)	0.003

NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor. **N=4459.**

Figure 1. Flow Chart of Inclusion Criteria

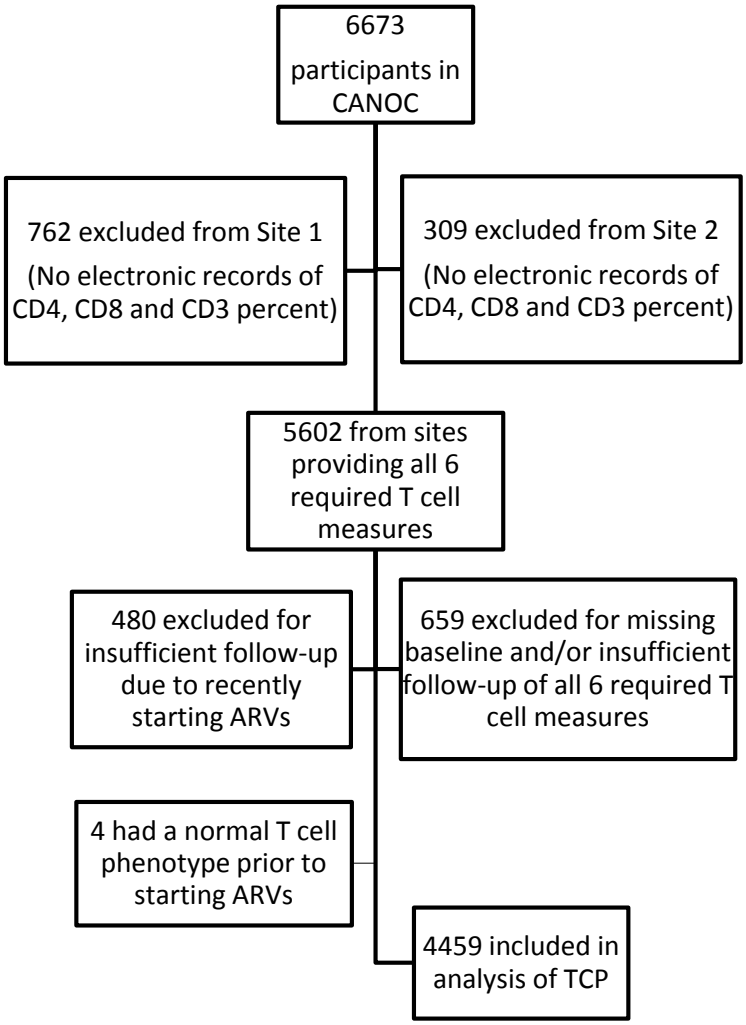
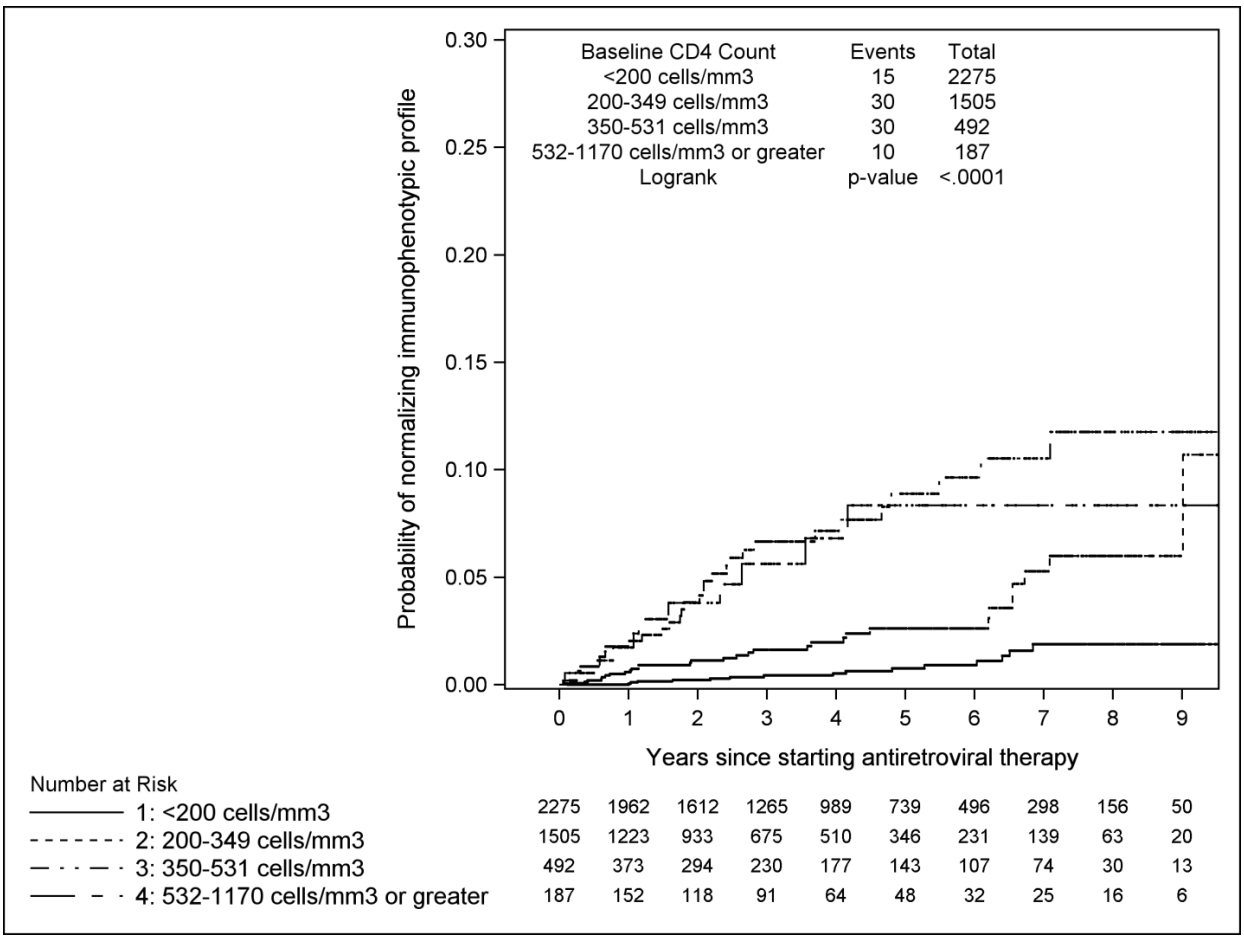
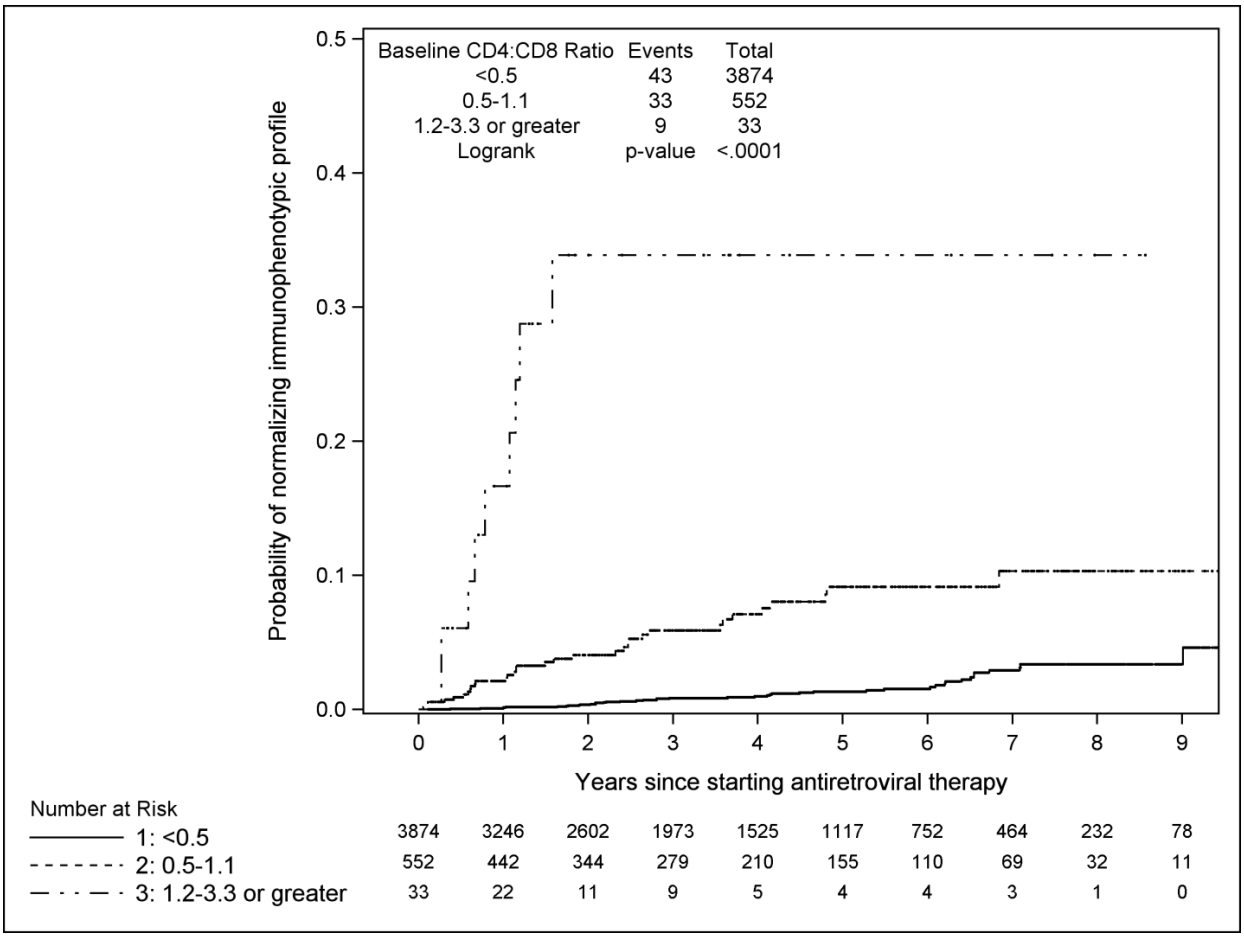


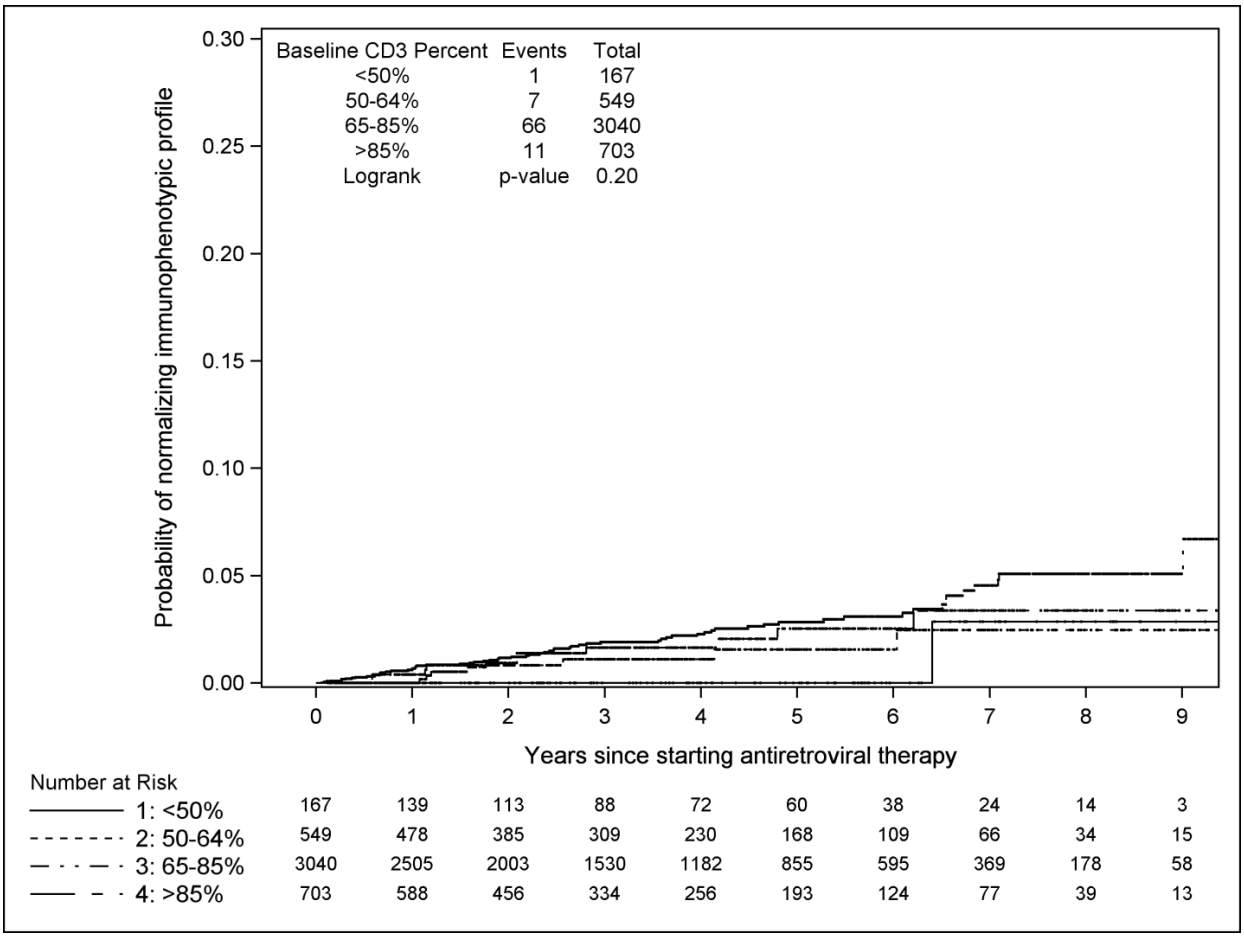
Figure 2. Time to T-cell phenotype normalization



1a. Kaplan-Meier Plot of time to T-cell Immunophenotype profile normalization by baseline CD4 T-cell count



1b. Kaplan-Meier Plot of time to T-cell Immunophenotype Profile Normalization by CD4:CD8 ratio



1c. Kaplan-Meier Plot of time to T-cell Immunophenotype Profile Normalization by CD3 percent

3.10 References

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Bridge from chapter 3 to chapter 4

The data presented in chapter 3 demonstrated that early treatment initiation and long-term viral suppression are both independent predictors of TCP recovery. Both persistent CD4 depletion and CD4:CD8 dysregulation have been associated with poor prognosis in HIV patients receiving ART. However, despite being associated with poor prognosis in untreated HIV disease, little is known about the clinical relevance of CD3+ T-cell homeostasis in successfully treated HIV patients. The study presented in chapter 4, aims to elucidate the characteristics and clinical impact of T-cell homeostasis failure in the context of treated HIV infection.

Chapter 4:

Clinical impact of altered T-cell homeostasis in treated HIV patients enrolled in a large Canadian Observational Cohort

Clinical impact of altered T-cell homeostasis in treated HIV patients enrolled in a large Canadian Observational Cohort

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4.1 Abstract

Objective(s): We investigated the probability of transitioning in or out of the CD3+ T-cell homeostatic range during antiretroviral therapy, and we assessed the clinical impact of lost T-cell homeostasis (TCH) on AIDS Defining Illnesses (ADI) or death.

Design: Within the Canadian Observational Cohort (CANOC), We studied 4463 ART-naïve HIV-positive patients initiating combination antiretroviral therapy between 2000 and 2010.

Methods: CD3 trajectories were estimated using a four states Markov model. CD3 percentage states were classified as: very low (<50%), low (50-64%), normal (65-85%) and high (>85%). Covariates associated with transitioning between states were examined. The association between CD3 states and time to ADI/Death from cART initiation was determined using Cox proportional hazards (PH) models.

Results: 4463 patients were followed for a median of 3 years. 2508 (56%) patients never transitioned from their baseline CD3 state; 85% of these had normal TCH. In multivariable analysis, individuals with time-updated low CD4 count, time-updated detectable HIV RNA, older age and HCV co-infection were less likely to maintain TCH. In the multivariable PH model both very low and high CD3 percentages were associated with increased risk of ADI/Death (adjusted HR=1.91 (95% CI: 1.27, 2.89) and HR=1.49 (95% CI: 1.13, 1.96), respectively).

Conclusions: Patients with very low or high CD3 percentages are at risk for ADIs/Death. To our knowledge this is the first study linking altered TCH and morbidity/mortality in cART-treated HIV-positive patients.

Key words: HIV;T-cell homeostasis;combination antiretroviral therapy;Immune reconstitution;AIDS defining illness

4.2 Introduction

Homeostatic regulation occurs in all living organisms and is critical for preserving a variety of biologic parameters within a normal physiologic range [384]. The maintenance of T-cell homeostasis (TCH) is essential for the functional integrity of the immune system. TCH is achieved through multiple complex and tightly regulated processes such as: thymic output, access to cytokines, interaction with self-peptide:MHC complexes and antigen-independent peripheral T-cell proliferation [192,201].

TCH is characterized by the maintenance of T-cell (CD3+) percentages within a normal physiologic range (NPR) representing 65-85% of peripheral blood lymphocyte [377]. The inability to maintain T-cells within that range denotes an impairment of TCH regulation. TCH imbalance, in certain situations, can be used as a diagnostic tool. For instance, T-cell lymphocytosis (abnormally high levels of T-cells) is associated with viral and bacterial infections, autoimmune diseases, lymphocytic leukemias and other lymphoprolypherative disorders [385-387]. On the other hand, T-cell lymphopenia (abnormally low level of T-cells) can be seen during acute infections, in hematologic disorders, and in both primary and secondary immune deficiency [172,388].

In a study by Margolick et al., a group of 372 HIV seroconverters enrolled in the MACS cohort were followed for 8 years post-seroconversion. It was found that individuals who did not progress to AIDS maintained an intact TCH, whereas those who progressed experienced a failure of their TCH approximately 18 months prior to the onset of AIDS [322]. This TCH failure was characterized by a substantial decline in the total level of T-cells. Therefore, although progressive CD4+ T-cell depletion is the hallmark of HIV disease, impaired TCH can also occur during the

course of the infection and is associated with progression to AIDS [321,389].

Impaired TCH has also been linked to deleterious clinical outcomes in non-HIV settings such as systemic lupus erythematosus, burn injury with subsequent decreased resistance to infections and active pulmonary tuberculosis [276,277,361,390].

Despite findings that impaired TCH is associated with negative clinical outcomes in both HIV and non-HIV settings, few, if any, studies have assessed the effect of long-term successful cART on TCH maintenance. The dynamics of CD3+ T-cell normalization in treated HIV+ patients are therefore unknown. We therefore investigated the probability and predictors of transitioning in or out of the NPR during the course of therapy. Furthermore, we also assessed the clinical impact of impaired TCH on AIDS Defining Illness (ADI) or death.

4.3 Methods

Cohort Description

The Canadian Observational Cohort (CANOC) collaboration is a study of antiretroviral-naïve HIV-positive patients initiating cART on or after January 1, 2000 [391]. CANOC participants represent nearly a quarter of Canadians currently on cART. This collaboration is open to all Canadian HIV treatment cohorts with more than 100 eligible patients and currently includes 8 participating cohorts across Canada. Eligibility criteria for inclusion into CANOC include: documented HIV infection, residence in Canada, aged 18 years and older, initiation of a first antiretroviral regimen comprised of at least 3 individual agents, and at least 1 measurement of HIV-1 RNA viral load and CD4+ T-cell count within 6 months of initiating cART. Patient selection and data extraction are performed locally at the data centers of the participating cohort studies.

Non-nominal data from each cohort on a predefined set of demographic, laboratory, and clinical variables are then pooled at the Project Data Centre in Vancouver, British Columbia. The last date of follow-up in the cohort for the current analysis was August 22, 2010. All participating cohorts have received approval from their institutional ethics boards to contribute non-nominal patient specific data.

Eligibility

Participants included in this study were first treated with at least 3 individual antiretroviral agents on or after January 1st, 2000. Participants were eligible for the analysis if a) they came from sites able to collect and provide electronic data on CD3+ T-cell percentages, b) had at least 1 CD3+ T-cell measurement within 2 years prior to starting cART, and c) had a at least two follow-up CD3+ T-cell measurements greater than 30 days apart following treatment initiation. Additionally, individuals from sites providing electronic ADI data without an ADI diagnosis prior to cART initiation were eligible for inclusion in the clinical impact analysis.

Study Design

TCH was defined as the maintenance of CD3+ T-cell percentages within an NPR, representing 65-85% of circulating blood lymphocytes. These values are based on the historical phenotyping of 124 healthy HIV seronegative controls recruited through the Montreal General Hospital. All controls received a thourough physical examination and were screened for immune deficiency and auto-immune diseases. Altered TCH was defined as having T-cell percentages that were lower or higher than the NPR. We followed patients with available baseline and follow-up CD3 measurements. Markov states were defined according to the CD3+ T-cell percentage intervals

at each visit as follows: *very low* (<50%), *low* (50-64%), *normal* (65-85%) and *high* (>85%). Patients required at least two consecutive values outside their CD3 state to be considered as having transitioned.

Primary Outcomes

Primary outcomes of interest were:

(a) Achievement of T-cell homeostasis – defined as remaining or transitioning into the NPR for CD3+ T-cell percentages (65-85%).

(b) Time from cART initiation to ADI or death – defined as developing an ADI or dying during the course of the follow-up.

Statistical Methods

Demographic and clinical characteristics

Baseline characteristics were summarized using medians and interquartile ranges (IQR) for continuous variables, and frequencies for categorical variables. Baseline values were defined as the closest values within 2 years of initiating cART. Duration of the follow-up period was measured from the time of cART initiation.

Longitudinal dynamics of CD3+ T-cell percentages

A Multi-State Markov model was used to model CD3+ T-cell dynamics in treated HIV-positive patients. Markov models depict the evolution of a disease as a gradual progression of mutually exclusive health states. These models are characterized by transition intensities, which represent the probability of transitioning from one state to another. Transition intensities are computed using the number of observed transitions between states and the time at which these

transitions took place [392,393].

In this paper we explored a continuous-time Markov process with four states defined by CD3+ T-cell percentage levels. We used a state structure that assumes an individual must pass through all intermediate states in order to move from the lowest state to the highest and vice versa (Figure 1). We estimated the mean amount of time that patients spent in a specific state prior to transitioning to a new state. We then performed univariate and multivariable analyses to assess the effect of covariates of interest on the transition probabilities between individual states. Based on previous studies analyzing the effect of HIV infection on the immune system, socio-demographic and clinical covariates potentially associated with immune dysregulation were included in the multivariable analysis [366,367]. These variables included: age, gender, region, baseline treatment regimen, time-dependent detectable HIV viral load and Hepatitis C virus (HCV) co-infection.

Clinical impact of CD3+ T-cell percentages on ADI or death

The primary covariate of interest was time-updated CD3+ T-cell state. Patients were stratified according to their CD3+ T-cell values, as detailed above, and time from first cART initiation to ADI or death was calculated. Individuals were censored at their last CD3+ T-cell measurement if they did not experience an event (ADI or death). The association between the patient's current CD3+ T-cell state and his progression to ADI or death was determined by Cox proportional hazards (PH) models [394].

All analyses were performed using SAS software version 9.3 (SAS Institute, Cary, North Carolina, USA). P-values<0.05 were considered significant.

4.4 Results

Demographic and clinical characteristics

Among the 6673 initially ART-naïve HIV-positive individuals followed within CANOC, 2210 were excluded from the analysis. Of these, 1071 came from 2 sites that did not have available electronic records of CD3+ T-cell percentages, 480 had insufficient follow-up data due to recent initiation of antiretroviral therapy and 659 had missing baseline and/or insufficient follow-up data. 4463 patients met the inclusion criteria for the multi-state analysis. Of these, 605 were diagnosed with ADI prior to cART initiation and were therefore excluded from the clinical impact analysis. The median follow-up times were 3.15 (IQR=1.48-5.47) years and 3.06 (3.63-6.54) years for the multi-state and the survival analyses, respectively. The median year of cART initiation was 2005 (IQR=2002-2007)**.** Most individuals were on an NNRTI-based or boosted PI-based regimen at the time of treatment initiation.

At baseline, for the multi-state analysis, median age was 40 (IQR=34-47) years old, median CD4 T-cell count was 190 (IQR= 106-280) cells/ul and median log10 viral load was 5.0 (IQR=4.0-5.0) copies/ml. 68% had CD3+ T-cell percentages within the NPR (65-85% of circulating lymphocytes), and 29% were co-infected with Hepatitis C virus (HCV). These values were similar in the survival analysis. The demographic and clinical baseline characteristics of the study population for both analyses are summarized in **Table 1**.

Multi-state modelling for transition between CD3 states

Transition intensities between states were estimated and summarized in **Figure 1**. Individuals in the high CD3+ T-cell percentage state were 1.7 times more likely to transition to the NPR than those in the low state (transition intensities: 0.055 and 0.032, respectively). The mean time spent in each specific state was also estimated. Patients in the normal CD3+ T-cell percentage state remained stable for the longest time, with a mean period of 87.3 months (CI: 82.90-91.93 months) prior to transitioning. Patients in the very low, low and high CD3+ T-cell percentage states transitioned after 15.34 months (95% Confidence Interval (CI): 13.52-17.39), 25.76 months (95% CI: 24.25-27.36) and 18.08 months (95% CI: 16.88-19.37) respectively.

Demographics and clinical characteristics by transition history are depicted in **Table 2**. Individuals were classified based on never transitioning from their baseline state or transitioning to the adjacent state. 2508 (56%) patients never transitioned from their baseline CD3+ T-cell percentage state; 85% of these had normal TCH at baseline. After adjusting for treatment regimen, multivariable analysis showed that individuals with time-updated low CD4+ T-cell count and detectable HIV RNA were less likely to maintain TCH, and more likely to transition to lower CD3+ T-cell percentage states. Older age (>50 years old) and HCV co-infection were also associated with increased likelihood of transitioning out of the NPR (**Figure 2**).

Survival analysis for time to ADI or death

Among the 3656 patients included in the survival analysis, we observed 438 events; of these, 217 were ADI diagnoses, and 221 were deaths. Survival analysis for time to ADI or death revealed that those in the very low CD3+ T-cell state (CD3+ T-cell percentages <50%) had the

fastest progression to ADI or death compared to the other groups. A Kaplan-Meier curve of time to ADI or death by CD3+ T-cell percentage states is shown in **Figure 3**.

In the multivariable PH model, after adjusting for region and gender, both *very low* and *high* CD3+ T-cell percentages were associated with increased risk of ADI or death (adjusted HR=1.91, $p<0.01$ and HR=1.49, $p<0.01$, respectively). Older age, HCV seropositivity, time-updated CD4+ T-cell count and detectable HIV RNA were also associated with poor clinical outcomes (**Table 3**).

4.5 Discussion

Previous reports of untreated individuals revealed that failure of T-cell homeostasis is an important landmark in HIV disease progression [321,324,389]. However, to our knowledge, no studies monitoring changes in circulating CD3+ T-cells exist in treated HIV-positive patients. We studied T-cell homeostasis in a well-described, large cohort of treatment naïve HIV seropositive patients initiating cART. We constructed a Markov multi-state model with reversible CD3+ T-cell percentage states, in order to evaluate covariates that influence the restoration and maintenance of T-cell homeostasis in these treated individuals. At baseline, two thirds of the study population had a normal T-cell homeostasis. Most of these patients never transitioned from that state. Those who transitioned, maintained their TCH for approximately 7 years prior to progressing to other states. This indicates that the TCH process is tightly regulated by an immune system that strives to maintain constant T-cell levels in the face of external perturbations from viral infections. Patients with baseline CD3+ T-cell percentage states outside the NPR spent less time in their respective state prior to transitioning. When they did transition, the most likely trajectory was toward the

NPR from both high and low states. Thus, following TCH disruption, the immune system seems to have a natural tendency to return to its equilibrium. Interestingly, the majority of the patients who never transitioned from the *very low* CD3+ T-cell percentage state had CD4+ T-cell counts <200 cells/mm^3. This is in line with previous findings from Gange et al, showing that TCH is generally lost during the last phase of the disease [346]. Previous studies measured T-cell homeostasis in terms of absolute CD3+ T-cell counts. However, in this study T-cell homeostasis was defined in terms of CD3+ T-cell percentages, as we consider this parameter to be a more reliable marker of the proportional stability of the circulating T-cell pool. Indeed, T-cell percentages vary less than absolute counts since they are not affected by fluctuations in total lymphocytes counts [348-350].

Our study revealed that sustained virological suppression and higher CD4+ T-cell counts are important factors for the achievement of TCH. On the other hand, older age and HCV co-infection appear to be detrimental to TCH. This is of particular interest given the increasing prevalence and incidence of HIV among individuals that are above 50 years old [395]. This group is expected to represent 50% of the United State HIV population by 2015 [396]. Furthermore, HCV co-infection occurs in approximately 20% of HIV-infected Canadians, with a prevalence of up to 90% among seropositive intravenous drug users [397-399].

Altered T-cell homeostasis has been associated with poor prognosis in non-HIV immune and infectious settings [276,277,361,390]. In untreated HIV patients, it has been associated with progression to AIDS [322]. However, the relationship between CD3+ T-cell percentages and clinical outcomes in treated HIV patients has not been investigated until now. Our survival analysis showed that individuals with very low or high CD3+ T-cell percentages were more likely to

develop an ADI or to die. It is possible that these two states reflect different immune pathologies: immune deficiency vs. chronic inflammation. Indeed, the persistance of very low CD3+ T-cell percentages might connote a permanent damage of regenerative processes. Patients with severe T-cell lymphopenia have a compromised immune system that renders them susceptible to recurrent infections [400,401]. On the other hand, high CD3+ T-cell percentages might reflect an underlying chronic antigenic stimulation leading to abnormal T-cell expansion. This is a phenomenon known as memory inflation. It occurs in the presence of persistent reactivating viruses such as CMV, where a large fraction of effector T-cells avoid apoptosis and become terminally differentiated memory T-cells [402,403]. These cells occupy the immunological "space" that would normally be allocated to naïve T-cells. The ensuing shrinkage of the T-cell repertoire diversity thus predisposes the host to increased risk of infections and poor response to vaccination [404]. In this study we could not assess the association between CMV co-infection and CD3+ T-cell expansion, because CMV serology is not available throughout all CANOC centers. Therefore the role of CMV in CD3+ T-cell homeostasis disruption remains to be elucidated.

The introduction of cART has resulted in a significant reduction in morbidity and mortality among HIV-positive patients. However, with HIV-positive patients living longer, a change in the mortality/morbidity profile of these individuals has been observed. Proportion of deaths attributable to Non AIDS Defining Illnesses (NADIs) rose from 13.1% in 1996 to 42.5% in 2004 [405]. Although CD4+ T-cell recovery might be adequate to control opportunistic infections and viral mediated neoplasms, residual immune dysregulation such as altered CD3+ T-cell homeostasis might impact on age related comorbidities that are linked to chronic inflammation. The specific immune pathways through which altered TCH may lead to death and/or comorbidities are still

unclear. Therefore, there is a need for future studies to identify the key immune and functional pathologies characterizing this phenotype.

One of the challenges faced in the evaluation of true immune recovery with cART stems from the limited number of clinically relevant and available surrogate markers. Monitoring CD3+ T-cell levels, as a supplement to CD4+ T-cell counts, may provide further insight into the immune reserve and restoration. To our knowledge this is the first study linking altered TCH and morbidity/mortality in a cohort of cART-treated HIV-positive patients.

4.6 Acknowledgement

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4.7 Figure legends

Figure 1. State structure and transition intensities for transitioning between CD3 states

Estimated transition intensities and confidence intervals for the four state reversible Markov model; size of arrow is proportional to effect size of the transition intensity.

Figure 2. Hazard ratios of transitioning between CD3 states in the multivariable model adjusting for baseline regimen

Arrows are proportional to the effect size of the hazard ratio. Dashed lines indicate non-significant hazard ratios.

Figure 3. Time from Initiation of combination antiretroviral therapy to AIDS defining illness or Death by time-updated CD3 state

Kaplan-Meier Plot of time to ADI or Death stratified by current CD3 state.

4.8 Tables and figures

Table 1. Demographics and clinical markers at baseline

		Multi-state Model of CD3 State N=4463	Time to ADI or Death N=3656
Province	British Columbia	2065 (46%)	1738 (48%)
	Ontario	1727 (39%)	1516 (41%)
	Quebec	671 (15%)	402 (11%)
Age		40 (34-47)	40 (34-46)
Male		3670 (82%)	3000 (82%)
Race	Black	231 (11%)	178 (11%)
	Caucasian	1368 (63%)	1010 (61%)
	First Nation	208 (10%)	173 (10%)
	Mixed	136 (6%)	114 (7%)
	Other	224 (10%)	179 (11%)
Risk Factors	MSM	1503 (50%)	1233 (50%)
	IDU	933 (31%)	779 (31%)
	Endemic	366 (12%)	310 (13%)

		Multi-state Model of CD3 State N=4463	**Time to ADI or Death N=3656**
	Heterosexual Contact	1159 (39%)	974 (39%)
Year starting ARVs		2005 (2002-2007)	2005 (2002-2007)
Baseline Regimen	NNRTI-based	2026 (45%)	1750 (48%)
	Boosted PI-based	2005 (45%)	1593 (44%)
	Single PI-based	352 (8%)	252 (7%)
	NRTI only	80 (2%)	61 (2%)
Baseline CD4	<200 cells/mm^3	2274 (51%)	1761 (48%)
	200-350 cells/mm^3	1535 (34%)	1366 (37%)
	351-500 cells/mm^3	435 (10%)	358 (10%)
	>500 cells/mm^3	219 (5%)	171 (5%)
Baseline CD3	<50%	167 (4%)	107 (3%)
	50-64%	549 (12%)	420 (12%)
	65-85%	3043 (68%)	2544 (70%)
	>85%	704 (16%)	585 (16%)

	Multi-state Model of CD3 State N=4463	**Time to ADI or Death N=3656**
Baseline Viral Load (log_{10} copies/ml)	4.92 (4.39-5.04)	4.90 (4.38-5.02)
Baseline ADI	605 (14%)	--
Hepatitis C Virus	965 (29%)	805 (30%)
Hepatitis B Virus	250 (17%)	212 (18%)

***** Baseline characteristics of patients included in the multi-state analysis and in the time to ADI or death analysis. IDU, intravenous drug users; MSM, men who have sex with men; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; VL, viral load; ADI, AIDS defining illness

Table 2. Demographics and clinical markers by transition history

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
Male	3670 (82%)	26 (87%)	119 (82%)	1767 (83%)	168 (85%)	106 (82%)	710 (82%)	620 (83%)	154 (73%)
Age	40 (34-47)	37 (33-43)	42 (34-47)	39 (33-46)	43 (37-48)	41 (34-47)	39 (34-45)	41 (35-48)	40 (34 -48)
Province									
British Columbia	2065 (46%)	17 (57%)	59 (41%)	914 (43%)	104 (53%)	72 (56%)	430 (50%)	361 (48%)	108 (51%)
Ontario	1727 (39%)	9 (30%)	57 (39%)	909 (43%)	66 (34%)	36 (28%)	268 (31%)	310 (41%)	72 (34%)

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
Quebec	671 (15%)	4 (13%)	29 (20%)	313 (15%)	27 (14%)	21 (16%)	166 (19%)	80 (11%)	31 (15%)
Risk Factors									
MSM	1503 (34%)	5 (17%)	42 (29%)	751 (35%)	78 (40%)	29 (22%)	284 (33%)	267 (36%)	47 (22%)
IDU	933 (21%)	11 (37%)	26 (18%)	389 (18%)	37 (19%)	33 (26%)	203 (23%)	164 (22%)	70 (33%)
Endemic	366 (8%)	6 (20%)	21 (14%)	173 (8%)	4 (2%)	17 (13%)	79 (9%)	43 (6%)	23 (11%)
Missing	1463 (33%)	7 (23%)	48 (33%)	750 (35%)	71 (36%)	38 (29%)	252 (29%)	248 (33%)	49 (23%)

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
Race									
Caucasian	1368 (31%)	6 (20%)	18 (12%)	645 (30%)	65 (33%)	35 (27%)	251 (29%)	278 (37%)	70 (33%)
Black	231 (5%)	5 (17%)	7 (5%)	112 (5%)	2 (1%)	6 (5%)	43 (5%)	34 (5%)	22 (10%)
First Nation	208 (5%)	1 (3%)	8 (6%)	76 (4%)	10 (5%)	8 (6%)	52 (6%)	32 (4%)	21 (10%)
Mixed	136 (3%)	0 (0%)	6 (4%)	57 (3%)	3 (2%)	5 (4%)	28 (3%)	27 (4%)	10 (5%)
Other	224 (5%)	3 (10%)	16 (11%)	94 (4%)	3 (2%)	13 (10%)	59 (7%)	26 (3%)	10 (5%)
Unknown/Missing	2296 (51%)	15 (50%)	90 (62%)	1152 (54%)	114 (58%)	62 (48%)	431 (50%)	354 (47%)	78 (37%)
Regimen									

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
NNRTI-based	2026 (45%)	12 (40%)	72 (50%)	962 (45%)	92 (47%)	45 (35%)	391 (45%)	372 (50%)	80 (38%)
Boosted PI-based	2005 (45%)	14 (47%)	62 (43%)	970 (45%)	96 (49%)	77 (60%)	386 (45%)	296 (39%)	104 (49%)
Single PI-based	352 (8%)	3 (10%)	10 (7%)	156 (7%)	9 (5%)	6 (5%)	75 (9%)	70 (9%)	23 (11%)
NRTI only	80 (2%)	1 (3%)	1 (1%)	48 (2%)	0 (0%)	1 (1%)	12 (1%)	13 (2%)	4 (2%)
Baseline VL									
>5.0log10 copies/ml	2007 (45%)	14 (47%)	52 (36%)	870 (41%)	79 (40%)	66 (51%)	447 (52%)	353 (47%)	126 (60%)

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
Baseline CD4 count									
<200 cells/mm^3	2274 (51%)	25 (83%)	83 (57%)	966 (45%)	63 (32%)	104 (81%)	559 (65%)	306 (41%)	168 (80%)
200-350 cells/mm^3	1535 (34%)	3 (10%)	47 (32%)	803 (38%)	84 (43%)	19 (15%)	242 (28%)	308 (41%)	29 (14%)
>350 cells/mm^3	654 (15%)	2 (7%)	15 (10%)	367 (17%)	50 (25%)	6 (5%)	63 (7%)	137 (18%)	14 (7%)
Has ADI at baseline									
Yes	605 (14%)	5 (17%)	15 (10%)	218 (10%)	20 (10%)	37 (29%)	130 (15%)	114 (15%)	66 (31%)

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
No	3656 (82%)	24 (80%)	123 (85%)	1822 (85%)	167 (85%)	87 (67%)	692 (80%)	605 (81%)	136 (64%)
Missing	202 (5%)	1 (3%)	7 (5%)	96 (4%)	10 (5%)	5 (4%)	42 (5%)	32 (4%)	9 (4%)
Hepatitis C Virus									
Yes	965 (22%)	10 (33%)	31 (21%)	396 (19%)	48 (24%)	34 (26%)	206 (24%)	169 (23%)	71 (34%)
No	2415 (54%)	15 (50%)	82 (57%)	1189 (56%)	92 (47%)	74 (57%)	468 (54%)	394 (52%)	101 (48%)
Missing	1083 (24%)	5 (17%)	32 (22%)	551 (26%)	57 (29%)	21 (16%)	190 (22%)	188 (25%)	39 (18%)

Variable	Total (n=4463)	Always <50% (n=30)	Always 50-64% (n=145)	Always 65-85% (n=2136)	Always >85% (n=197)	Between <50 and 50-65% (n=129)	Between 50-64 and 65-85% (n=864)	Between 65-85 and >85% (n=751)	Across all states (n=211)
Hepatitis B Virus									
Yes	250 (6%)	0 (0%)	13 (9%)	118 (6%)	11 (6%)	3 (2%)	52 (6%)	45 (6%)	8 (4%)
No	1236 (28%)	7 (23%)	44 (30%)	641 (30%)	37 (19%)	33 (26%)	219 (25%)	194 (26%)	61 (29%)
Missing	2977 (67%)	23 (77%)	88 (61%)	1377 (64%)	149 (76%)	93 (72%)	593 (69%)	512 (68%)	142 (67%)

***** Baseline characteristics of patients who never transitioned and patients who transitioned from their pre-treatment CD3 state. IDU, intravenous drug users; MSM, men who have sex with men; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; VL, viral load; ADI, AIDS defining illness.

Table 3. Univariate and multivariable Cox proportional hazards model for time to ADI or Death

			Univariate			Multivariable	
Variable	**Level**	**Hazard Ratio**	**95% Confidence Interval**	**p-value**	**Hazard Ratio**	**95% Confidence Interval**	**p-value**
CD3%	<50%	4.20	(2.81,6.28)	<.0001	1.91	(1.27,2.89)	<.01
	50-64%	1.32	(1.02,1.72)	0.04	1.09	(0.84,1.42)	0.52
	65-85%	1	--	--	1	--	--
	>85%	1.75	(1.33,2.30)	<.0001	1.49	(1.13,1.96)	<.01
CD4 count	>500	1	--	--	1	--	--
	350-500	7.35	(5.48,9.86)	<.0001	1.00	(0.70,1.43)	1.00
	200-350	1.85	(1.34,2.56)	<.001	1.38	(0.99,1.93)	0.06
	<200	1.14	(0.80,1.63)	0.48	4.18	(3.03,5.75)	<.0001
VL >50 copies/mL		4.19	(3.39,5.19)	<.0001	2.58	(2.03,3.28)	<.0001
Age	per 10 years	1.29	(1.18,1.42)	<.0001	1.45	(1.31,1.60)	<.0001

Male		0.78	(0.62,0.97)	0.03	1.04	(0.82,1.33)	0.74
Province	British Columbia	1	--	--			
	Ontario	0.40	(0.32,0.50)	<.0001	0.65	(0.51,0.83)	<.001
	Quebec	0.44	(0.31,0.65)	<.0001	0.59	(0.40,0.87)	<.01
Hepatitis C Status	Negative	1	--	--	1	--	--
	Positive	2.77	(2.29, 3.4)	<.0001	1.42	(1.13,1.78)	<.01

* VL, viral load

Figure 1. State structure and transition intensities for transitioning between CD3 states

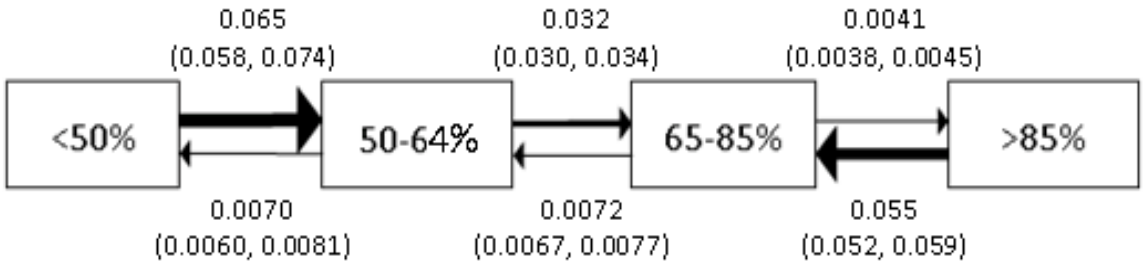


Figure 2. Hazard ratios of transitioning between CD3 states in the multivariable model adjusting for baseline regimen

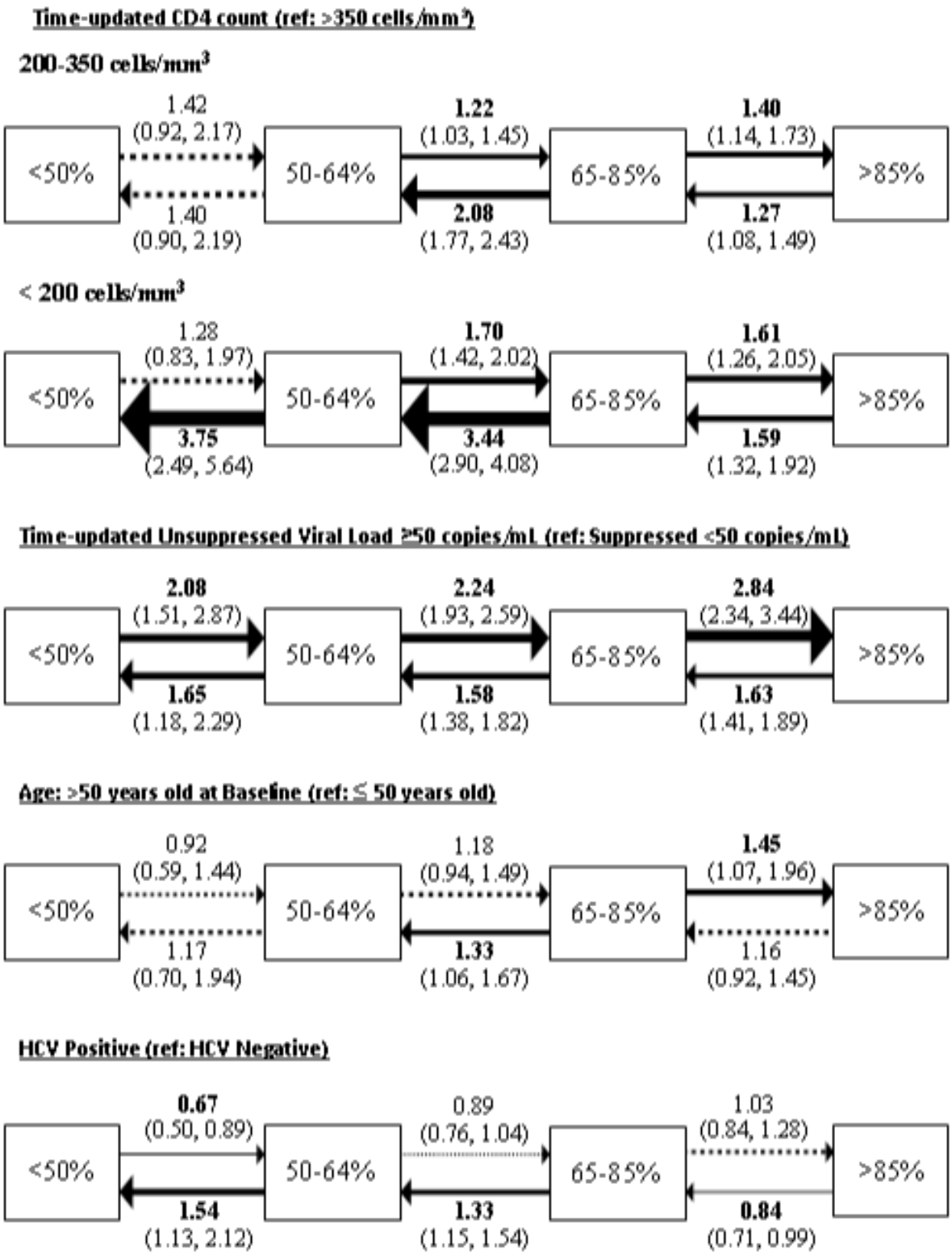
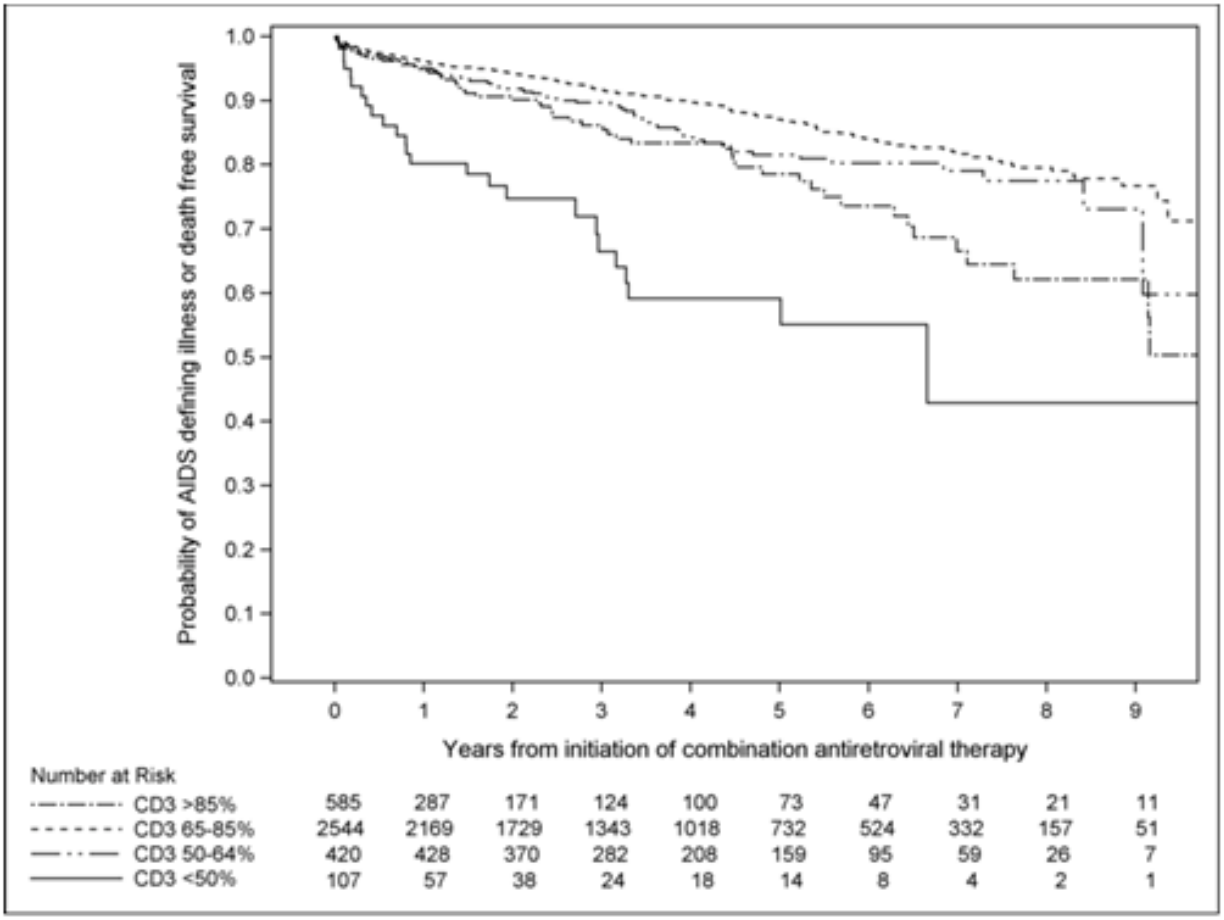


Figure 3. Time from Initiation of combination antiretroviral therapy to AIDS defining illness or Death by time-updated CD3 state



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Bridge from chapter 4 to chapter 5

Many of the persisting immune abnormalities described in previous chapters are reminiscent of the immune risk phenotype previously observed in uninfected octagerians. Despite being associated with an increased risk of morbidity and mortality, the IRP has never been evaluated in successfully treated HIV-infected individuals. In chapter 5, we evaluate the association of the IRP with phenotypic and functional markers of immune senescence in the context of treated HIV infection.

Chapter 5:

Comprehensive Evaluation of the Immune Risk Phenotype in Successfully Treated HIV-infected Individuals

Comprehensive Evaluation of the Immune Risk Phenotype in Successfully Treated HIV-infected Individuals

Running Title: IRP and HIV infection

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5.1 Abstract

Background: Despite successful treatment and CD4+ T-cell recovery, HIV-infected individuals often experience a profound immune dysregulation characterized by a persistently low CD4:CD8 T-cell ratio. This residual immune dysregulation is reminiscent of the Immune Risk Phenotype (IRP) previously associated with morbidity and mortality in the uninfected elderly (>85 years). The IRP consists of laboratory markers that include: a low CD4:CD8 T-cell ratio, an expansion of CD8+CD28- T-cells and cytomegalovirus (CMV) seropositivity. Despite the significant overlap in immunological phenotypes between normal aging and HIV infection, the IRP has never been evaluated in HIV-infected individuals. In this pilot study we characterized immune changes associated with the IRP in a sample of successfully treated HIV-infected subjects.

Methods: 18 virologically suppressed HIV-infected subjects were categorized into 2 groups based on their IRP status (no IRP, N = 8 and IRP, N = 10) and compared to 15 age-matched uninfected controls. HIV-infected individuals and controls were assessed for functional and phenotypic immune characteristics including: pro-inflammatory cytokine production, antigen-specific proliferation capacity, replicative senescence, T-cell differentiation and lymphocyte telomere length.

Results: Compared to HIV-infected subjects without an IRP, HIV-IRP subjects exhibited a higher frequency of TNF-α-producing CD8+ T-cells (p=0.05) and a reduced proportion of CD8+ naïve T-cells (p=0.007). The IRP status was also associated with a marked up-regulation of the replicative senescence markers CD57 and KLRG1, on the surface of CD8+T-cells (p=0.004). Finally, HIV-IRP individuals had a significantly shorter mean lymphocyte telomere length than their non-IRP counterparts (p=0.03).

Conclusions: Our findings suggest that, despite similar levels of treatment-mediated viral suppression, the phenotypic and functional immune characteristics of HIV-IRP individuals are distinct from those observed in non-IRP individuals. The IRP appears to identify a subset of treated HIV-infected individuals with a higher degree of immune senescence.

5.2 Introduction

Although the hallmark of HIV infection is progressive CD4+ T-cell depletion, other impairments in immune phenotype occur, including an inversion of the CD4:CD8 T-cell ratio [4]. In the short term, effective combination antiretroviral therapy (cART) increases CD4+ T-cell counts [336]. However, this increase may not accurately reflect long-term immune recovery, as the majority of cART-treated individuals maintain a profound and persistent immune dysregulation as defined by an abnormally low CD4:CD8 T-cell ratio [406,407]. Our previous study on a sample of 6673 HIV-infected adults enrolled in the Canadian Observational Cohort (CANOC) revealed that less than ten percent of those treated achieve normalization of their CD4:CD8 T-cell ratio [340]. The abnormally low CD4:CD8 T-cell ratio and other residual immune alterations found in successfully treated HIV-infected individuals resemble the immune risk phenotype (IRP) previously identified in very elderly uninfected individuals [329,408]. Longitudinal geriatric studies have shown an association between the IRP and increased risk of mortality among octagerians [292,409]. The IRP consists of a combination of phenotypic and serologic parameters that include: a low CD4:CD8 T-cell ratio, an expansion of the CD8+CD28- T-cell subset and the presence of cytomegalovirus (CMV) seropositivity [283,410,411]. Elderly individuals with an IRP have increased susceptibility to infections, reactivation of latent pathogens and

decreased responses to vaccination [412-414]; reflecting an age-related loss of T-cell function. The IRP thus defines the biological aging of the immune system, often referred to as immune senescence [415-417]. This phenotype has also been associated with markers of immune aging such as the depletion of naïve T-cells, the expansion of terminally differentiated memory T-cells, the expression of markers of replicative senescence such as CD57 and KLRG-1, as well as the production of pro-inflammatory cytokines [292,411,418,419]. The hallmark of cellular senescence is the shortening of telomeres at the end of chromosomes [298]. Chronic CMV infection contributes to telomere attrition in circulating T-cells [308]. However, telomere length has not yet been investigated in relation to the IRP. Considering the significant overlap in clinical and immunological phenotypes observed in the context of normal aging and HIV infection, we were interested in characterizing IRP-associated immune abnormalities in HIV-infected individuals. To our knowledge, an evaluation of the IRP that includes telomere changes has never been reported within the HIV population. Therefore, we assessed the IRP in a comprehensive fashion in successfully treated HIV-infected individuals, and investigated its relationship to phenotypic and functional markers of immune senescence.

5.3 Materials and methods

Ethics Statement

This research was approved by the institutional review board of the Montreal General Hospital (MGH). All participants provided written informed consent.

Participants

The IRP was defined as the combination of a low CD4:CD8 ratio (<1), an expansion of

CD8+CD28- T-cells of >50% of the peripheral blood lymphocytes and the presence of CMV-specific IgG antibodies. Eighteen men chronically infected with HIV were studied. All met the following study entry criteria: (1) absence of active clinical manifestations (2) successful treatment with cART for at least 2 years (<50 copies HIV-1 RNA/ml plasma). Subjects were excluded from participation if they had: (1) use of immunomodulatory therapy (2) evidence of any acute infection, active opportunistic infections, malignancy, or febrile illness. The 18 subjects were recruited and categorized based on their IRP status (IRP negative (neg) = 8 subjects, IRP positive (pos) = 10 subjects) and compared to 15 age-matched healthy male HIV uninfected controls that did not have an IRP, were negative for the presence of any immune deficiencies and autoimmune diseases.

Laboratory methods

CMV Serology. CMV IgG levels were determined in subjects' sera by using a commercial microparticle enzyme immunoassay (Abbott AxSYM; Abbott Laboratories).

Preparation of peripheral blood mononuclear cells (PBMC). PBMC were isolated by density gradient centrifugation (Ficoll-Paque^{TM}, Sigma-Aldrich) from whole blood obtained by venipuncture into tubes containing ACD anticoagulant. Proliferation assays were performed using fresh cells. Immune phenotype, intracellular cytokine detection and telomere measurement assays were performed at a later time point using PBMC that were cryopreserved in 10% DMSO (Sigma-Aldrich) with 90% fetal bovine serum (FBS, Wisent) and stored in liquid nitrogen.

Proliferation assays. A carboxyfluorescein succinimidyl ester (CFSE) dilution assay was used to measure the proliferative capacity of lymphocytes in response to antigen and mitogen stimulation. Fresh PBMC were labelled with 1 mM CFSE (Invitrogen, Molecular Probe) according to the manufacturer's instructions. Cells were resuspended at a concentration of 10^6/mL in RPMI 1640 medium supplemented with 15% human AB serum (Wisent), 2 mM L-glutamine (Wisent), 50 IU/ml penicillin (Wisent) and 50 μg/ml streptomycin (Wisent) (cRPMI-15). The cells were either left untreated (negative control) or stimulated with a panel of stimuli including: PHA (5 μg/ml, Sigma Aldrich), anti-CD3 monoclonal antibody (mAb) (1:1000, Research Diagnostics), anti-CD28 mAb (1:1000, Research Diagnostics), Pokeweed mitogen (5 μg/mL, Sigma Aldrich) and Tetanus toxoid (1LF/mL, Pasteur Merieux Connaught). After 7 days at 37°C in a humidified 5% CO_2 incubator, cells were washed twice in FACSflow buffer (BD Biosciences) and stained with anti-CD45 PerCP mAb. 20,000 events were acquired on a FACSCalibur flow cytometer (Becton Dickinson). Results were analyzed with the BD CellQuest software. BD Calibrite beads were used to set photomultuplier voltages and fluorescence compensation and to check instrument sensitivity before each experiment. The data obtained were corrected for background staining of unstimulated cells before statistical analysis.

Flow cytometry analysis. Cryopreserved PBMCs were thawed, washed in phosphate buffered saline (PBS) and stained with one of 2 mAb cocktails (Cocktail 1 – Maturation panel: Live/Dead UV-Blue (Life Technologies), anti-CD45-PerCP-Cy5.5 (Ebioscience), anti-CD3-PE-CF594 (BD Biosciences), anti-CD4-PE (Ebioscience), anti-CD8-APC (Ebioscience), anti-CD45RA-PE-Cy7 (Ebioscience), anti-CCR7-BV-421 (Biolegend) and

anti-CD28-FITC (EBioscience). Cocktail 2 – Senescence panel: Live/Dead UV-Blue (Life Technologies), anti-CD3-PerCP-Cy5.5 (Ebioscience), anti-CD4-BV 421 (Biolegend), anti-CD8-APC (Ebioscience), anti-CD57-PE-CF594 (BD Biosciences) and anti-KLRG1-PE (Biolegend). 10^6 PBMC were stained with each Ab panel for 30 minutes at RT and washed twice prior to acquisition. 200,000 events were acquired per condition with an LSRFortessa flow cytometer (BD Biosciences) using the Diva 4.1 software (BD Biosciences). CompBeads were used to set up voltage and compensation settings and fluorescence minus one (FMO) controls tubes were prepared for each run to establish gate settings between runs. Data analysis was performed using FlowJo software version 9.6 (TreeStar).

Intracellular cytokine staining (ICS). Cryopreserved PBMCs were thawed, washed twice in RPMI 1640 medium and resuspended in cRPMI-15. Cells were then stimulated for 24 hrs with 40 µL of staphylococcal enterotoxin B (SEB) (50µg/ml, Sigma) and 7 µL of a mAb cocktail to the co-stimulatory molecules CD28 and CD49d. After 2 hours of stimulation, intracellular protein transport was blocked by adding GolgiPlug™ (1 µL/mL, Becton Dickinson). Cells were then washed, fixed and permeabilized using the Fix and Perm kit (Becton Dickinson) according to manufacturer directions. PBMC were subsequently stained with 3 different mAb cocktails including Abs to cell surface markers CD3-PerCP, CD4-APC and CD28-FITC – all from EBiosciences) and a PE-conjugated Ab to one of the following intracellular cytokines: IFN-γ, TNF-α or IL-2. The data obtained were corrected for background staining of unstimulated cells before statistical analysis.

Telomere Length Analysis. The absolute telomere length (aTL) of genomic DNA from peripheral blood lymphocytes was determined by real-time quantitative polymerase chain reaction (qPCR) using a modification of a previously published protocol for measurement of relative telomere length [420,421]. Briefly, DNA was extracted from cryopreserved and thawed PBMC using a QIAamp DNA mini kit (Qiagen). The concentration and purity of DNA were assessed by UV spectroscopy (Nanodrop, Thermo Fisher Scientific Inc.). All DNA samples were diluted to a fixed concentration of 5 ng/µl. PCR reactions were performed using the LightCycler 480 real-time PCR detection system (Roche). PCR reactions were performed in triplicates using equal amounts of DNA (20 ng). For each reaction, 2 standard curves were made using serial dilutions of known amounts of telomeric (T) and a reference control gene 36B4 single copy gene (S) DNA oligonucleotide (Integrated DNA Technologies). Utilizing primers (Integrated DNA Technologies) specific for telomeric hexamer repeats and 36B4 SCG, the copy number of T DNA was compared to that of S DNA in order to generate a T/S ratio indicative of the relative telomere length for each DNA sample. Absolute telomere length was determined by dividing the telomere kilobase (kb) per reaction value by the number of diploid genome copies (estimated from the SCG 36B4 standard curve) to generate a total telomeric length in kb per diploid genome.

Statistical analysis

Statistical analyses and graphical presentations were performed using Graphpad Prism version 4. The Mann-Whitney U-tests were used for comparisons between independent groups. P-values <0.05 were considered significant.

5.4 Results

1. Demographic and clinical characteristics

All healthy controls and all but one of the HIV-infected subjects were Caucasian. The median (interquartile range [IQR]) age was 58 yrs [48-64], 78% were men who have sex with men (MSM) and 83% were on a regimen that included nucleosides. There were no significant differences in age, treatment regimen or risk group between controls, HIV-IRPpos and HIV-IRPneg groups.

2. Association between the IRP and the production of inflammatory cytokines

Pro-inflammatory cytokines play an important role in the induction and maintenance of immune senescence [422,423]. We therefore sought to evaluate the link between the IRP and chronic inflammation by monitoring the secretion of inflammatory mediators. Following antigenic stimulation with SEB, we assessed the frequencies of CD4+ and CD8+ T-cells producing IL-2, IFN-γ or TNF-α. Mean frequencies of cytokine-producing CD4+T-cells in response to SEB, were similar in HIV-infected and uninfected subjects (Figure 1a). Within the CD8+ T-cell subset, the frequency of IFN-γ producing T-cells was higher in HIV-IRPpos subjects compared with that in HIV-IRPneg and control subjects though this difference failed to achieve statistical significance ($p=0.07$ and $p=0.06$, respectively). The frequency of TNF-α producing CD8+ T-cells was also higher in HIV-IRPpos subjects than in HIV-IRPneg and control subjects. Differences between HIV-IRPpos and HIV-IRPneg were statistically significant ($p=0.05$). There was no difference in IL-2 production across the three groups in either CD4+ or CD8+ T-cell compartments. Thus, among successfully treated HIV-infected subjects, having an IRPpos status was associated with an increased frequency of CD8+ T-cells producing TNF-α (Figure 1b).

3. Association between the IRP and proliferative response

In the uninfected elderly, the IRP has been associated with reduced *in vitro* lymphocyte proliferative capacity in response to mitogens [329]. We therefore investigated lymphocyte proliferative responses to well-defined stimuli including: PHA, anti-CD3 and -CD28 mAbs, Pokeweed mitogen and Tetanus toxoid using a CFSE dilution assay. We observed no between-group differences in proliferation (Figure 2). In successfully treated HIV-infected subjects, being positive for the IRP did not influence lymphocyte proliferation responses.

4. Association between the IRP and T-cell subset distribution

CD45RA, CCR7 and CD28 are cell surface markers used to identify four phenotypically and functionally distinct subsets of CD4+ and CD8+ T-cells: naive (T_N: CD45RA+CD28+CCR7+), central memory (T_{CM}: CD45RA-CD28+CCR7+), effector memory (T_{EM}: CD45RA-CD28-CCR7-) and terminally differentiated effector memory (TEMRA: CD45RA+CD28-CCR7-). Figure 3a shows that, within the CD4+ T-cell compartment, no major between group differences in subset distribution was observed. In contrast, within the CD8+ T-cell compartment, the frequency of the T_N subset was significantly lower in the HIV-IRPpos than in age-matched controls (p=0.007) (Figure 3b).

5. Association between the IRP and replicative senescence

Increased levels of CD57 and KLRG1 expression on T-cells has been linked to immune replicative senescence and HIV-1 disease progression [256,424,425]. To determine whether these markers were differentially expressed in the populations studied here, we

compared their expression on both CD4+ and CD8+ T-lymphocytes from HIV-infected subjects with and without IRP and with age matched controls. Within the CD4+ T-cell subset, there was no difference in the proportion of CD57+KLRG1+ T-cells between HIV-IRPpos and HIV-IRPneg subjects. However, CD4+ T-cells from HIV-IRPpos subjects exhibited a significantly higher expression of senescence markers than those from age matched controls ($p=0.03$) (Figure 4a). Within the CD8+ T-cell compartment, the replicative senescence markers were higher on cells from HIV-IRPpos compared to HIV-IRPneg and control subjects ($p=0.004$ and 0.0003), respectively (Figure 4b). Overall, our data indicate that the upregulation of CD57 and KLRG1 expression occurred mainly on CD8+ T-cells from HIV-infected subjects with an IRP profile.

6. Association between the IRP and lymphocyte telomere length

Telomere length, a well-established marker of cellular senescence and telomere attrition, has previously been associated with several age related conditions such as cardiovascular disease [426]. In order to assess the relationship between the IRP and cellular senescence, we evaluated the absolute telomere length (aTL) of DNA from lymphocytes in the three study groups. The aTL in HIV-IRPpos subjects was significantly shorter than that in HIV-IRPneg ($p=0.03$) (Figure 5). HIV-IRPneg subjects appeared to have an aTL similar to that of uninfected controls. These results indicate, for the first time, that the IRP is associated with shorter telomere length in successfully treated HIV-infected individuals.

5.5 Discussion

The IRP was initially identified by Ferguson and colleagues in a longitudinal study of very old individuals (>85 years old) [329]. Subsequent studies indicated that this phenotype, which occurs in 15-20% of octagenarians, could predict long-term morbidity and mortality in the elderly [320]. Given that IRP-specific biomarkers can also be observed in the context of HIV, we examined here secretion of cytokines by CD4+ and CD8+ T-cells in responses to SEB, proliferation to a panel of mitogenic stimuli, the distribution of CD4+ and CD8+ T-cell subsets and the expression of markers of senescence on CD4+ and CD8+ T-cells in HIV infected individuals with and without an IRP and uninfected age and gender matched controls. We found that despite effective antiretroviral therapy, HIV-infected subjects with an IRP displayed an increased expression of phenotypic and functional markers of immune senescence compared to that in their non-IRP counterparts.

Chronic immune inflammation plays an important role in the onset and the maintenance of immune senescence; and has been linked to non-AIDS defining illnesses (NADIs) [417,427-429]. Our findings indicated a higher frequency of SEB stimulated TNF-α-secreting CD8+ T-cells among HIV-IRPpos compared with HIV-IRPneg and uninfected subjects. This observation suggests that the IRP is associated with a state of increased chronic inflammation despite well-controlled HIV infection as determined by controlled HIV viral load. Given the well-established association between chronic inflammation and NADIs, the presence of the IRP might identify a subset of treated HIV-infected individuals who are at risk for non-AIDS morbidity and mortality [79,430].

Interestingly, our analysis did not reveal any significant differences in the proliferative capacity of lymphocyte from the three study groups. These results are in accordance with previous findings that successfully treated asymptomatic HIV-infected individuals have T-cell responses to recall antigens and mitogens that are comparable to those of healthy controls [431]. Therefore, in treated HIV-infected individuals, the IRP does not seem to be associated with proliferative dysfunction.

Accumulating evidence suggests that a reduction in naïve T-cells and an accumulation of late-stage effector memory T-cells (TEMRA) is another key feature of immune senescence that is associated with poor immune status [284,432]. Although we did not find any IRP-related increase in late stage effector T-cells, we did note a lower naïve CD8+ T-cell frequency in HIV-IRPpos versus uninfected control subjects. As the development of immune responses against new pathogens is dependent on the availability of naïve T-cells, these responses in HIV-IRPpos individuals could be compromised by the lower frequency of this cell subset [433,434]. It will thus be important to determine whether HIV-IRPpos subjects are more likely to be susceptible to new infections or to fail to respond to vaccines involving cell-mediated immunity.

CD57 and KLRG1 are both putative markers of replicative immune senescence that are commonly used for the functional characterization of T-cells. CD57, also called human natural killer-1 (HNK-1) glycoprotein, is generally expressed on NK cells and on T-lineage lymphocytes, where it has been reported to identify terminally differentiated cells with reduced proliferative capacity, high susceptibility to apoptosis and shortened telomeres

[256]. Similarly, KLRG1 identifies replicative senescent cells that have lost their ability to proliferate but still produce high levels of IFN-γ [425]. Previous studies have demonstrated that some CD57+ T-cells are not ‘‘truly’’ senescent, because they can proliferate under specific conditions [435]. On the other hand, CD57+ KLRG1+ double-positive T-cells are thought to identify a subset of terminally differentiated cells that are unable to respond to antigens [436]. Our data indicate a marked expansion (approximately a 1.5 fold increase) of CD8+CD57+KLRG1+ T-cells in HIV-IRPpos subjects compared to both HIV-IRPneg and control subjects. The extent of replicative senescence is thus more pronounced in HIV-infected individuals with an IRP.

As a marker of biological senescence, telomere length has long been associated with the health and longevity of an individual. However, to our best knowledge, this marker has never been evaluated in the context of the IRP. In this study, we demonstrated for the first time that the HIV-IRPpos subjects have shorter telomeres than HIV-IRPneg subjects. Telomere length in HIV-IRPneg and uninfected controls were comparable. Taken together, these data indicate that HIV-IRPpos subjects have an increased degree of cellular senescence compared to their non-IRP counterparts. This is of particular importance considering that short lymphocyte telomere length has been repeatedly associated with poor clinical outcomes in other disease states such as; diabetes, cancer and cardiovascular disease [315,426,437]. HIV-IRPpos individuals might therefore be at higher risk for age-related co-morbidities. In fact among this small group of HIV infected individuals, we observed that while none of the HIV-IRPneg individuals experienced cardiovascular disease, 70 percent of those with an IRP had a documented cardiovascular event as defined

by the occurrence of an acute coronary syndrome (myocardial infarction, diagnosed unstable angina, or stroke).

Collectively, our findings suggest that the phenotypic and functional immune characteristics of HIV-IRPpos subjects are distinct from those observed in HIV-IRPneg subjects. Indeed, our data indicate a greater degree of immune senescence in those with an IRP, despite similar median ages and viral suppression levels in the two groups. It is important to note that the individuals included in this study are much younger than the octagenarians in which the IRP was initially identified as associated with poor clinical outcomes. It appears that the IRP occurs in HIV-infected individuals at a younger age and in association with functional, phenotypic and clinical aberrations similar to those observed in octagenarians.

One of the main limitations of this study is the small sample size of our populations. Therefore, larger cohort studies are needed to validate these data. Another limitation is that the study was restricted to men, as very few women attend our clinic. Finally we were not able to include HIV uninfected controls with an IRP status, because their prevalence is very low in this age group.

With the wide availability of potent successful therapy in many countries, individuals infected with HIV are able to live longer. Yet, despite successful and long-term viral suppression some individuals are at risk for age related co-morbidities such as cardiovascular disease. It is thus important to understand the relationship of age-associated

comorbidities and residual immune abnormalities. In this study we identified a subset of HIV-infected subjects with an IRP, who, despite being virologically suppressed exhibited phenotypic and functional T-cell aberrations associated with a pro-inflammatory and senescent immune system. The IRP may have potential as a useful marker of immune health and clinical outcomes in treated HIV-infected individuals. It also underscores the importance of understanding the role of CMV in the context of controlled HIV infection. It is clear that, larger studies are required to investigate the predictive value of the IRP as a marker of risk in the development of aging-related co-morbidities in those with HIV.

5.6 Acknowledgement

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5.7 Figure legends

Figure 1: Relationship between the Immune Risk Phenotype (IRP) and Staphylococcus Enterotoxin B induced pro-inflammatory cytokine secretion

Percentages of IL-2, IFN-γ or TNF-α producing T-cells in response to Staphylococcus Enterotoxin B (SEB) stimulation within the CD4+ (A) and CD8+ (B) T-cell compartments. Comparisons were made between uninfected controls (blue), HIV-IRPneg (purple) and HIV-IRPpos (red) subjects. Statistical significance was determined using the Mann–Whitney U test. *p≤0.05, **p<0.01, ***p <0.001.

Figure 2: Relationship between the IRP and proliferative capacity

The percentage of carboxyfluorescein succinimidyl ester (CFSE) positive cells was used to assess the proliferative capacity of lymphocytes after stimulation with phytohemagglutinin, (PHA), anti-CD3 and -CD28, Pokeweed mitogen and Tetanus toxoid. Comparisons were made between uninfected controls (blue), HIV-IRPneg (purple) and HIV-IRPpos (red). Statistical significance was determined using the Mann–Whitney U test. *p≤0.05, **p<0.01, ***p <0.001.

Figure 3: Relationship between the IRP and T-cell subset distribution

Percentages of naive (CD45RA+ CD28+CCR7+), central memory (CD45RA- CD28+CCR7+), effector memory (CD45RA- CD28-CCR7-) and terminally differentiated effector memory (TEMRA) (CD45RA+CD28- CCR7-) T-cells were assessed within the CD4+ (A) and CD8+ (B) T-cell compartments. Comparisons were made between the frequency of these subsets in uninfected controls (blue), HIV-IRPneg (purple) and HIV-

IRPpos (red). Statistical significance was determined using the Mann–Whitney U test. *p≤0.05, **p<0.01, ***p <0.001.

Figure 4: Relationship between the IRP and markers of replicative senescence

The frequency of CD4+ (A) and CD8+ (B) T-cells expressing a both CD57 and KLRG1 was measured and compared in uninfected controls (blue), HIV-IRPneg (purple) and HIV-IRPpos (red) sunjects. Statistical significance was determined using the Mann–Whitney U test. *p≤0.05, **p<0.01, ***p <0.001.

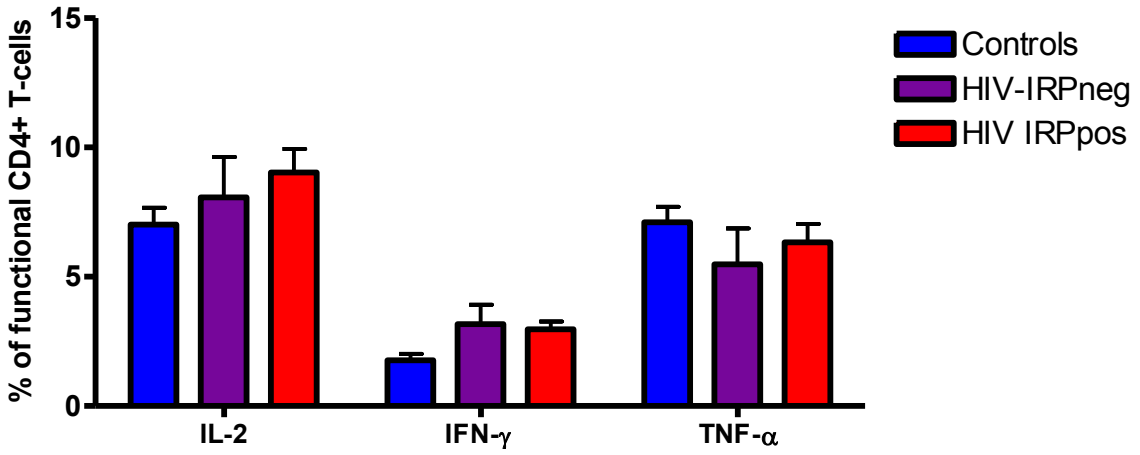
Figure 5: Relationship between the IRP and lymphocyte telomere length

Average telomere length (aTL) was measured in kilobases (kb) per diploid genome. Lymphocyte aTL comparisons were made between uninfected controls (blue), HIV-IRPneg (purple) and HIV-IRPpos (red). Statistical significance was determined using the Mann–Whitney U test. *p≤0.05, **p<0.01, ***p <0.001.

5.8 Figures

Figure 1: Relationship between the Immune Risk Phenotype (IRP) and Staphylococcus Enterotoxin B induced pro-inflammatory cytokine secretion

(A)



(B)

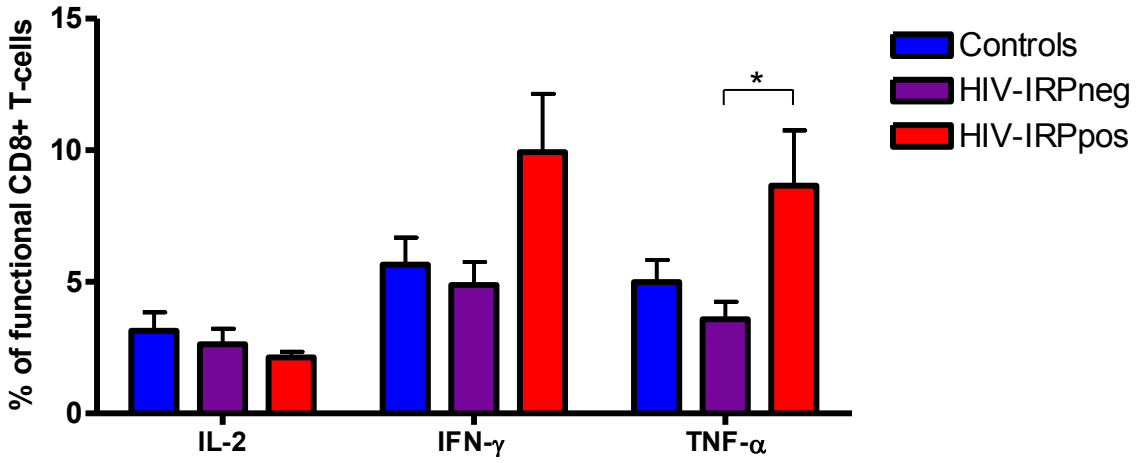


Figure 2: Relationship between the IRP and proliferative capacity

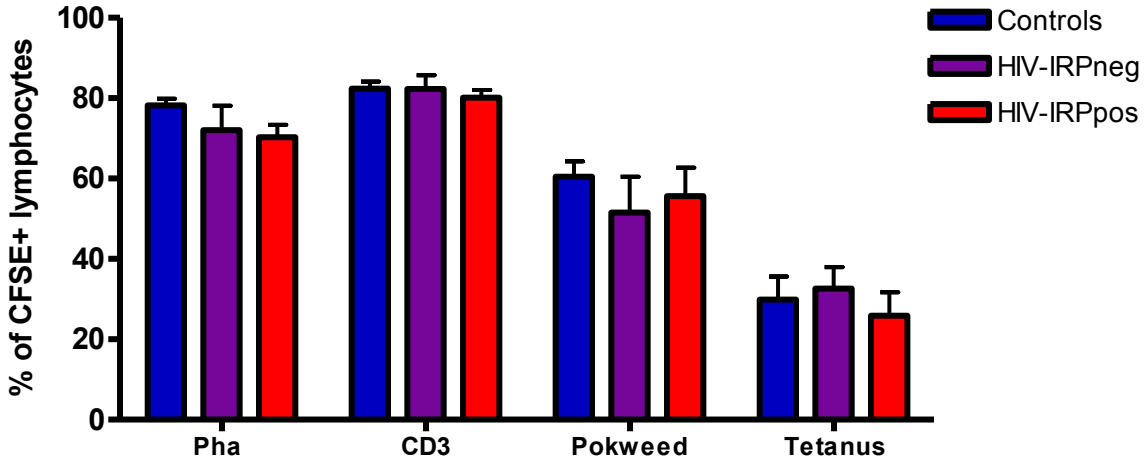
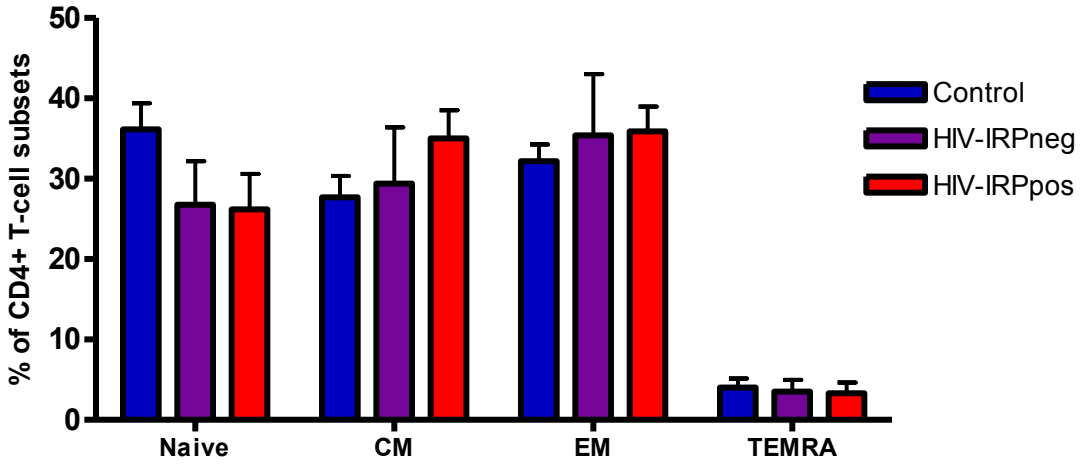


Figure 3: Relationship between the IRP and T-cell subset distribution

(A)



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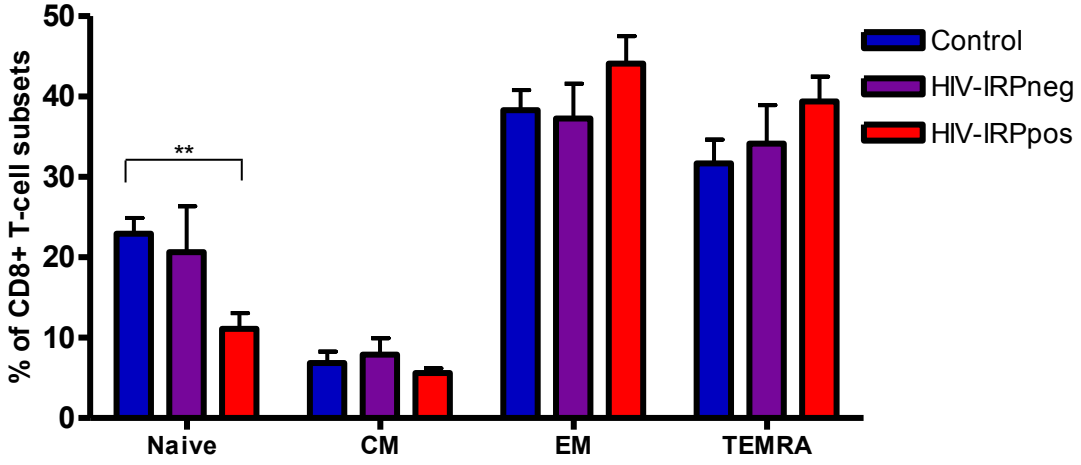
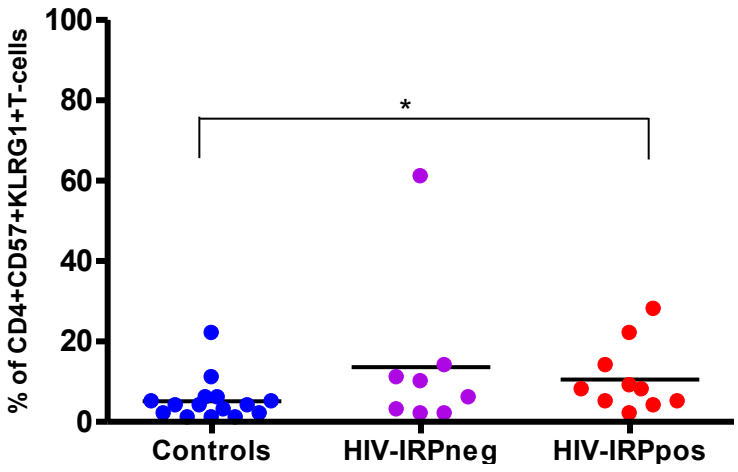


Figure 4: Relationship between the IRP and markers of replicative senescence

(A)



(B)

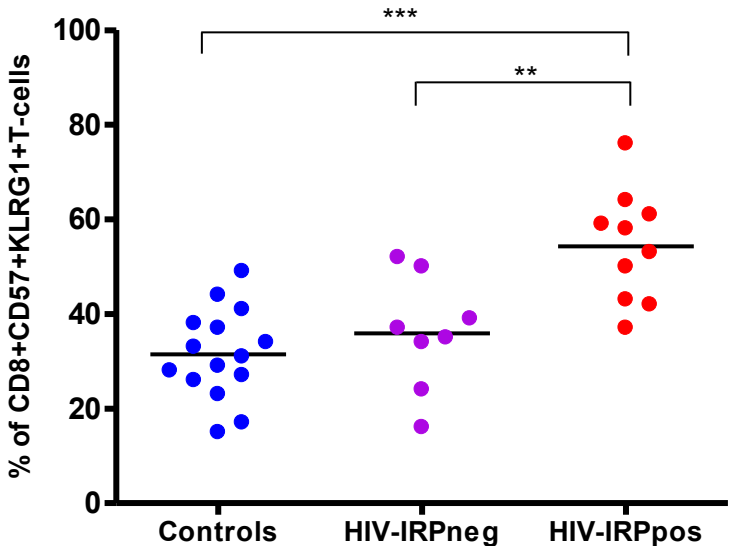
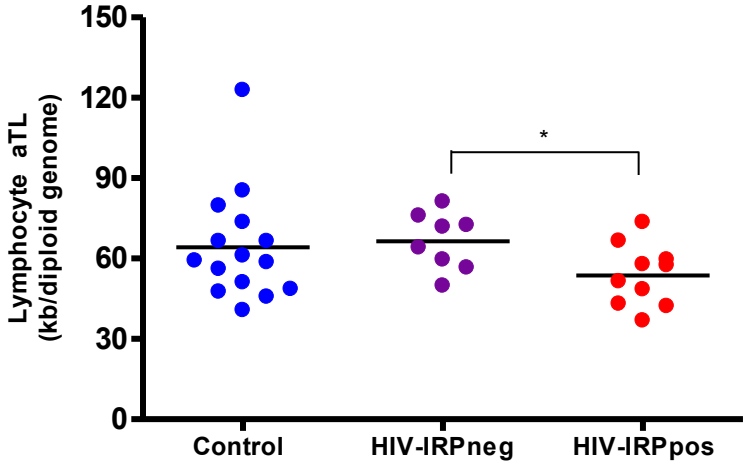
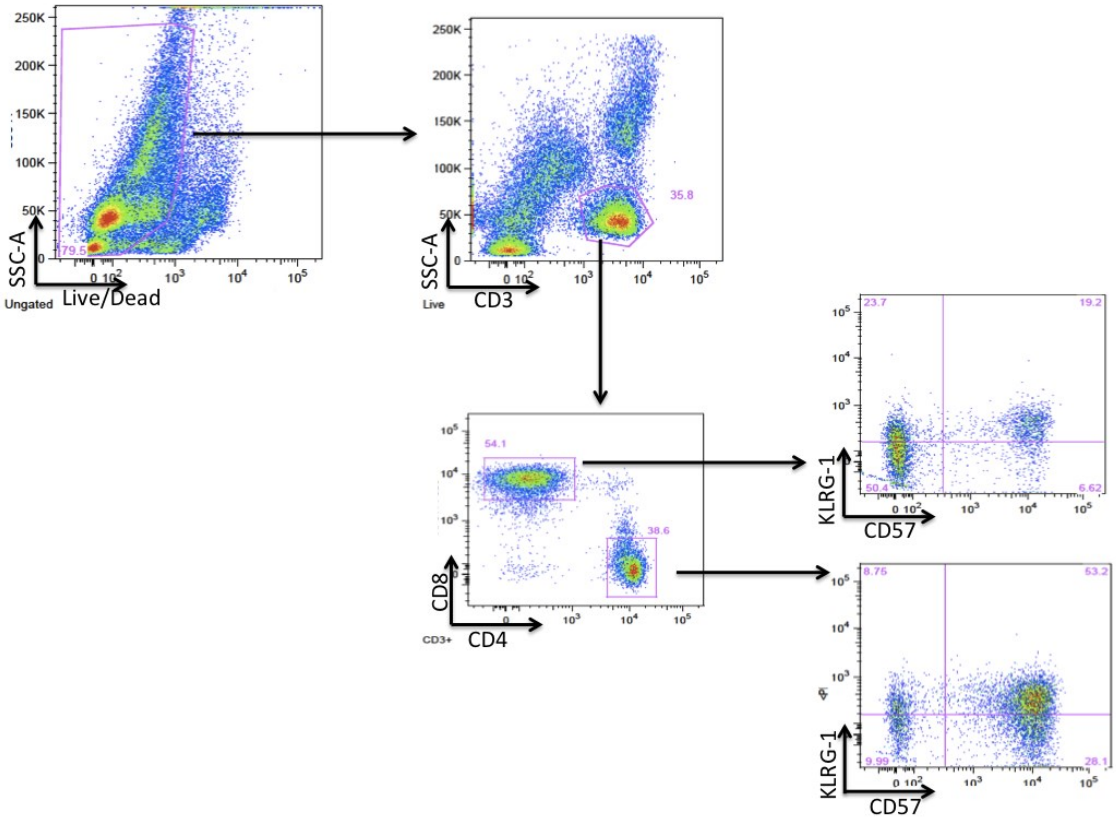


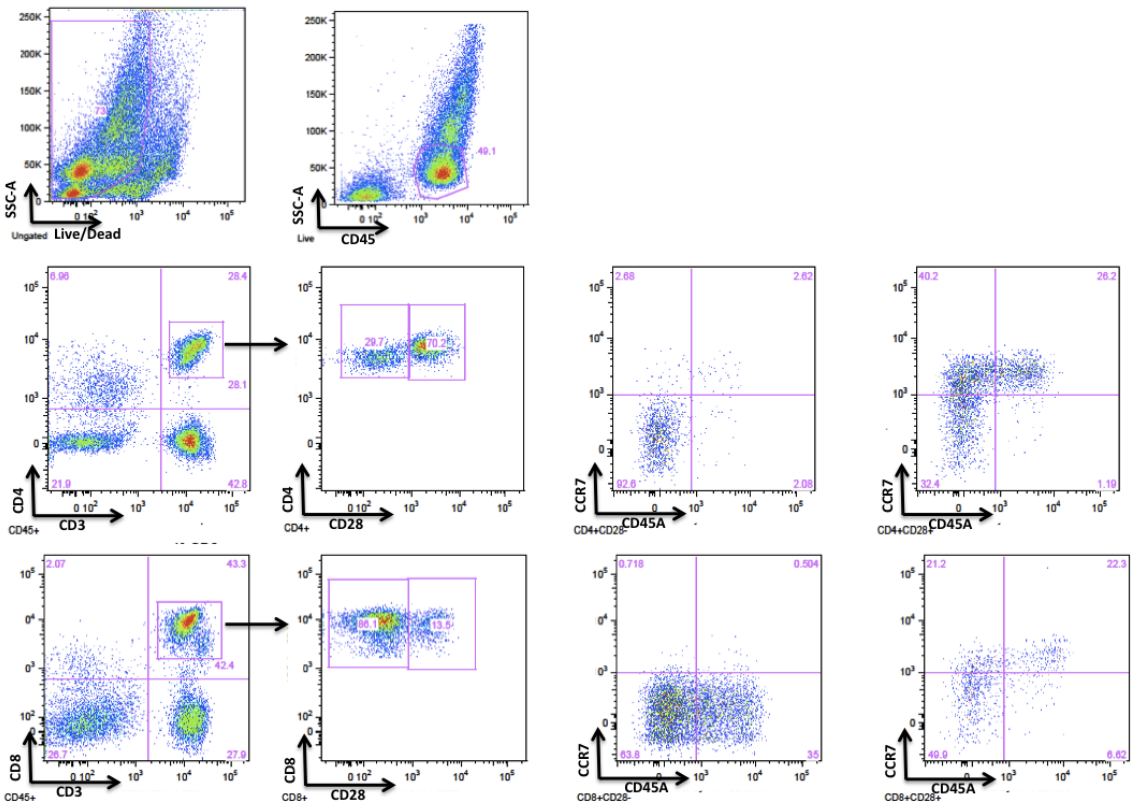
Figure 5: Relationship between the IRP and lymphocyte telomere length



Supplementary Figure 1: Gating strategy for senescence panel



Supplementary Figure 2: Gating strategy for T-cell subset panel



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Chapter 6:

Summary of original scholarship

From Chapter 2

In chapter 2, we evaluated the impact of a decade of effective ART on HIV-induced altered TCP.

1. The results presented here indicate that HIV infection causes profound TCP alterations that are not completely reversed despite long-term efficacious antiretroviral therapy.

2. Historically, the immune recovery of HIV-infected patients has been evaluated by monitoring the CD4+ T-cell count only. Our pilot study is the first to comprehensively evaluate immune recovery by monitoring a cluster of T-cell phenotypic markers including CD4+ T-cell counts, CD4:CD8 T-cell ratio, and CD3+ T-cell percentages.

3. An important strength and novelty of this study is its specific focus on patients with prolonged and optimal viral suppression (Plasma HIV viral load below 50 copies/mL for more than 10 years). This ideal clinical setting allowed us to investigate very long-term patterns of complete immune recovery in the context of highly effective viral suppression.

From Chapter 3

In chapter 3, we assessed the characteristics and determinants of TCP normalization among treated HIV patients.

1. Our findings demonstrated that high baseline CD4+ T-cell counts and viral load suppression can independently predict complete TCP normalization. This highlights the importance of early treatment initiation for long-term immune recovery.

2. This analysis, which was performed within a much larger population than our pilot study, also confirmed our original finding that HIV-induced alterations of the TCP are incompletely reversed by long-term ART.

3. Overall, the data presented here provided empirical evidence suggesting that the concept of immune reconstitution should not be restricted to CD4+ T-cell counts.

From Chapter 4

In chapter 4, we investigated the characteristics and clinical impact of altered T-cell homeostasis among HIV individuals receiving ART.

1. The data reported here are the first to demonstrate that sustained viral suppression and higher CD4+ T-cell counts are important for the maintenance of TCH in HIV patients under ART. On the other hand, older age and HCV co-infection were linked to impaired TCH.

2. This study also provided the first empirical evidence of an association between altered TCH and increased risk of morbidity and mortality in successfully treated HIV patients.

3. Collectively, the results of this study suggest that monitoring CD3+ T-cell levels, in addition to CD4+ T-cell counts, may provide further insight on immune and clinical outcomes in treated HIV individuals.

From Chapter 5

In chapter 5, we evaluated IRP-associated immune changes in the context of treated HIV

infection.

1. This study was the first to comprehensively evaluate the IRP in a population of treated HIV individuals.

2. The data reported here showed that despite having similar levels of ART-mediated viral suppression, HIV individuals with an IRP exhibited a higher degree of immune senescence than those without an IRP.

3 This study is also the first to indicate that the IRP status is associated with lymphocyte telomere attrition among treated HIV individuals.

Together these observations provide support for the importance of evaluating and monitoring novel markers of immune recovery among treated HIV patients. Whereas other studies have stressed the depletion of CD4+ T-cell counts as the hallmark of HIV-mediated immunologic dysfunction; the findings described in this thesis indicate that the immuno-pathogenic impact of HIV infection is multifaceted and only partially reversible with long-term suppressive treatment. This thesis also provided a basis for understanding the implications of the IRP in the context of treated HIV infection and a foundation for the rational monitoring of a composite measurement of immune status. This could represent a valuable tool for assessing improvement or deterioration of the immune system during the course of treated chronic HIV disease.

Chapter 7:

Discussion

Over the last three decades, considerable efforts have been made to understand how chronic HIV infection contributes to the progressive deterioration of the immune system. The discovery of HIV tropism for CD4+ T-cells has led to the establishment of CD4+ T-cell counts as the preeminent surrogate marker for HIV disease pathogenesis and clinical progression to AIDS. However, HIV-mediated immune pathology represents a far more complex scenario wherein the CD4:CD8 ratio balance and the CD3+ T-cell homeostasis are severely and gradually disrupted throughout the course of the infection. The initial premise of HIV treatment was to prevent the development of AIDS-related complications via ART-mediated viral load reduction and CD4+ T-cell restoration. This represented an important challenge in the past, as the ARV repertoire was limited in both quantity and quality. However, due to the increased efficacy and tolerability of modern ARV regimens, most treated HIV patients achieve virological suppression, improve CD4+ T-cell recovery and avoid AIDS-related clinical complications [438,439]. Although AIDS-related events no longer represent a primary threat, successfully treated HIV individuals are now at risk for non-AIDS conditions such as cardiovascular, renal, liver and autoimmune diseases [69-71]. Consequently, the changing clinical picture of HIV disease has led to a paradigm shift in the management of HIV infection. Although the root causes of these new HIV-associated clinical complications remain unclear, it has been suggested that the persistently aberrant immune profile is not restored with treatment and might play an important role in the onset of these non-AIDS diseases.

During the course of the work described here, novel markers of immune status have gained considerable interest among HIV researchers and clinicians. Recent studies have reported that persistent immune dysregulation is characterized by the upregulation of markers of

inflammation, activation and senescence [181,300]. These persistent changes in immunity have also been associated with an increased risk of non-AIDS morbidity and mortality [102]. Given the multifaceted nature of HIV-induced immune pathogenesis, focusing on a single pathologic marker such as CD4+ T-cells only provides a partial picture of immune health. Combinations of several markers might profile patterns of immune recovery versus patterns of persistent immune debility in those treated for HIV. To this end, this thesis has served to understand the long-term impact of ART in a comprehensive approach using a novel combination of available surrogate markers of immune recovery (defined in Chapter 2).

Our first research objective was to evaluate patterns of TCP recovery in a cohort of patients receiving suppressive antiretroviral therapy over very long periods of time (>10 years). In this respect, our work demonstrated that although the majority of patients recovered their CD4+ T-cell counts, very few were able to normalize their complete TCP despite prolonged viral suppression (Chapter 2, Table 2). Our findings were substantiated by a recent study by Serrano-villar and colleagues, reporting that immune dysregulation can persist in the presence of high CD4+ T-cell counts [181]. To our knowledge, the assessment of a combined markers of immune deficiency, immune dysregulation and lost T-cell homeostasis, was unique to the work described in this thesis. We are aware of only one other study, by Torti and colleagues, which assessed multiparametric T-cell recovery in treated HIV subjects [351]. However, that study failed to include the total CD3+ T-cell percentage, which in our view provides a more comprehensive measure of combined T-cell recovery as it takes into consideration both CD4+ and CD8+ T-cell levels. Furthermore, their analysis was based on a median follow-up of only 4 years. Our pilot study (Chapter

2) had a median follow-up period of 10 years, and therefore represents the longest evaluation of multiparametric T-cell phenotype recovery in HIV patients with consistently sustained virological suppression. Nonetheless, the findings independently reported by Dr. Torti's team reinforce and substantiate the theoretical basis of our work.

The interesting findings from our pilot study served as a foundation to address our second research objective, which was to characterize the determinants of complete TCP in treated patients. This study took place within a very large cohort, which allowed us to not only validate our preliminary findings but also to identify baseline CD4+ T-cell counts and viral load suppression as independent predictors of TCP recovery (Chapter 3, Table 3). Furthermore, our data indicated that the TCP parameter that was the least recovered among treated HIV-positive individuals was the CD4:CD8 T-cell ratio. Indeed, among over 4,000 patients followed in this study, less than ten percent normalized their CD4:CD8 T-cell ratio despite effective treatment. This continual ratio dysregulation appeared to be a result of persistenly elevated CD8+ T-cells. Recent data have supported our findings and have provided additional evidence showing that individuals with low CD4:CD8 T-cell ratios, independent of CD4 levels, demonstrate traits of a dysfunctional immune system [181,300].

It is unclear, to what extent HIV alone accounts for this persistent immune dysregulation. Studies have suggested that CMV co-infection could drive or exacerbate the expansion of terminally differentiated CD8+ T-cells that are responsible for ongoing CD4:CD8 T-cell dysregulation [283]. Indeed, CMV infection is prevalent in about 75% to 90% of HIV patients, and research has shown that responses to this virus can be accompanied with

dramatic phenotypic and functional changes to the immune system [264,282,440]. In a randomized controlled trial of valganciclovir (an antiviral drug used in the management of CMV infections), CMV was revealed to be a significant cause of persistent T-cell activation in treated HIV patients. Indeed, treatment with this drug was shown to result in a significant reduction in the frequency of activated CD8+ T-cells [83]. Furthermore, in the uninfected elderly, CMV is a well-known marker of immune senescence and is predictive of mortality [441,442]. Finally, Appay and colleagues, have recently shown that CMV induced immune responses were independently associated with altered CD4+ T-cell reconstitution in treated HIV patients [443]. Future studies quantifying the burden of low-level asymptomatic CMV replication could provide further insight into the long-term assessment of complete immune recovery.

Our third research objective was to assess the determinants and clinical impact of T-cell homeostasis failure in the context of treated HIV infection. The importance of TCH in HIV pathogenesis first became apparent when longitudinal studies reported a substantial decline in total CD3+ T-cell levels (reflecting a loss of homeostatic control of these cells) approximately 1.5-2 years prior to the development of AIDS [174]. It was later determined that CD3+ T-cell levels had predictive value for the onset of AIDS, independently of CD4+ T-cell counts [444]. Deficits in thymic function and progressive destruction of naive T-cells were suggested to play a role in the mechanisms underlying TCH failure [326]. Overall these findings indicate that TCH failure is an important landmark in the progression of HIV disease to AIDS. Despite being associated with poor prognosis in untreated HIV disease, little is known about the recovery or maintenance of TCH in the context of treated HIV infection. Our comprehensive evaluation of CD3+ T-cell dynamics within a large

cohort of treated HIV-infected adults provided the first in-depth characterization of TCH as a prognostic marker of health in ART-treated HIV patients. Our data indicated that high CD4+ T-cell counts and sustained viral suppression were important for TCH maintenance, while older age and HCV co-infections contributed to TCH disruption (Chapter 4, Figure 2). Our analysis also suggest that despite effective ART, individuals who fail to maintain physiologically normal levels of total T-cells are at higher risk of morbidity and mortality (Chapter 4, Figure 3, Table 3). This is the first time that altered TCH was linked to poor disease outcomes in the context of treated HIV infection.

Another novelty in our work stems from the fact that we defined TCH failure as having both abnormally high and abnormally low levels of CD3+ T-cells. Previous studies assessing this immune parameter in the context of HIV have mainly described TCH failure as a decline in T-cell levels [174]. This is not completely accurate, as our data indicate that some HIV patients can exhibit abnormally high T-cell proportions as a consequence of persistent CD8+ T-cell expansion. Our findings therefore provide a new understanding of HIV pathogenesis wherein abnormal levels of CD3+ T-cells could reflect two distinct immunopathological states: immune deficiency vs. chronic inflammation. Indeed, very low CD3+ T-cell levels might be indicative of severe irreversible damage to immune regenerative mechanisms, rendering the host immuno-compromised and susceptible to infections. Conversely, abnormally high CD3+ T-cell levels might reflect a state of activation induced-memory cell inflation, characterized by the presence of clonally expanded effector T-cells that are specific for immunodominant antigens such as CMV. The expansion of these clones not only affects the T-cell repertoire diversity, but also contributes to immune senescence via the release of inflammatory mediators [244]. Thus,

monitoring CD3+ T-cells dynamics in those treated for HIV may provide further insight into the state of T-cell immunocompetence.

Many of the persisting immune abnormalities that were observed in treated HIV patients are reminiscent of the IRP previously described in the uninfected elderly. The IRP essentially consists of a low CD4:CD8 T-cell ratio, an expansion of CD8+CD28- T-cells and CMV IgG-seropositivity. Accumulating evidence has led to a proposed model for the development of the IRP with age. According to this model, asymptomatic chronic CMV infection over several decades results in the gradual expansion of CMV-specific CD8+ T-cells. As these cells are continuously generated over the years, they acquire a senescent phenotype characterized by the loss CD28 and the shortening of their telomeres [445]. The apoptosis-resistant nature of these cells allows them to accumulate in the periphery and to compete for survival niches. This eventually leads to the loss of functional T-cells of other specificities, and an overall shrinkage of the T-cell repertoire [446,447]. The accumulation of these cells also correlates with the progressive immune dysregulation defined by a low CD4:CD8 T-cell ratio that is observed with age [283]. Furthermore these cells have been shown to contribute to systemic inflammation through the release of TNF-α and IL-6, which both correlate with reduced survival [448,449]. Interestingly, TNF-α has been associated with accelerated T-cell differentiation and reduced telomerase activity in CD8+ T-cells [450]. Thus, in the general population, the IRP correlates with markers of immunological aging and independently predicts all-cause mortality [445,451].

Nevertheless, the role of this marker in HIV-infected individuals under suppressive ART remains to be elucidated. Our final research objective was therefore to evaluate the

significance of the IRP in the context of treated HIV infection. Here, we have demonstrated for the first time that the IRP may help identify treated HIV individuals with persistent immune dysfunction and inflammation as reflected by: depleted CD8+ naïve T-cells, increased TNF-α secretion, increased levels of replicative senescence markers and shortened lymphocyte telomeres (Chapter 4, Figures 1-5). The loss of naïve T-cells has been associated with low responses to vaccination and recurrent infections [452]. Conversely, Inflammation and senescence markers have been shown to be key contributors of morbidity and mortality [292]. Finally telomere attrition is associated with an increased risk of mortality from cardiovascular disease and from infections [453]. Given the association of each of these pathologic markers with poor health outcomes, our finding of an increased prevalence among HIV-IRP positive patients is important. Indeed, monitoring the IRP may possibly be clinically useful in the screening and management of patients at higher risk of non-AIDS events. In fact, we found that more than two thirds of HIV patients with an IRP had experienced a cardiovascular event, which was not the case in those without an IRP. Thus, the IRP may represent a new clinical phenotype associated with persistent inflammation and immune activation despite effective ART.

Despite the tremendous clinical benefits of ART, the HIV virus cannot be eradicated and HIV disease persists. Outside of CD4+ T-cell dynamics, little is known about the evolution of HIV-induced T-cell phenotypic dysfunction subsequent to treatment with effective ART. The work presented in this thesis attempted to elucidate the nature and clinical implications of persistent immune dysregulation in treated HIV individuals.

Our results indicate that complete immune recovery is slow, variable, and partial. Whereas

previous studies have considered CD4+ T-cell depletion as the hallmark of immunologic destruction, the data reported here strongly suggest that additional immune mechanisms that are not captured by the CD4+ T-cell count and the HIV viral load might contribute to poor clinical outcomes. The use of a composite measurement of immune status could therefore be valuable for a more comprehensive evaluation of the immune recovery. Our findings that high baseline CD4+ T-cell counts and sustained viral load suppression independently predict TCP recovery and TCH maintenance, reinforces the importance of early treatment initiation for the preservation of immune functions.

Understanding the ongoing T-cell events that occur during the course of HIV infection is of particular interest for future therapeutic strategies aiming to improve clinical outcomes in those infected with HIV. Chronic inflammation, resulting from prolonged immune activation, is likely to be a significant contributor in both the initial establishment and maintenance of HIV-induced T-cell dysfunction. Given the nefarious effects of persistent inflammation on the T-cell phenotype and on clinical outcomes, identifying novel strategies that can prevent or reverse inflammation will be essential for improving the quality of life of those infected with HIV. Currently, a novel focus in HIV research is the evaluation of immune-modulatory drugs with anti-inflammatory properties that could be used as adjunct therapies in subjects on suppressive ART. There are several promising drug candidates with the capacity to reduce the risk of inflammation and inflammation-related morbidity. For instance, statins are well-known for their ability to prevent cardiovascular disease via their cholesterol-lowering properties. However, ample evidence indicates that these drugs also have potent anti-inflammatory and immune-modulating functions and may provide a survival benefit in the HIV population [454,455]. Additional findings have shown that

other anti-inflammatory drugs such as aspirin and Cox-2 inhibitors can help reduce excessive immune activation in HIV infected individuals [456,457]. Strategies attempting to reduce immune activation via the inhibition of microbial translocation are also of interest. In a study by Klatt and colleagues, SIV-infected pigtail macaques were treated with a combination of prebiotics/probiotics and ART vs. ART alone. Prebiotics and probiotics have been shown to benefit gut immunity by improving the gut microbiota and reducing microbial translocation. Interestingly, animals treated with ART in combination with prebiotics/probiotics exhibited a better immune recovery than animals treated with ART only [458]. Finally, the treatment of viral co-infections such as CMV has also been suggested as a means to decrease immune activation in HIV treated patients [83].

In conclusion, our work is novel and relevant to the development of new HIV treatment strategies in the era of effective ART. Given that the parameters that constitute the TCP are readily available in routine clinical practice, monitoring of the TCP could easily be implemented in clinical settings. An important take home message to consider for the clinical management of HIV patients is that the immune profile of treated HIV disease is fundamentally different from that of untreated disease. Indeed, untreated HIV infection is primordially characterized by an ultimate loss of immune function due to the depletion of CD4+ T-cells. This renders patients susceptible to AIDS-related morbidity and mortality. However, during treated HIV disease the remodelling of the immune system that was initiated during acute infection gains importance and impacts on natural aging, viral co-infections or ongoing microbial translocation. A state of chronic inflammation and altered immune phenotype contributes to a range of non-AIDS associated events that comprise a wide spectrum of age-related diseases. Our findings may improve the understanding of the

long-term residual consequences of HIV infection. Hopefully our work will reinforce the importance of early initiation of ART and encourage a new approach in the monitoring of immune recovery through the composite use of clinically available surrogate markers.

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