Inflammation and CMV-associated Lymphocyte Senescence in Chronic HIV Infection

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Abstract

While acute inflammation is important in immune responses to infection, chronic systemic inflammation intrinsically underlies age-associated pathologies. Lifelong cellular turnover equates biological age with long term accumulation of senescent cells, characterized by cell-cycle arrest and a pro-inflammatory senescence-associated secretory phenotype (SASP). Currently, the exact contributions of lymphocytes to the inflammatory landscape seen during aging remains understudied. Cytomegalovirus (CMV) infection creates an inflated, potentially senescent, $CD8^+$ T lymphocyte population, often exaggerated in age and in particular, human immunodeficiency virus (HIV) infection. Many living with long-term HIV infection often suffer "premature" age-related illnesses. Frequent rounds of antigen-driven expansion of CMV-specific lymphocytes could introduce a telomere-dependent SASP and a role in suspected age-related pathology. Using a 4-parameter flowFISH assay to quantify telomere length in peripheral blood lymphocytes, we show in HIV-infected CMV-seropositive individuals (n=32) that $CD57^+CD8^+$ T cells responding to CMV had the shortest telomere lengths compared to $CD57^+CD8^+$, global $CD8^+$ and $CD8^+$ T cells responding to HIV ($p < 0.001$, 0.001, 0.04, respectively). CMV-seropositive HIV-infected study groups (n=97) show higher circulating pro-inflammatory cytokine levels than those not infected with CMV (n=19), further linking CMV immunity, immune senescence and inflammation. Our data support consideration that CMV infection associated expansion of $CD8^+$ T cells compound the effects of natural aging on chronic inflammation.

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List of Abbreviations

AICD – activation induced cell death
AIDS – acquired immunodeficiency syndrome
APC – antigen presenting cell
ART – antiretroviral therapy
B2M – beta - 2 – microglobulin
BCL – B cell lymphoma protein
CCR – C-C chemokine receptor
CD – cluster of differentiation
CRP – C reactive protein
CXCR – C-X chemokine receptor
Cy - cyanine
DDR – DNA damage repair
DNA - deoxyribonucleic acid
DSB – DNA double strand break
FISH – fluorescent *in situ* hybridization
HCMV – human cytomegalovirus
HIV – human immunodeficiency virus
IFN – interferon
Ig – immunoglobulin
IL – interleukin
IRP – immune risk phenotype
ITAM – immunoreceptor tyrosine-based activation motif
ITIM – immunoreceptor tyrosine-based inhibitory motif
LTL – lymphocyte telomere length
MFI – median fluorescence intensity
MHC – major histocompatibility complex
MIP – macrophage inflammatory protein
NK – natural killer cell
PNA – peptidyl nucleic acid
RANTES - Regulated on Activation, Normal T Cell Expressed and Secreted
RNA- ribonucleic acid
SASP – senescence associated secretory phenotype
TCR – T cell receptor
TNF – tumor necrosis factor

1 Introduction

1.1 Biological Aging

Aging, broadly stated, is the long-term decline in physiological function occurring in a time-dependent manner. The biological process of aging is often thought of as an "accumulation of changes," however, the exact nature of these changes is widely disputed and often far more interconnected than appreciated. This age-associated accumulation is conceptualized as biological entropy, where damaged, less functional cells gradually collect in each tissue. This culminates through late ages as organ disease or dysfunction, effectively manifesting as a decline in physiological function. It is of great interest to define and discriminate the changes accumulated during aging to understand how these features could become accelerated or initiated prematurely, thereby promoting early morbidity and mortality. From a biological perspective, to understand the advancing detriment of aging on the human population, the compositional changes of the body during aging must first be better understood by more precisely identifying the key molecular and cellular "changes" that "accumulate."

1.1.1 Cellular Senescence

To senesce, from Latin *senescere,* is defined as "to grow old". Senescing, or becoming senescent, is the biological term originating from Hayflick et al. to describe the loss of replicative capacity, or replicative senescence, first observed in *in vitro* fibroblast cultures. This observation, however, addressed only one extrinsic aspect of "senescence" and merely fueled speculation of an internal process or underlying mechanism that inhibits cell replication, somehow, dependent on the length of time in culture[1]. To show

that this progressive decline in replication was not only restricted to cell lines *in vitro*, but reflected an in vivo process, Harley et al. demonstrated that cultured primary human fibroblasts showed the same decline in replication capacity over time and that this was physically reflected in fibroblast telomere length[2]. Furthermore, the duration of fibroblast replication *in vitro* and telomere length were both directly related to the age of the donor. Functionally, telomere sequences are noncoding and essentially serve as a protective cap to linear chromosomes, which encode all function and if damaged could incorporate harmful mutations and/or trigger cell death. This *in vivo* connection to ageing positioned telomere length as a surrogate cellular indicator of age, or 'biological clock.' The telomere is a hexameric repeat of TTAGGG with sequence length ranging from 18 kilobase pairs in cellular infancy to a few thousand-base pairs in old cells[3,4]. The position of the telomere is both incredibly strategic to stabilize chromosomal DNA and the "Achilles heel" of long-term stability of chromosomal DNA with cumulative replication. Most DNA polymerases utilized during cell division operate in a unidirectional 5`-3` direction and can only bind to existing primer regions, creating problems for the Okazaki fragments of the lagging strands (3`-5`) where RNA primers are required. Since there is no site for attachment preceding the most 5' RNA primer, when RNA primers are degraded post-DNA replication, a resultant 3' overhang of approximately 25 base pairs remains exposed and eventually is degraded. This telomere eroding process, termed the end-replication problem, occurs with every cell division[5]. While the chromosomal coding DNA is protected, it is at the expense of telomere length. An RNA-dependent DNA polymerase, telomerase, can restore sections of the TTAGGG sequence during DNA replication, effectively eliminating the end replication problem. While this is employed

by embryonic cells during intermittent cell division[6], telomerase is generally absent in differentiated cells *in vivo*[7], where the end-replication issue drives telomere erosion. Therefore, the absolute length of the telomere reflects both the history of -and the potential for further- cellular replication in somatic tissue.

Cell cycle arrest is often initiated as an anti-tumorigenic response to internal stresses, generally due to the detection of aberrations in nuclear or mitochondrial DNA. Momentary cell cycle arrest allows evaluation of said internal damage, its potential repair and eventual cell cycle progression. If internal cues for cell cycle arrest are interpreted as high risk for neoplastic transformation or oncogenesis, the cell becomes apoptotic or maintains cell-cycle arrest, acquiring a senescent phenotype. Cellular senescence is classically defined by terminal cycle arrest, resistance to apoptosis, and altered cellular function. A deeper understanding of cellular senescence now defines it as much more than the traditional tumor suppressor state[8]. In fact, cellular senescence is a dynamic process not only integral to cellular and tissue aging but unbeknownst to Hayflick et al, heavily involved in tissue and vascular homeostasis. Senescent cellular differentiation spans all cell types and tissue microenvironments, each cell encountering different extracellular cues and each with its own senescence-associated alterations in function, and therefore, differential contributions to organismal aging. Given the many instances of internal damages and stress our cells undergo throughout our lifetime, a better understanding of the complexity and pleiotropy of senescent processes could not only shed light on endogenous mechanisms of aging, but also on key homeostatic mechanisms such as cancer prevention and wound healing.

1.1.2 Senescent Cell Programing

Senescence can be viewed as a programmed response to stressful events that follows distinct acute and chronic scenarios. Much like the immune response to acute viral infections, acute senescence is rapid and well-orchestrated, ultimately including immune-mediated cell clearance. Chronic senescence, like the immune response to chronic infection, is the decelerated, deleterious, more detrimentally progressive state commonly associated with age-related diseases[9].

Acute cellular senescence contributes to tissue repair[10] and is integral to processes such as embryogenesis and maternal vascular remodeling[11,12]. The key feature of acute senescence is the rapidity of onset and subsequent clearance of senescent cells by the immune system. Acute senescent cells receive short high-grade extracellular damage signals, which trigger accumulation of stress signaling molecules that impose cell cycle arrest. Acute senescence rapidly invokes a distinct phenotypic change that empowers selective elimination of the senescent cells by the immune system. Acute senescent events terminate with cell clearance to maintain proper tissue function[9]. The absence of orchestrated cell clearance and subsequent senescent cell accumulation forms the canonical connection between chronic cell senescence and unhealthy aging.

As first demonstrated by Hayflick et al., long-term passage of fibroblasts eventually results in cell cycle arrest, reflected in chronic cellular senescence. Induction of senescence occurs via successive DNA damage responses due to critically shortened telomeres[13]. Due to the end replication problem, exhaustively proliferated cells have telomeres shortened to dangerous lengths that results in them becoming ‘uncapped.’ These exposed telomeres mimic double-strand DNA breaks (DSB) and trigger DNA

damage response (DDR) pathways involving the master regulator protein p53[14]. Cell cycle arrest is initiated through p53-dependent gene expression patterns resulting in expression of cyclin dependent kinase inhibitors p16 and p21 that impose irreversible cell cycle arrest. With cell cycle arrest, chromatin remodeling and genomic structural decline alter gene accessibility and expression[15–17]. Therefore, normal cellular processes suffer, often becoming aberrant or dysfunctional, and the chronic senescent cell develops a unique phenotype based on its cell type and previous functions. It is critical to identify how chronic senescent cell accumulation alters specific and general tissue microenvironments *in vivo*.

Processes such as telomere erosion create progressive endogenous microaggressions that persistently stress cells. This type of chronic, long-term cumulative stress on homeostatic cell function does not overwhelm the cell into acute senescence, but eventually culminates in chronic senescence. Chronic endogenous triggers have been mimicked using low dose irradiation to create genotoxic DSBs and chromosomal aberrations that generate similar senescence responses to those seen in previous in vitro studies. Irradiated young healthy mice respond with stable increases in tissue expression of the cell-cycle arrest marker, p16. The resulting increase in cell cycle arrest was accompanied by decreases in chromatin remodeling localized to tissue areas demonstrating extensive damage to the extracellular matrix[14].

Transcriptional analyses of telomere eroded cultures, irradiated cultures, and isolated murine $p16^{+}$ tissue-resident cells all indicate significant up-regulation of pathways triggering expression of active soluble factors such as interleukins, chemokines, growth factors, and proteases[18–21]. The prominent increase in production of these

mediators exhibited during cell-cycle arrest led to adoption of the term "senescence-associated secretory phenotype" or SASP to describe features associated with chronic cell senescence[8]. This senescence-initiated reprogramming positions chronic senescent cells as major sources of inflammatory and other soluble factors that potentially alter microenvironments, and contribute to age-related deleterious effects on tissues.

1.1.3 Chronic Inflammation and Aging

Triggering acute inflammation is an important mechanism through which damaged or infected cells of peripheral tissues communicate with the immune system. Cytokines, chemokines and other small signaling molecules are secreted upon insult to initiate vasodilation and thus, increased tissue accessibility for immune cells. However, this acute immune response is a highly regulated and beneficial process. Chronic inflammation involves a similar process, but in contrast, becomes persistent through failure to eliminate the triggering insult -or is a product of autoimmunity. Chronic sterile inflammation, such as results from autoimmunity, has recently been linked to the SASP acquired by senescent cells[22].

Chronic inflammation, distinct from the resolving inflammation generally seen during acute infection and insult, is the most commonly identified risk factor for mortality and morbidity in the elderly[23]. Chronic inflammation accompanies such age-related disorders as Alzheimer's disease, atherosclerosis, diabetes, osteoporosis, cardiovascular disease and cancer[16,24–29]. Age-related diseases are not only exacerbated by these inflammatory components, but may actually require them for their development, implying a causal relationship between inflammation and unhealthy aging termed "inflammaging"[30].

1.1.4 Soluble Mediators of "Inflammaging"

Longitudinal human cohort studies of inflammaging have identified striking changes in the levels of proteins involved in immune cell activation, recruitment and differentiation as part of various age-associated changes in plasma composition. Modest increases in pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, consistently occur with age[9,15,31]. These inflammaging components are produced mostly by endothelial cells that have either undergone damage, or have potentially become senescent, and are now attempting to communicate distress for clearance by immune cells. Acute senescence requires successful and immediate immune-mediated clearance and does so via chemokine/cytokine secretion or acute inflammation. In contrast, the chronic systemic pro-inflammation, seen during aging, reinforces a strong pleiotropic, and potentially deleterious, effect on immune cells by becoming a persistent immune stimulus. Interestingly, the role of anti-inflammatory cytokines has not yet been explored in the context of unhealthy age-promotion, however, conflicting evidence in such age-associated alterations in cytokines, such as IL-10, suggest that it requires further conclusive investigation[32–34]. This represents a much deeper relationship between chronic senescence and the immune system, indicating a role for the immune system in tissue decline.

In studies of parabiosis in murine models, aged mice appear to be rejuvenated by sharing the circulatory system of a much younger mouse. Blood components from the younger mouse reversed age-associated arterial thickening, prevented muscular degeneration and decreased the amount of DNA damage exhibited within muscle tissue

cells. Furthermore, these studies discriminate conserved "pro-geronic" factors between the donors, it was demonstrated that plasma transfusion alone had the same effect, confirming a role for soluble factors[35,36]. Key features of young plasma were low levels of pro-inflammatory molecules such as IL-1β, IL-6, and TNF-α[34,35]. These pleiotropic cytokines play a major role in immune cell signaling, including both activation and inhibition of immune cell functions.

1.1.5 Inflammatory Paracrine Senescence

Senescence can also be invoked through an alternate route dependent on proximity to chronic senescent cells. Co-culturing low passage fibroblasts with those exhibiting signs of senescence enforces a premature senescent phenotype and transcriptional pattern on the "younger" cells. The same results occurred when low-passage fibroblasts were cultured with supernatants from senescent cells, illustrating in this system that soluble factors secreted from senescent cells can effectively serve as promoters of senescence[37]. It is important to note that this paracrine-mediated senescence has not been distinguished as a representative feature of either acute or chronic senescence and may effectively contribute to both. Inflammation mediated by IL-1 family cytokines has been identified as integral to triggering and reinforcing paracrine senescence[38]. It has been recently identified that cells reaching senescence due to persistant DNA damage sensing, affect growth, transcriptional profiles, and cytokine production in nearby cells, effectively describing an act of paracrine senescence[39]. It is reasonable to suggest, although not reported in these studies, that the younger counterparts in the previously described parabiosis studies suffered premature

accumulation of senescent cells due to the influx of inflammatory mediators, and that this accelerated their aging.

Environmental stresses, such as paracrine stimulation, are now incorporated into the complex web of factors triggering acute and chronic senescence, thereby increasing the diversity of senescent phenotypes exhibited. These phenotypes depend not only on how senescence programming was initiated, but also on the cell type in question. The potential for SASPs to contribute towards the overall inflammaging environment and the influence of these feedback loops in instituting paracrine senescence are high priority subjects for studies on healthy aging. It should be noted that these inflammatory secretions are a form of distress signaling to immune cells to aid in senescent cell clearance. This increase in inflammaging is a direct product of senescent tissue accumulation that occurs with age, however, it also signifies the decline of immune mediated pro-inflammatory cell clearance. While the exact mechanism of age-related decline in senescent cell clearance is unknown, the senescence of immune cells themselves may be a critical benchmark in organismal aging.

1.2 T cell Immunity

In order to identify how an immune cell, particularly those of the adaptive T cell lineage, can undergo senescence, we will first describe how T cells arise in vivo and outline a unique biology that places these immune cells at risk for telomere-dependent chronic senescence.

1.2.1 Thymic Education

Large-scale expansion of adaptive immune cells occurs during almost every stage of their development. Thymocyte progenitors emerge in the bone marrow and

migrate to the thymus for further development. Upon entering the thymus, these progenitor cells do not express an antigen-specific T cell receptor (TCR) or either co-receptor, cluster of differentiation (CD) 4 or CD8. In the thymus, through a process known as thymic selection, these receptors are generated and ultimately represent the fate of the thymocytes bearing them. For simplicity, only TCR αβ–expressing T cells will be discussed in this thesis. The thymocyte systematically rearranges genes responsible for the clonotypic αβ–TCR, one at a time. Beginning rearrangement with the locus responsible for the β–chain, thymocytes undergo intense proliferation following a successful rearrangement, then proceed to TCR α–chain rearrangement, with proliferation ceasing once the successful αβ heterodimer is expressed. Thymocytes at this stage express both CD4 and CD8 and undergo successive positive and negative selection, to generate a final repertoire of T cells that can both sensitively and discriminately identify self-peptides in the context of self-human leukocyte antigen (HLA) molecules within an appropriate avidity threshold. As few as 2% of thymocytes survive this stringent thymic education, however, in each stage, high levels of proliferation occur to ensure sufficient numbers for the next round of selection[40]. IL-7, and other growth factors secreted by thymic stromal cells promote proliferation and survival of thymocytes, much like the stromal cells of the bone marrow niche[41,42]. Telomerase expression is directly enhanced by IL-7:IL-7R engagement to ensure these high levels of proliferation do not drive premature telomere erosion[43,44]. Only after surviving highly stringent selection processes do immature thymocytes develop into mature, yet antigen naïve, T cells and enter the periphery.

1.2.2 Peripheral T cells: Naïve, Effector and Memory

In the periphery, naïve T cells constantly traffic throughout the blood and lymph system in search of cognate antigen. The naïve T cell expresses a wide variety of chemokine and adhesion molecule receptors that control homing and extravasation into lymph organs. In the secondary lymph organs, antigen-presenting cells (APC) engage T cells by expressing major histocompatibility complexes (MHC, or HLA in humans) loaded with either self-peptides, or non-self-peptides that initiate T cell activation.

The signals needed for primary $CD8^+$ T cell activation are generated from the TCR:MHC class I complex and costimulatory CD28:CD80/86 interaction. The TCR complex is comprised of α:β chains and clustered CD3 proteins that possess intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) capable of initiating signalling cascades. Activated signalling proteins phosphorylate intracellular domains of proximal CD28 molecules, which generates signals resulting in nuclear factor - kappa beta (NF-κb), mammalian target of rapamycin (mTOR), and phosphoinositol-3-kinase (PI3K) pathway activation. Once initiated, these activation cascades prepare the transitioning T cell for differentiation to an effector phenotype. NF-κB translocation is essential to multiple T cell responses including effector cytokine production and memory formation[45,46]. mTOR activation results in production of B-cell lymphoma (BCL) family proteins that inhibit apoptosis and support T cell memory formation[47]. PI3K cascades control IL-2 production and secretion. Autocrine or paracrine engagement with IL-2R initiates telomerase expression and subsequent proliferation of activated T cells[48,49].

Activated T cells, as they proliferate, differentiate into effector $CD8^+$ T cells possessing cytotoxic effector functions. Effector $CD8^+$ T cells survey peripheral tissue to identify host cells that express cognate peptides. These peptides could be mutated self-proteins, indicative of cancer, or could be viral proteins, reflecting viral entry into and replication within the host cell. Once these cells have been identified, effector functions are triggered and target cells are selectively killed and removed. These effector functions utilize proteins such as cytotoxic granzymes and perforin to create pores in the extracellular membrane of target cells inducing apoptosis; antiviral cytokines, interferon (IFN)-γ and TNF-α to activate host cells and other immune cells; cell death receptors Fas/FasL to trigger pro-apoptotic caspase pathways[50].

Post-pathogen clearance results in contraction of the high numbers of effector T cells generated by antigenic exposure, a critical phase for protection against immunopathology. Regulatory contraction mechanisms depend on antigen withdrawal, falling cytokine and chemokine levels (in particular, IL-2 or other γ chain cytokines), and T cell:T cell engagement (Fas:FasL interaction). These processes eliminate more than 95% of effector $CD8^+$ T cells, leaving 5% or less to become long-lived memory T cells[51].

Memory $CD8^+$ T cells express a different phenotype and mediate different functions than naïve and effector T cells. Decreased expression of CD62L and variable C-C chemokine co-receptor (CCR) type 7 (CCR7) expression reflect that these cells have undergone antigen priming and no longer require migration to peripheral lymph nodes for activation. Memory T cells gain expression of CD27, which is needed to provide co-stimulatory signals when encountering antigen in the periphery. Memory T cells retain

expression of low affinity chains of IL-2R (CD122) and IL-7R (CD127), which are essential for survival, periodic homeostatic proliferation and antigen-driven proliferation[44,52]. These proliferative episodes are intermittent and may be due to any of a multitude of factors including re-infection, stress or periods of decreased immunity. In terms of memory T cell numbers, the absolute number of effector T cells generated in the effector phase is the strongest determinant of how many memory T cells persist. However, how the effector cells are eliminated and the manner in which differentiating effector $CD8^+$ T cells are maintained as memory cells, is also influential. Over time, memory T cells will decline in number if their survival requirements are not met. Conversely, persistence of memory T cells leads to further phenotypic changes. Expression of CD28, CD122, and CD127 decreases and with that, so does the capacity to activate pro-survival mechanisms such as telomerase expression[53].

From bone marrow to thymus to periphery, multiple regulatory processes involved in T lymphocyte proliferation, selection and survival ensure the sufficiency of adaptive immune responses against pathogens. However, once antigen is encountered, these T cells begin a slow progression towards the inability to proliferate, much like the *in vitro* fibroblasts described by Hayflick et al[54]. The associated decay in function is effectively defined by the extent of proliferation and differentiation previously undergone, which at least to a certain extent parallels biological aging. This feature of adaptive immune cells indicates that immune cell replicative senescence exists.

1.2.3 "Immunosenescence:" A Misnomer of Sorts…

In aging tissues, senescence broadly manifests as a lack of capacity for self-renewal, loss of organized function, decreased structural integrity and altered secretory profiles[8]. Fibrous tissue cell senescence has been thoroughly described, however, it is imperative to study senescence within the immune system as it comprises a large network within the body and is itself a tissue that ages and undergoes cellular senescence. The direct role of immune system senescence represents the largest unknown factor regulating the inflammaging-senescence relationship with unhealthy aging.

Immunosenescence was a term used originally to refer to an elderly individual's lack of an effective vaccine response[55,56], but today the term continues to be used to represent multiple other features the immune system exhibits in old age. This misnomer describes the gradual decline with age of naïve T cells and increases in adaptive memory T cells, increases in inflammation, and is somewhat unjustifiably associated with decreased resistance to infections and poor vaccine effectiveness[57]. Immunosenescence as defined by these popularized characteristics, applies only to macrophysiological changes in immune cell composition that occur with age, more specifically, an accumulation of cells representing adaptive immune memory. However, cells described by such broad criteria such as antigenic exposure comprise a largely heterogeneous population. Therefore, this view of immune senescence is not supported by aging at the level of individual cells, and thus, these organ level changes are not necessarily representative of the same underlying relationship with cellular senescence as seen in other tissues.

Indices of immune cell senescence can be extrapolated from studies of senescence in other tissues. The focus of our research will be restricted to the potential

development of chronic T cell senescence, rather than acute senescence, as the triggers are more readily related to aging. Therefore, we will outline a potential pathway to chronic senescence via the endogenous trigger of telomere erosion.

1.2.4. Underlying Immune Senescence: End Replication in Memory T cells

As Hayflick et al. identified excessive passaging of fibroblasts as a trigger of cellular senescence, it became of paramount importance to recognize the many proliferative phases in immune cell development. As outlined in **Figure 1.1**, lymphocytes undergo intermittent periods of increased cell division between long phases of quiescence, depending on homeostatic needs, antigenic burden and levels of immune activating molecules in the periphery. In cases of exhaustive lymphocyte proliferation, the opportunity arises to study the end-replication problem, and subsequent telomere erosion, as a cause of chronic immune cell senescence.

Like embryonic and stem cells, activated lymphocytes are one of the only somatic subsets of cells that selectively increase telomerase activity[7]. Most lymphocytes (excluding NK cells) originate from single cells selected in the thymus and the bone marrow based on affinity and specificity, and selected again in the periphery during primary antigenic exposure (denoted in **Figure 1.1** as a single color representing a single "clone"). Therefore, all cells expressing the same antigen recognition receptor (TCR) theoretically stem from proliferation of a single "clone." This concept, termed clonal expansion (denoted in **Figure 1.1** by increased size of cells), specifies each round of proliferation as an opportunity for telomere loss. During clonal selection of naïve lymphocytes in the primary and secondary lymphoid organs, telomerase activity is elevated via IL-7:IL-7R or IL-2:IL-2R engagement to compensate for telomere erosion

through heightened cell division[44]. In the periphery, memory cells receiving antigenic stimulation also increase telomerase expression; however, this is not a conserved quality, as long-lived memory cells decrease their level of telomerase expression with each successive round of activation[58] (**Figure 1.1 d, e**). Telomerase expression decreases virtually to zero in ex vivo mitogen activated memory T cells of the elderly, illustrating development of a large subset of T cells at risk for harmful telomere erosion[59].

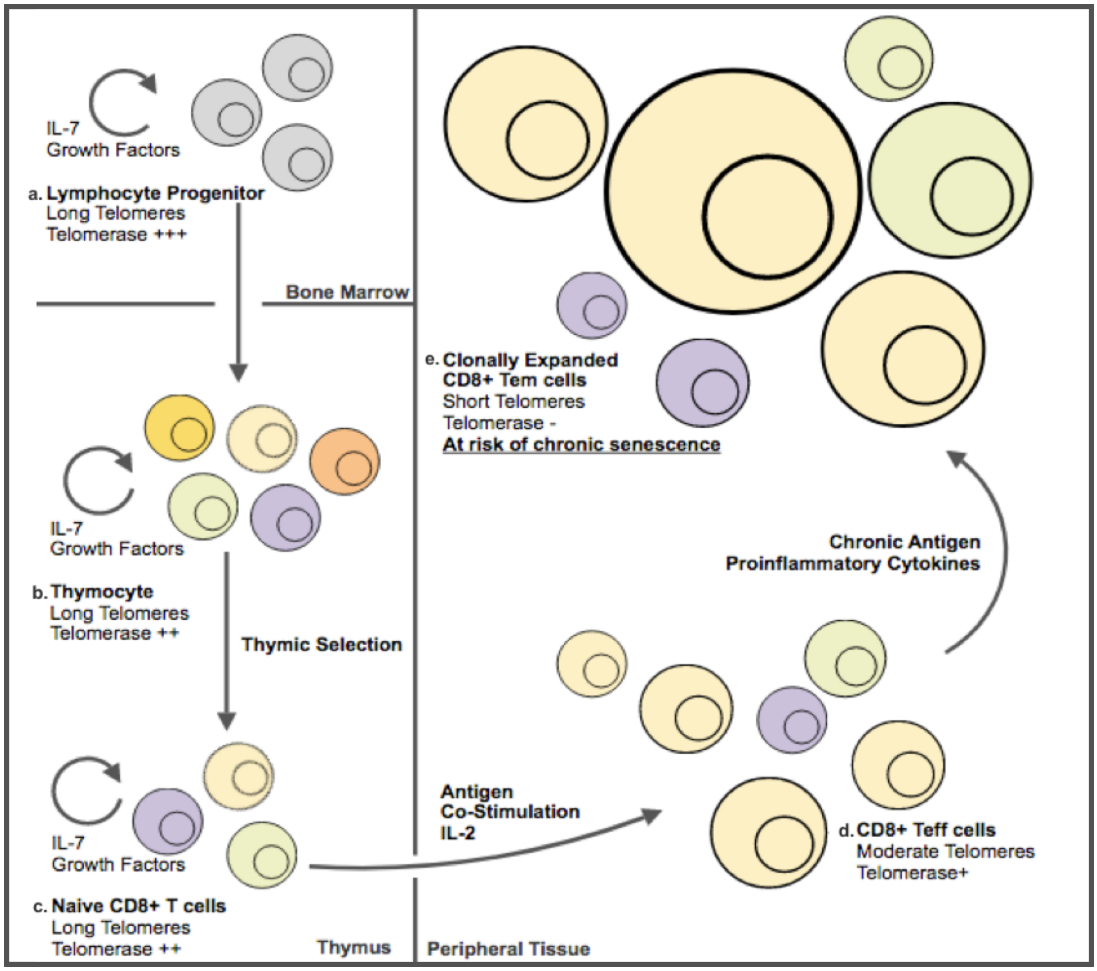

Figure 1.1. Factors involved in CD8$^+$ T cell telomere maintenance throughout phases of cell differentiation.

Lymphoid progenitor cells undergo cycles of quiescence and homeostatic proliferation **(a)**, the latter being maintained by high levels of telomerase expression initiated by cytokines and growth factors, like IL-7, secreted by bone marrow stromal cells. Similarly, thymic stromal cells supply IL-7 ensuring telomerase expression during proliferative stages of positive and negative selection **(b)**, and in antigen-naïve CD8$^+$ T cells **(c)**. In the periphery, naïve CD8$^+$ T cells encounter antigen, with successful activation triggering a proliferative response via an autocrine IL-2:IL-2R feedback loop and becoming CD8$^+$ T_{eff} cells **(d)**. IL-2R triggers telomerase expression, however, continual activation reduces telomerase levels, leaving clonally expanded CD8$^+$ T_{em} cells **(e)** with undetectable levels and at risk of critical telomere erosion and chronic senescence. IL, interleukin; Teff, effector T cell; Tem, effector memory T cell.

To investigate associations between immune cell telomere length and age-related illnesses, Cawthon et al. studied a large cohort of elderly patients stratified by leukocyte telomere length over a period of 15 years. The authors showed that individuals with the shortest leukocyte telomere lengths had the lowest rate of survival and the largest risk for developing age-related morbidities, most of which involved cardiovascular or immune complications[60]. Reflecting changes similar to those exhibited in elderly memory T cells, in vitro models employing successive rounds of mitogen stimulated T cell cultures recapitulate shortened telomeres, reduced proliferation, and altered cytokine generation[61]. These observations suggest a possible link between the telomere, exhaustive immune cell proliferation and in vivo immune senescence. Determining the exact role of telomere shortening in T cell functional decline with age could elucidate the mechanism validating use of the term immune, or more specifically lymphocyte, senescence and clarifying its role in inflammaging and age-related morbidity. Furthermore, identifying specific pathogens or other antigens that drive such progressive clonotypic expansion of memory T cell subsets, would be highly favorable for studies of immune senescence.

1.3 Cytomegalovirus (CMV) Infection

Human cytomegalovirus (HCMV), a member of the beta subfamily of Herpesviridae, infects ~75% of the developed world's population[62]. Large and robust immune responses incorporating natural killer (NK) cells, inflammatory cytokines, and adaptive B and T cells contain virus levels after primary infection, but HCMV always results in persistent latent infection. In healthy individuals, reactivation from latency remains asymptomatic, however, in persons with a weakened immune system, such as

transplant recipients, cancer patients and those living with human immunodeficiency virus, bouts of viral reactivation can result in serious clinical issues, ex. CMV retinitis[63].

1.3.1 $CD8^+$ T cell Responses to CMV

Adaptive immune responses to CMV have been extensively scrutinized, largely due to their atypical expansion into populations as large as 90% of total memory $CD8^+$ T cells[64]. Although they are thought to relate to lifelong infection and periodic reactivation from latency, the mechanisms behind these expansions and how they are maintained are currently unknown and the subject of considerable debate. Even if it is unknown exactly how this prolific expansion of CMV-specific memory $CD8^+$ T cells occurs, studying these cells and their associated maintenance could prove extremely useful for generating large, long lasting immune responses through vaccination.

This unconventional expansion of CMV-specific T cells has been suggested as a model to study T cell senescence. This CMV-specific population appears to possess both an altered phenotype and functional disposition. Although adaptive immune senescence, and its associated characteristics is a highly understudied and currently speculative topic, strong associations between CMV-specific T cell frequencies, markers of replicative senescence and age potentially position CMV immunity as a primary driver of chronic immune senescence with potential linkage to age-associated morbidities.

1.3.2 Atypical Clonality of CMV-specific $CD8^+$ T cells

CMV specific T cells in a single individual are predominantly restricted to one or two $V\alpha$ and $V\beta$ families of the entire TCR repertoire[65]. Upon stimulation with CMV

antigen the majority of the response is mounted by a highly TCR-restricted population, with a single clone accounting for up to 40% of directed effector response(s) and up to 15% of total $CD8^+$ T cells[66]. These data suggest that large T cell compartments targeted towards CMV mostly originate from only a few naïve T cells selected early during primary infection. How this immunodominant clonality arises is incompletely understood, but research draws connections to higher avidity/affinity selection during viral episodes, or selective maintenance of clones early in primary infection. Despite lacking a clear mechanism, to create such a large oligoclonal population, this extensive clonal expansion must rely on successive rounds of cell division with eventual creation of an altered phenotype and functionally distinct subset of $CD8^+$ T cells.

1.3.3 Altered Phenotype of CMV-specific $CD8^+$ T cells

Long-term cultured $CD8^+$ T cells lose CD27 and CD28 expression[67], thus, it is assumed that $CD8^+$ T cells, lacking expression of either *in vivo*, are long lived older cells. $CD28^-$ $CD27^-$ T cells dominate the CMV-specific subset[68] and exhibit no dependence on costimulatory signals[69], possibly relying solely on TCR engagement to manage antigen-specific effector responses. *Ex vivo* stimulation of $CD28^-$ $CD8^+$ T cells can restore expression of CD28, possibly to help initiate paracrine IL-2 activity once activated from quiescence. However, this was not achieved in $CD28^-$ CMV-specific T cells as only transduced CD28 expression in CMV-specific $CD8^+$ allowed successful paracrine IL-2 activity and proliferation[70]. Large fractions of $CD28^-$ CMV-specific $CD8^+$ T cells with atypical activation feedback mechanisms could signify manifestation of a pre-senescent or senescent T cell population with altered transcriptional programming.

CMV-specific CD8+ T cells typically express CD57[71], a cell surface marker associated with extensive rounds of proliferation and what is referred to as "terminal differentiation." CD57 expression has been well studied on NK cells, identifying populations that apparently are more functionally mature and that demonstrate heightened cytotoxicity in both natural and antibody-dependent responses, while undergoing lesser proliferation when exposed to activating cytokines such as IL-12 or IL-18[72]. This increased CD57 expression is not only strongly associated with loss of CD27/28[68], but also with loss of IL-2 family receptors CD122 (IL-2Rβ) and CD127[73,74] (IL-7Rα), all predisposing towards a lack of proliferative capacity. Given the decreased expression of CD28, IL-7R, and IL-2R on CMV-specific $CD8^+$ T cells and concomitant lack of a mechanism for telomerase induction, telomere erosion may underlie the replicative senescence exhibited by these cells.

It should be noted that the majority of $CD8^+$ T cells directed towards HCMV reside outside of the lymph nodes and are restricted to the circulation and tissue, a trafficking pattern reflected in the combination of chemotactic receptors expressed. CMV-specific $CD8^+$ T cells lose expression of CCR7 during memory development, but gain CCR5 and CXC3 chemokine receptor 1 (CX3CR1)[75,76]. CX3CR1 and CCR5 primarily home immune cells to sites of endothelial inflammation, normally in the initial stages of infection, via attraction to the chemokines: fractalkine (CX3CL1) and macrophage inflammatory protein (MIP) 1 α/β or RANTES, respectively[77]. CX3CR1:fractalkine activation pathways are also intimately involved in initiating pro-survival mechanisms in memory T cells through increased expression of BCL-2/xL[78].

Chronic activation of these pathways could potentially provide a mechanism for prolonged CMV-specific T cell survival and also possibly play a role in persistent inflammatory states of peripheral tissues in aging individuals.

1.3.4 CMV-specific $CD8^+$ T cells are Functionally Distinct

The secretory profile and antiviral responses of effector $CD8^+$ T cells are directly influenced by their stage of differentiation and the nature of the viral interaction. As frequencies of CMV-specific $CD8^+$ T cells rise over time, presumably with intermittent viral reactivation, recurrent bouts of successful antigenic clearance reinforce a pronounced multi-functionality in CMV-specific $CD8^+$ T cells optimized for effector function. This multi-functionality involves diverse cytokine production and heightened cytotoxicity; however, they also exhibit lack of a robust proliferative response[79].

The production of IFN-γ and TNF-α are fundamental characteristics of the antiviral immune response mounted by $CD8^+$ T cells. IFN-γ promotes T cell activation, enhances phagocytic activity of innate immune cells and primes infected host cells to increase expression of genes regulating antigen presentation[80]. TNF-α enhances the antiviral immune response in synergy with IFN-γ by directly triggering infected-cell death via the TNF superfamily of death receptors and by promoting $CD8^+$ T cell survival and heightened IFN-γ and granule production in positive feedback cascades involving classical NF-κB signaling[81]. CMV-specific $CD8^+$ T cells not only simultaneously produce both of these antiviral cytokines when exposed to antigen, but also produce them faster and at higher levels than other virus-specific effector $CD8^+$ T cells[82].

In addition to antiviral cytokines, the secretory profile of $CD8^+$ T cells engenders cytotoxic function through proximal secretion of serine protease granzymes and the pore-forming protein perforin. CMV-specific cells again demonstrate not only higher immediate production of granzyme/perforin, but also a higher level of pre-assembled granules, establishing their capacity to exert prompt cytotoxic activity against CMV-infected cells[79,83]. During chronic human immunodeficiency virus (HIV) and/or hepatitis C virus (HCV) infection, a majority of $CD8^+$ T cells recognizing viral epitopes produce antiviral cytokines such as IFN-γ, but have a reduced ability to lyse viral peptide-pulsed target cells. Those directed against CMV retain this function in the same host[84]. Selective maintenance of CMV-specific T cells with these features could represent adaptation towards greater viral control or an acquired secretory feature (SASP) representative of replicative T cell senescence.

Some studies of multifunctional $CD8^+$ T cells have incorporated IL-2 production, in addition to IFN-γ and TNF-α, as a third functional cytokine to gain insight on virus-specific T cell exhaustion. These studies have shown that simultaneous production of all 3 cytokines is generally associated with successful containment of viral or bacterial infections, like *M. tuberculosis* and even HIV[85,86]. Profiling of CMV-specific $CD8^+$ T cells shows that up to 75% are triple-cytokine producers, with as low as 2% of responding T cells producing only one cytokine (predominantly IFN-γ)[87]. These mono-cytokine producing T cells, although low in frequency, showed the highest IL-2/IL-2R expression and proliferation with Ag exposure. Poly-functional $CD8^+$ T cells that respond with multiple cytokine production showed little production/expression of IL-2/IL-2R and

underwent significantly less proliferation, despite high production of IFN-γ and TNF-α[69]. High effector functions and low proliferative capacity could potentially illustrate the phenotype acquired by CMV-specific $CD8^+$ T cells as they progress towards T cell senescence.

A key feature in contraction of expanded $CD8^+$ T cell populations is initiation of apoptosis. Studies comparing activation induced cell death (AICD), a mode of T-cell limiting apoptosis, in various antiviral T cell subsets suggest that CMV-specific cells harbour high levels of anti-apoptotic proteins B-cell lymphoma (BCL)-2 and BCL-xL, compared to HIV and HBV-specific $CD8^+$ T cells in the same host[88]. Normally upregulated during memory cell formation and triggered by CD28 or IL-2R engagement, levels of these anti-apoptotic proteins remain elevated in CMV-specific $CD8^+$ T cells independent of receptor ligation[78]. *Ex vivo* studies of CMV-specific $CD8^+$ T cells indicate that these T cells increase BCL-xl/2 protein expression in response to antigenic stimulation, in comparison to HIV-specific $CD8^+$ T cells, and remain resistant to a multitude of apoptotic triggers including protein kinase C (PKC) inhibition, FasL engagement and growth factor withdrawal[89]. Development of these pro-survival factors could arise due to unique features of CMV infection, but it is clear that CMV-specific T cells are long lived and become apoptosis-resistant, creating another potential hallmark feature of CMV-associated T cell senescence.

CMV-specific $CD8^+$ T cells represent a population that could be used as a model to understand T cell senescence. This particular effector memory subset possesses heightened functional specialization, which could represent features of an adaptive

immune cell SASP. CMV-specific T cells undergo extensive *in vivo* proliferation, however, they exhibit limited *ex vivo* replication, potentially representing subset-wide cell cycle arrest. Effectively dysfunctional in clonal retraction *in vivo*, this feature of CMV-specific T cells is paralleled *ex vivo* in their high resistance to apoptosis upon antigen stimulation. Possession of similar traits to those observed for senescent cells in tissue suggests that CMV-specific T cells could be a senescent immune cell subset and, therefore, accumulation of such could potentially drive relationships between CMV and age-associated morbidities.

1.4 Aging with CMV

As previously stated, aging is strongly associated with declining physiological function and whether due to decreased immunosurveillance or increased exposure, is strongly associated with prevalence of CMV infection[90]. In a study of a representative population of the developed world (n = 21 639) the prevalence of CMV infection rises from 36% in children under 11, to 91% in adults over the age of 80[91]. In all age groups, CMV infection creates signature alterations in adaptive immune memory profiles, however, in the old elderly, these changes related to CMV infection are associated with increased all-cause mortality[92].

Despite this clear association between CMV infection and unhealthy aging, the exact mechanism underlying this relationship remains unknown. It has been suggested that CMV infection accelerates the natural aging process by affecting immune system aging. Although it is governed by a multitude of known and unknown factors, the natural course of aging of the adaptive immune system primarily reflects declining thymic

function and immune stresses, ultimately progressing towards a steady state of immune senescence in the old elderly. This introduction will now focus on association of CMV infection with previously described features of immune senescence, including reduced clonal diversity in the T cell repertoire, increased inflammation, and onset of multiple age-related morbidities. Given the changing demographic of most populations and the social and economic costs of unhealthy aging, understanding how CMV influences unhealthy aging, and ultimately morbidity and mortality, is crucial for health-related research.

1.4.1 CMV-associated Repercussions on T Cell Diversity

Thymic involution reflects structural and functional degeneration of the thymus that begins around puberty and continues into old age. Thymic tissue gradually is replaced with adipose tissue, greatly reducing thymic output and, therefore, restricting the majority of naïve T cell production to prepubescent stages. This age-associated decline in thymopoiesis drives the ratio of naïve to memory T cells down with age and antigen exposure, and is, therefore, largely responsible for the immune profile seen in old age[93] and is a natural contributor to the common phenotypic profile of immune senescence.

Given the extensive thymic activity early in life, older children and even adults are generally considered to be unaffected by its surgical removal. However, a study by Sauce et al. showed that this actually holds true only if a thymectomized child is seronegative for CMV[94]. Monitoring T cell dynamics in thymectomized CMV-seropositive children showed progressive development of the characteristically low naïve:memory T cell ratios normally exhibited in old age. Deep-sequence profiling of the

TCR repertoires of these children also showed substantial restriction of TCR sequences, with a majority of sequences complementing immunodominant CMV antigens, another feature of immune senescence observed in the elderly[95]. These results represent an acceleration of effects occurring with CMV infection and natural thymic decline, with TCR diversity analyses of healthy CMV-seropositive adults revealing 33% reductions in TCR diversity compared to seronegative individuals of the same age[71]. Although not all the specific expansions were demonstrated to be CMV-epitope specific, the results of these studies clearly support the supposition that CMV infection reduces TCR diversity over time, to a much greater extent than natural aging alone.

To assess the impact of CMV infection on immune aging in an animal model, young mice were infected with MCMV and monitored longitudinally to assess age-related changes in immune proficiency. Challenges with secondary infections showed that successful pathogen clearance was more frequent in uninfected mice, however, rates of success did decrease with age in both groups of mice. MCMV-infected mice showed equivalent pathogen clearance when young, but their clearance rates declined at younger ages and more dramatically than in the uninfected mice[96]. TCR repertoire analysis indicated that the effects of MCMV-infection parallel those seen in human studies with a stark reduction in overall diversity, and a majority of expanded clonotypes being specific for MCMV epitopes. Interestingly, the MCMV-infected mice also suffered higher all-cause mortality and a shorter lifespan, suggesting that MCMV infection is a useful animal model for accelerated aging[96]. The mechanism by which CMV infection affects this

process remains speculative however, it is crucial to consider the role of extensive CMV-mediated clonal T cell expansion and explore its potential links to inflammaging.

1.4.2 CMV-associated Alterations in Systemic Inflammation

High frequencies of functionally intact CMV-specific $CD8^+$ T cells reveal the potential for cycles of inflammation, immune activation and homeostatic proliferation to contribute to aging of the immune system. "Healthy" CMV seropositive individuals have low-grade chronic inflammation characterized by increases in type 1 cytokines, growth factors, IL-6, and C reactive protein (CRP)[97]. Type 1 cytokines, such as IFN-γ, TNF-α, and IL-2 are a group of cytokines that facilitate cellular immunity, driving naive $CD4^+$ T cells to differentiate into Th1 cells while maintaining and reinforcing antiviral $CD8^+$ T cell immunity. Increased levels of these activating cytokines in the periphery could drive non-specific activation of nearby T cells opening the way for positive feedback loops closely resembling those suspected to be involved in paracrine senescence. CRP, as an acute phase protein produced in the liver due to increased IL-6, IL-1β or TNF-α, is elevated during acute infection, however, sustained increases occur in chronic inflammatory diseases and are indicative of increased risk for cardiovascular disease[98]. A study by van de Burg et. al associated serum levels of IFN-γ with CRP during CMV latency raising suggestions that the magnitude of CMV-specific T cell immunity is associated with increased risk for cardiovascular disease[97]. *In vivo* studies of vascular inflammation and arterial damage indicated no strong associations between detectable CMV DNA and CRP or other signs of cardiovascular risk[77], suggesting factors other than

CMV replication itself, such as CMV-immunity potentially play roles in vascular damage.

During arterial disease, cells within the inflamed vasculature can secrete high amounts of IL-6 and CX3CL1 (fractalkine) in attempts to communicate distress and promote immune-mediated clearance[99]. To investigate the possible link between CMV immunity and cardiovascular damage, Bolovan-Fritts et al exposed HCMV-infected endothelial cells to syngeneic lymphocytes from CMV-positive donors. While only minimal damage appeared to be directly due to CMV replication, blocking the CX3CL1:CX3CR1 interaction reduced overall endothelial cell death by 3-fold[100]. Since CX3CR1 expression is high on CMV-specific $CD8^+$ T cells, chemokines produced by endothelial cells may be intrinsically involved in invoking cell damage in response to CMV replication. Since CMV-immunity increases with age, it is reasonable to implicate the immune response against CMV as a major component of vascular degeneration, inflammation and increased age-associated cardiovascular risk. This specific situation of potential tissue senescence arising through multiple rounds of endothelial cell proliferation, due to viral replication and tissue homeostasis, with an exaggerated immune response leading to tissue damage could, provide a major link between CMV infection and pathogenesis of age-associated cardiovascular disease.

1.4.3 CMV and Risk of Age-related Morbidity

Despite extensive literature addressing the effect of CMV infection on both the adaptive immune system composition and pathogenesis of multiple age-related morbidities, none have effectively revealed a mechanistic connection. Pawelec et al. of

the Swedish OCTO and NONO longitudinal population studies, directly associated CMV infection with increased mortality in the elderly, but only alluded to immune senescence as a potential cause. The studies have uncovered immune parameters such as decreased ratios of naïve to memory T cells, decreased T cell repertoire diversity and increased levels of systemic inflammation, related to increases in mortality[56,71,92]. More specifically, studies in which the cause of death is identified have consistently shown increases in morbidity and drawn attention to dramatic increases in inflammation and cardiovascular-associated disease in CMV-infected elderly[101,102].

CMV-serostatus is linked to increased risk of multiple age-associated morbidities, especially atherosclerosis, diabetes, and cardiovascular disease[103]. CMV-seropositivity is associated with a 4-fold increase in new diabetes cases in transplant recipients, independent of age, a risk not seen with any other viral infection[104]. The inflammatory nature of diabetes, compounded by the apparent increase in inflammation that accompanies CMV infection, raises the risk of atherosclerotic events much higher in diabetic individuals and intensifies clinical repercussions[29,105]. In a later study exploring the additive nature of diabetes and CMV infection with regard to cardiovascular risk, increases in anti-CMV IgG titres paralleled increases in the frequency and severity of atherosclerotic events[106]. The systemic inflammation seen during CMV infection has been suggested to play an exacerbating role in other autoimmune or chronic inflammatory conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus, and systemic sclerosis[107]. Higher circulating levels of anti-CMV Ig have been associated with increased pathophysiological events during autoimmune diseases, often requiring surgical

intervention[108–110]. Pro-inflammatory CMV-specific $CD8^+$ T cells have been isolated from the synovial fluid of RA-affected joints and HCV-infected fibrotic livers[111,112]. CMV infection clearly plays a role in heightening risk of cardiovascular, autoimmune and metabolic disease, most likely through increases in systemic inflammation.

It should be noted that although these studies have used populations spanning generations, the majority have focused on the elderly or those living with chronic inflammation. A select population frequently identified as facing repercussions of immune senescence, are persons living with chronic human immunodeficiency virus (HIV) infection[113]. This population has an immune background that allows for dramatic growth of CMV-specific immunity in a virtually age-independent manner. Understanding the interaction between CMV and HIV-related immune parameters accelerating the pathogenesis of immune senescence during HIV infection could also help identify key immunological factors involved in unhealthy aging of the general population

1.5 Aging with CMV and HIV

It is now a common assumption that those living with HIV and having access to antiretroviral therapy (ART) will effectively suppress viral replication and prevent the onset of acquired immunodeficiency syndrome (AIDS). With the initial advent of ART, HIV-infected individuals remained subject to AIDS-defining conditions such as Kaposi's sarcoma, pneumocystis pneumonia, wasting syndrome, toxoplasmosis, a host of autoimmune conditions and high rates of opportunistic infections[114]. Through effective combination ART (cART), the HIV-infected population in developed countries has experienced a large decrease in the aforementioned conditions, progression to AIDS, viral

transmission and infectivity[115]. Despite reaching this landmark in HIV treatment, cART does not restore all aspects of health.

Longitudinal studies following HIV-infected individuals on cART, like that by Samji et al., have demonstrated dramatic increases in life expectancy[116]. The high effectiveness of cART and resulting increases in longevity for HIV-infected individuals, however, allows for increased frequencies of development of other adverse conditions not associated with progression to AIDS. Non-AIDS defining conditions like diabetes, cancer, cardiovascular disease, hypertension, bone disease, and most relevant to this project, immune senescence, can occur up to 15 years earlier during HIV infection than in the general population[117]. This increased risk for non-AIDS defining conditions follows a pattern similar to that of the more elderly non-HIV-infected population[118], suggesting that even well-treated HIV infection might accelerate the aging process. Given that accumulation of senescent cells is an intrinsic feature of aging, it is imperative that the age-associated regulatory processes involved in limiting senescence, including how the immune system is involved, become better elucidated in the context of HIV infection.

Early studies of CMV infection and its effect in HIV-infected individuals suggested it was a cofactor accelerating progression to AIDS in the pre-ART era. These studies primarily view CMV viremia as an AIDS-defining feature, and implied that CMV replication translates into increased inflammation, T cell death and an increased risk of opportunistic infections (not including CMV re-infection)[63]**.** However, this role of CMV as an opportunistic infection, in the context of HIV infection, differs in nature from the proposed deleterious age-associated effects CMV has on the immune system and

inflammation that occur in elderly HIV-negative individuals. Therefore, the burden of CMV infection and its effect on the immune system, its senescence and other sources of inflammation, should be re-addressed in order to elucidate a potential mechanism in the "premature" aging processes seen during HIV infection.

1.5.1 HIV-associated Inflammation

Inflammation that occurs despite ART-treatment renders virally supressed HIV infection a chronic inflammatory disease. Suggested contributors to the inflammatory landscape include microbial translocation and chronic immune activation, HIV replication, ART regimens and their side effects. However, it has recently also been suggested that unknown dysfunctional immune-regulatory components play a central role[119], including immune senescence and immune cell SASPs.

IL-6 levels, reportedly 40-60% higher in HIV-infected individuals than the general population, correlate with HIV viral load, $CD4^+$ T cell nadir and duration of HIV infection[120]. These levels peak during primary and early chronic HIV infection and although they decline with effective treatment, remain chronically higher than those of age-matched non-HIV-infected controls[118]. This peripheral inflammatory cytokine and downstream respondents like CRP can be twice as high in treated HIV-infected individuals as in the age-matched uninfected population[121]. These relatively low-grade increases in the concentrations of such pro-inflammatory cytokines are comparable to those occurring in aged (>70 years old) uninfected controls[23]. Although it is the most common marker for increased risk, it remains undetermined whether IL-6 elevation is

directly involved in the onset of the age-related morbidity in either group or if it is simply the most common marker of systemic inflammation.

Immune activation markers such as TNF-α, IFN-γ, and β–2 microglobulin (B2M) can all be elevated, even during treated HIV infection[122,123]. Irrespective of the underlying cause or route of generation, chronic increases in activating cytokines offers a potential explanation for the perpetually activated phenotype of $CD8^+$ T cells in HIV-infected individuals ($CD38^+$ HLA-DR^+ $CD28^-$ $CD27^-$)[124,125]. This chronic activating milieu could drive non-specific expansion of $CD8^+$ T cells, and accelerate the telomere attrition observed in $CD8^+$ T cells during HIV infection.

Inflammation and chronic immune activation are both associated with development of atherosclerosis, a major risk for the elderly. Recently, this risk was also implied in CMV^+HIV^+ individuals in a study of CMV/HIV co-infected subjects. Hsue et al. showed that the frequency of activated CMV-specific T cells, rather than levels of CRP or other markers of immune activation was associated with increased intima thickness, a marker of risk for development of atherosclerosis[121]. Pro-inflammtory IL-1 family cytokines, like IL-1α/β, are found in short-term culture supernatant of primary tissue samples (renal, adipose, mucosa, liver, vasculature) taken from HIV-infected individuals with high risk for cardiovascular disease[126,127]. Several studies involving ART-treated individuals state that production of these cytokines is due to dysfunctional immune cells, since no detectable HIV-RNA was identified (however, CMV was not included these studies)[128]. Thus, although CMV-related immunity may directly affect this

pro-atherogenic environment, it remains generally understudied as a vascular health factor in HIV infection.

Non-specific cytokine mediated immune activation and proliferation during HIV infection could increase the frequency of replicatively senescent effector $CD8^+$ T cells and fuel development of a comprehensive, yet uncharacterized, SASP phenotype with inflammatory components. Given the association of HIV infection and "premature aging," especially in the context of CMV infection, the role of an immune cell SASP and characterization of further complications of such, needs to be further addressed.

1.5.2 CMV/HIV Co-infection Impacts on T Cell Diversity

The most degenerative feature of progressive HIV infection is the decline of the adaptive immune system, namely selective loss of $CD4^+$ T cells. Untreated HIV infection is highly thymotoxic through direct and indirect effects of viral infection and through immune responses related to HIV infection. In ART clinical trials, researchers observed that a phenomenon known as "immune reconstitution" could occur, where low-level thymic activity and division of surviving/pre-existing immune cells lead to recovery, at least to some extent, from immune losses occurring during untreated infection[129]. Despite regaining high numbers of $CD4^+$ T cells during treatment, full recovery of immune function rarely occurs. This was reflected in a study by van den Berg et al., which identified the need to revaccinate HIV-infected individuals to restore hepatitis B virus (HBV) immunity after ART-treatment, demonstrating loss of -or loss of maintenance of- $CD8^+$ T cell memory responses[130]. HIV-infected individuals also showed reduced vaccine induced memory development, often generating only short-term $CD8^+$ T cell responses

against vaccine-encoded epitopes[131]. These studies show that despite the apparent reconstitution of lymphocyte numbers, this does not fully restore immune function, as illustrated by inconsistent $CD8^+$ T cell responses to secondary pathogens in HIV infection. Although the exact reasons for this are unknown it could play a role in the reduced $CD8^+$ T cell memory responses reported during treated HIV infection. Treated HIV-infected individuals often possess higher frequencies of CMV-specific $CD8^+$ T cells, than the general population[132,133].

HIV infection is associated with increased absolute numbers of $CD8^+$ T cells[133], and much like the CMV-infected elderly[134], these cells often express a memory phenotype. This persistent increase in $CD8^+$ T cells occurs despite effective treatment that reduces HIV replication and allows restoration of $CD4^+$ T cell counts[133]. HIV-infected individuals also show a 10-fold decrease in overall TCR diversity, particularly in the $CD8^+$ T cell compartments dedicated to memory[129], compared to the general population suggesting extensive clonal expansion or selective homeostatic proliferation. $CD8^+$ T cells in individuals treated for their HIV infection broadly show increases in CD57 expression, decreases in co-receptor expression (CD27, CD28), and a reduced capacity to proliferate and mount effector functions ex vivo compared to $CD8^+$ T cells isolated from uninfected individuals[68,132] . Increased numbers of $CD8^+$ T cells, acquisition of a senescent phenotype and decreased $CD8^+$ TCR diversity are all associated with increases in non-AIDS related diseases[118,129,135]. It is suggested that these deficits in the $CD8^+$ T cell compartment during HIV infection signify premature or accelerated immune senescence and mostly occur independent of age. Although generally similar to the immunological

abnormalities associated with the extensive clonal expansion of CMV-specific T cells in aged non-HIV-infected individuals, the mechanistic connection between these similar phenotypes remains under-examined.

With such a large increase in $CD8^+$ T cells and the marked reduction of $CD4^+$ T cells, it is common clinically to use the ratio of $CD4^+/CD8^+$ T cells as a surrogate marker for immune competence in HIV infection. When this ratio falls below 1.0, signifying increased expansion of $CD8^+$ T cells in relation to $CD4^+$ T cell recovery, there is a higher associated risk for non-AIDS related events[117]. Early-exposed HIV-infected children co-infected with CMV have higher frequencies of terminally differentiated $CD8^+$ T cells and lower CD4:CD8 ratios, when compared to CMV-seronegative children, regardless of detectable CMV DNA or HIV RNA in peripheral blood[118,135]. This observation indicates that CMV infection compounded by HIV infection produces an age-independent expansion of $CD8^+$ T cells. Barrett et al. further illustrate this interaction between CMV and HIV infection, showing that CMV-seropositive HIV-infected individuals have lower CD4:CD8 T cell ratios, independent of age and HIV viral load[136]. CMV infection clearly skews the $CD8^+$ T cell repertoire towards an immune senescent phenotype, in an age-independent manner during HIV infection.

1.5.3 Telomere Erosion and Aging in HIV Infection

To investigate the role of HIV infection in development of immune senescence and the aging processes, leukocyte telomere length (LTL) was used as a marker of cellular replicative history. The degree of telomere erosion is markedly pronounced during HIV infection, with general LTL comparable to that of uninfected individuals'

decades older. By compartment, $CD8^+$ T cells possess shorter average telomere lengths than $CD4^+$ T cells, indicating greater overall proliferation of $CD8^+$ T cells in HIV infection[137]. Effector memory $CD8^+$ T cells that re-express CD45RA, a subset comprised of pro-inflammatory terminal effector T cells, have shorter telomeres than other memory subsets[87]. To address the high genetic heterogeneity in cohorts screened for LTL, studies of HIV-discordant monozygotic twins showed that uninfected siblings had longer telomeres in their $CD8^+$ T cells. This effect was not apparent in $CD4^+$ T cells, indicating that the environment and operative mechanisms in HIV infection only override specific age and genetically determined effects on telomere erosion in $CD8^+$ T cells[138]. It is important to note that CMV infection was not taken into consideration for these studies, despite HIV-infected individuals having a higher rate of CMV infection[139]. LTL is also associated with the duration of untreated HIV disease and lower $CD4^+$ T cell nadir[140], the same factors associated with decreased TCR diversity, suggesting a relationship between LTL and TCR diversity, potentially through the effects of CMV-related T cell clonal expansion. LTL in HIV-infected populations is inversely associated with increased risk of COPD, heart disease, type 2 diabetes, cancer, and a host of other age-associated morbidities[141], reflecting the similar relationship occurring in older CMV-infected HIV-negative populations. These associations appear to suggest clonal expansion as a primary driver of lymphocyte telomere erosion, and affects the clinical presentation of age-related diseases in HIV infection.

A predominant feature of the aforementioned studies is that the cohorts consisted mainly of ART-treated participants, essentially excluding active HIV replication as a

mechanism of inflammaging. Increases in CMV-specific $CD8^+$ T cells, increases in immune activating cytokines or CRP, were all found to be consistently greater in HIV-infected individuals, regardless of specific ART regimen or duration of viral suppression[142]. Despite this, there is considerable debate over the role of ART in accelerated aging of HIV-infected individuals. Many antiretroviral drugs, particularly the older reverse transcriptase inhibitors, exhibit *in vitro* telomerase inhibition and have themselves been suggested as contributors to immune senescence[143]. LTL in HIV+ individuals does not change in relation to ART regimen, as reported in a study by Solomon et al, who longitudinally studied patients on various ART-regimens to address suggestions of ART-inhibition of telomerase[144,145]. Multiple studies of HIV-infected and uninfected adults consistently show that telomerase activity is virtually undetectable in $CD8^+$ T cells, therefore, ART should have little or no effect on telomere shortening, thereby implicating clonal expansion and homeostatic proliferation as the main cause of telomere erosion both in adults living with HIV and the general population[144]. While ART-toxicity and telomerase inhibition is potentially a contributor to telomere attrition *in vitro*, it is highly unlikely to induce senescence in immune cells *in vivo*. Accelerated telomere length decline, more-so than inhibition of telomerase activity, places the focus on drivers of expansion and exhaustive proliferation, such as CMV infection, as causes of senescence within the $CD8^+$ T cell population during HIV infection.

1.6 Exploring Aspects of Senescence in a HIV/CMV-co-infected Population

The protective role of the adaptive immune system in relation to aging processes is assumed to be in aiding senescent cell clearance, therefore, this regulatory role could be

compromised during HIV infection. The observed deficits in adaptive immunity, during HIV and CMV infection, and accompanying increased prevalence of age-associated pathologies, provide a unique setting within which to study the progress and impact of senescent cell accumulation. Decreased immune surveillance may allow cells to not only become chronically senescent, but also persist at high levels within tissues. This increases the likelihood of SASP-induced paracrine senescence of nearby cells. Peripheral $CD8^+$ T cells homing to this inflamed and highly stimulatory tissue could further fuel this feedback loop of activation, expansion and telomere attrition. Therefore, not only are immune cells indirectly contributing to senescent cell accumulation, but also could themselves reach senescence through this process, propagating a deleterious cycle that promotes "premature aging" in HIV infection.

We speculate that clonal expansion of CMV-specific $CD8^+$ T cells during HIV infection results in exhaustive proliferation and places this select T cell subset most proximal to replicative senescence. In line with this, we predict that this subset will have shorter telomeres than the general lymphocyte population, than other $CD8^+$ T cells, and other non-CMV-specific terminally differentiated $CD57^+$ $CD8^+$ T cells.

To test this, we will measure and compare telomere lengths of $CD8^+$ T cell subsets within the same individual to control for age and the highly inheritable genetic influence on telomere length. To assess whether replicative senescence is selectively approached by CMV-specific $CD8^+$ T cells, we will measure and compare the average telomere length of HIV-specific $CD8^+$ T cells in the same individual.

We will employ a combination technique, flow-FISH, comprised of fluorescence in situ hybridization (FISH) and flow cytometry to simultaneously quantify telomere lengths and identify antigen–specific $CD8^+$ T cells. The FISH procedure requires a high temperature and formamide treatments, therefore, we will develop a three-colour temperature-resistant fluorochrome-conjugated antibody panel to identify IFN-γ producing $CD57^+CD8^+$ T cells. The addition of a fourth colour, representing the fluorochrome-conjugated oligomer quantifying telomere repeat length, will complete the panel and allow us to properly discriminate subsets based on extracellular and intracellular proteins, as well as telomeric DNA content.

To identify any association between CMV-specific $CD8^+$ T cell telomere length and systemic inflammation, a suspected feature of immune senescence, we will quantify plasma levels of cytokines, chemokines and acute phase proteins. Plasma concentrations of IL-1β, IL-6, TNF-α, fractalkine (CX3CL1), and CRP will be compared between individuals distinguished by CMV serostatus. We will also assess biological age as a variable to determine if chronological aging outweighs the effects of CMV-related clonal expansion in HIV-infected individuals.

If CMV infection is a major factor in shifting the adaptive immune system towards chronic senescence, the most dramatic effects should be revealed in the context of HIV infection, where increased chronic inflammation and exaggerated anti-CMV immune responses may optimally support progressive age-independent chronic immune senescence.

2 Methods

2.1 Ethics

All subjects provided informed consent for whole blood collection, immunological studies and (when available) researcher access to medical laboratory records. The Newfoundland and Labrador Health Research Ethics Authority approved this study.

2.2 Study Subjects

Study subjects infected with HIV were recruited through the Newfoundland and Labrador Provincial HIV Clinic. HIV-infected subjects were screened for HIV-1 antibodies via ELISA and the presence of anti-HIV antibodies was confirmed by western blot. Routine clinical assessments; with lymphocyte subset analysis, including $CD4^+$ and $CD8^+$ T cell counts, and viral load were performed at least once every six months.

2.3 Peripheral blood mononuclear cell (PBMC) isolation

Whole blood (20 mL) was collected by forearm venipuncture into vacutainer tubes containing acid-citrate-dextrose (ACD) anticoagulant. Plasma was collected following centrifugation at 400*g* (10 min, room temperature (RT), aliquoted immediately and stored at -80°C**.** Packed cells and buffy coat were diluted to two times the original blood volume with phosphate-buffered saline (PBS), then layered over Ficoll-Hypaque density gradient separation medium (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and centrifuged at 400*g* (30 min, RT) *with the brake off*. Interface PBMC were collected, washed once in PBS with 1% fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA) and

resuspended in lymphocyte medium comprised of RPMI 1640 with 10% FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer solution and 2 x 10^{-5} M 2-mercaptoethanol (all from Invitrogen).

2.4 PBMC Cryopreservation/Thawing

Freshly isolated PBMC were resuspended in freezing medium, comprised of lymphocyte medium supplemented to 20% FCS, with 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MI, USA), at a minimum of 1 x 10^7 cells/mL and cooled at 1°C/min overnight reaching -80°C. Frozen PBMC were transferred to and maintained in liquid nitrogen until required. To thaw cells, cryopreserved PBMC were immediately immersed in 37°C water bath, gently agitated until almost thawed, then immediately transferred into, and washed 3 times in, 10 mL lymphocyte medium. Cells were resuspended in lymphocyte medium at 2 x 10^6 cells/mL and allowed to recover overnight at 37°C, 5% CO_2. Cells were counted after recovery and all PBMC used were >70% viable by trypan blue exclusion.

2.5 Peptide Stimulation of PBMC to Identify Antigen Specific $CD8^+$ T cells

Aliquots of 2 x 10^6 PBMC in 1 mL lymphocyte medium were stimulated with overlapping CMV-pp65 (0.5 μg/mL) and immediate early-1 (IE-1) peptides (0.5 μg/mL Miltenyi Biotec, San Diego, CA, USA), or overlapping HIV-1 Nef (1.0 μg/mL) and Gag (1.0 μg/mL) peptides (NIH AIDS Reagent Program, Germantown, ML, USA), for 60 minutes at 37°C (5% CO_2). Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 10.0 μg/mL to inhibit cytokine secretion and PBMC were

incubated at 37°C 5% CO_2 for an additional 4 hours after which they were immediately processed. The stimulated PBMC were first washed and stained for surface markers, using fluorescein isothiocyanate (FITC)-conjugated anti-human CD57 (TB03**,** Miltenyi-Biotec) and Quantum Dot (QD) 705-conjugated anti-human CD8 (3B5; Invitrogen). Samples were kept in the dark from this point on. Intracellular staining of PBMC was done using allophycocyanin-conjugated anti-human IFN-γ (4S.83, eBioscience, San Diego, CA, USA), after fixation and permeabilization using InsideStain (Miltenyi Biotec), according to manufacturer's instructions. Washes between steps were with flow buffer (PBS supplemented with 5 mM ethylendiaminetetraacetic acid (EDTA, Sigma-Aldrich), 0.5% FCS, 0.2% sodium azide, pH 7.2). Cells were resuspended in 1% paraformaldehyde (PFA) and were analyzed after acquiring a minimum of 1 x 10^5 events per sample within 48 hours, using a FACSCalibur Cell Analyzer (BD Biosciences, Mississauga, ON, CA). Isotype and fluorescence minus one (FMO) controls were used, when required, during optimization of multi-parametric panels. Unstained and unstimulated controls were used to account for background and endogenous activation, respectively.

2.6 FlowFISH Telomere Length Quantification of Antigen-specific $CD8^+$ T cells

2.6.1 Overview

The flow-FISH protocol is a technique that combines the high throughput and multi-parametric versatility of flow cytometry with cytogenetic fluorescence *in situ* hybridization techniques. The FISH technique is used in this instance to identify and

quantitate telomeric DNA repeats. Use of a fluorochrome-conjugated complement peptidyl-nucleic acid (PNA, $(CCCTAA)_3$) enables assessment of telomere repeat frequency, and thus length by flow cytometry. This FISH procedure uses PNA over oligonucleotides, for its high binding affinity in smaller sized oligomers, which results in less mismatch binding. The observed mean fluorescence intensity corresponds to the amount of fluorochrome-conjugated PNA bound during the hybridization process. Telomere length is first calculated relative to a control cell line (1301 T cell leukemia, Sigma-Aldrich) with extremely long telomeres (23,480 base pairs), that acts as an internal standard. From this relative value, the known telomere length of the 1301 cell line allows approximate telomeric base-pair calculation of sample(s) of interest. Building off this procedure, I developed and optimized a multiplex protocol to identify and phenotype various T cell subsets of interest and compare their average telomere length(s).

We first attempted to demonstrate efficacy of the flow-FISH assay by using a commercial (Dako) FITC-conjugated PNA-probe (already in hybridization solution) to quantify telomere length (TL) of whole peripheral blood mononuclear cells (PBMC) (*data not shown*). However, as illustrated by Akbar et al. (2015), the use of a commercial kit limits the optimization of fluorescent multiplexing[87]. Therefore, we quantified TL using an in-house Cy3-conjugated PNA system (Panagene, South Korea) with Cy3-probe preparations and hybridization solutions adapted from Akbar et al (Figure 2.1).

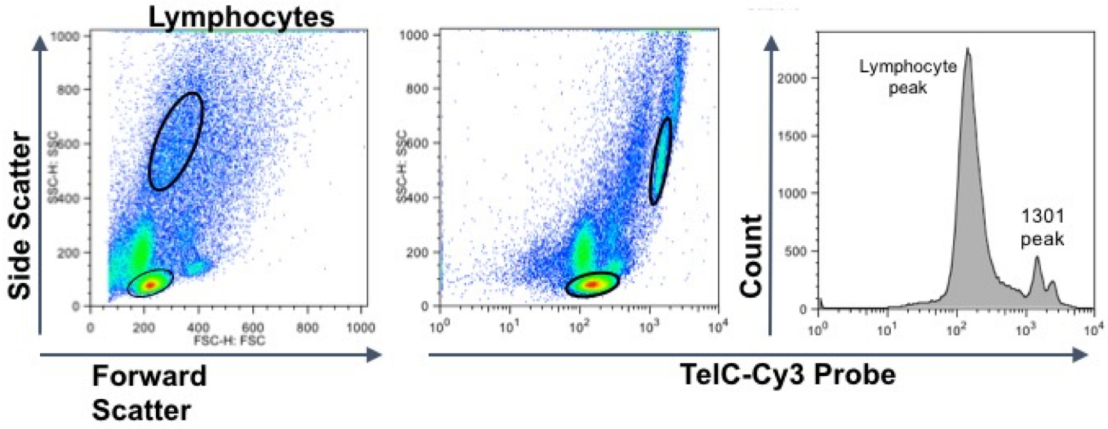

Figure 2.1. Flow cytometry events and Cy3-TelC MFI of gated subgroups.

Identification of an internal long telomere positive control, the 1301 T leukemia cell line, using FSC vs SSC event gating or solely Cy3-TelC PNA probe fluorescence. (FSC, forward scatter; SSC, side scatter; TelC-Cy3, Telomere C-rich peptide-nucleic acid cyanine-3 conjugated probe.

Given the high temperature of DNA denaturation, fluorochromes and antigen targets intended to be incorporated in the flowFISH assay must be either be high temperature resistant or have the antigen-antibody interaction stabilized via bissulfosuccinimidyl suberate (BS_3)-crosslinking. This process creates N-hydroxysulfosuccinamide (NHS) amide bonds with primary amines of antibody lysine residues, increasing stability of antigen-antibody-conjugate complexes to high temperatures.

We developed our flowFISH assay using FITC-conjugated antibodies, previously shown to have high temperature resistance, QDot-, and APC-conjugated antibodies and incorporated the crosslinking procedure as an essential step to maximize signal persistence. We first individually tested each fluorochrome (Figure 2.2) to validate preservation of fluorescent signals and to identify any spectral overlap or compensation issues. No issue developed that could not be solved by optimized compensations. We then used all fluorescent-conjugates together to establish optimal compensation and develop an efficient gating strategy (Figure 2.3).

Samples selected for flowFISH assays were stained, post-stimulation, as per section 3.1. and re-suspended in 1% FCS-PBS, rather than 1% PFA, after intracellular staining.

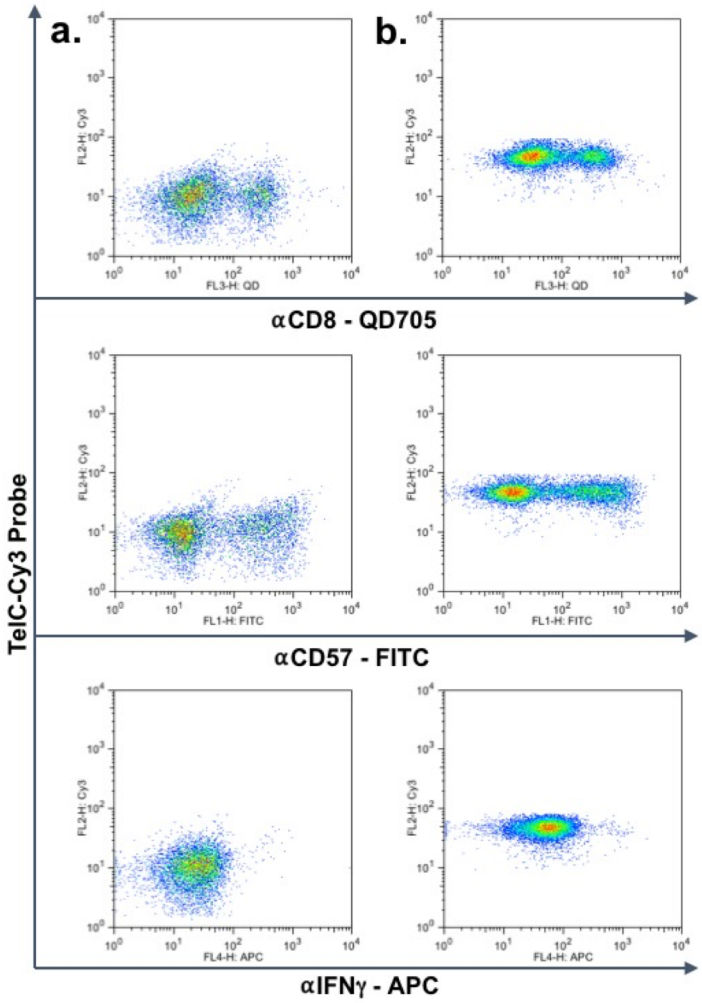

Figure 2.2. Flow cytometry events of gated subgroups showing preservation of antibody-conjugated fluorochromes during the flowFISH process.

(a) Identification of a positive fluorescent signal, post-hybridization. **(b)** Addition of the Cy3 probe to flowFISH hybridization indicates no large spectral overlap or compensation issues that would inhibit clear signal of either fluorochromes or Cy3-Tel C probe. TelC-Cy3, Telomere C-rich peptide-nucleic acid cyanine-3 conjugated probe; QD705, Quantum Dot 705; FITC, fluorescein isothiocyanate; APC, allophycocyanin, IFN- interferon.

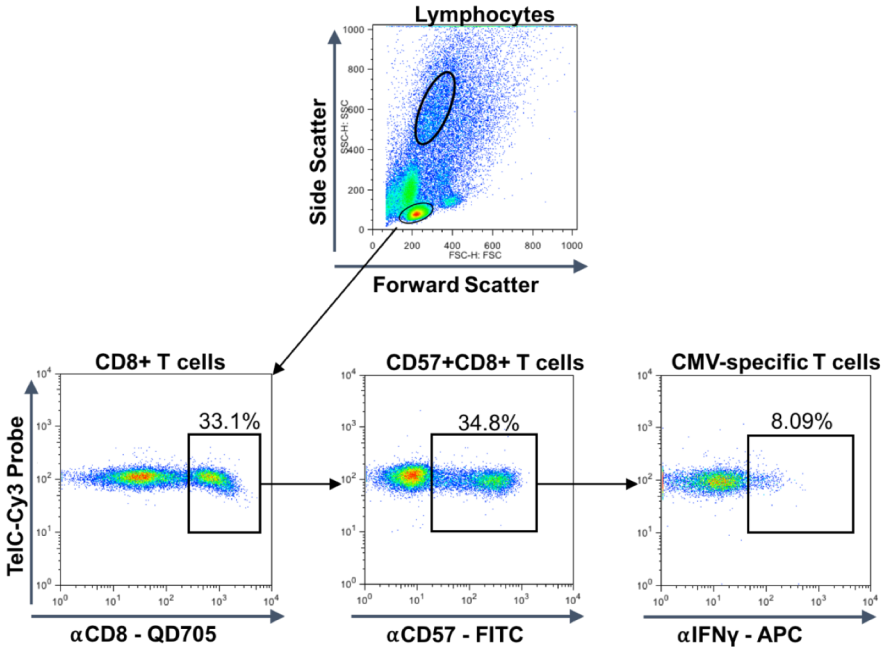

Figure 2.3. Gating strategy of representative flow cytometry events to determine telomere length of CMV-specific $CD57^{+}CD8^{+}$ T cell subsets.

PBMC samples were stimulated with overlapping peptides and subjected to flowFISH assay to quantify telomere length of different lymphocyte subsets. TelC-Cy3, Telomere C-rich peptide-nucleic acid cyanine-3 conjugated probe; QD705, Quantum Dot 705; FITC, fluorescein isothiocyanate; APC, allophycocyanin, IFN- interferon, CMV, cytomegalovirus.

2.6.2 Crosslinking

To increase stability of antigen-antibody-conjugate complexes, 200 μL of a 7.5 mM BS_3 (Thermofisher Scientific, Massachusetts, USA)-PBS crosslinking solution was added to PBMC to a final concentration of 5 mM. PBMC were incubated in cross-linker on ice for 30 minutes. Residual BS3 was quenched with 3 mL quenching solution (100 mM Tris-HCl, 50 mM NaCl in PBS). Samples were left to incubate on ice for a further 20 min. At this point, 1301 cells were added at a ratio of 1:4 to sample PBMC (approximately 0.5 x 10^6 cells). Samples were then washed in flow buffer, split into two aliquots of approximately 1.25 x 10^6 cells and added to clean 1.5 mL microcentrifuge tubes.

2.6.3 Hybridization

Samples were pelleted and all but 100 μL of the supernatant was removed via pipette to ensure pellet stability. To ensure maximal accuracy of signals and sample concentrations, 250 μL of pre-hybridization solution (70% formamide, 30 mM Tris HCl, 0.2 M NaCl, 1.5 % bovine serum albumin (BSA)) was added. Samples were then resuspended via wide bore 1000 μL pipetting and left to incubate for 10 min at RT. All subsequent resuspensions were done in this manner to avoid unnecessary shear force on fragile samples. Samples were then centrifuged at 1600*g* to ensure optimal pellet formation in formamide without compromising cellular integrity. All but 100 μL of supernatant was removed via pipette. Samples were then resuspended in 250 μl hybridization solution (70% formamide, 30 mM Tris HCl, 0.2 M NaCl, 1.5 % BSA) with

or without the addition of 0.75 μg/ml TelC-Cy3 (Panagene). An unprobed sample (FMO) to allow for proper correction for formamide-related auto-fluorescence that may artificially increase measured Cy3 fluorescence, was run with every experiment. All aqueous reagents were verified pH 7.2, followed by sterile filtration through a 0.45 μm nylon filter prior to formamide addition.

Samples were then incubated at 84°C for 10 minutes and immediately placed on ice for 5 minutes. Samples were left to hybridize in a dark chamber for 2 hours at RT. Samples were then diluted 3:1 with a post-hybridization solution (70% formamide, 15 mM Tris HCl, 0.2 M NaCl, 0.15% BSA, 0.15% Tween-20) and centrifuged at 1600*g*. Samples were pelleted and all but 100 μl of supernatant was removed via pipette. Samples were then washed twice with another wash solution (1.0% BSA, 0.5 mM EDTA, in PBS), and centrifuged twice, first at 900*g* and then at 500*g*, to account for varying concentrations of formamide. These washing steps ensure maximal removal of formamide that may damage the fluidic properties of the flow cytometer or cross-react with sanitizing bleach. Samples are re-suspended in the same wash solution and then analyzed immediately with a FACS Calibur Cell Analyzer (BD Biosciences). A minimum of 1 x 10^5 events were acquired per sample.

2.3.4 Calculation of Relative and Exact Telomere Length

Using the Cy3 MFI of the 1301 control cell line and sample(s), the relative telomere length of the subset is calculated and using the known 1301 telomere length, the exact telomere length (bp) of the subset is calculated.

$$Relative\ Telomere\ Length\ (RTL) = \frac{\left(\left(MFI_{Cy3}(Sample_{probe}) - MFI_{Cy3}(Sample_{unprobed})\right) x\ DNA\ Index\ of\ 1301\right)}{\left(\left(MFI_{Cy3}(1301_{probe}) - MFI_{Cy3}(1301_{unprobed})\right) x\ DNA\ Index\ of\ Sample\right)} = \frac{SampleTL_{MFI}}{1301TL_{MFI}}$$

$$Exact\ Telomere\ Length\ (TL) = \frac{SampleTL_{MFI}}{1301TL_{MFI}}\ x\ 1301TL_{bp}$$

2.7 Enzyme-linked Immunosorbent Assays (ELISA) for Serum Protein Quantification

Fresh plasma was aliquoted to smaller portions to avoid repeated freeze/thaw cycles. Previous exposure to CMV was confirmed by an in-house CMV lysate-ELISA described in Heath et al.[132] IL-1β (eBioscience, range = 2.00 – 200.00 pg/ml), IL-6 (eBioscience, 2.00 – 200.00 pg/ml), TNF-α (eBioscience, 4.00 – 500.00 pg/ml), fractalkine (R&D, Minneapolis, MN, USA, 0.63 – 20.00 ng/mL), C-reactive protein (R&D, 15.60 – 1,000.00 pg/mL) were quantified according to manufacturer's protocol. All ELISA sensitivity ranges covered physiologically appropriate levels such that plasma was added neat to the assay, except for CRP where plasma was diluted 1:10,000 with PBS. All samples were measured in duplicate, and each ELISA contained a control well with only sample diluent added. This value was then subtracted from all values to adjust for background. Absorbance was measured at 450 nm on a Biotek synergy HT ELISA reader (Gen5).

2.8 Statistical Analysis

Statistical analyses were carried out using Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). Normal distribution of data was assessed by the Shapiro–Wilk

test. If any test indicated deviation from normal distribution (all TL results, IL-1β, IL-6, TNF-α, fractalkine), data were represented with median ± interquartile range (IQR) and group medians compared by Mann–Whitney test or Wilcoxon signed rank test. Spearman correlation was used to assess relationships between variables. If data were normally distributed (age, CRP, CMV CD8 T cell response), mean ± SD was calculated and Student's *t*-test was used to compare means. Relationships were assessed using Pearson correlation matrices.

3 Results

3.1 Study Groups and Clinical Characteristics

To assess the prevalence of chronic inflammation in association with CMV infection, we measured IL-1β, IL-6, TNF-α, fractalkine and CRP in 116 HIV-infected individuals, 19 seronegative for HCMV and the remaining 97 HCMV-seropositive. Subjects selected for these analyses were on antiretroviral therapy (ART), possessed pro-calcitonin levels below 0.1 ng/ml (results not shown) and were of similar age range in each group. Responses to overlapping CMV peptides were significantly different between groups, complementing previously assayed CMV-seropositivity[126]. No significant differences were observed in duration of ART or $CD4^+$ nadir. These comparisons and other relevant demographic information, such as: age; $CD4^+/CD8^+$ differential counts; β2 microglobulin; are presented in Table 3.1. Subjects with detectable antibodies against hepatitis C virus (HCV) or who had undergone any type of chemotherapy were not included in the study.

3.2 Measurement of inflammatory cytokines, chemokines and acute phase protein C-reactive protein in plasma

Of the plasma components studied, median levels (+ IQR) of inflammatory cytokines that were significantly higher in the group co-infected with CMV in comparison to the CMV-uninfected group were IL-1β (6.04 [2.00-14.98] pg/mL vs 2.00 [2.00-7.15] pg/mL, p = 0.044), IL-6 (4.12 [2.00-12.06] pg/mL vs 2.22 [2.00-2.45] pg/mL, p = 0.0037) and TNF-α (11.39 [4.00-57.84] pg/mL vs 4.00 [4.00-9.89] pg/mL, p = 0.0185). The acute phase protein, CRP was also elevated in the group co-infected with

CMV (3.436[1.763-6.830] μg/mL vs 1.659 [0.659-4.193] μg/mL, p = 0.0385). There was no significant difference in levels of the chemokine fractalkine (CX3CL1) between groups. These data, outlined in Table 3.2 and Figure 3.1, demonstrate that multiple markers of systemic inflammation are significantly higher in the group of HIV-infected individuals co-infected with CMV. While there is considerable overlap in levels of these markers between groups, median levels are significantly higher with CMV co-infection.

To determine if HIV replication had any impact on the levels of these markers of inflammation, results for 25 individuals with detectable HIV viral load (log_{10} HIV RNA copies/mL plasma >1.3) were removed and data were then reanalyzed (Table 3.2 and Figure 3.1b). With this adjustment, upon comparison of the HIV-infected group co-infected with CMV to the uninfected group, the difference in median levels of IL-1β became insignificant, while differences in median levels of IL-6 (5.63 [2.00-13.74] pg/mL vs 2.23 [2.00-3.51] pg/mL, p = 0.0074), TNF-α (14.41[4.00-62.29] pg/mL vs 4.00 [4.00-19.83] pg/mL, p = 0.0456) and CRP (3.936 [1.862-7.210] μg/mL vs 1.932 [0.841-4.105] μg/mL, p = 0.0205) remained significant. Interestingly, despite the HIV-infected group co-infected with CMV having high plasma concentrations of inflammatory biomarkers, these levels did not correlate with frequency of CMV-specific $CD8^+$ T cells (data not shown). This indicates that the increased levels of IL-6, TNF-α, and CRP observed with CMV co-infection in HIV-infected subjects are independent of any effect due to HIV replication.

Table 3.1 General characteristics of CMV-seropositive and seronegative HIV-infected individuals.

General Characteristics	CMV-		CMV+		p	
n (% undetectable [#])	19	(84%)	97	(77%)	0.5459	ns
Age (years), Median (IQR)	44	(42-50)	48	(44-54)	0.1165	ns
B2M (μg/mL), Median (IQR)	2.63	(2.06-3.24)	2.64	(2.08-3.33)	0.9082	ns
% CMV Specific $CD8^+$ T cells, Mean (+/- SD)	0.01	0.03	3.94	4.15	<0.0001	***
$CD4^+$ T cell Count (cells/μL), Median (IQR)	742	(522-780)	648	(419-777)	0.7689	ns
$CD8^+$ T cell Count (cell/μL), Median (IQR)	648	(442-770)	869	(643-1217)	0.0093	**
$CD4^+$:$CD8^+$ T cell Ratio, Median (IQR)	1.05	(0.86-1.64)	0.68	(0.44-0.92)	0.1273	ns
Duration of ART (years), median (IQR)	13	(8-19)	15	(10-19)	0.3753	ns
Nadir CD4+ T cells/μL blood, median (IQR)	190	(89-325)	234	(121-404)	0.3655	ns

Patients chosen for experiments involving inflammatory biomarker and telomere length quantification. [#] Undetectable < 1.3 $\log_{10}$ HIV RNA copies/mL plasma. Two-tailed Student's unpaired t test. B2M, beta-2-microglobulin; CMV, cytomegalovirus.

Table 3.2 Inflammatory Biomarker Comparison

Inflammatory Biomarker	CMV-		CMV+		p	
	Median	IQR	Median	IQR		
IL-1β (pg/mL)	2	(2.00-7.15)	6.04	(2.00-14.98)	0.044	*
IL-6 (pg/mL)	2.22	(2.00-2.45)	4.12	(2.00-12.06)	0.0037	**
TNF-α (pg/mL)	4	(4.00-9.89)	11.39	(4.00-57.84)	0.0185	*
Fractalkine (CX3CL1) (ng/mL)	0.74	(0.533-0.970)	0.881	(0.590-1.187)	0.1781	ns
C-reactive Protein (CRP) (μg/mL)	1.659	(0.645-4.193)	3.436	(1.763-6.830)	0.0385	*
IL- 1β* (pg/mL)	2.14	(2.00-7.62)	5.64	(2.00-14.09)	0.1795	ns
IL- 6 * (pg/mL)	2.23	(2.00-3.51)	5.63	(2.00-13.74)	0.0074	**
TNF-α* (pg/mL)	4	(4.00-19.83)	14.41	(4.00-62.29)	0.0456	*
Fractalkine* (CX3CL1) (ng/mL)	0.789	(0.568-0.972)	0.919	(0.575-1.210)	0.2131	ns
C-reactive Protein (CRP)* (μg/mL)	1.932	(0.841-4.105)	3.936	(1.862-7.210)	0.0205	*

Inflammatory biomarker comparison between HIV-infected CMV-seropositive (n=97) and HIV-infected CMV-seronegative (n=19) study groups. Values were determined by ELISA, as per manufacturer's instructions (see Methods) and medians compared by two-tailed Mann-Whitney U Test. IL, interleukin; TNF, tumor necrosis factor; CMV, cytomegalovirus. * Only individuals with undetectable HIV viral load were considered (CMV-, n = 16; CMV+, n = 75).

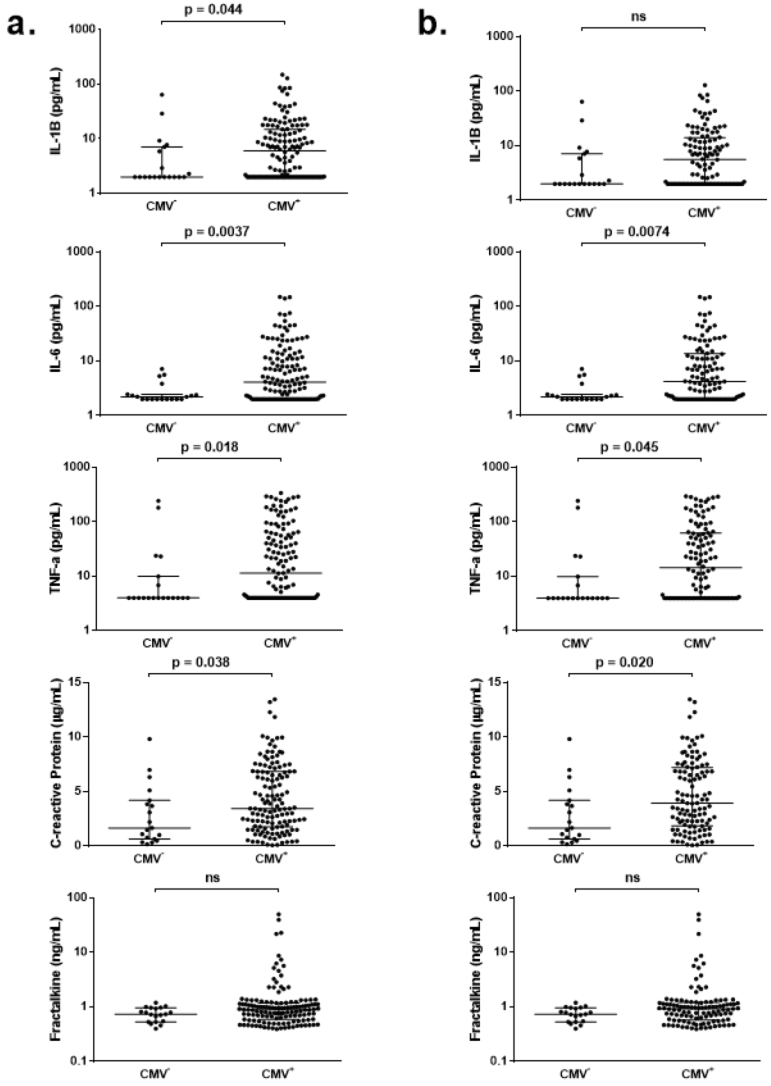

Figure 3.1. Inflammatory Biomarker Comparison of HIV$^+$CMV-seropositive and HIV$^+$CMV-seronegative individuals.

(a) Represents all individuals chosen for assessment (CMV-, n = 19; CMV+, n = 97). **(b)** Comparisons and analyses were performed again, with all individuals with a detectable HIV viral load excluded (> 1.3 log copies/µL) (CMV-, n = 16; CMV+, n = 75). Values were determined by ELISA as per manufacturer's instructions (see Methods 2.7). Median and interquartile range are represented with horizontal bars and medians compared by two-tailed Mann-Whitney U Test. IL, interleukin; TNF, tumor necrosis factor; CMV, cytomegalovirus.

3.3 Telomere Length (TL) of T Lymphocyte Subsets

Applying the gating strategy shown in **Figure 2.3** allowed us to acquire TL information from 6 defined subsets of lymphocytes from 32 CMV co-infected individuals infected with HIV. These sequentially more discrete subsets include global lymphocytes, $CD8^+$ T lymphocytes, $CD57^+$ and $CD57^-$ $CD8^+$ T lymphocytes. Within the $CD8^+$ and $CD57^+$ $CD8^+$ T lymphocytes CMV- and HIV-specific T lymphocytes were identified by IFN-γ production following PBMC stimulation with CMV or HIV peptides. Absolute TL values were obtained as outlined in the methods using the acquired Cy3 MFI and known TL of the 1301 control cell line. As this assay is not yet optimized for high-throughput format, 34 individuals tested is assumed representative. Paired comparisons of subset TLs, showed that the median lymphocyte telomere length (3133 bp, LTL) was the longest with a significant difference from that of $CD8^+$ T lymphocytes ($p < 0.0001$, **Figure 3.2a**). The median telomere length of $CD8^+$ T lymphocytes (2782 bp) and $CD57^-CD8^+$ T lymphocytes (3088 bp) was significantly greater than that of the $CD57^+CD8^+$ T lymphocytes (2594 bp, $p < 0.0001$, 0.0001, respectively, **Figure 3.3a, 3.2b**), corroborating a wealth of literature indicating CD57 expression is a marker of long term, extensive T-cell replication. The main aim of the assay was to determine the TL of the CMV-specific $CD57^+CD8^+$ T lymphocytes, and compare it to LTL, $CD8^+$ TL, and the reciprocal $CD57^+$ $CD8^+$ T cell population. In comparison to these identified subsets, the CMV-specific $CD57^+CD8^+$ T cell compartment showed the lowest median TL (2122 bp, $p < 0.0001$, 0.0001, 0.0001, respectively, **Figure 3.2c, 3.3b, c**). This demonstrates that CMV-specific

T lymphocytes residing in the highly proliferated $CD57^+$ $CD8^+$ T cell population are the $CD8^+$ T lymphocyte subset most proximal to telomere-dependent replicative senescence.

The genetic predisposition, age and environmental exposures of each HIV-infected individual with CMV co-infection contribute to the heterogeneity of telomere lengths within the T lymphocyte subsets presented in figures 3.2 and 3.3. Therefore, in figure 3.4 the ratio(s) of $CD8^+$ (0.9364 bp/bp), $CD57^+CD8^+$ (0.8938 bp/bp), and CMV-specific T lymphocytes (0.7944 bp/bp) to global lymphocyte TL are presented for each individual tested. These ratios demonstrate the increased telomere erosion exhibited within these $CD8^+$ T subsets compared to global lymphocytes, thereby masking individual differences.

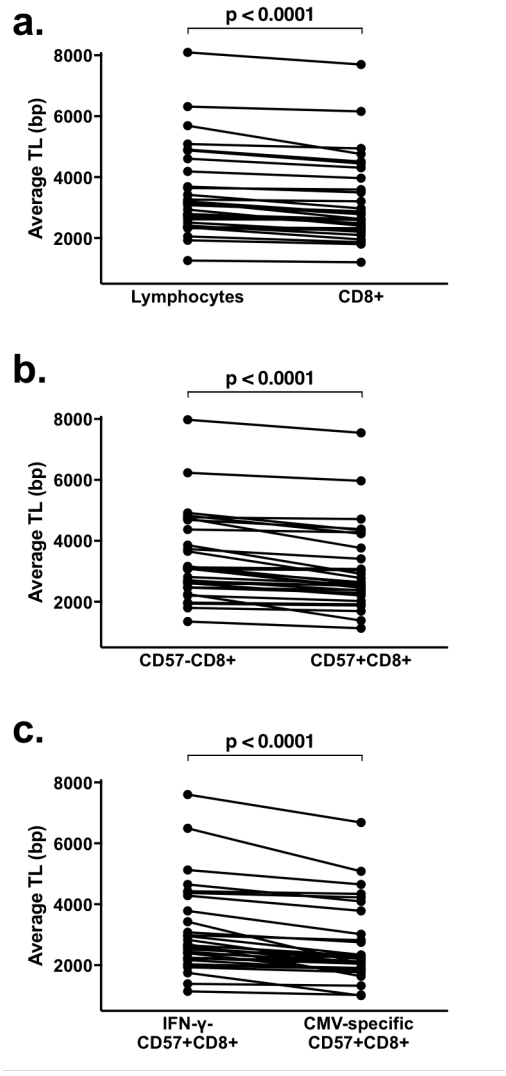

Figure 3.2. Telomere length of targeted lymphocyte populations

(a) $CD8^+$ T cells possess a shorter average telomere length than the general lymphocyte population. **(b)** $CD57^+CD8^+$ T cells possess a shorter average telomere length than the $CD57^-CD8^+$ T cell population. **(c)** $CD57^+CD8^+$ T cells not directed against CMV-peptides possess a longer median telomere length than those that produce IFN-γ when stimulated with CMV-peptides. All PBMC samples were stimulated with overlapping peptides and subjected to flowFISH assay to quantify telomere length (See Methods 2.6). Data is collected from 32 HIV-infected CMV-seropositive individuals and compared using Wilcoxon signed rank test. TL, telomere length; IFN, interferon; CMV, cytomegalovirus.

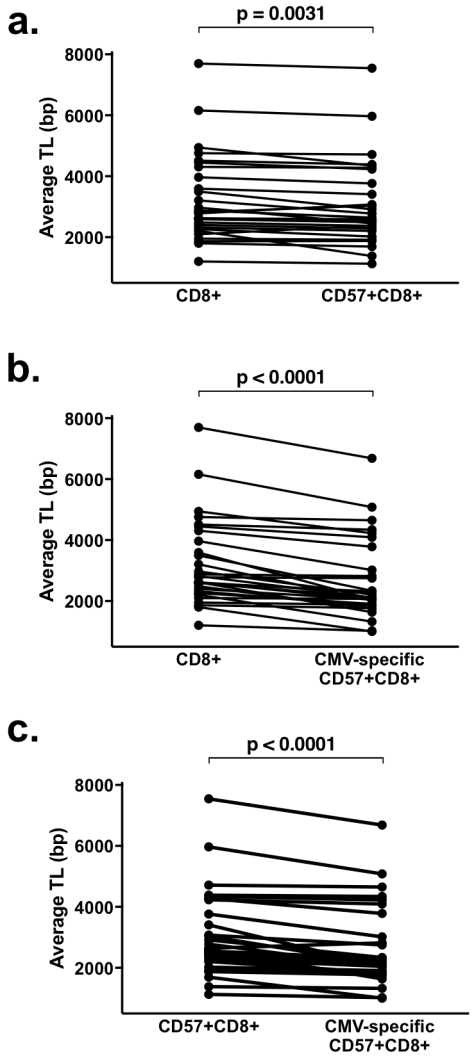

Figure 3.3. Telomere length of $CD8^+$ T cell subsets distinguished by functional markers

(a) $CD57^+CD8^+$ T cells, defined by the terminal differentiation marker CD57, possess a shorter average telomere length than the general $CD8^+$ population. **(b, c)** Both general $CD8^+$ and general $CD57^+CD8^+$ T cells possess a longer average telomere length than those that produce IFN-γ when stimulated with CMV-peptides, a population with a potentially senescent phenotype. All PBMC samples were stimulated with overlapping peptides and subjected to flowFISH assay to quantify telomere length (See Methods 2.6). Data is collected from 32 HIV-infected CMV-seropositive individuals and compared by Wilcoxon signed rank test. TL, telomere length; IFN, interferon; CMV, cytomegalovirus.

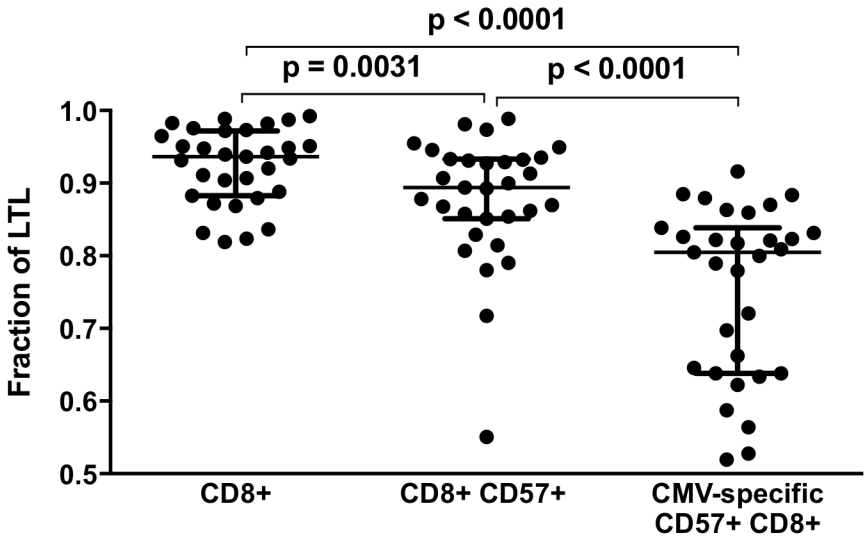

Figure 3.4. Average telomere length of selected subsets expressed as a fraction of total lymphocyte population average telomere length.

Median and interquartile range are represented with horizontal bars. Data were collected from 32 HIV-infected CMV-seropositive individuals. TL were determined by flowFISH (see Methods 2.7). (Although data is not graphically shown as paired tests, transformed data analyses were performed just as with the untransformed data using Wilcoxon signed rank test). TL, telomere length; LTL, lymphocyte telomere length; CMV, cytomegalovirus.

3.4 Correlation between CMV-specific $CD57^+CD8^+$ T cell TL and Frequency of CMV-specific $CD8^+$ Responses

It should be noted that absolute $CD8^+$ T cell counts are significantly higher in the HIV infected group co-infected with CMV (869 cells/μL vs 648 cells/μL, $p = 0.0093$, **Table 3.1**), a feature reflecting an underlying elevation of $CD8^+$ T cell clonal proliferation. To follow up the observation of highly eroded TL of clonally expanded, CMV-specific T lymphocytes, we investigated if there was an association between the degree of telomere erosion in CMV-specific $CD57^+CD8^+$ T cells and the magnitude of CMV-specific $CD8^+$ T cell responses in each individual. We chose to compare the ratios of CMV-specific $CD57^+$ $CD8^+$ T cell to LTL in order to minimize inter-individual age-related variations. **Figure 3.5** illustrates a co-ordinate increase in CMV-specific $CD8^+$ T cell-mediated responses and decreases in normalized CMV-specific $CD57^+$ $CD8^+$ T cell TL, however, this relationship was not statistically significant ($p = 0.0858$, $r = -0.3036$).

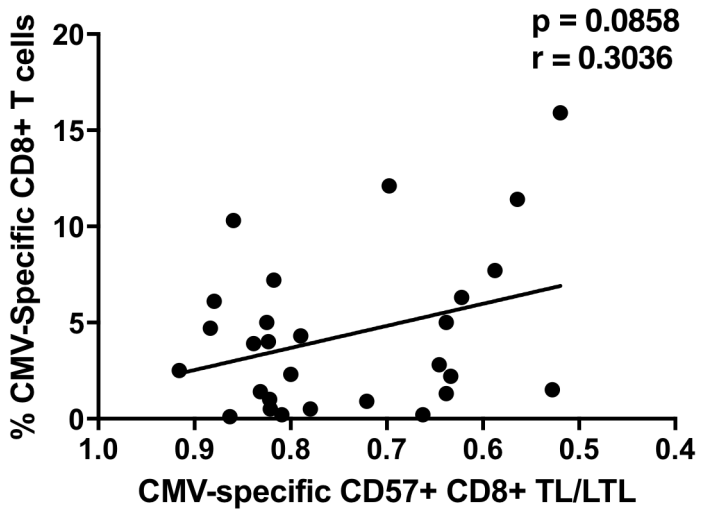

Figure 3.5. Median TL of CMV-specific $CD57^{+}CD8^{+}$ T cells versus magnitude of the anti-CMV response residing in this subset.

CMV-specific responses were determined as per Methods 2.6. TL was determined as per flowFISH in Methods 2.7. Data were collected from 32 HIV-infected CMV-seropositive individuals. Pearson Correlation with TL ratio(s). TL, telomere length; LTL, lymphocyte telomere length; CMV, cytomegalovirus.

3.5 Comparison of CMV-specific and HIV-specific $CD8^+$ T Cell TL

To test whether the dramatic TL erosion observed within the CMV antigen-specific $CD57^+CD8^+$ T cells was selective for the CMV response and not just an intrinsic quality of all antigen-expanded memory T cells, we compared the TL of another antigen-specific $CD8^+$ T cell population. We chose HIV-specific $CD8^+$ T cells for comparison as this population was large enough for similar analysis. However, it should be noted this comparison could only be made within $CD8^+$ T cells, since too few HIV-specific $CD8^+$ T cells expressed the terminal differentiation marker, CD57. **Figure 3.6** shows the differences in the ratios to overall LTL of HIV-specific $CD8^+$ and CMV-specific $CD8^+$ T cell TL for 6 HIV-infected individuals co-infected with CMV (0.9218 vs 0.8013, $p = 0.0461$). These data show that HIV-specific $CD8^+$ T cells possess similar TL to general $CD8^+$ T lymphocytes and overall lymphocytes and retain longer TL than CMV-specific $CD8^+$ T cells. Thus, the degree of telomere erosion present in the CMV-specific $CD8^+$ T cell compartment surpasses that seen in $CD8^+$ T cells specific for other chronic viral pathogens.

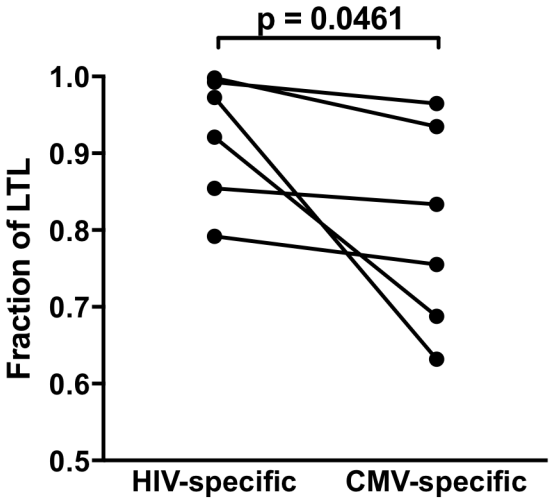

Figure 3.6. Comparison of average telomere length of HIV- and CMV-specific $CD8^+$ T cell subsets.

HIV-specific and CMV-specific telomere lengths were measured in 6 individuals with detectable anti-HIV responses using flowFISH (as per Methods 2.7). Wilcoxon signed rank test. TL, telomere length; LTL, lymphocyte telomere length; HIV, human immunodeficiency virus, CMV, cytomegalovirus.

3.6 Correlation between CMV-specific $CD57^+CD8^+$ T cell TL and Age

A general assumption of TL is that it decreases with biological age. In agreement with this assumption, we found a strong significant correlation between the TL of lymphocytes and biological age of the donor ($p = 0.0042$). How the TL of CMV-specific $CD57^+$ $CD8^+$ T lymphocytes TL varies with age has not yet been studied in the context of HIV infection. Data presented in **Figure 3.7**, show that CMV-specific $CD57^+CD8^+$ T cell TL does decline with age, however, only to a slightly lesser extent than total lymphocytes ($p = 0.0098$). In addition, when presented as a ratio of lymphocyte TL (**Figure 3.8**), there is no difference with respect to age. These data show that age has an impact on the decline in TL within the CMV-specific compartment, but that other factors extrinsic to chronology overwhelm the effect of aging.

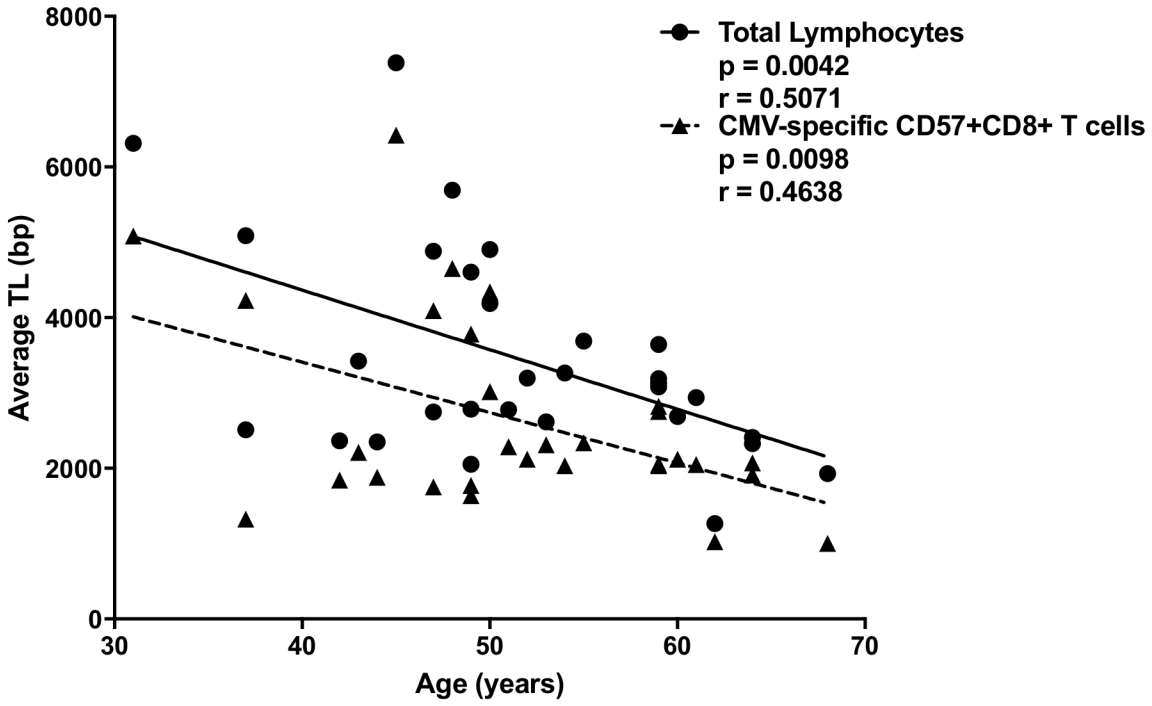

Figure 3.7. The average telomere length of general lymphocytes and the CMV-specific $CD57^{+}CD8^{+}$ T cell population are associated with biological age.

Data were collected from 32 HIV-infected CMV-seropositive individuals. TL are determined by flowFISH (see Methods 2.7). Pearson correlation with age. TL, telomere length; CMV, cytomegalovirus.

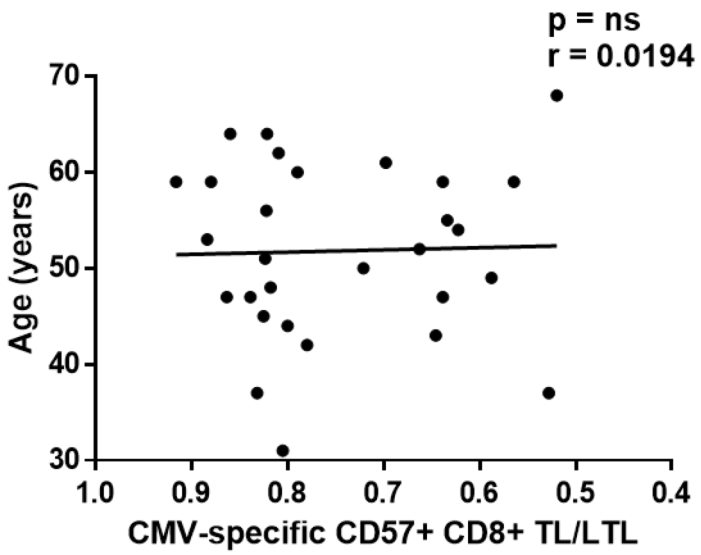

Figure 3.8. Ratio of telomere length of general lymphocytes and the CMV-specific $CD57^{+}CD8^{+}$ T cell population versus age.

Data were collected from 32 HIV^{+} CMV-seropositive individuals. TL are determined by flowFISH (see Methods 2.7). Pearson correlation with TL ratio(s). TL, telomere length; LTL, lymphocyte telomere length; CMV, cytomegalovirus.

3.7 Correlation between plasma markers of inflammation and CMV-specific $CD57^+$ $CD8^+$ T cell TL

To investigate a potential relationship between elevation of plasma markers of inflammation, and TL of $CD8^+$ T lymphocytes, particularly of CMV-specific $CD57^+$ $CD8^+$ T cells, we assessed correlation between levels of these parameters. The levels of IL-1β, IL-6, TNF-α, and fractalkine were not significantly correlated with any lymphocyte subset TL or frequency of CMV-specific $CD8^+$ T cells, however, CRP levels were significantly correlated with CMV-specific $CD57^+CD8^+$ TL ($p = 0.0122$, **Figure 3.9**). CRP also showed a significant direct correlation with age ($p = 0.0291$, **Figure 3.10**).

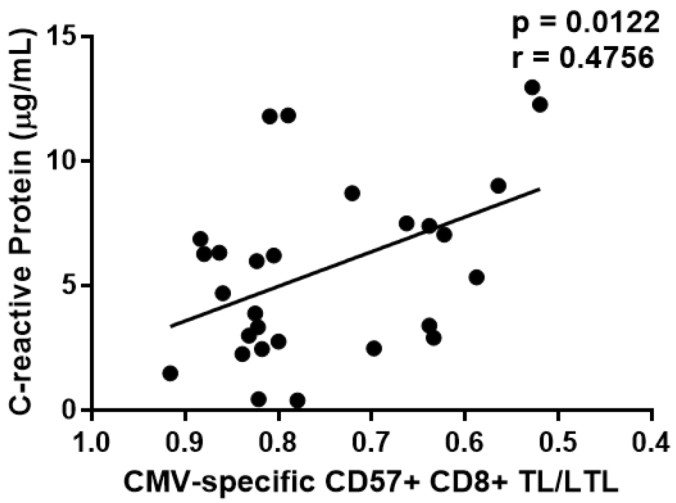

Figure 3.9. Relationship between $CD57^{+}CD8^{+}$ CMV-specific T cell: Lymphocyte telomere lengths and C-reactive protein levels in plasma.

Data were collected from 32 HIV^{+} CMV-seropositive individuals. CRP values are determined by ELISA as per manufacturer's instructions. TL are determined by flowFISH (see Methods 2.7). Pearson correlation with TL ratio(s). TL, telomere length; LTL, lymphocyte telomere length; CMV, cytomegalovirus.

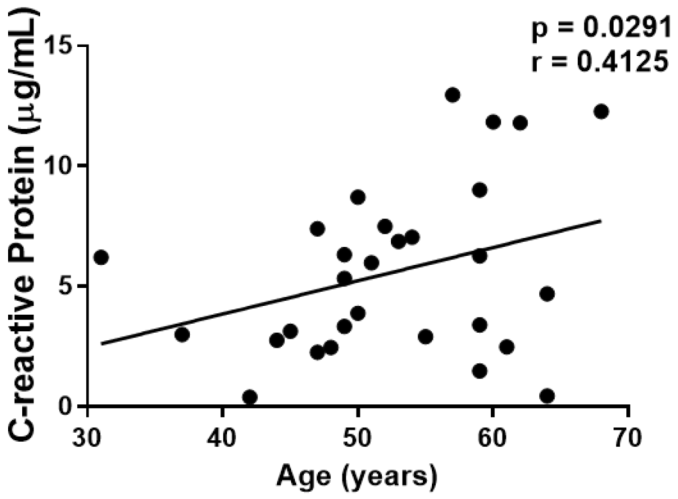

Figure 3.10. Relationship between biological age and C-reactive protein levels in plasma.

Data is collected from 32 HIV-infected CMV-seropositive individuals. CRP values were determined by ELISA as per manufacturer's instructions. Pearson correlation with age.

4 Discussion

In attempts to study mechanisms underlying physiological aging, researchers have uncovered that complex factors beyond just chronological age are involved. Like most homeostatic and regulatory cellular processes within the body, cellular senescence and its eventual culmination in organismal aging are intimately associated with the immune system. Short lived, acutely senescent cells arise during beneficial wound healing or vascular remodelling and are identified and cleared by immune cells such as T and NK cells. Conversely, long lived chronically senescent cells accumulate in high numbers in biologically aged tissue due to reduced immune clearance. These senescent cells acquire a pathological phenotype characterized by chronic production of inflammatory mediators, cell cycle arrest, and resistance to apoptosis. This chronic cellular senescence often develops through exhaustive division, during which eroding telomeres reach Hayflick's limit, triggering cell cycle arrest. Increased DNA damage repair pathway activation enforces the observed senescence, which often manifests a pro-inflammatory cellular phenotype in attempts to communicate internal cellular distress to nearby immune cells. While most somatic tissues exhibit homeostatic cellular turnover and repair, cells rarely reach this divisional limit and level of telomeric degradation before immune cell mediated clearance. However, situations where immune responses are insufficient or unavailable could lead to an increase in senescent cell accumulation.

It is well recognized that the elderly immune system provides less robust pathogen clearance, decreased vaccine responsiveness and reduced immunosurveillance in comparison to a young or middle aged individual. It is now speculated that these

observed clinical features are an illustration of the immune system itself undergoing a type of chronic senescence, a point at which age-related immune incompetence compounds the high tissue-resident senescent cell accumulation accompanying chronological aging. Much like cells of the peripheral tissue, loss of telomeric DNA in white blood cells has been proposed as a mechanism of immune senescence and was first associated with poor survival in elderly by Cawthon et al[60]. The highest observed causes of mortality were due to infectious disease and cardiovascular disease complications, with the lowest survival rates observed in individuals with the shortest telomere length. Cawthon et al. speculated that telomere length within peripheral blood subsets could indicate a progressive senescence that alters mortality rates, but such erosion occurred via an unknown mechanism and suggested a causal role of aging. Therefore, the aging immune system, once typically beneficial during youth in the prevention and amelioration of cellular and tissue aging, becomes -much like autoimmune disease- a pathogenic contributor to the physiological aging process. Senescent cell accumulation within tissue and downstream pathological sequelae such as increased inflammation and extracellular matrix destruction are, therefore, directly related to cellular immune senescence.

Despite the implications of this study, the operative mechanism and the specific subsets of immune cells directly involved in increasing mortality remain unidentified, although the immune cell senescence associated inflammatory immune phenotype (SASP) has recently been suggested as causal. However, the short half-life and high turnover of innate immune cells, such as neutrophils, suggest that long lived highly proliferated lymphocytes of the adaptive immune system are the leukocytes bearing

shorter telomere lengths. The adaptive immune system, including B and T lymphocytes, comprises a distributed organ that does not undergo simple cellular turnover and homeostasis. Strict selection of T lymphocytes within the thymus gives rise to a restricted set of TCR-defined clonal populations. These populations expand when cognate antigen is encountered, and only partially contract during antigen clearance to leave memory cells that have previously proliferated. TCR-restricted memory cells expand through additional rounds of proliferation during subsequent antigen exposures to replenish high numbers of specific pathogen-clearing effector T cells when needed. Each series of expansion and contraction sacrifices telomere length and presumably positions this specific clonal population closer to cellular senescence. Unique opportunities to study *in vivo* immune senescence can arise from the persistent antigenic stimulation that results from chronic infections.

CMV infection persists as a lifelong subclinical infection in healthy individuals, however, this typically generates a CMV-specific $CD8^+$ T cell population expanded to a unique degree. These large oligoclonal $CD8^+$ T cell expansions are restricted to several TCR epitopes and are predominantly of an effector memory phenotype. There is speculation that this extensive CMV-specific $CD8^+$ T cell population, which has undergone many rounds of proliferation, possesses many classical features of chronic senescence such as cell cycle arrest, heightened pro-inflammatory cytokine production, and resistance to apoptosis. Despite CMV infection remaining predominantly latent, at least from a clinical perspective, this CMV-specific $CD8^+$ T cell population grows with age, in parallel with the accumulation of senescent cells within aging tissue. Linking

aging with adaptive immunity, Pawelec et al. have shown that increases in B and T cell responses directed against CMV are strongly associated with "Immune Risk Profile" (IRP) parameters seen during aging[56]. Features of the IRP such as inversion of the $CD4^+/CD8^+$ T cell ratio, increased absolute numbers of $CD8^+$ T cells, poor T cell responses to mitogens, and interestingly, chronic inflammation, are common to the old elderly and to HIV-infected individuals co-infected with CMV. Thus, there is a theoretical and experimental framework positioning telomere attrition within expanded CMV-specific $CD8^+$ T cells, and the factors facilitating this expansion, as mechanisms contributing to chronic immune senescence, and the subsequent IRP, thereby accelerating the physiological aging process.

While a chronic manageable infection with the advent of HAART, HIV infection leaves individuals at heightened risk for early onset of age-associated diseases. Bestilny et al. cite a 15-year discrepancy (accelerated erosion) in leukocyte telomere length of HIV-infected individuals compared to an HIV-uninfected age-matched control group[146]. CMV-specific $CD8^+$ T cell populations typically become large in aging individuals, however, expansion of this population occurs earlier in life and is exaggerated in HIV-infected individuals. In the general population, CMV-specific $CD8^+$ T cell populations increase with age with immune cell telomere length decreasing, but these two features of immune cell replication have not yet been directly linked in HIV infection. We have previously shown that immune features seen in the old elderly (IRP), such as low $CD4^+/CD8^+$ T cell ratios and increased CD57 expression on $CD8^+$ T cells are exhibited to a greater extent in HIV-infected subjects co-infected with CMV compared to CMV-uninfected groups of

HIV-infected individuals.[127] To address the missing link between the uniquely altered immune cell composition seen exclusively in HIV-infected subjects living with CMV and the observed premature aging in the same group, we determined that the highly expanded CMV-specific $CD8^+$ T cells of HIV-infected individuals possess dramatically reduced telomeres in comparison to the larger $CD8^+$ T cell population, even in comparison to HIV-specific $CD8^+$ T cells. These are the first data positioning CMV co-infection as a driving factor of lymphocyte telomere erosion, equivalent to that occurring in elderly individuals, in HIV infection.

Previous studies addressing the role of telomere-dependent replicative senescence within CMV-specific $CD8^+$ T cells focused on traditional T cell effector function and phenotype.[81] In most cases, traditional multi-cytokine producing (IL-2, TNF-α, IFN-γ) CMV specific (using the public HLA-A2 restricted epitope NLVPMVATV) $CD8^+$ T cells did not possess the shortest telomeres. Furthermore, no association of enhanced cytokine producing $CD8^+$ T cells with telomere length was observed, contrasting the telomere-dependent SASP hypothesis. These studies involved *in vitro* selective expansion of CMV-specific $CD8^+$ T cells (expanded with IL-2 and NLV antigen, an HLA-A*02 restricted CMV tetramer), followed by re-stimulation with NLV peptide and assays for poly-functionality and telomere length via flowFISH. Conversely, we did not utilize IL-2 or MHC-tetramers to expand or identify CMV-specific populations. We aimed to limit the modes of activation involved in our procedures, as it is known that IL-2 (even via the low-affinity receptor, CD122) can trigger expression of telomerase. In order to identify the most representative of *in vivo* telomere lengths, we

chose to identify antigen-specific compartments strictly by IFN-γ production via overlapping peptide-stimulation ex vivo of primary PBMC. In addition, by focusing on the NLV epitope, the previously published data was restricted to HLA-A2 donors, while we were successful in measuring CMV-specific $CD8^+$ T cell telomere lengths of CMV-specific T cells specific to the public immunogenic regions of CMV proteins pp65 and IE-1 in individuals of diverse HLA types. Therefore, our method allows for a more accurate in vivo representation of telomere length dynamics within the CD8+ T cell subsets analyzed.

If and how CMV-specific T cell senescence contributes to the premature physiological aging exhibited in treated HIV infection remains unclear, however, several studies suggest that gradual increases of senescent, and often pro-inflammatory cells within tissue directly links aging to increased levels of chronic inflammation. Our data also show that coinfection with CMV is associated with higher plasma levels of inflammatory biomarkers in HIV infected individuals, such as TNF-α, IL-1β, IL-6, and CRP. Increased systemic CRP, a dominant indicator of risk for cardiovascular disease, is particularly prominent in individuals with shorter CMV-specific $CD8^+$ T cell telomere length. This correlation corroborates the link described by Pawelec et al., in the general population and emphasizes the connection between aging and CMV in the context of HIV infection, potentially linked by related repercussions apparent in the immune repertoire[147]. Persisting senescent cells within vasculature can often secrete IL-1β and IL-6 to inflict localized inflammation in a paracrine manner, but additionally can systemically trigger the acute phase response in an endocrine manner, resulting in CRP production.

Alternatively, CMV-infected cells within tissue can recruit highly cytotoxic CMV-specific $CD8^+$ T cells, which circulate in high numbers in the periphery. Secreted IFN-γ and TNF-α from recruited memory $CD8^+$ T cells can also contribute to systemic inflammation both directly and by acting on tissue resident innate immune cells, such as macrophages, to trigger inflammasome formation and reactive oxygen/nitrogen species secretion. Chronic increases in such systemic inflammatory mediators are positioned, clinically, as having the strongest predictive values of the onset of age-related morbidities. The results of this project provide the first statistical evidence of an association between inflammation and immune cell telomere length, a biomarker of lymphocyte cellular senescence, in the context of CMV infection. This potentially bridges the gap between extensive CMV-specific T cell expansion and increases in inflammation, highlighting a mechanistic role for CMV co-infection in premature aging of HIV-infected individuals.

Despite identifying links between chronic inflammation, lymphocyte telomere length and the CMV immune response, we have not shown any direct role in the aforementioned inflammation for CMV-specific $CD8^+$ T cells. The poorly described senescent $CD8^+$ T cell phenotype, in the context of both surface marker expression and effector function, is a large obstacle to definitive research in this area. It remains unknown if traditional markers of T cell effector function (ie. IFN-γ and TNF-α) are useful to identify a potential SASP-like activity pattern, or if single-cell transcriptional profiling is required to discover the unique reprogramming of senescent $CD8^+$ T cells. It has been recently described by Akbar et al. that $CD45RA^+$ effector memory $CD8^+$ T cells

express a secretory phenotype largely dominated by proteases more so than inflammatory cytokines/chemokines, much like the granzyme (serine proteases) secretory profile of NK cells.[141] Furthermore, as T cells reach stages of terminal differentiation, surface expression of NK-associated receptors is notably increased. However, it remains unknown whether this is indicative of any NK-associated behavior. In addition to the previously mentioned CD57 and CD16, other NK markers such as: killer-cell immunoglobulin-like receptors (KIR), killer-cell lectin-like receptors (KLR) and CD85j (LIR), are also expressed on CMV-specific T cells.[69,70,72] Understanding the parameters which not only CMV-specific, but global T cell senescence alters, translates, or augments to impact on classical T effector functions is crucial for addressing lymphocyte senescence.

Our results affirm involvement of the adaptive immune system in biological aging and further substantiate chronic viral infections as accelerators of immune senescence. A prominent feature of aging is reduced immune mediated cell clearance and subsequent accumulation of senescent cells within the peripheral tissue, suspected to reflect degenerating immune function. Reduced ratios of CD4:CD8 T cells often represents a negative restructuring within the immune system that occurs with aging, characterized mostly by polyclonal expansion of CD8+ T cells, or a large decrease in CD4+ T cells (i.e. HIV infection). Large oligoclonal populations of $CD8^+$ T cells can negatively affect the reciprocal $CD8^+$ T cell population via metabolite deficiency or "space competition." Populations affected by such inhibition could represent the $CD8^+$ T cell subsets responsible for senescent cell clearance from peripheral tissue. Highly

differentiated $CD8^+$ T cells, like the effector memory $CD8^+$ T cells specific to CMV-antigens, often possess regulatory functions and can suppress $CD8^+$ T cells of different specificity via Fas:FasL engagement and other fratricidal, autocrine or paracrine cell interactions leading to upregulation of exhaustion markers such as programmed cell death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and T cell immunoglobulin and mucin domain 3 (TIM3)[87]. Interestingly CD57 expression on the overall $CD8^+$ T cell population is increased during both CMV infection and HIV infection, indicating that increases in inflammation, including immune activating molecules such as IFN-γ, contribute to increases in homeostatic $CD8^+$ proliferation in addition to the extensive replication seen in the potentially replicative-senescent $CD8^+$ T cells directed against CMV. Enhancing replication of $CD8^+$ T cells, without TCR-engagement, could drive nearby $CD8^+$ T cells, and other lymphocytes, closer to replicative senescence. Inhibition of, and premature imposition of exhaustion on proximal $CD8^+$ T cells by CMV-specific T cells could compound the problem through decreased clearance of senescent cells from peripheral tissue, therefore it is of paramount importance to identify how CMV-specific CD8+ T cells interact *in vivo*.

Dominant suppressive effects on the overall $CD8^+$ T cell population due to highly expanded CMV-specific memory $CD8^+$ T cells could also explain reduced immune memory development and vaccine effectiveness within the elderly and ART-suppressed HIV-infected population. Despite poor development of vaccine-induced immunological memory in elderly, CMV-specific $CD8^+$ T cells remain highly cytotoxic and are functionally maintained throughout life, even in old age. Similarly, maintenance

of CMV-specific $CD8^+$ T cells remains undisturbed during HIV infection, despite dysfunction observed in most other immune subsets due to lack of $CD4^+$ T helper cell function and other factors.

The durable immune response developed during HCMV infection prompted Hansen et al. to develop a recombinant rhesus (Rh) CMV vector capable of expressing gag protein epitopes from simian immunodeficiency virus (SIV)[148]. Prophylactic administration of the RhCMV-SIV led to 50% protection in SIV-exposed macaques, and was the first recombinant vaccine shown to elicit protective responses predominantly composed of highly cytotoxic effector memory $CD8^+$ T cells. Much like HCMV-specific $CD8^+$ T cells, these SIV-specific $CD8^+$ Tem cells were found in high frequencies in the circulation and in the tissue, capable of immediate pathogen identification that allowed stringent viral clearance. The shared phenotype observed in the protective $CD8^+$ T responses during RhCMV-SIV vaccination and CMV infection may provide long lasting and efficacious immunity towards viral epitopes, but likely retain the capacity, like the CMV-specific $CD8^+$ T cells identified in our results, to accelerate immune senescence in vaccinated subjects. Therefore, identifying how this CMV-specific development and maintenance of effector memory $CD8^+$ T cells occurs in natural CMV infection and in current recombinant CMV vaccines is crucial to generate future vaccines with not only sustainable and efficacious protective immunity but no considerable long term effects on aging.

Many other chronic virus infections such as HIV infection, can establish latency in humans and then reactivate periodically or chronically, but none reinforce such a

dramatic $CD8^+$ T cell response or concomitant increase in inflammation as does CMV infection**.** CMV infections elicit high frequencies of terminally differentiated virus-specific T cells that can possibly minimize anti-senescent and homeostatic T cell responses. Senescent cells accumulate within the blood and tissue during aging and contribute to the development of age-associated disease, often by the acquisition of a pro-inflammatory phenotype. We have presented evidence that CMV-specific, and not HIV-specific, $CD8^+$ T cells are driven to critical telomere lengths in HIV-infected individuals and could illustrate excessive replication as a driving mechanism of lymphocyte senescence. Additionally, we show that CMV-infection is associated with increased levels of multiple biomarkers of inflammation, particularly CRP, during HIV infection, and speculate that this is observable due to increased senescence of the immune system. The association between CRP levels and CMV-specific $CD8^+$ T cell telomere erosion could introduce new directions for investigating the intimate link between CMV infection and enhanced risk of cardiovascular disease seen in HIV-infected and uninfected aging populations. Our results reinforce certain immune and inflammatory parameters, such as $CD8^+$ T cell telomere length and CRP, as biomarkers of senescence that should be considered during recombinant CMV vector vaccine trials as risk factors for CMV vaccine-related repercussions on aging, inflammation and immunity. There is still much unknown about how CMV can evade and influence the human immune system, however, given the high incidence of CMV infection in the global population, understanding how CMV infection can alter, or clearly accelerate, the aging process is key in addressing rapidly increasing rates of age-associated disease.

References

1. HAYFLICK L, MOORHEAD PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585–621.
2. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345:458–60.
3. Okuda K, Bardeguez A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, et al. Telomere Length in the Newborn. Pediatr Res. 2002;52:377–81.
4. Friedrich U, Griese E-U, Schwab M, Fritz P, Thon K-P, Klotz U. Telomere length in different tissues of elderly patients. Mech Ageing Dev. 2000;119:89–99.
5. Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. J Mol Biol. 1992;225:951–60.
6. Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. Dev Genet. 1996;18:173–9.
7. Yasumoto S, Kunimura C, Kikuchi K, Tahara H, Ohji H, Yamamoto H, et al. Telomerase activity in normal human epithelial cells. Oncogene. 1996;13:433–9.
8. Coppé J-P, Desprez P-Y, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol. 2010;5:99–118.
9. Howcroft TK, Campisi J, Louis GB, Smith MT, Wise B, Wyss-Coray T, et al. The role of inflammation in age-related disease. Aging (Albany NY). 2013;5:84–93.
10. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. Sci Transl Med. 2014;6:265sr6.
11. Muñoz-Espín D, Cañamero M, Maraver A, Gómez-López G, Contreras J, Murillo-Cuesta S, et al. Programmed cell senescence during mammalian embryonic development. Cell. 2013;155:1104–18.
12. Rajagopalan S. HLA-G-mediated NK cell senescence promotes vascular remodeling: implications for reproduction. Cell Mol Immunol. 2014;11:460–6.
13. Hewitt G, Jurk D, Marques FDM, Correia-Melo C, Hardy T, Gackowska A, et al. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. Nat Commun. 2012;3:708.
14. Rodier F, Coppé J-P, Patil CK, Hoeijmakers WAM, Muñoz DP, Raza SR, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol. 2009;11:973–9.
15. Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, et al. Molecular inflammation: underpinnings of aging and age-related diseases.1. Chung HY,

Cesari M, Anton S, et al. Molecular inflammation: underpinnings of aging and age-related diseases. Ageing Res Rev. 2009;8(1):18-30. doi:10.1016/j.arr.2008.07.002. Ageing Res Rev. 2009;8:18–30.

16. Zhang J, Rane G, Dai X, Shanmugam MK, Arfuso F, Samy RP, et al. Ageing and the telomere connection: An intimate relationship with inflammation. Ageing Res Rev. 2016;25:55–69.

17. Freund A, Orjalo A V, Desprez P-Y, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. Trends Mol Med. 2010;16:238–46.

18. Le ONL, Rodier F, Fontaine F, Coppe J-P, Campisi J, DeGregori J, et al. Ionizing radiation-induced long-term expression of senescence markers in mice is independent of p53 and immune status. Aging Cell. 2010;9:398–409.

19. Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, et al. p16INK4a induces an age-dependent decline in islet regenerative potential. Nature. 2006;443:453–7.

20. Coppé J-P, Rodier F, Patil CK, Freund A, Desprez P-Y, Campisi J. Tumor suppressor and aging biomarker p16(INK4a) induces cellular senescence without the associated inflammatory secretory phenotype. J Biol Chem. 2011;286:36396–403.

21. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature. 2011;479:232–6.

22. Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest. 2013;123:966–72.

23. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69 Suppl 1:S4-9.

24. Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). Annu Rev Immunol. 2009;27:165–97.

25. Parrinello CM, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, et al. Cytomegalovirus immunoglobulin G antibody is associated with subclinical carotid artery disease among HIV-infected women. J Infect Dis. 2012;

26. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003;112:1821–30.

27. Abu-Remaileh M, Bender S, Raddatz G, Ansari I, Cohen D, Gutekunst J, et al. Chronic Inflammation Induces a Novel Epigenetic Program That Is Conserved in Intestinal Adenomas and in Colorectal Cancer. Cancer Res. 2015;75.

28. Libby P, Okamoto Y, Rocha VZ, Folco E. Inflammation in atherosclerosis: transition from theory to practice. Circ J. 2010;74:213–20.

29. Tracy R. PAPER Emerging relationships of inflammation, cardiovascular disease and chronic diseases of aging. Int J Obes. 2003;27:29–34.

30. Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, et al. Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev. 2007;128:92–105.

31. Bartlett DB, Firth CM, Phillips AC, Moss P, Baylis D, Syddall H, et al. The age-related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection. Aging Cell. 2012;11:912–5.

32. Zhang B, Bailey WM, Braun KJ, Gensel JC. Age decreases macrophage IL-10 expression: Implications for functional recovery and tissue repair in spinal cord injury. Exp Neurol. 2015;273:83–91.

33. Spencer NF, Norton SD, Harrison LL, Li GZ, Daynes RA. Dysregulation of IL-10 production with aging: possible linkage to the age-associated decline in DHEA and its sulfated derivative. Exp Gerontol. 31:393–408.

34. Meador BM, Krzyszton CP, Johnson RW, Huey KA. Effects of IL-10 and age on IL-6, IL-1β, and TNF-α responses in mouse skeletal and cardiac muscle to an acute inflammatory insult. J Appl Physiol. 2008;104:991–7.

35. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature. 2005;433:760–4.

36. Phimister EG, Laviano A. Young Blood. N Engl J Med. 2014;371:573–5.

37. Nelson G, Wordsworth J, Wang C, Jurk D, Lawless C, Martin-Ruiz C, et al. A senescent cell bystander effect: senescence-induced senescence. Aging Cell. 2012;11:345–9.

38. Acosta JC, Banito A, Wuestefeld T, Georgilis A, Janich P, Morton JP, et al. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat Cell Biol. 2013;15:978–90.

39. Yang H, Wang H, Ren J, Chen Q, Chen ZJ, Miyamoto S, et al. cGAS is essential for cellular senescence.

40. Gray D, Abramson J, Benoist C, Mathis D. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. J Exp Med. 2007;204:2521–8.

41. de la Hera A, Marston W, Aranda C, Toribio M-L, Martinez-A C. Thymic stroma is required for the development of human T cell lineages *in vitro*. Int Immunol. 1989;1:471–8.

42. Nitta T, Suzuki H. Thymic stromal cell subsets for T cell development. Cell Mol Life Sci. 2016;73:1021–37.

43. Nasi M, Troiano L, Lugli E, Pinti M, Ferraresi R, Monterastelli E, et al. Thymic output and functionality of the IL-7/IL-7 receptor system in centenarians: implications for the neolymphogenesis at the limit of human life. Aging Cell. 2006;5:167–75.

44. Yang Y, An J, Weng N. Telomerase is involved in IL-7-mediated differential survival of naive and memory CD4+ T cells. J Immunol. 2008;180:3775–81.

45. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009;1:a001651.

46. Li Q, Verma IM. NF-κB regulation in the immune system. Nat Rev Immunol. 2002;2:725–34.

47. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, et al. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009;460:108–12.

48. Yamada O, Motoji T, Mizoguchi H. Up-regulation of telomerase activity in human lymphocytes. Biochim Biophys Acta - Mol Cell Res. 1996;1314:260–6.

49. Wu Y, Kawakatsu RB, Effros ST, Parish S, Kim RK, Sekhon JE, et al. Senescence in Human CD8 T Lymphocytes Telomerase Activity and Replicative Adenosine Deaminase Modulation of Adenosine Deaminase Modulation of Telomerase Activity and Replicative Senescence in Human CD8 T Lymphocytes. 2017;184:2847–54.

50. Charles A Janeway J, Travers P, Walport M, Shlomchik MJ. General properties of armed effector T cells. 2001;

51. Prlic M, Bevan MJ. Exploring regulatory mechanisms of CD8+ T cell contraction. Proc Natl Acad Sci U S A. 2008;105:16689–94.

52. Liao W, Lin J-X, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013;38:13–25.

53. Effros RB. Telomerase induction in T cells: a cure for aging and disease? Exp Gerontol. 2007;42:416–20.

54. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585–621.

55. Makinodan T, Kay MM. Age influence on the immune system. Adv Immunol. 1980;29:287–330.

56. Pawelec G, Adibzadeh M, Pohla H, Schaudt K. Immunosenescence: ageing of the immune system. Immunol Today. 1995;16:420–2.

57. Franceschi C, Valensin S, Fagnoni F, Barbi C, Bonafè M. Biomarkers of

immunosenescence within an evolutionary perspective: the challenge of heterogeneity and the role of antigenic load. Exp Gerontol. 1999;34:911–21.

58. Plunkett FJ, Franzese O, Finney HM, Fletcher JM, Belaramani LL, Salmon M, et al. The loss of telomerase activity in highly differentiated CD8+CD28-CD27- T cells is associated with decreased Akt (Ser473) phosphorylation. J Immunol. 2007;178:7710–9.

59. Saltzman RL, Peterson PK. Immunodeficiency of the elderly. Rev Infect Dis. 9:1127–39.

60. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. Lancet. 2003;361:393–5.

61. von Figura G, Hartmann D, Song Z, Rudolph KL. Role of telomere dysfunction in aging and its detection by biomarkers. J Mol Med. 2009;87:1165–71.

62. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol. 2010;20:202–13.

63. Jabs DA, Enger C, Bartlett JG. Cytomegalovirus Retinitis and Acquired Immunodeficiency Syndrome. Arch Ophthalmol. 1989;107:75.

64. van de Berg PJEJ, van Stijn A, Ten Berge IJM, van Lier RAW. A fingerprint left by cytomegalovirus infection in the human T cell compartment. J Clin Virol. 2008;41:213–7.

65. Link CS, Eugster A, Heidenreich F, Rücker-Braun E, Schmiedgen M, Oelschlägel U, et al. Abundant cytomegalovirus (CMV) reactive clonotypes in the CD8 $^{+}$ T cell receptor alpha repertoire following allogeneic transplantation. Clin Exp Immunol. 2016;184:389–402.

66. Trautmann L, Rimbert M, Echasserieau K, Saulquin X, Neveu B, Dechanet J, et al. Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses. J Immunol. 2005;175:6123–32.

67. Effros RB, Boucher N, Porter V, Zhu X, Spaulding C, Walford RL, et al. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: A possible cause for both in vivo and in vitro immunosenescence. Exp Gerontol. 1994;29:601–9.

68. Hoji A, Connolly NC, Buchanan WG, Rinaldo CR, Jr. CD27 and CD57 expression reveals atypical differentiation of human immunodeficiency virus type 1-specific memory CD8+ T cells. Clin Vaccine Immunol. 2007;14:74–80.

69. Ester Remmerswaal LB, ten Berge IJ, van Ester M van Leeuwen RA, Gamadia LE, Baars PA, van Leeuwen EM, et al. T Cells + Human CD8 Cytomegalovirus-Specific, Effector-Type Proliferation Requirements of Proliferation Requirements of Cytomegalovirus-Specific, Effector-Type Human CD8 ⁒ T Cells. J Immunol

Ref J Immunol. 2017;169:5838–43.

70. Topp MS, Riddell SR, Akatsuka Y, Jensen MC, Blattman JN, Greenberg PD. Restoration of CD28 expression in CD28- CD8+ memory effector T cells reconstitutes antigen-induced IL-2 production. J Exp Med. 2003;198:947–55.

71. Khan N, Shariff N, Cobbold M, Bruton R, Ainsworth JA, Sinclair AJ, et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol. 2002;169:1984–92.

72. Lopez-Vergès S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood. 2010;116:3865–74.

73. Mojumdar K, Vajpayee M, Chauhan NK, Singh A, Singh R, Kurapati S. Loss of CD127 & increased immunosenescence of T cell subsets in HIV infected individuals. Indian J Med Res. 2011;134:972–81.

74. Cicin-Sain L, Brien JD, Uhrlaub JL, Drabig A, Marandu TF, Nikolich-Zugich J. Cytomegalovirus Infection Impairs Immune Responses and Accentuates T-cell Pool Changes Observed in Mice with Aging. Mossman KL, editor. PLoS Pathog. 2012;8:e1002849.

75. Pachnio A, Ciaurriz M, Begum J, Lal N, Zuo J, Beggs A, et al. Cytomegalovirus Infection Leads to Development of High Frequencies of Cytotoxic Virus-Specific CD4+ T Cells Targeted to Vascular Endothelium. PLoS Pathog. 2016;12:e1005832.

76. Takiguchi Katsuhiko Fukada M, Sobao Y, Tomiyama H, Fukada K, Oka S, Takiguchi M. T Cells + Memory and Effector CD8 Receptor CCR5 on Virus Epitope-Specific Functional Expression of the Chemokine Functional Expression of the Chemokine Receptor CCR5 on Virus Epitope-Specific Memory and Effector CD8 ⁺ T Cells. J Immunol Ref. 2002;168:2225–32.

77. van de Berg PJEJ, Yong S-L, Remmerswaal EBM, van Lier RAW, ten Berge IJM. Cytomegalovirus-induced effector T cells cause endothelial cell damage. Clin Vaccine Immunol. 2012;19:772–9.

78. Wallace DL, Masters JE, De Lara CM, Henson SM, Worth A, Zhang Y, et al. Human cytomegalovirus-specific CD8(+) T-cell expansions contain long-lived cells that retain functional capacity in both young and elderly subjects. Immunology. 2011;132:27–38.

79. Chiu Y-L, Lin C-H, Sung B-Y, Chuang Y-F, Schneck JP, Kern F, et al. Cytotoxic polyfunctionality maturation of cytomegalovirus-pp65-specific CD4 + and CD8 + T-cell responses in older adults positively correlates with response size. Sci Rep. 2016;6.

80. Keskinen P, Ronni T, Matikainen S, Lehtonen A, Julkunen I. Regulation of HLA

class I and II expression by interferons and influenza A virus in human peripheral blood mononuclear cells. Immunology. 1997;91:421–9.

81. Ohmori Y, Schreiber RD, Hamilton TA. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. J Biol Chem. 1997;272:14899–907.

82. Sandberg JK, Fast NM, Nixon DF. Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8+ T lymphocytes. J Immunol. 2001;167:181–7.

83. Makedonas G, Banerjee PP, Pandey R, Hersperger AR, Sanborn KB, Hardy GAD, et al. Rapid up-regulation and granule-independent transport of perforin to the immunological synapse define a novel mechanism of antigen-specific CD8+ T cell cytotoxic activity. J Immunol. 2009;182:5560–9.

84. Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, et al. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med. 2000;192:63–75.

85. Arroyo L, Rojas M, Franken KLMC, Ottenhoff THM, Barrera LF. Multifunctional T Cell Response to DosR and Rpf Antigens Is Associated with Protection in Long-Term Mycobacterium tuberculosis-Infected Individuals in Colombia. Clin Vaccine Immunol. 2016;23:813–24.

86. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood. 2006;107.

87. Riddell NE, Griffiths SJ, Rivino L, King DCB, Teo GH, Henson SM, et al. Multifunctional cytomegalovirus (CMV)-specific CD8(+) T cells are not restricted by telomere-related senescence in young or old adults. Immunology. 2015;144:549–60.

88. Petrovas C, Mueller YM, Dimitriou ID, Bojczuk PM, Mounzer KC, Witek J, et al. HIV-Specific CD8+ T Cells Exhibit Markedly Reduced Levels of Bcl-2 and Bcl-xL. J Immunol. 2004;172.

89. Increased CD95/Fas-Induced Apoptosis of HIV-Specific CD8. Immunity. 2001;15:871–82.

90. Leng SX, Qu T, Semba RD, Li H, Yao X, Nilles T, et al. Relationship between cytomegalovirus (CMV) IgG serology, detectable CMV DNA in peripheral monocytes, and CMV pp65 495-503-specific CD8 + T cells in older adults. Age (Omaha). 2011;33.

91. Staras SAS, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of Cytomegalovirus Infection in the. United States. 1988;

92. Simanek AM, Dowd JB, Pawelec G, Melzer D, Dutta A, Aiello AE, et al. Seropositivity to Cytomegalovirus, Inflammation, All-Cause and Cardiovascular Disease-Related Mortality in the United States. Hernandez A, editor. PLoS One. 2011;6:e16103.

93. Palmer DB. The effect of age on thymic function. Front Immunol. 2013;4:316.

94. Sauce D, Larsen M, Fastenackels S, Duperrier A, Keller M, Grubeck-Loebenstein B, et al. Evidence of premature immune aging in patients thymectomized during early childhood. J Clin Invest. 2009;119:3070–8.

95. Torfadottir H, Freysdottir J, Skaftadottir I, Haraldsson A, Sigfusson G, Ogmundsdottir HM. Evidence for extrathymic T cell maturation after thymectomy in infancy. Clin Exp Immunol. 2006;145:407–12.

96. Smithey MJ, Li G, Venturi V, Davenport MP, Nikolich-Žugich J. Lifelong persistent viral infection alters the naive T cell pool, impairing CD8 T cell immunity in late life. J Immunol. 2012;189:5356–66.

97. van de Berg PJ, Heutinck KM, Raabe R, Minnee RC, Young SL, van Donselaar-van der Pant KA, et al. Human Cytomegalovirus Induces Systemic Immune Activation Characterized by a Type 1 Cytokine Signature. J Infect Dis. 2010;202:690–9.

98. Lagrand WK, Visser CA, Hermens WT, Niessen HWM, Verheugt FWA, Wolbink G-J, et al. C-Reactive Protein as a Cardiovascular Risk Factor. Circulation. 1999;100.

99. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol. 2009;78:539–52.

100. Bolovan-Fritts CA, Spector SA. Endothelial damage from cytomegalovirus-specific host immune response can be prevented by targeted disruption of fractalkine-CX3CR1 interaction. Blood. 2007;111.

101. Roberts ET, Haan MN, Dowd JB, Aiello AE. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. Am J Epidemiol. 2010;172:363–71.

102. Spyridopoulos I, Martin-Ruiz C, Hilkens C, Yadegarfar ME, Isaacs J, Jagger C, et al. CMV seropositivity and T-cell senescence predict increased cardiovascular mortality in octogenarians: results from the Newcastle 85+ study. Aging Cell. 2016;15:389–92.

103. Savva GM, Pachnio A, Kaul B, Morgan K, Huppert FA, Brayne C, et al. Cytomegalovirus infection is associated with increased mortality in the older population.

104. Dedinská I, Laca Ľ, Miklušica J, Kantárová D, Galajda P, Mokáň M. Correlation between CMV Infection and Post-transplantation New-onset Diabetes Mellitus. Int

J organ Transplant Med. 2016;7:173–82.

105. Shimamura M. The Contribution of Cytomegalovirus to Atherosclerotic Events after Kidney Transplantation. J Infect Dis. 2013;207:1487–90.

106. Blum A, Peleg A, Weinberg M. Anti-cytomegalovirus (CMV) IgG antibody titer in patients with risk factors to atherosclerosis. Clin Exp Med. 2003;3:157–60.

107. Halenius A, Hengel H. Human cytomegalovirus and autoimmune disease. Biomed Res Int. 2014;2014:472978.

108. Adam E, Probtsfield JL, Burek J, Mccollum CH, Melnick JL, Petrie BL, et al. HIGH LEVELS OF CYTOMEGALOVIRUS ANTIBODY IN PATIENTS REQUIRING VASCULAR SURGERY FOR ATHEROSCLEROSIS. Lancet. 1987;330:291–3.

109. Pierer M, Rothe K, Quandt D, Schulz A, Rossol M, Scholz R, et al. Association of anticytomegalovirus seropositivity with more severe joint destruction and more frequent joint surgery in rheumatoid arthritis. Arthritis Rheum. 2012;64:1740–9.

110. Söderlund C, Bratt GA, Engström L, Grützmeier S, Nilsson R, Sjunnesson M, et al. Surgical treatment of cytomegalovirus enterocolitis in severe human immunodeficiency virus infection. Dis Colon Rectum. 1994;37:63–72.

111. Tan LC, Mowat AG, Fazou C, Rostron T, Roskell H, Dunbar PR, et al. Specificity of T cells in synovial fluid: high frequencies of CD8(+) T cells that are specific for certain viral epitopes. Arthritis Res. 2000;2:154.

112. Rosa C La, Limaye AP, Krishnan A, Longmate J, Diamond DJ. Longitudinal Assessment of Cytomegalovirus (CMV)–Specific Immune Responses in Liver Transplant Recipients at High Risk for Late CMV Disease.

113. Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, et al. Immune Activation and CD8+ T-Cell Differentiation towards Senescence in HIV-1 Infection. Philippa Marrack, editor. PLoS Biol. 2004;2:e20.

114. Grabar S, Lanoy E, Allavena C, Mary-Krause M, Bentata M, Fischer P, et al. Causes of the first AIDS-defining illness and subsequent survival before and after the advent of combined antiretroviral therapy. HIV Med. 2008;9:246–56.

115. Ives NJ, Gazzard BG, Easterbrook PJ. The Changing Pattern of AIDS-defining Illnesses with the Introduction of Highly Active Antiretroviral Therapy (HAART)in a London Clinic. J Infect. 2001;42:134–9.

116. Samji H, Cescon A, Hogg RS, Modur SP, Althoff KN, Buchacz K, et al. Closing the Gap: Increases in Life Expectancy among Treated HIV-Positive Individuals in the United States and Canada. Okulicz JF, editor. PLoS One. 2013;8:e81355.

117. Serrano-Villar S, Pérez-Elías MJ, Dronda F, Casado JL, Moreno A, Royuela A, et al. Increased Risk of Serious Non-AIDS-Related Events in HIV-Infected Subjects

on Antiretroviral Therapy Associated with a Low CD4/CD8 Ratio. DeRiemer K, editor. PLoS One. 2014;9:e85798.

118. Serrano-Villar S, Moreno S, Fuentes-Ferrer M, Sánchez-Marcos C, Ávila M, Sainz T, et al. The CD4:CD8 ratio is associated with markers of age-associated disease in virally suppressed HIV-infected patients with immunological recovery.

119. Freeman ML, Lederman MM, Gianella S. Partners in Crime: The Role of CMV in Immune Dysregulation and Clinical Outcome During HIV Infection. Curr HIV/AIDS Rep. 2016;13:10–9.

120. Neuhaus J, Jacobs, Jr DR, Baker JV, Calmy A, Duprez D, La Rosa A, et al. Markers of Inflammation, Coagulation, and Renal Function Are Elevated in Adults with HIV Infection. J Infect Dis. 2010;201:1788–95.

121. Hsue PY, Hunt PW, Sinclair E, Bredt B, Franklin A, Killian M, et al. Increased carotid intima-media thickness in HIV patients is associated with increased cytomegalovirus-specific T-cell responses. AIDS. 2006;20:2275–83.

122. Roff SR, Noon-Song EN, Yamamoto JK. The Significance of Interferon-γ in HIV-1 Pathogenesis, Therapy, and Prophylaxis. Front Immunol. 2014;4:498.

123. Bedini JL, García F, Miró JM, Aznar E, Serrano J, Lozano L, et al. Serum levels of β2-microglobulin, neopterin, TNF-α and soluble receptors of TNF-α and interleukin-2 in intravenous drug abusers according to HIV-1 status and stage of the HIV-1 infection. Clin Microbiol Infect. 1998;4:4–10.

124. Jiménez VC, Wit FWNM, Joerink M, Maurer I, Harskamp AM, Schouten J, et al. T-Cell Activation Independently Associates With Immune Senescence in HIV-Infected Recipients of Long-term Antiretroviral Treatment. J Infect Dis. 2016;214:216–25.

125. Serrano-Villar S, Sainz T, Lee SA, Hunt PW, Sinclair E, Shacklett BL, et al. HIV-Infected Individuals with Low CD4/CD8 Ratio despite Effective Antiretroviral Therapy Exhibit Altered T Cell Subsets, Heightened CD8+ T Cell Activation, and Increased Risk of Non-AIDS Morbidity and Mortality. Silvestri G, editor. PLoS Pathog. 2014;10:e1004078.

126. Damouche A, Lazure T, Avettand-Fènoël V, Huot N, Dejucq-Rainsford N, Satie A-P, et al. Adipose Tissue Is a Neglected Viral Reservoir and an Inflammatory Site during Chronic HIV and SIV Infection. PLoS Pathog. 2015;11:e1005153.

127. Dau B, Holodniy M. The Relationship Between HIV Infection and Cardiovascular Disease. Curr Cardiol Rev. 2008;4:203–18.

128. Lederman MM, Funderburg NT, Sekaly RP, Klatt NR, Hunt PW. Residual immune dysregulation syndrome in treated HIV infection. Adv Immunol. 2013;119:51–83.

129. Baum PD, Young JJ, Schmidt D, Zhang Q, Hoh R, Busch M, et al. Blood T-cell receptor diversity decreases during the course of HIV infection, but the potential

for a diverse repertoire persists. Blood. 2012;119:3469–77.

130. van den Berg R, van Hoogstraten I, van Agtmael M. Non-responsiveness to hepatitis B vaccination in HIV seropositive patients; possible causes and solutions. AIDS Rev. 11:157–64.

131. Heath SL, Sabbaj S, Bansal A, Kilby JM, Goepfert PA. CD8 T-cell proliferative capacity is compromised in primary HIV-1 infection. J Acquir Immune Defic Syndr. 2011;56:213–21.

132. Heath J, Newhook N, Comeau E, Gallant M, Fudge N, Grant M. NKG2C^{+}CD57^{+} Natural Killer Cell Expansion Parallels Cytomegalovirus-Specific CD8^{+} T Cell Evolution towards Senescence. J Immunol Res. 2016;2016.

133. Barrett L, Fudge NJ, Heath JJ, Grant MD. Cytomegalovirus immunity and exhaustive CD8^{+} T cell proliferation in treated human immunodeficiency virus infection. Clin Infect Dis. 2016;62.

134. Blackman MA, Woodland DL. The narrowing of the CD8 T cell repertoire in old age. Curr Opin Immunol. 2011;23:537–42.

135. Lu W, Mehraj V, Vyboh K, Cao W, Li T, Routy J-P. CD4:CD8 ratio as a frontier marker for clinical outcome, immune dysfunction and viral reservoir size in virologically suppressed HIV-positive patients. J Int AIDS Soc. 2015;18:20052.

136. Barrett L, Stapleton SN, Fudge NJ, Grant MD. Immune resilience in HIV-infected individuals seronegative for cytomegalovirus. AIDS. 2014;28:2045–9.

137. Wolthers KC, Bea G, Wisman A, Otto SA, de Roda Husman AM, Schaft N, et al. T cell telomere length in HIV-1 infection: no evidence for increased CD4+ T cell turnover. Science. 1996;274:1543–7.

138. Palmer LD, Weng N, Levine BL, June CH, Lane HC, Hodes RJ. Telomere length, telomerase activity, and replicative potential in HIV infection: analysis of CD4+ and CD8+ T cells from HIV-discordant monozygotic twins. J Exp Med. 1997;185:1381–6.

139. Steele AK, Lee EJ, Vestal B, Hecht D, Dong Z, Rapaport E, et al. Contribution of Intestinal Barrier Damage, Microbial Translocation and HIV-1 Infection Status to an Inflammaging Signature. Bourgeois C, editor. PLoS One. 2014;9:e97171.

140. Dock JN, Effros RB. Role of CD8 T Cell Replicative Senescence in Human Aging and in HIV-mediated Immunosenescence. Aging Dis. 2011;2:382–97.

141. Zanet DL, Thorne A, Singer J, Maan EJ, Sattha B, Le Campion A, et al. Association Between Short Leukocyte Telomere Length and HIV Infection in a Cohort Study: No Evidence of a Relationship With Antiretroviral Therapy. Clin Infect Dis. 2014;58:1322–32.

142. Deeks SG. Immune dysfunction, inflammation, and accelerated aging in patients

on antiretroviral therapy. Top HIV Med. 17:118–23.

143. Strahl C, Blackburn EH. Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. Mol Cell Biol. 1996;16:53–65.

144. Solomon A, Tennakoon S, Leeansyah E, Arribas J, Hill A, Van Delft Y, et al. No Difference in the Rate of Change in Telomere Length or Telomerase Activity in HIV-Infected Patients after Three Years of Darunavir/Ritonavir with and without Nucleoside Analogues in the MONET Trial. Landay A, editor. PLoS One. 2014;9:e109718.

145. Leeansyah E, Cameron PU, Solomon A, Tennakoon S, Velayudham P, Gouillou M, et al. Inhibition of Telomerase Activity by Human Immunodeficiency Virus (HIV) Nucleos(t)ide Reverse Transcriptase Inhibitors: A Potential Factor Contributing to HIV-Associated Accelerated Aging. J Infect Dis. 2013;207:1157–65.

146. Bestilny LJ, Gill MJ, Mody CH, Riabowol KT. Accelerated replicative senescence of the peripheral immune system induced by HIV infection. AIDS. 2000;14:771–80.

147. Wikby A, Nilsson B-O, Forsey R, Thompson J, Strindhall J, Löfgren S, et al. The immune risk phenotype is associated with IL-6 in the terminal decline stage: findings from the Swedish NONA immune longitudinal study of very late life functioning. Mech Ageing Dev. 2006;127:695–704.

148. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat Med. 2009;15:293–9.