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Review

Role of CD8 T Cell Replicative Senescence in Human Aging and in HIV-mediated Immunosenescence

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ABSTRACT: As humans age, their immune systems undergo a process known as immunosenescence. This global aging of the immune system is associated with increased susceptibility to infectious diseases and cancer, reduced effectiveness of vaccination, increased autoimmune phenomena, and tissue damage due to dysregulated inflammation. One hallmark feature of immunosenescence is the accumulation of late-differentiated memory CD8 T cells with features of replicative senescence, such as inability to proliferate, absence of CD28 expression, shortened telomeres, loss of telomerase activity, and enhanced secretion of inflammatory cytokines. The proportion of senescent CD8 T cells increases progressively with age, and often consists of oligoclonal populations that are specific for cytomegalovirus (CMV) antigens. In addition, there is evidence that senescent memory CD8 T cells acquire suppressive functions and may also contribute to carcinogenesis. Chronic HIV disease, even when controlled through antiretroviral therapy (ART), is associated with accelerated immunosenescence, as evidenced by the higher numbers of senescent memory CD8 T cells and increased inflammatory milieu. Interestingly, even in HIV disease, a high proportion of late-differentiated, putatively senescent, memory CD8 T cells are specific for CMV antigens. As in age-related immunosenescence, these HIV-associated changes result in dysregulated immunity, chronic diseases linked to inflammatory damage, and increased morbidity and mortality. This review explores the evidence for CD8 T cell replicative senescence *in vitro* and *in vivo*, in the context of both chronological aging and HIV-mediated immunosenescence. We also highlight an important gap in our understanding of human immunosenescence, since all the studies to date have focused on peripheral blood, which contains a minority of the total body lymphocyte population.

Key words: CD8 T cells; HIV disease; Human Aging; immunosenescence; Replicative senescence

Immunosenescence is the global term used to describe the observed age-associated decline in immune competence characterized by functional and phenotypic alterations to the immune system as a whole [1]. Immunosenescence in the elderly human population is associated with increased susceptibility to infectious diseases and cancer, reduced effectiveness of vaccinations, increased autoimmunity, and damage to various organ systems through dysregulated inflammation [2-6]. Ultimately, these changes play a significant role in increasing morbidity and mortality [7, 8]. Although immunosenescence is a ubiquitous process, as with all complex diseases its progression is highly variable from individual to individual, and is undoubtedly influenced by a number of genetic and environmental factors.

Human immunosenescence is believed to be driven via systematic remodeling of the immune system over a lifetime of antigenic exposures and responses, mainly due to inflammatory processes. An in-depth discussion

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of inflammation and its role in immunosenescence, and aging in general, has been covered in depth in other reviews [9-11] and is outside the scope of this discussion. Instead, this review will focus on how immunosenescence, in both aging and HIV disease, influences the adaptive immune system, especially the CD8 T cell compartment, and how such alterations contribute to morbidity and mortality.

Some of the major immune alterations associated with immunosenescence include reduced number and function of hematopoietic stem cells, thymic involution, reduced circulating naïve T cells, decreased CD4/CD8 ratio, and increased levels of proinflammatory cytokines such as IL-6 and TNFα [9]. Another alteration strongly associated with immunosenescence is the accumulation of late-differentiated memory CD8 T cells which have undergone significant phenotypic and functional changes, and show features reminiscent of cellular replicative senescence characterized in long-term *in vitro* cultures [12]. *In vitro*, replicative senescence refers to the process by which cells reach an irreversible stage of cell cycle arrest following extensive replicative activity; these end stage cells show consistent changes in function and gene expression [13]. *In vivo*, the presence of oligoclonally expanded populations of similar late-differentiated cells is increasingly being implicated in advanced immunosenescence and predictive of poor prognosis [14, 15]. Although it cannot be definitively proven the cells have reached irreversible cell-cycle arrest *in vivo*, and in some cases have been shown to retain limited proliferative capacity[16], for the purpose of this review such cells are referred to as having reached replicative senescence, due to their overall similarity in function and phenotype to their *in vitro* counterparts and their putative role in immunosenescence.

This review will examine the evidence for CD8 T cell replicative senescence, both *in vitro* and *in vivo*, in the context of chronological aging and HIV-mediated premature immunosenescence. Evidence that CMV-mediated replicative senescence is part of an immune risk profile (IRP) predictive of morbidity and mortality in the very old will be summarized. Finally, studies implicating ‘inflammaging’ as an important contributor to immunosenescence will be briefly discussed, together with the role of CD8 T replicative senescence in this process.

CD8 T cell replicative senescence *in vitro*

The phenomenon of replicative (aka, cellular) senescence of cultured cells was first described by Hayflick 50 years ago [17], and has since been demonstrated in numerous cell types including fibroblasts, epithelial cells, hepatocytes, endothelial cells, and keratinocytes [12, 18]. Initially it was believed by immunologists that, with the addition of Interleukin 2 (IL-2), T cells in culture would be immortal and grow *in vitro* indefinitely [19]. However, it has since been repeatedly demonstrated that human T cells do, in fact, undergo replicative senescence *in vitro* [20, 21].

With proper activation via the T-cell receptor and constant exposure to IL-2, cultures of normal human T cells can undergo between 25 and 40 population doublings before reaching senescence and ceasing to proliferate [22], with an average lifespan of approximately 33 population doublings [23]. Although aging is correlated with the accumulation of cell types with senescence markers *in vivo*, there is no clear correlation between *in vitro* lifespan of T cell cultures and chronological age of the donor [24]. This holds true for both CD4 and CD8 T cells.

Characterization of CD8 T cell replicative senescence *in vitro*

As CD8 T cells progress toward replicative senescence *in vitro* they undergo predictable phenotypic and functional changes. In terms of cell surface markers, the most consistent and dramatic change is the loss of CD28 surface expression. CD28 is an essential T cell receptor (TCR) specific co-stimulatory molecule, and has been directly implicated in a number of critical T cell functions, such as lipid raft formation, IL-2 gene transcription, apoptosis, stabilization of cytokine mRNA, glucose metabolism and cell adhesion [25]. Thus, a T cell lacking CD28 is fundamentally different in numerous respects from a CD28+ T cell. It has been documented that during the progression of CD8 T cells to senescence in culture, the percentage of cells expressing CD28 decreases. Indeed, senescent cultures are > 95% CD28-, as compared to the starting population, which contains an average of 91% CD28+ T cells [26]; this initial value can vary, depending on such factors as chronological age and immune status of the blood donor. Although CD28 expression is regulated, at least in part, by protein turnover and transient transcriptional repression, there is evidence that by the time a cell reaches replicative senescence, CD28 transcription has been permanently silenced [16, 27].

Since CD28 is a critical co-stimulatory molecule and its transcription is repressed during cellular senescence, we hypothesized that sustained

transcription of CD28, via stable gene transduction, would affect the growth characteristics of primary CD8 T cells propagated in long term culture. Transduced cells did, in fact, demonstrate high initial CD28 transcription and cell surface expression, significantly increased proliferative potential, as well as a substantial delay in acquisition of certain features of replicative senescence, such as loss of telomerase activity and increase in inflammatory cytokine secretion [28]. Nevertheless, eventually CD28 cell surface expression was lost, CTLA-4 expression increased and replicative senescence was observed in the transduced cultures [28], indicating transcription of CD28 is important, but not in itself sufficient, to maintain replicative capacity indefinitely.

Another feature of senescent CD8 T cells in culture is resistance to apoptosis, consistent with observations on senescent fibroblasts [29]. In response to six different apoptotic stimuli, senescent and late passage cultures showed significantly lower levels of apoptosis as compared to quiescent early passage cultures derived from the same donor [30]. Senescent CD8 cells in culture also show a blunted response to stress, as measured by the ability to upregulate hsp70 [31], consistent with studies on other cell types such as fibroblasts [32]. Heat shock proteins are widely implicated in protecting cells from intrinsic and extrinsic damage, and their downregulation is believed to play a significant role in cellular aging [33].

Senescent CD8 T cells, although unable to replicate, still retain some functional capacity in response to antigenic stimulation. As CD8 T cell cultures progress towards senescence, they produce increasing amounts of inflammatory cytokines, such as IL-6 and TNFα [12]. Interestingly, addition of exogenous TNFα to long term T cell cultures has been shown to accelerate CD8 replicative senescence [34], suggesting a positive feedback role for senescent cells in promoting more rapid cellular senescence in pre-senescent cells within the same culture. Some senescent CD8 T cells (i.e., those driven to senescence by repeated alloantigen exposure) have been shown to retain antigen-specific cytotoxic function [24] in addition to acquiring major histocompatibility complex-unrestricted cytotoxicity [35]. By contrast, there is also evidence of compromised effector function. For example, senescent HIV-specific CD8 T cells show decreased antigen-specific production of IFNγ [12] and the progressive decline in ability to perform antigen specific lysis and suppress HIV replication *in vitro* [36], as well as a reduction in perforin and Granzyme B expression [37].

Telomere length and Telomerase

Telomeres are sequences of tandem $(TTAGGG)_n$ nucleotide repeats that protect the ends of chromosomes. An inevitable consequence of the end replication problem, originally hypothesized by Olovnikov [38], is that the free DNA ends of each chromosome are incompletely duplicated by DNA polymerase. The result is that the ends of human chromosomes can lose up to 200 base pairs per cell division [39]. When telomere length shortens to a critical minimum, a DNA damage signal leads to cell-cycle arrest and replicative senescence. It is believed this proliferative clock protects cells against malignant changes that can be brought about by chromosomal instability in dividing cells with shortened-telomeres. Consistent with this theory, a recent meta-analysis of 27 studies indicated a significant inverse correlation between telomere length of lymphocytes in surrogate tissues and cancer incidence [40], although it should be noted previous prospective studies have not shown a significant association. In addition to cell division, telomere length is also regulated by telomerase, an enzyme (consisting of the hTERT gene product, an RNA component TERC and other regulatory proteins) that can restore telomere ends during DNA replication, allowing cells to undergo proliferation with minimal telomere shortening. Most somatic cells do not have active telomerase, limiting proliferative capacity. However, cells of the immune system are able to upregulate telomerase in concert with early activation events, as will be discussed below.

At the beginning of culture, human CD8 T cells have mean telomere lengths of approximately 10-11 kb, but as the cells undergo multiple rounds of cell division and reach senescence, telomere length decreases to approximately 5-7 kb [41, 42], a value that is similar to reported telomere lengths in senescent fibroblast cultures [39]. Despite the overall decrease in telomere length during the progression to senescence *in vitro*, during initial activation, CD8 T cells (unlike most somatic cells) exhibit telomerase activity that is comparable with that of tumor cells [43], and which is associated with temporary maintenance of telomere length [44]. This initial high telomerase activity is consistent with the observed stable telomere length in virus-specific T cells during the early phase of adaptive immune responses to acute EBV infection *in vivo* [45]. As CD8 T cells are maintained in long-term cultures through repeated stimulation of the TCR, the telomerase activity upregulation associated with activation steadily decreases, until the 4th stimulation, at which time telomerase activity become undetectable

[44]. Interestingly, this trend is not seen in CD4 T cell cultures, which retain high telomerase activity through the 10th round of antigenic stimulation [44].

The loss of telomerase activity in senescing CD8 T cell cultures parallels the loss of CD28 cell surface expression [44, 46]. Blocking CD28 from binding to ligands on antigen-presenting cells (APC) during stimulation inhibits telomerase activity, indicating the key role in CD28 costimulation in upregulating telomerase activity during activation [44]. The importance of CD28 signaling in telomerase upregulation was further confirmed in the aforementioned experiments, in which the constitutive expression of CD28 via stable gene transduction in CD8 T cell cultures led to prolonged telomerase activity during multiple rounds of stimulation [28].

The importance of telomerase activity in regulating the process of replicative senescence has been documented via stable hTERT gene transduction in fibroblasts, endothelial cells and T cells [47-49]. Indeed, stable hTERT expression in HIV-specific CD8 T cells resulted in cultures that continued proliferating and maintained stable telomere lengths through many rounds of stimulation, well past the point at which the control vector transduced cultures had reached replicative senescence [49]. In addition, these transduced cultures showed enhanced inhibition of HIV replication and delayed loss of IFNγ production in response to HIV peptides [49]. Other studies, in which telomerase activity in T cell cultures was upregulated by exposure to a small molecule chemical telomerase activator (TAT2) demonstrated modest retardation of telomere shortening, increased proliferative potential, and enhanced functional cytokine/chemokine production and antiviral activity [50]. All these effects were ablated in the presence of a specific telomerase inhibitor. Overall, these *in vitro* experiments strongly implicate loss of telomerase activity upon repeated stimulation as a central player in telomere-shortening associated replicative senescence, and further confirm the importance of CD28 expression and co-stimulation in the regulation of telomerase activity.

CD8 T cell replicative senescence *in vivo*

Murine models are somewhat limited in terms of studying CD8 T cell replicative senescence in the context of immunosenescence. Although mice do undergo immunosenescence and their T cells show age-related reduced proliferative activity *in vitro*, the dynamics of both processes do not correlate with those of humans, even if one takes relative lifespan into account [51]. Additionally, human immunological aging is believed to be influenced by competition for space over several decades by lymphocytes that are specific for a variety of previously-encountered antigens [52], a situation that cannot be replicated in short lived animals that are subjected to minimal antigenic exposure, particularly with respect to persistent viral infections.

With current technology, there is no way to definitively prove that CD8 T cell replicative senescence occurs *in vivo*. Instead, researchers have conducted cross-sectional comparisons between young and old persons and short-term longitudinal studies with elderly cohorts to examine phenotypic and functional differences in selected cell populations and determine correlation to endpoints, such as morbidity and mortality. T cell replicative senescence probably occurs *in vivo* in an incremental fashion over the course of decades, and in a dynamic environment that molds cellular phenotype, making it difficult to pinpoint what exactly a senescent cell is, when senescence occurs, or the functionality of these cells. Indeed, whereas permanent *in vitro* loss of CD28 gene transcription is widely documented as a reliable indicator of senescence, *in vivo,* CD28- T cells represent a heterogenous population of cells, some of which still exhibit modest proliferative potential [16]. As stated above, for the purpose of this review replicative senescence will be used to describe *in vivo* late-differentiated cells implicated in immunosenescence that share features with their *in vitro* counterparts, which have undergone irreversible cell-cycle arrest, with the caveat that *in vivo,* such cells may not have reached strict terminal differentiation or completely exhausted their proliferative potential

Consistent with *in vitro* replicative senescence observations, perhaps the strongest indicator that CD8 T cell senescence occurs *in vivo* is the age-associated increase in T cells lacking CD28 expression. At birth, nearly 100% of human T cells express CD28 [53, 54]. As we age, the CD8+CD28- T cell population steadily increases, albeit at a variable rate, so that by age 80 these cells constitute up to 50%-60% of the peripheral blood CD8 T cell pool [55]. Cell culture and *ex vivo* work has clearly demonstrated that the CD28- population is derived from more early stage (CD28+) cells that have undergone multiple rounds of antigen-driven cellular division [56]. High proportions of CD8+CD28- T cells are correlated with reduced response to vaccinations in the elderly [57] and with autoimmune disease [58-60], providing evidence that their presence is an indicator, if not a cause, of dysregulated immunity. Moreover, despite the fact that CD28- T cells are not a homogeneous population,

overall telomere length of peripheral blood mononuclear cells (PBMC) has been shown to be significantly negatively correlated with the proportion of CD8+CD28- T cells [61]. Thus, the proportion of these late-differentiated (possibly senescent) T cells can serve as a surrogate marker for PBMC telomere length.

Cultures initiated from purified populations of CD8+CD28- T cells isolated *ex vivo* share multiple features with CD8+CD28- cells that arise in long-term repeatedly stimulated *in vitro* cultures. The signature feature of replicative senescence in cell culture is inability to enter the cell cycle, and *ex vivo* experiments on purified CD8+CD28- T cells isolated from peripheral blood are unable to proliferate *in vitro*, either in response to antigenic stimulation via the TCR or in response to mitogens, such as PMA and ionomycin [62]. Similar to CD8+CD28- cells that reach senescence *in vitro*, lymphocytes from elderly persons show attenuation in the molecular chaperone system hsp70, in steroid binding hsp90, and the chaperonin hsp60 [63], implicating reduced ability to respond to stress. *Ex-vivo* CD8+CD28- cell are also resistant to apoptosis, similar to their *in vitro* counterparts [56].

As stated earlier, CD8+CD28- T cells *in vivo* represent a very heterogeneous population, and it has been hypothesized that suppression of CD28 gene expression occurs prior to the ultimate state of differentiation/senescence [16]. Using analysis on *ex vivo* CD8 T cell populations, researchers have identified two other cell-surface markers, CD57 and CD27, which, when used in conjunction with loss of CD28, represent what is believed to be the most differentiated/senescent cell populations.

CD57 is an adhesion molecule, found on many cell types, that is believed to only be expressed on CD8 T cells that have undergone chronic proliferative activation, be it due to aging, persistent viral infection, autoimmune disease or cancer [64]. The CD8+CD57+ population increases with age, has been shown to have strong intracytoplasmic expression of cytotoxic granules [64], and has the shortest telomere lengths of any CD8 T cell subpopulation [65], indicative of an extensive proliferative history and a differentiative state close to replicative senescence. Short term culturing of CD8+CD57+ T cells indicates these cells respond to TCR stimulation with INFγ production, but are unable to proliferate [65]. T-cell receptor excision circle analysis documents that these cells have undergone more proliferative generations than other cell subtypes [65], consistent with the telomere length data. The frequency of CD8+CD57+ cells has been shown to increase in conditions associated with immune dysregulation, including HIV and CMV infection and autoimmune diseases [66]. These data constitute a compelling argument that CD8+CD57+ T cells have undergone proliferation-induced differentiation and replicative senescence.

CD8 T cells that have lost surface expression of the costimulatory molecule CD27 in conjunction with CD28 are also believed to be very late-differentiated/senescent cells. *Ex-vivo* experiments examining subpopulations of CD8+CD28+, CD8+CD28-CD27+ and CD8+CD28-CD27- T cells indicated that the CD28-CD27- subpopulation had the shortest telomeres, lowest telomerase activity, lowest IL-2 upregulation and highest IFNγ levels [67]. Despite being unable to proliferate or produce IL-2, primary CD28-CD27- CD8 T cells demonstrated high levels of cytotoxic molecules and enhanced toxicity *in vitro* [16, 68]. Initially it was believed such high cytotoxicity was a beneficial adaptation to fight infections, but recent studies have indicated the presence of CD28-/CD27- CD8 T cells is associated with poor disease control in persons infected with HIV and CMV [68, 69]. Interestingly, HIV-specific CD8 memory T cells have been shown to be predominately CD28-CD27+, whereas CMV-specific CD8 memory T cells are predominately CD28-CD27- [70]. This implies that different persistent viral infections drive memory T cells to different set-points, and that CMV may drive cells furthest along the path to replicative senescence, a notion to be addressed in depth later in this review.

HIV disease, CD8 replicative senescence and accelerated immunosenescence

With the success of antiretroviral therapy (ART) HIV infection has evolved into a chronic condition, allowing infected persons to survive into old age. Indeed, in the absence of ART, the median survival time following initial infection is 12 years [71], but with ART, patients are living up to several decades [72]. Coupling this increased survival with demographic trends showing an upward shift in the average age at initial infection, it is estimated that by 2015, more than 50% of all HIV-infected individuals in the U.S. will be older than 50 years of age [22].

Dramatically reducing CD4 T cell lymphopenia and progression towards AIDS in HIV-infected persons has increased our understanding of how the immune system responds to chronic infection over time, and the consequences of such a response propagated over several years. The results of such studies indicate that,

independent of peripheral blood CD4 lymphopenia, controlled HIV infection results in "premature immunosenescence," including accelerated CD8 T cell differentiation and replicative senescence.

HIV disease in relatively young cohorts is associated with changes in immune parameters that are remarkably similar to age-associated immune-senescence, including thymic involution, reduced circulating naïve T cells, decreased CD4/CD8 ratio, increased levels of proinflammatory cytokines, increased susceptibility to infectious disease and cancer, and reduced effectiveness of vaccines [9]. Moreover, new evidence, based on telomere and phenotypic studies, indicates that it is not only the CD8 T cell population that undergoes premature aging during HIV disease, but also specific naïve CD4 T cell subpopulations [73].

Additionally, there is strong evidence HIV disease results in accelerated increase in the number of CD8 T cell populations that have undergone replicative senescence. Compared to age-matched controls, HIV+ subjects have decreased average telomere length in the CD8 T cell compartment [74, 75], especially among CD28- T cells [62], indicating extensive cellular proliferation. Interestingly, these studies indicated no corresponding difference in CD4 T cell telomere length. Compared to age-matched controls, HIV-infected subjects also had a noticeable increase in CD28- and CD57+ CD8 T cell populations [70, 76, 77]. These late-differentiated populations tended to be highly oligoclonal and to exhibit senescent signatures, such as reduced capacity to proliferate in response to mitogens and high levels of perforin [78].

As age-associated CD8 T cell replicative senescence is associated with poor vaccine response and autoimmunity, it is not surprising that the accumulation of putatively senescent CD8 T cells in HIV disease is associated with poor prognosis. In one study, an increase in CD8+CD28-CD27- T cells was negatively correlated with CD4 T cell counts and positively correlated with disease progression [78]. In another study, an increased fraction of perforin expressing HIV-specific CD8 T cells, which were generally late differentiated, was indicative of disease progression [79]. Finally, a nested case-control study showed an increase in CD8+CD28- T cells early in disease was associated with more rapid progression to AIDS [80]. Interestingly, in that study, loss of CD28 proved to be a more accurate predictor of disease progression than proportion of CD57+ T cells.

Analysis of antigen specificity of late-differentiated oligoclonal CD8 T cell expansions in HIV disease indicates that many of the clones are specific for CMV antigens. One study that examined the differentiation state of CD8 T cells binding to tetramers for immunodominant epitopes of HIV, CMV, EBV and influenza in HIV-infected subjects noted that the greatest number of tetramer-binding clones that were CD27- recognized CMV antigens [78]. Another study analyzed CD8 T cells specific for two immunodominant CMV epitopes in HIV patients on ART, noting that a late-differentiated (CD27-CD28-CCR7-) phenotype predominated [81]. These data are consistent with previous studies indicating chronic HIV infection drives HIV specific CD8 T cells towards a moderately differentiated phenotype (CD28-/CD27+), in contrast to CMV-specific cells, which show a more late differentiated (CD28-/CD27-) phenotype [70]. In sum, these observations suggest that chronic activation and accelerated immunosenescence in HIV infected persons might be responsible for driving CMV-specific CD8 T cells into replicative senescence.

IRP, CMV and how CD8 senescent T cells may contribute towards immunosenescence

The Swedish OCTO/NONA studies were short-term longitudinal analyses that followed free-living persons > 85 years of age, with the goal of finding immune biomarkers representing a risk profile for morbidity and mortality in the elderly [15]. These studies identified an immune risk profile (IRP) present in 15-20% of 85 year olds associated with 2, 4, and 6 year mortality at follow-up. The initial IRP included an inverted CD4:CD8 ratio, caused by accumulation of CD8 cells lacking CD28 expression, poor T cell proliferative response to mitogen, and low B cell numbers [82]. It was subsequently shown that seropositivity for human CMV (but not for other persistent viruses, such as EBV, HSV or VZV) was also predictive of the IRP. Analysis of CMV infection showed that 100% of 85 year olds with the IRP group were seropositive for CMV, versus only 85% who were not in the IRP [15].

Follow-up studies have provided valuable insights into why CMV-specific CD8 T cell differentiation towards exhaustion/replicative senescence is a defining feature of immunosenescence in aging and HIV disease, and how such late-differentiated/senescent cells might contribute to the IRP and increased morbidity and mortality. It was demonstrated the accumulation of CD8 T cells responsible for the inverted CD4:CD8 ratio tended to be late-differentiated oligoclonal expansions specific for CMV antigens [14, 83, 84], comprising up to 45% of the total CD8 T cell pool [85]. These late-differentiated CMV-specific

clones in the elderly were subsequently shown to have reduced functionality *in vitro* compared to CMV clones from younger subjects [14, 84]. Interestingly, it was shown that for persons in the IRP group, an inverted CD4:CD8 ratio correlated with a relatively low number of total CD8 T cell clonal expansions and increased mortality [14]. A possible explanation for this observation is that large populations of late-differentiated immunodominant clones, often replicatively senescent or otherwise displaying impaired functionality, occupy immunological space and restrict the development of new clones specific for other epitopes/antigens, compromising overall immunity. Consistent with this idea, it is believed the association between immunosenescence and a decrease in naïve T cells is due, in part, to late-differentiated CD8 T cells crowding out naïve T cells in the context of a T cell pool restricted to a specific size through homeostatic mechanisms [86].

A consideration of all the above evidence allows one to construct a model for how the IRP is created, and why it might both contribute to immunosenescence and also be a predictor of mortality. During the course of chronic CMV infection over many decades, CD8 T cells recognizing immunodominant CMV epitopes are periodically stimulated, and over time they proliferate, differentiate and develop features of senescence, including accumulation of cell-surface senescence markers (CD28-/27-, CD57+), downregulation of telomerase, telomere shortening, and loss of proliferative capacity. As these cells accumulate, due, at least in part, to apoptosis resistance, they displace more functional cells that are specific for other antigens, reducing the overall T cell repertoire and immune competence. To compound matters, when these late-differentiated cells reach senescence, they may lose efficacy. The general loss of immune control results in a higher pathogen load and correspondingly higher levels of systemic inflammation. Additionally, late-differentiated CD8 T cells themselves contribute to the inflammatory milieu through secretion of TNFα and IL-6. This high level of systemic inflammation, termed "inflammaging" and itself a feature of immunosenenesence, is believed to further exacerbate age and HIV-mediated immunosenescence and contribute to mortality, as will be discussed below.

Thus far, CMV has primarily been viewed as a contributing factor — rather than an actual cause-- of age-related immunosenescence. However, there is some evidence that CMV may be a primary cause of immunosenescence, independent of age. For example, one study showed a strong association between CMV seropositivity and increased number of CD28- CD4 and CD8 T cells, irrespective of age, although age and CMV status were important determinants of other immune parameters, such as total T cell number, the number of CD8 T cells, and the number of CD8 T cells expressing CD45RA or CD28 [87]. Another study showed CMV seropositivity to be correlated with a 60% increase in the size of CD8 T cell memory pool and with a reduction in naïve CD8 T cells across *all* age groups [88]. A third study indicated that, in addition to driving the loss of naïve CD8 T cells, CMV seropositivity is associated with a Th1 polarization of the immune system, possibly resulting in increased inflammation [89]. Supporting this notion, two epidemiological studies have noted a correlation between CMV seropositivity, increased inflammatory biomarkers, and morbidity in the elderly [90, 91].

In future studies of immunosenescence, it will be important to design experiments to test the effects of CMV seropositivity across various age groups, to further our understanding how CMV infection contributes towards age related immunosenescence and may influence immunosenescence independent of age. One variable that is impossible to determine is the length of time persons have been infected with CMV. It should also be noted that the rare CMV-negative elderly persons do, in fact, show some characteristics of immunosenescence [82] and there is evidence that, in the absence of CMV infection, immunosenescence may be driven by other persistent viruses, such as EBV [92], indicating CMV seropositivity is not absolutely necessary for immunosenescence (although all persons in the IRP are CMV seropositive).

Similarly, as stated above, there is some evidence that HIV infection may accelerate immunosenescence by driving differentiation and replicative senescence of not only HIV-specific, but also CMV-specific CD8 T cells. To test this idea it would be necessary to compare the progression of immunosenescence between HIV+CMV+ and HIV+CMV- cohorts, an extremely challenging endeavor, due to the paucity of HIV+CMV- individuals. Only through careful design of future experiments testing CMV seropositive versus seronegative cohorts will we be able to accurately elucidate the precise influence of CMV seropositivity on age and HIV-mediated immunosenescence, and determine whether vaccinating against CMV infection, or aggressively treating CMV reactivation will affect immune status, morbidity and mortality.

Highly differentiated CD8+CD28- T cells: role in suppression and carcinogenesis?

In addition to contributing to immunosenescence through inflammatory cytokine secretion, impaired functionality against antigens, and decreasing overall T cell repertoire, late-differentiated CD8+CD28- T cells may have suppressor functions *in vivo*. CD8+CD28- cells have been found to suppress immune reactivity by inducing APC's to become tolerant to helper T cells [93, 94]. In addition, transplantation studies have identified donor-reactive CD8+28- cells in patients with successful organ transplants, but not in patients who experienced rejection, implicating a positive role for immune suppression, at least in this particular clinical situation [93]. In HIV-infected persons, CD8+57+ T cells were shown to be positively correlated with lymphocytic alveolitis disease progression and to have suppressive influences on HIV-specific CTL activity [95], indicating a role for these cells in immune suppression *in vivo*.

The effect of chronic antigenic stimulation in driving CD8 T cells to senescence may be relevant to tumor antigens as well as persistent viral infections. Indeed, CD8+CD28- T cells can be purified from various types of human tumors, including lung [96], colorectal [97], endometrial [98], ovarian [99], lymphoma [100] and breast [101, 102], as well as from metastatic satellite lymph nodes and peripheral blood of cancer patients [96, 103]. Interestingly, in patients with large granular lymphocyte leukemia, the CD8+CD28- T cells have acquired the ability to directly lyse arterial endothelial cells [104]. Expanded clonal populations of cytotoxic T cells with other features of replicative senescence (e.g., CD57 expression) are also present in melanoma and multiple myeloma patients [105, 106], and the abundance of senescent CD8 T cells correlates with tumor mass in head and neck cancer [107]. Moreover, in renal cancer, the proportion of CD8 T cells with features of replicative senescence is actually predictive of patient survival [108]. Tumor-reactive, memory CD8 T cells are also present in prostate cancer patients [109]. Importantly, immune evasion in these patients is associated with a population of quiescent CD8 T cells lacking perforin and inferferon-γ expression [110]—features that are identical to characteristics of *in vitro* senescent HIV specific CD8 T cell cultures [37]. At this time, it is not known whether late-differentiated CD8+28- actively repress anti-cancer immunity or passively fail to confer it.

Senescent cells have also been documented to contribute to cancer by creating a favorable tumor microenvironment. For example, senescent fibroblasts have been shown to enhance pre-malignant epithelial cell growth both *in vitro* and *in vivo* [111]. Interestingly, molecules released by CD8+CD28- T cells from HIV-1-infected patients promote endothelial-cell (EC) growth and induce ECs to acquire spindle cell morphology and upregulation of surface receptors that mimic the phenotype of Kaposi's Sarcoma, an AIDS-defining cancer [112]. This intriguing connection between T cell replicative senescence, cancer immunosurveillance, and tumor cell biology is an area that merits further investigation. Finally, there is clinical evidence that adoptive immunotherapy using CD8 T cells may ultimately be affected by the process of replicative senescence. Indeed, the degree of immune impairment of adoptively transferred tumor-specific CD8 T cells is directly related to the number of rounds of *in vitro* cell division prior to cell transfer [113]. Also, the persistence and expansion capacity of tumor-infiltrating CD8 T cells correlates with two key parameters of replicative senescence, namely, telomere length and CD28 expression [114-116].

"Inflammaging" and CD8 T cell replicative senescence

"Inflammaging," a term promoted by Fransceschi [117], describes how systemic low grade inflammation, a defining feature in age- and HIV-mediated immunosenescence, contributes to morbidity and mortality, often through damage to non-immune organs, resulting in chronic diseases. A common hallmark of aging and age-related immunosenescence is increased low-grade systemic inflammation, as evidenced by elevated serum CRP, IL-6, Amyloid A and TNFα [6, 118, 119], among other markers. Along with genetic interactions, this pro-inflammatory status is implicated as causative in a number of age-related conditions, including cardiovascular disease, atherosclerosis, metabolic syndrome, type 2 diabetes, obestity, neurodegeneration, arthrosis and arthritis, osteoporosis and osteoarthritis, sarcopenia, depression and frailty [6]. For example, CRP levels have been widely implicated as a moderate predictor of risk of coronary heart disease [120] and high levels of CRP, IL-6 and TNFα have been found to correlate with increased all-cause mortality in elderly men [121, 122].

Similar to aging, HIV disease is associated with relatively high levels of immune activation and systemic inflammation. Common biomarkers of this process include elevated levels of activated CD4 and CD8 T cells, high CD8 T cell counts, increased levels

of inflammatory cytokines such as IL-1β, IL-6 and TNFα, and activation of the coagulation pathway [9, 123]. An important factor believed to be driving HIV-mediated inflammation is the immune response directly against HIV antigens, both infectious and noninfectious particles. This is supported by observations that inflammatory markers decline with initiation of anti-retroviral therapy (ART) and suppression of HIV viral levels [9]. However, even in ART-suppressed subjects, inflammatory markers are elevated compared to non-infected controls [124, 125]. Possible causes for this sustained elevated inflammation include loss of inflammatory repressing regulatory CD4T cells, thymic atrophy, increased copathogen load, and loss of gut mucosal integrity and the resultant microbial translocation [9].

Recent evidence indicates this last point may be especially important in maintaining high systemic levels of inflammation, even when ART therapy successfully suppresses HIV replication. It is known the GALT (gut-associated lymphoid tissue) is an important site of acute viral replication, with severe local CD4 depletion [126], and that during ART therapy CD4 levels in the GALT do not recover nearly as well as they do in peripheral blood, presumably due to incomplete viral suppression and inflammatory effects [127]. Additionally, SIV models using rhesus macaques indicate Th17 CD4 cells in the GALT, which are particularly important in controlling microbial pathogens, are especially susceptible to SIV infection, leading towards a high rate of loss of these cells and increased microbial translocation from the gut into peripheral blood [128]. Supporting this evidence, additional studies on HIV-infected patients and SIV models indicate both conditions cause elevated levels of highly antigenic circulating microbial products, such as lipopolysaccharide [129]. As a whole, these data provide a compelling picture of how Th17 CD4 depletion, microbial translocation, and inflammatory responses against microbial products create an environment of chronic immune activation observed in HIV infection, even under conditions of ART therapy and peripheral blood viral suppression.

HIV disease is known to cause a 3-fold higher risk of death from all-cause mortality [130]. As expected, approximately 50% of the deaths in HIV patients on ART were attributed to AIDS-defining conditions, such as opportunistic infections [131, 132]. However, the remaining deaths are due to non-AIDS defining age-related illnesses associated with inflammation, including cardiovascular disease, kidney disease, liver disease, osteoporosis, non-AIDS cancers, neurologic disease and frailty [132-139]. Corroborating these data, studies indicate an increase in inflammatory markers, such as CRP, in HIV infected patients is independently correlated with accelerated progression to AIDS and an increase all cause mortality [140-142]. For this reason HIV infection is generally believed to be causing "accelerated immunosenescence," due in large part to effects from chronic immune activation and inflammation [143].

Late-differentiated, putatively senescent CD8 T cells are both affected by, and contribute to, inflammaging. Viral antigens (e.g., HIV and CMV) and the inflammatory mediators they elicit are implicated in driving CD8 T cell proliferation and differentiation, ultimately leading to replicative senescence. For example, there is evidence linking high levels of cellular activation with greater CD8 T cell differentiation towards a senescent phenotype [78]. Senescent CD8 T cells, in turn, secrete inflammatory cytokines, such as TNFα, which promote further CD8 T cell differentiation and loss of CD28 [34], among other effects. Linking the presence of senescent CD8 T cells with disease, *in vitro* culture experiments and studies in mice both document that activated T cells exposed to inflammatory mediators show increased mRNA and protein expression of RANKL, an osteoclastic mediator implicated in osteoporosis [144]. Interestingly, accumulation of CD28- T cells is associated with several autoimmune diseases such as rheumatoid, multiple sclerosis, and anklyosing spondylitis [16], in which premature bone loss is observed, suggesting a role for these cells in dysregulated inflammatory conditions.

Concluding Remarks

Antigen-driven differentiation towards the end stage of replicative senescence in CD8 T cells is an important component of both age- and HIV-mediated immunosenescence and the IRP. However, one important caveat regarding these observations is that most of our knowledge on immunosenescence in humans has been derived from studies on peripheral blood, which contains only 2% of total body lymphocytes. At this time, little is known about the dynamics of CD8 T cell aging in other tissues, especially the GI tract, which houses 60% of total body lymphocytes and is a major reservoir for HIV infection. As we move forward in defining the contribution of CD8 T cell replicative senescence to human immunosenescence, it will be critical to elucidate aging dynamics of lymphocytes in the GI tract. Our own preliminary studies suggest that T cells from the GI tract may be more antigen-experienced and further

differentiated than those in peripheral blood (J. Dock, unpublished observations). For example, as compared to the peripheral blood, the GI tract has a higher proportion of memory and effector memory CD8 T cells, increased expression of activation markers, and higher percentages of T cells lacking CD28 expression (J. Dock, unpublished observations). Thus, it is only via a systematic investigation of the process of lymphocyte aging dynamics in tissues outside the peripheral blood that we will be able to develop more effective therapies to combat both age- and HIV-mediated immunosenescence. Current research in our own laboratory is addressing this challenge.

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