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## The role of CD8<sup>+</sup> T-cell replicative senescence in human aging

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**Summary:** The strict limit in proliferative potential of normal human somatic cells – a process known as replicative senescence – is highly relevant to the immune system, because clonal expansion is fundamental to adaptive immunity. CD8<sup>+</sup> T cells that undergo extensive rounds of antigen-driven proliferation in cell culture invariably reach the end stage of replicative senescence, characterized by irreversible cell-cycle arrest and a critically short telomere length. Cultures of senescent CD8<sup>+</sup> T cells also show resistance to apoptosis, permanent loss of CD28 expression, altered cytokine profiles, reduced ability to respond to stress, and various functional changes. Cells with similar characteristics accumulate during normal aging as well as in younger persons infected with human immunodeficiency virus, suggesting that the process of replicative senescence is not an artifact of cell culture but is also occurring *in vivo*. Interestingly, in elderly persons, the presence of high proportions of CD8<sup>+</sup> T cells with characteristics of replicative senescence is correlated with reduced antibody responses to vaccines as well as with osteoporotic fractures. CD8<sup>+</sup>CD28<sup>-</sup> T cells also accumulate in patients with certain types of cancer. The emerging picture is that senescent CD8<sup>+</sup> T cells may modulate both immune and non-immune functions, contributing not only to reduced anti-viral immunity but also to diverse age-related pathologies.

### Introduction

The phenomenon of replicative senescence was first described by Hayflick and colleagues nearly 50 years ago (1). For the next few decades, gerontologists became singularly focused on what now seems like a naïve hypothesis, namely, that the proliferative behavior of cells propagated *in vitro* was somehow related to organismic lifespan. Numerous papers documented correlations between species lifespan and the *in vitro* proliferative potential of normal cells (2–4). Attempts were also made to relate donor chronological age to population doublings (PDs) that various types of human cells derived from that donor could achieve in cell culture (5).

It is now clear that the goal of relating the behavior of cells in tissue culture to *in vivo* lifespan was far too simplistic, and the notion that we die because our cells run out of divisions is certainly not correct. Interestingly, however, it turns out that

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within the immune system, there is, in fact, an intriguing relationship between replicative senescence and lifespan but not for the reasons originally posed by gerontologists. What has recently been shown is that proportion of senescent CD8<sup>+</sup> T cells present in the peripheral blood is a key component of the so-called 'immune risk phenotype', a cluster of immune measures obtained in elderly persons that is predictive of early all-cause mortality (6). In another study, lymphocyte telomere length in a cohort of elderly persons was significantly correlated with subsequent mortality (7). An interesting aspect of this study was that death from infection was seven to eight times more likely in individuals whose lymphocyte telomere length was in the shortest quartile, suggestive of a link with T-cell immunity. Thus, the study of the immune system cells, and T cells in particular, has generated renewed interest in the potential link between mechanisms by which replicative senescence might exert an influence on aging and lifespan.

In this article, we review our own research on the characterization of the process of CD8<sup>+</sup> T-cell replicative senescence *in vitro* and provide examples of the accumulation of cells with the same characteristics during aging as well as in certain disease states. We posit that, similar to replicative senescence in other cell types, the multiple functional attributes of senescent CD8<sup>+</sup> T cells implicate these cells in a variety of age-related pathologies. More complete characterization of the full spectrum of molecular, genetic, and functional characteristics associated with senescence in this cell population, therefore, has the potential to lead to novel telomere-based approaches at rejuvenating the immune system.

### Historical perspective on replicative senescence within the immune system

Our work was originally motivated by what seemed to be two parallel universes within the scientific community. For a period of at least a decade, there existed two major groups of investigators – cell biologists and immunologists – whose views on a critical aspect of cell behavior were incompatible. It appeared that each group was totally ignorant of the mutually exclusive and conflicting set of in basic theoretical tenets espoused by the other. On the one hand, beginning in the 1960s, cell biologists and gerontologists were generating a large number of publications documenting the characteristics of the so-called Hayflick Limit, the innate barrier to unlimited proliferation that is characteristic of normal human cells. Indeed, this process of replicative senescence, which was documented for a variety of cell types including fibroblasts, epithelial cells, hepatocytes, endothelial cells, and keratino-

cytes, soon became a cornerstone of gerontological thinking (8–12).

At about the same time that replicative senescence was being increasingly characterized at the genetic and molecular level by cell biologists and gerontologists, a new T-cell growth factor [now called interleukin-2 (IL-2)] was discovered, and the immunology literature abounded with reports describing seemingly unlimited growth of supposedly normal human lymphocytes (13–15). Phrases such as 'T cells can grow continuously *in vitro*', 'rapidly growing and apparently permanent cultures', and 'supernatant fluids . . . have been used to immortalize cells from mixed lymphocyte cultures' were typical of the extensive body of published reports (16, 17). Frequent descriptions of 'immortal T-cell lines', 'permanent T-cell cultures', and 'T cells capable of indefinite proliferation' suggested that cells of the immune system might be an exception to the rule of limited proliferative potential. Alternatively, it was also possible that the apparently immortal T-cell lines had undergone some sort of transformation and were therefore not necessarily subject to the limit defined by Hayflick, which is restricted to normal cells. In any case, it was this apparent conflict in certain fundamental notions of cell biology that motivated our own *entrée* as immunologists into the field of replicative senescence (18).

Based on our work and that of several other laboratories, it is now generally accepted that normal human T cells, like all other cell types, undergo replicative senescence in cell culture (19, 20). An immediate question arising from this observation is how the limited proliferative potential of T cells might be predicted to affect normal immune function. From the *in vitro* data obtained by various investigators, a preliminary estimate suggests that the number of PDs achievable by each T cell is so large that the finite replicative lifespan would not be biologically meaningful *in vivo*. For example, if a T cell has an average lifespan of 35 PDs (21) and if all daughter cells continue to grow unchecked, the end result would exceed 10<sup>10</sup> cells, a cell yield that seems at first glance to be more than sufficient. However, this may not be the case, because T-cell expansion includes both extensive proliferation and apoptotic removal of excess cells. Thus, the finite proliferative lifespan of T cells might only allow two or three rounds of antigen-driven expansion before the response is terminated due to the exhaustion of the ability to undergo further cell division. Even in the absence of antigen-specific restimulation, memory cells continue to divide *in vivo*, further accelerating the process of replicative senescence. The above crude estimate suggests that the limited proliferative potential might be particularly detrimental *in vivo*, particularly by old age (21).

In thinking about the potential effect of T-cell replicative senescence on the aging immune system and, indeed, on the aged organism in general, it is instructive to highlight some of the important concepts derived from the extensive research on senescence in other cell types (22). One of the major discoveries in fibroblasts is the extensive battery of genes whose expression is totally altered in senescent versus early-passage cells. Indeed, there are literally dozens of genes whose expression is either upregulated or downregulated in conjunction with senescence (23). These genetic changes are associated with drastic changes in function. For example, senescent fibroblasts cease producing factors that enhance intercellular matrix integrity and start secreting matrix-destroying enzymes, such as collagenase. Moreover, senescent fibroblasts can actually enhance the proliferation of epithelial cell tumor lines both in cell culture and in immunodeficient mice (24). Thus, although cell cycle arrest is the most easily identified phenotype associated with replicative senescence, the process involves multiple changes at the genetic and functional level.

#### Characterization of T-cell replicative senescence in cell culture

A systematic analysis of the long-term *in vitro* behavior of CD4<sup>+</sup> and CD8<sup>+</sup> T cells has been performed in numerous laboratories. The major overall observation from these studies is that in no case has a normal culture proliferated indefinitely. A rare instance of a seemingly spontaneously immortal culture was subsequently shown to have trisomy of chromosome 2 and to be infected with mycoplasma (25). Interestingly, in the two other reports of spontaneously arising immortal human T-cells lines, it happens that chromosome 2 trisomy was also the culprit (21).

Based on the extensive clinical data suggesting reduced control of viral infections in the elderly, our own work in the area of replicative senescence has focused on the CD8<sup>+</sup> T-cell subset. We initiated our studies by demonstrating that in over 100 T-cell cultures, derived from peripheral blood samples from both adults and neonatal donors, the number of PDs ranged from 11 to 57, with a mean of  $23 \pm 7$  (26). Although there was a significant difference in the PDs achieved by cells from adult versus neonatal donors ( $P < 0.025$ ), among the adult donors, there was no correlation between *in vitro* lifespan and chronological age. These alloantigen-stimulated cultures were shown to retain antigen-specific cytotoxic function as well as antigen-specific upregulation of the  $\alpha$  chain of the IL-2 receptor even at senescence, despite the inability to enter cell cycle when exposed to the same alloantigen (27).

Several other notable changes in cellular processes were documented for allospecific senescent cultures. First, as was reported for senescent fibroblasts (28), CD8<sup>+</sup> T-cell cultures that reach senescence become resistant to apoptosis (29). This diminished ability of senescent T cells to undergo apoptosis was observed using a variety of stimuli that initiated robust levels of apoptosis in their early-passage progenitors, such as antibody to Fas, IL-2 withdrawal, mild heat shock, galectin-1, and staurosporine. The reduced apoptosis was associated with increased bcl2 expression and reduced levels of caspase-3. Second, the ability to respond to stress by upregulation of the major mammalian stress protein, heat shock protein-70, was markedly diminished in senescent versus early-passage T-cell cultures (30). Interestingly, reduced ability to respond to physical and oxidative stress is believed to play a major role in normal aging (31, 32).

In addition to assessing functional characteristics of the senescent cultures, we compared the cell-surface expression of a variety of T-cell markers reflecting lineage, activation, adhesion, and memory, and found no change between early passage and senescent T cells (27). The single exception to date is the CD28 costimulatory molecule. As T cells progress to senescence in culture, the proportion of cells that are CD28<sup>-</sup> increases, and senescent cultures are >99% CD28<sup>-</sup> (33). The loss of CD28 expression is associated with complete suppression of gene expression of this key T-cell-signaling molecule. The intimate relationship between CD28 expression and replicative senescence is further underscored by the trisomy 2-related T-cell spontaneous immortalizations mentioned above, because the CD28 gene in fact is located on chromosome 2.

Defining the senescent phenotype using long-term cell culture has the advantage of allowing longitudinal analysis of the same population of cells over time, but is also hindered by the possibility of missing certain marker changes that might be influenced by the *in vivo* milieu. Indeed, whereas loss of CD28 expression has been defined in cell culture and presumed to be the ultimate senescence-associated change, it is possible that other cell-surface antigens identified on *ex vivo* samples may delineate a more precise phenotype for the true end-stage senescent CD8<sup>+</sup> T cells. Certain other putative senescent markers, such as CD57, have already been proposed, particularly for human immunodeficiency virus (HIV)-specific CD8<sup>+</sup> T cells (34). Indeed, there are many features that are shared by CD57<sup>+</sup> and CD28<sup>-</sup> T cells isolated *ex vivo* (35–43). It has also been shown that CD56, a natural killer (NK) cell marker bearing the human NK-1 (HNK-1) epitope, is expressed on some CD28<sup>-</sup> cells within the CD8<sup>+</sup> T-cell subset. Interestingly,

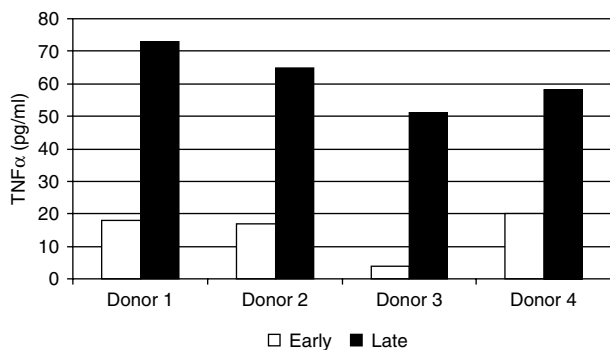
acquisition of major histocompatibility complex (MHC)-unrestricted NK function has been documented for CD8<sup>+</sup> T cells that reach senescence in cell culture (44).

As has been extensively documented in fibroblasts, CD8<sup>+</sup> T cells that progress to senescence in long-term culture undergo a variety of functional changes. Alloantigen-specific cultures from healthy donors produce increasing amounts of two pro-inflammatory cytokines, IL-6, and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (Fig. 1), and virus-specific CD8<sup>+</sup> T cells show decreased antigen-specific production of interferon  $\gamma$  (IFN $\gamma$ ) (Fig. 2).

HIV-specific CD8<sup>+</sup> T-cell cultures initiated from T cells of HIV-infected persons show a progressive decline in the ability to perform antigen-specific lysis of target cells pulsed with HIV peptides, a change that is accompanied by reduced expression of perforin (45, 46). Moreover, one of the key anti-viral activities believed to function protectively *in vivo* – inhibition of HIV replication – is markedly reduced in senescent HIV-specific CD8<sup>+</sup> T cells (47). One can envision that such functional changes might exert pleiotropic influences on a variety of physiological processes *in vivo*, both in the context of aging and chronic HIV infection.

### Telomere length is the clock that controls replicative potential in human cells

In human cells, the mechanism that controls the process of senescence is believed to be telomere shortening (11, 48). Due to the end-replication problem, normal somatic cells undergo progressive telomere shortening with cell division, and it is the



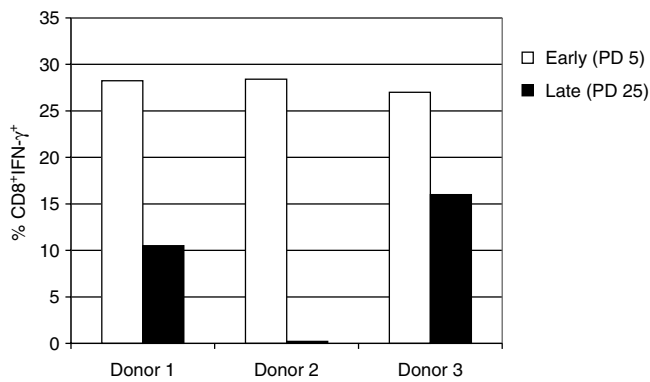
**Fig. 1. Increased levels of tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ) with culture age.** Long-term alloantigen-specific cultures were established as described (29) from four individual healthy donors. Culture supernatants were assessed by enzyme-linked immunosorbent assay (ELISA) at various stages for levels of TNF  $\alpha$ . Data are for early passage [ $<10$  population doubling (PD)] and late passage ( $>20$  PD), with TNF  $\alpha$  levels (pg/ml of culture supernatant) shown on the y-axis.

critically short telomere that signals DNA damage and cell-cycle arrest. Because of the fundamental differences between mouse and human cells in terms of both telomere length and telomerase activity, it is likely that if replicative senescence is a feature of mouse T cells, it is not under telomere regulation (49, 50).

In human T-cell cultures, telomere length undergoes progressive shortening, reaching a size of 5–7 kb at senescence (51), which is similar to the telomere length in senescent cultures of other cell types (11). We addressed the question of why telomeres undergo shortening in T cells despite the high levels of telomerase activity induced in these cells as a component of the activation process (52). Indeed, the levels of telomerase activity in antigen- or mitogen-stimulated T cells are comparable to those of tumor cells (53). Kinetic analysis of T-cell telomerase activity showed that the activation-induced telomerase activity peaks at 3–5 days, then undergoes a gradual decline, becoming undetectable at approximately 3 weeks. During the period of high telomerase activity, telomere length remains stable. A second wave of telomerase activity can be induced by a subsequent exposure to the same antigen. However, the antigen-induced upregulation of telomerase in response to stimulation with antigen is markedly reduced by the third stimulation, and is totally absent in all subsequent encounters with antigen (54).

Interestingly, the above pattern is true only in CD8<sup>+</sup> T cells. A comparison of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets from the same individuals cultured *in vitro* using the identical stimulatory schedule revealed a marked difference between the two cell types. The initial stimulation by alloantigen elicited a 45-fold and 53-fold increase in telomerase activity (compared to pre-stimulation levels) in the CD8<sup>+</sup> and CD4<sup>+</sup> cultures, respectively. However, by the fourth antigenic stimulation, telomerase activity was undetectable in the CD8<sup>+</sup> T cells, whereas CD4<sup>+</sup> T cells still showed robust telomerase activity even as late as the tenth round of antigenic stimulation.

The dichotomy in telomerase dynamics between the two T-cell subsets was mirrored by the pattern of CD28 down-regulation. By the seventh antigenic stimulation, 90% of the cells in the CD8<sup>+</sup> culture no longer expressed CD28, whereas at that same stage, 75% of cells in the CD4<sup>+</sup> cultures were still CD28<sup>+</sup> (54). The importance of CD28 signaling in T-cell telomerase activation was further demonstrated by the marked inhibition of this upregulation that resulted from pre-incubation of the antigen-presenting cells with antibodies to the CD28 ligands B-7.1 and B-7.2. Experiments using the T-cell activation inhibitor cyclosporine A also highlighted the distinct role of CD28 signaling in the CD8<sup>+</sup> T-cell telomerase pathway.



**Fig. 2. Decreased production of interferon  $\gamma$  (IFN  $\gamma$ ) with culture age.** Long-term human immunodeficiency virus (HIV)-specific cultures were established by repeated stimulation of CD8<sup>+</sup> T cells from HLA-A2<sup>+</sup> HIV-infected persons with the appropriate gag, pol, and env peptides. Early-passage and late-passage cultures from three different donors were assessed for intracellular IFN  $\gamma$  using flow cytometry.

Whereas telomerase upregulation was markedly reduced in the presence of cyclosporine, when the T cells were activated with anti-CD3, there was no telomerase inhibition in cultures stimulated with a combination of anti-CD3 and CD28, in agreement with findings reported for CD4<sup>+</sup> T cells (52).

### Targeting telomerase as a strategy to modulate replicative senescence

Because telomere loss is the major controlling factor in the replicative senescence program, our recent studies have been directed at evaluating strategies to enhance telomerase activity in CD8<sup>+</sup> T cells. In these experiments, we focused on virus-specific CD8<sup>+</sup> T cells isolated from persons infected with HIV. Our results demonstrate that gene transduction with the catalytic component of telomerase (hTERT) results in telomere length stabilization and reduced expression of the p16<sup>INK4A</sup> and p21<sup>WAF1</sup> cyclin-dependent kinase inhibitors, suggesting an important role for both proteins in mediating the senescence program (45). In addition, the telomerized HIV-specific CD8<sup>+</sup> T cells are able to maintain the production of IFN  $\gamma$  for extended periods and show significantly enhanced capacity to inhibit HIV replication. We also observed that although the loss of CD28 expression was significantly delayed in the transduced cultures, it was not prevented, suggesting that additional genetic manipulation of the CD28 gene itself may be required for full correction of this important senescence-associated alteration. Finally, the senescence-associated loss of virus-specific cytotoxicity was corrected by hTERT only in selected CD8<sup>+</sup> T-cell clones, but not in the bulk, uncloned CD8<sup>+</sup> T

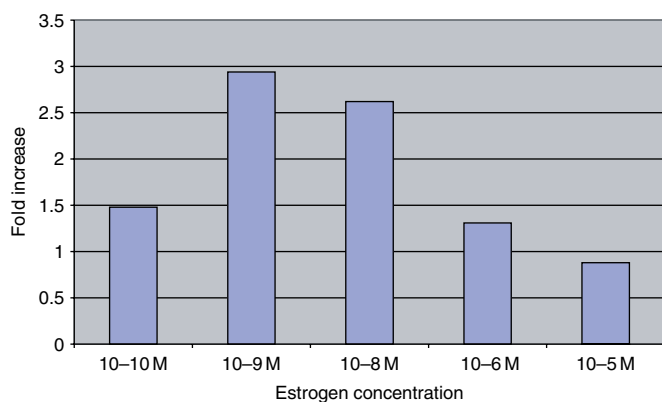
cells. Thus, hTERT corrects most, but not all, the alterations associated with replicative senescence in CD8<sup>+</sup> T cells isolated from HIV-infected persons. Whether the same spectrum of changes will be reproduced in hTERT-transduced cells from healthy donors is currently under investigation.

Non-genetic strategies that enhance telomerase activity in T cells may also cause telomere length stabilization and could possibly lead to therapies that modulate replicative senescence in vivo. For example, it is known that estrogen is able to enhance telomerase activity in reproductive tissues. The complex formed when estrogen binds to its receptors migrates to the nucleus and functions as a transcription factor. In normal ovarian epithelial cells, this complex actually binds to the hTERT promoter region (55). Because T cells also express estrogen receptors, we tested whether pre-incubation of T cells with 17 $\beta$ -estradiol prior to activation might augment telomerase activity. In preliminary studies, we have shown that estrogen does, in fact, enhance T-cell telomerase activity (Fig. 3). The enhancement is observed in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets, and it can also be seen when estrogen is conjugated to bovine serum albumin, indicating that surface estrogen receptor interaction may be sufficient to mediate the telomerase effect (Effros, manuscript in preparation).

These *in vitro* data showing estrogen-mediated telomerase enhancement accord with our earlier research on immune effects of post-menopausal hormone replacement therapy. This small cross-sectional comparison of women who were or were not taking hormone replacement suggested that estrogen was associated with a reversal of some of the age-related T-cell changes (56). In another set of preliminary experiments with small molecule activators of telomerase, we have shown a significant enhancement of telomerase activity in T cells from both healthy and HIV-infected persons (Fauce et al., manuscript in preparation). Thus, telomerase-based approaches seem to be promising candidates for therapies aimed at reversing or retarding the process of replicative senescence in T cells.

### Replicative senescence occurs *in vivo*

It is essentially impossible to formally prove that replicative senescence is occurring *in vivo*. However, the presence of peripheral blood T cells with characteristics that precisely mirror those observed in long-term culture provides cogent evidence in support of this notion. Initial demonstration that aging is associated with high proportions of T cells that lack CD28 expression was provided in our original report identifying the loss of CD28 as a marker of senescence in long-term



**Fig. 3. Estrogen enhances telomerase activity in activated T cells.**

Peripheral blood T cells from an adult female donor were pre-incubated for 1 h in media alone (control) or in increasing concentrations of estrogen ( $10^{-10}$  M to  $10^{-6}$  M), after which phytohemagglutinin (PHA) ( $10 \mu\text{g/ml}$ ) was added to each well. Cells were assayed after 72 h for telomerase activity, using the commercial Telomerase Repeat Amplification Protocol (TRAP-eze kit). Data are presented as the fold increase over the telomerase activity of control cultures.

culture (33). In that study, which compared 20 adults (age 25–69) and 21 healthy centenarians, there was a significant decrease ( $P < 0.01$ ) in the proportion of T cells that expressed CD28 in the elderly, with some aged individuals demonstrating values as low as 44, 53, and 54% CD28<sup>+</sup> T cells, compared to the mean young adult value of 91% CD28<sup>+</sup> T cells. The decrease in the percentage of CD28<sup>+</sup> T cells with age was not associated with an alteration in the intensity or standard deviation of mean fluorescence, suggesting that the expression of CD28 is normal on those cells that do score as CD28<sup>+</sup>. In addition, we showed a correlation between the reversal of the youthful CD4/CD8 ratio and the presence of high proportions of CD28<sup>-</sup> T cells. In other words, those elderly persons who had the highest proportion of CD28<sup>-</sup> T cells also had CD4/CD8 ratios that reflected an increased proportion of CD8<sup>+</sup> T cells. This observation accords with a report showing that altered CD4/CD8 ratios might be due to CD8<sup>+</sup> oligoclonal expansions in the elderly (57). These oligoclonally expanded populations were shown to resemble cells in senescent cultures by virtue of their level of activation, the absence of CD28 expression, and their inability to proliferate. In a subsequent study, we showed that there is a progressive increase over the lifespan in the proportion of T cells that lack CD28 expression (58).

A dramatic example of the probable occurrence of replicative senescence *in vivo* is in the context of chronic HIV infection. It had been observed for some time that HIV-infected persons had an unusual population of CD8<sup>+</sup> T cells that lacked CD28 expression (59–62). In some reports, this subset was suggested to have arisen as a distinct lineage, possibly related to some unusual aspect of HIV disease pathogenesis. However, based

on our cell-culture studies, we hypothesized that these cells were the descendants of cells that previously did express CD28 but which had undergone extensive proliferation and reached the end stage of replicative senescence. To test this idea, we isolated CD8<sup>+</sup> T cells from HIV-infected persons and sorted them further into CD28<sup>+</sup> and CD28<sup>-</sup> populations. The telomere length of the CD28<sup>-</sup> population was significantly shorter than that of the CD28<sup>+</sup> population from the same donor. In fact, the mean telomere length of the CD8<sup>+</sup>CD28<sup>-</sup> T cells from persons infected with HIV (mean age 43 years) was in the same range as that of peripheral blood mononuclear cells isolated from centenarians (63) as well as that of T cells that reach replicative senescence in culture (51). The sorted CD8<sup>+</sup>CD28<sup>-</sup> T cells tested immediately *ex vivo* showed minimal proliferative ability, even when the stimulation bypassed cell-surface-mediated activation signals. These data suggest that replicative senescence is a key component of the immunodeficiency associated with HIV disease (64). Interestingly, in addition to CD8<sup>+</sup> T-cell replicative senescence, several other aspects of the immune system in HIV-infected persons resemble features observed in the elderly, leading to the suggestion that accelerated aging of the immune system is a central feature of HIV disease (65).

In HIV-infected persons, CD8<sup>+</sup> T cells lacking CD28 expression accumulate progressively over time, and in some cases constitute >65% of the peripheral blood CD8<sup>+</sup> T-cell pool (63). The large proportion of senescent CD8<sup>+</sup> T cells may play a significant role in the ultimate immune collapse that is the hallmark of HIV disease. Indeed, the importance of functional and proliferative CD8<sup>+</sup> T cells in controlling HIV infection is highlighted by a number of clinical observations. For example, in long-term non-progressors, HIV-specific CD8<sup>+</sup> T cells maintain high levels of perforin as well as robust cytotoxic and proliferative capacity (66–69). Conversely, in other individuals, the progression to acquired immunodeficiency syndrome (AIDs) coincides with a decline in cytotoxic T-lymphocyte activity and reduced proliferative potential in HIV-specific CD8<sup>+</sup> T cells (70–77). Replicative senescence may also have a negative effect on the role of CD8<sup>+</sup> T cells in inhibiting HIV replication, an activity that is assumed to contribute to reduction of viremia *in vivo* (71, 78–81). Because this non-cytolytic effector mechanism has been attributed to CD8<sup>+</sup> T cells that express CD28 (82), it can be assumed that CD8<sup>+</sup> T cells that no longer express CD28 do not contribute to this particular anti-viral effect.

#### Antigen specificity of senescent T cells that arise *in vivo*

The specificity of the putatively senescent cells in HIV-infected persons as well as in elderly persons has been investigated. In

the case of HIV disease, CD8<sup>+</sup> T cells that recognize HIV peptides on infected cells would be one potential population that might undergo clonal exhaustion and senescence. During the asymptomatic phase of infection, viral replication has been estimated at approximately 10<sup>10</sup> virions per day, indicating that HIV-specific CD8<sup>+</sup> T cells are subjected to continuous antigenic stimulation over many years. The failure to eradicate the virus by even the most successful current therapy protocols suggests that even during treatment, HIV-specific CD8<sup>+</sup> T cells are subject to continued antigenic exposure. The presence of identical CD8<sup>+</sup> clonotypes within the CD28<sup>+</sup> and CD28<sup>-</sup> populations (83) confirms the relationship between these two subsets. Further, based on telomere measurements (63), one can conclude that the CD28<sup>-</sup> cells are the clonal progeny of CD28<sup>+</sup> cells that had undergone extensive cell division.

HIV is not unique in its property of driving CD8<sup>+</sup> T cells to the end stage of replicative senescence. Indeed, in some HIV-infected persons, there are substantial numbers of CD8<sup>+</sup>CD28<sup>-</sup> T cells that are specific for cytomegalovirus and Epstein–Barr virus. In the elderly as well, latent infection with herpesviruses seems to be the major driving force for the generation of senescent CD8<sup>+</sup> T cells. We have hypothesized that the cost of maintaining control over these latent infections is the progressive generation of senescent CD8<sup>+</sup> T cells (84). The apoptosis resistance of these cells leads to a situation where they occupy progressively more and more of the memory T-cell pool in the elderly, restricting the repertoire of the remaining T cells. Latent viruses associated with certain forms of cancer can also drive CD8<sup>+</sup> T cells to senescence, as illustrated by the accumulation in cervical cancer patients of CD8<sup>+</sup>CD28<sup>-</sup> T cells with reactivity to human papillomavirus E7 antigen (85).

#### Senescent CD8<sup>+</sup> T cells have regulatory roles *in vivo*

The presence of senescent CD8<sup>+</sup> T cells *in vivo* may have a variety of effects on immune function. First, they undoubtedly influence the quality and composition of the memory T-cell pool. Once generated, senescent CD8<sup>+</sup> T cells persist, due to their resistance to apoptosis, leading to their progressive accumulation over time. Because homeostatic mechanisms are believed to independently regulate the memory and naïve T-cell pools (86), a high proportion of senescent cells will result in the reduced proportion of proliferation-competent, non-senescent memory cells, a situation that may also indirectly influence other memory T cells of unrelated specificities. The fact that CD28<sup>-</sup> T cells are usually part of oligoclonal expansions (57, 87) would presumably also lead to a reduction in the overall spectrum of antigenic specificities within the T-cell

pool. The repertoire of antigenic specificities, in fact, is reduced in elderly persons who have high proportions of CD8<sup>+</sup> T cells lacking CD28 (88).

A more direct effect of CD8<sup>+</sup>CD28<sup>-</sup> T cells appears to be in the area of suppressor cell activity. It has been reported that a population of MHC class I-restricted CD8<sup>+</sup>CD28<sup>-</sup> T cells generated in the course of *in vitro* and *in vivo* immunizations functions to suppress immune reactivity by inducing antigen-presenting cells to become tolerogenic to helper T cells with the cognate antigen specificity (89). Interestingly, this same subset has been specifically implicated in the tolerance to allogeneic organ transplants. Donor-specific CD8<sup>+</sup>CD28<sup>-</sup> T cells are detectable in the peripheral blood of those patients with stable function of heart, liver, and kidney transplants, whereas no such cells were found in patients undergoing acute rejection (89). This observation suggests a possible suppressive function of the putatively senescent cells that may reduce reactivity against the allograft. It is possible that similar antigen presentation or helper cell effects mediated by CD8<sup>+</sup>CD28<sup>-</sup> T cells may explain the significant correlation between high proportions of these cells and poor antibody response to the influenza vaccination in the elderly (90, 91).

Additional immune suppressive activities of CD8<sup>+</sup> T cells with a senescent phenotype have also been reported. CD8<sup>+</sup> T cells that are CD57<sup>+</sup> (a phenotype associated with loss of CD28 expression) exert suppressive influences on effector functions of HIV-specific CTLs (92), and CD8<sup>+</sup>CD28<sup>-</sup> T cells also accumulate and mediate liver damage in hepatitis C infection (93). A novel cellular interaction between CD8<sup>+</sup>CD28<sup>-</sup> T cells and endothelial cells has recently suggested by *in vitro* experiments, which if confirmed *in vivo*, would have major implications on HIV pathogenesis. It has been reported that primary human endothelial cells that are exposed to culture supernatants from CD28<sup>-</sup>, but not CD28<sup>+</sup>, T cells show increased expression of intercellular adhesion molecule-1, E-selectin, and the vascular endothelial growth factor receptor, all of which are specific markers of Kaposi's sarcoma (KS) (94). The endothelial cells also acquire proliferative and morphological features of KS cells. The effect is mediated by soluble factors, such as TNF $\alpha$ , which we have also found to be enhanced in CD8<sup>+</sup> T cells that are driven to senescence in cell culture (Fig. 1).

Similarly, a population of TNF $\alpha$ -producing CD8<sup>+</sup>CD28<sup>-</sup> T cells has been reported in patients with cervical cancer (85). Expanded populations of CD8<sup>+</sup>CD28<sup>-</sup> T cells are present in ankylosing spondylitis patients, and, in fact, correlate with a more severe course of this autoimmune disease (95). Cells with the same phenotype accumulate in persons with coronary

artery disease, suggesting some sort of chronic antigenic exposure related to atherosclerosis (96). Finally, there is a progressive expansion of CD8<sup>+</sup>CD28<sup>-</sup> T cells in patients with head and neck tumors, and the proportion of these cells is reduced upon tumor resection (97). It is noteworthy that few of the above reports link the CD8<sup>+</sup>CD28<sup>-</sup> T cells with the process of replicative senescence, a possible reflection of the general lack of familiarity of many immunologists with the senescence literature. Nevertheless, the common theme in many of these reported accumulations of CD8<sup>+</sup>CD28<sup>-</sup> T cells is chronic antigenic stimulation, be it by virus, alloantigen, autoantigen, or tumor-associated antigen.

The regulatory effects of senescent CD8<sup>+</sup> T cells are not restricted to the immune system. For example, there is accumulating evidence indicating that bone biology is directly linked to immune system activity and that chronic immune activation is associated with bone loss (98). The CD8<sup>+</sup> T-cell subset, in particular, has been implicated in both bone resorption activity (99, 100) and osteoporotic fractures in the elderly (101). One of the central regulators of bone resorption is expressed on and secreted by activated T cells. This molecule, known as receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), binds to RANK on osteoclasts, inducing these bone-resorbing cells to mature and become activated (102). Under normal circumstances, the bone-resorbing activity induced by RANKL is kept in check by IFN $\gamma$ , a cytokine also produced by the activated T cells (103). However, as shown in Fig. 2, CD8<sup>+</sup> T-cell replicative senescence is associated with reduced ability to produce IFN $\gamma$  (104). A second type of defect relates to the fact that activated T cells affect not only osteoclasts but also can modulate bone mass by producing cytokines that inhibit the bone-forming activity of osteoblasts. Notably, IL-1 and TNF $\alpha$  inhibit osteoblast bone forming activity, and also affect bone mass by inducing formation of certain cytokines by osteoclasts that increase bone resorption (105). Senescent CD8<sup>+</sup> T-cell cultures contain high levels of TNF $\alpha$  (106), further implicating this class of T cells in the modulation of bone metabolism.

### Concluding remarks

Nearly 50 years after the process of replicative senescence was first documented in human fibroblasts cultured *in vitro*, it seems quite clear that CD8<sup>+</sup> T cells undergo a similar process *in vivo*, leading to both immune and non-immune consequences that become particularly evident by old age. It should be noted that although we have focused our research on telomere shortening that is driven by extensive proliferation, it is possible that other mechanisms may drive T cells to

senescence *in vivo* as well. Indeed, in cell-culture studies, oxidative stress accelerates telomere shortening and the types of DNA damage associated with senescence (107, 108). Thus, the increased oxidative stress associated with aging may function synergistically with antigenic stimulation to accelerate the generation of senescent T cells. Consistent with this notion, in hereditary hemochromatosis, a clinical condition associated with enhanced oxidative stress, there are increased proportions of CD8<sup>+</sup> T cells that lack CD28 (109).

Many of the effects of CD8<sup>+</sup> T-cell replicative senescence seem to be deleterious. However, it is possible that in some contexts, senescent CD8<sup>+</sup> T cells may exert beneficial effects. For example, acquisition of NK markers and function by these cells may compensate for diminished antigen-specific CD8<sup>+</sup> T-cell functions associated with aging. Moreover, the data from organ transplant patients (89) suggest that by suppressing the activity of other T cells that would otherwise be reacting against the allograft, the CD8<sup>+</sup>CD28<sup>-</sup> T cells are highly beneficial to the host. Further characterization of the full spectrum of changes associated with T-cell replicative senescence will undoubtedly provide a more complete picture of their role during aging.

Overall, the acquisition of regulatory functions by CD8<sup>+</sup> T cells that have the senescent phenotype is entirely consistent with a large body of data on replicative senescence in other cell types. These observations underscore the concept that the proliferative block associated with replicative senescence is just one aspect of the global changes in gene expression and function associated with this end stage cell. In T cells, the characteristics acquired by CD8<sup>+</sup> T cells that have reached senescence are often totally new and distinct from other stages through which the cells have transited since their original naïve cell activation. For this reason, we have proposed that replicative senescence might be appropriately viewed as the final stage of memory T-cell differentiation (110). It is clear that this end stage will probably never be reached by the majority of memory T cells, but rather only by those memory T cells which have antigen receptors that make them susceptible to long-term, chronic stimulation. However, because senescent CD8<sup>+</sup> T cells constitute a significant proportion of the T-cell repertoire in the elderly, elucidation of the full range of genetic, functional, and regulatory characteristics of these cells becomes an essential goal of immunologists and indeed all biologists interested in aging. Research on the interactions of senescent CD8<sup>+</sup> T cells with other cells and organ systems may provide novel insights into many of the pathologies of aging. Expanding the research on the influence that CD8<sup>+</sup> T-cell replicative senescence might exert on non-immune cells may therefore lead to possible immune-based therapies for a variety of age-related disease states.

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