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Extremely Differentiated T Cell Subsets Contribute to Tissue Deterioration During Aging

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Abstract

There is a dramatic remodeling of the T cell compartment during aging. The most notorious changes are the reduction of the naive T cell pool and the accumulation of memory-like T cells. Memory-like T cells in older people acquire a phenotype of terminally differentiated cells, lose the expression of costimulatory molecules, and acquire properties of senescent cells. In this review, we focus on the different subsets of age-associated T cells that accumulate during aging. These subsets include extremely cytotoxic T cells with natural killer properties, exhausted T cells with altered cytokine production, and regulatory T cells that gain proinflammatory features. Importantly, all of these subsets lose their lymph node homing capacity and migrate preferentially to nonlymphoid tissues, where they contribute to tissue deterioration and inflammaging.

INTRODUCTION

Aging impacts the immune function by reducing the ability to develop specific immune responses to both external and internal threats, including infections and cancer, as well as by limiting our vaccination response. The age-dependent deterioration of the immune system also compromises immune tolerance mechanisms, leading to engagement of futile proinflammatory and autoimmune reactions that trigger a plethora of noncommunicable diseases. This time-dependent decline of the immune system is referred to as immunosenescence. Although these age-related changes occur in both the innate and adaptive immune systems, the most remarkable alterations are observed in the adaptive immune system, particularly in T cells, probably due to the involution of the thymus. In fact, thymic involution is the first manifestation of aging. The thymus begins to degenerate during puberty, becoming almost nonfunctional in adulthood (1). As a result, there is a decline in the generation of new naive T cells after adolescence, so naive T cells in adulthood are maintained by homeostatic proliferation of the preexisting T cells (2). Replicative pressure, due to both homeostatic proliferation and clonal expansion after antigen recognition, triggers in naive cells the loss of stemness and acquisition of a memory-like and senescent phenotype. Aging also induces T cell–intrinsic and –extrinsic changes in spleen and lymph nodes involving germinal center (GC) alterations that disrupt adaptive immune responses, including vaccination and other humoral responses. Peripheral tissues accumulate terminally differentiated T cells, causing inflammaging and tissue damage. Consequently, there is a dramatic remodeling of the T cell compartment during aging (3) (**Figure 1**).

The most obvious change during aging is the reduction of the naive T cell pool (characterized by expression of CD62L) and the accumulation of memory-like T cells (characterized by loss of CD62L and upregulation of CD44), leading to a reduction in the size of the available T cell receptor (TCR) repertoire. The generation and heterogeneity of memory-like T cells that appear in older adults are complex. These include memory T cells that are generated due to clonal expansion after recognition of a foreign antigen and virtual memory T cells, which are generated as a result of homeostatic proliferation and have enhanced effector functions despite lacking stimulation by foreign antigens (4–6). Memory-like T cells in older people acquire a phenotype of terminally differentiated cells, lose the expression of costimulatory molecules like CD28 and CD27, and acquire properties of senescent cells, displaying signs of DNA damage, shortened telomeres, and senescence-associated signaling pathways (7). Importantly, during this transition from naive T cells with stem features to terminally differentiated cells with senescence characteristics, the migration pattern of T cells is modified. Naive T cells have the ability to migrate to secondary lymphoid organs, like spleen and lymph nodes, due to the expression of CD62L and the chemokine receptor CCR7. In contrast, terminally differentiated cells lose CD62L expression and express tissue-specific integrins and chemokine receptors that facilitate their migration to nonlymphoid organs. In the last years, thanks to the application of single-cell RNA sequencing (scRNA-seq) techniques, the phenotypic and functional complexity of the different subpopulations of these memory-like T cells that accumulate during aging is beginning to be unraveled (8), although the terminology and identification of each subpopulation are still confusing. Specifically, scRNA-seq analysis of T cells from spleen and other tissues of old mice revealed the accumulation of extremely differentiated T cells displaying exhausted, cytotoxic/natural killer (NK), and regulatory features. Although many publications have demonstrated the properties and markers of senescent T cells, a defined cluster of senescent T cells has not been identified yet in scRNA-seq studies, suggesting that the different subsets of age-associated T cells could harbor certain senescent characteristics.

Cellular senescence was first described in fibroblasts subjected to excessive stress or damage, including DNA damage, telomere dysfunction, or mitochondrial failure, and is characterized by

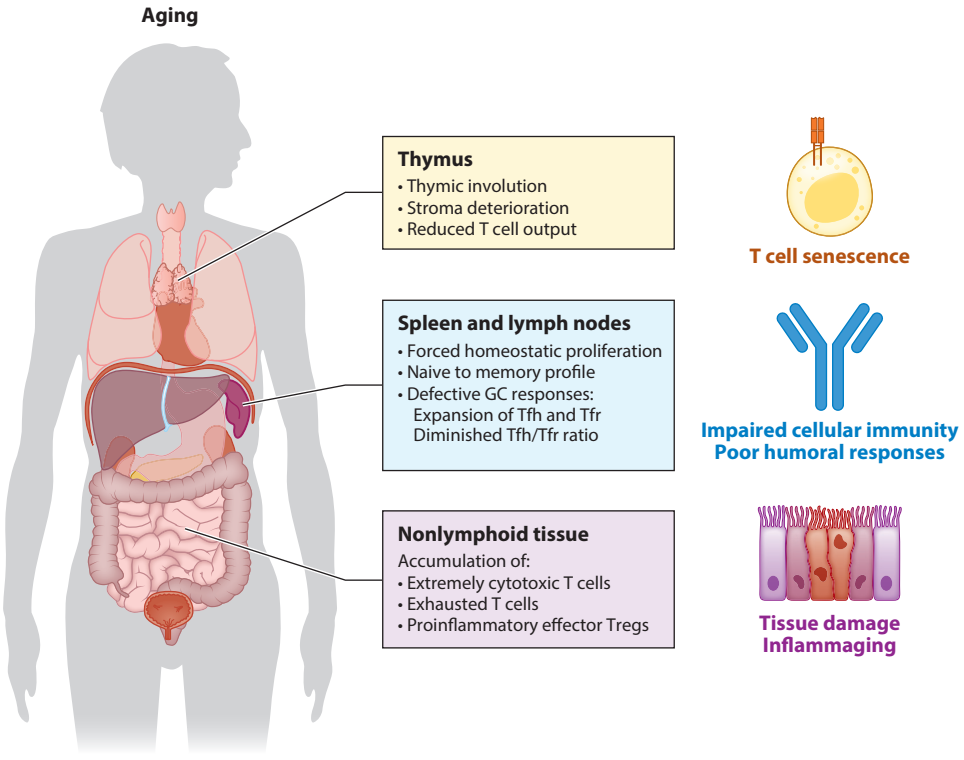

Figure 1

Consequences of age-associated changes in T cells among different tissues. Age-associated thymic involution reduces the generation of naive T cells. The number of naive T cells is then sustained by homeostatic proliferation of preexisting T cells. Replicative pressure, due to both homeostatic proliferation and clonal expansion after antigen recognition, promotes loss of stemness and acquisition of a memory-like and senescent phenotype. Aging also induces T cell–intrinsic and –extrinsic changes in spleen and lymph nodes involving germinal center alterations that disrupt adaptive immune responses, reducing vaccination efficiency and impairing humoral responses. Peripheral tissues accumulate terminally differentiated T cells that contribute to inflammaging and tissue damage. Abbreviations: GC, germinal center; Tfh, T follicular helper; Tfr, T follicular regulatory; Treg, regulatory T cell.

three features: (*a*) permanent proliferation arrest, (*b*) resistance to apoptosis, and (*c*) proinflammatory phenotype, known as the senescence-associated secretory phenotype (SASP). Since senescent cells are terminally growth-arrested, have shorter telomeres, and upregulate cell cycle regulators such as p53, p16, and p21, these hallmarks have been routinely employed in the detection of cellular senescence. Additionally, enhanced senescence-associated β-galactosidase (SA-βGal) activity at pH 6.0 has been extensively used as a marker of cellular senescence and reflects an alteration in lysosomal number or activity. Using a second-generation fluorogenic substrate for β-galactosidase and flow cytometry, researchers have discovered an age-dependent increase in SA-βGal activity in T cells from healthy individuals. The greatest age-associated increase has been observed in $CD8^+$ T cell populations, in which the fraction of cells with high SA-βGal activity reached average levels of 64% in individuals over 60 years old. This subset also exhibited telomere dysfunction and proliferation arrest and had a gene expression signature consistent with p16-mediated senescence (9).

Telomere length is reduced with aging and is considered to be a marker of biological aging. Thus, the flow-FISH (fluorescence in situ hybridization) method has been used to assess the

length of telomere repeats in circulating immune cells from healthy individuals (10). Naive T cells have the longest telomeres on average, whereas fully differentiated T cells and memory T cells have shorter telomeres (10). Chronic hepatitis C virus infection and HIV infection cause premature T cell aging, triggered by persistent immune stimulation and T cell overactivation. In fact, overactivation induces telomeric DNA damage due to telomere repeat–binding factor 2 (TRF2) inhibition, suggesting that restoring TRF2 might provide a novel approach to prevent telomeric DNA damage and premature T cell aging (11). Interestingly, it has been revealed that some T cells (naive or central memory) avoid senescence by receiving telomeres from antigen-presenting cells via extracellular vesicles, also corroborating that telomere shortening is an important factor in T cell senescence (12). In addition, T cells as well as many other cell types in the body display mitochondrial dysfunction correlating with aging. This mitochondrial damage could be due to cumulative mitochondrial DNA mutations, defects in mitochondrial biogenesis, or defective mitochondrial turnover (13–15), and it may also contribute to senescence and functional deterioration of T cells (**Figure 2**).

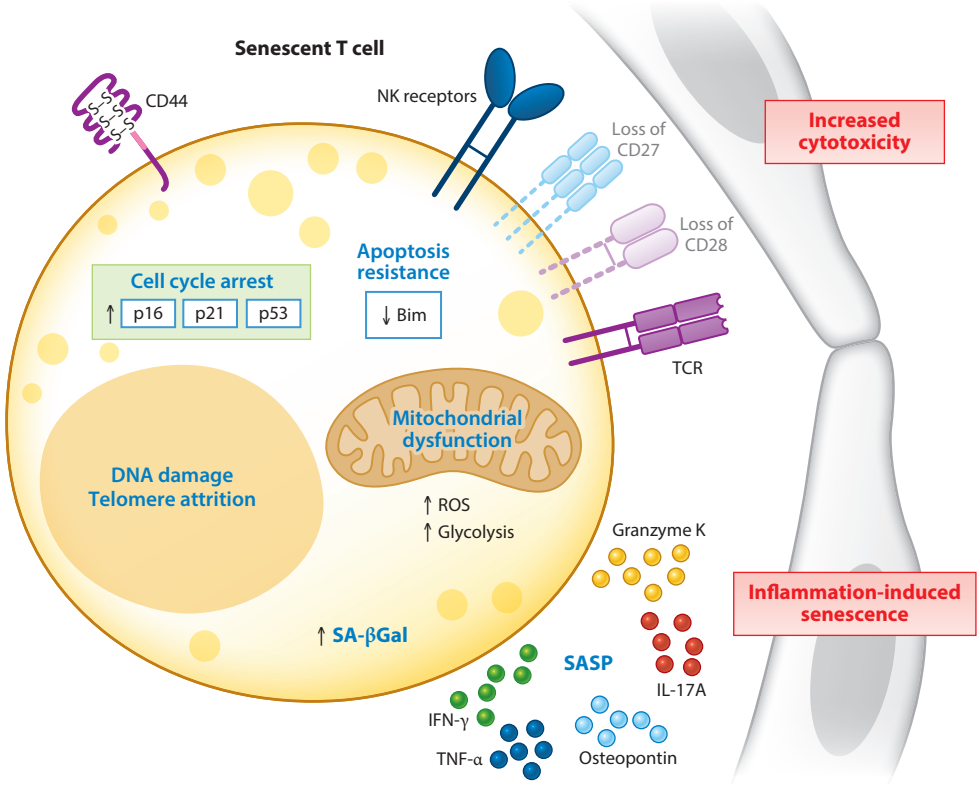

Figure 2

Old T cells acquire senescence features. During aging, T cells lose the expression of CD27 and CD28 and acquire the expression of NK receptors. Aged T cells show features of senescence including cell cycle arrest, resistance to apoptosis, mitochondrial dysfunction, DNA damage, shortening of telomeres and secretion of proinflammatory molecules. As a consequence, old T cells can contribute to tissue senescence by cytotoxicity or inflammation. Abbreviations: NK, natural killer; ROS, reactive oxygen species; SA-βGal, senescence-associated β-galactosidase; SASP, senescence-associated secretory phenotype; TCR, T cell receptor.

The loss of the expression of costimulatory molecules like CD27 and CD28 seems to be the age-related change that better correlates with T cell aging. CD28 plays a central role in the regulation of IFN-γ production, since several studies have reported that $CD4^+CD28^-$ T cells secrete large amounts of IFN-γ and are associated with Th1 (T helper 1)-driven autoimmune diseases such as multiple sclerosis or rheumatoid arthritis (16–19). Importantly, a population of cytotoxic $CD4^+$ T cells dominantly regulated by transcription factors associated with Th1 polarization has recently been identified in old mice (20). Additionally, a subset of $CD27^-CD28^-$ cytotoxic $CD4^+$ and $CD8^+$ T cells that express NK receptors and several senescent features, including impaired activation and proliferation, reduced telomerase activity, higher DNA damage, impaired calcium flux, and reduced IL-2 synthesis, has been described in humans (21, 22). Besides these cytotoxic T cells with NK properties, exhausted T cells also accumulate during aging. Exhausted T cells are characterized by the expression of inhibitory molecules such as PD-1, TIM-3, and LAG-3 and the diminished ability to produce cytokines (23). In contrast, exhausted T cells have been considered important for limiting immunopathology or autoreactivity (24). Remarkably, a recent report has shown that exhausted $CD8^+$ T cells, which are increased in several tissues in an age-dependent manner, secrete high amounts of granzyme K (GzmK), a factor that induces senescence in other cells (25), supporting the idea that exhausted T cells might also participate in tissue damage. Finally, regulatory T cells (Tregs) are especially important in maintaining tissue homeostasis. Nonetheless, there is an increase of terminally differentiated effector Tregs during aging that, in addition to expressing Foxp3 and IL-10, acquire proinflammatory markers and can contribute to maladaptive responses and tissue deterioration (26) (**Figure 3**).

In addition to the accumulation of terminally differentiated T cells in nonlymphoid tissues, dramatic changes occur in lymph nodes during aging. Importantly, there is a decline in the number and function of their GCs supporting poor humoral responses and consequent impaired vaccination response in older people (>65 years) (27–31). Within GCs, dysfunctional and immature $CD4^+$ T follicular helper (Tfh) cells and, to a greater extent, their negative regulators $Foxp3^+$ T follicular regulatory (Tfr) cells are increased in aged mice (27–31). Furthermore, there is an expansion of IL-10-producing Tfh cells (32), and a subset of $CD153^+$ Tfh cells with senescent properties characterized by the secretion of osteopontin during aging (33), which could fuel inflammaging as well as limit immune responses. These changes in GCs that weaken antigen-specific antibody responses in aged mice and humans have recently been reviewed (34).

In this review, we focus on the characteristics, development, and function of the different age-associated T cell subsets with senescence features that appear in nonlymphoid tissues during aging and that contribute to their deterioration, including exhausted T cells, cytotoxic T cells, and activated Tregs.

NAIVE T CELLS ACQUIRE A MEMORY-LIKE PHENOTYPE DURING AGING

A key feature of age-related changes in the T cell compartment is the drastic reduction of naive T cells. There are different causes of this reduction, including homeostatic proliferation or the priming of naive T cells by antigens throughout life. During homeostatic proliferation, naive T cells acquire memory-like features while retaining a phenotypically naive state. Thus, naive T cells in older individuals (>65 years) lose their stemness and their functional plasticity, shifting toward a more memory-like state (35). This aging signature has epigenetic and transcriptional profiles of differentiation, mostly driven by increased accessibility to bZIP (basic leucine zipper) family transcription factors, such as BATF, and to a lesser extent, T-box family transcription factors such as T-bet and Eomes (36). In addition, naive $CD8^+$ T cells display an age-dependent loss of

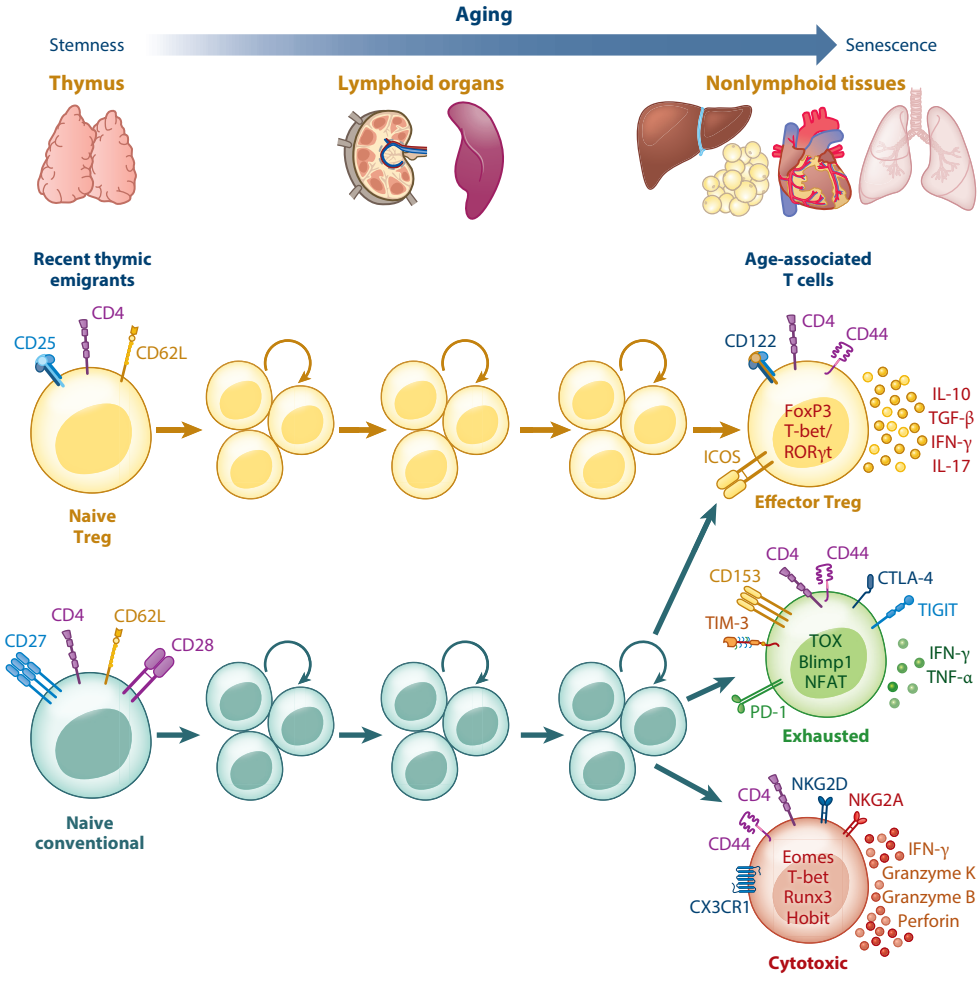

Figure 3

Age-associated changes of CD4 T cells, from the thymus to the periphery. During aging, due to replicative pressure caused by both homeostatic proliferation and clonal expansion, naive CD4 T cells lose their stemness and acquire features of terminally differentiated T cells, becoming activated regulatory T cells, exhausted T cells, and cytotoxic T cells and displaying senescence properties. They simultaneously lose lymphoid organ tropism and gain the capacity to migrate to several tissues and organs. Abbreviation: Treg, regulatory T cell.

chromatin accessibility of nuclear respiratory factor 1 (NRF1), a transcription factor that controls expression of mitochondrial proteins and could be involved in the age-related mitochondrial decline.

Supporting that differentiation pathways are involved in the aging of naive T cells, old naive T cells are characterized by a lower expression of miR-181a and an increased expression of miR-21 and miR-146a, a pattern that is also seen during T cell differentiation (37, 38). miR-181a is considered to act as a rheostat of TCR activation by controlling the expression of several phosphatases, including DUSP6 (39). Due to reduced expression of miR-181a and increased DUSP6, naive $CD4^+$ T cells from older people have reduced ERK phosphorylation upon TCR stimulation (37). Mice with conditional deletion of miR-181ab1 in peripheral T cells exhibit characteristics of immune aging, including impaired expansion of antigen-specific T cells in antiviral responses

and delayed viral clearance (40). Increased expression of miR-21 induces inhibition of negative feedback loops involving several signaling pathways, thereby causing degradation of FOXO1 and sustained mTORC1 signaling (38, 41).

CD8$^+$ T CELLS AGE FASTER THAN CD4$^+$ T CELLS

Although both CD4$^+$ and CD8$^+$ T cells undergo changes associated with age, these alterations are much more severe and appear earlier in CD8$^+$ than in CD4$^+$ T cells. The decline in the absolute numbers of circulating T cells over time, the reduction of the naive T cell pool, and the accumulation of T cells with a terminally differentiated memory-like phenotype occur in both the CD4$^+$ and CD8$^+$ T cell compartments. However, these changes occur earlier in CD8$^+$ than in CD4$^+$ T cells in both humans and mice. In fact, reduced numbers of circulating CD8$^+$ T cells are the most consistent and prominent marker of immune aging in older adults (>65 years), independent of comorbidities and chronic latent infections (42, 43). Accordingly, it has recently been shown that age-related TCR repertoire attrition occurs earlier in CD8$^+$ than CD4$^+$ T cells (44). However, greater sensitivity of CD8$^+$ T cells to age-dependent deterioration is still a matter of debate. Different mechanisms have been proposed to explain it, such as poorer survival of CD8$^+$ T cells or less ability to maintain quiescence. Furthermore, naive CD8$^+$ T cells have greater homeostatic proliferation (45). A shorter period of antigen exposure is required to initiate the proliferative program in naive CD8$^+$ T cells than in naive CD4$^+$ T cells. Consequently, CD8$^+$ T cells also divide earlier and have a higher rate of cell division than CD4$^+$ T cells upon activation, probably due to different costimulatory requirements (46). Indeed, naive CD8$^+$ T cells tend to differentiate into virtual memory T cells faster than CD4$^+$ T cells in mice, indicating that they are more easily driven into differentiation (47). Altogether, these features could explain the reasons CD4$^+$ T cells exhibit increased resilience to aging than CD8$^+$ T cells.

CD8$^+$ T CELLS GAIN NATURAL KILLER FUNCTIONS DURING AGING

CD8$^+$ T cells exert a cytotoxic function. Their killing of infected or damaged cells is dependent on antigen-MHC-I. Among the subpopulations of activated CD8$^+$ T cells in humans, terminal effector memory CCR7$^-$CD45RA$^+$ (TEMRA) T cells increase with aging (48). These cells are characterized by loss of the costimulatory markers CD28 and CD27; reexpression of CD45RA; and expression of the exhaustion marker PD-1, the chemokine receptor CX3CR1, or the NK receptor CD57 (49–51). These CD8$^+$ subpopulations have been considered senescent due to their impaired proliferation and decreased TCR activation. Over time, CD8$^+$ T cells gain other NK receptors, such as NKG2D and NKG2A (52–54). Importantly, the acquisition of NK receptors changes T cells' function by giving them the ability to kill altered or stressed cells in an antigen-independent manner (55). These old CD8$^+$ T cells (CD27$^-$CD28$^-$) can be then activated by both the TCR and NK receptors but show a propensity to respond in an antigen-independent manner. NKG2D stimulation alone is sufficient to induce expression of GzmB and secretion of the cytokine IFN-γ (22). NK receptors can activate or inhibit T cell function (56), but importantly, inhibitory NK receptors inhibit only complex cellular functions, such as proliferation, while leaving effector functions intact in CD8$^+$ T cells (57). Recent research associates the acquisition of an NK-like phenotype in CD8$^+$ T cells with sestrins (22). Sestrins are stress-sensing proteins that bind and inhibit MAP kinases, including p38, ERK, and Jnk, promoting inhibitory effects and senescence characteristics in aged T cells (21). Importantly, sestrin expression is increased in CD8$^+$ T cells from older people (>65 years). The genetic silencing of sestrins leads to a recovery of the proliferation capacity and function in these senescent T cells and enhances vaccination response in old mice (21, 22). Importantly, sestrins participate in the acquisition of NK receptors, since CD8$^+$

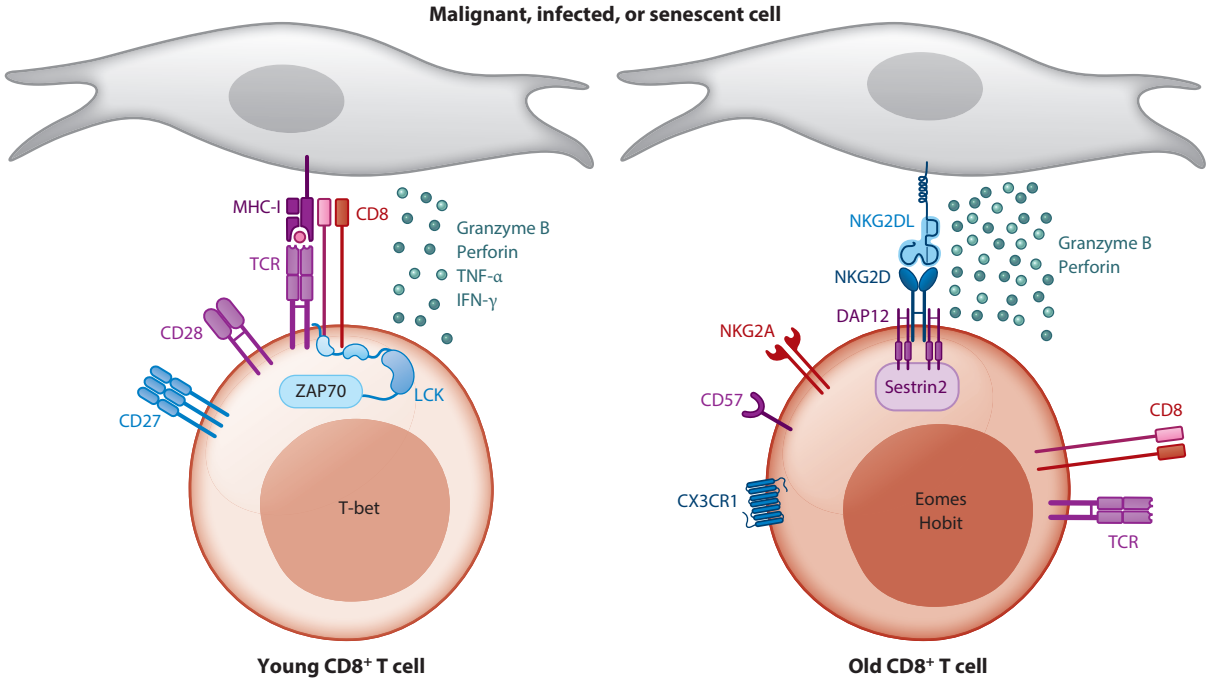

Figure 4

Old CD8$^+$ T cells acquire an NK phenotype and function with aging. CD8$^+$ T cells lose expression of costimulatory TCR molecules and express NK receptors, such as NKG2A or NKG2D, changing their cytotoxicity from antigen-dependent to antigen-independent target recognition. Abbreviations: NK, natural killer; TCR, T cell receptor.

T cells assemble NK cell receptor signaling machinery through their interaction with sestrins and the scaffold protein DAP12. Very recently, the coexpression of TIGIT with the transcription factor Helios was identified as a marker of senescent CD8$^+$ T cells with impaired activation and proliferation. Interestingly, TIGIT$^+$Helios$^+$CD8$^+$ senescent T cells accumulate predominantly in the CD27$^-$CD28$^-$ population but also in less terminally differentiated CD27$^+$CD28$^-$ T cells (58). All of this indicates that CD8$^+$ T cells change their phenotype with aging, showing innate properties, losing antigen-dependent recognition, and acquiring senescence features (**Figure 4**).

OLD CD4$^+$ T CELLS ACQUIRE CYTOTOXIC FEATURES

Apart from CD8$^+$ T cells, there is a rare subpopulation of CD4$^+$ T cells that possesses cytolytic activity. These CD4$^+$ cytotoxic T lymphocytes (CTLs) were identified many years ago (reviewed in 59). They were initially thought to be an artifact of excessively long in vitro cultures but were then found in vivo in both mice and humans (60, 61). CD4$^+$ CTLs are antigen-experienced cells that differentiate in response to repeated activation signals or continuous stimulation and thus were first described in chronic viral infections (62), autoimmune diseases (63), chronic inflammation (64), and cancer (65, 66). Recently, they have further been associated with aging, especially in scRNA-seq studies in humans and mice (20, 67). In one of these works, Elyahu and colleagues (20) performed scRNA-seq of splenic CD4$^+$ T cells from young and old mice (2–3 and 22–24 months old). They characterized different subpopulations of CD4$^+$ T cells, revealing that one of these subpopulations expresses cytotoxic molecules such as CCL5, GzmK and GzmB. Although CD4$^+$ CTLs accumulate in the spleen during aging, their presence is higher in the bone marrow and

blood than in typical lymphoid organs. $CD4^+$ CTLs can also be found in basal conditions in the intestine as intraepithelial $CD4^+$ T cells in mice (61). In humans, the comparison of blood samples from young and older people (>65 years) showed that circulating $CD4^+$ CTLs increase with age (68), and these cells are even more numerous in blood samples from supercentenarians (>100 years) (67).

$CD4^+$ CTLs are characterized by the expression of specific markers that are absent in canonical $CD4^+$ Th cells. Some of them are markers of classical cytotoxic $CD8^+$ T cells, including the molecule CD8a (61). During thymic development, ThPOK and Runx3 are expressed in $CD4^+CD8^+$ double-positive T cells and are antagonistic, driving the differentiation of double-positive cells into helper $CD4^+$ T and cytotoxic $CD8^+$ T cells, respectively. However, $CD4^+$ CTLs lose expression of ThPOK and reexpress Runx3 (69–72), reprogramming their activity from helper to CTL (73). $CD4^+$ CTLs lose expression of CCR7, CD27, and CD28 and display senescent characteristics such as reduced proliferation, decreased telomerase activity, and DNA damage (22, 74). Other markers that have been related to $CD4^+$ CTLs are CD57, CD29, LAMP (or CD107a), and CX3CR1 (75, 76). The function of $CD4^+$ CTLs is killing infected cells by releasing cytotoxic molecules and secreting proinflammatory cytokines. Therefore, apart from their surface markers, $CD4^+$ CTLs can be identified by the cytokines that they produce. Cytokine production (at transcriptional level) of $CD4^+$ CTLs is similar to that of Th1 cells; they produce the canonical Th1 cytokines IFN-γ, TNF-α, and IL-2 (20, 76). However, they also produce cytotoxic molecules such as perforin, granzymes, and granulysin, which induce the death of infected target cells (20, 61, 67, 68, 77, 78). In addition, $CD4^+$ CTLs express intracellular chemokines like CCL3, CCL4, and CCL5, in both humans and mice (20, 79).

As $CD4^+$ T cells recognize antigens via MHC-II, the function of $CD4^+$ CTLs might be killing infected antigen-presenting cells to maintain the healthy ones (80). Additionally, some viruses use the downregulation of MHC-I to avoid the immune response, and thus antigen presentation through MHC-II (and recognition by $CD4^+$ CTLs) might be an additional mechanism to fight against infections (81). Importantly, $CD4^+$ CTLs express markers of NK cells such as NKG2A, NKG2D, 2B4, and KLRG1 (61, 68, 75, 82–84). Similar to the case of $CD8^+$ T cells, recent research associates sestrins with $CD4^+$ T cell acquisition of an NK-like phenotype (22). Whether $CD4^+$ CTLs acquire an innate recognition pattern, as occurs with $CD8^+$ T cells, should be further explored.

Although a lot of research has been done to elucidate the process of differentiation of $CD4^+$ CTLs, it is yet not fully understood. Interestingly, the intensity of antigen signaling is important to the differentiation of $CD4^+$ CTLs, and low levels of antigen generate higher cytotoxicity (85). Supporting their antigen-dependent development, two studies combining scRNA-seq with TCR-seq found that $CD4^+$ CTLs had less TCR diversity, thus defining them as the result of clonal expansion (67, 79). There are some similarities between $CD4^+$ Th1 cells and $CD4^+$ CTLs that suggest that $CD4^+$ CTLs derive from Th1 cells. $CD4^+$ CTLs express the Th1 transcription factor T-bet and produce the proinflammatory cytokines IFN-γ, TNF-α, and IL-2 (81). In addition, Eomes, which is indispensable for $CD8^+$ T cell function (86), has been associated with the development of $CD4^+$ CTLs in immunotherapy treatments in cancer models (69–71). Consequently, a single copy of Eomes transfected into $CD4^+$ T cells results in increased expression of IFN-γ, FasL, perforin, and GzmB (87). Apart from T-bet and Eomes, Blimp1 is required for differentiation in viral infections and cancer (88). In contrast, BCL-6 is a key factor in the differentiation of $CD4^+$ T cells into Tfh cells and inhibits the generation of cytotoxic $CD4^+$ T cells, suggesting that $CD4^+$ CTL differentiation and Tfh cell differentiation are mutually exclusive (89). The transcription factor Hobit was also found in $CD4^+$ CTLs (90, 91). Hobit is an analog of Blimp1 that had previously been associated with cytotoxicity in $CD8^+$ T cells (92) and in NK

T cells (93). In $CD4^+$ T cells, Hobit induces expression of T-bet, IFN-γ, perforin, GmzA, and GmzB but not GmzK in human cytomegalovirus infection (90).

Different cytokines have been related to $CD4^+$ CTL differentiation. For example, IL-2 induces Eomes expression, driving the $CD4^+$ CTL program (64, 69, 71, 88). TGF-β and retinoic acid added to in vitro cultured T cells suppress expression of ThPOK and increase expression of Runx3, T-bet, and GzmB, differentiating T cells toward $CD4^+$ CTLs. Blocking the signaling of TGF-β or retinoic acid reduces differentiation of $CD4^+$ CTLs (73). Very recently, the class I–restricted T cell–associated molecule (CRTAM) has arisen as an essential player in the differentiation process toward CTLs, because CRTAM intracellular signaling is required for expression of Eomes, IFN-γ, and GzmB. Therefore, the CRTAM-expressing T cells, which accumulate in lungs and intestines, are the precursors of CTLs (94). Thus, $CD4^+$ T cells lose their helper identity over time and secrete cytotoxic molecules contributing to tissue damage.

AGING INCREASES THE AMOUNT OF EXHAUSTED T CELLS

Exhausted T cells lose their ability to produce cytokines after encountering an antigen and performing their effector function. In fact, exhausted T cells recapitulate three interconnected characteristics: resistance to activation signals, decreased cytokine expression (IFN-γ, IL-2, and TNF-α), and impairment in their effector function (95, 96). Furthermore, the exhaustion program has been related to high TCR signaling strength and/or continuous TCR stimulation (97–99), suggesting that it is oriented toward downregulation of T cell effector function in order to protect tissues from an excessive immune response. Although exhausted T cells have been classically studied in cancer, viral infections, and autoimmunity, they are now known to accumulate in aging.

Exhausted T cells are characterized by expression of inhibitory receptors in their surface membrane such as PD-1, LAG-3, TIM-3, TIGIT, and CTLA-4 (100, 101). Among these markers, PD-1 has been described as the major inhibitory receptor related to exhausted T cells (95). When activated, PD-1 attenuates TCR signaling by recruiting SHP1 and SHP2 tyrosine phosphatases, which inhibit phosphorylation of downstream effector molecules such as ZAP70 (102, 103). PD-1 also interrupts CD28-induced activation of the PI3K (phosphatidylinositol 3-kinase)/AKT/mTOR pathway, leading to inhibition of T cell metabolism, survival, and cell cycle (104). PD-1 signaling further enhances regulation of the transcription factor NFAT, which can promote expression of exhaustion-related genes (105). Thus, anti-PD-1 therapy has been used to reinvigorate exhausted T cells in cancer and viral infections (106–108). Although PD-1 is one of the most-used markers to determine the $CD4^+$ and $CD8^+$ exhausted T cell populations in mice, it has also been observed in up to 60% of memory $CD8^+$ T cells in healthy volunteers (109). This fact brings up the necessity of using a combination of other exhausted T cell features, such as complementary surface markers, cytokines, or transcription factors, to accurately identify these cells in mice and, more importantly, in humans (98).

The development of exhausted T cells is driven by the characteristic transcription factors TOX, NFAT, and Nr4a (105, 110, 111). There are three described states of exhausted $CD8^+$ T cells: progenitor, transitory, and terminally exhausted (112–114). Progenitor exhausted T cells express the chemokine receptors CXCR5, SLAMF6, and CCR7; costimulatory molecules like CD28, Tnfsf14, and ICOS; and the transcription factors TCF-1, LEF1, BCL-6, and ID3. Some of these molecules are also found in naive T cells. They maintain stem-like properties, which means that they may proliferate into more progenitor exhausted T cells or differentiate toward transitory or terminally exhausted T cells (113, 115). This feature has been related to TCF-1, whose expression decreases with age (115), suggesting an increase of the further differentiated exhausted subsets in aging. Functionally, although to a lesser extent than effector T cells, progenitor exhausted $CD8^+$

T cells are still able to produce and secrete IFN-γ, TNF-α, and IL-2 (112, 113). Transitory exhausted T cells express the chemokine receptor CX3CR1 and the transcription factors T-bet, Zeb2, Klf2, Runx1, and ID2. They have shown a higher cytotoxic profile with higher expression of GzmB and IFN-γ in vivo, resembling cytotoxic T cells (116, 117). Interestingly, IL-21 seems to be determinant for the differentiation of the progenitor pool toward the transitory one (117). Finally, terminally exhausted T cells express the surface protein CD101 (116, 117) and have higher expression of the coinhibitory surface markers TIM3, 2B4, and CD39 (112, 113, 118) and the transcription factors Eomes and Blimp1. Functionally, their production and release of cytokines are impaired. They express GzmB and IFN-γ, however, to a lesser degree than the other subsets, and their capacity for degranulation is impaired. They also lack proliferative potential even upon TCR restimulation after PD-1 blockade (112, 113, 116, 117). Interestingly, Mogilenko and colleagues (25) recently discovered a new subset of exhausted $CD8^+$ T cells by using scRNA-seq. They found an exhausted $CD8^+$ subpopulation that, besides exhibiting terminally exhausted features such as the inhibitory surface molecule PD-1 and transcription factors TOX, T-bet, and Eomes, also showed increased expression of GzmK. Interestingly, the development of these PD-1^+CD8^+ T cells was driven by extrinsic components of an aged environment, and the production of GzmK by these cells promoted senescence in adjacent fibroblasts, boosting inflammaging (25).

Although $CD8^+$ exhausted T cells have been more deeply studied, $CD4^+$ T cells also become exhausted. Exhausted $CD4^+$ T cells have higher PD-1 expression, lower T-bet expression, and altered expression of GATA-3, BCL-6, and Helios compared with $CD8^+$ exhausted T cells (101, 119). Exhausted $CD4^+$ and $CD8^+$ T cells share similar general features, but inhibitory receptors 2B4, TIM3, and Lag3 have been observed to be biased toward exhausted $CD8^+$ T cells whereas CTLA-4, CD200, and BTLA are rather biased toward the exhausted $CD4^+$ T cells in lymphocytic choriomeningitis virus–infected mice (119). In addition, $CD4^+$ exhausted T cells tend to differentiate toward a Tfh phenotype, maybe due to changes in BCL-6 expression, and do not produce IFN-γ, TNF-α, and IL-2 (101, 119). Importantly, the three subsets described for exhausted $CD8^+$ T cells have not been reported among $CD4^+$ T cells. Finally, CXCL13-producing $PD1^{hi}CXCR5^-CD4^+$ T cells are related to the formation of ectopic lymphoid-like structures that stimulate autoantibody production in autoimmune diseases such as arthritis (120). The size, presence, and functionality of these structures, also known as tertiary lymphoid tissues, have been related to aging (121). These facts suggest that exhausted T cells are present in age-related diseases and structures, and in fact, scRNA-seq has detected exhausted $CD4^+$ T cells in aged mice. Old $CD4^+$ T cells showed upregulation of the exhaustion genes *Pdcd1* (encoding PD-1), *Tigit*, and *Lag3* (25), and a differential increment of a PD-1^+CD4^+ population was present in old mice spleen, peritoneum, lungs, liver, white adipose tissue, and blood. Moreover, a similar study reported that old $CD4^+$ T cells had upregulated expression of exhaustion markers (*Pdcd1*, *Lag3*, *Tbc1d4*, *Sostdc1*, and *CD153*) compared to the young $CD4^+$ population and that the splenic exhausted PD-1^+CD4^+ T cells increased in old mice from 6 months on (20). In these mice the exhausted T cells tend to accumulate in nonlymphatic immune compartments, and the activity of the NFAT regulon is involved in the exhaustion process. Strikingly, in this work the $CD4^+$ T cells expressing *Runx3*, *Eomes*, *TOX*, *Tbx21*, *Ifng*, and *GzmB* were not included in the exhausted population but rather in $CD4^+$ CTLs. This observation suggests that the $CD4^+$ CTLs described by Elyahu et al. (20) may share similar functional and phenotypic features with the transitory exhausted $CD8^+$ T cells found in chronic viral infections and cancer.

Exhausted T cells are considered hyporeactive; thus, reinvigorating them could be a therapeutic approach for cancer and chronic viral infections (107, 108, 122). Therefore, different strategies to reinvigorate this T cell population have been developed. The use of antibodies against specific inhibitory receptors (such as PD-1 or CTLA-4), known as checkpoint blockade therapy, may be

the most extended treatment to reinvigorate exhausted T cells in cancer. Nonetheless, the latest discoveries about exhausted T cells have uncovered new alternative approaches, such as neutralization of IL-10 (123) and TGF-β (124) or IL-21 supplementation (125). Some of these may also be helpful in combination with checkpoint inhibitors, as they might block differentiation of progenitor exhausted T cells toward terminally exhausted T cells, maintaining progenitor exhausted T cells that are involved in the proliferative burst in response to anti PD-1 immunotherapy in both cancer (126) and viral infection (113). A recent report proposed manipulation of miR-29a as a new strategy to reinvigorate exhausted T cells, since miR-29a plays an important role in the regulation of Eomes. In addition, miR-29a overexpression reduces transcription of inhibitory receptors like CD160 and 2B4, although it does not impact PD-1 expression (127).

Interestingly, therapeutic strategies involving exhausted T cells in the context of aging may be completely opposite to those for cancer and viral infections. The physiological aims of exhaustion in T cells are to avoid an excessive immune response that may damage tissue and to promote resolution of the inflammation process. It is tempting to speculate that a novel approach to drive terminal exhaustion of the proinflammatory T cells that accumulate with age might help delay inflammaging and age-associated diseases.

OLD Tregs ACQUIRE A TERMINALLY DIFFERENTIATED PHENOTYPE WITH PROINFLAMMATORY PROPERTIES

Tregs are a subset of $CD4^+$ T cells that attenuate the immune response, playing a central role in immune tolerance and tissue homeostasis by preventing inflammatory and autoimmune diseases. Since aging is characterized by chronic and constant inflammation, understanding the mechanisms underlying the age-related dysfunction of Tregs is critical to address immunosenescence and inflammaging. Tregs are commonly characterized by expression of CD25 (IL-2 receptor α chain) and their master transcription factor Foxp3. The Treg compartment comprises thymic-derived Tregs, which are $Helios^+Nrp\text{-}1^+$ Tregs that emigrate from the thymus and provide wide-range specificity to avoid unwarranted self-responses; and peripheral Tregs, generated from naive $CD4^+$ T cells upon environmental cues (128). Tregs are also able to differentiate into Tfr cells, which play a central role in controlling GC B cells and Tfh cells, therefore tailoring antibody production (129).

Tregs in aged mice are augmented in lymphoid organs (e.g., spleen and lymph nodes) (20, 25, 130–133) and other nonlymphoid tissues such as visceral fat (134) and lungs (26, 135), but not in muscle (136). Similarly, humans aged ≥70 years manifest an increased percentage of Tregs in peripheral blood (131, 137) and nonlymphoid tissues including the skin (138) and lacrimal glands (132).

Age-associated changes in the thymus may impact the Treg pool. Although some authors declare that thymic Treg generation is not intrinsically affected by the age of the thymus in mice (139), it has been observed that activated Tregs return to the thymus from the periphery and limit the supply of IL-2, thus suppressing the formation of new thymic Treg precursors (140, 141). In accordance with the decline in IL-2 during aging, old Tregs reduce their dependency on IL-2 and become more dependent on IL-15 by expressing low levels of CD25 (IL-2 receptor) but high levels of CD122 (IL-2/IL-15 receptor chain β) (142–145). Additionally, there is diminished extrathymic peripheral Treg differentiation during aging, supporting the idea that the naive Treg compartment may be reduced overall (146).

Consistently, accumulating data highlight that there is an expansion of Tregs with a memory-like profile during aging characterized by downregulation of CD62L; upregulation of CD44, CD69, Blimp1, and KLRG1; and expression of immunoregulatory molecules such as IL-10, ICOS,

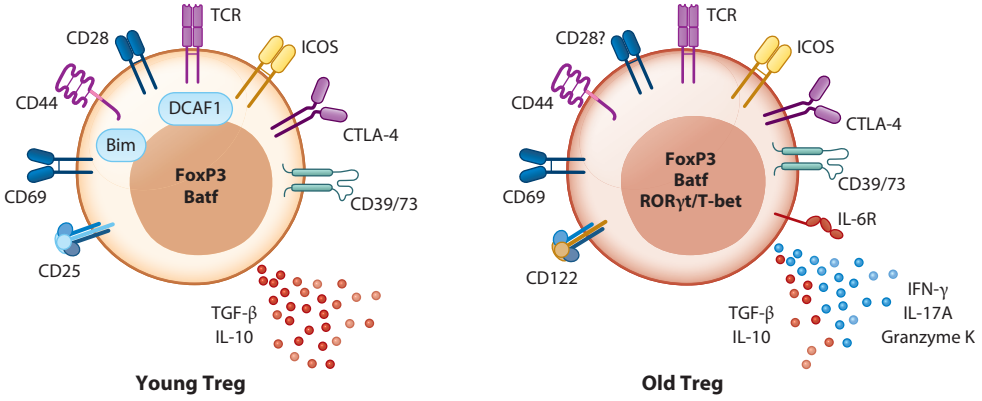

Figure 5

Tregs acquire a terminally differentiated phenotype with proinflammatory properties during aging, contributing to inflammaging and age-related diseases. Old Tregs that accumulate during aging are characterized by increased expression of memory/effector markers such as CD44 and CD69, as well as immunomodulatory molecules like ICOS, CTLA-4, and CD39/73. However, they become defective and gain proinflammatory features through the expression of RORγt or T-bet, which governs the secretion of IFN-γ and IL-17A, fueling inflammaging and other age-associated pathologies. Interestingly, these old Tregs upregulate CD122, becoming more dependent on IL-15 in response to the declining levels of IL-2 during aging. Abbreviations: TCR, T cell receptor; Treg, regulatory T cell.

and CTLA-4. Intriguingly, the transcriptional signature of aged Tregs is compatible with effector/memory Tregs from young mice and includes expression of *Batf*, a transcription factor involved in tissue residence (25). Furthermore, Elyahu et al. (20) have recently revealed that age-associated changes in the $CD4^+$ T cell landscape include the accumulation of $CD81^+$ Tregs displaying a highly activated profile. The transcriptional fingerprint of these aged Tregs is enriched in genes related to Treg activation (*Il6ra*, *Gmzk*, *Cd81*) and to Th1, Th17, and Tfr differentiation pathways (*Tbx21*, *Rorc*, and *Sostdc1*, respectively) (26, 132). Accordingly, there is an expansion of $T\text{-bet}^+Foxp3^+$ Tregs and Tfr cells during aging (27, 132). Cytokine profiling of old Tregs reveals enhanced production of IFN-γ, IL-17A, and IL-10 (26, 132, 147, 148), showing that aged Tregs acquire a harmful phenotype by upregulating an inflammatory transcriptomic signature while losing repair-associated gene pathways (**Figure 5**). In addition to the proinflammatory phenotype of old Tregs, they are unable to suppress aged conventional T cells, so these secrete greater amounts of IFN-γ and IL-17A, which could exacerbate chronic inflammation (132). Interestingly, data indicate that the in vitro and in vivo functioning of old Tregs is equivalent to that of young Tregs during acute inflammation; however, old Tregs fail to restrain $IL\text{-}17^+$ T cells during chronic inflammation due to an intrinsic defect in STAT3 activation (149).

The progressive accrual of Tregs during aging could be explained by the increase in circulating IL-6. IL-6 represses the proapoptotic protein Bim, thus increasing Treg resistance to apoptosis (20, 144, 150). Accordingly, the age-linked expansion of activated Tregs is highly correlated with serum levels of IL-6 in mice (20). In addition to apoptosis resistance (150), aged Tregs acquire other signs of T cell senescence such as increased SA-βGal activity and upregulation of an age-related transcriptomic signature enriched in genes associated with cell cycle arrest ($p16^{Ink4a}$, $p19^{Arf}$, $p21^{Cip1}$), T cell anergy (*Rnf128*), or DNA damage response (*Xrcc5* and *Rm1*) (26, 147). Interestingly, Tregs become more severely senescent than their conventional T cell counterparts (147). Moreover, old Tregs show reduced proliferation capacity and suppressing activity in vitro and in vivo (147, 151). One of the main players in regulating the acquisition of this senescent

program in aged Tregs seems to be DDB1- and CUL4-associated factor 1 (DCAF1), the loss of which culminates in elevated levels of reactive oxygen species (ROS) in human and mouse Tregs during aging (147).

Dysfunctional Tregs displaying a senescent profile have been described in age-related inflammatory and autoimmune diseases (152). For instance, a population of Tregs in rheumatoid arthritis patients harbors signs of T cell senescence and is unable to control conventional T cell responses, perpetuating inflammation (153). Accordingly, other works have pointed out that Tregs that lose Foxp3 and CD25 expression, termed ex-Tregs, acquire a Th17-like profile under arthritic conditions, mediating inflammation and joint destruction (154). The Th17/Treg ratio in humans diminishes in an age-dependent fashion (155), and in accordance, other age-related osteolytic conditions such as osteoporosis exhibit an imbalanced Th17/Treg ratio leading to loss of bone mass (156). Dysregulation of Treg homeostasis during aging also impairs muscle repair and physical performance in aged mice, as tissue-resident Tregs fail to accumulate in the lesion due to age-related reduction in local IL-33 (136, 157). The study of age-associated cardiometabolic and neurological diseases has shed light on the detrimental role of aged Tregs. Tregs accumulate with age in adipose tissue, which correlates with metabolic dysfunction in patients with type 2 diabetes (134, 158). Importantly, either Treg depletion (134) or deletion of their insulin receptor (159) protects mice from developing age-related metabolic syndrome by improving insulin sensitivity. Moreover, although the frequency of Tregs and levels of TGF-β increase in healthy older individuals (>65 years), they are markedly reduced in older patients with cardiovascular and metabolic disease, which may aggravate inflammation (137). Intriguingly, the accelerating aging SAMP1 mouse model manifests an increased percentage of Tregs that mediate a systemic hyperoxidative state inducing neurodegeneration of cochlear neurons and, consequently, loss of hearing. Interestingly, this could be reversed by depleting Tregs (160) or by overcoming T cell immunosenescence using fetal thymus transplantation (161). Experimental autoimmune encephalomyelitis (EAE) severity is increased in old mice and correlates with accumulation of Tregs expressing IFN-γ and IL-17A transcripts in the central nervous system. Supporting a pathogenic role of old Tregs in EAE, transient depletion of Tregs mitigates this disease in old mice (162). Interestingly, patients with Alzheimer disease or Parkinson disease have an increased percentage of peripheral Tregs that correlates with disease severity (163).

On the other hand, mouse models have unveiled that the percentage of Tregs increases during infectious diseases, and this increase is associated with an exacerbated inflammatory response (164) and reactivation of chronic infections (131) in aged hosts. For example, old mice infected with *Candida albicans* show a diminished ratio of IL-1β/IL-6 that mediates a dysregulated mTOR signaling pathway in Tregs and the persistence of damaging IFN-γ^+ and IL-17A$^+$ CD4$^+$ T cells in the oral mucosa. Interestingly, these results were mirrored in humans aged more than 60 years (164). Likewise, *Listeria monocytogenes*–infected, old mice display a higher percentage of immunosuppressive CD39/CD73$^+$ Tregs in the spleen but fail to control IFN-γ^+ and IL-17A$^+$CD4$^+$ T cells compared to infected young controls, leading to increased immunopathology (83). This age-associated enrichment of Tregs has also been analyzed in geriatric patients with *Mycobacterium tuberculosis* infection (165). Moreover, the expansion of skin-resident Tregs in older individuals (>70 years) could favor the increased susceptibility to cutaneous infections during aging (138). Thereby, imbalanced pro- and anti-inflammatory responses during aging may underpin the susceptibility to these bacterial infections. In addition, aged Tregs lose their pro-repair properties during infectious diseases. Influenza virus–infected old mice exhibited a higher percentage of activated Tregs in the lung, linked to deficient repair of the tissue (26, 135). Finally, the role of Tregs during tumoral processes is undeniable, as they favor an anti-inflammatory microenvironment that drives immune evasion and, thus, cancer development. As such, Tregs also dampen

antitumoral immune responses during aging since Treg depletion reduces the tumor burden in old mice (130). In line with this, the age-associated increased percentage of Tregs in the skin of old individuals (>70 years) may explain their susceptibility to cutaneous malignancies (138).

Other nonclassical cells exerting regulatory roles in the organism are $Foxp3^+CD8^+$ T cells, termed $CD8^+$ Tregs, which accumulate with age in lymphoid tissue of mice (130). Interestingly, older individuals (>70 years) exhibit a heightened frequency of peripheral $CD28^-CD25^+CD8^+$ T cells, which have features resembling those of old $CD25^+CD4^+$ Tregs and express high levels of CD122 (145, 166). Aged $CD8^+$ Tregs have diminished immunosuppressive activity since they downregulate the expression of checkpoint inhibitory molecules (167) and during aging show impaired secretion of NADPH oxidase 2 (NOX2)-containing exosomes, which normally abrogate $CD4^+$ T cell activation (168).

Given the profound changes that Tregs undergo during aging and their active role in inflammaging and its related pathologies, it is tempting to speculate that targeting this subset may foster healthier aging. In this regard, the antioxidant *N*-acetylcysteine reinvigorates aged Tregs (147, 165). Moreover, anti-IL-6 and proapoptotic strategies (144, 169) seem to be promising tools to reverse the age-associated increase of dysfunctional Tregs to delay immunosenescence.

Overall, the accumulation of extremely differentiated Tregs secreting both resolving and proinflammatory cytokines during aging could have a detrimental role in age-related diseases not only by causing tissue damage but also by inducing age-related programs such as exhaustion or senescence in conventional T cells, placing Tregs in the center of interest during aging.

CROSS TALK BETWEEN OLD TISSUES AND T CELLS

Heterochronic adoptive transfer and parabiosis experiments suggest that the function and differentiation state of T cells change depending on their surrounding microenvironment (29, 170, 171). Aged lymph organs negatively impact the homing of circulating T cells, impairing their activation and differentiation (29). Adoptive transfer of T cells from young to aged mice demonstrated that the aged host is sufficient to convert young T cells into exhausted $PD\text{-}1^+TOX^+$ T cells producing GzmK (25). In addition, Tregs are affected by the microenvironment since TGF-β secreted by senescent tissues increases the amount of Tregs (28, 135). Consequently, depleting senescent cells by using senolytics or TGF-β-neutralizing antibodies alleviates the expansion of Tregs during infection in geriatric mice (135).

Finally, cumulative evidence from others and our lab supports the idea that both innate and adaptive old immune cells can act as drivers of tissue senescence, contributing to organ dysfunction during aging (15, 172, 173). Recent reports provide evidence that accumulation of all these age-associated T cells contributes to sustaining inflammaging and promoting accumulation of senescent cells in aged tissues (15, 173). The different mechanisms by which age-associated T cells might contribute to inflammaging have recently been reviewed (174). Briefly, old T cells, characterized mainly by constant production of IFN-γ, TNF-α, or GzmK, promote the activation of a senescence program in bystander cells. Besides the acquisition of this pathogenic phenotype, old T cells could also contribute to senescence and inflammaging by losing their protective function against senescent cells. Functional T cells play an essential role in senescence immunosurveillance, which is the recognition and clearance of senescent cells (175–177). Recently, engineered chimeric antigen receptor (CAR)-T cells designed to recognize senescent cells have been proposed as a new senolytic approach to delete senescent cells in old tissues. In fact, Amor et al. (178) identified the urokinase-type plasminogen activator receptor (uPAR) as a cell surface marker of senescent cells and demonstrated that uPAR-specific CAR-T cells efficiently ablate senescent cells, restoring tissue homeostasis in mice with liver fibrosis. However, similar to cancerous cells, senescent cells

can develop strategies to avoid the immune response. Senescent dermal cells express the nonclassical MHC molecule HLA-E, which inhibits cytotoxic activity in T cells through the interaction with NKG2A. HLA-E is induced by the SASP and regulated by p38 signaling in vitro, explaining how the aged tissue could evade senescence immunosurveillance (179). Thus, the decline of T cell activity during aging could contribute to an indirect accumulation of senescent cells due to defects in this immunosurveillance of old tissues. Finally, functional T cells are necessary to maintain intestinal homeostasis and gut microbiota. As such, defective T cell activity could contribute to age-related changes in gut microbiota leading to dysbiosis and increased gut permeability and bacterial translocation, which foster inflammaging (174). Altogether, these results begin to unravel the intimate cross talk between T cells and tissue senescence during aging, illuminating an emerging field based on T cell therapy to develop strategies to delay tissue deterioration.

CONCLUDING REMARKS

The immune system is highly affected by aging. The thymus may be the most affected tissue since its degeneration starts during puberty. Thus, the homeostatic proliferation caused by reduced generation of naive T cells together with clonal expansion produced by the exposure to antigens triggers the acquisition of terminal differentiation and senescence features. Although both $CD4^+$ and $CD8^+$ T cells age, $CD8^+$ T cells exhibit earlier and more profound changes. Characterization of aged T cells by scRNA-seq has exposed different subsets of age-associated T cells comprising exhausted, cytotoxic, and activated Tregs. $CD8^+$ T cells lose their function and become exhausted with aging, probably due to persistent antigen stimulation. These exhausted cells stop producing proinflammatory and cytotoxic molecules and secrete GzmK, which causes senescence in neighboring cells. In addition, other $CD8^+$ T cells gain expression of NK receptors that change their recognition pattern from antigen-dependent recognition to antigen-independent killing of infected or damaged cells. Aged $CD4^+$ T cells experience a similar process that shifts their function from classical helper to cytotoxic characterized by the expression of NK receptors (NKG2A, NKG2D) and cytotoxic molecules (GzmB, GzmK). Again, some other $CD4^+$ T cells become exhausted and inactive, reducing their cytokine production. Theoretically, T cell exhaustion programs could be a protective mechanism to prevent tissue damage, whereas the acquisition of cytotoxic and proinflammatory phenotypes may contribute to tissue deterioration and worsen inflammaging. This dichotomy is fascinating. Contrary to cancer treatment, triggering exhaustion may be a suitable approach to decrease chronic inflammation and age-associated diseases. However, it is important to remark that transitory exhausted T cells do not lack cytokine production; they produce alternative cytokines such as GzmK. Regarding Tregs, during aging they are converted into terminally differentiated effector Tregs that exert both anti- and proinflammatory roles. Importantly, aged Tregs promote senescence or exhaustion in conventional T cells (123, 147, 180–182); thus, both Tregs and conventional T cells have key roles in the age-related deterioration of tissues.

The different stimuli driving the development of age-associated T cells are not perfectly known yet. The memory-like and terminally differentiated phenotype suggests that age-associated T cells develop upon antigen recognition, but the identification of virtual memory T cells supports the idea that some of these subsets could emerge in an antigen-independent manner as a consequence of their long replicative history. Importantly, signals from the microenvironment are emerging as an essential factor in this process.

The increasing knowledge of checkpoint regulators, immunometabolism, CAR-T cells, and other T cell–based therapies will allow the development of new strategies not only to treat cancer and immune disorders but also to prevent inflammaging and age-associated diseases.

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