Telomerase and primary T cells: biology and immortalization for adoptive immunotherapy

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Abstract

Telomeres are specialized repeats, present at the end of chromosomes, whose loss during cell division is followed by growth arrest, a central mechanism of replicative senescence in human cells. Telomere length in stem cells is maintained by telomerase, a specialized reverse transcriptase, whose function is to restore shortening telomeres. Unlike most somatic cell types, human T lymphocytes are capable of briefly reactivating telomerase expression at the time of stimulation. Telomerase expression in T lymphocytes is modulated by a variety of external stimuli and by viral infections. However, telomerase reactivation in stimulated, proliferating human T lymphocytes is limited and cannot prevent the ultimate onset of senescence. Ectopic telomerase expression can rescue human and macaque antigen-specific T cells from senescence. Primary T cells have been engineered with telomerase to have substantially extended replicative lifespans without the loss of primary cell functions or malignant transformation. 'Immortal' antigen-specific T-cell lines and clones overexpressing telomerase are an invaluable source of well-characterized quasi-primary T cells for research of T-cell biology and are potentially useful for immunotherapy of cancer and AIDS.

Keywords

adoptive transfer; antigen-specific; immortalization; immunotherapy; retroviral vectors; T cells; telomerase

In mammals, somatic cells continuously differentiate from pluripotent stem cells to acquire specific biological functions. Typically, cells lose the ability to proliferate when they reach their terminally differentiated state. Terminally differentiated somatic cells ultimately enter a phase of replicative senescence, during which the cells withdraw from the cell cycle and undergo specific molecular and physiological changes. Replicative senescence is the fundamental feature of all mammalian cells, which ensures tight regulation of cell growth and the integrity of structure and function of tissues and organs.

Somatic cells of different types and developmental origins differ in their capacity to proliferate and have variable proliferative lifespans; the cells stop dividing after reaching a certain number of population doublings (the Hayflick limit) [1], and then enter a replicative senescence phase owing to shortened telomeres that reach a critical length. Telomeres are specialized structures at chromosome ends that consist of repetitive DNA sequences of varying length (telomeric repeats). Telomeres are associated with several proteins that form a complex, which protects chromosomes from fusion and chromosome ends from being improperly recognized as DNA break/damage signals [2–4]. With each DNA replication cycle, telomeres are shortened [5] and ultimately reach a critical length, at which a p53-mediated growth arrest mechanism is induced and cell proliferation is ceased [4]. While young cells tend to have longer telomeres, telomere length in aged and senescing cells is much shorter. Thus, telomere shortening is thought to function as a molecular clock, which imposes a limit on proliferative capacity and population expansion of most cells and serves as a signal for replicative senescence.

Shortened telomeres can be stabilized or restored by the enzyme telomerase reverse transcriptase (TERT), which is a specialized DNA polymerase, capable of extending telomere repeats by synthesizing DNA strands on the template of specialized telomerase RNA (TR). TERT, TR and several associated proteins form a complex on the telomere ends. TERT is exclusively expressed in the cells that are normally capable of long-term proliferation (e.g., embryonic stem cells), but not in normal differentiated somatic cells, except for lymphocytes [6–10]. TERT expression is tightly regulated and is repressed to undetectable levels during the differentiation of embryonic stem cells into mature somatic cells; however, TR and telomere-associated proteins are still expressed in terminally differentiated cells, making TERT the limiting factor in telomere length maintenance. TERT expression is upregulated in the majority (>85%) of cancers [11–17]. This, and other observations, indicate that telomere maintenance is a central mechanism of cell immortalization associated with malignant transformation.

Telomere shortening, TERT & senescence of human T lymphocytes

The immune system is highly complex and consists of many different types of cells that, together, ensure its functioning as a tightly regulated and highly efficient mechanism of protection against pathogens. T lymphocytes are a core cell type in the immune system, and perform important functions in the immune surveillance (such as immunologic memory, elimination of pathogens and regulation of the immune response). T cells mostly circulate in a quiescent nonproliferating state yet vigorously proliferate when activated with antigens or nonspecific stimuli. *In vivo*, naive antigen-specific T cells proliferate and undergo a limited clonal expansion during the primary immune response. Conversely, central memory T cells do not proliferate and can persist in the immune system through the lifespan of the host, maintaining the ability to differentiate into rapidly proliferating and expanding effector T cells during the secondary immune response to a cognate antigen. *In vitro*, T cells can be activated and proliferate in response to a specific (antigen) or nonspecific (mitogenic anti-CD3 antibodies, lectins) stimulation. TERT activity is sharply elevated in activated human [18–22] and mouse [23] T cells, and may play a role in supporting the survival, proliferation and expansion of antigen-responding T cells *in vivo*. B cells can also be activated and proliferate in response to external stimuli, which is accompanied by upregulation of TERT expression [24]. Thus, T and B lymphocytes are unique among other differentiated somatic cell types in terms of their ability to respond to specific or nonspecific stimuli by proliferation and continued expansion, accompanied by TERT upregulation.

Proliferating activated T cells can be expanded and maintained in culture for extended periods of time. However, similar to the other somatic cell types, they ultimately stop

dividing, undergo replicative senescence and become resistant to apoptosis [25,26]. Several disorders and experimental observations highlight the role of telomeres in T-cell biology, and ample evidence has been accumulated that the replicative potential of human T cells is determined by the length of their telomeres and by the ability to upregulate TERT.

TERT expression is rapidly activated in both CD8^{+} and CD4^{+} human T cells stimulated by antigens [27]. While dramatic increase of TERT activity occurs upon primary stimulation, subsequent stimulation cycles induce less TERT expression, which eventually becomes nearly undetectable as the cells progress towards senescence. This suggests that TERT activity in human T cells is tightly regulated. The decline in TERT activity parallels the loss of CD28 costimulation molecule expression. Stringent TERT regulation thus contributes to telomere shortening and finite replication potential of antigen-specific T cells, which may play a role in the loss of immune control of some pathogens (such as HIV) that cause persistent T-cell activation.

Endogenous telomerase activity levels are not sufficient to completely block telomere loss and to extend the replicative lifespan of T cells beyond their natural Hayflick limit [28]. Human T cells, expressing dominant-negative TERT, have a decreased lifespan in culture and develop cytogenetic abnormalities such as chromosome fusions and the lack of telomeric DNA at the chromosome ends [29]. The inverse correlation between TERT activity levels and replicative history suggests that TERT expression in human T cells is limited and insufficient to sustain extended periods of proliferation.

T cells from the patients with Nijmegen breakage syndrome (NBS; a rare genetic abnormality associated with a high incidence of lymphoid malignancies) were found to frequently undergo spontaneous immortalization in culture [30]. Immortalized T-cell lines from NBS patients invariably have stabilized telomere length and high TERT activity. Upregulation of TERT expression in the spontaneously immortalized T cells from NBS patients was recently discovered to be a late event in the immortalization process [31].

In vivo, antigen-responding, proliferating T cells can quickly loose telomeric DNA and move towards replicative senescence. Injection of purified protein derivative into the skin of individuals immune to tuberculosis mobilizes purified protein derivative-specific CD4^{+} T cells, which proliferate and differentiate extensively in the skin. These cells develop significant telomere erosion as compared with the T cells found in the blood from the same individuals [32]. Type I interferon inhibits TERT expression in these cells, demonstrating that extracellular signals, such as cytokines, can regulate the replicative lifespan of antigen-specific T cells, modulating TERT expression levels and (indirectly) telomere length.

Further evidence that TERT plays a pivotal role in maintaining telomere length and proliferative potential of human T cells comes from the study of the regulatory CD4^{+}CD25^{+} T cells (Tregs), isolated from peripheral blood of cancer patients. Tregs, presumably, are an important factor causing an inefficient immune response against tumor cells [33–38]. Treg numbers are often elevated in the blood of cancer patients, but it remains unclear whether this elevation is due to redistribution of Tregs or to an induction of their active proliferation. Tregs isolated from blood were found to have shortened (but stable) telomere length, suggesting that induction of TERT *in vivo* helps prevent replication senescence [39]. In the Tregs expanded *in vitro*, TERT activity was readily inducible, but was not sufficient to prevent further telomere shortening. These data further support the notion that the replicative lifespan of human T cells critically depends on the length of telomeric DNA and that endogenously induced TERT may support contained expansion but cannot protect the cells from senescence.

Telomere length and capability to reactivate TERT activity are factors that determine the lifespan and antitumor activity of tumor-infiltrating lymphocytes (TILs), which mediate the regression of tumors in patients with meta-static melanoma [40–42]. TILs isolated from resected tumors are frequently represented by several specific persistent and nonpersistent clonotypes. TIL clonal persistence was found to correlate with telomere length and TERT activity [43]. Some TIL clonotypes extensively proliferate *in vivo* following adoptive transfer, but fail to upregulate TERT, undergo rapid decrease in telomere length within days after transfer and are driven into senescence. Other TIL clonotypes with longer telomeres are able to persist and mediate antitumor effects. Thus, TERT upregulation and telomere length may be two central factors determining the effectiveness of adoptive immunotherapy of cancer by influencing the replicative lifespan of transferred T cells.

In patients with rheumatoid arthritis (RA), T cells are chronically stimulated, are prone to premature aging and exhibit accelerated telomere loss [44,45]. While T-cell activation and cell-cycle progression are not affected, both naive and memory T cells are defective in TERT induction upon activation and are highly sensitive to apoptosis, which can be reversed by ectopic TERT overexpression. Thus, TERT deficiency and increased T-cell aging resulting from chronic stimulation of T cells, reveal the critical role that telomere length and TERT upregulation may play in determining T-cell fate *in vivo*.

T cells that are at the end stage of replicative senescence accumulate in elderly persons and patients chronically infected with HIV [46], which potentially leads to immunodeficiency. These cells are irreversibly cell-cycle arrested, have shortened telomeres and are unable to upregulate TERT upon stimulation. Clonal exhaustion of HIV-specific CD8^{+} T cells has been proposed as a mechanism for failure of antigen-specific immune responses, but the molecular basis for it is still not well understood. Telomere length is abnormally short in the T cells from persons infected with pathogens such as HIV-1 [47,48], potentially due to high chronic cell turnover [49,50]. Since chronic antigenic stimulation can induce long-term T-cell proliferation followed by telomere shortening and replicative senescence, it is possible that clonal exhaustion may be caused by TERT downregulation and increased T-cell senescence. Constitutive overexpression of TERT in HIV-1-specific cytotoxic T-lymphocyte (CTL) clones transduced with TERT-expressing retroviral vector protects the cells from the onset of senescence [51] and the cells exhibit an increased proliferative capacity in culture, and elevated cytolytic and antiviral functions. This suggests that the loss of antigen-specific response in HIV-1-infected individuals may be a consequence of an increased rate of replicative senescence of virus-specific effectors. Furthermore, constitutive TERT expression alone can stabilize telomere length and protect human T cells from senescence.

In summary, telomere length is the primary factor affecting the lifespan of human T cells. The ability of activated T lymphocytes to upregulate TERT distinguishes them among other somatic differentiated cell types; however, this naturally occurring TERT upregulation is transient and is not sufficient to prevent the onset of replicative senescence in the long-term cultures. Telomere loss, aging and replicative senescence of T cells are accelerated *in vivo* by chronic antigenic stimulation and extended proliferation of T-cell populations in patients with chronic inflammatory diseases, such as RA, and viral infections, such as AIDS. Accelerated aging of chronically activated T cells may be directly linked to the development of immunodeficiency.

Telomere length & cell-cycle arrest

When telomeres in proliferating human cells become critically short, the cells recognize chromosomal ends as a DNA damage event. This triggers upregulation of p53, which, in turn, induces irreversible cell-cycle arrest, followed by the onset of senescence [4]. What

protects the cells from inappropriately inducing the DNA damage signaling when the telomeres are still sufficiently long? In addition to TERT, six other proteins are associated with the ends of telomeres as a complex known as shelterin and, together with TERT, participate in regulating replicative senescence [52]. Three proteins, TRF1, TRF2 and POT1, directly recognize vertebrate telomeric repeat sequences (TTAGGG) and are interconnected by three additional proteins, TIN2, TPP and Rap1. Telomere ends not capped with shelterin complex are not hidden from DNA damage surveillance and inappropriately induce DNA repair mechanisms and cell-cycle arrest, resulting in replicative senescence. In long-term human T-cell cultures, telomere shortening occurs preferentially in long telomeres and is interrupted at each stimulation by a transitory increase in length [53]. In senescing T cells, the number of cells exhibiting DNA damage foci in telomeres increases, presumably owing to the continuous increase of p16INK4a cell-cycle inhibitor protein upon cell aging [53]. The expression of shelterin genes decreases at each stimulation, suggesting that, in addition to TERT, a deficit of shelterin proteins and subsequent telomere uncapping contribute to the telomere dysfunction during T-cell senescence.

Induction of TERT expression in human T lymphocytes

TERT expression in human T cells is tightly regulated. In long-term T-cell cultures, the levels of TERT induced upon initial T-cell activation reach a peak at 3–5 days and then decline over the following 2 weeks [19] (reviewed in [54]). A second antigenic stimulation still causes upregulation of TERT expression but, by the third and all subsequent stimulations, the T cells are unable to upregulate TERT. Proliferation in the absence of TERT results in progressive telomere shortening and, ultimately, in cell-cycle arrest and senescence, accompanied by altered cytokine expression profiles, resistance to apoptosis and down-regulation of CD28 costimulatory molecule. Accumulation of senescent T cells has been associated with poor responses to vaccines and with increased synthesis of proinflammatory cytokines [55,56].

TERT expression in normal human primary T cells can be upregulated by extracellular signaling or intracellular events, which can be induced either by normal immune system functioning or by viral pathogens.

Cytokines

Cytokines are directly involved in the regulation of T-cell proliferation and expansion *in vivo* and regulate TERT expression through the signal transduction pathways. IL-2 is the major growth factor for T cells; hence its involvement in regulating TERT activity is not surprising. IL-2 increases TERT transcription in human T cells [57]. At least in some types of human lymphocytes (such as human NK cells), IL-2 affects TERT activity post-translationally through the PI3K/Akt pathway [58]. IL-2 is capable of inducing transcriptional activation of TERT promoter in an IL-2-dependent quiescent human T-cell line; this activation coincides with the G1 to S phase transition of the cell cycle, and is independently mediated by two distinctive elements within the TERT promoter region [57].

IL-7 and IL-15 are two major growth factors implicated in the maintenance, survival and homeostatic control of CD8+ and CD4+ T-cell pools [59–61]. Both cytokines were shown to upregulate TERT expression in human T cells. IL-7 mediates T-cell survival through the regulation of Bcl2 family proteins (Bcl2, Bcl-xL, Bax and Bad), which are equally induced by IL-7 treatment of naive and memory CD4+ T cells in culture [62]. However, IL-7 induces higher levels of TERT in naive cells than in memory cells and considerably increases naive T-cell protection from cell death, implying TERT involvement in differential regulation of survival of naive and memory CD4+ T cells.

IL-15 plays an important role in long-term survival and homeostasis of human memory T lymphocytes [59–61]. IL-15 is capable of activating TERT expression in memory CD8^{+} T cells through Jak3 and PI3K signaling pathways; IL-15-induced TERT levels are sustainable over long periods of time in culture, which minimizes telomere shortening and significantly increased the proliferative lifespan over a number of cell divisions [63]. Furthermore, both IL-7 and IL-15 induce delayed proliferation of human naive T cells in culture without marked differentiation into effectors, accompanied by an upregulation of TERT expression and stable telomere length [64]. Retroviral transduction of a primary human CD8^{+} T-cell clone with the *IL-15* gene generated a cell line that constitutively expressed TERT and exhibited external cytokine-independent growth for more than 1 year while preserving the capability for antigen-specific activation, suggesting that the IL-15 autocrine loop alone is sufficient to ensure TERT-dependent extension of the T cell replicative lifespan [65]. Thus, participation of IL-7 and IL-15 in the maintenance and survival of human naive and long-term memory T cells is not limited to regulating proliferation and protecting from apoptosis; both cytokines can increase the longevity of the T cells by supporting stable and sustainable telomere length through upregulated TERT expression.

TNF-α rapidly induces TERT activity and translocation of TERT protein to the nucleus in peripheral blood mononuclear cells (PBMCs) through the PI3K/Akt/NF-κB signaling pathways [66]. Interestingly, data from another study suggests that TNF-α inhibition or neutralization in long-term cultures of human CD8^{+} T cells can enhance TERT activity and delay the nonproliferative end stage of replicative senescence [67].

Signal transduction pathways

TERT upregulation by extracellular growth factors is mediated by the specific signal transduction pathways (such as the PI3K/Akt pathway, or the NF-κB pathway). Among the components of the NF-κB pathway, protein kinase C (PKC) has been shown to have an essential function in the upregulation of TERT activity in stimulated human T cells. PKC-ζ is required for both the induction of TERT expression and the post-transcriptional control of its enzymatic activity [68]. Overexpression of PKC-θ (but not the other PKC isoforms) activates transcription from the TERT promoter in resting T cells [69]. PKC-θ acts through NF-κB pathway via c-Myc.

In addition to the PI3K/Akt and NF-κB pathways, other signal transduction mechanisms may contribute to the regulation of TERT activity in human T cells. MAPK cascade-mediated phosphorylation of H3 histone Ser10 was found to be involved as a link between the T-lymphocyte proliferation and TERT activation [70]. In normal human T cells, H3 Ser10 phosphorylation through the MAPK pathway occurs in response to growth stimulation and/or stress. H3 Ser10 phosphorylation alone results in weak transient TERT activation. However, Ser10 phosphorylation, followed by Lys14 acetylation, strongly activates TERT activity and results in constitutive TERT expression in normal and transformed T cells, while H3 acetylation alone has a weak TERT activation effect. Thus, H3 phosphorylation/acetylation through the MAPK signaling appears to be a fundamental mechanism of TERT regulation in T cells.

Interestingly, signal transduction pathways may be involved in modulating human T cell replicative lifespan and senescence by mechanisms other than direct TERT regulation. Highly differentiated CD8^{+}CD28^{−}CD27^{−} senescing T cells, with short telomeres, low TERT activity and reduced proliferation capacity, express elevated levels of the senescence-associated inhibitory receptor, KLRG1, and exhibit defective Akt Ser473 phosphorylation upon activation. Blocking KLRG1 signaling by antibodies against its ligand, E-cadherin, results in a significant increase in Akt Ser473 phosphorylation and Akt-mediated induction of cyclines D and E, downregulation of cell-cycle inhibitor p27 and induced T-cell

proliferation, while TERT levels in growing cells remain low and are unaffected by KLRG1 signaling blockade [71]. This suggests that while TERT regulation is critically important in modulating human T-cell lifespan and senescence, certain functional defects in differentiated aging T cells can be induced and maintained via inhibitory receptor signaling and may be independent of telomere length and TERT activity levels.

CD28 is a costimulatory molecule, which is involved in T-cell activation by antigens and in the upregulation of TERT activity. T cells usually lose CD28 expression together with TERT activity after repeated activation and prolonged proliferation in culture. However, restoration of CD28 signaling by introducing exogenous CD28 does not reverse loss of TERT activity [72]. In T cells that lost CD28 expression, TERT is still inducible but not enzymatically active; this defect is associated with decreased phosphorylation of Akt kinase that phosphorylates TERT into an enzymatically active protein. Thus, TERT activity during extended T-cell proliferation can be downregulated by inhibiting external phosphorylation, rather than by the loss of TERT expression.

Transcription factors & telomerase complex proteins

The molecular mechanism of TERT upregulation upon T-cell activation is still not completely understood. Transcription factors may play a central role in the tight regulation of TERT promoter activity. Several lymphoid cell-specific transcription factors were found to be implicated in regulating TERT expression in human T cells.

Nuclear factor of activated T cells (NFAT1) is directly involved in transcriptional activation of TERT expression [73]. The TERT promoter has five putative NFAT1 binding sites and two sites for SP1, and is activated by overexpressed NFAT1. Mutations in one of these sites cause a considerable reduction in the promoter transcriptional activity. SP1 transcription factor apparently activates TERT promoter in cooperation with NFAT1, as simultaneous mutations in NFAT1 responsive element, and in one or both flanking SP1-binding sites, lead to a more dramatic promoter activity decrease, suggesting a functional synergy between NFAT and SP1 in TERT transcriptional regulation.

Interferon regulatory factors (IRF)-4 and -8 induce TERT activity in stimulated proliferating T cells by activating transcription from the TERT promoter [74]. IRF-4 binds the interferon-response-stimulated element and the IFN-γ-activated sequence element in the TERT promoter. IRF-4 also activates coordinated binding of Sp1, Sp3, USF-1, USF-2 and c-Myc transcription factors to the promoter. Furthermore, transcriptional activation is the result of synergistic cooperation of IRF-4 with Sp1 and Sp3.

In addition to transcription factors, TERT expression can be regulated by the other proteins of the telomerase complex. Premature telomere shortening, cell aging and death in the hematopoietic compartment are seen in the patients with dyskeratosis congenita (DC), an inherited multi-system disorder characterized by bone marrow failure and a predisposition to cancers. Patients with DC have mutations in genes encoding the components of the telomerase complex (DKC1, TERC, TERT, NOP10 and NHP2) and telomere shelterin complex (TINF2) that are important in telomere maintenance. Lentiviral transduction with exogenous TERC alone was shown to rescue this defect by preserving telomere length, increasing TERT activity and improving survival and growth in human T-cell lines derived from DC patients [75], suggesting an important role of telomerase complex proteins in regulating TERT activity.

Viral pathogens

Viruses, such as HIV-1 and human T-cell leukemia virus (HTLV)-1, can infect T cells and can modulate TERT levels and replicative lifespan of the T cells by several mechanisms.

Viral proteins can directly downregulate TERT function, thus contributing to the decrease in the T-cell lifespan and promoting immunodeficiency. Both TERT expression and phosphorylation are impaired in human CD4+ cells infected with HIV-1, potentially contributing to HIV-1-associated immune dysfunctions [76]. HIV-1 transcriptional regulator protein, Tat, has been directly associated with TERT activity downregulation [77]. TERT transcription is not affected, but enzymatic activity is increased in the cytoplasmic compartment, and decreased in the nuclear compartment of HIV-1-infected T-cell lymphoid Jurkat cells [78], suggesting direct involvement of viral infection in the regulation of TERT activity. Telomere length and TERT activity in HIV-1-specific CD8+ T cells from progressors or controllers of HIV-1 infection were studied [79]. T cells from controllers had significantly longer telomeres than those from progressors, which corresponded to higher levels of constitutive TERT activity in controller T cells, demonstrating an important role of telomere shortening in the functional deficiencies of HIV-specific CD8+ T cells in chronic progressive infection.

HTLV-1 immortalizes human primary T cells and induces adult T-cell leukemia/lymphoma (ATLL) in infected individuals. The HTLV-1 oncogene, *Tax*, can transform human cells and is a main contributory factor in HTLV-1-induced leukemogenesis. In T-cell lines derived from ATLL patients, TERT transcription is upregulated by *Tax* [80]. The role of *Tax* in the regulation of TERT expression in T cells remains controversial. Interestingly, *Tax* can exert two opposite effects on TERT expression. It can impair TERT induction in mitogenically stimulated primary T cells and in the T cells infected with HTLV-1 [81]. However, transduction of *Tax* into primary T lymphocytes activates and maintains TERT expression through NF-κB signaling and promotes telomere length stabilization, thus contributing to T-cell immortalization and transformation associated with ATLL [80,82]. In nondividing T cells, *Tax* activates TERT transcription. By contrast, *Tax* represses TERT transcription in proliferating Jurkat cells [82], suggesting that the outcome of Tax action on TERT expression may depend on the cell cycle state of the T cells. Both upregulation and downregulation by *Tax* are mediated through the same element in the TERT promoter. *Tax* is also able to affect the expression of several shelterin genes. *Tax*-mediated upregulation of TERT transcription in activated CD4+ T cells correlates with downregulation of transcription of *TRF1*, *TRF2* and *POT1* genes encoding shelterin complex proteins, while in HTLV-1-infected or *Tax*-transformed T cells, downregulation of TERT is accompanied by a significant upregulation of *TRF2* and *POT1*. This suggests that *Tax*-mediated modulation of *TERT* and shelterin gene expression is tightly coordinated [83]. *Tax*-mediated immortalization of human T cells is accompanied by a sustained TERT activity. *Tax* also activates PI3K/Act signaling pathway, one of the mechanisms of TERT upregulation [84].

TERT-immortalized human T cells maintain antigen specificity & primary cell characteristics

Human antigen-specific T cells are finding increased use as a prime tool for adoptive immunotherapy [85–90]. One prominent example is cancer immunotherapy by adoptive transfer of autologous tumor-specific T cells expanded *in vitro* [41,91–94] Recent success in redirecting antigen specificity of the T cells by retroviral vector-mediated transfer of cloned cancer antigen-specific T-cell receptor (TCR) genes has opened the way for a new direction in the gene therapy of cancer. It is now possible to modify patient's autologous T cells with cancer-specific TCR genes, followed by the adoptive transfer of the modified and *in vitro*-expanded T cells back to the host; this strategy has been recently used to treat metastatic melanoma [95–98].

Although immunotherapy protocols are being developed to treat cancer and infectious diseases such as AIDS, the success of these strategies is hindered by the limited *in vitro*

replicative lifespan of human primary T cells. Antigen-specific human T cells can be isolated and cloned from blood, lymph nodes, or resected tumor tissues; however, upon prolonged periods of culturing and expansion *in vitro* the cells ultimately enter a phase of replicative senescence, which results from TERT deficiency and consecutive progressive loss of telomere DNA. Since senescing cells have rather limited potential for use in immunotherapy, a technology providing the means to efficiently protect T cells from telomere loss and replicative senescence would be highly advantageous for successful clinical application of antigen- specific T cells. One such technology is engineering of the T cells to express TERT at levels that are sufficient to protect T cells from senescence.

Immortalization of human T-cell lines & clones by TERT overexpression

The proliferative lifespan of human antigen-reactive T cells can be significantly extended by introducing the ectopically expressed *TERT* gene. The human papillomavirus type 16-derived oncoproteins, E6 and E7, are responsible for the onset and maintenance of the transformed phenotype in cervical carcinoma cells [99]. Low frequency CD8^{+} E7-specific CTLs can be isolated and cloned from healthy donors and their lifespan can be considerably extended by transducing the cells with a retroviral vector constitutively expressing TERT [100].

TERT overexpression can be used to increase the survival of the T cells and to protect them from apoptosis [101,102]. The longevity of human CD4^{+} helper type-1 or -2 T cells in culture was increased by *TERT* gene overexpression [103]. T cells, ectopically expressing TERT, were found to expand more vigorously than untransduced cells of the same replicative age. Importantly, in comparison with the untransduced T cells, TERT-transduced cells expressed elevated levels of antiapoptotic Bcl2 protein, contained lower active caspase-3, and were resistant to telomere DNA-damaging oxidative stress. Thus, in addition to protecting the T cells from replicative senescence, conferring resistance to apoptosis afforded by ectopically expressed TERT could further increase the survival of T cells.

As described previously, premature senescence of HIV-1-specific T cells and associated loss of the expression of effector molecules (such as granzyme and perforin) has been implicated as a potential mechanism of immune system failure in AIDS. Senescence of HIV-1-specific CTL and the loss of effector functions have been shown to be preventable by transducing the cells with TERT-expression vectors [104].

Immortalized T cells & animal models

In addition to maintaining antigen recognition and effector functions *in vitro,* TERT-overexpressing human tumor-reactive T cells were found to maintain full antitumor reactivity in an *in vivo* murine cancer model [105]. Human melanoma cell line melAKR was transduced with a retrovirus expressing the influenza virus epitope and the resulting melAKR-Flu cells were transplanted in RAG-$2^{-/-}$ IL-$2R\gamma^{-/-}$ double-knockout mice, where they developed tumors. Adoptively transferred TERT-transduced influenza virus-specific human CTL clones inhibited the growth, and caused regression of, melAKR-Flu tumors, but not the parental melAKR tumors in mice, with the same efficiency as observed with untransduced CTL clone, suggesting that TERT-transduced T cells can maintain antigenic specificity and a full set of effector functions *in vivo*. Thus, human T cells, overexpressing TERT, can potentially be used to eradicate tumors by adoptive immunotherapy.

Nonhuman primates (NHPs) provide an attractive model for research of immune responses to lentiviral infections. Rhesus monkeys (RMs) infected with simian immunodeficiency virus (SIV) are widely used to model the pathology of human AIDS and cellular immune responses to HIV-1. Similar to human T cells, *in vitro*-expanded RM T lymphocytes enter a

phase of replicative senescence and have a limited replicative lifespan. TERT-immortalized RM antigen-specific T cells might provide an invaluable source for studies of SIV immune responses and for experimental adoptive immunotherapy. CD8^{+} T-cell clones with effector memory surface marker profiles, specific for immunodominant SIV gag and tat epitopes [106], were derived from MamuA*01 RMs and transduced with a retroviral vector expressing human TERT [107]. Transduced cell lines exhibited substantially better survival in long-term *in vitro* cultures in comparison with their untransduced parental clones, responded specifically to antigenic stimulation, and displayed comparable proliferation rates, cytokine expression profiles and cytolytic activities. Furthermore, these T-cell lines constitutively expressing TERT were shown to respond to the SIV-infected autologous CD4^{+} target T cells by IFN-γ production and degranulation and were able to significantly suppress SIV replication [108]. Thus, primary NHP antigen-specific T cells can be successfully immortalized by human TERT. Immortalized NHP T cells retain full potential for antigen recognition and effector functions and may prove to be a valuable tool for developing experimental immunotherapy protocols. Whether TERT-immortalized RM T cells have an extended lifespan while maintaining their antigen recognition characteristics and effector functions when adoptively transferred *in vivo*, remains to be seen.

Selective capture & TERT-mediated immortalization of human T cells

TERT overexpression from retroviral vectors can be applied to simultaneously genetically tag and immortalize human T cells responding to a specific antigen. Infection with Moloney murine leukemia virus-derived retroviral vectors, expressing TERT, allows selective capture and immortalizing of human antigen-specific T cells from a complex cell population such as blood [109,110]. Such vectors can only integrate in dividing cells [111,112], thus, being able to selectively transduce only those T cells in a population that are specifically activated and dividing. Human PBMCs, stimulated with alloantigen and infected with TERT-expressing vectors, gave rise to CD8^{+} antigen-specific T-cell lines with considerably extended replicative lifespan. The lines were generated by passaging the transduced cells for extended periods of time with no other selection steps involved, demonstrating that antigen-responding T cells could be selectively captured, genetically marked and immortalized by TERT vector transduction. TERT-immortalized CD8^{+} allospecific T-cell lines, maintained IL-2-dependent growth, specifically responded to cognate alloantigen by proliferation and production of cytokines and exhibited antigen-specific cytolytic activity.

Are TERT-immortalized T cells transformed?

Permanent TERT activation and subsequent cell immortalization are critical steps for malignant transformation. The majority of tumor-derived cell lines or cancer cells derived from resected tumor tissues express high TERT levels. Are TERT-immortalized human primary T cells on the way to transformation? Human CD8^{+} T-cell clones, overexpressing TERT, have identical growth rates with their untransduced counterparts and have no differences in gene expression patterns [113]. However, at later passages, despite an increased expression of genes involved in the cell-cycle progression, TERT-transduced T cells exhibit reduced proliferative responses and accumulate cell cycle inhibitor proteins p16INK4a and p21Cip, which are associated with growth arrest. Thus, TERT-expressing T cells do not appear to develop genetic changes characteristic of the malignant transformation and the mechanisms regulating growth remain intact and are apparently still able to limit proliferation rates of immortalized T cells, independently of the telomere status.

To be considered as a practical, safe and useful cell resource for adoptive immunotherapy, TERT-transduced T cells must maintain their antigen specificity and stably retain the full spectrum of their effector functions. CD8^{+} HIV-1-specific T lymphocytes, transduced with TERT expression vectors, exhibit an enhanced ability to suppress HIV-1 replication *in vitro*,

as well as the ability to produce IFN-γ and TNF-α in response to stimulation with HIV-1 peptides [114]. Interestingly, in CD28^{+} T cells overexpressing TERT, the loss of CD28 costimulatory molecule expression can be significantly delayed in comparison with untransduced control cells. Thus, TERT-mediated immortalization of the T cells does not directly lead to malignant transformation. However, long-term consequences of constitutive TERT expression in the T cells must be further investigated.

In summary, engineering of human and NHP T cells to overexpress TERT can provide a significant replicative lifespan extension to valuable antigen-specific T-cell lines and clones without the loss of their primary T-cell characteristics, thus providing cellular immunologists with a lasting source of well-characterized T cells. In addition, protection from replicative senescence *in vivo,* afforded by TERT overexpression, may make immortalized T cells an attractive tool for adoptive transfer-based immunotherapy.

Potential problems using TERT-immortalized T cells for immunotherapy

While the TERT-mediated protection of antigen-specific T cells from replicative senescence may assist in developing novel immunotherapy approaches, two major problems may potentially hamper the use of TERT-engineered T lymphocytes in clinical-grade adoptive transfer protocols.

TERT itself is an antigen recognizable by the host immune system

Telomerase reverse transcriptase is expressed in the majority of human cancers and can be recognized by the immune system as a tumor-associated antigen. Human CTLs, specific for TERT-derived epitopes, have been isolated and were reactive against TERT-expressing human cancer cells [115]. These CTLs were able to recognize endogenously produced TERT depending on its expression level. Furthermore, TERT-reactive T cells were found in most patients with cancer and were shown to be capable of killing autologous TERT-positive tumor cells [116,117]. The role of TERT in the anti-tumor immune response is becoming clearer, and targeting TERT as a way to treat cancer has been proposed [118,119]. The potential for using TERT for anticancer vaccination has been recently demonstrated in mouse models [120,121]. Thus, although no studies on the adoptive transfer of TERT-overexpressing T lymphocytes in NHPs or in humans have so far been published, adoptively transferred TERT-expressing T cells could potentially be rejected by the host, thus limiting the applicability of these cells in immunotherapy protocols.

TERT-immortalized T cells can develop genomic instability & become transformed

Extending the longevity of T lymphocytes, TERT overexpression may cause genomic instability in transduced cells. Furthermore, extending the lifespan of the T cells may give these cells a chance to develop secondary genetic changes (e.g., the loss of heterozygocity of tumor suppressor genes), which can ultimately lead to their malignant transformation. TERT-transduced human primary CD4^{+} T cells exhibited significantly increased replicative lifespan in culture, but eventually developed signs of genomic instability and senesced [122]. This suggests that constitutive TERT expression is not always sufficient to completely stave off senescence and that the genomic changes may develop at the end of an active proliferation phase. Extensive cytogenetic analysis of TERT-transduced melanoma antigen recognized by T cells (MART-1) and human papilloma-virus type 16 E7-specific human CD8^{+} CTLs, revealed the development of minor or severe chromosomal aberrations [123]. The frequency and biological significance of chromosomal aberrations in TERT-transduced T cells remain unclear and warrants further investigation. Besides maintaining telomere length, additional telomere-independent functions of TERT in eukaryotic cells (antiapoptotic activity, promoting survival in many cell types, enhancing tumorigenesis and

affecting the expression of genes that control cell growth) were discovered (reviewed in [124,125]). Thus, extensive studies of the fate of TERT-overexpressing T cells *in vivo* are necessary before any clinical application of these cells becomes a reality.

Conclusion & future perspective

Before TERT-mediated immortalization of human T cells finds its place in the arsenal of tools available for immunotherapy, several important questions must be answered.

Lifespan extension or true immortalization?

TERT overexpression protects human T cells from replicative senescence in long-term *in vitro* cultures. T cells, constitutively expressing TERT, maintain characteristics of primary lymphocytes: they specifically recognize and respond to cognate antigens and maintain their natural effector functions, such as production of physiologically appropriate cytokines and antigen-specific cytolytic activity. We and others were able to maintain human and RM TERT-transduced antigen-specific T-cell lines and clones in culture for periods over 1 year without the loss of specific T-cell functions, which far exceeds the normal replicative lifespan of primary human and NHP T cells [107–109,114]. However, it remains to be established that TERT-transduced T-cell lines and clones are truly immortal, rather than protected from senescence for an extended, but still finite, period of time. Long-term observations of TERT-immortalized T-cell lines are necessary to determine their actual longevity *in vitro* and *in vivo*, and their ability to maintain primary T-cell functions.

Can TERT-transduced T cells eventually become transformed?

Typically, overexpression of TERT does not lead to transformation in human cells [126]. Human and RM T-cell lines overexpressing TERT display a behavior that is characteristic for normal primary T lymphocytes, for long periods of time [109]. Importantly, similar to their nontransduced counterparts, and in contrast to transformed T-cell lines, proliferation of TERT-transduced T cells can be induced by activation and their growth is strictly dependent on cytokines. However, the potential for a malignant transformation of TERT-immortalized T cells needs to be thoroughly investigated by long-term studies.

What is the fate of adoptively transferred TERT-transduced T cells?

The most advanced use of human TERT-immortalized T cells can be their application for adoptive immunotherapy. However, adoptively transferred immortalized T cells may potentially behave differently *in vivo* than in long-term *in vitro* cultures. As of yet, no data has been obtained on the fate of the immortalized T cells *in vivo.* Research of adoptively transferred, TERT-transduced T cells in NHP models will allow the study of the longevity of TERT-immortalized T lymphocytes *in vivo* and their ability to home, persist and function in the appropriate compartments of the immune system. Importantly, these studies will also help to determine the potential for a malignant transformation, which remains a major safety concern.

Can TERT-overexpressing T cells sustain efficient immune responses during chronic infections?

Telomere loss and premature senescence of chronically stimulated antigen-specific T cells may be the primary cause for clonal exhaustion and for a failure of immune responses against persistent viral pathogens such as HIV-1. HIV-1-specific TERT-immortalized CTLs are antigen-reactive and can proliferate and expand without senescence *in vitro* [114]. Engineering these cells to express ectopic TERT, followed by an adoptive transfer, may be

an attractive approach to create a senescence-resistant antigen-reactive CTL pool that can efficiently protect the host against HIV-1 infection.

To conclude, ectopic TERT expression can significantly extend the lifespan of human and RM antigen-reactive T cells, without affecting their function. TERT-mediated immortalization can be used as an important tool in cellular immunology and can potentially enable the development of safe and efficacious adoptive immunotherapy to treat cancer and AIDS.

Acknowledgments

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Executive summary

Telomerase reverse transcriptase & replicative senescence

- Mammalian somatic cells have limited replicative lifespan in culture or *in vivo*.

- The onset of replicative senescence is regulated by the length of telomeres, which are specialized structures at chromosome ends that progressively become shorter with each DNA replication cycle.

- Shortening of telomeres beyond a critical length induces p53-mediated growth arrest and senescence.

- Telomerase reverse transcriptase (TERT) is a specialized reverse transcriptase, capable of restoring telomere length and preventing replicative senescence.

- TERT is expressed in long-living stem cells and in immortal cancer cells, but not in normal differentiated somatic cells, except for T and B lymphocytes.

Telomere shortening is a central mechanism of human T-cell senescence

- Human T cells circulate in the blood as a nonproliferating population but can be stimulated to proliferate by antigens.

- In proliferating T cells, telomeres progressively shorten with each cell division and the cells eventually enter a phase of replicative senescence.

- Human T cells differ from other types of somatic cells in that they upregulate TERT expression during activation.

- TERT induction is transient and is lost after several consecutive stimulations.

- Abnormally short telomeres are found in T cells from persons infected with some viral pathogens such as HIV-1, or in chronically stimulated T cells from patients with inflammatory diseases, such as rheumatoid arthritis.

Regulation of TERT expression in human T cells

- TERT expression levels in T cells can be modulated by cytokines and T-cell-tropic viral pathogens, such as HIV-1 and human T-cell leukemia virus 1.

- Specific signal transduction pathways have been found to participate in telomerase expression regulation:

    – PI3K/Akt pathway;

    – NF-κB pathway;

    – MAPK pathway.

- Certain functional defects in senescing T cells may be induced through the inhibitory receptor signaling, independently of telomere shortening and TERT activity levels.

- In T cells, transcription factors such as NFAT1, Sp1, Sp3, USF-1, USF-2, c-Myc, IRF-4 and IRF-8 may play a central role in the regulation of TERT promoter activity.

- TERT expression can be regulated by the proteins of a telomerase complex and a shelterin complex.

- HTLV-1 *Tax* oncogene can both impair and activate TERT induction in primary human T cells.

Ectopic TERT expression significantly extends replicative lifespan of human primary T lymphocytes, while preserving primary T-cell characteristics

- Antigen-specific T-cell lines and clones, transduced with TERT expression vectors, exhibit better survival and increased replicative lifespan and are protected from senescence-associated loss of effector functions.

- In transduced T cells, antiapoptotic proteins are upregulated and the cells can be resistant to telomere DNA-damaging oxidative stress.

- TERT-transduced primary antigen-specific human and rhesus monkey T cells maintain the full spectrum of primary T-cell characteristics:

  – Ability to respond specifically to antigenic stimulation;

  – Production of cytokines in response to stimulation;

  – Antigen-specific cytolytic activity.

- Retroviral vectors, expressing TERT, can be used to selectively capture and immortalize antigen-specific T cells directly from complex cell populations such as blood.

- TERT-overexpressing human tumor-specific T cells maintain full antitumor reactivity in an *in vivo* cancer model.

Potential problems using TERT-immortalized T cells for immunotherapy

- TERT-mediated immortalization can provide an attractive source of well-characterized, long-lasting, antigen-reactive T cells to use in research or in adoptive immunotherapy.

- Several problems may hinder the use of TERT-overexpressing T cells in the clinic:

  – TERT can be recognized as an antigen; hence, adoptively transferred T cells may be rejected by the host immune system;

  – TERT-mediated replicative lifespan extension may give T cells critical time necessary for developing genomic instability or picking up mutations in cancer-related genes, thus paving the way for a malignant transformation.

- Further studies are necessary to establish long-term survival and fate of TERT-immortalized T cells *in vivo*.