

Telomerase Activation and Rejuvenation of Telomere Length in Stimulated T Cells Derived from Serially Transplanted Hematopoietic Stem Cells

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Abstract

Telomeres shorten in hematopoietic cells, including hematopoietic stem cells (HSCs), during aging and after transplantation, despite the presence of readily detectable levels of telomerase in these cells. In T cells, antigenic stimulation has been shown to result in a marked increase in the level of telomerase activity. We now show that stimulation of T cells derived from serially transplanted HSC results in a telomerase-dependent elongation of telomere length to a size similar to that observed in T cells isolated directly from young mice. Southern analysis of telomere length in resting and anti-CD3/CD28 stimulated donor-derived splenic T cells revealed an increase in telomere size by ~ 7 kb for the population as a whole. Stimulation of donor-derived T cells from recipients of HSCs from telomerase-deficient mice did not result in regeneration of telomere length, demonstrating a dependence on telomerase. Furthermore, clonal anti-CD3/CD28 stimulation of donor-derived T cells followed by fluorescent in situ hybridization (FISH) analysis of telomeric signal intensity showed that telomeres had increased in size by $\sim 50\%$ for all clonal expansions. Together, these results imply that one role for telomerase in T cells may be to renew or extend replicative potential via the rejuvenation of telomere length.

Key words: T cell • hematopoietic stem cell • transplantation • telomere • mouse

Introduction

Telomeres are genetic elements that are essential for the stability of chromosomal ends. The critical shortening or loss of one or more telomeres leads to the formation of unstable end-to-end fusions and chromosomal instability (1–3). Telomeric chromatin is composed of a number of different telomeric binding proteins and tandem arrays of simple DNA repeats, (TTAGGG) $_n$ in mammals (4) ranging in length from <100 bp in ciliates (5) to 5,000–8,000 bp in humans (6–8) and, in some mouse strains, $>100,000$ bp (9).

The telomerase complex, composed of an essential RNA component (10) and several different protein components including an essential catalytic component (11) is required for the complete replication of telomeres in most dividing eukaryotic cell populations (12). Immortal cell populations, including germ line cells and tumor cell lines, express telomerase and maintain a stable telomere length (2, 13–15). Genetic ablation of the telomerase RNA gene in yeast (16) and mouse embryonic stem cells (17) or inhibition of te-

lomerase in tumor cell lines (18) leads to the continuous attrition of telomere length as cells divide, culminating in growth arrest and/or cell death. Telomere length also shortens during replicative aging in many types of human somatic cells in which telomerase is repressed (2, 6, 7, 19).

Many mitotically active somatic cells in humans have a finite replicative capacity, up to ~ 100 population doublings (pd), when grown in vitro. The state of irreversible growth arrest that subsequently ensues is termed replicative senescence (20). Studies have now demonstrated that replicative senescence is the ultimate effect of continuous telomere attrition, as activation of telomerase via ectopic expression of the catalytic component of telomerase, telomerase reverse transcriptase (TERT;* reference 11) in primary cell strains prevents telomere shortening and leads to cell immortalization (19, 21, 22).

A perplexing feature of hematopoietic cells, including hematopoietic stem cells (HSCs), is the presence of readily detectable levels of telomerase activity (23, 24) and yet di-

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*Abbreviations used in this paper: FISH, fluorescent in situ hybridization; HSC, hematopoietic stem cell; TERT, telomerase reverse transcriptase; TRF, terminal restriction fragment.

vision of these cells, is accompanied by extensive telomere shortening both *in vitro* (22, 25) and *in vivo* (8, 26, 27). It has also recently been shown that the continuous erosion of telomeres and limited replicative capacity observed in long term cultures of T cells from humans (22, 25) can be prevented by the ectopic expression of TERT (22, 28). Thus, for reasons unknown at present, telomerase appears to be present in hematopoietic cells, but not fully functional.

Recently, we have shown that telomere length shortens in HSCs and other hematopoietic cells of donor type during serial transplantation of HSCs in mice (27). We now show that stimulation of splenic T cells isolated from HSC transplant recipients results in a telomerase-dependent restoration of telomere length to a size found in young mice.

Materials and Methods

Mice. The derivation of the mTR knockout mice and mTERT knockout mice has been described previously (3, 29). The mTR^{-/-} mice and mTERT^{-/-} mice were backcrossed 6 and 4 times, respectively, to the C57Bl/Ka-Thy1.1(Ly5.1) strain at the Stanford University animal facility before use in this study. In all transplant experiments, the Thy1.1/Ly5.1 mice were used as HSC donors and the congenic C57Bl/Ka-Thy1.2(Ly5.2) strain was used as recipients. The initial donor mice and all the recipient mice were 2–3 mo old. The major histocompatibility class I gene promoter (H2K^b-GFP) transgenic mice colony was developed and maintained at Stanford University. All mice were bred and maintained on acidified water (pH 2.5).

HSC Detection and Transplantation. Bone marrow cells were isolated and stained with fluorophore-conjugated antibodies as described previously (27). The antibodies used in the immunofluorescence staining for HSC detection are as described previously (27). The HSC population is defined as c-kit^{hi}Sca-1^{hi}Thy1.1^{lo}lineage⁻. Whole bone marrow aliquots containing either 100 or 200 HSCs were used in each round of transplantation.

Splenic T Cell Sorting and Stimulation. Spleens were collected and single cell suspensions prepared followed by lysis of red blood cells using ACK solution (150 mM NH₄Cl, 10 mM KHCO₃). Splenocytes were stained with an antibody cocktail (CD3^{PE}, B220^{APC}, Ly5.1^{FITC}, and Ter119^{TR}) in staining media (Hank's balanced salt solution plus 3% fetal bovine serum, pH 7.2) on ice for 30 min. Cells were then washed once and resuspended in staining media containing propidium iodide (0.5 μg/ml). The donor type splenic T cells and splenic T cells from H2K-GFP transgenic mice were defined as (CD3^{hi}B220⁻Ly5.1^{hi}Ter119⁻) and (CD3^{hi}B220⁻), respectively, and were purified by FACS[®] on a dual-laser Vantage (Becton Dickinson) FACS[®] machine. Cells were either sorted into growth media (RPMI 1640 plus 10% FBS (GIBCO BRL), glutamine, nonessential amino acids, Pen/Strep, sodium pyruvate, β-mercaptoethanol, ml-2 (200 U/ml), and CD28 antibody [a kind gift from Dr. Tien Chin, Department of Immunology, Stanford University]) in 24-well plates (mass culture) or 96-well V-bottom plates (oligoclonal culture) precoated with CD3 antibody (BD Biosciences). During oligoclonal culture, cells were split at a 1:2 ratio on days 7, 11, and 14, and donor-derived cells were harvested on day 17.

Detection of Telomerase Activity. The TRAP assay and quantification of telomerase activity was performed using the TRAP assay kit from Intergen, as described (15) but with following modi-

fications. The final concentration for all dNTPs was 10 mM, and 0.2 μg of TS primer was used per 25 μl reaction.

DNA Isolation and Terminal Restriction Fragment Length Analysis by Field Inversion Gel Electrophoresis. Splenic T cells were collected for DNA isolation at the time of the initial transplantation and from secondary HSC transplant recipients 4 mo or more post-transplant. The DNA was isolated, digested with restriction enzymes, and subjected to southern analysis of terminal restriction fragment (TRF) length using a field inversion gel electrophoresis (FIGE) apparatus as described previously (27). Mean TRF length was calculated as described previously (25).

Fluorescent In Situ Hybridization. Cells were fixed and telomeres stained with a FITC conjugated peptide nucleic acid telomeric oligomer, (CCCTAA)₃ (Applied Biosystems), as described previously (27, 30). Images were collected and quantitative analysis of the fluorescent signal intensity was performed using a ZEISS confocal microscope. Control slides of unstained cells were used to correct for background fluorescence.

Immunostaining. The telomerase specific K-370 antibody was used to detect mTERT as described previously (31). Briefly, cells were fixed in methanol, blocked in 10% goat serum, and stained with a 1/500 dilution of the K-370 antibody in 1% serum for 1 h at 37°C. Cells were washed in PBS, and stained with biotin-conjugated anti-rabbit IgG (Vector Laboratories) for 1 h at 37°C. After a brief wash in PBS, cells were stained with Texas Red-conjugated avidin (Sigma-Aldrich) for 1 h at 37°C. Nuclei were stained with Hoechst 33258.

Results

Southern Analysis of Terminal Restriction Fragment Length in Resting and Stimulated T Cells from HSC Recipients. To assess the effect on telomere length of antigenic stimulation of T cells of donor type from adult mice and HSC transplant recipients, we collected donor-derived splenic T cells by FACS[®] for anti-CD3/CD28 stimulation *in vitro* and performed southern analysis of TRF length on the resting and stimulated T cells (Fig. 1). The TRF length for resting splenic T cells isolated from secondary HSC recipients (mean ~16 kb) was significantly shorter than that observed for resting T cells from young adult mice (mean ~23 kb; P = 0.005). 1 wk after anti-CD3/CD28 stimulation, no change in the TRF length was detected for splenic T cells isolated from young adult mice. However, the TRF length of the stimulated splenic T cells isolated from HSC transplant recipients had increased significantly (Fig. 1; P = 0.002), to an average size approximately equal to that observed for splenic T cells from young adult mice.

Activation of Telomerase Is Required for Telomere Length Increase in Stimulated T Cells. To assess the potential role of telomerase in the restoration of telomere length in stimulated T cells derived from transplanted HSCs, we performed the TRAP assay on resting and anti-CD3/CD28 stimulated splenic T cells (Fig. 2). Similar to that reported in previous studies (32–35), we observed a large (~45 fold; Fig. 2 B) increase in telomerase activity 2 d after anti-CD3/CD28 stimulation of donor-derived T cells from adult mice and from HSC transplant recipients. No difference in the level of telomerase activity was observed for resting T cells or stimulated T cells isolated from young adult mice as

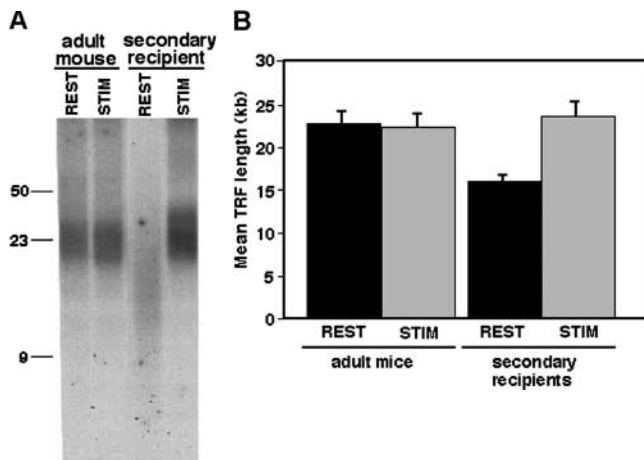


Figure 1. Analysis of TRF length of resting and stimulated donor-derived T cells. (A) Splenic T cells (5×10^4) from young adult mice and secondary HSC recipients were collected via FACS[®] and either transferred to growth media for stimulation or used for isolation of high molecular weight DNA. Anti-CD3/CD28 stimulated T cells were collected for isolation of DNA after 1–2 wk of growth. The extraction and restriction enzyme digestion of DNA was performed as described previously (reference 27). 1 μ g of each DNA sample was resolved in a 0.75% agarose gel by field inversion gel electrophoresis (pulse conditions: 180 V forward; 120 V reverse for 16 h). The gel was dried and the DNA hybridized to a ³²P-end labeled telomeric oligomer overnight followed by 3 \times 5 min washes at 37°C in 0.35 \times SSC buffer. Images were collected using a Phosphor-Imager screen. Size of molecular weight standards (kilobases) are shown on the left. REST, resting; STIM, anti-CD3/CD28 stimulated. (B) The mean TRF length was calculated and averaged for all resting and stimulated T cell samples taken from a total of five adult mice and seven secondary recipients. Error bars (standard deviation) and P values (Student's *t* test) are shown.

compared with secondary HSC recipients. To begin to assess the mechanism as to how telomerase is activated after antigenic stimulation of T cells, we stained splenic T cells with an antibody to mTERT before and after anti-CD3/CD28 stimulation. TERT appeared to be localized primarily in the cytoplasm of resting cells and in the nucleus of stimulated cells (Fig. 2 C), as previously observed by others (36). To exclude the possibility of nonspecific binding of the mTERT antibody, splenic T cells from mTERT^{-/-} mice were also stained. Only a very faint, nonspecific nuclear signal was observed in both resting mTERT^{-/-} T cells (Fig. 2 C) and activated mTERT^{-/-} T cells (data not depicted).

To verify the essential role of telomerase in telomere length rejuvenation after activation of T cells, we analyzed telomere length in T cells from young adult mice and secondary HSC recipients in which the gene encoding the RNA component of telomerase (mTR) was knocked out (3). Telomere length was analyzed using fluorescent in situ hybridization (FISH) as opposed to southern analysis of TRF length due to the large, heterogeneous, multi-modal nature of the TRFs in this mouse strain (unpublished data). Telomere signal intensity increased after antigenic stimulation of donor-derived T cells from secondary recipients of HSC from mTR^{+/+} mice (Fig. 3; *P* = 0.001), in agreement with the increase in TRF length observed for wild-

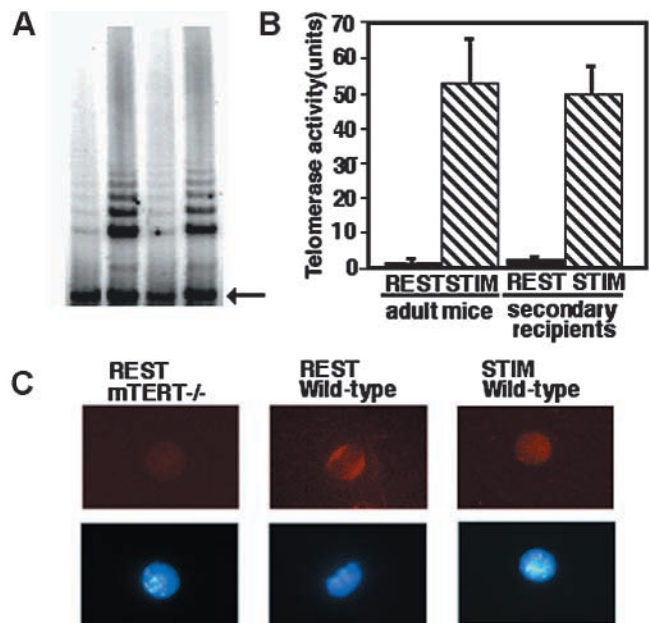


Figure 2. Analysis of telomerase activity in resting and stimulated donor-derived T cells. (A) Splenic T cells (2×10^5) from young adult mice and secondary HSC recipients were collected via FACS[®] and either lysed in CHAPS buffer for extraction of telomerase, or transferred to growth media for stimulation. After 2 d of growth, anti-CD3/CD28 stimulated splenic T cells were harvested for extraction of telomerase. Telomerase activity was measured for 500 cell equivalents of each sample extract by the TRAP assay. (B) Telomerase activity was measured for resting (REST) and stimulated (STIM) T cells from a total of five adult mice and seven secondary recipients and averaged for all samples. The mean level of activity and error bars (standard deviation) are shown. (C) Analysis of TERT localization in resting and stimulated T cells. Resting and anti-CD3/CD28 stimulated T cells from mTERT wild-type mice and resting T cells from mTERT knockout mice were fixed and stained with an mTERT antibody (top panel). Corresponding Hoechst 33258 staining for each cell is also shown (bottom panel). Original magnification: $\times 60$.

type C57Bl/Ka Thy1.1 mice (Fig. 1). However, no change in telomere signal intensity was observed following stimulation of donor-derived T cells from secondary recipients of HSCs from mTR^{-/-} mice (Fig. 3), thereby confirming the necessity of telomerase for extension of telomere length.

Telomere Length Increase in Stimulated T Cells Is Due to Elongation of Short Telomeres by Telomerase, Not Selection of Cells with a Long Telomere Length. The increase in telomere size in stimulated splenic T cells isolated from HSC transplant recipients could be accounted for by selection of T cells with a long initial telomere length and/or the synthesis of new telomeric DNA after stimulation of T cells with a short initial telomere length. The former possibility implies that there is a rare population of T cells with long telomeres in the spleens of HSC transplant recipients. This population must exist at a frequency of $\sim 10\%$ or less of the reconstituted splenic T cells since we are able to detect two distinct modes of longer and shorter TRFs when DNA from splenic T cells of donor type from adult mice and secondary HSC transplant recipients are mixed at a ratio as low as 1:10 and analyzed for TRF length, but only observe

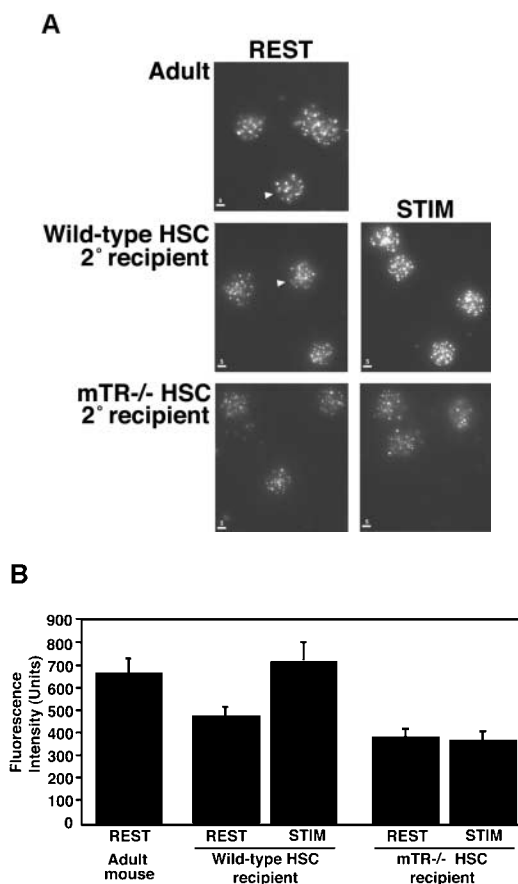


Figure 3. FISH analysis of telomere length in resting and stimulated donor-derived T cells from transplant recipients of HSC from mTR knockout mice. (A) Splenic T cells (5×10^4) from young adult mTR wild-type or knock-out mice and secondary HSC recipients were collected via FACS[®] and either transferred to growth media for stimulation or fixed. 1 wk after anti-CD3/CD28 stimulation, cells were cytospun onto glass slides and fixed. Telomeres were detected by FISH using a FITC-conjugated peptide nucleic acid telomeric oligomer. Individual interphase nuclei are indicated by arrowheads. Original magnification: $\times 60$. The size scale (μm) is indicated in the bottom left. (B) The fluorescent telomeric signal intensity was calculated and corrected for background for 20 well isolated individual resting or stimulated mTR^{+/+} or mTR^{-/-} donor-derived splenic T cell nuclei. Telomeric signal intensity was also measured for resting splenic T cells from an adult wild-type mouse. The mean fluorescent signal intensity and standard deviation are shown. Telomeric signal intensity increased significantly ($P < 0.005$; Student's *t* test) after stimulation of donor-derived splenic T cells from secondary recipients of mTR^{+/+} HSCs.

the shorter TRF mode in T cells from secondary recipients (unpublished data). To test for selection of T cells with a long initial telomere length after stimulation, we costimulated 10 pools of 9 T cells from transgenic mice which express GFP throughout the hematopoietic system, plus a single donor-derived T cell from a secondary HSC recipient. 2 1/2 wk after stimulation, cells derived from the secondary HSC recipient T cell were isolated by FACS[®] for FISH analysis of telomere length (Fig. 4). For all pools containing cells derived from the secondary recipient (6 of 10), telomere signal intensity increased significantly ($\sim 50\%$), becoming approximately equal to that observed for resting T

cells from adult mice (Fig. 4 B). In addition, we also stimulated 10 pools of 10 T cells from an independent secondary HSC recipient and observed a similar increase in telomere signal intensity in all pools (unpublished data). Thus, while we cannot completely exclude the existence of a rare population of T cells with a long initial telomere length, the increase in telomere length following stimulation of donor-derived T cells from HSC transplant recipients is, at least in part if not entirely, a direct result of extension of the shortened telomeres in these cells.

Discussion

Results from a number of studies have indicated that extension of telomere length can occur in normal somatic cells. Lengthening of telomeres has been observed during development in the offspring of mice in which the set of telomeres inherited from one parent are longer than those inherited from the other (37). Compared with adult tissues, telomerase activity is relatively high in the germ line (15) and the developing embryo (38, 39) including embryonic stem cells (17) and therefore the increase in telomere length observed in this study is likely telomerase dependent. Previous studies have also provided strong evidence for telomere lengthening in B cells: germinal center (GC) B cells have longer telomeres than either precursor naive B cells or more mature memory B cells (40), and stimulation of murine splenocytes *in vivo* has been shown to be accompanied by an increase in telomere length in wild-type mice but not early generation (G1) mTR^{-/-} mice (41). However, the selection of a rare subpopulation of B cells with an initial long telomere length, as opposed to true extension of telomeres, was not ruled out in these studies. Here we show that antigenic stimulation of T cells derived from serially transplanted HSCs with an initial short telomere length directly results in a telomerase-dependent extension of telomere length to a size roughly equal to that observed in T cells from young animals.

Although we believe our data favors a scenario in which telomere length is restored via a telomerase-dependent mechanism in most if not all resting T cells that have acquired shortened telomeres, other possible mechanisms warrant discussion. One alternative explanation for the restoration of telomere length that we observe in stimulated T cells from secondary HSC recipients is that HSCs and/or resting T cells with increased levels of telomerase activity are being selected for during transplantation or after stimulation, respectively. However, we have compared telomerase activity between HSCs isolated directly from donor animals and from primary and secondary recipients, and found no change in the level of activity with successive rounds of transplantation (unpublished data). Furthermore, we have previously shown that telomere length decreases in HSCs during serial transplantation, which would not be expected if cells with higher levels of telomerase were being selected for. Although we cannot rule out the possibility of selection of resting T cells with high initial levels of telomerase, a mechanism to explain the inability of telom-

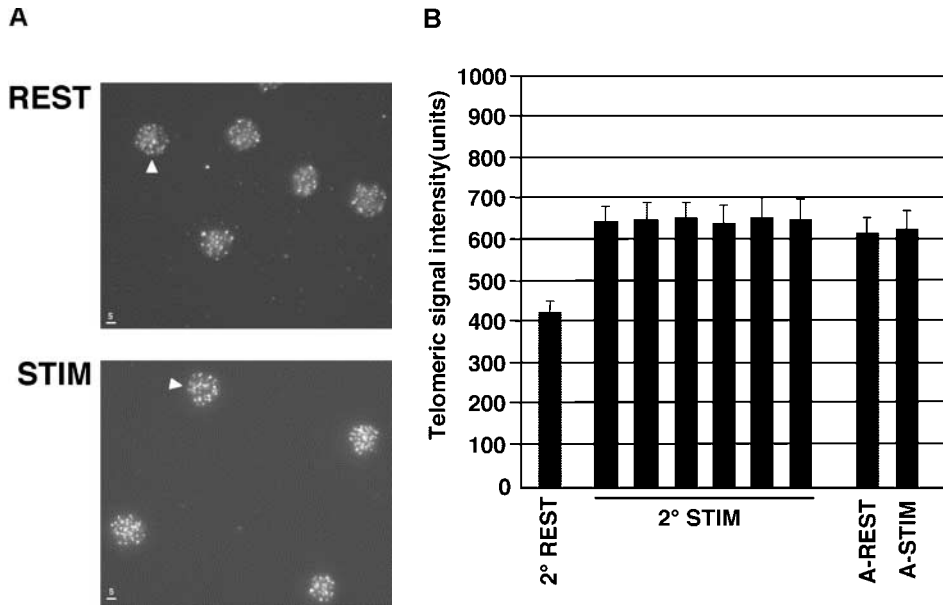


Figure 4. FISH analysis of telomere length after clonal stimulation of donor-derived T cells. (A) Splenic T cells were sorted into 10 pools of 10 cells, 9 cells from a H2K-GFP transgenic mouse, and 1 cell of donor type from a secondary HSC recipient, in growth media in a 96-well V-bottomed dish for stimulation. Resting splenic T cells were also collected via FACS, cyto-spun onto slides, and fixed at this time. 17 d after stimulation, T cells derived from the secondary recipient (i.e., non-GFP cells) were collected via FACS[®] from each stimulated pool in which they could be detected, and either cyto-spun onto glass slides, and fixed or used for confirmation of T cell functionality by TCR clonotype analysis (reference 48; unpublished data). The telomeres were detected by FISH using a FITC-conjugated peptide nucleic acid telomeric oligomer. Individual interphase nuclei are indicated by arrowheads. Sample images

of stained nuclei collected from resting splenic T cells (top panel) and of one clonal pool of anti-CD3/CD28 stimulated splenic T cells from a secondary recipient (bottom panel) are shown. Original magnification: $\times 60$. The size scale (μm) is indicated in the bottom left. (B) The fluorescent telomeric signal intensity was calculated and corrected for background for 20 well isolated individual resting or stimulated splenic T cell nuclei from a secondary recipient. Telomeric signal intensity was also measured for resting and clonally stimulated splenic T cells ($n = 20$ for each) from a C57Bl6/Ka Thy1.1 mouse. The mean fluorescent signal intensity and standard deviation are shown. For all clonal expansions derived from T cells from the secondary HSC recipient, the telomere signal intensity increased significantly relative to resting T cells from the same mouse ($P \leq 0.005$; Student's *t* test).

erase to restore telomere length in these cells before stimulation would have to be presumed, as these cells almost certainly have a shortened telomere length before stimulation (Fig. 4; see Results). Furthermore, it would also have to be assumed that any T cells with higher levels of telomerase would either have to be a rare population or be able to further increase their levels of telomerase upon activation, as telomerase activity increases dramatically following antigenic stimulation of T cells (32–35; Fig. 2). It is also possible that the telomerase-independent ALT (alternative lengthening of telomeres) mechanism (42) for extending telomeres may be contributing to the telomere elongation in activated donor-derived T cells. The rate of telomere length increase, ~ 7 kb over 16–17 d or ~ 8 –12 population doublings, that we observe is relatively fast, akin to the rapid increase in telomere length observed in ALT-positive tumor cell lines (42). Also, after immunization, telomere length in splenocytes from late generation (G5) $\text{mTR}^{-/-}$ mice have been observed to increase by ~ 12 kb (41), which may very well be explained by the previous activation of ALT in these mice. However, we have not observed, in resting or activated T cells (Fig. 1 A), the large, heterogeneous distribution of TRFs that is characteristic of ALT-positive cells (42), nor have we observed an increase in telomere length after activation of T cells from telomerase-deficient mice (Fig. 3). Nevertheless, it will be of interest to further assess the possible contribution of ALT to the restoration of telomere length following activation of donor-derived T cells.

The observations reported here suggest that one function of telomerase in some or all subsets of T cells may be to re-

store telomere length upon antigenic stimulation in cells that have acquired shortened telomeres. In agreement with this notion is the positive correlation previously observed between telomerase activity level and telomere length after antigen stimulation of human CD4^+ T cells (43). One consequence of the ability to replenish telomere length in T cells with short telomeres is a concomitant increase in replicative capacity. This could perhaps be important not only in any rare naive or memory T cells in young individuals which may have acquired one or more critically short telomeres, but also in the elderly in which hematopoietic cells, including T cells, have very short telomeres (25). Specifically, it may be possible for these cells, upon antigenic stimulation *in vivo*, to thwart a premature replicative senescence induced by further telomere shortening via the regeneration of telomere length to a size observed in young individuals. To confirm this, it will be necessary to assess changes in telomere length after stimulation of T cells from elderly individuals, or, if they can be identified, T cells with short telomeres from young individuals.

As previously noted by Liu et al. (36) we find that TERT, surprisingly, appears to be predominantly present in the cytoplasm in resting T cells and translocates to the nucleus after antigenic stimulation (Fig. 2 C). It is quite likely that TERT translocation, as well as other events, are essential for the activation of telomerase in stimulated T cells. Although full details of the signaling mechanism leading to the nuclear translocation TERT have yet to be worked out, it may involve association of TERT with 14–3–3 proteins (44). The 14–3–3 family of signaling proteins act as molecular chaperones and have been shown to asso-

ciate with TERT (44). In addition, TERT contains a NES-like motif in close proximity to the 14–3–3 binding site, suggesting that the binding of 14–3–3 proteins to TERT may inhibit the interaction of the exportin CRM1 with the TERT NES-like motif (44). The signaling mechanism for TERT translocation may also involve phosphorylation of TERT (36). It will be of great interest to identify all of the factors involved in the activation of TERT in resting T cells, as these factors may perhaps provide a novel target in therapies to treat T cell leukemias. It will also be important to assess the physiologic significance of the localization of TERT in the cytoplasm of resting T cells, and to assess whether TERT is also localized in the cytoplasm of other hematopoietic cells, including HSCs.

The data reported here suggests that telomerase can extend telomere length in T cells during the first few doublings after stimulation, but only to a size equal to that observed in resting T cells in young animals. The mechanism which limits the amount by which telomerase can extend telomere length, although not well understood, may involve the interaction of the newly assembled telomeric chromatin with telomerase in a negative feedback loop. One telomeric binding protein in particular that probably has an important role in this feedback loop is the Myb-related protein TRF1, which binds at numerous sites along the telomeric DNA tract (45). Overexpression of TRF1 or inhibition of its normal association with telomeric chromatin leads to a decrease or increase in telomere length, respectively (46). Furthermore, TRF1 induces bending in telomeric DNA upon binding (47) which may in turn affect the enzymatic activity of telomerase. Future in vivo studies as to the effect of TRF1 function and expression on telomere length maintenance in embryonic stem cells and germ line cells, and telomere length rejuvenation in lymphocytes, should help shed more light on this subject.

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