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The Loss of Telomerase Activity in Highly Differentiated CD8+CD28−CD27− T Cells Is Associated with Decreased Akt (Ser⁴⁷³) Phosphorylation¹

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The enzyme telomerase is essential for maintaining the replicative capacity of memory T cells. Although CD28 costimulatory signals can up-regulate telomerase activity, human CD8⁺ T cells lose CD28 expression after repeated activation. Nevertheless, telomerase is still inducible in CD8⁺CD28− T cells. To identify alternative costimulatory pathways that may be involved, we introduced chimeric receptors containing the signaling domains of CD28, CD27, CD137, CD134, and ICOS in series with the CD3 ζ (ζ) chain into primary human CD8⁺ T cells. Although CD3 ζ-chain signals alone were ineffective, triggering of all the other constructs induced proliferation and telomerase activity. However, not all CD8⁺ CD28− T cells could up-regulate this enzyme. The further fractionation of CD8⁺ CD28− T cells into CD8⁺ CD28− CD27− and CD8⁺ CD28− CD27⁺ subsets showed that the latter had significantly shorter telomeres and extremely poor telomerase activity. The restoration of CD28 signaling in CD8⁺ CD28− CD27⁻ T cells could not reverse the low telomerase activity that was not due to decreased expression of human telomerase reverse transcriptase, the enzyme catalytic subunit. Instead, the defect was associated with decreased phosphorylation of the kinase Akt, that phosphorylates human telomerase reverse transcriptase to induce telomerase activity. Furthermore, the defective Akt phosphorylation in these cells was specific for the Ser⁴⁷³ but not the Thr³⁰⁸ phosphorylation site of this molecule. Telomerase down-regulation in highly differentiated CD8⁺ CD28− CD27− T cells marks their inexorable progress toward a replicative end stage after activation. This limits the ability of memory CD8⁺ T cells to be maintained by continuous proliferation in vivo. The Journal of Immunology, 2007, 178: 7710–7719.

Memory T cell pools can persist for over 8 decades in humans (1). Because thymic involution occurs, these cells have to be maintained by periodic stimulation and expansion of pre-existing populations of Ag-specific T cells (2). However, after a finite number of cell divisions, human lymphocytes experience growth arrest due to chromosomal aberrations resulting from telomere erosion. Telomeres are repeating hexameric sequences located at the ends of chromosomes that are lost when cells divide (1, 3). The induction of telomerase activity can compensate for telomere loss (4–6), however, upon repeated restimulation, T cells lose the capacity to up-regulate this enzyme leading eventually to telomere erosion and replicative senescence (1, 3). The question that remains is whether excessive telomere erosion in Ag-specific T cell populations, especially during persistent infections (7–9), may lead to growth arrest and loss of these cells. This is particularly important because the human life expectancy is increasing (1, 10). Furthermore, T cells that have been expanded by specific stimulation in vitro have been used successfully to treat infections and tumors in vivo (11). However, excessive expansion of T cells from older subjects who have shorter telomeres to begin with (7) may limit the persistence of these cells after adoptive transfer in vivo and thus reduce their therapeutic efficacy (12).

Previous studies suggest that CD28 cosignals may be important for telomerase induction in human T cells (1, 3, 13, 14). Although CD8⁺ CD28− T cells accumulate during aging (15) and telomerase activity in CD8⁺ T cells from old subjects is reduced (4), it is unclear whether either or both changes are directly linked and are direct consequences of aging, or due to lifelong Ag stimulation. Furthermore, it is not known whether CD28 is the only costimulatory signal that can induce telomerase in human T cells.

To address this, we first investigated CD8⁺ T cells from patients with two different genetic defects. The first group have an immunodeficiency known as X-linked lymphoproliferative syndrome (XLP) due to defective SLAM-associated protein (SAP), a molecule coded for by the SH2D1A gene (16, 17). These young individuals experience excessive T cell stimulation after activation both in vitro and in vivo and enable the distinction between the

¹ Abbreviations used in this paper: XLP, X-linked lymphoproliferative syndrome; DKC, dyskeratosis congenita; flow-FISH, flow cytometric-fluorescent in situ hybridization; hTERT, human telomerase reverse transcriptase; rh, recombinant human; PKCζ, protein kinase C; SAP, SLAM-associated protein; scFv, single-chain variable fragment; TRAP, telomeric repeat amplification protocol.

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effects of aging and those resulting from excessive proliferation on CD28 expression. The second group of patients with the X-linked form of an inherited disease known as dyskeratosis congenita (DKC) which results in a defect in a telomerase-associated protein known as dyskerin (18). These patients have significantly reduced telomere length in PBMC that is linked to defective telomerase activity (19). In addition, to directly test the role of CD28 and other costimulatory molecules on telomerase induction, we introduced and triggered chimeric signaling receptors consisting of a single Ab chain (scFv) specific for CD33 coupled via a transmembrane spacer region to intracellular signaling domains for CD28, ICOS, CD27, CD137, and CD134 into primary human CD8+ T cells (20). These receptors enable the study of intracellular signaling pathways independently of extracellular receptor engagement.

We demonstrate that loss of CD28 on CD8+ T cells is due to chronic stimulation and not aging per se and that other costimulatory pathways apart from CD28 can induce telomerase activity. Nevertheless, the further differentiation of CD8+CD28-CD28+ T cells into CD8+CD28+CD27- populations leads to loss of enzymatic activity that cannot be reversed by alternative costimulation. This defect is associated with defective Akt phosphorylation which has been shown to be essential for telomerase up-regulation (21). The loss of telomerase inducibility limits the extent to which human CD8+ T cells can be maintained by continuous proliferation and heralds the onset of end-stage differentiation of these cells in vivo.

Materials and Methods

**Patient and control sample collection and isolation**

Heparinized peripheral blood samples from 20 young (age range 20–39, median age 26), 11 healthy old (age range 69–89, median age 77) healthy volunteers, 3 XLP patients (age 15, 10, and 32 years), and 3 DKC patients (age 32, 39, and 17). Diagnosis of XLP was confirmed by Western blotting for SAP protein (4). SAP protein was undetectable in patients 1 and 2. Low levels of SAP protein were detected in patient 3 but genetic analysis of cells from this individual showed a point mutation at cDNA base 117c→t. This creates an alternative splice site and the mRNA showed a deletion consistent with this. All three patients had pan-hypogammaglobulinemia consistent with this genetic disorder (details in Plunkett et al. Ref. 4). For DKC, patients were diagnosed at Hammersmith Hospital (London, U.K.) and mutations were G202E for patient 1, E350T for patient 2, and P40R for patient 3 (22). All samples were obtained in accordance with the Ethical Committee of Royal Free and University College Medical School. Old volunteers did not have any comorbidity and were not on any immunosuppressive drugs and retained mobility and independence. For some experiments, cells were obtained from either blood packs or leukocyte concentrates that are from leukocyte-depletion filters via The National Blood Service (Colindale, U.K.).

**CD8+ T cell purification and cell sorting**

CD8+ T cells were purified by negative selection using the VARIOMACS system (Miltenyi Biotec) according to the manufacturer’s instructions. Negatively selected CD8+ T cells were stained with anti-CD28 biotin (BD Biosciences), washed, and then incubated with antibody microbeads according to the manufacturer’s instructions. Positively selected cells were CD8+ 2827. The CD8+ 28 negative fraction was further separated into CD8+ 2827 and CD8+ 2827 using CD27 microbeads (Miltenyi Biotec). Analysis of each subpopulation showed purity to be >95%. In some experiments, negatively selected CD8+CD28+ T cells were stained with anti-CD28 FITC and anti-CD27 PE and the subpopulations sorted on the MoFlo (DakoCytomation).

**Flow cytometric analysis of cell phenotype**

Four-color flow cytometric (BD Biosciences) analysis of T cell phenotype was performed as previously described using various combinations of Abs against CD3, (DakoCytomation), CD8, CD28, CD27, CD137, CD134, CD69 (BD Pharmingen), CD45RA (Caltag Medsystems), and ICOS (eBioscience). All samples were run using FACSCalibur and analyzed using CellQuest software (BD Biosciences). For intracellular staining of perforin expression, PBMC were stained for surface expression and then fixed and permeabilized (Fix and Perm Cell Permeabilization kit; Caltag Medsystems) before staining with perforin (BD Pharmingen). PBMCs were stained directly ex vivo.

**Telomere length measurement by flow cytometric-fluorescent in situ hybridization (flow-FISH)**

Telomere length of MoFlo-sorted CD8+ T cell populations defined by expression of CD28 and/or CD27 were measured using a modified version of the flow-FISH method that was previously described (7, 23). In brief, CD8+ subpopulations were washed in PBS and fixed in BS3 (final concentration 1 m; Perbio Science) for 30 min at 4°C. The reaction was quenched using 1 ml of 50 mM Tris (pH 7.2) in PBS and incubated in the dark for 20 min at room temperature. After washing in PBS followed by hybridization buffer, cells were incubated in 0.25 µg/ml of the protein nucleic acid telomeric probe (C3TA2) conjugated to Cy5 (Applied Biosystems). Samples were then heated for 10 min at 82°C, rapidly cooled, and left to hybridize in the dark at room temperature for 1 h. Samples were washed in posthybridization buffer followed by PBS and analyzed immediately by flow cytometry. Fluorescently labeled beads (DakoCytomation) were used to standardize the flow cytometer and no probe controls were analyzed to allow for differences in background between samples. All samples were run in triplicate alongside previously characterized cryopreserved PBMC to ensure consistency of results. Kilobase length was determined from mean fluorescence intensity values using a standard curve.

**Measurement of telomerase activity**

Purified cell populations (2 × 106 cells) were snap-frozen at different times after activation in culture. Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP; TRAPEze telomerase detection kit; Serologicals, distributed through Chemicon Europe) as previously described. In some experiments, absolute numbers of CD8+ subsets were enumerated using Tru-count tubes (BD Biosciences) and Ki67 analysis. PCR was performed with samples adjusted to 500 Ki67+ T cells per reaction (24).

**Cell culture**

Purified CD8+ subsets were activated in the presence of anti-CD3 Ab (purified OKT3 0.5 µg/ml) and PBMC irradiated with 40 Gy gammaradiation, as a source of multiple costimulatory ligands provided by B cells, dendritic cells, and macrophages found in these populations, in the presence or absence of recombinant human (rh) IL-2 (5 ng/ml; R&D Systems) and/or IL-15 (5 ng/ml; R&D Systems) at 37°C in a humidified 5% CO2 incubator. Proliferation was measured on indicated days following stimulation by [3H]thymidine incorporation and expressed as the mean [3H]thymidine incorporation (cpm) of triplicate wells ± SD.

**Cytokine secretion and perforin expression by CD8+ T cell subsets**

For intracellular staining of perforin expression, PBMC were stained for surface expression and then fixed and permeabilized (Fix and Perm Cell Permeabilization kit; Caltag Medsystems) before staining with IFN-γ and IL-2 (BD Pharmingen). PBMCs were stained directly ex vivo. For cytokine expression, sorted CD8+ subpopulations were stimulated with anti-CD3 (purified OKT3, 5 µg/ml) and irradiated APC for 15 h at 37°C in a humidified 5% CO2 incubator. Brefeldin A (5 mg/ml; Sigma-Aldrich) was added after 1.5 h. Unstimulated cells were used as controls. Cells were stained for surface expression and then fixed and permeabilized (Fix and Perm Cell Permeabilization kit; Caltag Medsystems) before staining with IFN-γ and IL-2 (BD Pharmingen).

**Transfection of primary CD8+ T cells**

Purified CD8+CD28/27 populations of T cells were transfected with chimeric signaling receptors containing either the TCR-ζ signaling regions only, or the TCR-ζ signaling regions in series with those from the costimulatory domains of CD28, CD27, CD137, CD134, or ICOS by electroporation as previously reported (20). Briefly, each chimeric signaling receptor containing both TCR and costimulatory domains was cloned into an expression vector derived from pQBI-AdCMV5 (QBioGene). Plasmid DNA was prepared by p500 columns according to the manufacturer’s instructions (Qiagen). A total of 3–6 µg of DNA was added to 5 × 106 T cells that were resuspended in 100 µl of Nucleofector solution for T cells (Amaxa Biosystems) and electroporated using the U-13 program of the Nucleofector device (Amaxa Biosystems). Transfected T cells were immediately transferred into prewarmed complete medium and left to rest for 3 h at 37°C in a humidified 5% CO2 incubator. A total of 2 × 106 transfected T cells were then stimulated using 96-well plates (Nunc) precoated (Fix and Perm Cell Permeabilization kit; Caltag Medsystems) before staining with perforin (BD Pharmingen). PBMCs were stained directly ex vivo.
with anti-CD3 (5 μg/ml in 0.1 M NaHCO₃). Cells were analyzed for proliferation and telomerase expression at times indicated. Cells expressing the chimeric receptors were identified by flow cytometry using CD33-FTTC at 3 and 24 h after transfection.

Inhibition of PI3K, protein kinase C (PKC), and Akt

Purified CD8⁺ CD28/27 T cell populations were incubated for 30 min with inhibitors of PI3K (LY 294002, final concentration 20 μM), PKC (bisindolylmaleimide I; 1 μM) or Akt (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; 5 μM) all from Calbiochem. Cells were activated as before with anti-CD3 (0.5 μg/ml) and irradiated APC in the presence or absence of rhIL-2 (5 ng/ml; R&D Systems). Cells were analyzed for proliferation and telomerase expression at times indicated. The inhibitors at the concentrations used in these experiments did not result in a loss of cell viability.

Western blot analysis pAkt

Purified CD8⁺ CD28/27 populations were activated in the presence of anti-CD3 (0.5 μg/ml) irradiated APC in the presence or absence of rhIL-2 (5 ng/ml; R&D Systems). Cells were isolated at times indicated and lysates obtained by sonicating cells in 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 0.1% Triton X-100 buffer. Lysates from 2 × 10⁶ cells were fractionated on SDS-polyacrylamide electrophoresis gels and analyzed by immunoblotting with either anti pAkt1/2/3 (Ser473), anti-pAkt (Thr308), Akt, or human telomerase reverse transcriptase (hTERT) (all from Santa Cruz Biotechnology) for 3 h at 4°C. Samples were separated by 10% SDS-polyacrylamide electrophoresis gels and analyzed by immunoblotting with anti-CD33 (5 ng/ml, Sigma-Aldrich) overnight at 4°C on a rotary shaker. This indicated that the loss of CD28 and CD27 was related to cell proliferation and unlikely to be due directly to aging. We confirmed this by showing that telomere length of CD8⁺ T cells from XLP individuals was significantly shorter than that of the young age group and in the same range as that of old subjects (Fig. 1C). The mean expression is depicted as a horizontal bar. Telomere length assessed using flow-FISH, in different CD8⁺ T cells populations was assessed using two-color flow-FISH (C). Each dot represents a different CD8⁺ T cells population. The mean expression of CD8⁺ T cells was 11 young, 11 old volunteers, and 3 XLP and 3 DKC patients (B). The mean expression is depicted as a horizontal bar. Telomere length assessed using flow-FISH, in different CD8⁺ T cells populations was assessed using two-color flow-FISH (C). Each dot represents a different individual with the mean telomere length shown as a horizontal bar. Telomere length assessed using flow-FISH, in different CD8⁺ T cells sorted that were sorted on the basis of CD28 and CD27 expression (D). Graph shows the mean ± SE for 3 young and 3 old individuals.
major subsets defined on the basis of CD28 and CD27 expression (Fig. 1D). Telomeres were longest in the CD28+CD27+ population followed by that in the CD28+CD27− subset, while the CD28−CD27− subset had the shortest telomeres of all three populations (Fig. 1B). This suggests that CD8+ T cells progressively differentiate from a CD28+CD27+ to a CD28+CD27− and finally to a CD28−CD27− phenotype upon repeated stimulation. Although this scheme holds true for freshly isolated and unactivated CD8+ T cells, there is low and transient re-expression of CD28 in CD28−CD27+ and to a lesser extent CD28+CD27− T cells after activation in vitro in young subjects, but there is less re-expression of these molecules in old individuals and XLP patients (data not shown). The rare population of CD8+CD27− cells, that are not found in sufficient numbers for isolation in most subjects (Fig. 1A), had short telomere lengths like the CD28−CD27− subset (data not shown) and may represent highly differentiated cells that had been activated in vivo. Highly differentiated CD8+ T cells that reexpress CD45RA (28–30) are found exclusively within the CD28−CD27− subset (9).

We investigated whether the CD8+ subsets in young subjects that lost expression of CD28 and/or CD27 were functional by activating whole CD8+ T cells with anti-CD3 and APC (irradiated PBMC). The vast majority of IL-2-producing CD8+ T cells were found within the CD28−CD27− subset whereas the majority of IFN-γ-producing CD8+ T cells were found within the CD28+CD27− population (Fig. 2A). The intermediate CD8+CD28+CD27− subset did not secrete large levels of either IL-2 or IFN-γ but showed high perforin expression directly ex vivo (Fig. 2B). However, the CD27−CD28+ population showed the greatest expression of perforin with the CD28+CD27− cells showing least expression. Therefore, the CD27−CD28+ population can up-regulate effector mediators and exhibit the characteristics of functional differentiation.

**Telomerase induction in different subsets of CD8+CD28− T cells**

We next examined the ability of CD8+CD28− and CD8+CD27− T cell subsets to up-regulate telomerase upon activation with a
range of different stimuli (Fig. 3). We found that the CD8\(^+\)CD28\(^-\) T cells contained significant levels of telomerase irrespective of the stimulus used. It should be noted that these cells still had residual Ab to CD28 on their surface that was used for their positive selection. The high telomerase activity with anti-CD3 Ab alone therefore includes a component of CD28 costimulation (Fig. 3, A and B). In contrast, CD8\(^+\)CD28\(^-\) T cells did not up-regulate significant telomerase activity after anti-CD3 activation in the presence of CD28 costimulatory signals confirming previous results (31, 32). However, the use of irradiated PBMC as a source of multiple costimulatory ligands provided by B cells, dendritic cells, and macrophages (33, 34) induced significant telomerase activity compared with anti-CD3 stimulation alone (Fig. 3C). The mean telomerase induction in the CD8\(^+\)CD28\(^-\) T cells after stimulation with anti-CD3 and irradiated APCs was less, however, than that observed in the CD8\(^+\)CD28\(^+\) T cells (Fig. 3B). This raised the question of which alternative costimulatory signals could induce telomerase in CD8\(^+\) T cells that have lost CD28 expression.

Costimulatory molecule expression on CD8\(^+\) populations

We next determined whether CD8\(^+\) T cells that down-regulate CD28 and/or CD27 are capable of expressing alternative costimulatory molecules. We investigated a limited number of costimulatory molecules including CD137, CD134, and ICOS have all been shown to provide potent costimulatory signals for CD8\(^+\) T cells (35–37). These molecules were not constitutively expressed on the CD8 population, but all were rapidly induced after activation with anti-CD3 and APCs (Fig. 4A) and expression was maintained for over 7 days (Fig. 4B). In addition, the different CD8 populations were all capable of up-regulating CD137, CD134, and ICOS (Fig. 4C). The up-regulation of ICOS, CD137, and CD134 could be induced by the stimulation of CD8\(^+\) T cells with anti-CD3 Ab alone after 24 h (data not shown) that makes these receptors available for subsequent T cell triggering by their ligands on APCs. However, it is very likely that other costimulatory molecules, that may be expressed constitutively on T cells, are also involved in telomerase induction in CD8\(^+\) T cells. This indicates that loss of CD28 and/or CD27 on CD8 cells does not equate to a global loss of costimulatory receptor expression.

Stimulation of primary human CD8\(^+\) T cells by chimeric signaling receptors

To test directly whether costimulatory signals other than those provided by CD28 can induce telomerase, we introduced chimeric signaling receptors containing the TCR\(\zeta\)-signaling domain in series with signaling regions of a limited number of costimulatory molecules including CD28, CD27, CD137, CD134, or ICOS into primary unstimulated CD8\(^+\) T cells using Amaxa nucleofector technology (20) (Fig. 5A). The control consisted of a chimeric receptor containing the TCR\(\zeta\)-signaling domain alone and the construction and use of these receptors has been described previously (20). The extracellular portion of these receptors is a P67 anti-CD33 scFv and triggering of these constructs in cells was achieved by plate-bound CD33 Ag.

Expression of functional chimeric receptors was assessed at 4 h after nucleofection and was found to be >40% for all constructs (Fig. 5B). Upon anti-CD33 binding, chimeric receptors providing TCR\(\zeta\) signaling alone were not capable of inducing either proliferation (Fig. 5C) or telomerase activity (Fig. 5, D and E). However, the chimeric receptors that included both the TCR\(\zeta\) and signaling regions from the different costimulatory molecules could all induce both proliferation (Fig. 5C) and telomerase activity (Fig. 5, D and E) in CD8\(^+\) T cells. This confirms that CD28 can directly up-regulate telomerase expression and extend these observations to show that there is redundancy in the costimulatory pathways that are available to induce this enzyme. We also anticipate that other costimulatory molecules in addition to the ones that we have tested are also involved in telomerase up-regulation in CD8\(^+\) T cells.

Telomerase induction in different subsets of CD8\(^+\)CD28\(^-\) T cells

CD8\(^+\)CD28\(^-\) T cells are heterogenous and can be further divided into CD8\(^+\)CD28\(^+\)CD27\(^-\) and CD8\(^+\)CD28\(^-\)CD27\(^+\) populations that are at different stages of differentiation (Fig. 1). We next examined whether both subsets could induce telomerase to the same extent. We activated the different subsets of CD8\(^+\) T cells with anti-CD3 and irradiated PBMC as a source of multiple costimulatory ligands that enabled the activation of CD8\(^+\) T cells in the absence of CD28 (Fig. 3A). Telomerase is undetectable in unactivated CD8\(^+\) T cells (4). However, high levels of activity was observed in both the CD28\(^+\)CD27\(^-\) and CD28\(^-\)CD27\(^+\) T cells after 3 days of stimulation that was maintained until day 6 of culture (Fig. 6A). By day 9, telomerase activity declined in both these populations (Fig. 6A). In contrast, the CD28\(^-\)CD27\(^-\) subset had...
The triggering of the TCR receptors did not induce telomerase in any subset tested (Fig. 6). Reduced ability to up-regulate this enzyme compared with the other subsets even when we standardized the telomerase assay to include an equivalent number of Ki67+ cells for each CD8+ T cell subpopulation, but were able to proliferate after stimulation, albeit at a lower level compared with the other populations studied (Fig. 6B).

We next investigated whether the reintroduction of the CD28 signaling pathway in CD8+ T cells at different stages of differentiation could reinstate their capacity to up-regulate telomerase activity. After nucleofection with CD28ζ chimeric receptor constructs, up to 20% of the CD8+CD28–CD27– T cells were induced to express the TCRζ chain alone construct while 25% of these cells expressed the TCRζ/CD28 receptor in the experiment shown (Fig. 6C). We found that ligation of the TCRζ chain alone receptors did not induce telomerase in any subset tested (Fig. 6D). The triggering of the TCR/CD28 receptor in contrast induced telomerase and proliferation in both CD8+CD28+CD27+ and CD8+CD28–CD27– T cells (Fig. 6, D and E). However, the triggering of these receptors in CD8+CD28+CD27– T cells induced neither telomerase nor proliferation in these cells (Fig. 6, D and E). This does not indicate that the CD8+CD28–CD27– subset is totally unresponsive, because proliferation occurred after stimulation with anti-CD3 and irradiated APCs as a source of multiple costimulatory ligands (Fig. 6B). This observation indicates that the lack of telomerase activity in CD8+CD28+CD27– T cells is not due to a lack of costimulation.

The effect of IL-2 and IL-15 on telomerase induction in CD8+ T cells

Because CD8+CD28+CD27– T cells were not able to synthesize IL-2 after activation (Fig. 2A), we investigated whether exogenous IL-2 or IL-15 could increase telomerase activity in these cells after activation (Fig. 7). We found that CD8+CD28+CD27– T cells had reduced capacity to survive in culture (Fig. 7A) and to proliferate after anti-CD3/APC stimulation (Fig. 7B) compared with CD8+CD28+CD27+ and CD8+CD28–CD27+ T cells. However, the addition of either IL-2 or IL-15 enabled the CD8+CD28+CD27+ T cells to survive and proliferate to levels observed in the other populations. The CD8+CD28–CD27– T cells also had reduced ability to up-regulate telomerase compared with the other subsets, but this was only partially reconstituted by the addition of these cytokines (Fig. 7C). This indicated that while CD8+CD28+CD27– T cells are dependent on exogenous factors for survival and proliferation, additional changes contribute to the telomerase defect in these cells.

Telomerase defect in CD8+CD28–CD27– T cells is associated with decreased Akt signaling

We investigated whether decreased telomerase activity in CD8+CD28–CD27– T cells was due to decreased synthesis of telomerase reverse transcriptase (hTERT), the catalytic protein...
component of this enzyme in these cells. After activation, hTERT was found in all the different CD8\(^+\)/H11001 T cell subsets, which was only slightly increased by the addition of IL-2 (Fig. 8). This is consistent with previous observations that telomerase activity in human T cells is not only regulated at the level of hTERT expression (38). Although highly differentiated CD8\(^+\)/CD28\(^-\)/CD27\(^-\) T cells have decreased telomerase activity, they are still able to proliferate

**FIGURE 6.** Telomerase expression in CD8\(^+\)/CD28/CD27 populations. CD8\(^+\)/CD28/CD27 subpopulations were purified using the VARIOMACS and activated with anti-CD3 plus APC. A, A representative blot for telomerase activity in CD8\(^+\)/CD28/CD27 populations on days 3, 6, and 9 following activation. The blot is representative of eight separate experiments. The CD8\(^+\)/CD28\(^-\)/CD27\(^-\) cells were a rare population that was only found in a few individuals. There were only sufficient numbers of these cells for telomerase analysis on day 3 in the representative experiment shown. B, Proliferation of CD8\(^+\)/CD28/CD27 subpopulations was assessed on day 3 following stimulation by tritiated thymidine incorporation. Error bars represent the SE from the mean of triplicates. These experiments are representative of 11 separate experiments. Purified CD8\(^+\)/CD28/CD27 populations were transected with chimeric receptors as described in Materials and Methods. C, Chimeric receptor expression was analyzed by flow cytometry and representative histograms are shown for CD8\(^+\)/CD28\(^-\)/CD27\(^-\) T cells. Following stimulation via plate-bound CD33, proliferation and telomerase expression was determined. D, A representative blot for telomerase activity in CD8\(^+\)/CD28/CD27 populations on days 2 and 5 following stimulation. E, Proliferation of transfected CD8\(^+\)/CD28/CD27 populations cells was assessed on day 5 by tritiated thymidine incorporation following stimulation. These data are representative of two separate experiments.

**FIGURE 7.** The effect of IL-2 and IL-15 on telomerase activity of CD8\(^+\)/T cells. CD8\(^+\)/CD28/CD27 populations were purified using the VARIOMACS and activated with anti-CD3 plus irradiated APC alone (\(\square\)) or with the addition of IL-2 (\(\square\)) or IL-15 (\(\square\)). A, Viable cell recovery at day 5 was determined using a combination of trypan blue exclusion and Trucount analysis and is shown as percent cells recovered relative to day 0. B, Proliferation was assessed on day 3 following stimulation by tritiated thymidine incorporation and error bars represent the SE from the mean of triplicate determinations. These results are representative of four separate experiments. C, Relative telomerase activity was assessed on day 3 following activation and was normalized by using 500 Ki67\(^+\) proliferating T cells for each subset in the TRAP assay. Data shown are representative of four separate experiments.
albeit to a lesser extent than the other CD8\(^+\) subsets (Fig. 6B). This suggests that the defective cell-signaling pathway involved inhibits telomerase to a greater extent than proliferation. To identify the potential signaling pathway that was involved, isolated CD8\(^+\)T cell subsets were activated with anti-CD3 and irradiated APCs in the presence or absence of inhibitors of PI3K, Akt, and PKC, all of which have key roles in intracellular T cell signaling (39–41). The inhibition of PI3K and PKC induced a decrease in both proliferation and telomerase activity in all three populations (Fig. 9, A and B). In contrast, the inhibition of Akt at the concentration used had very little effect on the levels of proliferation but induced inhibition of telomerase activity in all the CD8\(^+\) T cell subsets (Fig. 9, A and B). This confirmed and extended observations that Akt signaling is crucial for pathway in telomerase induction (21). However, this data also suggested that Akt inhibits telomerase in preference to proliferation and recapitulates the defect observed in CD8\(^+\)CD28\(^-\)CD27\(^+\) T cells.

We therefore investigated whether there were changes in Akt expression in CD8\(^+\)CD28\(^-\)CD27\(^+\) T cells and found that telomerase down-regulation after activation was not due to lack of this molecule per se (Fig. 9C). Total Akt was unchanged in any of the CD8\(^+\) T cell subsets studied (Fig. 9C), Akt itself has to be phosphorylated at two different sites (Ser\(^{473}\) and Thr\(^{308}\)) for activation (42, 43). We found that there was a selective lack of Akt phosphorylation at the Ser\(^{473}\) site in the CD8\(^+\)CD28\(^-\)CD27\(^+\) T cell subset that was not reversed by the addition of IL-2 (Fig. 9C). This was consistent in three totally separate experiments (data not shown). Furthermore, it is of note that that the level of Akt (Ser\(^{473}\)) phosphorylation was also reduced in CD8\(^+\)CD28\(^-\)CD27\(^+\)
compared with the CD8⁺ CD28⁺ CD27⁺ T cells, suggesting that CD8⁺ T cells may lose ability to phosphorylate Akt (Ser⁴⁷³) during progressive differentiation. This suggests that the deficient Akt (Ser⁴⁷³) phosphorylation, that is independent of the presence of IL-2, may contribute to the defective telomerase induction in CD8⁺ CD28⁺ CD27⁻ T cells.

Because telomerase activity in T cells requires hTERT phosphorylation (44) and because hTERT is a substrate for Akt (21), one prediction is that CD8⁺ CD28⁺ CD27⁺ T cells would also have decreased hTERT phosphorylation. Indeed, we found decreased hTERT phosphorylation relative to the expression of another serine-phosphorylated protein, β-catenin (25) and the ratio of p-Ser-hTERT to p-Ser-β-catenin was reduced in CD8⁺ CD28⁻ CD27⁻ T cells compared with CD8⁺ CD28⁺ CD27⁺ and CD8⁺ CD28⁺ CD27⁺ subsets (Fig. 9D). This strongly suggests that the decreased telomerase activity is secondary to changes in Akt function in highly differentiated CD8⁺ T cells.

Discussion
Old humans have an increased incidence of infections compared with young subjects, including some to which they were previously immune (1). This suggests that immune memory to certain organisms may be lost during aging. It is therefore important to understand the control of replicative lifespan in T cells, with a view to pre-empt the predicted decline in immunity and increased rate of infection in the future. The enzyme telomerase is inextricably linked to the maintenance of memory T cell pools throughout life (1, 4–6, 32). The observation that humans have greater lifespan but 10-fold shorter telomeres than found in mice (45) indicates that the impact of telomere erosion on T cell memory will be more profound in the former species (46). Another difference between both species is that the costimulatory molecule CD28 is lost on CD8⁺ T cells in humans but not mice during differentiation in vivo (2). This is important because CD28 signals are considered to be crucial for telomerase induction in T cells (31, 32). The central aim of the present study was to clarify the link between CD28 signaling, telomerase induction, and end-stage differentiation in human CD8⁺ T cell populations.

Because XLP patients who are young have CD8⁺ T cells with an identical phenotype to those from old individuals, it suggests that CD28 and CD27 receptors are lost because of excessive proliferation and not aging of the cells per se. Furthermore, we demonstrated unequivocally that the costimulatory molecule CD28 is lost on CD8⁺ T cells in humans but not mice during differentiation in vivo (2). This is important because CD28 signals are considered to be crucial for telomerase induction in T cells (31, 32).

The telomerase down-regulation in CD8⁺ CD28⁺ CD27⁻ T cells was not related to reduced expression of hTERT, however, the phosphorylation of this molecule has been shown to be essential for up-regulating telomerase activity in human T cells (31). Because hTERT is a substrate for Akt and there is a specific defect in Akt phosphorylation at the Ser⁴⁷³ site in CD8⁺ CD28⁺ CD27⁻ T cells, our results suggest that decreased telomerase activity in these cells is due to defective Akt (Ser⁴⁷³) phosphorylation. However, this remains to be tested directly. Furthermore, although IL-2 and IL-15 can partially restore telomerase activity in CD8⁺ CD28⁻ CD27⁻ T cells, these cytokines do not alter Akt phosphorylation after stimulation (9, 53). Nevertheless, it will be of interest to determine whether the blockade of PD-1 signaling, that reverses the exhausted phenotype (48, 54, 55) in T cells, re-establishes the ability of CD8⁺ CD28⁺ CD27⁻ T cells to up-regulate telomerase activity.

The telomerase down-regulation in CD8⁺ CD28⁺ CD27⁻ T cells that have been described in mice (52) because we find that CD8⁺ CD28⁺ CD27⁻ T cells can synthesize IFN-γ and perforin after stimulation (9, 53). It may be the result of interest to determine whether Akt phosphorylation, which is restored by IL-2 and IL-15 in human T cells, is altered in human CD8⁺ T cells.

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Disclosures
The authors have no financial conflict of interest.
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