p53 isoforms regulate aging- and tumor-associated replicative senescence in T lymphocytes

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Introduction
Cellular senescence contributes to aging and decline in tissue function. p53 isoform switching regulates replicative senescence in cultured fibroblasts and is associated with tumor progression. Here, we found that the endogenous p53 isoforms Δ133p53 and p53β are physiological regulators of proliferation and senescence in human T lymphocytes in vivo. Peripheral blood CD8+ T lymphocytes collected from healthy donors displayed an age-dependent accumulation of senescent cells (CD28−CD57+) with decreased Δ133p53 and increased p53β expression. Human lung tumor-associated CD8+ T lymphocytes also harbored senescent cells. Cultured CD8+ blood T lymphocytes underwent replicative senescence that was associated with loss of CD28 and Δ133p53 protein. In poorly proliferative, Δ133p53-low CD8+CD28− cells, reconstituted expression of either Δ133p53 or CD28 upregulated endogenous expression of each other, which restored cell proliferation, extended replicative lifespan and rescued senescence phenotypes. Conversely, Δ133p53 knockdown or p53β overexpression in CD8+CD28− cells inhibited cell proliferation and induced senescence. This study establishes a role for Δ133p53 and p53β in regulation of cellular proliferation and senescence in vivo. Furthermore, Δ133p53-induced restoration of cellular replicative potential may lead to a new therapeutic paradigm for treating immunosenescence disorders, including those associated with aging, cancer, autoimmune diseases, and HIV infection.

Due to alternative splicing and usage of alternative promoters (13), among them are p53β, a C-terminally truncated isoform that cooperates with p53FL, and Δ133p53, an N-terminally truncated isoform that inhibits p53FL in a dominant-negative manner (14). In normal human fibroblasts cultured in vitro, p53β accelerates and Δ133p53 represses replicative senescence (15), consistent with their modes of functional interaction with p53FL. Premalignant colon adenomas with pathologically induced senescent cells in vivo also showed a specific profile of p53 isoform expression (i.e., elevated levels of p53β and reduced levels of Δ133p53), the loss of which was associated with malignant progression to colon carcinomas (15). We recently discovered that SRSF3, a member of a highly conserved family of splicing factors, regulates the generation of p53β and Δ133p53 in regulation of cellular proliferation and senescence in vivo. Furthermore, Δ133p53-induced restoration of cellular replicative potential may lead to a new therapeutic paradigm for treating immunosenescence disorders, including those associated with aging, cancer, autoimmune diseases, and HIV infection.

The p53 signaling network plays a critical role in the induction of cellular senescence (12). The human TP53 gene encodes, in addition to full-length p53 protein (p53FL), at least 13 natural isoforms due to alternative splicing and usage of alternative promoters (13). Among them are p53β, a C-terminally truncated isoform that cooperates with p53FL, and Δ133p53, an N-terminally truncated isoform that inhibits p53FL in a dominant-negative manner (14). In normal human fibroblasts cultured in vitro, p53β accelerates and Δ133p53 represses replicative senescence (15), consistent with their modes of functional interaction with p53FL. Premalignant colon adenomas with pathologically induced senescent cells in vivo also showed a specific profile of p53 isoform expression (i.e., elevated levels of p53β and reduced levels of Δ133p53), the loss of which was associated with malignant progression to colon carcinomas (15). We recently discovered that SRSF3, a member of a highly conserved family of splicing factors, regulates the generation of p53β and Δ133p53 in regulation of cellular proliferation and senescence in vivo. Furthermore, Δ133p53-induced restoration of cellular replicative potential may lead to a new therapeutic paradigm for treating immunosenescence disorders, including those associated with aging, cancer, autoimmune diseases, and HIV infection.

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naive T cells (most proliferative and least differentiated) to central memory, effector memory, and effector (least proliferative and terminally differentiated) T cells. Repeated or chronic antigen stimulation throughout the normal lifespan or under pathological conditions (e.g., patients with HIV infection, autoimmune diseases, and cancer; refs. 18–20) drives progression of these differentiation states and results in a large population of late-differentiated CD8+ T lymphocytes that are approaching or have reached replicative senescence (21). These cells are characterized by loss of CD28 (a costimulatory receptor; ref. 20) and gain of CD57 (also known as human natural killer–1; ref. 22), as well as shortened telomeres (23), and directly contribute to immunosenescence (20, 24). CD8+ T lymphocytes with these characteristics can also be a cause of functional impairment of tumor-specific T cell immunity (25).

Figure 1
Senescent phenotypes in CD28–CD57+ subsets of CD8+ T lymphocytes in vivo. (A) CD28+CD57− subsets decreased and CD28−CD57+ subsets increased with donor age. (B) High-throughput quantitative FISH revealed shortened telomeres in CD28−CD57+ subsets. n represents total number of telomere spots analyzed per subset. (C) Summary of SA-β-gal activity (donors 4, 6, and 7). (D) Representative images for HP1γ foci by immunofluorescence staining (donor 4). (E) Quantitative analysis of HP1γ foci per cell (donors 4, 6, and 7). (F) Representative images for γ-H2AX foci (donor 26). Irradiated (2 Gy) CD28+CD57− cells were used as a positive control. IR, irradiation. (G) Summary of γ-H2AX foci per cell (donors 26–28). (H) Quantitative RT-PCR analysis for SASP factors (donors 1–6). B2M was used for normalization. Data are mean ± SEM (B) or mean ± SD (C, E, G, and H). Scale bars: 5 μm (D and F). *P < 0.05; **P < 0.01; ***P < 0.001.
Our present study shows for the first time that in vivo accumulation of senescent CD8+ T lymphocytes in blood during physiological aging and in the tumor microenvironment involves changes in endogenous expression of Δ133p53 and p53β, and that manipulated expression of these p53 isoforms can control proliferation and senescent phenotypes of blood CD8+ T lymphocytes.

Results

In vivo accumulation of senescent CD8+ T lymphocytes during physiological aging. Multiparameter flow cytometric analysis of circulating CD8+ T lymphocytes isolated from 36 healthy donors (age, 21–74 years) showed decreased frequency of the CD28+CD57− subset (r = −0.8096; P < 0.0001) and increased frequency of the CD28−CD57+ subset (r = 0.7285; P < 0.0001) with advancing age (Figure 1A, Supplemental Figure 1A, and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI70355DS1), while no or weaker association was found in the CD28−CD57− (r = −0.0953; P = 0.7883) or CD28 CD57 (r = 0.3934; P = 0.0019) subsets (Supplemental Figure 1B). Consistently, when CD28 and CD57 were separately analyzed, CD8+ T lymphocytes tended to lose CD28 (r = −0.8224; P < 0.0001) and gain CD57 (r = 0.5813; P < 0.0001) expression as a function of donor age (Supplemental Figure 1C and refs. 20, 22).

Fluorescence-activated cell sorting (FACS)-sorted individual CD28/CD57 quadrants were examined for cell proliferation by a CFSE proliferation assay (Supplemental Figure 2A). The CD28−CD57− subset, which decreased with age, showed the highest proliferation rate (proliferation index, 2.61 ± 0.23), while the CD28−CD57+ subset, which increased with age, showed the lowest (proliferation index, 1.19 ± 0.09) (Supplemental Figure 2B). As previously reported for replicatively senescent CD8+ T lymphocytes (23, 26, 27), the CD28−CD57− subset had shortened telomeres and showed an increase in cells positive for senescence-associated β-galactosidase (SA-β-gal) compared with the CD28−CD57+ subset (Figure 1B, C, and Supplemental Figure 3). Other senescence markers previously used with other cell types, including formation of H2A-H2AX foci (28, 29), spontaneous induction of γ-H2AX foci (30), and mRNA expression of SASP factors, such as IL6, IL8, CXCR1, and CXCR2 (8), were also increased in the CD28−CD57+ subset (Figure 1, D–H, Supplemental Figure 4, and Supplemental Figure 5, A–D). These results indicate that the CD28−CD57+ subsets within CD8+ T lymphocytes, which accumulate in vivo during physiological aging in humans, are characterized by the senescent phenotypes.

Reduced Δ133p53 and elevated p53β expression in senescent CD8+ blood T lymphocytes in vivo. Endogenous protein expression of Δ133p53 and p53β was examined in FACS-sorted CD28/CD57 quadrants of CD8+ blood T lymphocytes using MAP4 (Supplemental Figure 6A and refs. 14, 15) and TLQ19 (14, 15) antibodies, respectively (Figure 2A; see complete unedited blots in the supplemental material). In donors 1 and 2, in which all 4 quadrants were examined, Δ133p53 expression was decreased from the CD28−CD57+ (most proliferative) subset to the CD28−CD57−, CD28−CD57+, and CD28+CD57− (senescent) subsets (Figure 2A). In all 6 donors examined, the expression level of Δ133p53 in the CD28−CD57+ subset was 1%–22% (average, 10%) of that in the CD28−CD57− subset (P < 0.0001; Figure 2, A and B). The expression level of p53FL was not significantly different between CD28−CD57− and CD28−CD57+ subsets (Supplemental Figure 7). In contrast, the expression level of p53β in the CD28−CD57− subset was 1.23- to 2.06-fold (average, 1.72-fold) higher than that in the CD28−CD57− subset (P = 0.0020; Figure 2, A and B). These results were confirmed by immunoblot analysis using a full blot, which was probed with the Δ133p53-specific antibody MAP4 or the antibody CM1 (31), which recognizes p53FL, Δ133p53, p53β, and other p53 isoforms (Supplemental Figure 6, B and C). The elevated levels of p53β protein in the senescent CD28−CD57+ subsets were associated with increased p53β mRNA levels (Figure 2C and Supplemental Figure 5E). SRSF3, a splicing factor that restricts the alternative splicing generating p53β (16), was downregulated in the CD28−CD57− subsets (Figure 2D and Supplemental Figure 5F), consistent with the increased p53β mRNA (Figure 2C). In contrast, the decreased levels of Δ133p53 protein in the CD28−CD57− subsets were not associated with a decrease in Δ133p53 mRNA (Figure 2E and Supplemental Figure 5G), indicative of Δ133p53 downregulation at the protein level in this senescent population of CD8+ T lymphocytes.

To examine a mechanism for the downregulation of Δ133p53 at the protein level, we first used a proteasome inhibitor, MG-132. Whereas treatment with MG-132 resulted in increased p53FL levels in the whole population of CD8+ T lymphocytes and the CD28−CD57− and CD28−CD57+ populations, the same treatment did not increase Δ133p53 protein levels in any of these populations (Supplemental Figure 8, A and B), which suggests that Δ133p53, unlike p53FL, is not subject to proteasome-mediated protein degradation. We next inhibited autophagy, an alternative mechanism for protein degradation, by treatment with bafilomycin A1 (Figure 2F and Supplemental Figure 8C). This treatment did not stabilize p53FL in either CD28−CD57− or CD28−CD57+ subsets. While the abundant levels of Δ133p53 in CD28−CD57− subsets were not affected by this treatment, the reduced levels of Δ133p53 in both CD28−CD57− subsets were upregulated approximately 2- to 3-fold in response to bafilomycin A1 in all 3 cases examined. These results indicate that autophagic degradation of Δ133p53 contributes to its downregulation in senescent CD28−CD57− subsets of CD8+ T lymphocytes.

Senescence-associated phenotypes and p53 isoform expression profile in tumor-associated CD8+ T lymphocytes. Tumor-associated CD8+ T lymphocytes were isolated from lung cancer tissues of patients who received surgery and were examined for the CD28−CD57− and CD28−CD57+ subsets. A considerable fraction of CD28−CD57+ (mean, 60.3% ± 12.7%) and CD28−CD57− (mean, 8.7% ± 6.7%) subsets were observed in the tumor-associated CD8+ T lymphocytes, similar to blood T lymphocytes (Supplemental Tables 1 and 2). The CD28−CD57− subsets, similar to those from blood CD8+ T lymphocytes, showed switching of p53 isoform abundance, i.e., elevated p53β and diminished Δ133p53 protein levels, and senescence phenotypes, including increased γ-H2AX foci and elevated SASP factors, such as IL6 and IL8 (Figure 2, G–I, and Supplemental Figure 9, A–D). The elevated levels of p53β protein in the CD28− CD57− cells were again coincident with increased p53β mRNA and decreased SRSF3 mRNA (Figure 2, J and K, and Supplemental Figure 9, E and F). In contrast, Δ133p53 mRNA expression was similar in these 2 subsets (Figure 2L and Supplemental Figure 9G), again suggesting regulation at the protein level. These results indicate that CD8+ T lymphocytes in the tumor microenvironment may undergo differentiation and senescence processes similar to those observed in the same cell type in blood during aging.

In vitro replicative senescence of CD8+ T lymphocytes reproduces CD8+ T lymphocyte loss and diminished Δ133p53 expression observed in vivo. FACS-sorted CD8+ T lymphocytes were stimulated with anti-CD3/2/3/28 cocktail beads and recombinant human IL-2 (rIL-2) and expanded in culture. The CD8+ T lymphocytes from all 3 donors examined ceased to proliferate after 7–8 weeks and remained quiescent (Figure 3A). In these proliferation-arrested cells, an increase in SA-β-gal–positive...
Diminished Δ133p53 and elevated p53β in the CD28-CD57+ subset of CD8+ T lymphocytes in vivo. (A) Immunoblot of CD28/CD57 quadrants of blood CD8+ T lymphocytes showing Δ133p53 and p53β proteins (donors 1–6). The CD28+CD57+ and CD28–CD57– subsets from donors 3–6 did not give enough amounts of protein because of low cell counts. p53β bands are denoted by thin lines at right of lanes and arrowheads. Lanes were run on the same gel but were noncontiguous (black lines). Densitometric values (normalized to β-actin, expressed relative to the CD28+CD57– subset) are shown below. (B) Quantitative data summary of Δ133p53 and p53β protein expression (donors 1–6). (C–E) Quantitative RT-PCR analysis for p53β (C), SRSF3 (D), and Δ133p53 (E) (donors 1–6). B2M was used for normalization. (F) Immunoblot of bafilomycin A1–treated (Baf A1; 100 nM for 6 hours) CD28+CD57– and CD28–CD57+ subsets of blood CD8+ T lymphocytes (donors 34 and 35). Δ133p53, p53FL (detected by DO-1 antibody), p62, and LC3B proteins were examined. Inhibition of autophagy was confirmed by increased amounts of p62 and LC3B-II. Densitometric values (expressed relative to untreated cells) are shown below. (G) Quantitative data summary of Δ133p53 and p53β protein expression in the CD28+CD57– and CD28–CD57+ subsets of tumor-associated CD8+ T lymphocytes (tumors 1–3; see Supplemental Figure 9A). (H–L) Quantitative RT-PCR analysis for IL6 (H), IL8 (I), p53β (J), SRSF3 (K), and Δ133p53 (L) (tumors 5–7). B2M was used for normalization. Data are mean ± SD (B–E and G–L). *P < 0.05; **P < 0.01; ***P < 0.001.
tive cells and increased numbers of HP1-γ and γ-H2AX foci were observed (Figure 3, B–E, and Supplemental Figure 10, A and B), similar to the senescent CD8+ T lymphocytes that accumulated in vivo (Figure 1, C–G). The frequency of the CD28+ population decreased gradually toward the end of the replicative lifespan (week 12; Figure 3F), reproducing its donor age–dependent decrease in vivo, although loss of CD57+ population during in vitro culture contradicted the age-dependent increase in CD57+ population in vivo (compare Supplemental Figure 1C and Supplemental Figure 10C). All 3 isolates of CD8+ T lymphocytes showed a decrease in Δ133p53 expression associated with proliferation arrest at week 8, with or without further decrease at week 12 (Figure 3G and Supplemental Figure 10D). While p53FL was upregulated during in vitro culture, p53β expression showed only a marginal increase or remained constant through week 8 (Figure 3H and Supplemental Figure 10, D–F). These results indicate that CD8+ T lymphocytes undergoing replicative senescence in vitro largely recapitulated their aging-associated in vivo phenotypes, including loss of CD28 and diminished expression of Δ133p53, although the lack of increase in CD57 and the undetectable or marginal induction of p53β in vitro may also imply differences between in vivo and in vitro processes of replicative exhaustion of CD8+ T lymphocytes.
Reconstitution of either CD28 or Δ133p53 restores cell proliferation, extends replicative lifespan, and rescues senescence phenotypes in CD8\(^+\)CD28\(^-\) T lymphocytes. Given that CD28 loss occurs during both in vivo and in vitro replicative senescence, we chose CD8\(^+\) T lymphocytes sorted based on CD28 expression (CD28\(^+\) and CD28\(^-\) populations) as recipient cells in gene transduction experiments to examine the mechanistic role of the p53 isoforms. When CD28\(^+\) and CD28\(^-\) populations were compared, the former showed remarkably higher rep-

Figure 4
Reconstitution of CD28 or Δ133p53 expression extends replicative lifespan in CD8\(^+\)CD28\(^-\) T lymphocytes. (A) Cumulative PDLs of CD28-reconstituted CD28\(^-\) cells (donors 29 and 30) after puromycin selection. Empty vector was used as control. (B) Immunoblot analysis of Δ133p53 and p53FL (donor 29, days 27 and 55). β-actin was the loading control for normalization. Expression level relative to control is shown below. (C) Quantitative RT-PCR analysis for IL6 and IL8 in CD28-reconstituted CD28\(^-\) cells (donors 29 and 30, day 20). B2M was used for normalization. (D) Reconstitution of Δ133p53 expression in CD28\(^-\) cells (donor 29). Immunoblot analysis was performed 12 days after blasticidin selection. p53\(\beta\) and p53FL levels were not affected. p53\(\beta\) bands are denoted by arrowheads. (E) Cumulative PDLs of Δ133p53-reconstituted CD28\(^-\) cells after blasticidin selection (donors 29 and 30). Cells with control vector are also shown. (F) Quantitative RT-PCR of CD28 mRNA (donor 29, day 19). B2M was used for normalization. (G) Representative dot plots for CD28 expression by flow cytometry (donor 29, day 19). (H) Frequency of CD28\(^+\) cells at days 3, 12, 19 (as in G), and 27 (donor 29). (I) Quantitative RT-PCR analysis for IL6 and IL8 in Δ133p53-reconstituted CD28\(^-\) cells (donors 29 and 30, day 19). B2M was used for normalization. Data are mean ± SD (C, F, and I), from triplicate assays (F). *P < 0.05; **P < 0.01; ***P < 0.001.
licative potential than the latter (~14 versus ~5 PDLs; Supplemental Figure 11A), as expected from the CFSE cell proliferation assay (Supplemental Figure 2) and previous reports (32). Compared with CD28+ cells, CD28- cells expressed decreased Δ133p53 protein and elevated p53β protein levels, again with unchanged Δ133p53 mRNA, increased p53β mRNA, decreased SRSF3 mRNA, and elevated IL6 and IL8 levels (Supplemental Figure 11, B–G), consistent with the results from CD28/CD57 quadrants (Figures 1 and 2).

To examine the functions of CD28 and Δ133p53 in blood CD8+ T lymphocytes, we reconstituted their expression in Δ133p53-low CD8+CD28+ cells (Figure 4). When CD28 expression was reconstituted by transducing a CD28 retroviral expression vector (Supplemental Figure 12, A and B), the cells reproducibly had an extended replicative lifespan by more than 4 PDLs compared with vector-transduced control cells (Figure 4A), in agreement with a previous report using whole CD8+ T lymphocytes (17). Reconstitution of CD28 restored the expression of Δ133p53 protein without changes in Δ133p53 mRNA levels, p53FL levels, or CD57- cell frequency (Figure 4B and Supplemental Figure 12, C–E). These CD28-reconstituted CD28- cells also showed decreased expression of IL6 and IL8 (Figure 4C). When CD28- cells were transduced with a lentiviral construct for Δ133p53 expression (Figure 4D), the transduced cells showed higher proliferation rate, bypassed the senescence point of vector control cells, and continued to proliferate for 4 or 5 more PDLs (Figure 4E). A similar extension of replicative lifespan was also observed when Δ133p53 was overexpressed in whole CD8+ cells (Supplemental Figure 13, A and B). Upon Δ133p53 expression in CD28- cells, Δ133p53 mRNA expression was induced, and up to 30% of cells were converted to CD28+ (Figure 4, F–H), whereas CD57 expression was unaltered (Supplemental Figure 14, A and B). Expression of IL6 and IL8 was reduced in Δ133p53-rescued CD28- cells (Figure 4I), similar to the CD28-rescued cells (Figure 4C). In addition to the attenuation of senescence factors, these Δ133p53-rescued CD28- cells also increased expression of central memory markers like CD27 and CD62L and decreased expression of late-differentiated markers such as PD-1 and LAG-3 (Supplemental Figure 14, C–J). These findings indicate that Δ133p53, through upregulation of the costimulatory receptor CD28, plays a physiological role in regulating CD8+ T lymphocyte proliferation, senescence, and function.

Since Δ133p53 dominant-negatively inhibits the transcriptional activity of p53FL for p53 target genes, including p53-repressed genes (15), and CD28 contains a p53 response element within its intron 1 (p53FamTag; http://p53famtag.ba.itb.cnr.it/), we hypothesized that the transcriptional repression of CD28 by p53FL is a mechanistic basis for the Δ133p53-induced upregulation of CD28 at the mRNA level. In whole CD8+ T lymphocytes from 3 donors, treatment with nutlin-3a (an inhibitor of MDM2), but not nutlin-3b (an inactive enantiomer), resulted in the activation of p53FL, evidenced by increased amounts of p53FL protein, increased p53 phosphorylation at the serine 15 residue, and induced expression of a p53 target, p21WAF1 (Supplemental Figure 15A). Under this condition, CD28- cell frequency was decreased, which was attributed to decreased expression of CD28 mRNA (Supplemental Figure 15, B–D). To examine the effect of Δ133p53 on the p53FL-mediated repression of CD28, Δ133p53 was overexpressed in whole CD8+ T lymphocytes. While nutlin-3a treatment in control vector-transduced cells resulted in significant repression of CD28 mRNA (60% decrease), similar to that observed in untransduced cells, Δ133p53 overexpression largely abrogated the repressive effect of nutlin-3a on CD28 mRNA expression (19% decrease) (Supplemental Figure 15, D and E). These results suggest that Δ133p53 counteracts the p53FL-mediated transcriptional repression of CD28.

Knockdown of Δ133p53 or overexpression of p53β induces cellular senescence in CD8+ T lymphocytes. Highly proliferative CD28+ populations with abundant levels of Δ133p53 (Supplemental Figure 11, A and B) were used in a further mechanistic experiment, in which endogenous Δ133p53 expression was knocked down. A siRNA oligonucleotide against Δ133p53, which was transfected every 3–4 days by nucleofection, efficiently downregulated Δ133p53 expression with no effect on p53FL (Figure 5A). The cells with Δ133p53 knockdown underwent proliferation arrest within 10 days of induction of SA-β-gal activity, whereas control cells were proliferating well (Figure 5, B and C, and Supplemental Figure 16A). Knockdown of Δ133p53 decreased CD28 mRNA expression and CD28+ cell number, with no remarkable change in CD57+ cells (Figure 5, D and E, and Supplemental Figure 16, B–D), again indicating that Δ133p53 positively regulates CD28 at the mRNA level.

Upregulation of endogenous p53β during in vivo senescence (Figure 2A) was mimicked in vitro by lentiviral overexpression of Flag-tagged p53β in the CD28+ population (Figure 5F), which were p53β-low (Supplemental Figure 11B). While vector control cells were proliferating well, the p53β-overexpressing cells stopped proliferating after 10 days, with an increase in SA-β-gal activity (Figure 5, G and H, and Supplemental Figure 17A), similar to the results using whole CD8+ T lymphocytes (Supplemental Figure 13). Overexpression of p53β was associated with a significant increase in CD57+ cells, but not CD28+ cells (Supplemental Figure 17, B–E), suggesting coregulation of p53β and CD57. IL6 and IL8 were consistently upregulated in the p53β-overexpressing senescent CD28+ cells (Figure 5I). Conversely, PD-1 and LAG-3 were reproducibly upregulated in the senescent CD28+ cells overexpressing p53β (Figure 5, J and K, and Supplemental Figure 17, F and G). These findings suggest that p53β overexpression leads cells toward senescence, with expression of senescence-associated factors as well as terminally differentiated markers such as PD-1 and LAG-3.

Discussion

In this study of CD8+ T lymphocytes from healthy human donors, we observed age-dependent in vivo accumulation of late-differentiated cells, which were associated with specific changes in cell surface antigens (loss of CD28 and gain of CD57) (20, 22), as well as various senescence markers and phenotypes, such as SA-β-gal activity, shortened telomeres (23, 33), poor proliferation (21, 34), increased HP1-γ foci (29), increased γ-H2AX foci (35), and increased SASPs (8). In these physiologically senescent or near-senescent CD8+ T lymphocytes, Δ133p53 expression was diminished and p53β expression was increased, similar to our previous observations with senescent human fibroblasts in vitro and pathologically induced in vivo senescence in premalignant tumors (15). The CD28 CD57+ populations of tumor-associated CD8+ T lymphocytes from lung cancer tissues also showed downregulation of Δ133p53 and upregulation of p53β. The regulatory mechanisms for the p53 isoform expression may be conserved in CD8+ T lymphocytes (present study) and fibroblasts (15). p53β is likely to be regulated by SRSF3-mediated alternative splicing in both cell types (16), and the change in Δ133p53 occurs at the protein level, but not the mRNA level, in both cell types. There is unlikely to be a direct relationship between the regulation of p53β and Δ133p53 expression, because overexpression of either one did not affect the expression level of the other (Figure 4D and Figure 5F).
Successive in vitro culture of isolated CD8+ T lymphocytes from blood resulted in senescent proliferation arrest with CD28 loss and Δ133p53 downregulation, consistent with their regulation during in vivo aging, enabling in vitro functional analyses of the costimulatory receptor and the p53 isoforms. The ability of Δ133p53 to extend replicative lifespan was shown in CD8+ T lymphocytes from blood. When Δ133p53 expression was restored in the Δ133p53-low CD28− population that corresponded to cells undergoing or

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**Figure 5**

Δ133p53 knockdown or p53β overexpression induces cellular senescence in CD8+CD28+ T lymphocytes. (A) Δ133p53 siRNA− or control siRNA− nucleofected CD28+ cells were analyzed by immunoblot analysis of Δ133p53 and p53FL (donor 30, day 7). (B) Nucleofections were performed every 3–4 days, 5 consecutive times. Cumulative PDLs were plotted against days after the first nucleofection (donors 29–31, day 18). (C) Representative dot plots for CD28 expression by flow cytometry (donors 30, day 18). (D) Cumulative PDLs were plotted against days after the first nucleofection (donors 29–31, day 18). (E) Flag-tagged p53β− or control vector−transduced CD28+ cells were analyzed by immunoblot analysis of the indicated proteins after FACS sorting based on vector-derived GFP expression (donor 29, day 7). Overexpression of Flag-p53β was confirmed by TLQ9 and DO-1 antibodies. Δ133p53 was not substantially affected. (G) Cumulative PDLs of Flag-p53β−overexpressing CD28+ cells (donors 29 and 31). Cells with control vector are also shown. (H) Quantitative analysis of SA-β-gal activity (donors 29 and 31, day 19). (I) Quantitative RT-PCR analysis for IL6 and IL8 in p53β-reconstituted CD28+ cells (donors 29 and 31, day 19). B2M was used for normalization. (J and K) Frequency of PD-1+ (J) and LAG-3+ (K) cells (donors 32 and 33, day 19). Data are mean ± SD (B, C, E, H, and I), from triplicate assays (H). *P < 0.05; **P < 0.01.
approaching senescence (20, 21), Δ133p53 delayed the onset of senescent proliferation arrest, extended the replicative lifespan, and rescued cells from senescence phenotypes as efficiently as the reconstitution of CD28. The extension of the replicative lifespan by Δ133p53 restoration was coincident with restored expression of CD28 along with CD27 and CD62L, suggestive of a functional conversion to a less-differentiated, more-proliferative state, such as that of central memory T cells (25). When the in vivo senescence-associated changes in the p53 isoform expression (i.e., p53β upregulation and Δ133p53 downregulation) were reproduced by p53β overexpression or Δ133p53 knockdown, cellular senescence was induced in an otherwise highly proliferative CD28+ population, further validating the physiological roles of the p53 isoforms in regulating proliferation and senescence of CD8+ T lymphocytes.

Our data provide a mechanistic basis for the regulatory interaction between CD28 and Δ133p53, which mutually upregulated the expression of each other. The autophagic degradation of Δ133p53 is in contrast to the proteasome-mediated degradation of p53FL and p53β (15), thus leading to the isoform-specific expression profile of Δ133p53. This finding links CD28 function to Δ133p53 upregulation at the protein level, since NF-κB and its activation of mTOR, a major downstream pathway activated by the CD28 signaling, may repress autophagy (36–40). Our data also suggest that an additional mechanism may exist for the CD28-mediated regulation of Δ133p53, in that the bafilomycin A1 only partially restored Δ133p53 protein levels in CD28+CD57− subsets compared with the levels in CD28+CD57− subsets (Figure 2F and Supplemental Figure 8C). We showed that the activation of p53FL repressed CD28 mRNA expression, which was abrogated by Δ133p53 overexpression. Together with our previous finding that Δ133p53 functions as a dominant-negative inhibitor of p53FL (15), these data suggest a mechanism by which Δ133p53, which lacks the transactivation domain (14), transcriptionally activates CD28 mRNA expression.

Tumor-associated CD8+ T lymphocytes may undergo differentiation and senescence in response to chronic exposure to tumor antigens, as blood CD8+ T lymphocytes respond to chronic infectious agents. In addition, cross-talk between these T lymphocytes and tumor cells likely exists in the tumor microenvironment. Tumor-induced senescence of T lymphocytes is also mediated by tumor-derived soluble factors (41). Senescent CD8+ T lymphocytes not only contribute to tumor immune evasion through their functional unresponsiveness (42) and their suppressor function toward responder T cells (41, 43), but may also actively promote tumor progression through SASP factors such as IL6 and IL8. The senescence-associated change in Δ133p53 and p53β expression in tumor-associated CD8+ T lymphocytes, together with the similar change in premalignant tumors and its reversion during malignant progression in our previous study (15), suggests that these p53 isoforms regulate tumorigenesis in vivo in both tumor cell–autonomous and non-autonomous manners.

This study not only improves understanding of the regulation of CD8+ T lymphocytes, but may also suggest a strategy to overcome immunosenescence. Restoration of cell proliferation and function in terminally differentiated or senescent CD8+ T lymphocytes in blood could reinstate systemic cell-mediated immunity in the elderly and patients with chronic antigen exposure (e.g., patients with HIV) (18, 19, 44, 45). Tumor-specific, tumor-infiltrating CD8+ T lymphocytes that are terminally differentiated or senescent could be functionally restored for adoptive cell transfer therapies against cancer (25). The Δ133p53-induced restoration of the costimulatory receptor CD28 and the central memory markers CD27 and CD62L, as well as the attenuation of the terminal differentiation markers PD-1 and LAG-3, suggests that enhanced expression of Δ133p53 may be a strategy for dedifferentiating and expanding CD8+ T lymphocytes toward future clinical applications. Of particular relevance is that the blockade of PD-1 and its ligand has recently emerged as a novel, promising immunotherapy against lung cancer and other cancers (46, 47).

A growing body of evidence, including the present study and a prior report of reprogramming of senescent human fibroblasts into induced pluripotent stem cells (iPSCs; ref. 48), suggests that near-senescent or senescent cells can be induced back into a proliferative state. Recently, iPSCs were derived from human CD8+ T lymphocytes (49). Direct reprogramming or iPSC reprogramming and subsequent redifferentiation may convert terminally differentiated cells to naive or early-differentiated states (50). However, these methods usually involve an oncogenic factor such as c-Myc or SV40 large T antigen (49, 51–53). Since Δ133p53 is a natural isoform of p53 and is physiologically expressed in normal proliferative cells at high levels, enhanced expression of Δ133p53 may lead to a safe method for functional restoration of CD8+ T lymphocytes with minimum concern for malignant transformation.

Methods

Cell cultures and reagents. Mononuclear cells from blood or single-cell dissociated lung tumors (54) were obtained by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich) and then used for flow cytometric staining or stored in liquid N2 with freezing media (Invirotogen) until use. CD8+ T lymphocytes and their CD28+ and CD28− subsets were isolated from PBMCs using FACS or by magnetic bead–activated cell sorting (MACS) using the EasySep CD8 enrichment kit (Stemcell). The CD28/CD57 quadrants (CD28+CD57+, CD28–CD57−, CD28+CD57−, and CD28−CD57−) of CD8+ T lymphocytes were isolated by FACS. Cell cultures were established as described previously (17, 55, 56). Briefly, cells were cultured at 1 × 106/ml in AIM-V complete medium (Invirotogen) supplemented with 300 IU/ml rIL-2 (PeproTech) and 5% heat-inactivated human AB serum (Valley Biomedical) unless otherwise specified. RPMI-1640 complete medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 10 mM HEPES (Invitrogen), 2 mM glutamine (Invitrogen), 50 IU/ml penicillin/streptomycin (Invitrogen), and 20 IU/ml rIL-2 was used in Supplemental Figure 2. Stimulation was either with 1 μg/ml phytohemagglutinin-L (PHA-L, Sigma-Aldrich) for 48 hours or with anti-CD2/3/23/32 microbead cocktail (Miltenyi Biotech) for 72 hours. Cell viability was determined by trypan blue exclusion every 3–4 days, and when the cell density reached ≥2 × 105/ml, cells were subcultured at 1 × 106/ml. Population doubling levels (PDLs) were calculated as log10(number of cells counted after expansion) − log10(number of cells seeded)/log102. Bafilomycin A1 and MG-132 were from Cayman Chemical Company.

Flow cytometry and cell sorting. Mononuclear cells were washed with cold PBS plus 0.5% BSA or with RPMI-1640 complete medium and incubated with FcR blocking reagent (Miltenyi Biotech) for 15 minutes at 4°C. Cells were then stained with APC-Cy7– or FITC-conjugated anti-human CD8 (BD Biosciences), APC-conjugated anti-human CD28 (BD Biosciences), PE-conjugated anti-human CD57 (Abcam), PE-Cy7–conjugated anti-human CD27 (BD Biosciences), APC-conjugated anti-human CD62L (BD Biosciences), PE-conjugated anti-human PD-1 (BD Biosciences), and FITC-conjugated anti-human LAG-3 (LifeSpan Biosciences) for 30 minutes on ice. 7-AAD (BD Biosciences) was used to assess the viability of cells. Fluorescence data from at least 10,000 cells were acquired using FACSDiva software in LSRII (BD Biosciences). Data were analyzed using FlowJo (Tree Star Inc.). The purity of each population was checked and >90% pure cells were used for all experiments.
Telomere length analysis. High-throughput quantitative FISH was performed to compare the telomere lengths of the CD28+CD57– and CD28–CD57+ subsets as described previously (58). The 4′,6-diamidino-2-phenylindole channel was used for nuclear staining and the CY3 for telomere detection. Telomere specific knockdown Δ133p53 protein; ref. 14) was used here because it caused more efficient knockdown via nucleofection. The Δ133p53 siRNA oligonucleotide and its scrambled control were synthesized by Invitrogen. The siRNA oligos were nucleofected at a final concentration of 300 nM using P3 Primary Cell 96-well Nucleofector Kit (Lonza). In experiments where cellular replicative lifespan was examined, nucleofection was repeated every 3–4 days, 5 consecutive times.

Real-time qRT-PCR. RNA samples were prepared using TRIzol (Invitrogen). Reverse transcriptase reaction was performed using SuperScript III First Strand Synthesis System (Invitrogen). Taqman Universal PCR Master Mix (Applied Biosystems, catalog no. 4304437) was used with the following probes and primers (all from Applied Biosystems): Δ133p53 (forward, 5′-ACTCT-GTCTCCTCCCTCTCTCCTACAG-3′; reverse, 5′-GGTGGATACACAAA-CAAGCT-3′; probe, 5′-TCCCTGTCCCTAACAAGATGTGGTCCG-3′); CD28 (Hs01007422_m1); CD57 (Hs00128629_m1); IL6 (Hs00174146_m1); IL8 (Hs00174103_m1); CXC1R1 (Hs00174146_m1); CXC2R1 (Hs01011557_m1). SYBR green PCR Master mix (Applied Biosystems, catalog no. 4367659) was used with Δp53 (forward, 5′-CTTGGACTCTTGCTGTGTTGC-3′; reverse, 5′-TTGAAAGCTCTGCTGGTCTCCTACAG-3′; ref. 16) and SRSF3 (forward, 5′-AGCTGATGCTACCCGGAG-3′; reverse, 5′-GTTGGGCGCAAGTCTGC-3′; ref. 16) primers. The internal control was B2M (Applied Biosystems, catalog no. 4326319E). Normalized expression was calculated using the ΔΔCt method according to the supplier’s protocol (Applied Biosystems, protocol no. 4310255B, user bulletin no. 4303859B).

Statistics. Unless otherwise indicated, statistical analyses were carried out using 2-tailed Student’s t test for paired and unpaired samples as appropriate. A P value less than 0.05 was considered significant.

Study approval. Human peripheral blood samples were collected from healthy donors after informed consent and in accordance with the NIH IRB (study no. 99-CC-0168). Lung tumors were obtained through the CIN Biospecimen Repository Services under UMDNJ IRB approval (study no. 0220100267).

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Proliferation assay. Cells were stained with CFSE using the Vybrant CFDA SE Cell Tracer kit (Invitrogen) as described previously (57). CFSE-stained cells were washed twice with RPMI-1640 complete medium before they were cultured at 1 × 10^6 cells/ml. Stimulation was performed with PHA-L at day 0. Cells were immunostained with anti-CD8-APC-Cy7, anti-CD28–APC, and anti-CD57–PE at day 5, and fluorescence data were acquired as described above. The proliferation index was calculated from the data (total number of cell divisions that took place/number of cells that went into division) using Flowjo (Tree Star Inc.).
The serendipitous observation of the limited in vitro lifetime of human diploid fibroblasts by Hayflick and Moorhead opened the field of cellular senescence. The key players in this process are the tumor suppressor protein p53 and its downstream effector, the retinoblastoma protein (Rb). The discovery of p53 set off a wave of interest in understanding mechanisms of aging and cancer. The field of cellular senescence has grown tremendously over the last two decades with the recognition that cellular senescence has a role in the aging process and in repressing carcinogenesis.

In human cells, p53 is a transcription factor that responds to various stresses by activating genes that control cell cycle arrest, apoptosis, and gene expression. Downregulation of splicing factor 
