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Mechanisms That Limit the In Vitro Proliferative Potential of Human CD8+ T Lymphocytes

Marco Migliaccio,* Kenneth Raj,† Olivier Menzel,* and Nathalie Rufer2*†

Human T lymphocytes can be numerically expanded in vitro only to a limited extent. The cyclin-dependent kinase inhibitor p16INK4a is essential in the control of cellular proliferation, and its expression, in epithelial cells, is associated with irreversible growth arrest. Using long-term cultured CD8+ T lymphocytes, we have investigated the role of the p16/pRb pathway in the regulation of T cell proliferation and senescence. In this study, we describe at least two mechanisms that cause replicative growth arrest in cultured lymphocytes. The first one depends on the expression of p16INK4a and is directly responsible for the exit of a number of restimulations, T lymphocytes cease to respond. This state, termed replicative senescence, appears to be irreversible even though the cells remain alive so long as IL-2 is periodically added (reviewed in Ref. 1). Replicative senescence imposes a limit to the numerical expansion of human T cells in vitro, which constitutes a major barrier in the development of adoptive transfer approaches based on the use of Ag-specific cytolytic CD8+ T lymphocytes (CTLs), in which substantial numbers of T cells must be generated in vitro (2).

Many studies have focused on the identification of the molecular mechanisms that trigger cell cycle entry, progression, and exit, thereby regulating lymphocyte proliferation and their numerical expansion. The E2F transcription factors are required for T lymphocytes to enter the cell division cycle. E2Fs play a pivotal role in the timely activation of a number of genes coding for proteins that participate in DNA synthesis (reviewed in Ref. 3). The pRb family proteins (pRb, p107, and p130) prevent cellular division by binding to and inhibiting the transcriptional activity of E2F. Therefore, to enter the cell division cycle, proteins of the pRb family, especially pRb itself, must be inactivated, by cyclin-dependent kinases (CDKs), which phosphorylate pRb (reviewed in Refs. 4 and 5). Together, the core members of the pRb pathway (pRb, E2F, and cyclin CDKs) regulate the progression of cells into the cell division cycle.

The factors that cause cultured T lymphocytes to cease responding to stimulation and enter a state of irreversible growth arrest have not been clearly identified. In mesenchymal and epithelial cells grown under optimal culture conditions, replicative senescence involves telomere shortening. In most human cell types, some telomere repeats are lost during each cell division. Cells in which one or more telomeres have become too short to prevent the chromosome end from being treated as a double strand break undergo a proliferation arrest mediated by the p53 and pRb pathways (6). In various primary human cell types, the causal link between telomere shortening and cellular senescence has been established by showing that transduction of the human telomerase reverse transcriptase (hTERT) gene results in extension of telomeres and an increase in the proliferative life span (7, 8). Apart from critically short telomeres, tissue culture conditions that inadvertently stress cells may also lead to the irreversible growth arrest observed in vitro cultures. This has been most clearly shown in epithelial cells, which stop to proliferate even though the lengths of their telomeres are well beyond the critical limit (9, 10).

In some cell types, the presence of the p16INK4a (hereafter p16) protein is expressed in response to various forms of cell stress and is associated with irreversible growth arrest (11–13). High levels of p16 protein are detected in keratinocytes that senesce despite having long telomeres (9, 10, 14, 15). High levels of p16 have also been found in senescing human fibroblasts (16–18), and, together with the p53 pathway, the p16/pRb pathway has been shown to be involved in the response to uncapped telomeres in such cells (19). Thus, p16 can mediate senescence induced either by critically

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short telomeres or inadequate culture conditions. The p16 protein is a CDK inhibitor that prevents phosphorylation and inactivation of the pRb protein by cyclin D-Cdk4/6 (20) and inhibits the transition of cells from the G_1 to the G_2 phase of the cell cycle (21). The importance of p16 in normal cell proliferation is reflected in the high frequency of mutations that inactivate the function of p16 in numerous types of human cancers, including the majority of T cell leukemias (22, 23). Recent analysis of p16-deficient mice demonstrated the importance of p16 in restraining the incidence and development of tumors (24).

At present, there is no consensus as to why cultured T lymphocytes cease to proliferate after a certain number of stimulation cycles. Proliferation arrest could be due to inadequate culture conditions, or to telomere shortening. In either case, we expect an involvement of p16. We have assessed the status of the p16/pRb pathway in human CD^{8+} T lymphocytes that were subjected to repeated rounds of mitogenic stimulation until replicative growth arrest. We observed that p16 expression was directly induced as a consequence of T cell activation. A large fraction of T cells expressed high levels of p16 in response to each round of stimulation and was not related to cells that underwent activation-induced cell death. Under the culture conditions used, replicative senescence of CD^{8+} T cells was observed after 30 population doublings (PD), but curiously, it did not correlate with high p16 levels or telomere attrition. Taken together, our results indicate that the in vitro replicative potential of mitogen-activated human T lymphocytes is limited by at least two independent mechanisms, neither of which is associated with telomere shortening, and only one of which is associated with p16 expression.

Materials and Methods

Long-term culture of human CD^{8+} T lymphocytes

PBMC were obtained from healthy donors by density centrifugation on Ficoll-Hypaque (Pharmacia), and CD^{8+} T lymphocytes were purified by magnetic sorting using a miniMACS device (Miltenyi Biotec). Bulk cultures of human T cells were obtained by seeding them onto 24-well plates (10^6 cells in 2 ml/well) in RPMI 1640 medium supplemented with 8% human serum and 150 U/ml human rIL-2 (a gift from GlaxoSmithKline) and 100 U/ml PHA (Sodiag) plus 0.5 × 10^5/ml irradiated allogeneic PBMC (3000 rad) as feeder cells. Culture medium was checked daily and changed when required. The stimulation procedure was repeated every 10 or 15 days of culture. PDs were determined by counts of cells alive once or twice per week. When indicated, samples of 10^6 cells were resuspended in 1 ml of CFSE (Molecular Probes) solution (2 μg/ml in PBS), incubated for 10 min at 37°C, washed twice, and seeded into 24-well plates, as described (25).

Western blot analysis

A total of 5 × 10^6 cell samples was spun down, and cell extracts were obtained by resuspending the pellets in a lysis buffer (1% SDS, 1 mM sodic orthovanadate, and 10 mM Tris-HCl, pH 7.4). The protein concentration of supernatants recovered after centrifugation was measured by spectrophotometry with a protein assay kit (Bio-Rad). A total of 20 μl of extract corresponding to 8 × 10^6 cells was loaded in each well, and the proteins were resolved on an 8% (for detection of pRb, p130, and p107) or a 12% (for the other proteins) polyacrylamide gel by SDS-PAGE and electroblotting to polyvinylidene difluoride membranes by semidry transfer. The membranes were blocked and probed with the indicated Abs: anti-pRb, anti-p16 (BD Pharmingen), anti-p27Kip1, anti-p107, anti-p130, and anti-α-actin (Santa Cruz Biotechnology). Ab-Ag interaction of the primary Abs was detected by incubation with secondary HRP-conjugated Abs and subsequent detection by ECL (Amersham).

Annexin V staining, intracellular staining, and flow cytometry analysis

Collected samples of fresh or cultured T lymphocytes were washed and stained with Cy-5-conjugated annexin V (BD Biosciences), according to manufacturer instructions. Cell pellets were then washed, fixed in PBS/2% paraformaldehyde, permeabilized in PBS/3% FCS, 0.5% saponin (Sigma-Aldrich), and 50 μl of cell suspension was mixed with 5 μl of PE-conjugated mAbs against human p16, pRb, hypophosphorylated, active form of pRb, or corresponding isotype controls (BD Biosciences). Cells were incubated 1 h at room temperature, washed with permeabilization solution, resuspended in 400 μl of PBS/3% FCS, and kept at 4°C until analyzed on a FACS caliber flow cytometer (BD Biosciences). Early apoptotic cells (annexin V-positive) and allogeneic PBMC cells (FITC-negative) were discarded by gating the cells in the corresponding plots. For BrdU incorporation studies, 10^6 cell samples were incubated in the presence of 10 μg/ml BrdU during 1 h at 37°C. Cells were then fixed, permeabilized, treated with DNsase to expose BrdU epitopes, and stained with an anti-BrdU Ab and 7-aminoactinomycin D (7-AAD) using the BrdU flow kit (BD Biosciences).

Data were expressed as: 1) normalized mean fluorescence (Figs. 2 and 7) or as 2) percentage of p16-positive cells (Figs. 3–6). 1) Histograms obtained from cells stained with each Ab and the corresponding isotype control were generated. The normalized mean fluorescence for every mAb in respect to its isotype control was first calculated using the geometric mean values of each histogram (X_{mab} and X_{iso}), according to the following formula: (X_{mab}/X_{iso}). To facilitate the comparison between samples, these mean fluorescence values were then normalized with the mean fluorescence value obtained from fresh cells that did not proliferate at day 4 after stimulation (CFSE^{norm}). This normalization was arbitrarily set to 1 (total pRb and active pRb) or 10 (p16) and expressed as normalized mean fluorescence. 2) For quantifying the percentage of p16-positive cells (%), histograms obtained with anti-p16 or isotype control were overlaid. In these p16 histograms, the area that overlapped the isotype histogram represented the p16-negative area (area p16^-), while the area that did not overlap the isotype histogram represented the p16-positive area (area p16^+). Both areas were determined using Adobe Photoshop software (Adobe Systems), and the percentage of p16-positive cells was calculated as % of p16^+ = (area p16^-)/(area p16^- + (area p16^-) × 100. The specificity of the anti-human p16 mAb was confirmed using HeLa (p16^-) and U2OS (p16^-) cell lines (American Type Culture Collection).

Knockdown of p16 expression with specific short hairpin RNA (shRNA) oligonucleotides

Small interfering RNAs directed against two target sequences of the first exon of INK4a (p16shRNA-A (GAGGGAGGTGCAGGCCTGC) and p16shRNA-B (GTGCTCGGAGTTAATAGCA)) have been previously reported to knock down p16 gene expression (26, 27), and were thereby chosen to create two lentiviral vectors. Oligonucleotides designed to form shRNA targeting these sequences (28) were subcloned into a pSUPER vector under the control of the H1 promoter. DNA cassettes containing the H1 promoter and each of the specific shRNA were then subcloned in a third generation lentiviral vector containing the enhanced GFP under the control of the CMV promoter, kindly provided by L. Naldini (Laboratory of Gene Transfer and Therapy, Institute for Cancer Research and Treatment, Can- diolo, Italy). Vesicular stomatitis virus-pseudotyped lentivirus was produced by transfecting 293T cells with the corresponding plasmids, as described (29), and 10 μl of concentrated viral supernatant (~10^6 transduction U/μl) was used to infect 10^6 resting lymphocytes in 0.5 ml of complete medium with 1 μg/ml polybrene (Sigma-Aldrich). Four to 12 h after infection, cells were washed and stimulated with PHA and allogeneic feeder cells, as described above. The transduction efficiency typically ranged between 30 and 60%.

Long-term culture of hTERT-transduced CD^{8+} T cell clones

Human CD^{8+} T cell clones expressing either hTERT/GFP or GFP alone were obtained and cultured following serial rounds of subcloning steps, as previously described (30). Briefly, transduced T cells expressing high levels of GFP were sorted by a FACStar® flow cytometer (BD Biosciences) and further recloned by limiting dilution using PHA and feeder cells, as described for bulk cultures. This subcloning procedure was repeated until no further subclones could be obtained (GFP control clones) or until the ninth recloning step (hTERT/GFP clones).

Telomere fluorescence in situ hybridization (FISH) and flow cytometry (flow FISH)

The average length of telomere repeats at chromosome ends from in vitro cultivated T lymphocytes was measured by FISH and flow cytometry (flow FISH), as previously described (31, 32). Telomere fluorescence was calculated by subtracting the mean fluorescence of the background control (no probe) from the mean fluorescence obtained from cells hybridized with the telomere probe after calibration with FITC-labeled fluorescent beads.
(Quantum TM-24 Premixed; Bangs Laboratories) and conversion into molecules of equivalent soluble fluorochrome units. The following equation was used to estimate the telomere length in base pair: bp = molecules of equivalent soluble fluorochrome × 0.495 (33).

**Results**

**Expression of p16 protein does not coincide with replicative senescence of CD8+ T lymphocytes cultured in vitro**

Human CD8+ T lymphocytes isolated from peripheral blood are predominantly small quiescent cells in the G0 phase of the cell cycle. When subjected to stimulation with PHA and irradiated allogeneic PBMC, the CD8+ T cells became large blasts and proliferated, as reflected by the high proportion of cells in the S/G2/M phases of the cell cycle. During seven to eight consecutive rounds of mitogenic stimulation over 80 days, these cells underwent >30 PD until they reached a state in which net increase in the total cell number was no longer observed upon further stimulation (Fig. IIA). This state was previously described for lymphocytes and has been defined as replicative senescence by analogy with the term used to designate the growth arrest of diploid human fibroblasts after a finite number of cell divisions (1).

To study the role of CDK inhibitors during in vitro culture of CD8+ T lymphocytes from the time of their isolation from human blood, up to the point when they senesced, we analyzed the levels of p16 and p27Kip1 (hereafter p27) proteins on Western blots (Fig. 1B). Data obtained from two independent experiments showed a progressive increase of p16 protein levels during culture, with a peak after ~20 PD. Surprisingly, this did not coincide with senescence of these cells. Instead, p16 expression had decreased to very low levels by the time the cells became senescent. p16 was undetectable in freshly isolated CD8+ T cells and in cells rendered quiescent by IL-2 starvation. In contrast, p27 levels were particularly high in these two populations, clearly above the levels in senescent cells. These results suggest that neither p16 nor p27 is responsible for the senescence of CD8+ T lymphocytes in vitro.

**De novo expression of p16 is a delayed response of some T lymphocytes to mitogen stimulation**

To investigate the role of the transient increase in the levels of p16 during the proliferative life span of the CD8+ T cell population as a whole, we analyzed these populations by flow cytometry after staining them intracellularly with anti-p16 and anti-pRb Abs. When we stained ex vivo isolated CD8+ T cells with CFSE before stimulation and analyzed their response to mitogen by flow cytometry, it became apparent that more than one-half of the cells failed to divide at all, whereas ~11% underwent a single division and then arrested. The remainder of the cells divided from two to at least seven times during the 7-day culture period (Fig. 2A; data not shown).

When we combined CFSE staining with intracellular staining for p16, we found (although not detectable by Western blotting) a low level of p16 in freshly isolated CD8+ T cells (day 0; Fig. 2B). Four days after mitogenic stimulation, p16 was undetectable, but after a further 3 days it was again detectable to levels comparable to that in unstimulated cell. Because at this time point ~99% of the population was made up of cells that had undergone several divisions, most of p16 in these cells must have been synthesized after day 4. To define more precisely the dynamics of p16 expression, we determined the levels of this protein in cells that have undergone different number of divisions according to their CFSE concentration. A representative example of histograms obtained 4 days after stimulation is depicted in Fig. 2C. High levels of p16 were found in cells that did not divide in response to stimulation. In contrast, p16 was barely detectable in cells that had undergone at least four divisions, indicating that the apparent decrease in p16 expression in the bulk population 4 days after stimulation is the result and increase in proliferating, p16-negative cells.

**High proportions of cycling T lymphocytes accumulate p16 during the successive rounds of mitogen stimulation**

The findings described above were made with cells obtained during the first cycle of mitogenic stimulation of CD8+ T cells in vitro. When we determined p16 expression on days 4, 7, and 10 of each of the following seven stimulation cycles before the cells senesced, we observed a similar pattern in each cycle (Fig. 3A). The percentage of p16-containing cells declined during the first 4 days after stimulation (see day 4, Fig. 3A), due to an increase in p16-negative proliferating cells (see day 4, Fig. 3B). As observed during the first cycle of stimulation, the percentage of p16-expressing cells began to increase from day 5 onward. This augmentation was particularly stark at days 7 and 10 after stimulation. The size of the fraction of p16-positive cells increased after each consecutive mitogenic stimulation up to the fourth to fifth stimulation (Fig.
when stimulated. This is also true for CD8 T lymphocytes. Our data indicate that the accumulation of p16 may be related to the proliferative state of T lymphocytes, as assessed by 7-AAD costaining of the DNA content (Fig. 3A). Comparable data were obtained from two additional independent experiments.

Expression of p16 has been associated, in epithelial cells, with irreversible growth arrest (33, 34). Using BrdU incorporation as a measure of cell division, we next investigated the consequence of p16 expression during the G1 phase of the cell cycle. As shown in Fig. 3A, almost all T cells that had incorporated BrdU and progressed through the cell cycle were also p16 negative. In contrast, a significant proportion of cells that failed to divide in response to stimulation and were thus BrdU negative expressed the p16 protein (Fig. 4A). These p16-negative T cells were found exclusively within the G0/G1 phase of the cell cycle, as assessed by 7-AAD costaining of the DNA content (Fig. 4B). Our data indicate that the accumulation of p16 may be responsible in preventing a fraction of cells from proliferating when stimulated. This is also true for CD8 T lymphocytes in which p16 was expressed as the result of de novo synthesis (see day 7; Fig. 4B).

**Transient knockdown of p16 expression by specific shRNA resulted in increased proliferation of T lymphocytes**

To further investigate the causal link between activation-induced p16 expression and the proliferative potential of mitogen-activated CD8 T lymphocytes, we knocked down p16 gene expression with two lentiviral vectors, each expressing a different p16-specific shRNA (p16shRNA-A or p16shRNA-B) (26, 27). In vitro cultured T cells were transduced once with the lentiviral supernatant p16shRNA-A or p16shRNA-B. The ability of each specific shRNA to knock down the expression of p16 protein was monitored, as shown in Fig. 5A. Following p16 shRNA treatment, a 2-fold reduction in the amount of p16 expression was observed in transduced T lymphocytes at day 4 after stimulation. However, knockdown of p16 expression was only transient, as there was no significant difference in p16 levels between transduced and control T cells from day 7 onward (data not shown). We next assessed the number of PDs (Fig. 5B) as well as the proportion of cells in the S/G2/M phases of cell cycle (Fig. 5C). Transient reduction of p16 gene expression resulted in a significant increase of the numerical expansion of transduced cells from day 4 to 7 following mitogenic stimulation in comparison with control cells. Moreover, this net growth advantage was accompanied by a higher proportion of cycling cells within the fraction of transduced GFP-positive cells when compared with the one present among GFP-negative cells (53 vs 38%; Fig. 5C). Although the p16 knockdown appears at first to be disappointingly transient, in a roundabout way, it has actually created a quasi p16 off-on situation, which is closely paralleled by a transient boost in PD of the cells. Hence, the transientness of the p16 knockdown has inadvertently lent even more weight to the conclusion that reduction of p16 level increases CD8 T cell expansion, and re-establishment of the p16 levels.
CFSE dot plots. CFSEhigh) or the lowest (L, CFSELow) CFSE fluorescence regions from p16-positive cells in histograms was derived after gating in the highest (H, at days 4 and 7 after the fifth cycle of stimulation. The percentage of histograms of combined p16 and CFSE analysis were obtained from samples described in stimulation, and the percentage of p16-positive cells was calculated, as cultivated following eight cycles of stimulation until they senesced. Levels following repeated rounds of mitogen stimulation.

Expression and telomere loss in in vitro cultured CD8

Expression can be also induced by the presence of critically short stress signals due to suboptimal culture conditions (9), but its ex-

One question that our findings raise is what event or stimulus is responsible for the onset of p16 synthesis during a stimulation cycle. In cultured cells, p16 has been described as a mediator of stress signals by suboptimal culture conditions (9), but its expression can also be induced by the presence of critically short telomeres (19). To explore a possible relationship between p16 expression and telomere loss in in vitro cultured CD8+ T cells, we monitored the average length of telomere repeats using flow FISH. As shown in Fig. 6A, a strong decline in telomere fluorescence was observed between the third and fifth cycles of stimulation, corresponding to a loss of ~3.7-kb telomeric DNA. However, from the fifth stimulation onward until replicative senescence telomere fluorescence remained stable and corresponded to ~5.5 kb (Fig. 6A). In contrast, human T cell clones that senesced after cultivation by serial recloning for extended periods of time (30) displayed extremely short telomeres (2 kb; see control clones, Fig. 6A). This comparison indicates that telomere attrition is unlikely to be involved in the replicative senescence of bulk population of CD8+ T cells, in agreement with previous reports (35). Intriguingly, these results do not corroborate those obtained from senescent T cells that were subjected to consecutive rounds of recloning (30). To date, we do not have a rational explanation for the apparently contradictory findings concerning the role of telomere shortening in the senescence of CD8+ T cells cultured in a different manner.

Our data suggest that induction of p16 expression in bulk cultures of CD8+ T cells is independent of telomere attrition. Further evidence for this comes from the analysis of p16 in CD8+ T cell clones that ectopically express the human telomerase catalytic subunit (hTERT) (30). These hTERT-transduced T clones express high levels of telomerase and have elongated their telomeres up to 13-kb average length (see control cells, Fig. 6A). Their replicative life span exceeds 200 PD compared with 108 PD in control clones. Nevertheless, a significant fraction of p16-positive cells was generated as a consequence of mitogen stimulation of T cell clones transduced with either control or hTERT vectors (Fig. 6B). As in primary CD8+ T cell bulk cultures, p16 expression increased after 7 days of stimulation to comparable levels (Fig. 3A). Taken together, these results indicate that mitogen-induced p16 expression can be mediated by a mechanism that is independent of telomere length.

The active form of pRb is predominant in senescent CD8+ T lymphocytes

We observed irreversible growth arrest or replicative senescence in our bulk culture system after seven to eight consecutive rounds of mitogenic stimulation during ~80 days of culture (Fig. 1A). Careful analysis using CFSE staining revealed that 99.9% of the senescing CD8+ T cells failed to proliferate in response to mitogenic stimulation (PHA) or when challenged with PMA and ionomycin (our unpublished data). Our findings that the senescent cells contained no or very low levels of p16 and had telomeres the average length of which considerably exceeded that of senescing clones.
Recent studies have suggested that in senescent CD8+ T cells, the pRb protein remains active in its hypophosphorylated form, whereas in proliferating T cells, pRb is in its inactive, hyperphosphorylated state (36). These findings are consistent with the observations made with other human cell types and suggest that a high ratio of active vs total pRb is associated also with lymphocyte senescence. What is unclear, at present, is what controls pRb phosphorylation in these cells. It does not appear to be p16.

**Discussion**

In vitro expansion of human cytolytic CD8+ T lymphocytes by repeated mitogenic stimulation is widely used for clinical and research purposes. Adoptive immunotherapy of cancer has been limited by the fact that under currently used tissue culture conditions, tumor-infiltrating lymphocyte populations or tumor-specific CTL clones can be expanded at most to $10^9$-$10^{11}$ cells. As reinfused T cells often do not persist longer than a few days in the host, the efficiency of adoptive immunotherapy might be enhanced if culture methods that allow the generation in vitro of sufficient numbers of CTLs for multiple reinfusions into patients can be developed (2).

This complex task would be made easier by a better understanding of the molecular mechanisms that limit the proliferative potential of in vitro cultured CTLs. The culture system used in the experiments described in this work allows bulk CD8+ T lymphocytes to undergo ~30 PD in eight rounds of stimulation with PHA and IL-2. This means that in our culture conditions we can produce $10^8$ cells per initial T cell. However, analysis by CFSE staining showed that a fraction of the cells undergoes at least eight divisions during one stimulation cycle. If all of the cells divided eight times per stimulation, then we would obtain $2 \times 10^{19}$ descendants from each initial cell! Understanding the extrinsic and intrinsic factors that prevent a T cell from realizing this maximum proliferation potential of eight divisions per cycle should provide us with clues as to how we can...
improve culture conditions to increase the fraction of cells that proliferate maximally. In the present study, we describe that there are at least two mechanisms that lead to irreversible growth arrest of bulk-cultured CD8^+ T lymphocytes.

Activation-induced p16 expression limits the in vitro expansion of human CD8^+ T lymphocytes

The first mechanism is characterized by the appearance of p16 protein in a subset of cells during each cycle of stimulation. Our data indicate that the cells that are induced to synthesize p16 in a given cycle will not proliferate in response to the next mitogen stimulation. Thus, during the early phase of each cycle, the p16-positive cells were diluted out by proliferating p16-negative cells, a fraction of which subsequently expressed p16. During the early cycles, this fraction was small, but it gradually increased and reached a maximum during the fifth round of stimulation when 35% of the proliferating population was p16 positive at the end of the cycle (Fig. 3).

Our observations reveal several important points. First, others have reported p16 accumulation in long-term culture of fibroblasts (6, 17, 18). We demonstrate that in cultured lymphocytes this is not a process that uniformly occurs in all cells, but that de novo p16 appears only in a fraction of T cells that had been actively cycling (Fig. 3). Moreover, activation-induced p16 expression is not related to activation-induced cell death and occurs in a different cell subpopulation (data not shown). Second, this subset of p16-expressing cells is found exclusively within the G0/G1 phase of the cell cycle (Fig. 4). Third, in line with these results, transient knocking down of p16 expression led to the concomitant increase in the proportion of actively cycling cells (Fig. 5). Thus, our data clearly indicate that accumulating p16 is responsible for the exit of a fraction of cells from the proliferating population following each stimulation. These results also suggest that the number of divisions that a cell will undergo after stimulation is determined by the onset of de novo synthesis of p16. The earlier synthesis starts the fewer divisions the cell can make. Such a process would whittle the number of proliferating cells down after each cycle, and make it difficult to achieve eight PD per stimulation. Our observations are in agreement with a recent report showing that T lymphocytes from p16 knockout mice proliferate more than cells from wild-type animals in response to anti-CD3 plus anti-CD28 (24). Recently, Maus et al. (39) demonstrated that artificial APCs expressing ligands for the TCR, CD28, and 4-1BB enhance the expansion of T cells in vitro. Interestingly, the efficiency of this system in amplifying T cell numbers appears to be comparable to that of our system. Although the levels of p16 in the former were not measured, it would not at all be surprising if p16 was also exerting a limit to their system of expansion.

What causes a fraction of mitogen-stimulated cells to express p16 after stimulation? The average length of telomeres in these populations makes it unlikely that p16 expression is triggered by a disruption of telomeric structures. The finding that hTERT-transduced T cell clones with long telomeres also expressed increased amount of p16 following mitogen stimulation (Fig. 6) reinforces this conclusion. There is some evidence that p16 appearance can be induced by stress due to inappropriate culture conditions (9); the resulting growth arrest is referred to as culture-induced senescence or culture shock (40). It is possible that inadequate culture conditions contribute to the appearance of p16 in bulk-cultured T cells. However, it is intriguing that although all cells were grown under the same conditions, only a fraction of them expressed p16. Should culture shock be the cause of p16 expression, it would imply that the culture conditions were inappropriate for some cells while being appropriate for others. Moreover, one would expect a gradual and linear increase of p16 expression following each cycle of stimulation with high levels of p16 at replicative senescence. Instead, we found that from the sixth round of stimulation onward, the amount of p16-positive cells significantly diminished and senescent T cells expressed extremely low to undetectable levels of p16 (Figs. 1 and 3). It may be suggested as another view that the expression of p16 in a fraction of bulk T cells could be the result of an intrinsic mechanism that regulates the number of times that these cells can divide. The notion of a physiological role for p16 in regulating the proliferative potential of human T lymphocytes is consistent with recent observations that will be discussed in the next section.

Does p16 play a physiological role in regulating the proliferative potential of human T lymphocytes?

As in keratinocytes (41) or fibroblasts (16, 17), p16 expression cannot be detected in freshly isolated ex vivo CD8^+ T lymphocytes by Western blot analysis (Fig. 1B). However, by using intracellular staining techniques, we have found low, but readily detectable levels of p16 in the latter cells (Fig. 2B), a result that is consistent with a recent comparative microarray analysis that revealed that p16 mRNA levels were 2 times higher in freshly isolated human T cells than in the same population 3 days after in vitro activation (42). These observations suggest a physiological role for p16 in CD8^+ T lymphocytes, in line with the thymic hyperplasia in mice lacking p16 (24). That p16 indeed regulates the proliferative potential of T cells in vivo is further suggested by the elevated p16 RNA levels in the differentiated subset of peripheral blood T cells (43).

The entry into or exit of CD8^+ T lymphocytes from the cell cycle may be determined not merely by the levels of CDK inhibitors such as p16, but also by the levels of the CDKs themselves. Indeed, Veiga-Fernandes and Rocha (44) recently reported that elevated levels of active CDK6 in mouse CD8 memory T lymphocytes favor rapid cell division. Another important parameter involves the subtype of CD8^+ T lymphocytes, as bulk T cells can be phenotypically and functionally divided into naive, memory, and effector subsets. For instance, it is possible that memory T cells are maintained by high levels of CDK6 in a preactivated stage, allowing them to progress more rapidly through the cell cycle than naive cells (44). Similarly, human memory T cells do also present different proliferation potentials in response to homeostatic cytokines when compared with other subsets (45). In that context, single cell analysis of naive, memory, and effector CD8^+ T lymphocyte subpopulations will reveal whether levels of p16 or

**FIGURE 7.** pRb expression correlates with the proliferative state of T lymphocytes, and senescent cells express high levels of the active form of pRb. Total and hypophosphorylated (active) pRb expression were measured by intracellular staining in CD8^+ T populations at days 4, 7, 10, and 22 after the third cycle of stimulation (day 20), as well as at replicative senescence (day 85) and after additional 10 days (day 95). Both isotype control (open) and total or active pRb (gray) histograms are shown. The normalized mean fluorescence for each histogram and the active/total pRb ratio were calculated, as described in Materials and Methods.
CDK6 expression can be differentially regulated in those subsets and may reflect potential differences in their in vivo and in vitro proliferative capacity.

Replicative senescence of CD8\(^+\) T lymphocytes after extended in vitro culture is independent of p16

In the bulk cultures of CD8\(^+\) T cells used in this study, p16 limited the fraction of cells that responded to the next cycle of stimulation, but the fraction of p16-positive cells never exceeded 35% and diminished after the fifth cycle. Nevertheless, eventually the culture reached a stage at which <0.1% of the cells divided in response to mitogenic stimulation (Fig. 3; day 85, eighth cycle of stimulation). In analogy to a similar phenomenon in fibroblasts (46, 47), we refer to this permanent growth arrest as replicative senescence. Senescence is thought to be a protective mechanism against the development of tumors, and in fibroblasts, is mediated by the activation of DNA damage signaling (p53 and pRb) pathways triggered by critically short telomeres (48, 49). Like senescent fibroblasts (36), senescent T lymphocytes expressed moderate levels of pRb, most of which were in the hypophosphorylated active form (Figs. 1B and 7). But, unlike some strains of senescent fibroblasts or epithelial cells (16, 17, 41, 50), senescent bulk-cultured CD8\(^+\) T cells express almost no p16 protein. Thus, senescence of CD8\(^+\) T cell bulk cultures does not appear to be mediated by p16. It occurs at a stage when the average telomere length has not yet been reduced to the level at which critically short telomeres are expected to induce senescence, and we have previously reported that extension of telomeres by ectopic expression of telomerase does not prevent such cultures from becoming senescent (35). Moreover, it has been recently shown that several components of the DNA damage response machinery, including phosphorylated H2AX, 53bp1, and NBS1, form foci at dysfunctional telomeres in senescent fibroblasts (48). In contrast, we have found that senescent T lymphocytes do not show a significant increase in the frequency of nuclear foci of phosphorylated histone H2AX (our unpublished data), suggesting that a DNA damage response does not take place in these cells. Altogether, senescence of bulk-cultured CD8\(^+\) T cells seems independent of telomere shortening and of p16.

What is unclear, at present, is what controls pRb phosphorylation in senescent T lymphocytes. Western blot analysis revealed only moderate levels of p27 protein in senescent T lymphocytes, while they were particularly high in nonsenescent quiescent cells infected by IL-2 deprivation (Fig. 1B). The role of other CDK inhibitors such as p21\(^{Cip1}\) or p57\(^{Kip2}\) in preventing pRb phosphorylation and hence triggering irreversible growth arrest observed in senescing CD8\(^+\) T cells requires further investigations, when appropriate Abs become available for intracellular staining. An alternative explanation that may be tested is that CD8\(^+\) T cells, upon reaching the end of their proliferative potential, are guided into senescence by reduction of CDK levels (51).

In summary, our data show that the proliferative potential of bulk-cultured CD8\(^+\) T cells that are expanded by periodic stimulation with mitogen and IL-2 is limited by at least two processes: 1) p16-induced growth arrest that affects a fraction of the cells during each stimulation cycle, and 2) a phenomenon that resembles fibroblast senescence, but is independent of p16 and does not appear to be triggered by telomere shortening. At present, we do not know what mediates the latter senescence-like growth arrest. Comparison with other cell culture systems raises the possibility that p16 induction after mitogen stimulation is a response to suboptimal culture conditions, but the finding that a very small fraction of freshly isolated T cells is p16 positive suggests that this molecule also plays a role as an intrinsic regulator of T cell proliferation in vivo. Further work is needed to elucidate completely the signals and pathways that limit T lymphocyte proliferation in vivo and in vitro. A better understanding of the factors that restrict T cell expansion in vitro will contribute to the design of standard culture conditions that make efficient production of these cells as a therapeutic tool against infections or cancer possible.

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