Tumor-Induced Senescent T Cells with Suppressor Function: A Potential Form of Tumor Immune Evasion

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Abstract

Senescent and suppressor T cells are reported to be increased in select patients with cancer and are poor prognostic indicators. Based on the association of these T cells and poor outcomes, we hypothesized that tumors induce senescence in T cells, which negatively effects antitumor immunity. In this report, we show that human T cells from healthy donors incubated with tumor for only 6 h at a low tumor to T-cell ratio undergo a senescence-like phenotype, characterized by the loss of CD27 and CD28 expression and telomere shortening. Tumor-induced senescence of T cells is induced by soluble factors and triggers increases in expression of senescence-associated molecules such as p53, p21, and p16. Importantly, these T cells are not only phenotypically altered, but also functionally altered as they can suppress the proliferation of responder T cells. This suppression requires cell-to-cell contact and is mediated by senescent $CD4^+$ and $CD8^+$ subpopulations, which are distinct from classically described natural T regulatory cells. Our observations support the novel concept that tumor can induce senescent T cells with suppressor function and may effect both the diagnosis and treatment of cancer. [Cancer Res 2008;68(3):870–9]

Introduction

Tumor immune evasion is now increasingly understood to be an important process in carcinogenesis. One of the best-characterized forms of immune evasion is tumor-induced apoptosis (TIA) of immune effector cells, resulting in an inadequate immune response (1, 2). Similar to apoptosis, other forms of cell death, such as senescence, are recognized as important in limiting immune cell survival (3, 4). T-cell senescence was originally a concept of aging. However, others have observed senescence in T lymphocytes during chronic infections and some tumor processes (5–7). Vallejo (8) recently proposed that during aging, immune remodeling occurs, leading to T-cell senescence. Similarly, this remodeling leads to premature T-cell senescence in younger patients with chronic diseases and cancer. Premature senescence is a type of cellular dysfunction that can be triggered by multiple signals such as oxidative stress, DNA damage, oncogene activity, and telomere uncapping (9). Despite the recognition of senescent T cells in cancer patients, the cause and ramifications remains poorly understood.

Tumor-infiltrating lymphocytes (TILs), upon entering the tumor microenvironment, encounter very high tumor to T-cell ratios and result in TILs undergoing TIA (1, 10). Models have been created to reproduce this process *in vitro* by coincubating tumor with T cells at relatively high tumor to T-cell ratios and for prolonged time intervals (1). Similarly, chemotherapies induce apoptosis at relatively high concentrations of drug. In the field of chemotherapy, lower concentrations of these chemicals induce a senescence-like phenotype in which the cells continue to be metabolically active but do not proliferate (11). We hypothesized that tumor coincubated with T cells at a low tumor to T-cell ratio can induce a state of T-lymphocyte senescence similar to that seen in the low-dose chemotherapy model.

Senescent T cells are usually characterized by loss of costimulatory molecules such as CD27 and CD28 (12). Another mechanism considered an important marker of senescence is telomere shortening (13). Moreover, there are many proteins implicated in cellular senescence including the cell cycle regulation molecules p53, p21, and p16 (9). In addition, activation of ATM DNA damage response and heterochromatinization of the nuclear genome are found in senescent cells (14). Despite numerous known molecular markers of senescence, the signal that triggers senescence in T lymphocytes is not well-understood.

Interestingly, $CD8^+$ T cells with senescent phenotypes have significant suppressor function (5, 15). These $CD8^+$ suppressor cells are up-regulated in various cancers studies, but no direct connection to cellular senescence has been made (6). Unlike $CD8^+$ T cells, little is known about the suppressor function of $CD4^+$ T cells with senescent features. Finally, it is not clear whether $CD4^+$ or $CD8^+$, $CD28^-$ senescent T lymphocytes shown to be increased in cancer patients have suppressor function (4).

In this study, we sought to understand the mechanisms by which tumors induce senescent T cells and the downstream effects of this form of immunosuppression on the tumor host interaction. We show a tumor-induced senescence (TIS) process of T cells after a single brief coincubation in conditions of a low tumor to T-cell ratio. Furthermore, we show that both $CD8^+$ and $CD4^+$ senescent T lymphocytes suppress proliferation of normal T cells. We propose that tumors directly induce senescent features in T cells, changing them functionally into suppressor cells, and that this process is a novel mechanism of tumor immune evasion.

Materials and Methods

Purification of T Cells

Human peripheral blood mononuclear cells (PBMC) from healthy donors (ages 25– 40 years) were isolated by centrifugation over Ficoll-Hypaque gradients (GE Healthcare Bio-Science AB). T cells were purified from PBMCs by negative selection using the Pan T-Cell Isolation kit II (Miltenyi Biotech), according to the manufacture's instructions. For some experiments, $CD4^+$

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and $CD8^+$ were purified by positive selection from total T cells using CD4 or CD8 Microbeads (Miltenyi Biotech). The purity of each population was routinely checked and was >95% pure as determined by fluorescence-activated cell sorting (FACS) staining.

Cell Lines

Squamous cell carcinoma of the head and neck 012SCC, breast carcinoma cell line MCF-7, human colon carcinoma cell line HCT-116, and melanoma cell line MEL-624 were cultured in DMEM (Cellgro). Jurkat T leukemic cell line and T cells derived from healthy donors were cultured in RPMI 1640 (Cellgro). All medium was supplemented with 10% fetal bovine serum (FBS; Atlanta biologicals), 1% glutaMax (Life Technologies), 25 mmol/L Hepes (Life Technologies), and 1% penicillin/streptomycin (Life Technologies). Human dermal fibroblasts (HDF; ScienCell Research Laboratories) from healthy donors were cultured in fibroblast medium containing 10% FBS and Fibroblast Growth supplement.

Induction of Senescence by Tumor Cell Lines

To induce senescent T cells, tumor cell lines (012SCC, MCF-7, HCT-116, and MEL-624) or HDF were cultured for 24 h, then $CD3^+$, $CD4^+$, or $CD8^+$ T lymphocytes, or Jurkat T cells were added to the wells containing complete culture medium (referred as control T cells) or tumor cells at a tumor:T-cell ratio of 1:1 (TIS-T group) and incubated for 6 h. As apoptotic controls, the same conditions were repeated except with a tumor:T-cell ratio of 40:1, or T cells were cultured with the apoptotic-inducing chemotherapy, Etoposide (Vp16; 30 μmol/L) for 12 h. Then, the T cells were collected, washed, and cultured for 7 days in complete medium; no additional cytokines were added. Routinely on day 7, cell viability of control T cells (89 ± 7.5) or TIS-T (62 ± 3.2) was checked by Trypan Blue. T cells collected after coincubation with tumor cell lines were analyzed by FACS and found to be 99% $CD3^+$.

Flow Cytometry

Apoptosis in T cells or Jurkat cells was assessed by staining cells with FITC-labeled Annexin V and propidium iodide (BD PharMingen) according to the manufacture's instructions.

TIS-T or control T cells were incubated with allophycocyanin-labeled anti-human CD28, phycoerythrin-labeled anti-human CD27, or isotype control (BD PharMingen) for 30 min at 4°C in the dark.

TIS-T and control T cells were fixed with ethanol and incubated at −20°C for 2 h. The cells were incubated with FITC-labeled anti-human p16 monoclonal antibodies (mAb) or isotype control (BD PharMingen) for 30 min at room temperature in the dark.

The length of telomere repeats at chromosome ends in TIS-T and control T cells were measured using Telomere PNA kit/FITC for Flow Cytometry (DakoCytomation) following the manufacture's instructions. FITC-labeled fluorescent calibration beads (Quantum TM-24 Premixed; Bangs Laboratories) were used to convert telomere fluorescence data to molecules of equivalent soluble fluorescence (MESF) units. The following equation was performed to estimate the telomere length in bp from telomere fluorescence in MESF units (bp = MESF × 0.495; ref. 16).

All samples were analyzed on a BD LRSII with FACSDiva Software (BD Bioscience).

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, as described previously (17). The membranes were incubated with antibodies against either p53 (Santa Cruz), p21 (Abcamb), p16 (Santa Cruz), or β-actin (Cell Signaling) and then with horseradish peroxidase–conjugated secondary antibody. The protein bands were detected by enhanced chemiluminescence (Pierce).

Evaluation of DNA Damage

To analyze DNA damage foci, the expression of known senescence-associated markers, ataxia telangiectasia–mutated (ATM) kinase ATM (Ser^{1981}), phosphorylated histone H2AX (γ-H2AX), and a marker for histones, heterochromatin protein 1 (HP1)-β, were assessed. TIS-T or control T cells were fixed for 20 min in 4% paraformaldehyde. After permeabilization, the cells were blocked with 3% bovine serum albumin. The samples were first incubated with anti-ATM (Ser^{1981}; Abcam) or anti–HP1-β (Abcam) overnight. After a fixation with 4% paraformaldehyde and inactivation with 25 mmol/L glycine PBS, the samples were incubated with anti–γ-H2AX (S^{139}; Upstate) for 1 h. Samples were incubated with Alexa 488 Fluor goat anti-mouse and Alexa 647 Fluor goat anti-rabbit secondary antibodies. The cells were centrifuged onto microscopic glass slides and examined using fluorescent microscopy.

In vitro Proliferation, Immunosuppression Assay, and Mixed Lymphocyte Reaction

Proliferation analysis. To analyze the proliferative capacity of TIS-T cells in response to polyclonal activation, plates were coated overnight at 4°C with 1 μg/mL of anti-CD3 mAb (BD PharMingen). Then, TIS-T or control T cells (2×10^5 cell/well) were cultured for 3 days. The cells were then pulsed for 18 h with 1 μCi (0.037 MBq)/well [^{3}H]-thymidine, and [^{3}H]-thymidine uptake was evaluated.

Immunosuppression assay. Plates were coated overnight at 4°C with 1 μg/mL of anti-CD3 mAb. Then, fresh autologous T cells (referred to as responders) were plated with TIS-$CD3^+$, $CD4^+$, or $CD8^+$ cells or control T cells at various ratios for 3 days. Then, wells were either pulsed for 18 h with 1 μCi/well, and the proliferative response was evaluated as describe above, or the supernatants were collected for cytokine analysis. The levels of INF-γ (Diaclone), interleukin (IL)-6 (Diaclone), IL-10 (Diaclone), and transforming growth factor (TGF)-β (R&D system) were measured by ELISA following the manufacturer's instruction.

To assess whether TIS-T cells exert their regulatory function through direct cell contact or through release of soluble factors, we used Transwell inserts. TIS-T or control T cells were added in the upper chamber at a ratio of 1:3 to autologous anti-CD3–stimulated responder cells seeded in the lower chamber. After 3 days, the proliferative response was evaluated as described.

Mix lymphocyte reaction. Autologous T cells (1.5×10^5/well) were seeded with irradiated (3,000 rad) allogeneic PBMC (1.5×10^5/well) and irradiated TIS or control T cells (0.5×10^5/well) for 4 days. Then, the proliferative response was evaluated as described.

Statistical Analysis

The statistical significance was analyzed using unpaired Student's t test. Values were considered statistically significant when P value is <0.05. All experiments were repeated at least four times and error bars represent the SD.

Results

Tumor-induced senescence — a distinct phenomenon from TIA. To test our hypothesis that a low tumor to T-cell ratio could induce senescence, we coincubated the 012SCC cell line with a T-cell line Jurkat at both low and high tumor to T-cell ratios. We observed that Jurkat cells pretreated at a tumor:T-cell ratio of 40:1 for 6 h were apoptotic after 2 days of culture as indicated by increased staining with Annexin V and propidum iodide (Fig. 1*A*). However, Jurkat cells pretreated at tumor:T-cell ratios of 5:1 to 0.5:1 also for 6 h did not show the augmentation of Annexin V/propidum iodide staining after 2 days of culture, indicating the absence of apoptosis (Fig. 1*A*). To evaluate the senescence phenotype, we used loss of CD28 as an indicator of senescence (18). On day 2, the loss of expression of CD28 was similar in Jurkat cells preexposed to high and low tumor to T-cell ratios (Fig. 1*B*). As expected, Jurkat cells pretreated with a high number of 012SCC did not survive beyond 2 days of culture. However, Jurkat cells pretreated at a low tumor to T-cell ratio survived and showed a significant loss of CD28 expression by day 7, suggesting that these cells became senescent (Fig. 1*D*). Consistent with models of low dose chemotherapy inducing a senescent-like process, these TIS-T cells showed increased staining with Annexin V (Fig. 1*C*; ref. 19). In addition, this is in line with studies showing normally associated

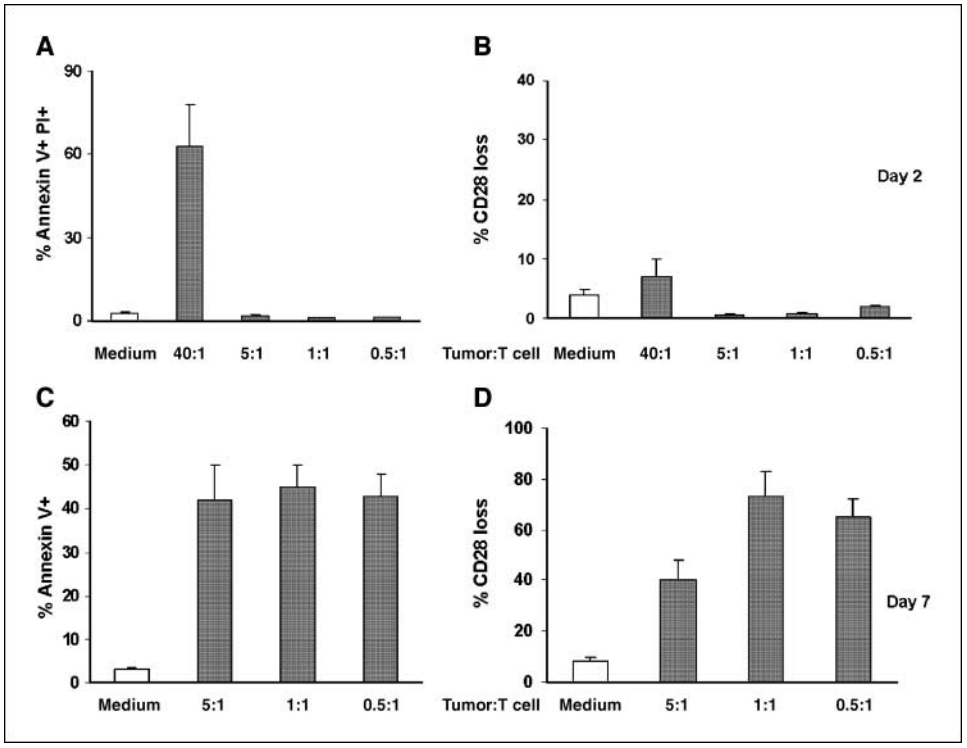

Figure 1. 012SCC can induce apoptosis or senescence depending on the tumor to T-cell ratio of coincubation. The T-cell line Jurkat was coincubated with 012SCC cell line in tumor:T-cell ratios of 40:1, 5:1, 1:1, or 0.5:1 for 6 h. Jurkat T cells were washed and cultured in complete medium for 7 d. *A* to *B,* after 2 d, using flow cytometry, we studied the apoptotic markers Annexin V and propidum iodide (*PI*), as well a senescent marker (*% CD28 loss*) in each group of T cells. *C* to *D,* on day 7, Annexin V and CD28 loss were evaluated. *Columns,* mean; *bars,* SE.

apoptotic molecules functioning in nonapoptotic processes (20). Taken together these results show that short term exposure of Jurkat cells to lower tumor to T-cell ratios induce a senescence-like phenotype.

TIS is induced in T cells from healthy donors. To evaluate if the TIS process could occur in normal T cells, we purified $CD3^+$ cells from healthy donors and placed them with complete medium alone, O12SCC tumor cell line at a low tumor to T-cell ratio (1:1), or a high ratio (40:1) for 6 h. Parallel studies were performed with VP16, an apoptotic inducing reagent. After 2 days, only 10% of T lymphocytes cultured with medium alone were Annexin V+/propidum iodide+. As expected, we observed increased percentage of Annexin V+/propidum iodide+ T cells preincubated with a high tumor to T-cell ratio or with etoposide (46% and 43%, respectively). In contrast, only 15% of T cells preincubated with O12SCC tumors at a low tumor:T-cell ratio (1:1) were Annexin V+/propidum iodide+ (Fig. 2*A*).

Because normal T cells express CD27 and CD28, we used the loss of both of these cell surface markers as an indicator of senescence. After 2 days of culture, no group of T cells exhibited a senescent phenotype (Fig. 2*A*). After 7 days, T cells that had been exposed to apoptotic conditions were no longer in culture. However, as shown in Fig. 2*B*, T lymphocytes preincubated at a low tumor to T-cell ratio exhibited a significant decrease (55% $\pm$ 7%) of CD27/CD28 expression compared with control T cells (10% $\pm$ 4%). In addition, by day 7, T lymphocytes preincubated with a low tumor to T cell low ratio exhibited an increment in the expression of Annexin V compared with control T cells.

Interestingly, we observed that anti-CD3–stimulated T cells were able to undergo TIS at a higher extent than unstimulated T cells (data not shown).

We have observed that TIS-T cells on day 7 exhibit not only an increment in Annexin V but also the activation of caspase 3 and 7. The addition of a pan-caspase inhibitor (QVD-OPH) did reduce the expression of these caspases in TIS-T cells but did not block the senescence induced by tumor cells, but amplified it instead (data not shown).

One of the major indicators of senescence is telomere shortening. Using a flow-fluorescence *in situ* hybridization (FISH) cytometric assay, we determined telomere length in T lymphocytes incubated with medium alone (control T cells) or pretreated for 6 h with either VP16 or O12SCC tumor cells at a 1:1 tumor:T cell ratio. As shown in Fig. 2*C*, after 2 days of culture, all groups exhibited similar telomere lengths. Importantly, at day 7, control T cells grown in medium alone had no significant change in telomere length; however, T cells pretreated at a low tumor to T-cell ratio had a statistically significant decrease in telomere length compared with the control group ($P < 0.03$).

Lack of proliferative capacity is another hallmark of senescence. Freshly isolated T cells, control T cells (cultured with medium), or T cells pretreated with a low ratio of O12SCC tumor were stimulated by immobilized anti-CD3 antibody, and proliferation was measured by ^{3}H-thymidine uptake. TIS-T cells showed a significant reduction in proliferation compared with fresh or control T cells (Fig. 2*D*). Taken together, these results indicate that pretreatment of T lymphocytes with a low tumor to T-cell ratio for 6 h induces a senescence-like phenotype.

T-cells senescence is induced by tumor cells but not by normal cells and requires soluble factor(s). To assess whether the TIS process was isolated to the 012SCC cell line or a more generalized phenomenon, we examined other tumor cell lines. T lymphocytes were pretreated with the following tumor cell lines: MEL-624, MCF-7, and HCT-116, as described. T lymphocytes pretreated with these tumor cell lines exhibited similar or even greater losses of CD27/CD28 (50% $\pm$ 4% for Mel-624, 60% $\pm$ 3% for MCF-7, and 61% $\pm$ 3% for HCT-116). In contrast, HDF, under the

same conditions, did not induce down-regulation of CD27/CD28 expression (12% ± 3%; Fig. 3*A*), indicating that senescence is induced only by tumor cells.

To evaluate whether the induction of a senescence-like phenotype requires cell-to-cell interaction, we repeated our TIS model but separated the tumor cells from T lymphocytes with a Transwell insert. We observed no change in the level of the down-regulation of CD27/CD28 expression, indicating that TIS of T cells is mediated by soluble factors (Fig. 3*B*).

Cell cycle–regulating and DNA damage–responding molecules are involved in TIS-T cells. Various pathways leading to senescence have been described, suggesting a direct connection to cell cycle machinery. For this reason, we analyzed the expression of the cell cycle–controlling molecules, p53, p21, and p16, in TIS-T cells. Using Western blot analysis, we detected distinct increases in p53, p21, and p16 on day 7 in T cells pretreated with a 1:1 tumor:T-cell ratio versus control or fresh T cells. Although p53 was most strikingly increased, there were clearly differences in the expression of p21 in the TIS group (Fig. 4*A*). Because others have shown p16 changes are best seen by flow cytometry, and our results by Western blot were modest, we next studied the expression of p16 by FACS on TIS-T cells and control T cells and found that the former group had a significant increase in the expression of this molecule (Fig. 4*B*; ref. 21).

There have been a number of recently discovered DNA damage changes that cause heterochromatinization that are indicative of senescence (14). Figure 4*C* shows that TIS-T cells consistent with reported senescence DNA damage changes displayed activated ATM (Ser^{1981}) and higher expression of γ-H2AX with colocalization compared with control T cells. In models of DNA damage in

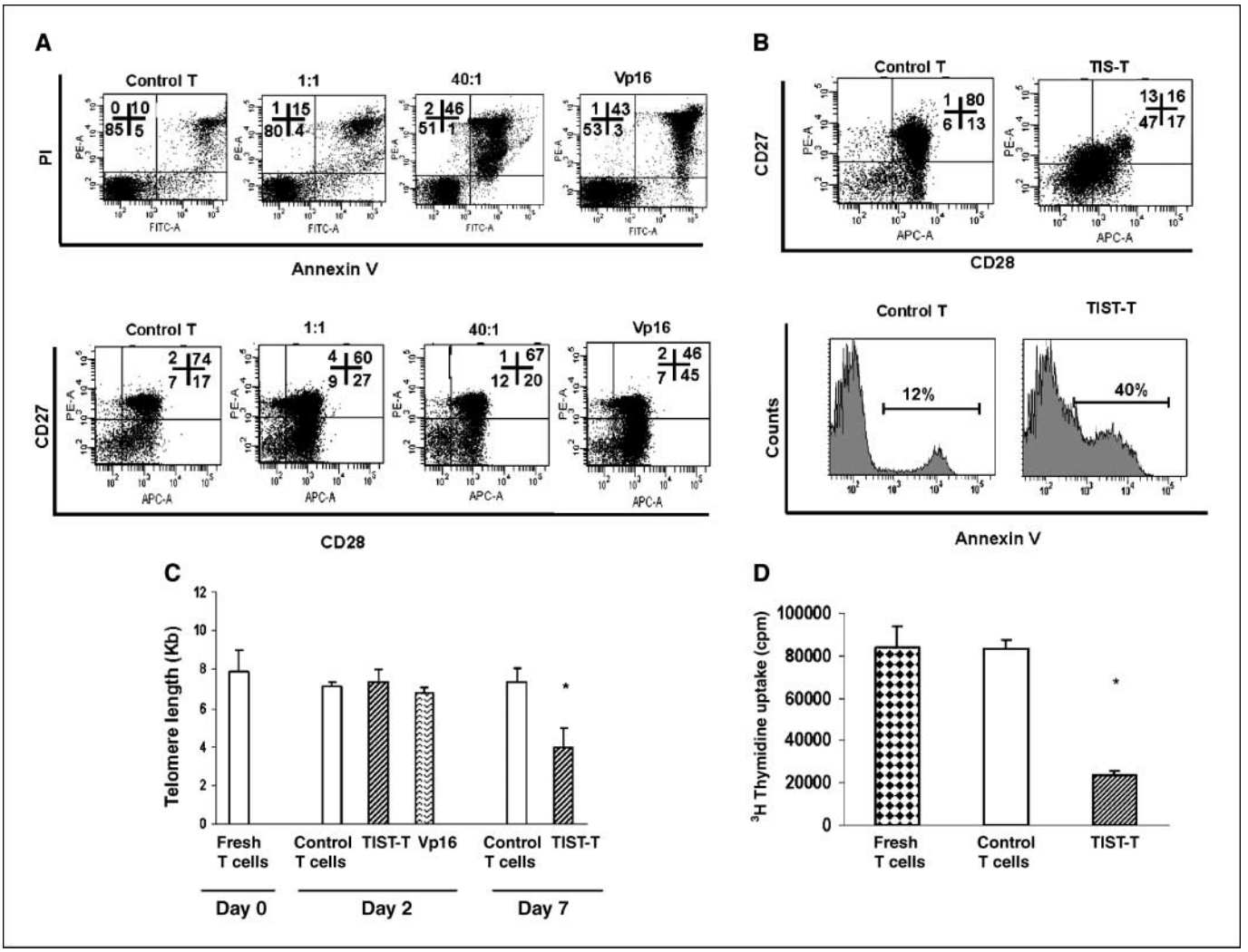

Figure 2. Induction of TIS-T cells. Peripheral T cells from healthy donors were coincubated with the tumor cell line 012SCC in tumor:T-cell ratios of 1:1 or 40:1 for 6 h, or with VP16 for 12 h. T cells were washed and cultured in complete medium for 2 or 7 d. *A,* after 2 d, by flow cytometry, we studied the apoptotic markers Annexin V and propidium iodide, as well as senescent markers (loss of CD28 and CD27 represented as % $CD28^-$ $CD27^-$ T cells in $CD3^+$-gated cells) in each group and in T cells cultured with medium alone (control T). *B,* on day 7, we evaluated loss of CD28 and CD27 and the expression of Annexin V in TIS-T cells incubated with tumor in a 1:1 ratio and in T cells cultured with medium alone (control T). *Inserts,* percentage of cells within respective quadrants. *C,* telomere length of freshly isolated T cells or T cells cultured in medium (control T) or T cells previously incubated with tumor at a 1:1 ratio (TIS-T) or with VP16. Telomere length is represented as mean of telomere length + SD. *D,* after 7 d, T cells cultured in medium (control T) or previously incubated with tumor at a 1:1 ratio (TIS-T) were stimulated with immobilized anti-CD3 antibody (1 μg/mL) for 3 d and pulsed with ^{3}[H] dTh. Fresh T cells were processed in parallel as control. T-cell proliferation was evaluated by ^{3}H Thymidine incorporation and represented as mean counts per minute (cpm) + SD. *, $P = 0.027$ versus control T cells (*C*); *, $P < 0.0002$ versus control T cells (*D*). Results are representative of four independent experiments from different donors.

stimulated lymphocytes, phosphorylated histones have been shown to be diffusely expressed as opposed to controls that are usually seen in focal distribution (14, 22). The expression of phosphorylated histones in TIS-T cells, similarly, were seen diffusely as opposed to the punctate expression in the control T cells. Consistently, we observed that only TIS-T cells display diffuse staining for another distinct marker of heterochromatin, HP1β, another DNA damage indicator (Fig. 4*D*). These results indicate that TIS induces DNA damage that may activate heterochromatization, a hallmark of cellular senescence.

TIS-T cells are able to suppress antigen nonspecific and allogeneic-induced proliferation of T cells. Both senescent and some suppressor T cells have similar phenotypes, suggesting that they may be the same population. We thus evaluated the ability of TIS-T to suppress T-cell proliferation. Figure 5*A* depicts that control T cells did not significantly alter the anti-CD3–induced proliferation of responder T cells (freshly purified autologous CD3+ cells) at any control T cell to responder ratio tested. In contrast, TIS-T cells even at a 1:30 TIS-T cell:responder ratio were able to induce a profound reduction of anti-CD3–induced proliferation of responder cells. We further confirmed this phenomenon by a mixed lymphocyte reaction (MLR). Freshly isolated responder T cells were incubated with irradiated allogeneic PBMCs in the presence of irradiated TIS-T or control T cells, and proliferation was measured by ^{3}H-Thymydine uptake. As shown in Fig. 5*B*, contrary to control cells, TIS-T cells strongly suppressed the proliferation of responder cells induced by MLR.

The expression of particular cytokines has been associated with specific T suppressor cells (23). For instance, although $CD4^{+}CD25^{high}Foxp3^{+}$ T regulatory cells (T-regs) secrete IL-10 and TGF-β, $CD8^{+}CD28^{-}$ suppressor cells secrete INF-γ and IL-6 (24, 25). Our data (Fig. 5*C*) indicate that the levels of INF-γ and IL-6 production was significantly increased only in the supernatant of TIS-T cells in coculture with anti-CD3–stimulated responder cells. In contrast, IL-10 and TGF-β production was similar in all groups.

TIS occurs comparably in $CD4^{+}$ and $CD8^{+}$ T cells and causes the loss of natural T-regs. Because senescence changes have been shown to preferentially occur in $CD8^{+}$ versus $CD4^{+}$ T lymphocytes (4), we analyzed TIS susceptibility in these subpopulations. $CD4^{+}$ or $CD8^{+}$ T lymphocytes isolated from PBMC of healthy donors were preincubated with O12SCC at a low tumor to T-cell ratio and expression of senescence markers was analyzed by FACS. Figure 6*A* shows that under this condition, both $CD4^{+}$ and $CD8^{+}$ T cells lost CD27 and CD28 expression, indicating that both populations were susceptible to TIS. We also showed (Fig. 6*B*) that telomere shortening occurred similarly between the $CD4^{+}$ and $CD8^{+}$ groups, confirming their senescence status. In addition, we observed no significant changes in the CD4:CD8 ratio in TIS-T cells compared with control T cells after 7 days of culture (data not shown).

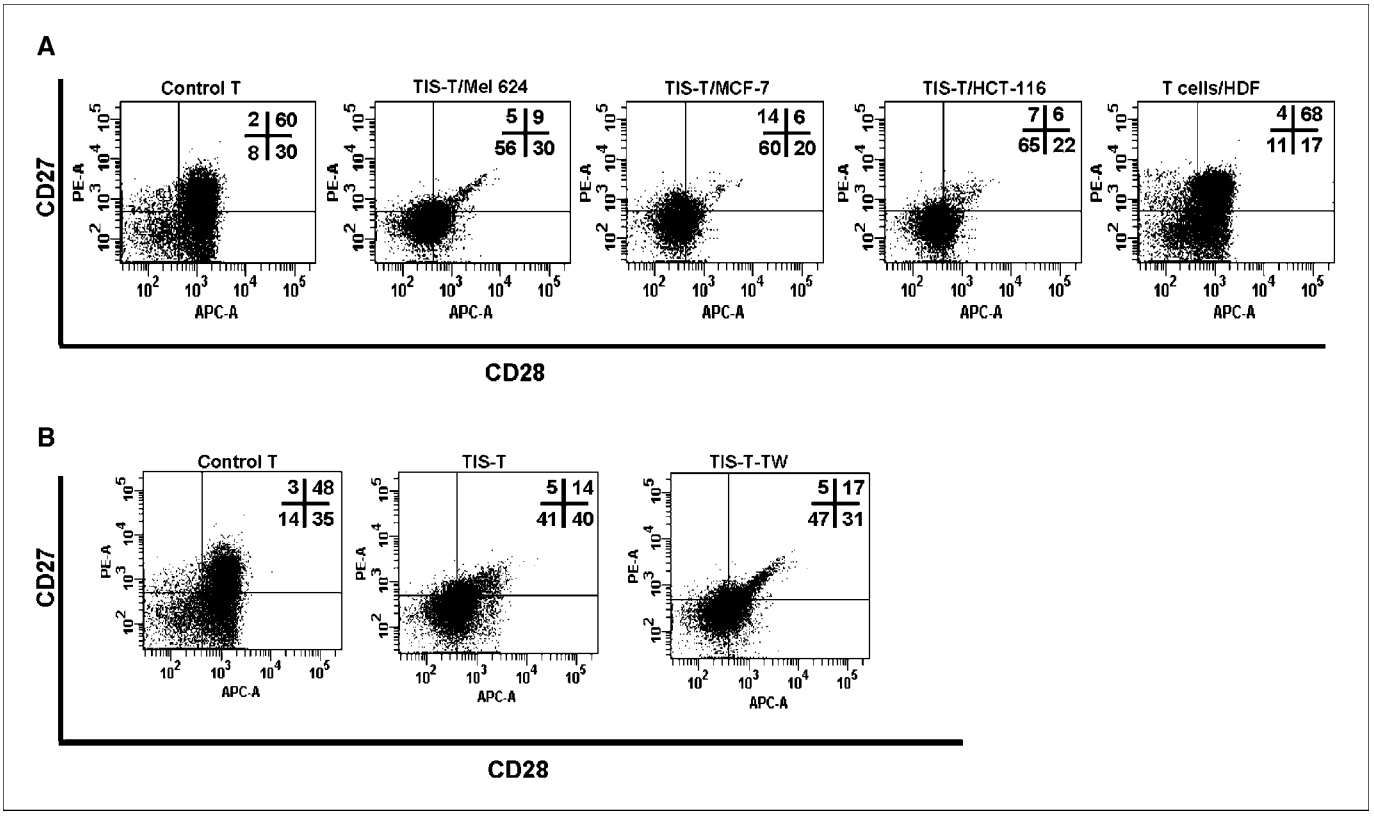

Figure 3. TIS of T cells is induced by several tumor cell lines and does not require cell-to-cell contact. *A,* peripheral T cells from healthy donors were coincubated with the tumor cell lines MEL-624, MCF-7, and HCT-116 or HDF from healthy donor in a 1:1 tumor:T-cell ratio for 6 h. T cells were washed and cultured in complete medium for 7 d. After 7 d, using flow cytometry, we evaluated loss of CD28 and CD27 in each group of T cells. *B,* T cells were coincubated in direct contact with 012SCC tumor cell line (TIS-T) or separated by a Transwell semipermeable membrane (*TIS-T-TW*) in a 1:1 tumor:T-cell ratio for 6 h. The cells were washed and cultured in complete medium. After 7 d, loss of CD28 and CD27 was evaluated by flow cytometry in these two groups and in T cells cultured with medium (control T). *Inserts,* percentage of cells within respective quadrants. Results are representative of four independent experiments from different donors. *APC-A,* allophycocyanin-area.

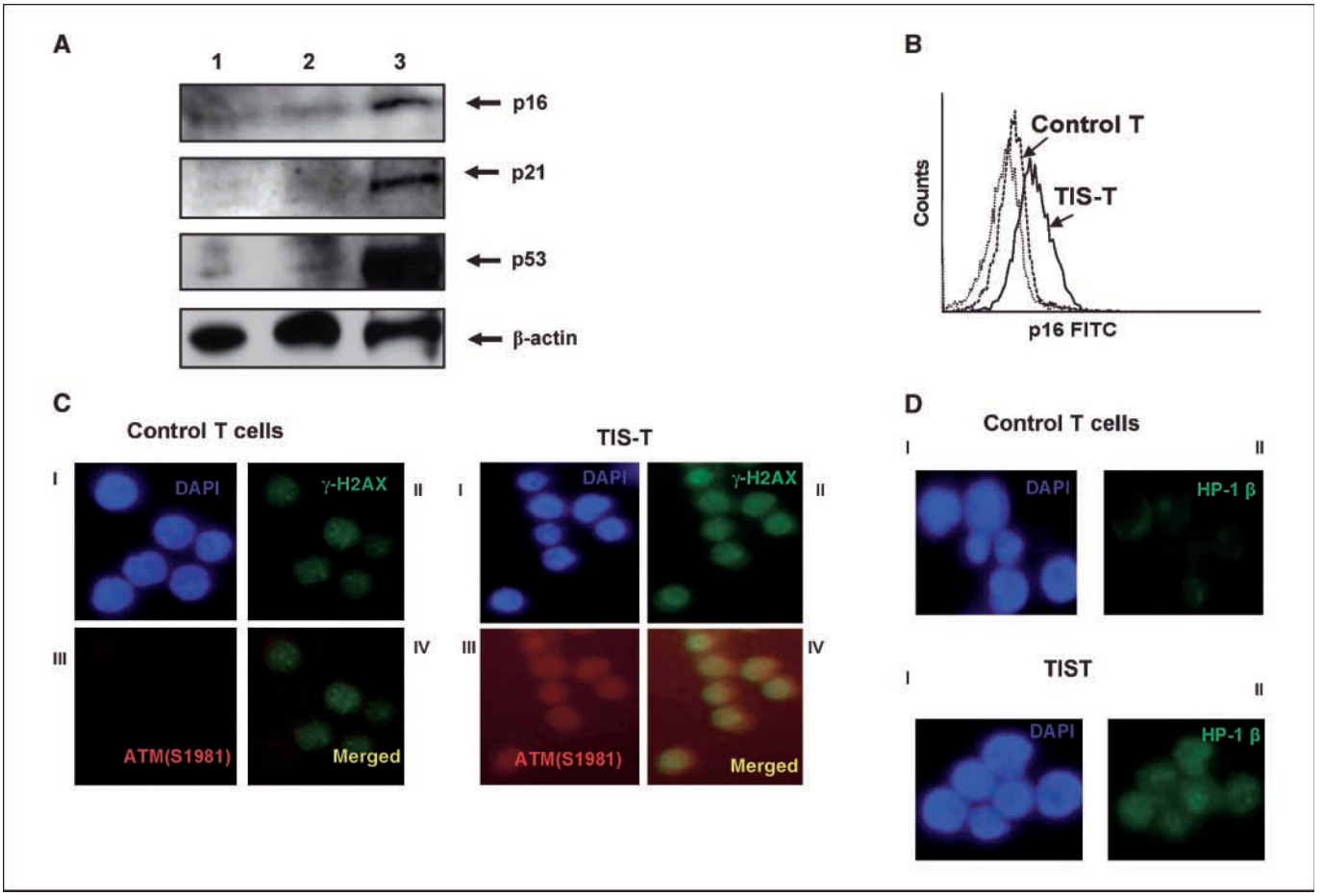

Figure 4. Cell cycle–regulating and DNA damage–responding molecules are involved TIS of T cells. *A,* Western analysis of TIS-T cells. T cells from healthy donors were either used fresh (*1*), cultured in complete medium for 7 d (*2*), or cocultured with the O12SCC tumor line at a 1:1 ratio for 6 h and then cultured in complete medium for 7 d (*3*). T cells from each group were lysed, and proteins were separated by SDS-PAGE. Gels were blotted to nitrocellulose membranes and then first probed with anti-p53 before stripping and reblotting the membrane three more times with antibodies specific for p21, p16, and β-actin (protein-loading control). *B,* FACS analysis of intracellular expression of p16 in TIS-T cells (*solid black line*), in control T cells (*dash line*), or isotype control (*dotted line*). *C* to *D,* expression of DNA damage–responding molecules. T cells from healthy donors were cultured in complete medium for 7 d (control T) or cocultured with the O12SCC tumor line at a 1:1 ratio for 6 h and then cultured in complete medium (TIS-T). On day 7, DNA damage–responding molecules were evaluated by immunofluorescence. *C,* staining with 4′,6-diamidino-2-phenylindole (DAPI) to label DNA (*I*); *II,* expression of γ H2AX; *III,* expression of ATM (S1981); *IV,* merging of γ H2AX and ATM (S1981). *D,* cells stained with DAPI (*I*); *II,* expression of HP1-β. Magnification, ×40 (*C*) and ×60 (*D*). Results are representative of four independent experiments from different donors.

We next tested whether immunosuppression by TIS-T lymphocytes is mediated by $CD4^+$ or $CD8^+$ cells. Figure 6*C* shows that both TIS-$CD4^+$ and -$CD8^+$ cells produced an equal and significant abrogation of proliferation of responder T cells. Taken together, our data indicate that in TIS of T cells, there is a parity of changes within both $CD4^+$ and $CD8^+$ populations.

We next decided to assess whether TIS-$CD8^+$ and -$CD4^+$ T suppressor cells exert their regulatory function through direct contact or release of soluble factors. For that, we separated the TIS-T cells and responder T cells with Transwell insert. We observed (Fig. 6*C*) that the suppression induced by TIS-T cells, TIS-CD4, or TIS-CD8 cells was abrogated when they were separated by a semipermeable membrane from the anti-CD3–stimulated T responders. Consistently neutralizing anti–IL-6 or anti–INF-γ mAbs had little effect on the suppression of T cells cultured in direct contact with TIS-T cells (data not shown). These results indicate that both TIS-CD4 and TIS-CD8 T cells inhibit the proliferation of responder cells through direct cell-to-cell contact. Although TIS-T cell suppressor function was associated with expression of specific cytokines (Fig. 5*C*), they were not necessary.

To exclude the possibility that the suppression observed was mediated by natural T-regs, we studied FACS phenotypic markers expressed by T-regs ($CD4^+CD25^{high}Foxp3^+$) in TIS-T cells. We found that 2.8% of T lymphocytes in control T cells were $CD4^+CD25^{high}$ and expressed Foxp3. In contrast, TIS-T cells lost all $CD4^+CD25^{high}$ cells (Fig. 6*D*). Although our data provide direct evidence that TIS-T cells can suppress proliferation of responder T cells, this process causes the loss of natural T-regs.

Discussion

Recent successes of immunotherapy has made the immune system a realistic target for cancer treatment, but its limitations has highlighted the dysfunction of immune effector cells in these patients (26). Despite the potential significance of T-cell senescence as a form of cancer-induced immunosuppression, its pathophysiology is poorly understood (4). Based on models of tumor immune evasion through apoptosis of T cells, we designed an *in vitro* tumor microenvironment to test whether tumors could directly induce senescence in T cells. We showed that lowering the

tumor to T-cell ratio could switch the T-cell response from apoptosis to senescence. We also showed that a variety of cancers, but not fibroblasts, can induce these changes in T cells. Furthermore, tumors can induce senescence in T cells without cell-to-cell contact, implying that soluble factor(s) may be responsible. We are currently investigating whether these soluble factors may be the same that induce T-cell apoptosis including both proteins, such as FasL and other molecules such as

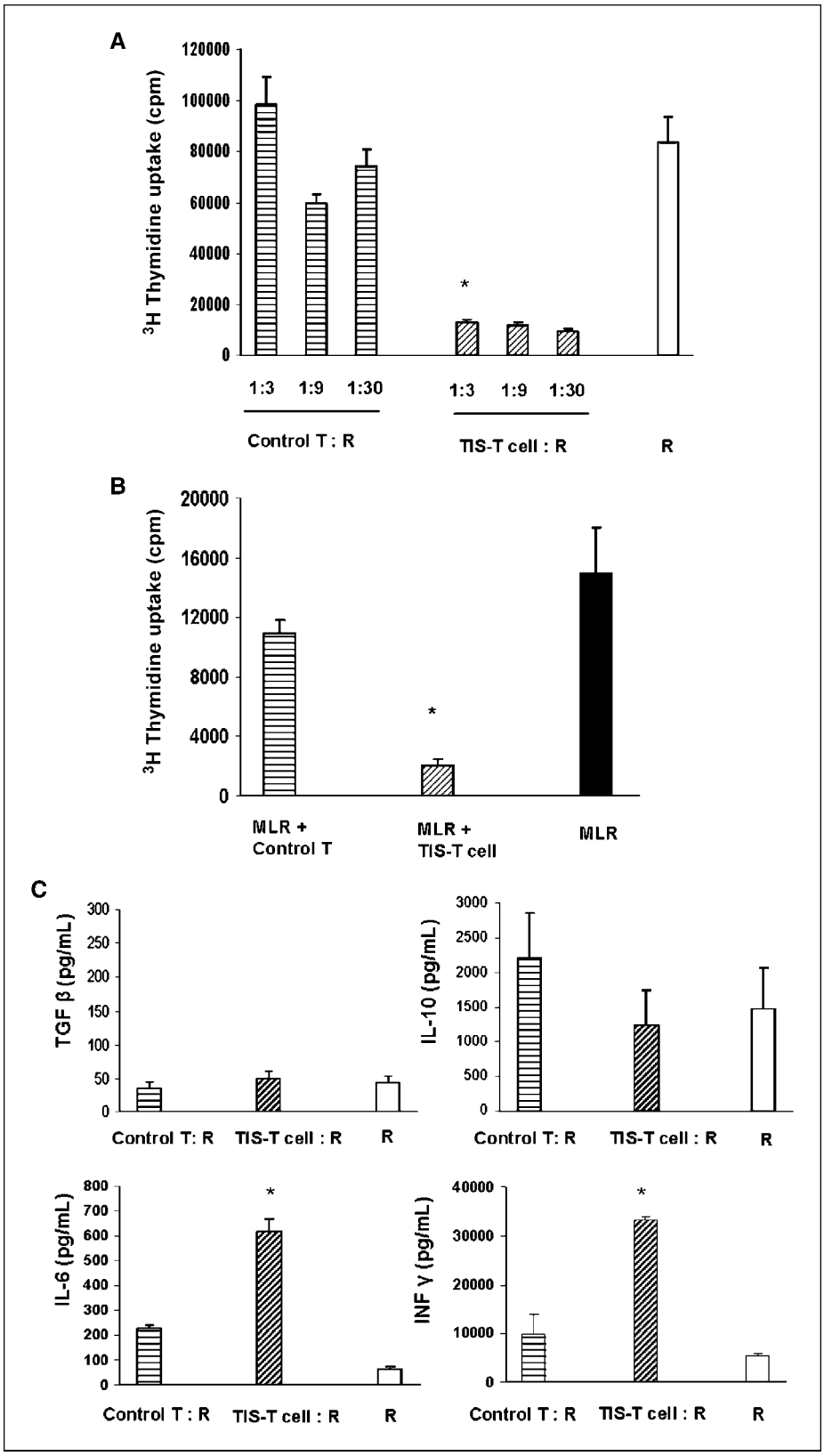

Figure 5. TIS-T cells suppress T-cell proliferation *in vitro*. *A,* T cells from healthy donors were incubated with the tumor cell line 012SCC in a 1:1 tumor:T-cell ratio for 6 h and then washed and cultured in complete medium. After 7 d, TIS-T cells or T cells cultured in medium for 7 d were added to fresh T cells (responders) stimulated with immobilized anti-CD3 antibody (1 μg/mL) at the ratios indicated in the figure. Responders T cells stimulated with immobilized anti-CD3 antibody were also used alone as control. After 3 d of culture, the cell proliferation was evaluated by ^{3}H Thymidine incorporation and represented as mean of cpm + SD, or the culture supernatants were collected for cytokine detection. *B,* TIS-T cells obtained as described above or T cells cultured alone in medium for 7 d were irradiated and then incubated with fresh responder T cells in the presence of irradiated, allogenic peripheral mononuclear cells (*MLR*). Responder T cells were incubated with irradiated allogenic T cells only as a control. After 4 d, responder T-cell proliferation was evaluated by ^{3}H Thymidine incorporation and represented as mean of cpm + SD. *C,* TIS-T cells are associated with the induction of cytokine production. After 3 d of culture in the condition mentioned above, TGF-β, IL-10, IL-6, and INF-γ production was evaluated in the culture supernatant by ELISA. *, $P < 0.002$ versus control T:responder (1:3; *control T:R*; *A*); *, $P < 0.0001$ versus MLR control T (*B*); *, $P < 0.016$ versus control T cell:responder (*C*); **, $P < 0.034$ versus control T cell:responder (*C*). Results are representative of four independent experiments from different donors. *R,* responder.

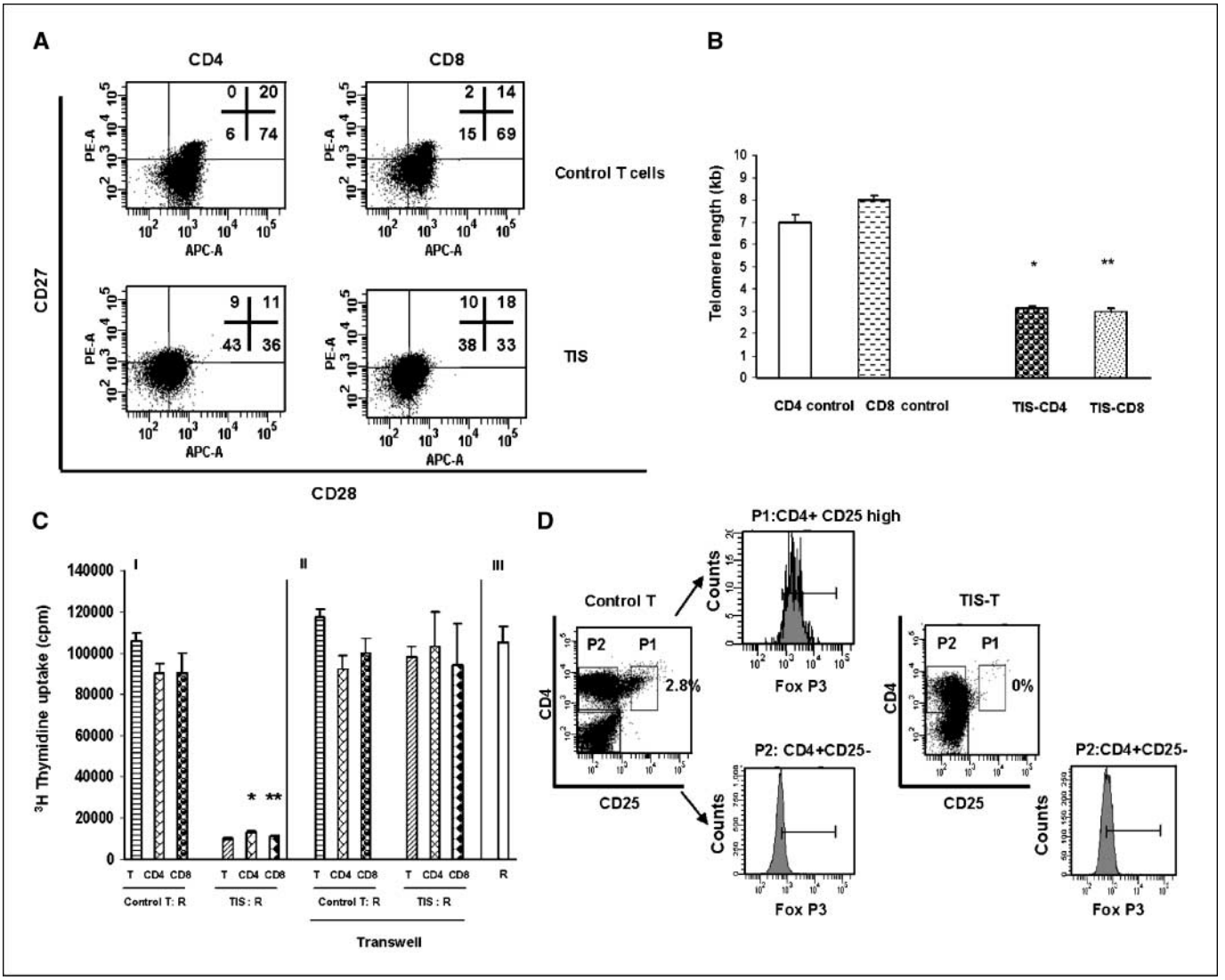

Figure 6. Effects of the TIS process on CD4$^+$ and CD8$^+$ T cells and T-regs. *A* to *B,* purified CD4$^+$ or CD8$^+$ T cells from healthy donors were incubated with the tumor cell line 012SCC in a 1:1 tumor:T-cell ratio for 6 h and then washed and cultured in complete medium. CD4$^+$ or CD8$^+$ T cells cultured in medium were used as control. *A,* on day 7, loss of CD28 and CD27 expression was evaluated by flow cytometry in CD4$^+$ or CD8$^+$ T cells placed in medium or previously incubated with tumor (TIS). *Inserts,* percentage of cells within respective quadrants. *B,* on day 7, telomere length of CD4$^+$ or CD8$^+$ T cells cultured in medium or previously incubated with tumor was measured by flow-FISH assay and represented as mean of telomere length + SD. *C,* control T, CD4$^+$ or CD8$^+$ T cells, or TIS-T, TIS-CD4, and TIS-CD8 were cultured in direct contact with immobilized anti-CD3–stimulated T cells (responder; *I*) or separated from responder T cells with a semipermeable membrane (*II*) and as control responder T cells alone (*III*). After 4 d, T-cell proliferation was evaluated by ^{3}H Thymidine incorporation and represented as cpm + SD. *D,* TIS-T cells (*right*) or T cells cultured alone in medium for 7 d (*left*) were evaluated by flow cytometry for the expression of Foxp3 in the CD4$^+$CD25^{high} and CD4$^+$ CD25$^-$ subpopulations. *, $P < 0.008$ versus CD4 control (*B*); **, $P < 0.0036$ versus CD8 control (*B*); *, $P < 0.003$ versus control CD4:responder (*C*); **, $P < 0.0012$ versus control CD8:responder (*C*). Results are representative of four independent experiments from different donors.

gangliosides (1, 27). Strikingly, tumors induce senescence in T cells after a brief interaction, which is in contrast to other models of T-cell senescence where multiple repetitive signals are required (28).

One of the markers to characterize T-cell senescence is the loss of the cell surface expression of CD27 and CD28. Many studies have shown these T-cell senescent features in cancer patients both in the tumor microenvironment and in the periphery (6, 29). The number of CD28$^-$ T cells, at least in the periphery, is an important negative prognostic indicator in many cancer types (7, 30). Furthermore, the loss of CD28 expression is associated with immune dysfunction observed in cancer patients (31).

The loss of CD27 and CD28 in T cells has also been correlated with their function as cytotoxic immune effectors (32). Interestingly, unlike in the peripheral blood where CD27 and CD28 loss is a poor prognostic indicator in the tumor microenvironment, the opposite has been shown (33, 34). A potential explanation is that patients with a better prognosis have higher rates of TIL-TIS, whereas those with a poorer prognosis have a more aggressive cancer leading to TIA. In a corroborating paper, higher rates of TIL apoptosis correlated with a poorer prognosis in patients with colon cancer (35). Another group recently showed that CD28$^-$ T cells with suppressor function can represent >50% of all of the TILs in many different cancer types and correlate with a poorer prognosis (36). The difference in these articles most likely represents emphasis and the heterogeneity that can be found in TILs some with more detrimental qualities and others with appropriate antitumor capabilities.

Our data indicate a delayed gain of Annexin V staining in TIS conditions. The timing of Annexin V positivity correlates with other models of senescent-like phenotypes (19). Increases in Annexin V have been documented in $CD27^-$ or $CD28^-$ peripheral blood T cells in patients with cancer and in the aging population (37, 38). One group found that after successful resection of head and neck tumors, the number of $CD3^+CD28^-$ cells decreased (7). Their explanation was that extirpation of the tumor removed the TIA process. Alternatively, removal of the tumor could have caused an abrogation of the TIS process. Our current model of TIS-T cells may explain how patients with cancer can have increases in both apoptotic and senescent T-cell phenotypes in the same peripheral blood lymphocytes (39).

We confirmed the TIS process through other assays, such as lack of proliferation, expression of senescence-associated molecules, and telomere shortening. Telomere shortening occurs in older individuals and in diseases that cause cellular senescence, and is a diagnostic measure for this process (40). A recent publication using TILs from melanoma patients for adoptive T-cell therapy showed a correlation between longer telomeres and response to therapy (41). Interestingly, the same group showed that TILs with lower CD27 and CD28 levels were less efficacious when used for immunotherapy (42).

Like senescent T cell, T suppressor cells are increased in patients with cancer and in aging populations (4, 43). It has been proposed that senescent T cells may have suppressor activity (4). A subset of T suppressor cells that are $CD8^+CD28^-$ has been described in both the transplant and cancer literature (6, 44). Our results show a strong ability of TIS-T cells to suppress proliferation of normal T cells stimulated through a variety of inducers. Thus, the importance of tumors inducing senescent T cells after a single brief interaction is amplified by their the ability to, in addition, cause the generation of T suppressor cells (18).

To characterize these TIS-T suppressor cells, we evaluated potential immunosuppressive cytokines secreted during immunosuppression of normal T cells. We observed increases in INF-γ and IL-6 but not other cytokines such as TGF-β or IL-10. These former two cytokines have already been shown to be associated with $CD8^+CD28^-$ suppressor cells (25). Although we found an association between the expression of these cytokines and TIS-T suppressor cells, these cytokines were not necessary but do have diagnostic potential.

The TIS process is generated similarly in both $CD4^+$ and $CD8^+$ T-cell populations. Interestingly, in our TIS model, there was an almost complete loss of natural T-regs ($CD4^+CD25^{high}Foxp3^+$). One possibility is that although $CD28^-$ suppressor/senescent cells, which are apoptosis resistant, are induced, other cell types such as $CD4^+CD25^{high}Foxp3^+$ T cells undergo cell death instead (45). Others authors have shown expansion of T-regs by coculture T cells with various tumors. However, most of those studies were in mouse models, and the expansion of T-regs required the addition of local immature myeloid dendritic cells (46, 47). Our results reflect a single interaction between human cancer cells and T cells, and highlight only one form of immune evasion.

No data on $CD4^+CD28^-$ suppressor cells exist in the literature to date (23). One possibility is although $CD4^+CD28^-$ cells are increased in some cancer patients, they were not recognized as suppressor cells (48). For instance, in resected melanoma specimens, both $CD4^+$ and $CD8^+$ cells were found to have reduced CD28 levels but only in less dense areas of tumor. The authors argued that these $CD28^-$ $CD4^+$ and $CD8^+$ cells were effective cytotoxic cells. However, this may also be as a result of TIS as these T cells were further from the tumor and potentially became unrecognized senescent/suppressor cells (49).

Another plausible explanation for the lack of $CD4^+$ suppressor T cells *in vivo* is that there is a differential susceptibility to senescence (and, thus, to become suppressor T cells) of $CD8^+$ versus $CD4^+$ cells (50). It has been shown that after a repetitive set of stimuli, $CD8^+$ T cells are induced preferentially to senesce versus their $CD4^+$ counterparts (4). Potentially, *in vivo*, unlike our model, there would be opportunity for repetitive stimuli from the tumor, and thus, $CD8^+$ cells may be more likely to have senescent/suppressor features. Considering the importance of T suppressor cells, confirming the existence of these $CD4^+CD28^-$ suppressor cells has ramifications not just for cancer but many others pathologies.

In conclusion, senescent T cells with suppressor function observed to be increased in cancer patients can be reproduced *in vitro*. If senescence is one of the major mechanisms of T-cell dysfunction, this model then offers an environment to study this phenomenon to understand the major pathways and potential points of intervention. By reversing processes such as tumor-induced T-cell senescence, modern immunotherapeutic modalities can become a realistic approach as an alternative to traditional cancer treatments.

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