Cytotoxic T Lymphocytes Block Tumor Growth Both by Lytic Activity and IFNγ-Dependent Cell-Cycle Arrest

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Abstract

To understand global effector mechanisms of CTL therapy, we performed microarray gene expression analysis in a murine model using pmel-1 T-cell receptor (TCR) transgenic T cells as effectors and B16 melanoma cells as targets. In addition to upregulation of genes related to antigen presentation and the MHC class I pathway, and cytotoxic effector molecules, cell-cycle-promoting genes were downregulated in the tumor on days 3 and 5 after CTL transfer. To investigate the impact of CTL therapy on the cell cycle of tumor cells in situ, we generated B16 cells expressing a fluorescent ubiquitination-based cell-cycle indicator (B16-fucci) and performed CTL therapy in mice bearing B16-fucci tumors. Three days after CTL transfer, we observed diffuse infiltration of CTLs into the tumor with a large number of tumor cells arrested at the G1 phase of the cell cycle, and the presence of spotty apoptotic or necrotic areas. Thus, tumor growth suppression was largely dependent on G1 cell-cycle arrest rather than killing by CTLs. Neutralizing antibody to IFNγ prevented both tumor growth inhibition and G1 arrest. The mechanism of G1 arrest involved the downregulation of S-phase kinase-associated protein 2 (Skp2) and the accumulation of its target cyclin-dependent kinase inhibitor p27 in the B16-fucci tumor cells. Because tumor-infiltrating CTLs are far fewer in number than the tumor cells, we propose that CTLs predominantly regulate tumor growth via IFNγ-mediated profound cytostatic effects rather than via cytotoxicity. This dominance of G1 arrest over other mechanisms may be widespread but not universal because IFNγ sensitivity varied among tumors. Cancer Immunol Res; 3(1); 26–36. ©2014 AACR. See related commentary by Riddell, p. 23

Introduction

Adoptive T-cell immunotherapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) can be highly effective for treating melanoma (1). The recent development of genetically engineered T cells stably expressing exogenous T-cell receptors (TCR) or chimeric antigen receptors (CAR) specific for tumor-associated antigens offers the possibility of testing the efficacy of ACT against a wide range of cancer types in addition to melanoma (2, 3). Many clinical trials have now been conducted using genetically engineered T cells specific for tumor antigens as well as TILs, and some objective responses have been achieved (4, 5). It is clear from mouse models that adoptively transferred antigen-specific T cells are capable of eradicating established cancer (6–8), and the ability of CTLs to directly kill tumor and/or stromal cells is thought to be important for tumor elimination (9–11). Nonetheless, cytokines such as IFNγ and TNFs produced by T cells are also likely to contribute to the prevention of tumor growth by ACT via mechanisms other than cell lysis (12–14).

IFNγ is a critical cytokine for antitumor immunity under natural and therapeutic conditions (15, 16). It enhances tumor immunogenicity by upregulating components of the MHC antigen processing and presentation pathway. It also induces the expression of chemokines, including the angiostatic chemokines CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), that block neovascularization in the tumor and recruit effector immune cells (17–19). Furthermore, IFNγ has been reported to exert antiproliferative effects on the developing tumor (20, 21), and it triggers apoptosis of tumor cells by inducing proapoptotic molecules (22, 23).

To understand the global antitumor effect mediated by ACT, we used the B16 melanoma pmel-1 TCR-transgenic T-cell model to perform a gene expression analysis of ACT-treated tumors. On the basis of these results, we focused on genes controlling the cell cycle and arresting growth of B16 tumor cells in this model. We examined the effects on tumor cells of the IFNγ produced by the CTLs in situ using cell-cycle status indicators and investigated the mechanism of cell-cycle arrest. Furthermore, we demonstrate the importance of cell-cycle arrest induced by CTL-derived IFNγ in the regulation of tumor growth.
Materials and Methods

Mice, tumor cells, and peptides
Six-week-old male C57BL/6 mice were purchased from Japan SLC. Mice transgenic for the pmel-1-TCR, which recognizes the H-2D\(^{b}\)-restricted epitope EGSRRQDWL from gp100 (gp100\(^{25–33}\)), were obtained from The Jackson Laboratory. All mice were housed in a pathogen-free environment, and all animal procedures were conducted in accordance with institutional guidelines. All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments (10-P-127). The H-2D\(^{b}\)-restricted peptide human gp100 (hgp100\(^{25–33}\), KVRPRQDWL) was purchased from GenScript Japan at a purity of \(>90\%\), with free amino and carboxyl terminals. B16F10, FBL3, and 3L cell lines were maintained in culture medium consisting of DMEM with 10% FCS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. EL4, P815, and CT26 were cultured in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. All cell lines were tested for Mycoplasma by the MycoAlert Mycoplasma Detection kit (Lonza). Cellular morphology and growth curve in vitro were checked in all cell lines. B16F10 and B16-fucci cells were authenticated by transplantation for assessing growth ability in vivo.

Dendritic cell preparation and CTL stimulation
Dendritic cells (DC) were obtained by 8-day culture of C57BL/6-derived bone marrow cells with granulocyte-macrophage colony-stimulating factor (GM-CSF), as described previously (24). Briefly, bone marrow cells obtained from tibias and femurs of C57BL/6 mice were cultured in RPMI-1640 medium supplemented with 10% FCS, 10 mmol/L HEPES, 5 \(\times\) \(10^{-3}\) mol/L 2-mercaptoethanol, 1 \(\times\) \(10^{-3}\) mol/L sodium pyruvate, 1% nonessential amino acids, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 20 ng/mL GM-CSF (PeproTech) for 8 days. On days 3 and 6, half of the medium was replaced with fresh medium containing GM-CSF. DCs were further incubated with 1 \(\mu\)g/mL lipopolysaccharide for 16 hours and then pulsed with 1 \(\mu\)g/mL hgp100\(^{25–33}\) peptide for 3 hours to obtain mature DCs. To prepare CTLs, 1 \(\times\) \(10^{8}\) spleen cells from pmel-1 TCR-transgenic mice were cocultured with 2 \(\times\) \(10^{5}\) DCs in a medium containing 50 U/mL IL2 (Chiron Corporation). After 3 days of in vitro stimulation, approximately 90% of the harvested cells were CD3\(^{+}\)CD8\(^{+}\) CTLs.

ACT and anti-IFN\(\gamma\) mAb treatment
C57BL/6 mice were inoculated subcutaneously with \(1 \times 10^{6}\) B16 tumor cells followed by adoptive CTL transfer (1 \(\times\) \(10^{5}\) or \(4 \times\) \(10^{5}\)) 9 days later. Tumor growth was monitored every 2 to 3 days with calipers in an anonymous fashion. On the day of, and 2 days after, CTL transfer, mice received intraperitoneal injections of 500 \(\mu\)g anti-IFN\(\gamma\) mAb (clone XMG1.2; BioXCell) or rat IgG1 isotype control (BioXCell). Tumor volume was calculated as described previously (24).

Cell preparation and flow cytometry
Tumors were harvested from mice at scheduled time points, cut into pieces, and resuspended in Hank’s Balanced Salt Solution (HBSS) supplemented with 0.1% collagenase D (Roche Diagnostics) and DNase I (Roche Diagnostics) for 60 minutes at 37°C. The entire mass of the material was pressed through a 70-\(\mu\)m cell strainer (BD Falcon; BD Biosciences) using a plunger to obtain single-cell suspensions of tumor-infiltrating cells. For flow cytometry, the cells were first stained with the Fixable Viability Dye eFluor450 (eBioscience) to label dead cells, and pretreated with Fc Block (anti-CD16/32 clone 2.4G2; BD Pharmingen). The cells were then stained with antibodies and analyzed on a Gallios flow cytometer (Beckman Coulter). The following mAbs were obtained from BioLegend: PerCP/Cy5.5-conjugated anti-CD45, Alexa Fluor647-conjugated anti-CD90.1, and APC-Cy7-conjugated anti-CD8. Data were analyzed with the Kaluza software (Beckman Coulter).

Comprehensive gene expression analysis
Gene expression profiling data of B16 tumor tissues on different days were obtained by Agilent whole-mouse genome microarray. Total RNA was extracted with TRIzol (Invitrogen) from B16 tumor tissues and fluorescently labeled using a One-Color Agilent Quick Amp Labeling Kit. The microarray slides were hybridized, washed, and read on an Agilent Microarray scanner following the manufacturer’s instructions, and raw fluorescence signal intensities were generated by Agilent Feature Extraction Software v9.5. The signals were normalized to align at 75th percentile, and then turned into log2 ratio against day 1 in untreated and CTL-treated groups. We began with 45,018 probes, and removed probes if their gsWellAboveBG flag values were 0 at all samples, and then filtered out log2 ratio values that were unvarying (between –1 and 1) at all time points. We obtained 10,855 probes and ran hierarchical clustering (standard correlation, UPGMA) on them. All data were analyzed with the Subio Platform and Basic Plug-in v1.16 (Subio Inc.). The microarray data are available from the Gene Expression Omnibus (GEO) database (series accession number GSE57304; sample accession numbers GSM1379331–GSM1379344).

For quantitative gene panel–based PCR, Cell Cycle RT2 Profiler PCR arrays (SABioscience; http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-020Z.html) were used to simultaneously examine the mRNA levels of 84 genes in 96-well PCR arrays. Total RNA was prepared using TRIzol according to the manufacturer’s instructions (Invitrogen), and reverse-transcribed using RT2 First Strand kits (SABioscience). Real-time PCR was performed as instructed by the supplier on an ABI PRISM 7900HT Sequence Detection System (Life Technologies). Data were analyzed by a \(\Delta\Delta\) cycle threshold method to determine the fold changes of the mRNA levels (http://www.SABiosciences.com/pacrnaadataanalysis.php).

Expression vectors
CSII-EF-MCS/mAG-hGeminin and CSII-EF-MCS/mKO-cdt1 vectors were kindly provided by Dr. Atsushi Miyawaki (RIKEN, Wako, Japan; ref. 25). cDNA encoding mouse IFN\(\gamma\)R1 lacking the intracellular component of the receptor (26) was generated by PCR using the primer pair 5’-ATCTCCTACTCGAGATGGCCCGGAGCGGGGCGAGCT-3’ and 5’-ATCTCCTAGATCGCTTCTTCTTAGTTACAACATACGACCTG-3’ and subcloned into the Xho-I and Eco-DIC sites of the RV-GFP vector (designated RV-IFNGR1\(\Delta\)IC; ref. 27).

Production of B16-fucci and B16-fucci\(\Delta\)IC tumor cells
mAG-hGeminin and mKO-cdt1 were expressed in B16 tumor cells using lentiviral vectors (designated B16-fucci). IFN\(\gamma\)R1 lacking the sequence encoding the intracellular component of the receptor was expressed in B16-fucci tumor cells in the same way (designated B16-fucci\(\Delta\)IC).
Figure 1.
Gene expression analysis of the tumor in ACT. A, C57BL/6 mice were injected with $1 \times 10^6$ B16 tumor cells, and 9 days later (designated as day 0), tumor-bearing mice received $1 \times 10^7$ in vitro activated B16-specific (gp100-specific) CD80.1+ CTLs (designated ACT mice). Tumor volumes were measured on days 1, 3, 5, and 7 after CTL transfer ($n = 5$). B, tumor tissues from untreated or ACT mice were harvested on days 1, 3, 5, and 7. Total RNA extracted from 3 to 4 tumor tissues in each group was pooled and used for gene expression analysis. Heatmaps of hierarchical clustering analysis based on fold changes of gene expression on days 3, 5, and 7 relative to day 1 are shown (left). Some groups of genes that were upregulated (top) or downregulated (bottom) after CTL transfer were extracted (right). C, cell-cycle PCR array performed using tumor tissues from untreated or ACT mice ($n = 4$) on day 3. Seven cell-cycle genes that were significantly downregulated in the tumors from ACT mice are shown. The fold regulation is the negative inverse of the fold change.
Quantitative RT-PCR
Total RNA was extracted using TRizol and converted into cDNA using the SuperScript III First-Strand Synthesis System according to the manufacturer’s instructions (Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions were carried out using EXPRESS SYBR GreenER qPCR SuperMix Universal (Invitrogen). Primer sequences are listed in Supplementary Table S1. PCR reactions were run in a Thermal Cycler Dice Real-Time (TaKaRa) using the following program: one cycle 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 30 seconds. Results are expressed as ratios. The quantity of target mRNA was normalized to the level of GAPDH in each sample. PCR was performed in duplicate for each experiment, and PCR products were monitored by electrophoresis in 1.8% agarose gels and visualized with ethidium bromide.

Histologic analysis
Cryosections were fixed in 4% paraformaldehyde (PFA) at 4°C overnight and then transferred into 30% sucrose/PBS. After incubation for more than 24 hours, they were embedded in an optimal cutting temperature (OCT) compound (Sakura Finetek Japan) in liquid nitrogen. Sections measuring 8–10 μm were incubated with primary antibodies, followed by secondary antibodies and streptavidin. Polyclonal anti–Azami-Green antibody (PM011) was purchased from MBL. Polyclonal anti–single-stranded DNA was purchased from IBL-America. APC-conjugated anti-Cy3.50.1 antibody was purchased from BD Biosciences. Alexa 647–conjugated polyclonal secondary antibodies and streptavidin were from Life Technologies. Anti–APC-biotin was from BioLegend. The samples were analyzed using a BZ-9000 fluorescence microscope with BZ-H image processing software (Keyence). The number of cells in the necrotic/apoptotic area was estimated by calculating the surface area of the region using BZ-H1M software (Keyence).

Cytology
Cultured B16-fucci tumor cells treated with IFNγ were examined using bright-field or fluorescence microscopy (Olympus IX71; Olympus; magnification, ×200).

Senescence-associated β-galactosidase activity assay
Senescence-associated β-galactosidase (SA-β-gal) activity in cancer cells was assessed using the Senescence Detection Kit (BioVision). SA-β-gal–positive cells were identified using bright-field microscopy (Olympus IX71; Olympus; magnification, ×400).

Protein extraction and Western blotting
B16-fucci tumor cells were harvested from untreated or ACT mice receiving either rat IgG (control for treatment) or anti-IFNγ mAb on day 3 after CTL transfer. Protein extracts were prepared from each tissue using RIPA buffer (Thermo Scientific) with the protease inhibitor cocktail Complete Mini (Roche). Protein extracts (50 μg) were used for immunoblotting. Protein extracts (30–50 μg) from B16-fucci, B16-fucciΔIC cells, FBL3-, or EL4-treated with IFNγ (10 U/mL) for the indicated time were used for immunoblotting. The following antibodies, all from Santa Cruz Biotechnology, were used: rabbit anti-pSTAT1 (sc-79888-R), rabbit anti-Skp2 (sc-7164), mouse anti-ATM (sc-23921), rabbit anti-p53ser15 (sc-101762), and rabbit anti-p21 (sc-397). Mouse anti-p27 (kip1) antibody was purchased from BD Biosciences. All antibodies were used at a final concentration of 0.2 to 1.0 μg/mL. After incubation with anti-rabbit IgG or anti-mouse IgG antibodies conjugated with horseradish peroxidase, proteins were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences).

Statistical analysis
Comparison of results was performed by an unpaired, two-tailed Student t test with GraphPad Prism 5 (GraphPad Software, Inc.).

Results
Gene expression analysis in CTL transfer therapy
To understand the effector mechanism whereby ACT inhibits tumor growth, we assessed tumor-cell gene expression in a B16 melanoma model of pmel-1 TCR-transgenic CTL transfer. Tumors grew progressively in untreated B16-bearing mice, which was prevented between days 3 and 7 after the animals had received 10 million CTLs (Fig. 1A and Supplementary Fig. S1A). Tumor tissues were harvested from untreated mice and ACT mice on days 1, 3, 5, and 7 after CTL transfer, and gene expression was analyzed. Upregulation of genes related to CD8+ T cells, the MHC class I pathway, IFNγ signaling, cytotoxic effector molecules, and others was observed in tumors from treated but not untreated mice (Fig. 1B). These data are consistent with our previous findings (24, 28) that adoptively transferred CTLs infiltrated into the tumor and that mRNA encoding IFNγ, Perforin, Granzyme B, and Fasl was expressed on days 3 to 7, with kinetics reflecting the infiltration of the CTLs (Supplementary Fig. S1B and S1C). Interestingly, some genes positively regulating the cell cycle, such as Skp2, E2f2, Ccnf, Mib67, and Wee1, were downregulated in tumors from ACT mice on days 3 and 5 (Fig. 1B). This was not the case in the untreated controls. We confirmed these data by a cell-cycle PCR array (Fig. 1C). Thus, gene expression analysis revealed profiles related to cell-cycle regulation, as well as cytotoxicity, in tumors from mice with ACT treatment.

CTL therapy induces G₁ cell-cycle arrest
Using the fucci (fluorescent ubiquitination-based cell-cycle indicator) system (25), we investigated the impact of ACT on the cell cycle of B16 tumor cells. To this end, we generated B16 tumor cells expressing fucci (designated B16-fucci), which emit red fluorescence in the G₁-phase, but otherwise fluoresce green. We then treated B16-fucci tumor-bearing mice with ACT. Tumor growth was not affected by the transduction of fucci into B16 tumor cells, but ACT inhibited their growth (Fig. 2A). On day 3 after CTL transfer, tumors were harvested from untreated or ACT mice for histologic analysis. As shown in Fig. 2B, CTLs had infiltrated into the tumors and were visible as blue spots. Whereas green cells were dominant in the growing tumor cells, the majority of tumor cells from ACT mice were red, suggesting that CTL therapy induced tumor cell-cycle arrest in the G₁-phase. Expressing the cell-cycle state as a green:red (G:R) ratio (Fig. 2C) showed that this was lower in the ACT mice (0.26 ± 0.12; n = 3) than in the untreated control mice on day 3 (1.1 ± 0.05; n = 3; P = 0.0032). This difference remained up to day 5 after CTL transfer, but on day 7, the G:R ratio increased again, together with the disappearance of CTLs, and green cells became dominant once more after day 10 (Supplementary Fig. S1B).
IFNγ is critical for tumor growth inhibition and cell-cycle arrest
IFNγ is important for antitumor immunity. We have shown that it is critical for tumor growth inhibition in this model using IFNγ neutralizing antibody (anti-IFNγ mAb; ref. 28). Because IFNγ is involved in MHC class I upregulation, antigen processing, and trafficking of T cells into the tumor site by promoting chemokine production, the number of T cells infiltrating into the tumor was decreased by neutralizing IFNγ (data not shown). It was necessary to inject 4-fold more T cells to achieve the same level of CTL infiltration in anti-IFNγ Ab-treated animals (Fig. 3B). Nevertheless, anti-IFNγ treatment still prevented tumor growth blockade, despite the presence of equivalent levels of CTL in the tumor (Fig. 3A). Strikingly, this was the case even though the expression of mRNA encoding the effector molecules IFNγ, Perforin, Granzyme B, and FasL in ACT mice treated with anti-IFNγ mAb was the same or even higher than that in control ACT mice treated with rat IgG (Fig. 3C and D). A major difference in the anti-IFNγ mAb-treated mice was that the expression of mRNA encoding STAT1 and IFNγ-inducible genes such as MIG, IP10, or I-TAC was suppressed. This suggests that IFNγ signaling was blocked by the treatment with anti-IFNγ mAb.

As shown in Fig. 4A, all tumor cells fluoresced either green or red. In growing tumors, the majority of B16 tumor cells were in the S–G2–M phase (Fig. 4A, left). After CTL transfer, most of the tumor cells became red (Fig. 4A, middle), but in the anti-IFNγ mAb-treated ACT mice, the tumor cells remained green (Fig. 4A, right). Diffused infiltration of CTLs into the tumor accompanied by massively infiltrated mononuclear cells and destruction of tumor cells, corresponding to spotty necrotic/apoptotic areas, was seen in ACT mice whether or not they received anti-IFNγ mAb treatment. Furthermore, apoptotic cells positive for single-stranded DNA (ssDNA), detected as white spots, were rare, but were present equally in ACT mice with or without anti-IFNγ mAb treatment (Fig. 4B and C). This, therefore, suggests that the transferred CTLs actually mediated relatively little tumor cell killing, which was unaffected by anti-IFNγ mAb administration.

The numbers of CTLs, tumor cells in necrotic/apoptotic areas, and tumor cells in the G1 or S–G2–M phase were compared systematically in these mice. More green than red cells were observed in untreated tumors (Fig. 4D). In CTL-treated tumors, as described above, the G:R ratio was inverted, but the ratio was restored by the abrogation of IFNγ signaling. The surface area of the part of the tumor with necrotic/apoptotic cells was similar in the two CTL-treated groups (with or without anti-IFNγ mAb treatment), and the estimated number of dead cells was always smaller than that of the live cells (whether green or red; Fig. 4E). These results indicate that G1 cell-cycle arrest, and not cytolytic killing, was primarily responsible for the CTL-induced suppression of tumor growth.

IFNγ directly suppress B16-fucci tumor cell growth through cell-cycle arrest
We constructed B16-fucci tumor cells expressing an IFNγ receptor lacking the intracellular component (B16-fucciΔIC). ACT did not suppress the growth of these cells even when 4-fold more CTLs (4 x 10^7) were transferred (Supplementary Fig. S2A). Although a similar number of CTLs infiltrated into B16-fucciΔIC tumor sites, as in mice with B16-fucci tumors receiving 4-fold less CTLs, no IFNγ production was observed (Supplementary Fig. S2B and S2C), and therefore the effect of IFNγ could not be evaluated in this system. This might be due to limited recognition of B16-fucciΔIC tumor cells by the CTL because of their low level of MHC class I expression (Supplementary Fig. S2D). As expected, B16-fucciΔIC did not upregulate MHC class I molecules after exposure to IFNγ.

Because we could not evaluate the effect of IFNγ on tumor cells in vivo in this manner, we tested its effects directly on B16 tumor cells in vitro. As shown in Fig. 5, proliferation of B16-fucci cells, but not B16-fucciΔIC cells, was inhibited completely when they were treated with IFNγ (Fig. 5A). These cells were arrested in G1 (Fig. 5B), showing that IFNγ directly inhibits the growth of B16-fucci tumor cells through G1 cell-cycle arrest.

Recently, it was reported that a combination of IFNγ and TNFα produced by CD4+ T cells can drive tumor cells into senescence by inducing G0–G1 cell-cycle arrest through the activation of p16INK4a (14). Therefore, we tested the effect of IFNγ and/or
TNFα on B16 tumor cells. Whereas IFNγ alone inhibited cell proliferation by G1 arrest, TNFα alone had a limited inhibitory effect on B16 proliferation even at a high concentration (10 ng/mL; Supplementary Fig. S3). When B16 tumor cells were cultured in the presence of both IFNγ and TNFα at a high concentration, a synergistic effect on cell growth inhibition and cell senescence was observed (Supplementary Fig. S3).

IFNγ production by transferred CTLs induces G1 cell-cycle arrest by a mechanism involving Skp2/p27-related cell-cycle regulation

We next investigated the mechanism of G1 cell-cycle arrest by IFNγ. B16-fucci tumor tissues were harvested from untreated mice, ACT mice treated with rat IgG, or ACT mice treated with anti-IFNγ mAb on day 3 after CTL transfer. Proteins were extracted from each tissue for Western blot analysis. As shown in Fig. 6A, downstream of IFNγ signaling, STAT1 was phosphorylated in tumors from ACT mice treated with rat IgG, but not in tumors from anti-IFNγ mAb-treated ACT mice. To confirm the gene expression data that Skp2 was significantly downregulated in tumors from ACT mice (Fig. 1B and C), we examined the protein expression of Skp2. As shown in Fig. 6A, Skp2 expression was suppressed in tumors from control ACT mice, but not in those from mice treated with anti-IFNγ mAb. Conversely, the cyclin-dependent kinase inhibitor (CKI) p27 accumulated in the former but not in the latter. We also investigated the ataxia telangiectasia mutated (ATM)–p53–p21 pathway involved in G1 cell-cycle arrest following DNA damage. We found that ATM was not upregulated as a result of CTL therapy, p53 was not activated, and no subsequent accumulation of p21 was observed. This shows that the ATM–p53–p21 pathway is not involved in this model (Fig. 6A). We also investigated the expression of these molecules in vitro (Fig. 6B). B16-fucci and B16-fucciΔIC tumor cells were treated with 10 U/mL IFNγ and
harvested at the indicated times. STAT1 phosphorylation was observed at early time points (15 and 30 minutes after IFNγ treatment) in B16-fucci, but not in B16-fucciΔIC. Skp2 expression was downregulated gradually, and p27 accumulated by 48 hours after IFNγ treatment in B16-fucci but not in B16-fucciΔIC cells (Fig. 6B). We confirmed that the ATM–p53–p21 pathway was also not involved in G1 cell-cycle arrest in vitro. These results suggest that G1 cell-cycle arrest by CTL therapy is likely due to Skp2/p27-related cell-cycle regulation by IFNγ.

Inhibition of FBL3 cell proliferation by IFNγ

We next investigated whether proliferation of other murine cell lines is inhibited by IFNγ. FBL3, p815, CT26, 3LL, and EL4 tumor cells were treated with IFNγ (10 U or 100 U/ml) for 4 to 6 days (Fig. 7A). The proliferation of FBL3 tumor cells was inhibited by IFNγ treatment in a manner similar to that of B16 tumor cells. The proliferation of P815, CT26, and 3LL tumor cells was moderately inhibited. No inhibition was observed in IFNγ-treated EL4 tumor cells. In Western blot analyses, using tumor lysates from FBL3 and EL4 tumors at the indicated time point, Stat1 phosphorylation was observed in FBL3 lysates, but the phosphorylation was very weak in EL4 tumor lysates. Skp2 expression was downregulated 24 to 48 hours after IFNγ treatment in FBL3 but not in EL4 tumors, and p27 accumulated (Fig. 7B). Again, the ATM–p53–p21 pathway was not involved. These results suggest that inhibition of FBL3 tumor cell proliferation by IFNγ might involve Skp2/p27-related cell-cycle regulation, as in B16.

Discussion

In this study, we demonstrated that the mechanism of tumor growth inhibition by adoptive CTL therapy was largely dependent on IFNγ-induced G1 cell-cycle arrest rather than on tumor cell lysis. In microarray analysis, the upregulation of genes related to CD8+ T cells, the MHC class I pathway, IFNγ signaling, cytotoxic effector molecules, and others was observed in tumors from ACT mice. At the same time, a decrease was found in the expression of some genes positively regulating the cell cycle in these tumors. Therefore, we focused on cell-cycle control in the B16 adoptive immunotherapy model and used the fucci system, which allows the visualization of cell-cycle stage of tumor cells in situ in mice receiving CTL.
Histologic analysis following ACT showed that the number of CTLs in the tumor was far lower than that of tumor cells. On average, only 140 CTLs per mm² tumor tissue on day 3 after transfer were found. In contrast, this area contained 1,911 tumor cells (both green and red cells). Thus, it seems a priori unlikely that this small number of CTLs infiltrating the tumor would be sufficient to prevent tumor growth by direct cytotoxicity 3 to 7 days after CTL injection. Consistent with this observation, we also found that the area of the tumor undergoing necrosis/apoptosis was relatively small. Instead, a larger area consisting of tumor cells had undergone cell-cycle arrest at G₁. Therefore, transient tumor suppression from days 3 to 7 seems to be largely due to cell-cycle arrest rather than due to CTL killing. Using mAbs that neutralize IFNγ and completely block IFNγ signaling, we demonstrated that IFNγ is required for tumor growth inhibition and G₁ cell-cycle arrest but not for CTL killing. Thus, IFNγ-dependent G₁ cell-cycle arrest makes a major contribution to tumor growth suppression in this model. This would explain why tumor growth was suppressed despite the low ratio of CTLs to tumor cells in this system, and how T cells can suppress the growth of bystander tumor cells that may not express the target antigen. This could also explain some examples to tumor suppression by CD4⁺ T cells that can also make IFNγ even if they are not lytic and even if the tumor is MHC class II negative, as long as antigen-presenting cells are infiltrating and can present antigen, as the soluble IFNγ can target neighboring cells.

IFNγ inhibits cell proliferation via cell type–specific pathways that involve CKIs, such as p21Cip1 (29, 30) and p27Kip1 (31, 32). It has been shown that STAT1 interacts directly with cyclin D1/Cdk4 and mediates the cell-cycle arrest of human U3A cells (33). Here, we investigated the involvement of CKIs in G₁ cell-cycle arrest, and found that p27Kip1, but not p21Cip1, accumulated in B16 tumor cells following CTL therapy in vivo or IFNγ treatment in vitro. Another CKI, p16, is involved in senescence-like G₁ cell-cycle arrest (14), but this factor is not expressed in B16 tumor cells due to a p16Δexon1 allele deletion (34). Thus, p27Kip1 appeared to be the major CKI involved in G₁ arrest in this model. Skp2 is an oncogene; Skp2 inactivation induces cell senescence independent...

Figure 5.
IFNγ directly inhibits tumor cell growth through G₁ cell-cycle arrest. A, proliferation of B16-fucci cells or B16-fucci expressing an IFNγ receptor lacking the intracellular component (B16-fucciΔIC) was assessed after exposure to IFNγ (10 U/mL) for 6 days. B, B16-fucci or B16-fucciΔIC cells were incubated with IFNγ (10 U/mL). Two days later, the cell-cycle state was determined by fluorescence microscopy (left). Original magnification, ×200. The G:R ratio in fluorescence images is shown (n = 2 per group; right). Samples were compared using an unpaired, two-tailed Student t test (*, P < 0.01).

Figure 6.
G₁ cell-cycle arrest by ACT was mediated by IFNγ-dependent Skp2/p27-related cell-cycle regulation. A, B16-fucci tumors were harvested from untreated or ACT mice given control rat IgG or anti-IFNγ mAb on day 3 after CTL transfer. Protein extracts (50 μg) from tumor tissues in each group were used for immunoblotting of the indicated proteins. B, protein extracts from B16-fucci or B16-fucciΔIC cells treated with IFNγ (10 U/mL) for the indicated time were used for immunoblotting of the indicated proteins.
of the p53 pathway (35). STAT1 has been shown to repress Skp2 gene transcription by binding to its promoter region and stabilizing p27Kip1 in Ras-transformed cells (36). In this report, we showed that Skp2 expression was downregulated after either CTL therapy or IFNγ treatment; thus, STAT1 may repress Skp2 expression and promote p27Kip1 stabilization.

G1 cell-cycle arrest is also known to be induced by ATM-dependent activation of p53 and induction of p21Cip1 (37). Because ATM is a key molecule in the cellular response to DNA damage (38), we investigated its expression by Western blot analysis. We found that the ATM protein was not highly expressed in the tumor after CTL therapy. We also confirmed this finding in vitro in B16 cells cultured with IFNγ. Furthermore, p53 was not phosphorylated at Ser15, and the CKI p21Cip1, which is downstream of phospho-p53 (Ser15), was not upregulated. Therefore, we conclude that ATM expression and the subsequent activation of the phospho-p53–p21 pathway was not involved in this model.

We tested the effect of IFNγ on other murine tumor cell lines, and found that the proliferation of FBL-3 cells was strongly inhibited by IFNγ, similar to that of the B16 tumor cells. On the other hand, EL-4 cells were insensitive to IFNγ, whereas p815, CT26, and 3LL cells were moderately sensitive. IFNγ sensitivity and the mechanisms involved in the inhibition of cell proliferation may differ in different tumor cell lines. It is important to know whether IFNγ insensitivity is due to the downregulation of IFNγ receptors on these tumors, or defects in their IFNγ signal transduction.

Braumuller and colleagues (14) reported that IFNγ together with TNFα reduced the proliferation of different cancer cell lines in both mice and humans. Here, we showed that the combination of IFNγ and TNFα strongly inhibited B16 tumor cell proliferation and induced cell senescence (Supplementary Fig. S3). Because pmel-1 CTLs produce large amounts of IFNγ, but not TNFα, when they are cultured with B16 tumor cells in vitro (Supplementary Fig. S4), and IFNγ alone is enough to suppress tumor cell proliferation (Supplementary Fig. S3), the transient suppression of tumor growth from days 3 to 7 in vivo in this model may be entirely due to IFNγ, as there is only a small amount of TNFα at the tumor site. Th1 CD4+ T cells or Toll-like receptor (TLR)-stimulated macrophages might be able to produce enough TNFα, but these cells are not present in our system. Alternatively, a strategy to induce polyfunctional CD8+ T cells producing IFNγ, TNFα, and IL2 might be important to enhance further the antitumor effects in this model (39, 40).

IFNγ is a critical molecule in cancer immunosurveillance or immunoediting in primary mouse tumor models (41–44). In our study, as long as high concentrations of IFNγ were present...
in the tumor, its growth was controlled through G1 arrest (Supplementary Fig. S1). Thus, our study suggests that deregulation of the cell cycle due to insufficient availability of IFNγ or IFNγ insensitivity developed by tumor cells may be one mechanism by which tumor cells escape from CTL therapy (Supplementary Figs. S1 and S2).

Our study indicates that a small number of infiltrated CTLs can cause a large number of tumor cells to arrest in G1, rather than dying. On the basis of this finding, we propose that the development of an appropriate strategy to maintain tumor cells in a quiescent, dormant state for extended periods (immunotherapy-induced equilibrium/dormancy), or to induce apoptosis/senescence, would be highly desirable.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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Cytotoxic T Lymphocytes Block Tumor Growth Both by Lytic Activity and IFN γ-Dependent Cell-Cycle Arrest

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