Increased IFN\(\gamma\)\(^{+}\) T Cells Are Responsible for the Clinical Responses of Low-Dose DNA-Demethylating Agent Decitabine Antitumor Therapy

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Abstract

Purpose: Low-dose DNA-demethylating agent decitabine therapy is effective in a subgroup of cancer patients. It remains largely elusive for the biomarker to predict therapeutic response and the underlying antitumor mechanisms, especially the impact on host antitumor immunity.

Experimental Design: The influence of low-dose decitabine on T cells was detected both in vitro and in vivo. Moreover, a test cohort and a validation cohort of advanced solid tumor patients with low-dose decitabine-based treatment were involved. The activation, proliferation, polarization, and cytolytic capacity of CD3\(^{+}\) T cells were analyzed by FACS and CCK8 assay. Kaplan–Meier and Cox proportional hazard regression analysis were performed to investigate the prognostic value of enhanced T-cell activity following decitabine epigenetic therapy.

Results: Low-dose decitabine therapy enhanced the activation and proliferation of human IFN\(\gamma\)\(^{+}\) T cells, promoted Th1 polarization and activity of cytotoxic T cells both in vitro and in vivo, which in turn inhibited cancer progression and augmented the clinical effects of patients. In clinical trials, increased IFN\(\gamma\)\(^{+}\) T cells and increased T-cell cytotoxicity predicted improved therapeutic responses and survival in the test cohort and validation cohort.

Conclusions: We find that low-dose decitabine therapy promotes antitumor T-cell responses by promoting T-cell proliferation and the increased IFN\(\gamma\)\(^{+}\) T cells may act as a potential prognostic biomarker for the response to decitabine-based antitumor therapy. Clin Cancer Res; 1–13. ©2017 AACR.

Introduction

The deregulation of epigenetic modifications is one of the hallmarks of cancer, especially for DNA methylation (1, 2). DNA methyltransferase (DNMT) inhibitors, such as 5-aza-2’-deoxycytidine (AZA) or decitabine (DAC), have clinical benefits in both hematologic malignancies and solid tumors, especially myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML; refs. 3, 4). Previously, AZA and DAC were regarded as cytotoxic drugs, and more than 500 mg/m\(^2\)/cycle high-dose DAC was used in antitumor treatment, showing strong antiproliferative effects by inducing DNA strand breaks (5, 6). However, intolerable toxicity and primarily hematologic inhibition have greatly limited the clinical application of DNMT inhibitors in solid tumors. Owing to knowledge of their DNA demethylation effects, a lower dose schedule (100–135 mg/m\(^2\)/cycle) was used, and encouraging activity was determined in myeloid leukemia (3). An even lower dose of DAC (50–90 mg/m\(^2\)/cycle) was recommended as the “optimal dose” for the treatment of solid tumors (7–10). Notably, low-dose DAC is more effective than a highly cytotoxic dose of DAC in both blood neoplasms and solid tumors (10–12). Investigations of antitumor mechanisms of low-dose DAC therapy have previously focused mainly on its effects on cancer cells, including the upregulation of genes associated with cell-cycle control, cytoskeletal remodeling, apoptosis, cell lineage commitment, anaerobic glycolysis, antigen exposure, and inflammation (7, 13, 14). However, as critical roles in T-cell–mediated responses in antitumor immunity, whether low-dose DAC treatment has a direct influence on T-cell activation and cytolysis-mediated antitumor responses remains unknown.

Recently, epigenetic therapy has been an important approach to treat cancer patients. Although low-dose DAC therapy showed beneficial clinical responses in approximately 50% of patients with MDS, the overall response rate was 20%–30% in refractory solid tumor patients (3). Thus, exploring the effective biomarkers to predict responses to low-dose DAC therapy is of primary importance in clinical epigenetic therapy for cancer. Numerous studies have sought to illuminate the alterations in a wide range of genes and pathways following low-dose DAC therapy in both peripheral blood mononuclear cells (PBMC) and cancer cells, and found a set of gene expression changes associated with the cell cycle, DNA damage, and the immune and inflammatory responses (12, 15). Although the number of these genes may be...
patients, specimens, and DAC-based therapy

For the antitumor activity of DAC, which was presumably not positively correlated with the doses of DAC (17). Other biomarkers to predict clinical responses to low-dose DAC therapy have been observed, the clinical effects were dose-dependent DNA demethylation (16). Furthermore, although dose-dependent DNA demethylation activity of DAC, has been observed, the clinical effects were not positively correlated with the doses of DAC (17). Other mechanisms besides DNA demethylation may be responsible for the antitumor activity of DAC, which was presumably due to enhancement of the antitumor immune response. Therefore, investigating the immune regulation and elucidating the immune-related biomarkers for the identification of low-dose DAC therapy responses, especially the biomarkers in the blood of patients, are an important and promising topic that remains unresolved.

We conducted a phase I/II clinical trial at the Chinese PLA general hospital (www.clinicaltrials.gov: NCT01799083) and reported that low-dose DAC treatment prominently prolonged the progression-free survival (PFS) of patients with advanced refractory solid tumors (9, 18). In our clinical trial, the disease control rate exceeded 70% for advanced ovarian cancer and gastrointestinal cancer, demonstrating a significant improvement compared with first-line chemotherapy. As the effects of low-dose DAC on antitumor immune responses are unknown, we aimed to examine the roles and mechanisms of DAC on the host antitumor immune response, especially T-cell activation and function, to suggest effective biomarkers, especially immune-related biomarkers in the blood, for the identification and prediction of responses to low-dose DAC therapy.

Materials and Methods

Patients, specimens, and DAC-based therapy

This was a single-center, open-label, phase I/II study conducted at Chinese PLA general hospital (www.clinicaltrials.gov: NCT01799083). This study was undertaken in accordance with the principles of good clinical practice and was approved by the appropriate institutional review boards and regulatory agencies. In this article, there were two cohorts of patients with solid tumors: the Test Cohort (20 solid tumor patients) and the Validation Cohort (38 refractory/recurrent ovarian cancer patients) from Chinese PLA general hospital (Beijing, China), who were enrolled from April 12, 2012 to April 12, 2016. Participants in this study had refractory advanced solid tumors and included patients with ovarian cancer, lung cancer, and colorectal cancer, among others. The detailed eligibility criteria were as previously reported in our clinical trials (9, 19). Patients were given 7 mg/m²/d decitabine via intravenous push for 5 days, followed by chemotherapy regimen as they previously received, in 28-day cycles. Baseline patient demographic and clinical characteristics were listed in Supplementary Table S1 (Test Cohort) and Supplementary Table S2 (Validation Cohort). All patients underwent a complete medical interview and a physical examination that included a blood profile and a CT of the disease lesions. The patients were restaged by CT every two cycles. The clinical feasibility evaluation was performed using the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). The primary end points were disease control rate (percentage of patients with CR+PR+SD), objective response rate (percentage of patients with CR+PR) and PFS. Secondary evaluation included safety assessment, overall survival (OS) and biological research. A quarterly follow-up evaluated survival until the patient either died or withdrew from the trial by November 12, 2016, and the median follow-up time was 14 months (for test cohort) and 12 months (for validation cohort).

Sample size was evaluated by PASS software (α 0.05, precision 0.15, proportion 0.3).

The peripheral blood was collected before (Day 0) and after DAC treatment (Day 6) during each cycle. Normal peripheral T cells were obtained from healthy donors. Peripheral blood mononuclear cells were isolated according to standard protocol and immediately analyzed or frozen for posterior analysis. All donors provided written informed consent as approved by the ethics committee of the General Hospital of PLA, Beijing, China.

Reagents

Antibodies against CD3 (ab16669), CD4 (ab25475), and CD8 (ab22378), were from Abcam. The following antibodies and reagents were obtained from BD Biosciences: anti-CD3-PerCP (347334, 553067), anti-CD4-APC (340443, 553051), anti-IFNγ-FTC/IL4-PE (340456), anti-IFNγ-PE (554412), anti-IL-4-PE (554435), anti-CD25-APC (340393), anti-CD69-APC (555533), anti-CD107a-APC (560664), anti-Granzyme B-APC (561999), isotype-matched anti-rat IgG1, and the IntraSure Kit (641776). T-cell stimulation cocktail (plus protein-transport inhibitors; 00-4975-93) was purchased from ebioscience.

Isolation and treatment of T cells

T cells were isolated from PBLS from healthy donors or cancer patients. CD3+ T cells were sorted using anti-CD3 antibody by fluorescence-activated cell sorter (FACS). For in vitro treatment, T cells were treated with PBS or 10 nmol/L DAC (or other doses as indicated) for 3 days in a plate pre-coated with 2 µg/mL anti-CD3 (or the indicated concentration) and soluble IL-2 (1,000 U/mL).

In vivo tumor studies

All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Scientific Investigation Board of PLA General Hospital, Beijing, China. To evaluate the antitumor capacity of low-dose DAC in vivo, 5 × 10⁶ murine colon carcinoma CT-26 cells were injected subcutaneously into the back region of BALB/c mice. After
Adoptive T-cell therapy

CD3⁺ T cells were sorted from splenocytes of BALB/c mice using anti-CD3 antibody by FACS and treated with 10 nmol/L DAC for 72 hours \textit{in vitro}. PBS or DAC-treated T cells (5 × 10⁷) were intravenously transfused into CT26 colon cancer-bearing BALB/c mice (n = 8/group). Tumor growth was monitored and recorded. For analysis of the adoptive T cells, both the DAC-treated T cells and control T cells were labeled with CFSE, and the CFSE-positive T cells in tumor tissues 48 hours post infusion were collected and analyzed by flow cytometry.

Statistical analysis

Data are presented as the mean ± SD. Statistical comparisons between groups were analyzed using the unpaired Student t test, and a two-tailed P < 0.05 was used to indicate statistical significance. The median time of PFS and OS were calculated from the date of first decitabine treatment. For FACS, patients who were alive without progressive disease (PD) were censored at the time of last contact. To analyze the correlation between T-cell activity and clinical parameters, the χ² test and one-way ANOVA were applied using SPSS 18.0 (Chicago, IL), and the P values were shown as indicated. Kaplan–Meier survival analysis was performed to compare patient survival based on the peripheral T-cell status, and the log-rank test was used to generate P values with SPSS 18.0. Hazard ratios (HR) with 95% CI were estimated using a Cox regression model.

Detection of T-cell proliferation, immunofluorescence analysis, CCK8 cytotoxicity test for T cells, and quantitative real-time PCR

Please see Supplementary Methods.

Results

Low-dose DAC promotes the activation and proliferation of human T cells

Because the direct effects of low-dose DAC therapy on host antitumor immune responses are still unknown, here we intended to investigate the impact of low-dose DAC on antitumor immunity, especially T-cell activation and proliferation because of their crucial roles in antitumor responses. First, human peripheral CD3⁺ T cells from four healthy donors were sorted and treated with 10 nmol/L DAC because 10 to 300 nmol/L \textit{in vitro} DAC treatment was commonly regarded as a low dose, and the 10 nmol/L very low doses of DAC produced minimal cytotoxicity in leukemia cells (13, 20). First, the expression levels of CD69 and CD25 (early and later T-cell activation markers) were examined following stimulation with anti-CD3 antibody and low-dose DAC as indicated. We observed enhanced T-cell activation following low-dose DAC treatment, especially at early stage of T-cell activation as observed significant CD69 upregulation (Fig. 1A and B and Supplementary Fig. S1A). CD69 was induced both in CD4⁺ and CD8⁺ T cells, whereas the alteration was more marked in CD4⁺ T cells (Fig. 1C). The increased phosphorylation levels of CD3-zeta, LAT, and Lck in DAC-treated T cells further confirmed the increased intracellular T-cell signaling activation (Fig. 1D).

We further evaluated the cell proliferation and found that low-dose DAC treatment enhanced T-cell viability (Fig. 1E and F). Moreover, the CFSE dilution assay analysis validated the increased proliferation of T cells following low-dose DAC treatment (Supplementary Fig. S1B). In addition, cell-cycle progression was also promoted by low-dose DAC treatment (Supplementary Fig. S1C). These results suggested that low-dose DAC treatment enhanced T-cell activation and proliferation \textit{in vitro}.

We further investigated the effects of low-dose DAC treatment on T cells in vivo. As shown in Fig. 1G and Supplementary Fig. S1D, low-dose DAC (0.2 mg/kg) treatment (21) significantly increased CD3⁺ T cells in the spleen and peripheral blood of mice. In agreement with the increased T-cell frequency, the absolute number was also increased (Fig. 1H and Supplementary Fig. S1E). Other immune cells were examined and were not influenced by low-dose DAC, including CD19⁺ B cells, F4/80⁺Ly-6C⁺ macrophages and CD11b⁺CD11c⁺ dendritic cells (DC; Fig. 1G and H and Supplementary Fig. S1D and S1E). Furthermore, after \textit{in vivo} low-dose DAC treatment, the activation and viability of T cells was enhanced, as analyzed by the CCK8 assay and CD69 expression (Fig. 1I and J and Supplementary Fig. S1F and S1G). These results showed that low-dose DAC treatment enhanced T-cell activation and proliferation \textit{in vivo}. However, in the human T-cell leukemia cell lines Jurkat and Molm4, 10 nmol/L low-dose DAC treatment showed no effect on the proliferation of these leukemia cell lines (Supplementary Fig. S1H–S1I), which differed from the effects on T lymphocytes. Taken together, low-dose DAC promoted the activation and proliferation of T lymphocytes but not T-cell leukemia cells.

Low-dose DAC promotes Th1 polarization and CTL cytolytic activity

Because T-cell activation was promoted by low-dose DAC treatment, we further examined its properties. Cytokine production by these human T cells was examined, and we observed that IFNy production was significantly upregulated by low-dose DAC treatment, whereas IL4 production was significantly inhibited using a real-time PCR array (Fig. 2A). The increased IFNy production and decreased IL4 production were validated by flow cytometry analysis (Fig. 2B and C). The intracellular cytokine staining assay confirmed the increase in IFNy-producing T cells following low-dose DAC treatment. In addition, the number of IFNy-producing CD8⁺ cytotoxic T lymphocytes (CTL) was prominently augmented (Fig. 2D). These results
indicate that low-dose DAC can promote IFN-γ-producing Th1 cells and CTLs. In addition, the frequency of potential regulatory T (Treg) cells was detected by intracellular staining of Foxp3 in CD4⁺CD25⁺ cells, and both the frequency and total number of CD4⁺CD25⁺Foxp3⁺ cells were deceased following low-dose DAC treatment (Fig. 2E and F). These data suggest

Figure 1.
Low-dose DAC promotes the activation and proliferation of T cells. 

A, CD3⁺ T cells were sorted using anti-CD3 antibody by FACS from the peripheral blood cells of 4 healthy donors. The cells were treated with PBS or 10 nmol/L DAC for 48 hours, followed by stimulation with different doses of anti-CD3 antibody for 12 hours. Cell activation was measured by staining with antibody against CD69, gated on CD3-positive T cells. 

B, Peripheral CD3⁺ T cells were treated with PBS (control) or 10 nmol/L DAC for 48 hours, followed by stimulation with 2 μg anti-CD3 antibody for the times indicated. Cell activation was measured by staining with antibody against CD69, both gated on CD3-positive T cells. 

C, CD3⁺ T cells were treated with different doses of DAC for 48 hours, followed by stimulation with anti-CD3 antibody for 12 hours. Cell activation was assessed in CD4⁺ and CD8⁺ T cells by detecting the level of CD69. 

D, CD3⁺ T cells were treated with PBS or 10 nmol/L DAC for 48 hours, followed by stimulation with anti-CD3 antibody for 2 minutes, and the cells were analyzed by immunoblotting using the indicated antibodies. 

E, CD3⁺ T cells were treated with different doses of DAC in the presence of immobilized anti-CD3 antibody, and the cell viability was measured at the indicated time points using CCK8 assays. 

F, CD3⁺ T cells were treated with different doses of DAC in the presence of immobilized anti-CD3 antibody for 3 days, and Ki67 levels were detected by flow cytometry, and the frequencies of Ki67⁺ CD3⁺ T cells were shown. 

G, B, and H, BALB/c mice were treated with PBS (Con group) or 0.05 mg/kg DAC (DAC group) for 5 days, and immunocytes were collected from splenocytes 10 days after DAC treatment using the indicated antibodies. The frequencies (G) and absolute numbers (H) are shown. 

I, BALB/c mice were treated with different doses of DAC (0, 0.05, 0.2 mg/kg) for 5 days; splenic T cells were collected 10 days after DAC treatment. The percentage of CD3⁺CD69⁺ T cells was measured in response to anti-CD3 stimulation. 

J, Splenic CD3⁺ T cells as in (H) were isolated, and cell viability was determined in vitro using the CCK8 assay following stimulation with anti-CD3 antibody. Data represent the mean ± SD (n = 3) or images of representative experiments. Similar results were obtained in three independent experiments; *, P < 0.05.
Low-dose DAC promotes Th1 polarization and CTL cytolytic activity. A, CD3⁺ T cells were sorted using anti-CD3 antibody by FACS from peripheral blood cells of healthy donors. Quantitative PCR analysis of a series of T/B cell activation-associated genes (Qiagen, PAHS-053Z RT² Profiler PCR Array Human T-Cell & B-Cell Activation) in 10 nmol/L DAC-treated T cells compared with control cells was performed, and the relative cytokine expression levels are shown. B and C, CD3⁺ T cells were sorted using anti-CD3 antibody by FACS from peripheral blood cells of healthy donors. Cells were treated with different doses of DAC in the presence of immobilized anti-CD3 antibody for 3 days, and the production of Th1 (IFNγ and TNFα, B) and Th2 (IL4, C) cytokines in the culture supernatant was detected by flow cytometry using the human Th1/Th2 cytokine CBA kit. D, CD3⁺ T cells were treated with DAC for 3 days, followed by re-stimulation with cell stimulation cocktail (including PMA, ionomycin plus protein transport inhibitors), and intracellular staining with antibodies against IFNγ and IL4. CD3⁺CD4⁺ cells (green dots, CD4 T cells) or CD3⁺CD8⁺ cells (violet dots, CD8 T cells) were gated, and the percentage of T cells expressing IFNγ or IL4 was analyzed by flow cytometry and shown. E and F, The percentage (E) and absolute number (F) of FoxP3⁺CD25⁺CD4⁺ Treg cells was determined by flow cytometry using the human Foxp3/CD25/CD4 FITC/PE/Cy7 CBA kit. G, The 10 nmol/L DAC-primed CD3⁺ T cells were treated with IFNγ neutralization antibody, and T-cell activation was evaluated using anti-CD69 antibody. H, The 10 nmol/L DAC-primed CD3⁺ T cells were treated with IFNγ neutralization antibody, and T-cell viability was assessed by the CCK8 assay. I, qRT-PCR analysis of the expression levels of IFNγ-induced genes is shown as indicated. J, The cytotoxicity of different doses of DAC-treated CD3⁺ T cells against SMMC7721 cells. K, Control or DAC-treated CD3⁺ T cells were co-cultured with SMMC7721 cells for 24 hours. IFNγ and TNFα secretion was detected using the Th1/Th2 cytokine CBA kit by flow cytometry. L and M, Control or 10 nmol/L DAC-treated CD3⁺ T cells were co-cultured and re-stimulated with SMMC7721 for 24 hours. The amounts of Granzyme B-expressed (L) or CD107a-expressed (M) cells were detected by flow cytometry. Data represent the mean ± SD (n = 3) or images of representative experiments. Similar results were obtained in three independent experiments; *, P < 0.05. N.S., not significant.
that low-dose DAC treatment promoted Th1 polarization and CTL cytolytic activity but not Treg cell activation.

The molecular mechanisms responsible for low-dose DAC-induced Th1/CTL activation were then investigated. Because IFNγ production played pivotal roles in the Th1/CTL response and was significantly increased by low-dose DAC treatment, we examined whether low-dose DAC-induced Th1/CTL responses were dependent on the production of IFNγ. As shown in Fig. 2G and H, low-dose DAC failed to promote human T-cell activation and proliferation when IFNγ was blocked using neutralizing antibodies against IFNγ. In addition, the downstream IFNγ-stimulated genes, such as IFI27, IFI44, IFIT3, IFITM1, PSMB9, and IRF1, were all induced by low-dose DAC treatment in human T cells (Fig. 2I). Thus, low-dose DAC-induced Th1/CTL responses were dependent on the enhanced IFNγ production.

The enhanced Th1 and CTL activation in response to low-dose DAC treatment inspired us to investigate the roles of these T cells in antitumor immunity. When co-cultured with human hepatocellular carcinoma SMMC7721 cells, low-dose DAC-treated human CD3+ T cells exhibited elevated cytolysis compared with control T cells (Fig. 2J and Supplementary Fig. S2A). In addition, the secretion of IFNγ and TNFα and the percentage of activated CTL cells also increased in low-dose DAC-treated T cells (Fig. 2K–M). Moreover, the increased T-cell cytolytic activity induced by low-dose DAC treatment was validated in several types of cancer cell lines as targets (Supplementary Fig. S2B). Thus, low-dose DAC-treated T cells inhibited cancer progression in vitro.

**Low-dose DAC-treated T cells inhibit cancer progression in vivo**

To examine the antitumor capacity of low-dose DAC-treated T cells in vivo, we used a mouse colon cancer CT26 cell tumor-bearing xenograft model. We found that treatment with low-dose DAC inhibited colon cancer growth and prolonged survival in mice (Fig. 3A and B). Strikingly, we observed that both the frequency and absolute number of CD3+ T cells were prominently increased in the spleen and peripheral blood following low-dose DAC treatment (Fig. 3C and Supplementary Fig. S3A–S3C). Moreover, the activation of T cells and their infiltration in cancer were significantly increased by low-dose DAC administration (Fig. 3D and E). Intracranial staining analysis suggested that the IFNγ-producing effector cells in the tumor draining lymph node (TDLN) and spleen were augmented in the low-dose DAC-treated group (Fig. 3F, Supplementary Fig. S3D and S3E). Thus, low-dose DAC treatment inhibited cancer progression and promoted antitumor T-cell responses in vivo.

To mimic human tumor development, we used the diethyl-nitrosamine (DEN)-induced hepatocellular carcinoma mouse model. As multiple liver tumors were developed in control-treated mice, low-dose DAC-treated mice presented liver tumors that were markedly decreased in number and size (Fig. 3G). Moreover, significantly greater number of T cells had infiltrated the tumor following low-dose DAC treatment, particularly increased IFNγ-producing Th1 and CTL cells in tumors, TDLN and spleen (Fig. 3H and Supplementary Fig. S3F). These results suggested that low-dose DAC treatment increased the antitumor properties of T cells and inhibited cancer progression in vivo.

To further confirm the suppression of cancer development by low-dose DAC treatment was dependent on its direct impact on T cells, we performed an adoptive transfer experiment of in vitro low-dose DAC-treated T cells. Remarkably, in a BALB/c mouse model carrying CT26 tumors, tumor development was inhibited to a greater extent by transferring 10 nmol/L DAC-treated T cells compared with control T cells (Fig. 3I and J). By labeled with CFSE, we observed more tumor infiltrated T cells with enhanced activation (analyzed by CD69 expression) and IFNγ-producing capacities in mice infusion with DAC-treated T cells, as compared with control T cells (Supplementary Fig. S3G and S3H). Moreover, to exclude the possibility of cancer immune activation, we constructed a CT26 or SMMC7721 tumor-bearing nude mouse model and found that the immunogenic properties of cancer tissues were not significantly altered by low-dose DAC treatment, including the expression of MAGE tumor antigens, MHC molecules or co-stimulatory factors (Fig. 3K and Supplementary Fig. S3I). Thus, low-dose DAC-induced activation of T lymphocytes was less likely due to enhanced cancer immunogenicity but to a direct impact on T lymphocytes.

**Improved antitumor T-cell activity in patients following low-dose DAC therapy**

Next, we explored whether low-dose DAC therapy could enhance the antitumor activity of T cells in patients with solid tumors. In our clinical trials of low-dose DAC therapy, DAC was administered at a dose of 7 mg/m² for 5 days of each 28-day treatment cycle (9, 19). We collected peripheral blood cells from patients before (Day 0) and after DAC administration (Day 6) in the first cycle and sorted CD3+ T cells. In patients with a good response to low-dose DAC therapy (UPN1 and UPN2), the number and viability of T lymphocytes was increased following low-dose DAC therapy (Fig. 4A and B). We also analyzed serum cytokine levels before and after DAC infusion in the first cycle. In UPN1 and UPN2, elevation of the cytokine IFNγ and TNFα, and reduced production of IL6 and IL4, were observed in both patients (Fig. 4C). Moreover, we also detected the enhanced T-cell proliferation and Th1 cytokine secretion in clinical benefit patients among the other 18 patients in the test cohort (Supplementary Fig. S4A–S4C). Consistently, increased numbers of IFNγ-secreting Th1 and CTL cells were observed (Fig. 4D). Next, we evaluated the antitumor cytotoxicity properties of T cells in low-dose DAC-treated patients. Using different tumor cell lines as target cells, we observed that the cytotoxicity of peripheral CD3+ T cells from these patients was significantly enhanced following low-dose DAC therapy (Fig. 4E). By comparing the T-cell cytotoxicity ability and clinical responses in a total of 20 patients (Test Cohort), we observed that patients with prominently enhanced T-cell cytotoxicity (n = 11) showed beneficial clinical responses to low-dose therapy [including complete response (CR), partial response (PR) and stable disease (SD)]. Moreover, in 6 patients with a SD clinical response, we did not observe marked cytotoxicity enhancement of T cells. For the other 3 patients with progressive disease (PD), there was no improvement in T-cell cytolytic ability (Fig. 4F and Supplementary Fig. S4D). Hence, low-dose DAC-activated T cells can inhibit cancer progression in patients. In addition, the increased frequency of IFNγ+ T cells may be important for the clinical effects in solid tumor patients (Fig. 4G). These data suggest that the cytotoxicity of peripheral T cells is enhanced following low-dose DAC-based therapy in solid tumor patients, which may be important for the response of tumor patients.
Figure 3.
Low-dose DAC-treated T cells inhibit cancer progression in vivo. A, Subcutaneous growth of CT26 tumors in BALB/c mice treated with 0, 0.05, or 0.2 mg/kg DAC as indicated from days 12 to 16 \((n = 8/\text{group})\). The arrows indicate the DAC treatments. B, Survival curves of CT26-bearing mice treated with different doses of DAC as indicated \((n = 8/\text{group})\). C, CT26-bearing mice were treated with PBS (Con group) or 0.05 mg/kg DAC (DAC group) for 5 days, and immunocytes were collected from splenocytes 10 days after DAC treatment and detected by flow cytometry using the indicated antibodies. The frequencies are shown as indicated. (Continued on the following page.)
An increased frequency of IFNγ+ T cells and T-cell cytotoxicity predict improved responses to low-dose DAC therapy

Because the increase in IFNγ+ T-cell frequency and T-cell cytotoxicity were determined to be important for the clinical effects of low-dose DAC therapy, we next examined the correlation between IFNγ+ T-cell frequency or T-cell cytotoxicity and the clinical response to low-dose DAC therapy in solid tumor patients. In the test cohort of 20 patients, patients with an increased frequency of IFNγ+ T cells or T-cell cytotoxicity following low-dose DAC therapy demonstrated a significantly higher disease control rate (DCR) and objective response rate (ORR) compared with patients without a significant increase in IFNγ+ T cells or T-cell cytotoxicity (Fig. 5A). In addition, patients with an increased frequency of IFNγ+ T cells or T-cell cytotoxicity had both improved PFS and OS following low-dose DAC therapy compared with patients without a significant increase in IFNγ+ T cells or cytotoxic activity (Fig. 5B–E). These data suggest that the increase in IFNγ+ T cells or cytotoxicity in T cells is significantly correlated with the response to low-dose DAC therapy. We also included a validation cohort of 38 patients with refractory/recurrent ovarian cancer and determined that patients with an increased frequency of IFNγ+ T cells or T-cell cytotoxicity following low-dose therapy had significantly higher DCR and ORR (Supplementary Table S2 and Fig. S5F), and improved PFS and OS compared with patients without a significant increase in IFNγ+ T cells or cytotoxicity (Fig. 5G–I). Further statistical analysis showed that the enhanced T cell function was correlated with the best clinical response to DAC therapy in both test cohort and validation cohort, while no significant correlation was observed in other parameters (Supplementary Tables S3 and S4). Thus, these data suggest that the increased IFNγ+ T cell frequency or T-cell cytotoxicity was positively correlated with the clinical response to low-dose DAC therapy in solid tumor patients.

Detection of peripheral IFNγ+ T-cell frequency following low-dose DAC treatment in vitro predicts the clinical response to low-dose DAC therapy in solid tumor patients

To present a very predictable pattern for the response to low-dose DAC therapy in solid tumor patients, we evaluated the effects of in vitro low-dose DAC treatment on peripheral T cells from solid tumor patients. The peripheral CD4+ T cells from solid tumor patients before DAC therapy were treated with low-dose DAC in vitro, and the frequencies of IFNγ+ T cells were measured. We defined increased IFNγ+ T cells as the ΔIFNγ+ T-cell frequency >10%, and found that patients with an increased frequency of IFNγ+ T cells following in vitro low-dose DAC treatment had significantly higher levels of DCR and ORR than patients without a significant increase in IFNγ+ T cells in both the Test Cohort and the Validation Cohort (Fig. 6A). In addition, patients with an increased frequency of IFNγ+ T cells with in vitro DAC treatment showed improvements in both PFS and OS than patients without a significant increase in IFNγ+ T cells in both the Test Cohort and the Validation Cohort (Fig. 6B–E). Importantly, the changes in IFNγ+ T-cell frequency after in vitro DAC therapy were positively correlated with those following in vitro DAC treatment in both cohorts (Fig. 6F and G). In addition, univariate and multivariate Cox regression identified that the increased IFNγ-producing T-cell frequency with in vitro DAC treatment might be associated with longer PFS and OS in both test cohort and validation cohort (Supplementary Tables S5 and S6). Therefore, pre-detection of the IFNγ+ T-cell frequency following low-dose DAC treatment in vitro may be a useful approach to predict and determine the clinical response to low-dose DAC therapy in solid tumor patients (Fig. 6H).

Discussion

DNMT inhibitors including DAC have been widely studied in antitumor treatment. Currently, low-dose DAC therapy has been used for the treatment of both blood neoplasms and solid tumors, demonstrating effectiveness and less toxicity (9–11). Concerning the mechanisms of low-dose DAC therapy, previous reports have determined that it induces the expression of genes involved in cell cycle arrest, apoptosis, DNA damage responses, defense responses, antigen exposure and lineage commitment, leading to decreased tumorigenicity and self-renewal. However, these previous anti-tumor effects of DAC were mainly focused on its influence on cancer cells; it is still unknown whether low-dose DAC therapy has a direct effect on T cell-mediated antitumor responses. Here, we found that low-dose DAC therapy could directly enhance the activation and expansion of IFNγ-producing Th1 and CTL cells. Furthermore, increased numbers of IFNγ-producing T cells following DAC therapy was suggested to be an effective predictor of improved responses to low-dose DAC therapy in solid tumor patients. Thus, we presented the important roles of low-dose DAC therapy on host antitumor immune responses in this study.

IFNγ is a Th1 cytokine and plays critical roles in driving naive CD4+ T cells toward a Th1 cell phenotype. Here, we found that low-dose DAC therapy significantly enhanced the numbers of IFNγ-producing T cells. Th2 type cytokine production was suppressed, and CD4+ CD25+ Foxp3+ Treg cells were also inhibited, which might be mediated by the enhanced Th1 and CTL activation because IFNγ production is known to block the expansion of Th2

(Continued) D and E, Numbers (D) and immunohistochemical (IHC) analysis (E) of the tumor infiltration of CD3+ T cells in CT26 colon cancer tissues from PBS or 0.05 mg/kg DAC-treated mice are shown (n = 8/group). F, Tumor-draining lymph nodes (TDLN) from control or 0.05 mg/kg DAC-treated mice were collected after 2 weeks of treatment with ex vivo cell stimulation cocktail for 4 h. Flow-cytometry analysis was performed to detect CD3+PerCP, CD4+APC, IFNγ-PE and Granzyme B-FITC. G, DAC-treated mice displayed decreased numbers of tumor nodules. The mice were sacrificed at 4 weeks after the initial DAC treatment, which was approximately 11.5 months after DEN injection. The results were displayed as dots representing the numbers of tumor nodules per mouse (n = 8). The maximum tumor nodule diameter per mouse was measured. H, TILs from DEN mice were collected as in (G), which were stimulated ex vivo with cell stimulation cocktail for 4 h. Flow-cytometry analysis was performed to detect CD3+PerCP, CD4+APC and IFNγ-PE. I and J, Tumor sizes (I) and survival curves (J) for CT26-bearing mice that received PBS (Con), control T cell (5 × 10⁶) transfections (T) or 10 nmol/L DAC-treated T cell (5 × 10⁶) transfections (DAC; n = 8/group). K, Human hepatocellular carcinoma SMMC7721 cells were injected subcutaneously into nude mice and treated with control or 0.05 mg/kg DAC for 5 days. The expression levels of CTAs (MAGE A1/A3, NY-ESO-1), co-stimulatory molecules (CD80, CD86), and MHC molecule (B2M) in tumor cells were detected by qRT-PCR at 10 days after DAC treatment. Data represent the mean ± SD (n = 3) or images of representative experiments. Similar results were obtained in three independent experiments; *, P < 0.05. N.S., not significant.
Figure 4.
Improved antitumor T-cell activity in patients following low-dose DAC therapy. A, Peripheral CD3+ T cells from solid tumor patients before and after DAC infusion in the first cycle were sorted by FACS using anti-CD3 antibody, and the numbers of T cells are shown. These cells were used for the functional analysis as in (B-G). B, T-cell proliferation in patients was measured using the CCK8 assay before and after DAC treatment during the first cycle. The representations (UPN1 and UPN2) are shown. C, Cytokine production in the sera from patients before and after DAC treatment during the first cycle was detected using the human Th1/Th2 cytokine CBA kit by flow cytometry. D, Intracellular IFNγ staining was used to identify CD4+ and CD8+ T cells in representative patients. E, The cytotoxicity of peripheral T cells before and after DAC therapy against tumor cells, including WT and drug-resistant colon cancer tumor cells as indicated. F, The cytotoxicity of peripheral T cells before and after DAC therapy during the first cycle against HCT116 tumor cells was determined in all 20 patients. Cytolysis = post cytotoxicity – pre cytotoxicity. “*” refers to a beneficial clinical response (including a complete response, partial response, and stable disease), and “/C0” refers to progressive disease. Cytolysis >10% (the average cytolyis of the 20 patients) refers to enhanced cytolysis. G, The percentages of IFNγ-producing CD8 T and CD4 T cells were detected by flow cytometry. “*” refers to a beneficial clinical response (including a complete response, partial response and stable disease), and “/C0” refers to progressive disease. Data represent the mean ± SD (n = 3) or images of representative experiments. Similar results were obtained in three independent experiments; *, P < 0.05.
and Treg cells (22, 23). In addition, CD4⁺ T cells can differentiate into Treg cells in the presence of TGF-β, and DAC has been reported to suppress TGF-β signaling (16, 24). Moreover, although the frequency and absolute number were not markedly altered, the function of other immune cells, such as macrophage or DCs might be influenced following low-dose DAC treatment, probably due to changed profiles of cytokines or tumor antigens. Thus, the roles for epigenetic modifying agent may be multifaceted, and meaningful antitumor immune responses were induced by low-dose DAC therapy, although whether and how low-dose DAC therapy alters the immunosuppressive microenvironment in tumors as well as other immune cells requires further investigation. Interestingly, we also found that low-dose DAC treatment showed growth-promoting effects in normal human T cells but not T-cell leukemia cell lines. Because increasing evidence has suggested that DAC-regulated genes are different depending on the cell types and concentrations (25), the distinct genomic or epigenomic profiles of normal T cells and T leukemia cells may

Figure 5.
Increased frequency of IFNγ⁺ T cells and cytotoxicity of T cells following 5 days of in vivo DAC therapy predict a good response to low-dose DAC therapy in solid tumor patients. A, The correlation between clinical responses and peripheral T-cell activity in the Test Cohort (frequency of IFNγ⁺ T cells and T-cell cytotoxicity). P values were calculated using the χ² test in SPSS 18.0. Because the average cytotoxicity in the Test Cohort was 10%, we defined an increased IFNγ⁺ T or cytotoxicity as an IFNγ⁺ T-cell frequency or cytotoxicity after DAC therapy greater than 10% higher compared with before DAC therapy. The absence of an increase in IFNγ or cytotoxicity indicated that the frequency of IFNγ⁺ T cells or cytotoxicity after DAC therapy were less than 10% higher or lower than the values before DAC therapy. B and C, Kaplan-Meier survival curves of PFS (B) and OS (C) based on the frequency alteration of peripheral IFNγ⁺ T cells in the Test Cohort. Ten percentages higher was used as the cutoff, using the log-rank test to evaluate significance. D and E, Kaplan-Meier survival curves of PFS (D) and OS (E) based on the cytotoxicity alteration of peripheral T cells in the Test Cohort, using the same cutoff value and statistical analysis as described in (B, C). F, Correlation between clinical responses and peripheral T-cell activity in the Validation Cohort (frequency of IFNγ⁺ T cells and T-cell cytotoxicity). P values were calculated using the χ² test in SPSS 18.0. G and H, Kaplan-Meier survival curves of PFS (G) and OS (H) based on the frequency alteration of peripheral IFNγ⁺ T cells in the Validation Cohort, using the same cutoff value and statistical analysis as described in (B, C). I and J, Kaplan-Meier survival curves of PFS (I) and OS (J) based on the cytotoxicity alteration of peripheral T cells in the Validation Cohort, using the same cutoff value and statistical analysis as described in (B, C).
contribute to the distinct effects mediated by DNMT inhibitors. For example, we observed that the expression levels of cell cycle-related proteins, such as p21 and p15, were increased in Jurkat T leukemia cells following low-dose DAC treatment, but the expression of these genes was slightly reduced in normal human T cells (Supplementary Fig. S1I). Therefore, low-dose DAC therapy may favor the enhancement of antitumor T-cell responses but not the proliferation of cancer cells.

Low-dose DAC therapy has been determined to have promising therapeutic effects for some types of solid tumors, such as ovarian
cancer, colorectal cancer, and esophageal cancer (8–10). However, only a subgroup of patients has demonstrated a good response to low-dose DAC therapy, and thus the identification of biomarkers for the prediction of responses to low-dose DAC therapy is of prominent importance but remains unsolved. Substantial research has focused on the changes in DNA methylation and gene expression in tumor cells or tissues during epigenetic therapy, or the basal levels of DNA methylation of specific genes in tumors. Although both global DNA demethylation and the reactivation of some genes have been determined, such as p15, p16, MLH1, and BRCA1 (26), the relationship between the demethylation of specific genes in tumors and the responses to low-dose DAC therapy was identified to be ineffective or even not determined. Since the doses of DAC and changes in specific genes in tumors were poorly correlated with the responses of patients, we focused on the effects of low-dose DAC therapy on antitumor immune responses and attempted to analyze whether the enhance numbers of IFN-$\gamma$ T cells was correlated with the response group. On the basis of our clinical trials of low-dose DAC therapy, we found that both an increased frequency of IFN-$\gamma$ T cells and cell cytotoxicity determined in the peripheral blood of patients following the first cycle of DAC therapy were significantly correlated with better clinical responses and improved survival after low-dose DAC therapy. Moreover, we found that in vitro low-dose DAC treatment in peripheral T cells from solid tumor patients exhibited similar effects compared with low-dose DAC therapy in vivo, indicating that the pre-detection of T-cell responses to low-dose DAC treatment in vitro would guide the therapeutic regimen in solid tumors. These data suggest that the detection of peripheral T-cell alterations in response to low-dose DAC in vitro may be a useful approach for the prediction of responses to low-dose DAC therapy. However, why T cells from the other subgroup of patients respond poorly to low-dose DAC therapy remains unknown. Next, we will focus on the mechanisms underlying this phenomenon and hope to define new approaches to enhance low-dose DAC-induced T-cell antitumor immune activation.

In conclusion, we have examined the effects of low-dose DAC therapy on host antitumor immune responses, including enhanced T-cell expansion and activation, increased IFN-$\gamma$-producing Th1 and CTL cells and augmented T-cell cytotoxicity, which were correlated with clinical responses following low-dose DAC therapy in solid tumor patients.

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

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