



Published in final edited form as:

Cancer Res. 2006 January 15; 66(2): 1105–1113.

De novo Induction of a Cancer/Testis Antigen by 5-Aza-2'-Deoxycytidine Augments Adoptive Immunotherapy in a Murine Tumor Model

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Abstract

Recent studies suggest that immunotherapy targeting specific tumor-associated antigens (TAAs) may be beneficial in cancer patients. However, most of these TAAs are tumor type specific and heterogeneous among patients, thus limiting their applications. Here, we describe the *de novo* induction of a cancer/testis antigen (CTA) for immunotherapy of tumors of various histologies. The murine CTA P1A, normally expressed only in a few tumor lines, could be induced *de novo* in all P1A-negative cancer lines of eight histologic origins *in vitro* and in various murine xenografts by systemic administration of 5-aza-2'-deoxycytidine. The induction of P1A expression correlated strongly with demethylation of the CpG island in the promoter region of this gene. The induced antigen was processed and presented properly for recognition by H-2L^d-restricted P1A-specific CTLs. The combination of a demethylating agent and adoptive transfer of P1A-specific CTL effectively treated lung metastases in syngeneic mice challenged with P1A-negative 4T1 mammary carcinoma cells. These data show a novel strategy of combined chemoimmunotherapy of cancer targeting a CTA induced *de novo* in a broad range of tumor histologies, and support further evaluation of chromatin-remodeling agents for human cancer therapy.

Introduction

Pioneering work a decade ago showed the existence of human tumor-associated antigens (TAAs) using patient CTLs that recognized peptides derived from these antigens (1–3). However, the scarcity of clinically significant tumor-specific immune responses in cancer patients had cast doubt for many years that antigen-specific immunotherapy would play an important role in treating human cancer. Although earlier studies focused on melanoma, TAAs that react with T cells have been characterized in several other types of cancer (1–3), suggesting that most if not all tumors express antigens that allow recognition and attack by antigen-specific CTLs. Consequently, clinical efforts proceeded to target these TAAs in using vaccination and adoptive T-cell therapy in cancer patients (4–9). Recently, adoptive T-cell therapy has achieved

significant clinical results, including cancer regression in patients with metastatic melanoma (10,11).

Unfortunately, the expression of most known TAAs that are reactive with autologous T cells is restricted to one or a few types of tumors and to a fraction of patients with these malignancies and the expression can vary among metastases obtained from the same patient. Immune selection of antigen loss variants may be an additional obstacle for targeting most known tumor antigens for cancer immunotherapy. In addition, immune tolerance is one of the major obstacles in immunotherapy. This may be related to low levels of antigen expression in solid tumors (12). Due to these factors, clinical studies have progressed slowly because strategies have been tested one malignancy at a time and, in some cases, patient by patient (13). To circumvent these obstacles, investigators have attempted to find universal TAA that could trigger CTL responses against a broad range of tumor types (14).

To address some of these important issues, we have turned our attention to cancer/testis antigens (CTA). The cancer/testis genes are regulated, at least in part, by epigenetic mechanisms. DNA methylation has been identified as one of the predominant epigenetic mechanisms to modulate gene expression in cancer, aging, and normal development (15–17). Patterns of DNA methylation and chromatin structure are profoundly altered in neoplasia, which include genome-wide losses of and regional gains in DNA methylation. CTAs are expressed in a wide range of human malignancies (3). Genes encoding CTAs are expressed in a stage-specific manner in germ cells yet are strictly silenced in normal somatic cells (17). During malignant transformation, cancer/testis genes are derepressed via complex epigenetic mechanisms (18,19). Numerous cancer/testis genes map to the X-chromosome and encode proteins, such as MAGE-3 and NY-ESO-1, that are recognized by CTL from cancer patients (3). Despite the fact that most human malignancies simultaneously express multiple CTAs, immune response to those antigens seems limited. In part, this is due to levels of expression that appear below the threshold for immune recognition *in vivo* (20,21). Conceivably, innovative treatment regimens that enhance CTA expression in primary malignancies may facilitate the development of efficacious immunotherapy protocols with broad applicability in cancer patients (22). Our group has shown previously that NY-ESO-1 and MAGE-3 can be induced *in vitro* in thoracic cancer cell lines by 5-aza-2'-deoxycytidine (5-azadC) alone or in combination with the histone deacetylase inhibitor depsipeptide (23–25). Others have also shown that MAGE antigens and LAGE-1 can be induced by 5-azadC in certain cancer cell lines *in vitro* (26,27).

Recently, we utilized murine models to address many basic scientific questions regarding the induction of CTAs and their suitability as targets for cancer immunotherapy. The mouse CTA P1A, originally identified in mastocytoma P815 cells, is encoded by a single gene located in the X-chromosome (28,29). A single peptide named P1A 35–43 (NH₂-Leu-Pro-Tyr-Leu-Gly-Trp-Leu-Val-Phe-COOH) is presented to anti-P815 CTL clones by MHC H-2L^d molecules (29). P1A is a nonmutated self-protein expressed in mastocytoma P815 and in several other tumors. It was unclear why P1A is expressed at high levels in testes and a few cancer lines. Previous studies suggested that P1A is silent in normal tissues, except testis and placenta (28,30), a generalized concept for CTAs. However, recent meticulous studies have indicated that P1A is expressed at extremely low levels in normal tissues, including hematopoietic cells (31) and medullary thymic epithelial cells, along with a wide range of tissue-specific antigens (32). These low expression levels, however, do not prevent safe induction of CTL against P1A-expressing tumors (30). Immunization with P1A-expressing vaccinia virus or tumor cells can induce CTL that provide protection against challenge of P1A-expressing tumors (33) indicating that P1A can function as a tumor rejection antigen (34).

Here, we describe a novel phenomenon of inducing the CTA P1A *de novo* in tumors of multiple histologies, and show that the induced CTA can be effectively used as a target for adoptive immunotherapy of cancer in a murine tumor model. The results of these studies suggest that combined chemoimmunotherapy may represent a novel strategy for human cancer treatment.

Materials and Methods

Cell lines

Most tumor cell lines were obtained from American Type Culture Collection (Manassas, VA), or were available in cell line repositories at the National Cancer Institute (NCI). 4T1 mammary tumor cells (35) were obtained from Dr. Fred Miller (Wayne State University, Detroit, MI). Tumor cells were propagated *in vitro* as recommended by the respective providers.

Tumor cells treated with 5-azadC

5-AzadC (Sigma Chemical Co., St. Louis, MO) was dissolved as 1.0 mmol/L stock solution in HBSS and stored at -20°C . 5-AzadC was added to tissue culture medium daily at a final concentration of 1.0 $\mu\text{mol/L}$ for 48 hours unless specified otherwise. Drug-treated cells were cultured for an additional 24 hours in normal medium before harvesting for analysis of P1A expression.

Reverse transcription-PCR reactions

Total RNA was prepared using an RNeasy mini kit (Qiagen, Inc., Valencia, CA). Reverse transcription was done using a Reverse Transcription System at the suggested conditions (Promega, Madison, WI). One microgram of total RNA was used in each 20 μL reaction. PCR was done using the following primers and thermal cycle conditions: 5'-CCCTTCATTGACCTCAACTACATGG-3' [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward], 5'-CCTGCTTCACCACCTTCTTGATGTC-3' (GAPDH reverse), 5'-CGGAATTCTGTGCCATGTCTGATAACAAGAAA-3' (P1A forward), 5'-CGTCTAGATTGCAACTGCATGCCTAAGGTGAG-3' (P1A reverse), $94^{\circ}\text{C} \times 5$ minutes ($94^{\circ}\text{C} \times 1$ minute, $58^{\circ}\text{C} \times 1$ minute, and $72^{\circ}\text{C} \times 1$ minute) $\times 30$ cycles, and $72^{\circ}\text{C} \times 7$ minutes. PCR products were separated on 1% agarose gels and visualized by ethidium bromide techniques.

Methylation-specific PCR

Methylation status of a CpG island in the 5' regulatory region of the P1A gene was evaluated by methylation-specific PCR techniques as described by Herman et al. (36) using CpGenome DNA modification and CpG Wiz amplification kits (Serologicals Corp., Norcross, GA). CpG WareTM software (Serologicals) was used to design the following PCR primers that would specifically amplify methylated or unmethylated templates following bisulfite modification of genomic DNA: 5'-TTAAGTGC GTTATTACGTTTGGTTTTTAC-3' (methylated forward), and 5'-ATAACCGATTATTTAATACAAAAATCGACG-3' (methylated reverse), 5'-GATTAAGTGTGTTATTATGTTTGGTTTTTAT-3' (unmethylated forward), and 5'-ACATAACCAATTATTTAATACAAAAATCAACA-3' (unmethylated reverse). The methylation-specific PCR thermal cycle conditions were 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 30 seconds, for a total of 40 cycles. The PCR products were analyzed by gel electrophoresis with 2.0% agarose gel.

Generation of CTL

Initially, H-2L^d-restricted CTL recognizing P1A or β -galactosidase were generated by vaccinating BALB/c mice twice with 2×10^7 plaque-forming units/mouse of vv.ES-P1A or vv.lacZ, as described (33). Splenocytes from immunized mice were isolated and pulsed with

1.0 $\mu\text{mol/L}$ of L^d -restricted synthetic peptides derived from P1A (P1A 35–43: LPYLGWLVF) or β -galactosidase protein (β -galactosidase 876–884:TPHPARIGL). These cells were cultured *in vitro* for 1 week before adding rhIL-2 (Chiron Corp., Emeryville, CA) to the medium for T-cell expansion. The specificity of the CTLs was confirmed by assaying their activities against appropriate targets. Later in the study for immunotherapy *in vivo*, P1A-specific CTLs were generated from splenocytes isolated from P1A_{35–43} T-cell receptor transgenic mice (31). These splenocytes were pulsed with 1.0 $\mu\text{mol/L}$ of the H-2L^d-restricted peptide (P1A 35–43). Three days later, rhIL-2 was added to growth medium for T-cell expansion.

Cytotoxicity assays

The cytotoxicity of H-2L^d-restricted P1A and β -galactosidase CTL was measured in an improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described by Ribeiro-Dias et al. (37). Briefly, target cells were cultured in normal medium with or without 1.0 $\mu\text{mol/L}$ 5-azadC for 48 hours and were rested for 24 hours. Normal splenocytes were used as controls. Target cells (1×10^4) in 50 μL of culture medium were added to effector cells suspended in 50 μL culture medium in 96-well flat-bottomed culture plates at effector-to-target cell ratios of 0.2:1, 1:1, 5:1, and 25:1. After 16 hours of incubation at 37°C, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Promega), was added to each well, and the absorbance at 492 nm was measured 1 hour later. Percent lysis was calculated as follows: $[1 - \{(\text{absorbance of effector} + \text{target cells}) - (\text{absorbance of effector cells})\} / (\text{absorbance of target cells})] \times 100$.

IFN- γ release assays

Target cells were prepared as described for cytotoxicity assays. Five microliters of 10^4 target cells in 100 μL culture medium were added to effector cells in 100 μL medium in 96-well flat-bottomed culture plates at an effector-to-target cell ratio of 1:10. Following 16 hours incubation at 37°C, culture supernatants were harvested and IFN- γ levels were determined using a murine IFN- γ ELISA kit (Pierce, Rockford, IL).

S.c. tumor models

Female BALB/c and C57BL/6 (B6) mice (6 weeks of age) were purchased from the NCI-Frederick facility (Frederick, MD). Lewis lung carcinoma (LLC), B16, MC38, MCA102, or 4T1 tumor cells grown in log phase were washed thrice in cold HBSS, and 1.0×10^5 cells in 100 μL HBSS were injected s.c. into the flank of syngeneic mice (day 0). Commencing on day 10, 5-azadC (1.0 mg/kg body weight or other specified concentrations in 100 μL HBSS) or HBSS alone was injected i.p. twice daily for 5 consecutive days. Two days after completion of drug treatments, mice were euthanized and tumors as well as a variety of normal tissues were collected for analysis of *P1A* gene expression.

4T1 Mammary tumor lung metastasis model and treatment

On day 0, 2.0×10^5 4T1 mammary tumor cells in 200 μL HBSS were injected into tail veins of syngeneic BALB/c mice. Commencing on day 7, mice were injected i.p. with 5-azadC (0.8 mg/kg body weight) or HBSS once daily for 6 consecutive days. Subsequently, the mice were rested for 1 day. On day 13, 1×10^6 H-2L^d-restricted P1A-specific CTL or β -galactosidase-specific CTL were injected i.v.; rhIL-2 (50 K CU/mouse) was administered i.p. twice during the first 24 hours. On day 21, mice were euthanized, and mediastinal organs harvested. Lungs were perfused with 15% India ink solution, and metastases were enumerated. For doses of 5-azadC to be used in mice, maximal tolerable doses vary among different strains of mice and need to be determined empirically.

Statistics

All data from animal experiments were analyzed by using Student's *t* test (SigmaPlot), where $P < 0.05$ indicated that the value of the test sample was significantly different from that of the relevant controls.

Results

P1A gene induction by 5-azadC is dose and time dependent

Initial screening by reverse transcription-PCR (RT-PCR) indicated that P1A was expressed in only 5 of 21 tumor cell lines (Table 1). To examine if P1A could be induced *de novo* in cultured cells, a P1A-negative tumor cell line LLC was treated with 5-azadC at varying doses and exposure durations. As shown in Fig. 1, LLC cells grown under normal conditions did not express P1A. Under 48-hour exposure conditions, 0.3 $\mu\text{mol/L}$ 5-azadC was sufficient to induce P1A in LLC cells; higher levels of P1A expression were observed with increasing doses of 5-azadC (Fig. 1A). A significant percentage of cells exhibited growth arrest or apoptosis following treatment with 3 or 10 $\mu\text{mol/L}$ 5-azadC, a phenomenon observed previously (38). As such, a dosage of 1.0 $\mu\text{mol/L}$ 5-azadC was chosen for additional *in vitro* studies.

The relationship between duration of drug exposure and P1A gene expression was also investigated. At a dose of 1.0 $\mu\text{mol/L}$, 6-hour 5-azadC exposure was sufficient to induce P1A expression; longer exposure times increased the expression of this CTA until 48 hours when the level of gene induction seemed to plateau (Fig. 1B). These results showed that 5-azadC induced P1A expression *de novo* in a dose- and time-dependent manner in LLC cells.

The persistence of induced P1A antigen expression was also examined in 5-azadC-treated cells. LLC cells were mock-treated or treated with 5-azadC at 1.0 $\mu\text{mol/L}$ for 48 hours. Then, drug was removed and cells were washed with $1 \times$ PBS and fed with fresh growth medium and split when necessary. Aliquots of cells were taken at different time points and stored until analysis. As shown in Fig. 2, P1A was peaked within 2 to 6 days and remained at significant levels for 1 month. Its expression gradually reduced to background level in 2 months. These results showed that the induced P1A expression was quite stable for several weeks, which might be a sufficient time window for immunotherapy targeting this CTA.

De novo induction of P1A *in vitro* is universal

We were interested in knowing whether *de novo* P1A induction as seen in LLC cells was universal among various types of tumors. Therefore, 21 murine tumor cell lines representing cancer of eight different histologies were selected for study. These tumor cells were untreated or treated with 5-azadC at 1.0 $\mu\text{mol/L}$ for 48 hours. The results obtained with RT-PCR are summarized in Table 1. Under normal growth conditions, no P1A expression was detected by RT-PCR, except five tumor lines—EL4, TIMI.4, Hepa1-6, A20, and CA51, which naturally expressed P1A at very low levels. It is interesting to note that three of the five P1A weakly positive cell lines are lymphomas, and only 2 of 16 tumor lines derived from other solid tumors expressed P1A. Treatment with 5-azadC induced P1A expression to significant levels in all P1A-negative tumor lines and further enhanced P1A expression in those P1A weakly positive tumor cell lines. These results were consistent with induction of CTAs in cultured human cancer cells (23–27).

P1A induction correlates with demethylation of the P1A promoter region

A series of experiments were done to ascertain if 5-azadC mediated induction of P1A via direct modulation of chromatin structure within the promoter region of P1A. Using the generally accepted definition (39,40) and the CpG Plot software online,⁵ a region extending from nucleotides -798 to -551 that fulfilled current criteria for CpG island was identified ~300 bp

proximal to the major initiation site of transcription in the gene (41). Methylation-specific PCR assays were used to examine the status of this CpG island in untreated as well as 5-azadC-treated tumor cells. Results are depicted in Fig. 3. PCR products corresponding to methylated template were detected in bisulfite-treated genomic DNA from normal murine liver cells that do not express P1A. In contrast, unmethylated template was detected in P815 cells that express high levels of P1A. From cells grown under normal conditions, methylated as well as unmethylated templates were observed in CA51 cells that normally exhibit very low level P1A expression. The P1A CpG island seemed to be completely methylated in P1A-deficient MC38 and 4T1 cells. When the three cancer lines were treated with 5-azadC, a concurrent switch from hypermethylation to hypomethylation of the CpG island with induction of P1A was noted. Collectively, these results strongly suggest that 5-azadC mediates P1A induction via direct chromatin remodeling mechanisms targeted to the 5' regulatory region of this cancer/testis gene.

5-AzadC-treated cancer cells were recognized specifically by H-2L^d-restricted P1A-specific CTL

Expression of P1A, as well as the integrity of antigen processing and presentation pathways in 5-azadC-treated tumor cells, were assessed in two functional assays using P1A epitope-specific CTL. First, CTL recognition of tumor cells was assessed by cytokine release assays. When cultured with H-2L^d-negative tumor cells, P1A-specific CTL released very little IFN- γ regardless of whether target cells were treated with 5-azadC (Fig. 4A). In contrast, when cultured with untreated H-2L^d-positive P815 cells expressing high levels of P1A, H-2L^d-restricted P1A-specific CTL released a significant amount of IFN- γ (Fig. 4B). Much less IFN- γ release was observed when these CTL were cultured with CA51 or A20 cells (Fig. 4B). Furthermore, very little IFN- γ was detected when M109, CT26, or 4T1 cells were used as targets. Increased levels of IFN- γ release (2,000 to 16,000 pg/mL) were detected following culture of P1A-specific CTL with 5-azadC-treated 4T1, CA51, A20, or P815 cells (Fig. 4B). In contrast, minimal (<20 pg/mL) IFN- γ release was detected when these 5-azadC-treated targets were incubated with H-2L^d-restricted β -galactosidase-specific CTL (data not shown). These results were consistent with P1A expression data derived from RT-PCR experiments (Table 1). Interestingly, very little IFN- γ release was observed when P1A-specific CTLs were cultured with 5-azadC-treated M109 or CT26 cells, despite induction of P1A in these targets (Table 1). Whereas the mechanisms responsible for this phenomenon were not fully investigated, these results may have been attributable to deficiencies regarding antigen processing/presentation that are known to occur frequently in cancer cells (42).

Cytotoxicity assays were next done to further examine recognition of 5-azadC-treated tumor cells by the P1A-specific CTL (Fig. 5). Two H-2L^{d+} cancer lines (A20 and 4T1) were selected for this analysis with MC38 (H-2L^{d-}) and P815 (H-2L^{d+}) serving as negative and positive controls, respectively, for MHC class I expression. CTL-mediated cytotoxicity was observed only at high effector-to-target cell ratio when untreated A20 cells, which naturally express low levels of P1A (Table 1), were used as targets. However, when A20 cells were treated with 5-azadC, high-level cytotoxicity was observed even at a low effector-to-target cell ratio (Fig. 5A). No cytotoxicity was noted when untreated P1A-negative 4T1 cells were used as targets. In contrast, when 4T1 cells were treated with 5-azadC, an enhancement of CTL-mediated cytotoxicity was observed at the highest effector-to-target cell ratio. Little if any specific activity was observed against MC38 cells (H-2L^{d-}), irrespective of drug treatment. High levels of CTL-mediated cytotoxicity was observed against P815 cells (H-2L^{d+} and P1A+); this cytotoxicity was further augmented by pretreatment of these target cells with 5-azadC (Fig. 5D). Collectively, these data showed that 5-azadC-induced P1A was properly processed and

⁵<http://www.ebi.ac.uk/emboss/cpgplot/>.

presented in most H-2L^d-positive cancer cells enabling their recognition by L^d-restricted CTL specific for this CTA.

De novo induction of P1A in tumor xenografts

Logically, we then asked if P1A would be induced in P1A-negative tumor cells *in vivo*. Preliminary dose-escalation experiments were conducted with C57BL/6 mice bearing syngeneic, s.c. LCC xenografts to examine 5-azadC-mediated toxicity and P1A induction. Mice were divided into four groups (five per group) and treated with 5-azadC at 0, 0.4, 2.0, or 10 mg/kg body weight administered i.p. twice daily for 5 consecutive days. Forty-eight hours after completion of 5-azadC treatment, mice were euthanized and tumor xenografts as well as various normal tissues were collected for further examination. Throughout the duration of the experiment, mice were observed frequently for signs of systemic toxicity. All of the five animals receiving the maximal 5-azadC dose exhibited significant toxicity with three mice dying before completion of the experiment. However, only mild toxicity was observed in animals receiving 5-azadC at a dose of 2.0 mg/kg. No toxicity was observed at doses of 0 and 0.4 mg/ kg. RT-PCR analysis revealed no P1A expression in tumor xenografts from mice treated with HBSS, results that were consistent with *in vitro* data (Table 1). P1A expression in tumor xenografts from 5-azadC-treated animals increased in a dose-dependent manner, a phenomenon we observed *in vitro* (data not shown). On the basis of toxicities and levels of P1A induction, a dose of 5-azadC ranging from 0.5 to 1.5 mg/kg body weight was selected for use in subsequent studies.

Additional experiments were done to examine if the 5-azadC treatment was sufficient to mediate P1A induction in cancer cells of various histologies *in vivo*. Five P1A-negative cancer lines, comprising melanoma (B16), lung carcinoma (LLC), colon carcinoma (MC38), sarcoma (MCA102), and mammary adenocarcinoma (4T1) were grown as s.c. xenografts in syngeneic BALB/c or C57BL/6 mice. Eleven days after inoculation, mice with tumor xenografts ~5 × 5 mm in size were treated with 5-azadC at 1.5 mg/kg using the treatment regimen described above. P1A expression in tumor and normal tissues was examined using RT-PCR techniques. As shown in Fig. 6A, no P1A expression was detected in xenografts from mice treated with HBSS or untreated controls. In contrast, P1A was expressed in all of the xenografts from mice treated with 5-azadC.

The 4T1 tumor model was used to examine P1A expression in normal tissues relative to tumor xenografts following 5-azadC exposure (Fig. 6B). As anticipated, no P1A expression was detected in tumors from control mice. However, P1A expression was readily detected in tumor tissues from 5-azadC-treated animals. The 5-azadC treatment regimen did not induce P1A expression in a variety of normal tissues, including heart, kidney, liver, or lung. However, low-level P1A expression was observed in bone marrow from 5-azadC-treated mice. Collectively, these results indicated that P1A could be induced in murine tumor xenografts of diverse histologies using a tolerable 5-azadC treatment regimen. Under these treatment conditions, induction of P1A *in vivo* was restricted primarily to cancer cells.

Systemic 5-azadC administration followed by infusion of H-2L^d-restricted P1A CTL effectively treated naturally P1A-negative 4T1 tumors

Finally, experiments were done to ascertain the effects of combining the 5-azadC treatment regimen with i.v. infusion of P1A-specific CTL in mice bearing P1A-negative tumors. A well-established lung metastasis model was used to examine this issue. 4T1 mammary carcinoma cells are poorly immunogenic and highly metastatic in syngeneic BALB/c mice. These cells exhibit *in vivo* growth characteristics resembling human metastatic breast carcinoma and are typically refractory to chemotherapy or immunotherapy (35,43). Lung metastases were established by injecting 4T1 tumor cells i.v. into syngeneic hosts. Seven days later, tumor-

bearing mice commenced a 6-day treatment regimen of 5-azadC administered at a dose approximating 0.8 mg/kg i.p. For these experiments, the 5-azadC dose was reduced somewhat to minimize cumulative toxicity from the interleukin 2 (IL-2), which was administered in conjunction with H-2L^d-restricted P1A-specific or β -galactosidase-specific CTL following the P1A antigen induction regimen. On day 21, animals were euthanized and the size and number of lung metastases were determined. Representative data pertaining to these experiments are presented in Fig. 7. As shown in Fig. 7A, control mice developed numerous, large lung metastases. 5-AzadC treatment significantly reduced the number and size of lung metastases. Treatment with H-2L^d-restricted P1A CTL alone had little effect on the number or size of these tumors. However, the combination of 5-azadC treatment and adoptive transfer of P1A-specific CTL significantly reduced the number and size of lung metastases. Data from a representative experiment are summarized in Fig. 7B. In this experiment, the average number of lung metastases in untreated control mice was 72, whereas the average number of metastases in 5-azadC-treated mice was 32. Transfer of P1A-specific CTL alone did not diminish the number of lung metastases relative to those observed in untreated control animals. Similarly, transfer of β -galactosidase-specific CTL did not further reduce the number of pulmonary metastases following 5-azadC treatment. However, systemic administration of 5-azadC followed by transfer of P1A-specific CTL reduced the average number of lung metastases per animal to three. Interestingly, three of five mice receiving 5-azadC- and P1A-specific CTL seemed to be tumor-free after treatment. Collectively, these results suggest that 5-azadC mediated direct cytotoxic effects in 4T1 cancer cells *in vivo*, and that induced P1A expression in the cancer cells was sufficient for recognition and lysis by adoptively transferred P1A-specific CTL. The effects of 5-azadC and adoptive transfer of P1A-specific CTL seemed synergistic in this pulmonary metastasis model.

Discussion

We as well as others have previously shown that some human CTAs can be up-regulated by 5-azadC treatment in certain cancer cells *in vitro*. These antigens included MAGE in melanoma cells (26), LAGE-1 in lymphoblastoid cancer cells (27), and NY-ESO-1 plus MAGE-3 in thoracic malignancies (22,23–25). De Smet et al. (44) showed that DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter.

Because they can be induced in cultured human cancer cells but not normal cells following exposure to chromatin remodeling agents under conditions achievable in clinical settings (22,24,25), the CTAs represent potential targets that can be exploited not only for immunotherapy of melanoma and renal cell carcinomas but also for the treatment of more common epithelial malignancies that to date have seemed refractory to immunologic interventions. In this regard, NY-ESO-1 is a particularly attractive target for the immunotherapy of thoracic malignancies (22). NY-ESO-1 is the most immunogenic CTA identified to date. Nearly 50% of patients whose tumors express NY-ESO-1 exhibit serum antibodies to this CTA, which fluctuate with extent of disease (3,45,46). Vaccines using either CD4 or CD8 T-cell-restricted peptide epitopes, or full-length recombinant NY-ESO-1 protein, have enhanced anti-ESO-1 reactivity in cancer patients, some of whom have exhibited disease regression following immunization (47,48). Whereas NY-ESO-1 is frequently expressed in pulmonary carcinomas (23,49), immune response to this CTA seems limited in lung cancer patients (21). Nevertheless, our experience concerning induction of NY-ESO-1 in tumor tissues from lung cancer patients, and detection of NY-ESO-1 antibodies in several of these individuals following exposure to chromatin remodeling agents,⁶ attests to the potential utility of gene

⁶D.S. Schrupp, et al., submitted for publication.

induction regimens for enhancing the immunogenicity of lung cancer cells *in vivo*. However, presently, there are no published data indicating that CTAs induced by chromatin remodeling agents *in vivo* can serve as bona fide targets for adoptive immunotherapy of cancer.

PIA may serve as an excellent model to address many basic scientific questions regarding the induction of CTAs and their potential use for immunotherapy. PIA is a well-characterized CTA in mice. It is naturally expressed in some cancer cell lines, mostly leukemia (ref. 50; this study). Immunization with PIA-expressing vaccinia virus or tumor cells can induce CTL that provide protection against challenge of PIA-expressing tumors (33) and can function as a tumor rejection antigen (34). In addition, PIA epitope-specific T-cell receptor transgenic mice have been generated (31), making the study of immunotherapy *in vivo* using this model antigen very feasible. All these properties of PIA, our extensive knowledge of the antigen, and availability of relevant biological reagents made it an ideal model for our study.

Our current study showed that PIA could be induced *de novo* by 5-azadC treatment in all PIA-negative cancer cell lines derived from eight different histologies. In addition, PIA expression was further enhanced in the four cancer cell lines in which this CTA was expressed naturally but weakly. *in vivo*, PIA was induced *de novo* in all five types of tumor we tested after the tumor-bearing mice were treated with systemic 5-azadC. The PIA induction strongly correlated with demethylation of the CpG island in the *PIA* gene promoter region as assessed by MSP technique. Indeed, the CpG island of the *PIA* gene promoter region is hypermethylated in mouse liver and those tumor cell lines where the gene is silenced but hypomethylated in P815 tumor cells where the gene is strongly activated. The induction of the gene expression in those PIA-negative tumor cells by 5-azadC displayed a concurrent switch from hypermethylation to hypomethylation of the CpG island in the *PIA* gene promoter region.

This study has generated a number of new findings pertaining to PIA. First, similar to human CTAs, PIA can be induced by a demethylating agent. Second, the *de novo* induction of PIA is universal. All cancer cell lines exhibited significant levels of PIA expression *in vitro* after treatment with 5-azadC. PIA was induced *de novo* in all five different cancers representing melanoma, lung carcinoma, colon carcinoma, sarcoma, and mammary carcinoma in two strains of mice bearing those tumors when treated with systemic 5-azadC. Third, cancer cells treated with 5-azadC maintained the integrity and functionality of the antigen processing and presentation pathways. This is important for the subsequent application of immunotherapy. Fourth, the induction of PIA was achievable in tumor-bearing mice *in vivo* and was quite tumor specific. Fifth, the induced CTA can be used as a target for adoptive T-cell transfer-based immunotherapy. Finally, the direct tumoricidal effect of 5-azadC and adoptive transfer of PIA-specific CTL seemed synergistic in this pulmonary metastasis model.

Results derived from our current study may have direct translation regarding the development of TAA induction regimens for human cancer therapy. First, if a TAA is constitutively expressed in tumor tissues, anergic/suppressive CD4⁺CD25⁺ T cells may be generated in the periphery as a consequence of repeated antigenic encounter, thus anergy or tolerance to the antigen (51) may also result in the tumor variants generating “tumor escape” phenotype (52). *De novo* induction of CTAs in solid tumors may represent a novel means to break antigen-specific tolerance, which is a major impediment to immune-mediated cancer regression (4). Second, intratumoral heterogeneity of CTA expression seems related to methylation status; 5-azadC treatment can revert this phenomenon (53). 5-AzadC, particularly when administered in conjunction with depsipeptide, mediates robust CTA induction as well as apoptosis in cancer cells (24,25), which may enhance antitumor immunity. Third, self-antigens expressed by solid tumors do not efficiently stimulate naïve or activated T cells (54). However, increased levels of CTAs induced by demethylating agents may be sufficient for cross-presentation by bone marrow-derived stromal cells, and may overcome immunologic “ignorance” to solid tumors.

Fourth, 5-azadC can enhance the expression of HLA class I antigens and restoration of antigen-specific CTL response in cancer cells in which HLA class I antigens are down-regulated by hypermethylation (55,56). This property may extend immunotherapy to those cancers that are otherwise untreatable by this approach. Finally, an immunogenic TAA that can be induced in many types of tumors may enable treatment of cancer patients with standardized, potentially well-defined, and efficacious immunotherapy regimens (4–9,13,57). Collectively, these data support and provide guidance for further evaluation of chromatin remodeling agents for cancer immunotherapy.

Acknowledgments

We thank Dr. Steve A. Rosenberg for his insight and helpful suggestions during the course of this study; Pawel Kalinski, Lisa Butterfield, and Mike Gorry for critical reading of the manuscript; and Arnold Mixon for help in flow cytometry.

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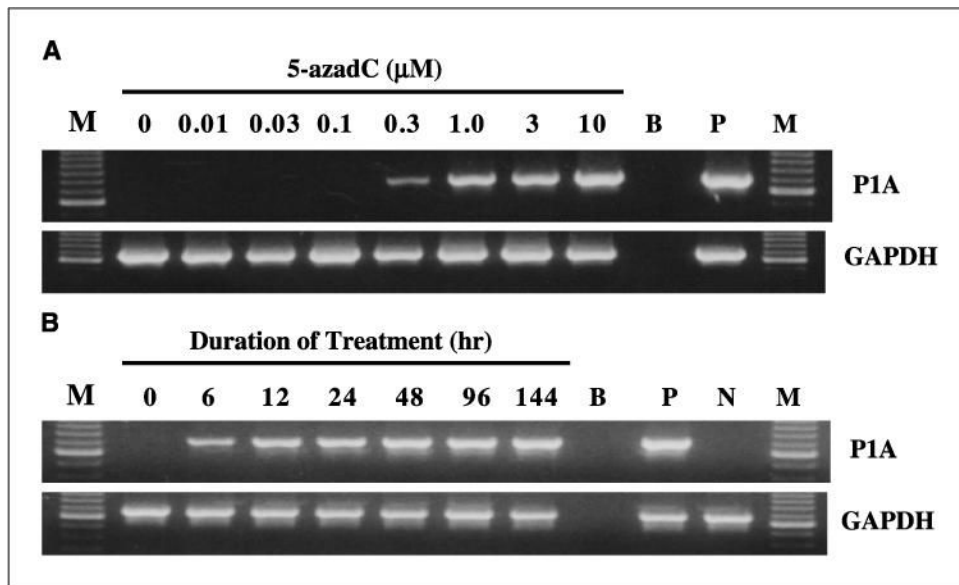


Figure 1.

Dose- and time-dependent induction of P1A by 5-azadC in LLC tumor cells, as determined by RT-PCR assays. *A*, 5-azadC dose response. LLC cells were treated for 48 hours with 5-azadC at concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3, and 10 $\mu\text{mol/L}$. *B*, blank; *P*, positive control. *B*, duration of 5-azadC treatment with a fixed concentration of 5-azadC at 1.0 $\mu\text{mol/L}$. LLC cells were incubated with 5-azadC for 0, 6, 12, 24, 48, 96, and 144 hours. The treated cells were harvested and total RNA was purified and used as templates for RT-PCR to determine the expression of the antigen P1A and control gene *GAPDH*. *N*, negative control for P1A; *M*, DNA molecular weight markers.

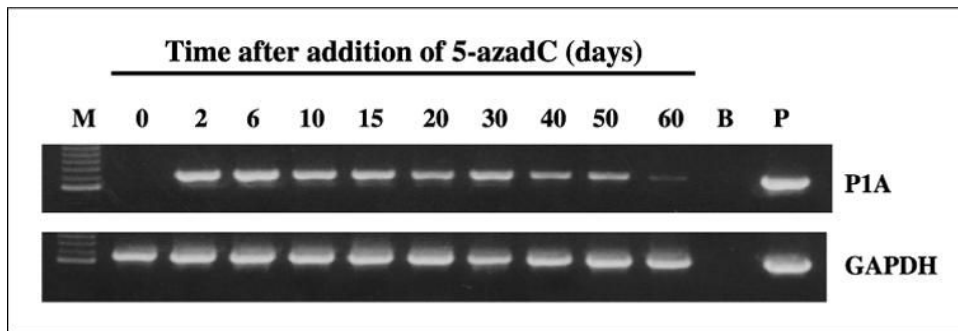


Figure 2. Duration of induced P1A expression by 5-azadC in LLC cells. The cells were treated with 5-azadC at 1.0 $\mu\text{mol/L}$ at day 0 for 2 days. After 2 days, the drug was removed and cells were split when necessary. Aliquots of cells were collected and stored at -80°C . Total RNA was extracted and RT-PCR was done as described.

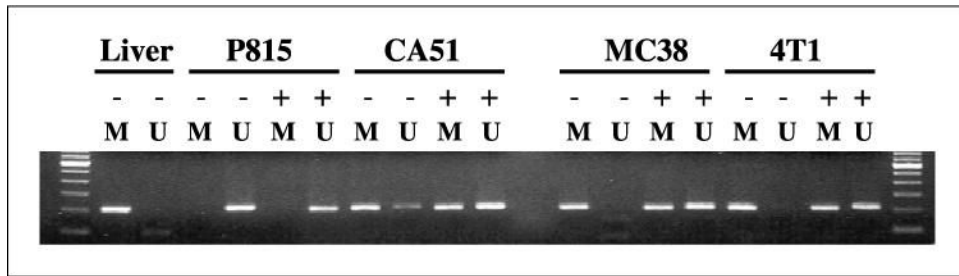


Figure 3.

Methylation status of the CpG island in the *P1A* gene promoter region as determined by methylation-specific PCR. Two controls are genomic DNA from mouse liver cells that express no *P1A*, and P815 tumor cells that express high level of *P1A*. The three tested tumor cell lines are CA51, MC38, and 4T1. -, not treated with 5-azadC; +, 5-azadC treatment of cells at 1.0 $\mu\text{mol/L}$ for 48 hours. *M*, methylated DNA; *U*, unmethylated DNA.

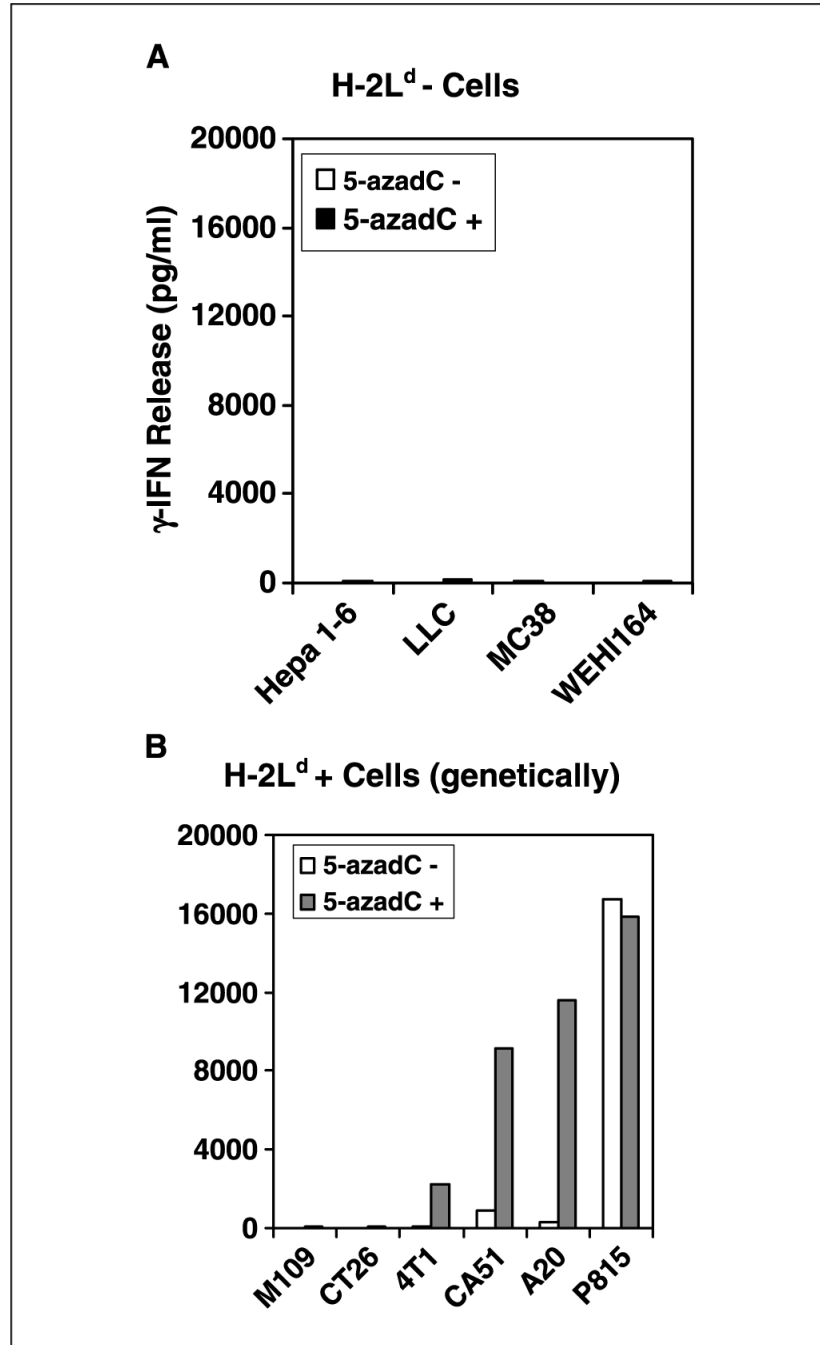
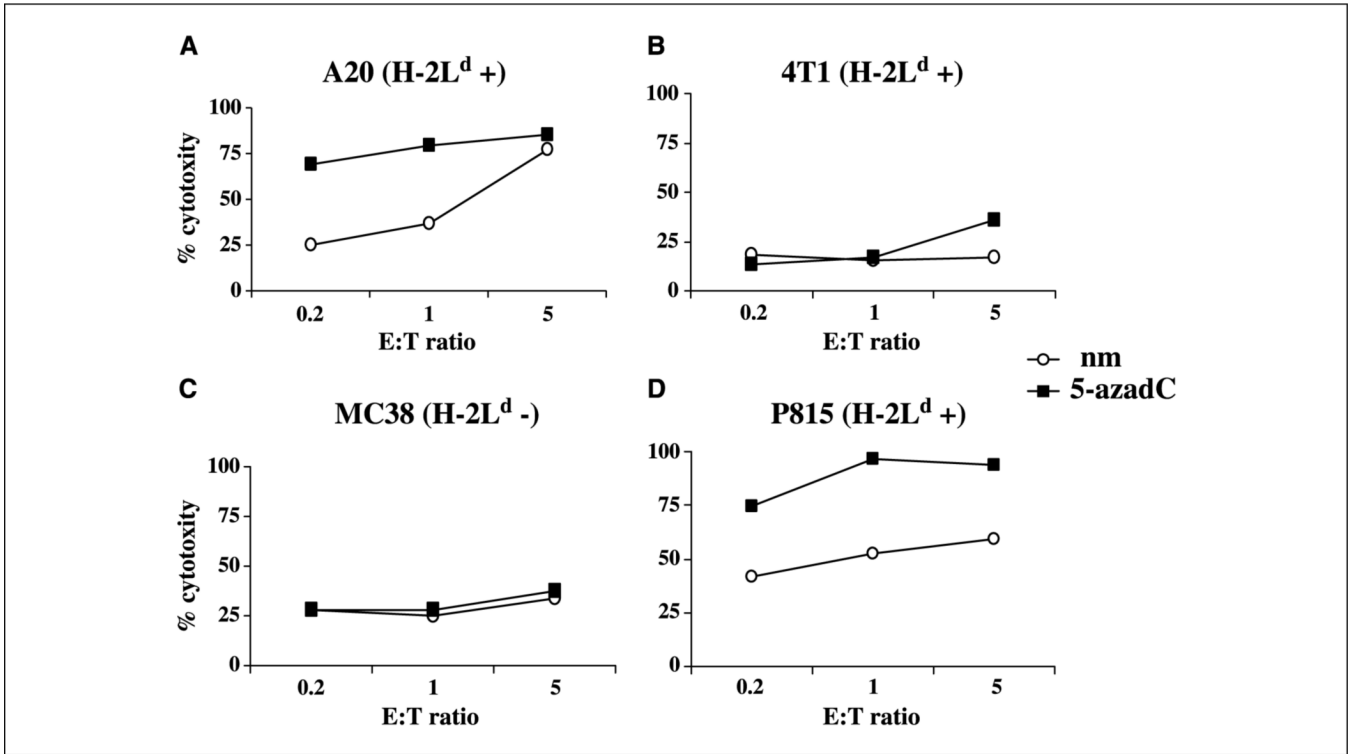


Figure 4. IFN- γ release assays with H-2L^d-restricted P1A-specific CTL incubated with either 5-azadC-treated or untreated tumor cells. *A*, H-2L^d-negative tumor lines. *B*, tumor lines derived from H-2L^d-positive mice, which may include H-2L^d antigen down-regulated or negative tumor cell lines. The experiments were done in duplicates and repeated thrice. Similar results were obtained.

**Figure 5.**

Specific lysis of tumor cells mediated by H-2L^d-restricted P1A-epitope-specific CTL. Two MHC-matched tumor cell lines, A20 (H-2L^{d+}) and 4T1 (H-2L^{d+}), were tested, whereas MHC-mismatched MC38 (H-2L^{d-}) served as negative control, and MHC-matched, P1A-positive P815 cells (H-2L^{d+}) served as positive control. ■, 5-azadC-treated target cells (5-azadC+); ○, untreated target cells (*nm*). The experiments were done with CTL generated from splenocytes isolated from BALB/c mice vaccinated with vv.ES-P1A. *Points*, mean of duplicates. Representative of at least three independent experiments. *E:T ratio*, effector-to-target cell ratio.

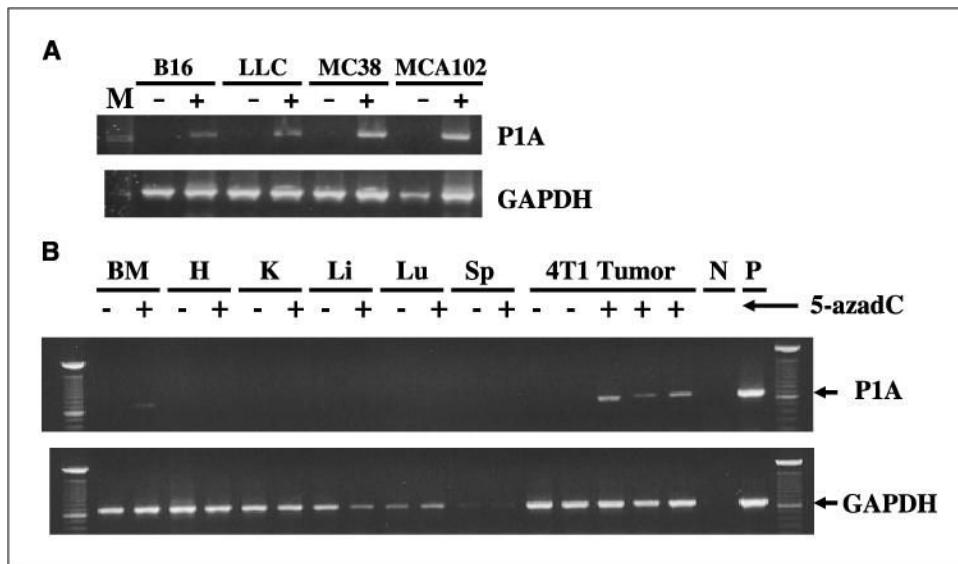


Figure 6.

Induction of P1A in five different tumors in syngeneic BALB/c or C57BL/6 tumor-bearing mice. The five tumors represent melanoma (B16), lung carcinoma (LLC), colon carcinoma (MC38), sarcoma (MCA102), and mammary adenocarcinoma (4T1). The s.c. tumor-bearing mice were untreated or treated with 2-deoxy-5'-azacytidine systematically as described in Materials and Methods. P1A expression was detected by RT-PCR assays (30 cycles of PCR). *A*, tumors of B16, LLC, MC38, and MCA102 from mice untreated (-) or treated (+) with 5-azadC. *B*, 4T1 tumor and normal tissues from tumor-bearing BALB/c mice untreated (-) or treated (+) with 5-azadC. *BM*, bone marrow; *H*, heart; *K*, kidney; *Li*, liver; *Lu*, lung; *Sp*, spleen; *N*, non-input RNA; *P*, positive control of P815 cells. We have noticed that lower levels of GAPDH detected by RT-PCR in the tissues of lung and spleens represented real situations in those tissues.

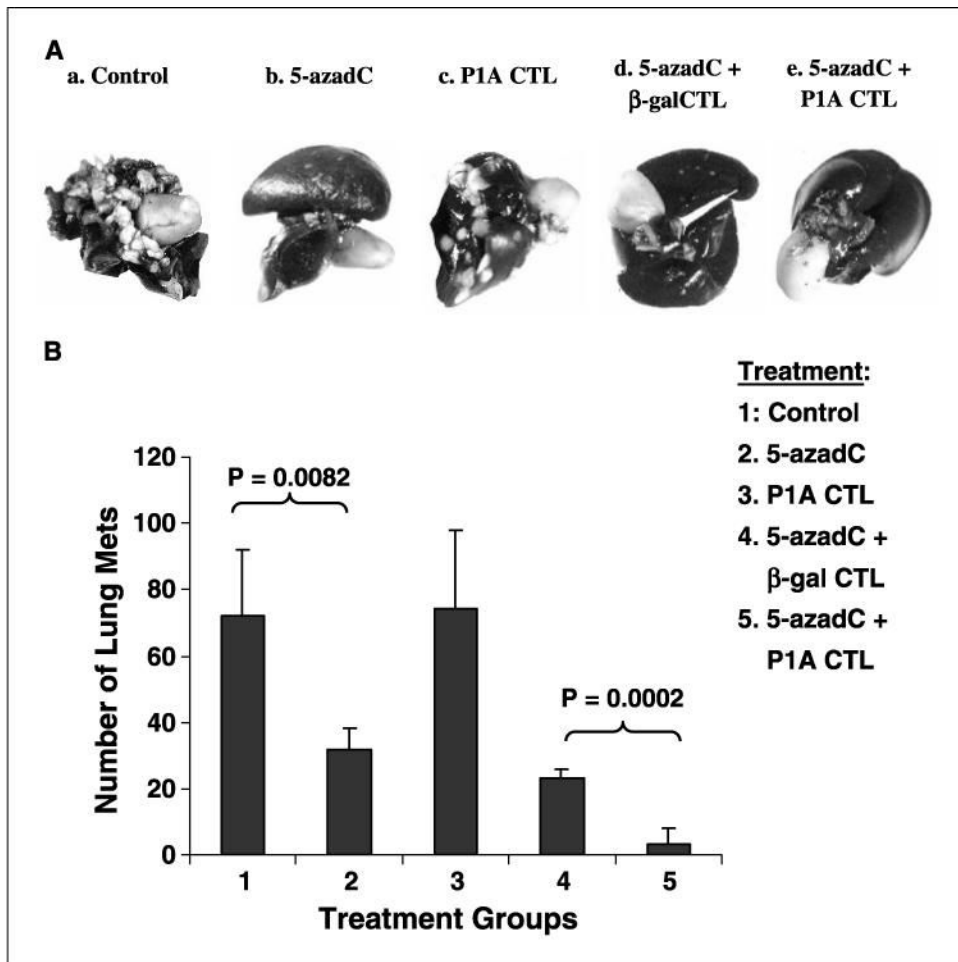


Figure 7. The 4T1 mammary tumor lung metastases in syngeneic BALB/c mice treated by 5-azadC and P1A-specific CTL. BALB/c mice with 7-day lung metastases of 4T1 mammary tumor were sequentially treated with 5-azadC and/or H-2L^d-restricted CTL specific to P1A or β -galactosidase epitope. The CTLs against P1A antigen were derived from P1A T-cell receptor transgenic mice. The mice were sacrificed on day 21 and lungs were perfused with India ink solution to distinguish between the tumor nodules from the lung tissue proper. Representative of at least three experiments. *A*, lungs from treatment groups, along with heart organ as a marker. The tumor nodules appeared as white nodules in the background of black lung tissue. *B*, the numbers of lung metastases in treatment groups, five mice per group, were quantified. Columns, mean; bars, SD. *P* values between the most important groups are given.

Table 1
P1A gene expression as detected by RT-PCR assays

Tumor type	Cancer cells	Relative P1A expression	
		-5azadC	+5azadC
Mastocytoma	P815	+++	+++
Colon carcinoma	CA07/A	-	+++
	CA51	+/-	+++
	CT26	-	+++
	MC38	-	+++
Lung carcinoma	LC12	-	+++
	LLC1	-	+++
	LM2	-	+++
	M109	-	+++
	A20	+/-	+++
Lymphoma	CH-1	-	+++
	EL4	+	+++
	TIM1.4	+	+++
	YAC-1	-	+++
	MCA 102	-	+++
Sarcoma	MCA 205	-	+++
	WEHI 164	-	+++
	4T1	-	+++
Mammary tumor	C127I	-	+++
	Hepa 1-6	+	+++
Hepatoma	Hepa 1-6	+	+++
Melanoma	B16	-	+++
Neuroblastoma	Neuro-2a	-	+++

NOTE: The relative expression of P1A mRNA was determined by a semiquantitative RT-PCR assay using total RNA extracted from cells. +++, levels of mRNA detected easily by 30 cycles of PCR after reverse transcription; +, a faint band of PCR product by 30 cycles of PCR; +/-, extremely low levels of expression, barely detectable by 35 cycles of PCR. -, no DNA product detected by 35 cycles of PCR.