Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumors

A. Francesca Setiadi,[1,2,3] Kyla Omilusik,[1,2,5] Muriel D. David,[1] Robyn P. Seipp,[1,2,3] Jennifer Hartikainen,[1,2,5] Rayshad Gopaul,[1,2,3,4,5] Kyung Bok Choi,[1,2,3,4,5] and Wilfred A. Jefferies[1,2,3,4,5]

[1]Biomedical Research Centre, [2]Michael Smith Laboratories, Departments of [3]Zoology and [4]Medical Genetics, University of British Columbia, and [5]Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

Abstract

Histone deacetylase inhibitors (HDACi) have been hailed as a powerful new class of anticancer drugs. The HDACi, trichostatin A (TSA), is thought to interfere with epigenetic control of cell cycle progression in G_1 and G_2-M phase, resulting in growth arrest, differentiation, or apoptosis. Here, we describe a novel mechanism of action of HDACis in promoting immune responses against tumors. We report that treatment of carcinoma cells with TSA increases the expression of many components of the antigen processing machinery, including TAP-1, TAP-2, LMP-2, and Tapasin. Consistent with this result, we found that treatment of metastatic carcinoma cells with TSA also results in an increase in MHC class I expression on the cell surface that functionally translates into an enhanced susceptibility to killing by antigen-specific CTLs. Finally, we observed that TSA treatment suppresses tumor growth and increases *tap*-1 promoter activity in TAP-deficient tumor cells *in vivo*. Intriguingly, this *in vivo* anti-tumoral effect of TSA is entirely mediated by an increase in immunogenicity of the tumor cells, as it does not occur in immunodeficient mice. These novel insights into the molecular mechanisms controlling tumor immune escape may help revise immunotherapeutic modalities for eradicating cancers. [Cancer Res 2008;68(23):9601–7]

Introduction

In antigen-presenting cells, peptides processed by the proteasome are transported by the transporters of antigen processing (TAP-1 and TAP-2) into the endoplasmic reticulum, where they are loaded onto MHC class I. Stabilized peptides/MHC I complexes translocate to the cell surface for presentation to $CD8^+$ CTLs. Only MHC molecules loaded with peptides derived from tumor-associated antigens expressed by malignant cells, or peptides derived from foreign pathogens, activate CTLs. Thus, cellular immunity can function as an effective tumor detection and elimination system (1).

In a wide variety of human carcinomas, loss of expression of antigen-processing machinery (APM) components is strongly correlated with disease progression and metastasis (1–4). A prominent feature of metastatic cells is deficiency in TAP-1 expression, resulting from deletions within the *tap-1* gene, decreases in TAP-1 mRNA stability or, more frequently, decreases in TAP-1 promoter activity (3–6). Interestingly, impaired TAP-1 expression leads to additional defects in the expression or functionality of other APM components (3, 7). Consistent with this notion, stable transfer of the *tap-1* gene in carcinoma cells that are deficient in multiple APM components is sufficient to restore MHC I surface expression and antigen presentation, although this is not the case when LMP-2 or TAP-2 alone are re-expressed (3, 7, 8).

Deregulation of genes involved in the modulation of chromatin structure has been closely linked to immune evasion, uncontrolled cell growth, and development of tumors (5, 9–11). Trichostatin A (TSA), an inhibitor of histone deacetylases activity (HDACi), confers anti-tumor effects *in vitro* and *in vivo* (12, 13). Although TSA was shown to selectively alter transcription of ~2% of the genes (12), the mechanism underlying its effect on tumor antigen presentation is unknown. To date, the effects of HDACi on tumor growth *in vivo* have been linked to their ability to induce growth arrest and apoptosis (12, 13). Here, we provide a novel, alternative explanation for their antitumoral activity.

Materials and Methods

Cell lines, plasmids, cell transfection, and Luciferase assays. The TC-1, D11, A9, PA, LMD, B16F10, and B16F10/rTAP-1 cell lines were cultured as previously described (5, 8). When indicated, cells were treated with 100 ng/mL TSA (Sigma) for 24 h (PA, LMD, B16F10, and B16F10/rTAP-1 cells) or 48 h (TC-1, D11, and A9 cells), or with 50 ng/mL IFN-γ for 48 h. Transfection of cells with the pTAP1-Luc construct and selection of stable transfectants were performed as previously described (5). Luciferase activity in stable transfectants was assessed by Bright-Glo luciferase assay (Promega), and the resulting values were normalized to the copy numbers of genome-integrated plasmids as previously described (5).

Chromatin immunoprecipitation assays. Five micrograms of anti-RNA pol II (N-20, sc-899; Santa Cruz Biotechnology, Inc.) or anti–acetyl-histone H3 (Upstate Biotechnology, Inc.) antibodies were used for immunoprecipitation. Chromatin immunoprecipitation experiments were done as previously described (5).

Reverse transcription-PCR analysis. Total cellular RNAs were extracted using Trizol Reagent (Invitrogen), treated with DNase 1 (Ambion, Inc.), and reverse transcribed using the SSII RT kit from Invitrogen. The resulting cDNAs were used as a template for PCR using the following primers (written 5′ to 3′): TGGCTCGTTGGCACCCTCAAA and TCAGTCTGCAGGAGCCGCAAGA for TAP-1; GCTGTGGGGACTGCTAAAAG and TATTGGCATTGAAAGGGAGC for TAP-2; CGACAGCCCTTTACCATCG and TCACTCATCGTAGAATTTTGGCAG for LMP-2; ATGGCTCGCTCGGTGACC and TCACATGTCTCGATCCCAGTAGA for

Note: A.F. Setiadi, K. Omilusik, and M.D. David contributed equally to this work.

Current address for M.D. David: Institut National de la Sante et de la Recherche Medicale U749, Faculté de Pharmacie-Université Paris-Sud, #5 rue Jean Baptiste Clement, 92296 CHATENAY-MALABRY Cedex, France.

Requests for reprints: Wilfred A. Jefferies, Biomedical Research Centre, 2222 Health Sciences Mall, Vancouver, British Columbia, V6T 1Z3, Canada. Phone: 604-822-6961; Fax: 604-822-6780; E-mail: wilf@brc.ubc.ca.

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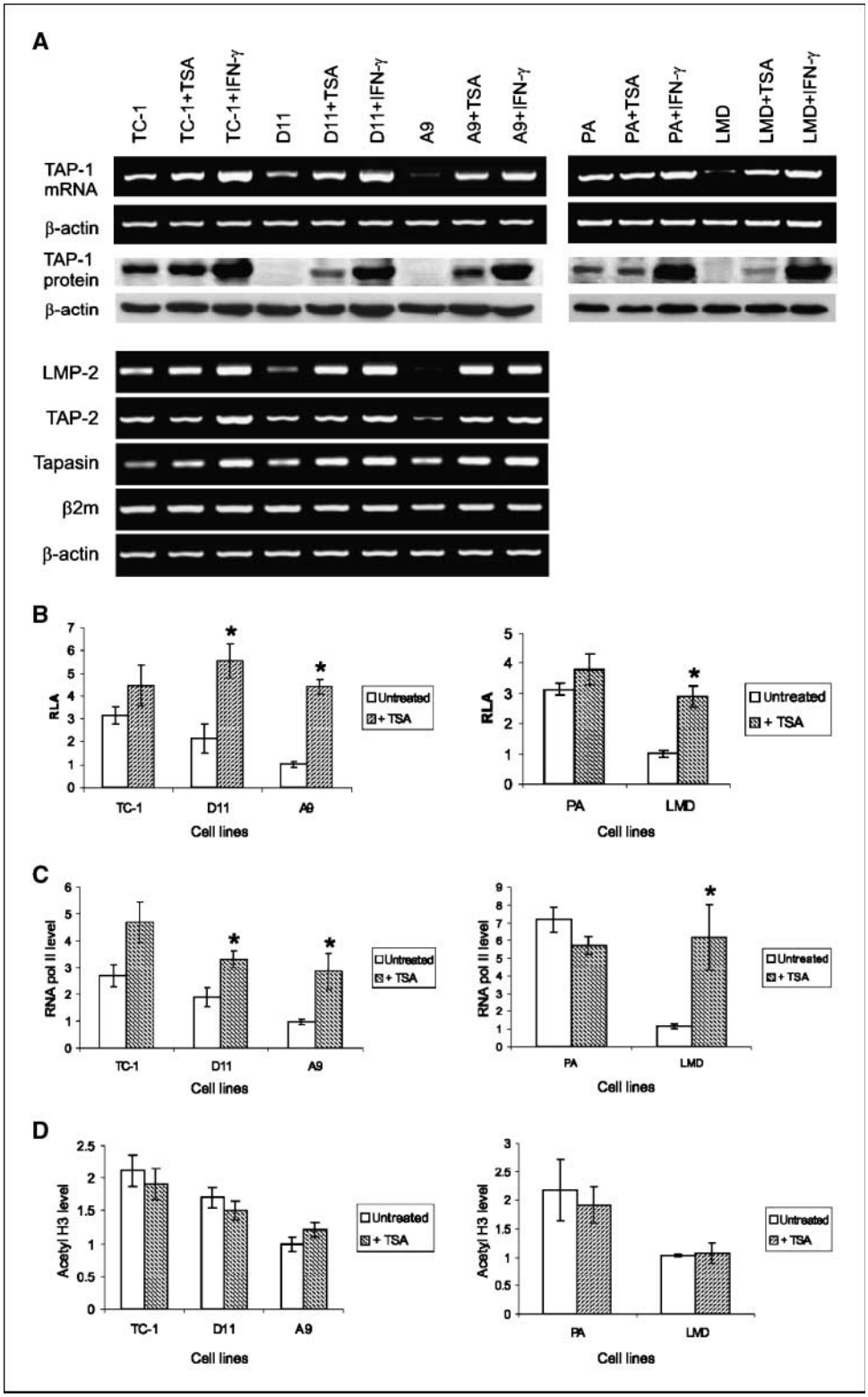

Figure 1. Treatment of TAP-deficient cells with TSA enhances expression of multiple APM components. TAP-expressing (TC-1 and PA) and TAP-deficient (D11, A9, and LMD) cells were treated with TSA or IFN-γ, or left untreated. *A,* levels of TAP-1, TAP-2, LMP-2, Tapasin, and β2m mRNA were monitored by reverse transcription-PCR. TAP-1 protein levels were assessed by Western Blot. To confirm equal loading, expression of β-actin was assessed in each sample. Data are representative of three independent experiments. *B,* TAP-expressing (TC-1 and PA) and TAP-deficient (D11, A9, and LMD) cells stably transfected with the pTAP1-Luc construct were treated with TSA or left untreated. Levels of TAP-1 promoter activity in these cells were determined as described in Materials and Methods. Values [expressed in relative luciferase activities (RLA)] represent the average of three to six independent experiments ± SEM. *, statistically significant differences in RLA between TSA-treated and untreated cells (Student's *t* test, $P < 0.05$). *C* and *D,* the levels of RNA Pol II (*C*) or acetyl-histone H3 (*D*) at the TAP-1 promoter locus were assessed by chromatin immunoprecipitation using anti-RNA pol II or anti–acetyl-histone H3 antibody, respectively. *, $P < 0.05$ compared with untreated cells (Student's *t* test). Results indicate that TSA-mediated increase in TAP-1 promoter activity correlates with an increase in RNA pol II to the TAP-1 promoter, in the absence of modification of the Histone H3 acetylation level at this locus.

β2M; ATGGATGACGATATCGCTGC and TTCTCCAGGGAGGAAGAGGAT for β-actin. All PCR reagents were obtained from Invitrogen and Fermentas (Burlington, ON).

Western blots. Protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Blots were incubated sequentially with the rabbit anti-mouse TAP-1 antibody (8) and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) and then were developed using Lumi-light ECL reagents (Pierce). Anti–β-actin mouse antibody (Sigma) and the HRP-conjugated goat anti-mouse antibody (Pierce) were used for loading controls.

Flow cytometry. H-2K^b expression was assessed by staining with PE-conjugated anti–H-2K^b mouse monoclonal antibody (mAb; BD Pharmingen), followed by analysis on a FACScan cytometer (Becton Dickinson), as previously described (6).

Cytotoxicity assays. The target cells were treated for 24 h with IFN-γ (50 ng/mL) or TSA (100 ng/mL), or left untreated. The CTL assay was performed as previously described (8). The specific ^{51}Cr release was calculated as follows: [(CTL-mediated release − background release)/(total release − background release)] × 100%.

Injection of tumor cells in mice and *in vivo* treatment with TSA. Four hundred thousand TC-1 or A9 or 1.5×10^5 B16F10 or B16F10/rTAP-1 cells in PBS were injected s.c. into C57BL/6 syngeneic mice. From the seventh day after injection of the tumor cells, mice were subjected to daily i.p. injections with TSA (500 μg/kg) or DMSO (vehicle control) for 20 d. TC-1-, A9-, B16F10-, and B16F10/rTAP-1–derived tumors were measured every 2 to 4 d. Tumor volume was calculated using the formula: tumor volume = length × width × height × π/6. All procedures were performed in compliance with the guidelines of the University of British Columbia and the Canadian Council on Animal Care.

Measurement of TAP-1 promoter activity in A9 tumor cells isolated from mice. The *in vivo* experiment described above was also performed with A9 cells stably transfected with the pTAP1-Luc construct. Mice were sacrificed 26 d after tumor injection and the tumors were removed. Tumor tissues were treated with 1 mg/mL collagenase D (Roche Applied Science)

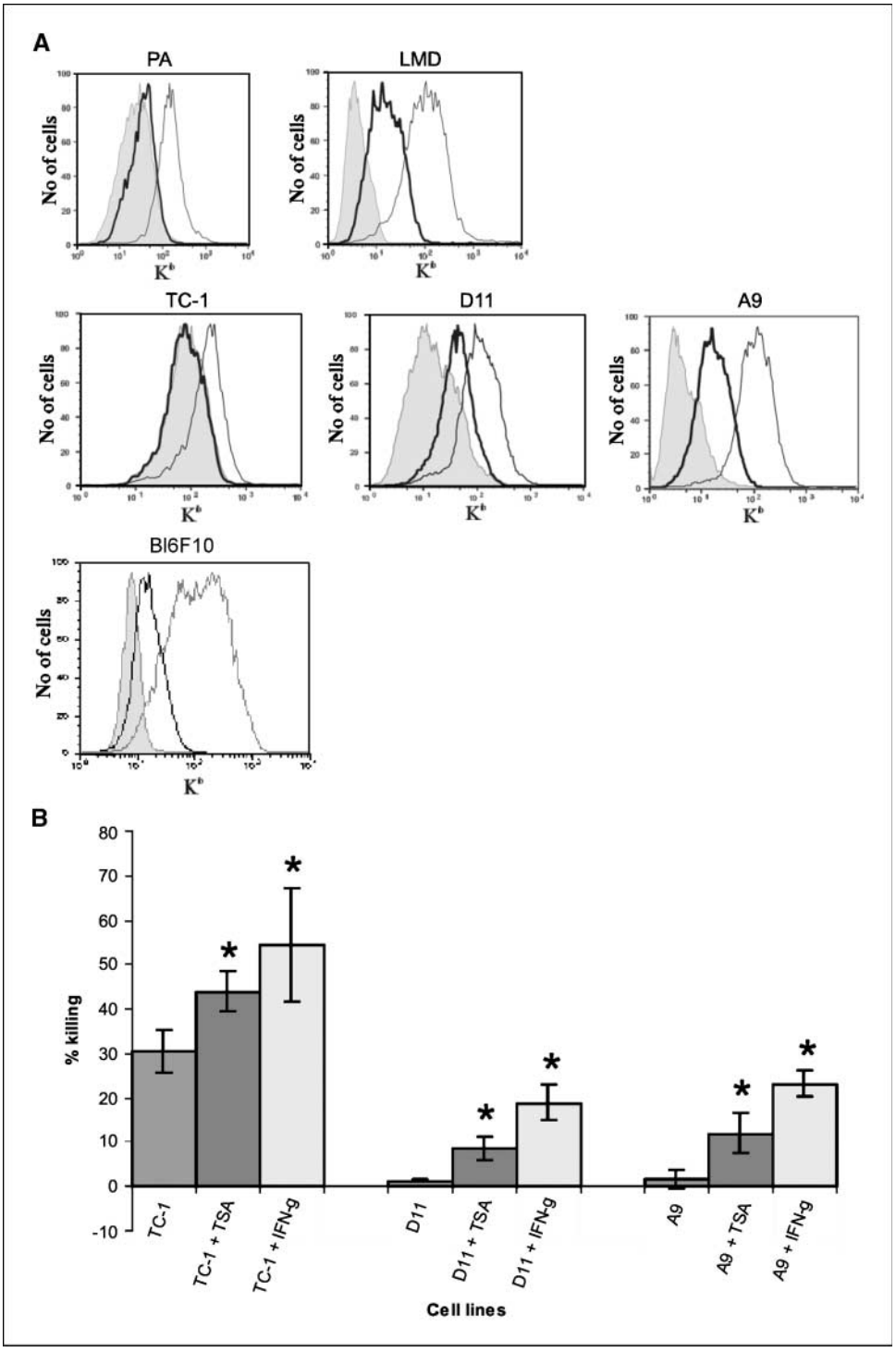

Figure 2. Treatment of TAP-deficient cells with TSA increases MHC class I antigen presentation and promotes killing of tumor cells by specific CTLs. *A,* TAP-expressing (TC-1 and PA) and TAP-deficient (D11, A9, LMD, and B16F10) cells were treated with TSA or IFN-γ, or left untreated. Levels of MHC I at the surface of untreated cells (*shaded areas*), TSA-treated cells (*black lines*), and IFN-γ–treated cells (*gray lines*) were assessed by fluorescence-activated cell sorting (FACS) using a PE-conjugated anti–H-2K^b mAb. Data are representative of three independent experiments. *B,* to assess the effect of TSA treatment on the ability of cells to present antigen in the context of MHC class I, TC-1, D11, and A9 cells treated with TSA or IFN-γ for 24 h or left untreated were infected with VSV at a multiplicity of infection of 7.5 for 16 h, and then subjected to CTL assays using effector/target ratios of 1:1 to 200:1. Representative data using the 22:1 effector/target ratio are shown in this figure. *Columns,* mean (expressed in % killing of target cells) of three independent experiments; *bars,* SE. *, statistically significant differences in % killing between untreated cells and TSA-treated or IFN-γ treated cells (Student's *t* test, $P < 0.05$).

for 40 min at 37°C. Luciferase activity was measured by Bright-Glo luciferase assay (Promega).

Results and Discussion

Treatment of TAP-deficient metastatic carcinomas cells with TSA increases expression of TAP-1 as well as many other components of the APM. Down-regulation of TAP-1 in metastatic carcinoma cells leads to deficiency in presentation of tumor-specific antigens and escape from immune surveillance. As we had previously shown, the existence of epigenetic control of TAP-1 expression (5), we hypothesized that TSA could restore TAP-1 expression in TAP-deficient metastatic cells. For our studies, we used two experimental models. The first model consisted of the TAP-expressing cell line (TC-1), derived from murine lung cells

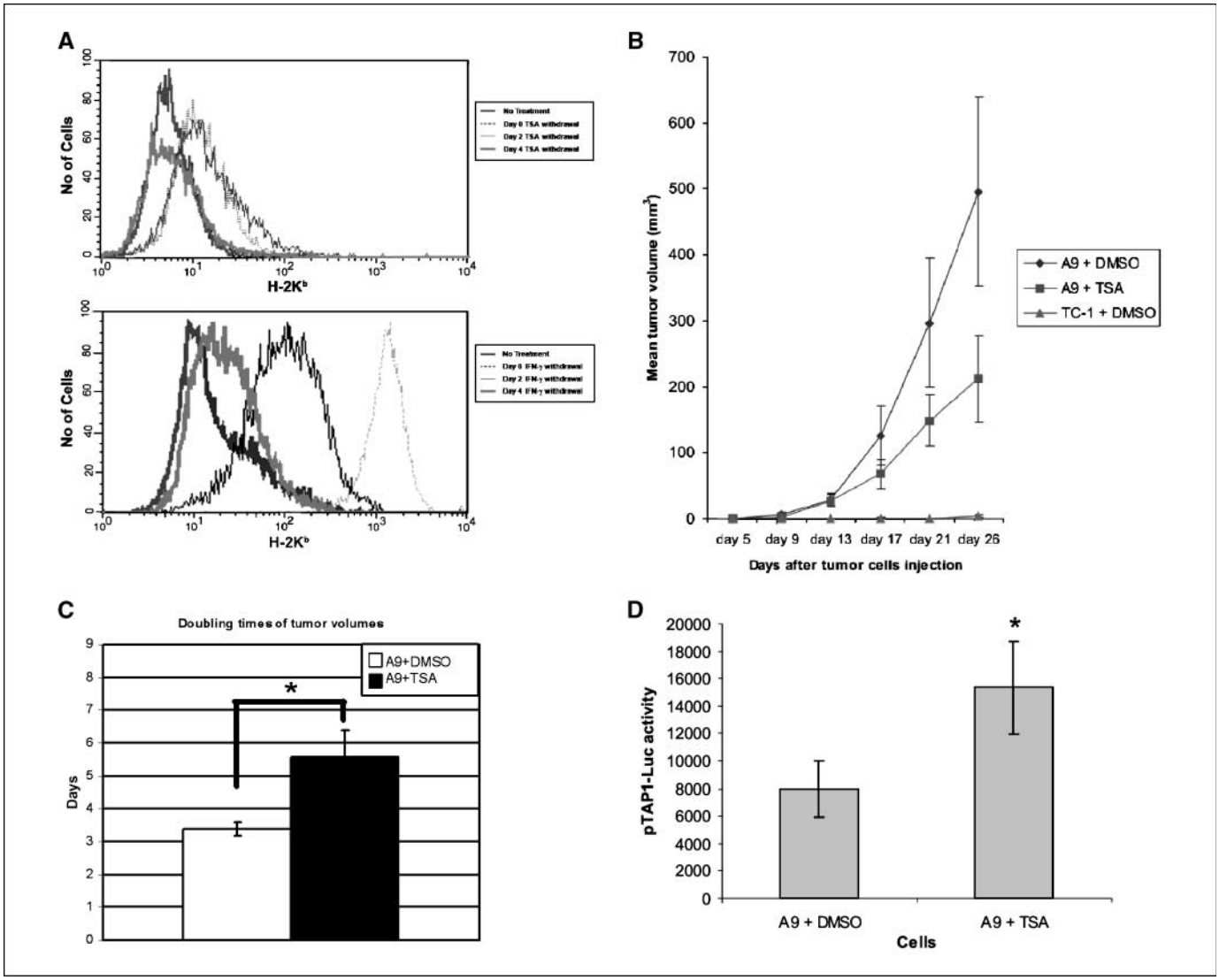

Figure 3. TSA treatment *in vivo* suppresses growth of tumors derived from TAP-deficient cells. *A,* TAP-deficient, A9 cells were treated with TSA or IFN-γ, as indicated. Forty eight hours later (day 0), cells were washed and cultured in the absence of TSA and IFN-γ for 2 to 4 additional d. Levels of MHC I at the surface of untreated cells (*thick dark lines*), cells treated with TSA or IFN-γ for 48 h (day 0; *dotted lines*), cells cultured for 2 d after TSA and IFN-γ withdrawal (day 2; *thin lines*) and cells cultured for 4 d after TSA and IFN-γ withdrawal (day 4; *thick gray lines*) were assessed by FACS using a PE-conjugated anti–H-2K^b mAb. These experiments indicate that the restoration of MHC I surface expression in TAP-deficient tumor cells treated with TSA or IFN-γ is a reversible phenomenon. *B,* four hundred thousand TAP-deficient (A9) or TAP-expressing (TC-1) cells were injected s.c. into C57BL/6 syngeneic mice. From the 7th day after cell injection, mice were subjected to daily i.p. injections with TSA (500 μg/kg) or DMSO (vehicle control) for 20 d. TC-1– and A9-derived tumors were measured every 4 d. *Points,* mean volume of eight tumors per group (A9 cells/DMSO-treated mice; A9 cells/TSA-treated mice; TC-1 cells/DMSO-treated mice) over the indicated periods of time after tumor cells injection; *bars,* SE. This experiment indicated that the TAP-expressing (TC-1) cells are less tumorigenic than the TAP-deficient (A9) cells, and that daily treatment of mice with TSA hampers A9-derived tumor progression. *C,* The effect of *in vivo* treatment with TSA on the rate at which A9-derived tumors grew was further assessed by calculating the tumor volume doubling times in each mouse individually. Before this analysis, we determined the period of time over which tumors grew exponentially with a constant growth rate (i.e., the natural log of tumor volume (V) was a linear function of time (t): $V = V_0e^{kt}$, with k being the growth rate constant). We found that the rates of exponential growth of all tumors remained constant between day 6 and day 20 after the first administration of TSA. Therefore, the corresponding growth rate constants were used to calculate the tumor volume doubling times (T_d), according to the formula: $T_d = \ln(2)/k$. *Columns,* mean T_d of eight tumors per group (A9 cells/DMSO-treated mice; A9 cells/TSA-treated mice); *bars,* SEM. *, statistically significant difference in tumor volume doubling times between TSA-treated mice and DMSO-treated mice (Student's *t* test, $P < 0.05$). *D,* tumors from TSA-treated or DMSO-treated mice that had been injected with TAP-deficient A9 cells stably transfected with the pTAP1-Luc construct were isolated. RLAs in the A9-derived tumor cells were assessed as described in Materials and Methods. *Columns,* mean of four independent experiments; *bars,* SEM. *, statistically significant difference in RLA between cells from TSA-treated mice and cells from untreated mice (Student's *t* test, $P < 0.05$).

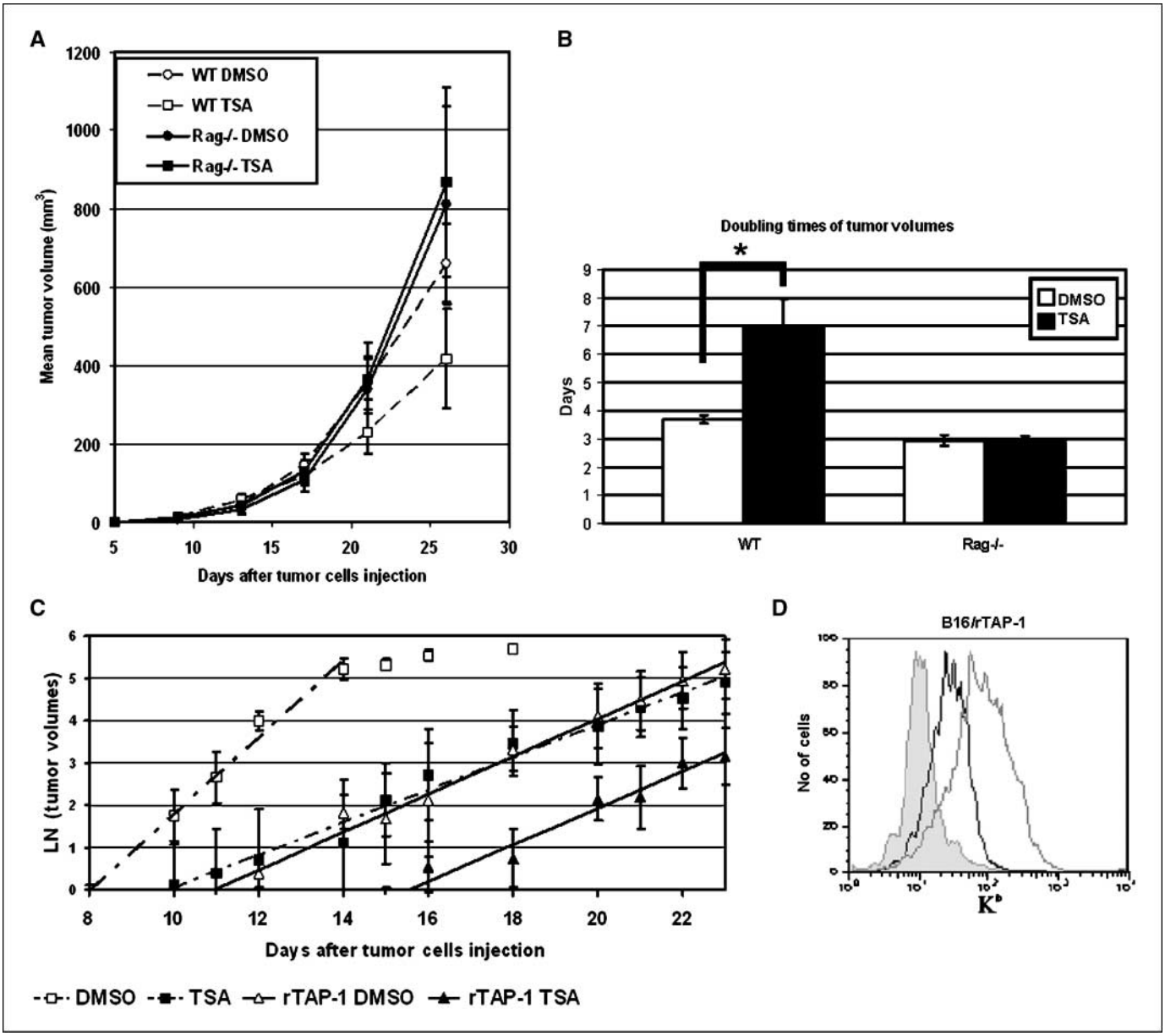

Figure 4. The inhibitory effect of TSA administration on growth of tumors derived from TAP-deficient cells does not occur in immunodeficient Rag$^{-/-}$ mice. *A,* C57BL/6 and Rag$^{-/-}$ mice were injected s.c. with four hundred thousand TAP-deficient A9 cells and subjected to daily i.p. injections with TSA or DMSO for 20 d, as described in Fig. 3. *Points,* mean tumor volume (A9 cells/DMSO-treated C57BL/6 mice; A9 cells/TSA-treated C57BL/6 mice; A9 cells/DMSO-treated Rag$^{-/-}$ mice; A9 cells/TSA-treated Rag$^{-/-}$ mice); *bars,* SEM. *B, columns,* mean tumor volume doubling time of the tumors shown in *A*; *bars,* SEM. *, statistically significant difference in tumor volume doubling times between TSA-treated C57BL/6 mice and DMSO-treated C57BL/6 mice ($n = 4$; Student's t test, $P < 0.05$). In contrast, TSA administration to Rag$^{-/-}$ mice did not induce significant changes in tumor volume doubling times. *C,* the effect of *in vivo* TSA treatment on the growth rate of B16F10- or B16F10/rTAP-1–derived tumor was assessed as described in Fig. 3. *Points,* mean LN (tumor volume) of four tumors per group (B16F10 tumor/DMSO-treated C57BL/6 mice; B16F10 tumor/TSA-treated C57BL/6 mice; B16F10/rTAP-1 tumor/DMSO-treated C57BL/6 mice; B16F10/rTAP-1 tumor/TSA-treated C57BL/6 mice); *bars,* SE. *D*, TAP-deficient B16F10 cells genetically reconstituted with TAP-1 (B16/rTAP-1) were treated with TSA or IFN-γ, or left untreated. Levels of MHC I at the surface of untreated cells (*shaded areas*), TSA-treated cells (*black lines*), and IFN-γ–treated cells (*gray lines*) were assessed by FACS using a PE-conjugated anti–H-2K^b mAb. Data are representative of three independent experiments. These results indicate that the TSA-mediated increase in TAP-1 expression is not the sole mechanism underlying the antitumoral effect of TSA.

transformed with HPV16, and two of its TAP-deficient, metastatic derivatives: D11 and A9 (14). The second model consisted of the TAP-expressing cell line (PA) derived from a murine primary prostate tumor and its TAP-deficient, metastatic derivative (LMD; ref. 15). Expression levels of β2-microglobulin (β2m) were not impaired in the A9 and D11 TAP-deficient cells (Fig. 1*A*). However, these cells displayed down-regulated levels of TAP-1, LMP-2, and TAP-2. Indeed, levels of TAP-1, LMP-2, and TAP-2 mRNA were low in A9 cells, moderate in D11 cells, and the highest in TC-1 cells (Fig. 1*A*). We observed that treatment of the A9 and D11 TAP-deficient cells with TSA increased the levels of TAP-1 mRNA and protein, as well as the levels of *LMP-2, TAP-2*, and *Tapasin* mRNA (Fig. 1*A*). The TSA-induced increase in expression of TAP-1 mRNA and protein was also observed in TAP-deficient prostate tumor (LMD; Fig. 1*A*), murine lung carcinoma (CMT.64; data not shown) and melanoma (B16F10)

cells. In all experimental models, TAP-1 protein expression in TSA-treated TAP-deficient cells was nevertheless lower than in naturally TAP-expressing control cells (Fig. 1*A*). Thus, treatment of TAP-deficient cells with TSA increases expression of components of the APM.

TSA increases the expression of TAP-1 by enhancing recruitment of RNA polymerase II to the TAP-1 promoter. We observed that treatment of the TAP-deficient cells with TSA *in vitro* fully restored TAP-1 promoter activity to levels observed in TAP-expressing cells (Fig. 1*B*). The fact that levels of TAP-1 protein in TSA-treated, TAP-deficient cells were lower than those in TAP-expressing cells, despite full restoration of TAP-1 promoter activity, may reflect posttranscriptional defects in TAP-deficient cells that specifically impair TAP-1 expression. In line with this notion, we and others reported that the stability of TAP-1 mRNA is often decreased in TAP-deficient cells (6, 16).

We previously reported that, in metastatic cancer cells, a defect in histone acetylation resulting from a decrease in CBP recruitment to the TAP-1 promoter leads to a compact nucleosome structure that acts as a physical barrier to prevent access of RNA pol II to the TAP-1 promoter (5). To test the hypothesis that the TSA-induced up-regulation of TAP-1 promoter activity occurs via enhancement of histone H3 acetylation and RNA pol II recruitment to the TAP-1 promoter, we performed chromatin immunoprecipitation assays. We found that treatment of TAP-deficient cells with TSA enhanced the recruitment of RNA pol II complex to the TAP-1 promoter to levels similar to those in TAP-expressing cells (Fig. 1*C*). Based on our observation that levels of histone H3 acetylation at the TAP-1 promoter correlated with TAP-1 expression in physiologic conditions and that an increase in acetyl-H3 at the TAP-1 promoter locus accompanied IFN-γ–induced TAP-1 expression (5), it seemed likely that the effect of TSA on TAP-1 promoter activity occurred via an increase in histone H3 acetylation. However, we observed that TSA treatment did not significantly alter the levels of acetyl-histone H3 in TAP-1 promoter in any of the cell lines tested (Fig. 1*D*). Thus, although TSA-induced relaxation of the chromatin structure allowed recruitment of RNA pol II to the TAP-1 promoter, this phenomenon did not involve an increase in histone H3 acetylation. We also failed to detect increases in acetyl-H4 levels upon IFN-γ or TSA treatment (data not shown). Histone H3 and H4 are not the only core histone whose modification has been shown to influence gene expression (17, 18). Additional experimentation has yet to identify a histone modification in the TAP-1 promoter region that is directly modified by TSA. It is also entirely feasible that yet to be identified nonchromatin organization proteins, which are themselves acetylated, may be altered by TSA treatment, and play a role in the regulation of TAP-1 promoter activity. These observations are tantalizing and further investigation will be required to delineate the exact mechanism underlying the effect of TSA on TAP-1 promoter activity.

Treatment of TAP-deficient metastatic cells with TSA enhances surface expression of MHC I and susceptibility to CTL-mediated killing. We tested whether the TSA-induced increase in expression of antigen processing components would result in an increase in surface MHC I expression on the metastatic carcinoma cells. Flow cytometric analysis showed that TSA treatment increased H-2K^b surface expression by ~10-fold in TAP-deficient cells, whereas the levels were unchanged in PA and TC-1 cells that naturally express high levels of surface H-2K^b (Fig. 2*A, thick lines*). Similar induction of MHC I expression by TSA was also observed in the TAP-deficient CMT.64 and B16F10 cell lines (data not shown; Fig. 2*A*). IFN-γ treatment increased the surface H-2K^b expression in all cell lines.

To test whether the TSA-induced increase in H-2K^b surface expression would improve antigen presentation and increase cell susceptibility to antigen-specific CTLs, we infected the TAP-expressing TC-1 and the TAP-deficient D11 and A9 cells with vesicular stomatitis virus (VSV) and assessed the efficiency of recognition and killing by VSV-specific CTLs. As expected, D11 and A9 cells, which express lower surface H-2K^b than the TC-1, were less susceptible to killing by the CTLs (Fig. 2*B*). Treatment of all VSV-infected cell lines with TSA or IFN-γ enhanced the CTL-mediated killing. However, this increase was much more drastic with TAP-deficient D11 and A9 cells than with TAP-expressing TC-1 cells (~10-fold in D11 and A9, versus 1.5-fold in TC-1; Fig. 2*B*). Similar trends were also observed using VSV-infected TAP-deficient CMT.64 and B16F10 cells as targets (data not shown). Infection with VSV itself was not sufficient to increase CTL-mediated killing of the TAP-deficient tumor cell lines we used (data not shown).

The fact that treatment of VSV-infected TAP-expressing cell lines with TSA or IFN-γ enhanced the CTL-mediated killing despite the apparent lack of increase in MHC I surface expression suggests that mechanisms other than TAP-1/MHC I up-regulation may be involved. It was shown that treatment of lung cancer cells with TSA resulted in a significant increase in Fas and a decrease in expression of antiapoptotic Bcl-2 and Bcl-XL proteins (19). In addition, TSA was shown to render genetically-transformed mouse embryonic fibroblasts (MEF) cells less tumorigenic *in vivo*, through a mechanism involving an increase in Fas levels on the MEF (20). Of note, untreated MEF already displayed MHC I at their surface, and TSA did not further increase cell surface expression of MHC I (alike the TAP-expressing TC-1 cells we used in our study).

Taken together, these results indicated that the TSA-mediated increase in expression of APM components functionally translates into enhancement of CTL-mediated recognition of virus-infected TAP-deficient carcinoma cells. These findings suggested that treatment of TAP-deficient carcinoma cells with TSA would result in decreased growth rate of TAP-deficient–derived tumors *in vivo*.

Administration of TSA *in vivo* suppresses the growth of TAP-deficient tumors in syngenic mice and correlates with an increase in TAP-1 promoter activity *in vivo*. As a prerequisite to test whether TSA-induced increase in MHC I surface expression and increase in CTL-mediated killing of TAP-deficient cells could contribute to the antitumoral effect of TSA *in vivo*, we investigated whether TSA-induced MHC I expression was sustained even after TSA withdrawal. We found that the restoration of MHC I surface expression in TAP-deficient tumor cells treated with TSA (or IFN-γ) was a reversible phenomenon. Indeed, upon TSA withdrawal, TAP-1 expression level (and thus cell-surface expression of MHC I) was sustained for 2 days but decreased back to background level by 4 days after TSA withdrawal (Fig. 3*A*). These observations indicated that the regulation of TAP-1 expression is a dynamic process. Therefore, our protocol of *in vivo* tumor growth assay was designed so that daily injection of TSA would allow sustained MHC I surface expression.

We found that the TAP-expressing TC-1 cells were significantly less tumorigenic than the TAP-deficient A9 cells. This was shown by the low occurrence of tumor formation when TC-1 cells were injected s.c. into mice (only four of nine mice injected with TC-1 cells developed tumors, whereas all mice injected with TAP-deficient A9 cells did), as well as by the relatively small size of the TC-1–derived tumors (Fig. 3*B*). Using this experimental model,

we observed that daily treatment of mice with TSA hindered A9-derived tumor progression (Fig. 3*B* and *C*).

To assess whether treatment of mice with TSA enhanced TAP-1 promoter activity in tumor cells, we also performed this *in vivo* experiment using A9 cells stably transfected with a reporter construct in which the transcription of a luciferase gene was under the control of the TAP-1 promoter (pTAP1-Luc). We found that the decrease in A9-derived tumor growth observed in TSA-treated mice correlated with a significant increase in TAP-1 promoter activity (Fig. 3*D*). These observations supported our hypothesis that the mechanism underlying the anticancer effect of TSA involves up-regulation of TAP-1 expression in cancer cells.

The antitumor growth effect of TSA results from an increase in immunogenicity of the tumor. We found that TSA no longer dampened tumor growth when TAP-deficient cells were inoculated into RAG1$^{-/-}$ B6 mice (Fig. 4*A* and *B*). The fact that the TSA effect *in vivo* disappeared in mice lacking T cells formally shows that the effect of TSA on tumor growth is entirely mediated through increased tumor immunogenicity rather than through a direct TSA-induced apoptosis of TAP-deficient cells or through inhibition of cellular proliferation. This is a departure from the explanations given in the literature for the action of HDACis and therefore provide a new framework for understanding the action of these drugs.

Genes or other factors in addition to TAP-1, TAP-2, and Tapasin contribute to the immuno-therapeutic effect of TSA. Finally, we investigated whether the TSA-induced up-regulation of TAP-1 was the sole mechanism underlying the antitumor growth effect of TSA or whether other TSA-induced events also contributed to this phenomenon. We showed previously that restoration of TAP1 expression in TAP-deficient cells (by transfecting B16F10 cells with a plasmid encoding TAP-1) was sufficient to increase expression or stabilization of TAP-2 and Tapasin expression to the levels observed in TAP-expressing cells, resulting in increased MHC I surface expression, enhanced CTL-mediated cell killing, and subsequently, decreased tumor growth *in vivo* (8). In the current study, we used the same cellular model to investigate whether TSA was capable of further increasing immunogenicity of TAP-deficient cells genetically reconstituted with TAP-1. In accordance with our previous study, restoration of TAP-1 expression in B16F10 cells was sufficient to decrease the *in vivo* tumor growth rate (Fig. 4*C*). We observed, however, that injection of TSA *in vivo* further enhanced immune recognition of syngeneic tumors that were genetically reconstituted with TAP-1. This was correlated with the ability of TSA to further increase cell surface expression of MHC I on TAP-deficient cells reconstituted with TAP-1 (Fig. 4*D*). This shows that genes/factors in addition to TAP-1 are induced by TSA and that these significantly contribute to reducing tumor growth by promoting immune responses against the tumor. In contrast to genetic etiology of cancer, the possibility of reversing epigenetic codes may provide new targets for immuno-therapeutic intervention in cancer. This study should guide oncologists in reassessing and reformulating their approaches for effective anticancer immunotherapy.

Disclosure of Potential Conflicts of Interest

A.F. Setiadi, K. Omilusik, M.D. David, R.P. Seipp, J. Hartikainen, R. Gopaul, K.B. Choi, W.A. Jefferies: The University of British Columbia has filed a PCT application based on the information described in this manuscript.

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A. Francesca Setiadi, Kyla Omilusik, Muriel D. David, et al.

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