

Functional Up-regulation of Human Leukocyte Antigen Class I Antigens Expression by 5-aza-2'-deoxycytidine in Cutaneous Melanoma: Immunotherapeutic Implications

Ester Fonsatti,¹ Hugues J.M. Nicolay,^{1,2} Luca Sigalotti,² Luana Calabrò,¹ Laura Pezzani,² Francesca Colizzi,² Maresa Altomonte,¹ Massimo Guidoboni,¹ Francesco M. Marincola,³ and Michele Maio^{1,2}

Abstract Purpose: To investigate the potential of the DNA hypomethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR) to improve the effectiveness of immunotherapeutic approaches against melanocyte differentiation antigens.

Experimental Design: The effect of 5-aza-CdR on the constitutive expression of gp100 was investigated in 11 human melanoma cell lines by real-time reverse transcription-PCR and indirect immunofluorescence (IIF) analyses. 5-aza-CdR-mediated changes in the levels of expression of human leukocyte antigen (HLA) class I antigens and HLA-A2 allospecificity, intercellular adhesion molecule-1 (ICAM-1), and leukocyte-function-associated antigen-3 were investigated by IIF analysis on melanoma cells under study. The recognition of gp100-positive Mel 275 melanoma cells, treated or not with 5-aza-CdR, by HLA-A2-restricted gp100₍₂₀₉₋₂₁₇₎-specific CTL was investigated by ⁵¹Cr-release assays, IFN- γ release and IFN- γ ELISPOT assays.

Results: The constitutive expression of gp100 was not affected by 5-aza-CdR on all melanoma cells investigated. Compared with untreated cells, the exposure of Mel 275 melanoma cells to 5-aza-CdR significantly ($P < 0.05$) up-regulated their expression of HLA class I antigens and of ICAM-1. These phenotypic changes significantly ($P < 0.05$) increased the lysis of 5-aza-CdR-treated Mel 275 melanoma cells by gp100-specific CTL and increased their IFN- γ release. 5-aza-CdR treatment of Mel 275 cells also induced a higher number of gp100-specific CTL to secrete IFN- γ .

Conclusions: Treatment with 5-aza-CdR improves the recognition of melanoma cells by gp100-specific CTL through the up-regulation of HLA class I antigens expression; ICAM-1 also contributes to this phenomenon. These findings highlight a broader range of therapeutic implications of 5-aza-CdR when used in association with active or adoptive immunotherapeutic approaches against a variety of melanoma-associated antigens.

Human leukocyte antigen (HLA) class I antigens play a crucial role in antitumor cellular immune responses (1, 2), and levels of expression of both HLA class I antigens and tumor-associated antigen (TAA) contribute to determine the extent of TAA-specific

CTL recognition of neoplastic cells (3, 4). Thus, the identification of new strategies potentially able to up-regulate the expression of HLA class I antigens and/or of therapeutic TAA on neoplastic cells may improve the clinical effectiveness of T cell-based immunotherapeutic approaches in cancer patients.

Along this line, we have shown that the DNA hypomethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR, Dacogen) up-regulates the constitutive cell surface expression of HLA class I antigens and of HLA class I allospecificities and concomitantly induces or up-regulates that of different cancer testis antigens (CTA) in cultured human melanomas (5). Furthermore, 5-aza-CdR administration has also proven to induce a persistent up-regulation of HLA class I antigens, along with a long-lasting induction and up-regulation of CTA expression, in human melanomas grafted into BALB/c *nu/nu* mice (6). Of prospective clinical value, these phenotypic changes significantly improved the recognition of melanoma cells by CTA-specific CTL (7).

These evidences have clearly identified novel immunologic properties of 5-aza-CdR that represents a powerful pharmacologic agent to design new clinical strategies of CTA-based immunotherapy; however, due to its concomitant effect on the expression of HLA class I antigens and of CTA, the relative

Authors' Affiliations: ¹Division of Medical Oncology and Immunotherapy, Department of Oncology, University Hospital of Siena, Istituto Toscano Tumori, Siena, Italy; ²Cancer Bioimmunotherapy Unit, Department of Medical Oncology, Centro di Riferimento Oncologico, Istituto di Ricovero e Cura a Carattere Scientifico, Aviano, Italy; and ³Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, Maryland
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Requests for reprints: Michele Maio, Division of Medical Oncology and Immunotherapy, Department of Oncology, University Hospital of Siena, Strada delle Scotte 14, Siena 53100, Italy. Phone: 39-577-586335; Fax: 39-577-586303; E-mail: mmaio@cro.it.

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contribution of either molecules in improving CTL recognition of melanoma cells could not be dissected. This aspect is in fact crucial to define the therapeutic potential of 5-aza-CdR also in non-CTA-based immunotherapeutic approaches.

Among known therapeutic TAA, melanocyte differentiation antigens (i.e., tyrosinase, tyrosinase-related protein-1 and tyrosinase-related protein-2, Melan-A/MART-1, gp100/Pmel17) are widely used as targets for immunotherapy of melanoma patients (8, 9). Therefore, this study was designed to investigate the perspective potential of 5-aza-CdR in improving the clinical efficacy of immunotherapeutic approaches that target differentiation antigens in melanoma. To this end, the effects of 5-aza-CdR on the expression of the differentiation antigen gp100 in melanoma cells and on the functional interaction of gp100-positive melanoma cells with gp100-restricted CTL were investigated.

Our results show that 5-aza-CdR allows for a more efficient recognition of melanoma cells by CTL directed against gp100, which is not modulated by the drug. These findings provide further support to the immunomodulatory role of 5-aza-CdR and strongly encourage for its broader clinical use to design novel immunotherapeutic approaches against different therapeutic TAA, regulated or not by DNA methylation, expressed in human melanoma.

Materials and Methods

Cells. Melanoma cell lines were obtained from gp100-positive metastatic melanoma lesions surgically excised from patients with no history of chemotherapy as previously described (10). All melanoma cell lines and K562 human leukemia cell line were grown in Iscove Medium (Biochrome KG) supplemented with 10% heat-inactivated FCS (Biochrome KG) and 2 mmol/L L-glutamine (Biochrome KG).

HLA-A2-restricted gp100₍₂₀₉₋₂₁₇₎-specific CTL were generated and characterized as previously described (11). CTL were seeded at 10^6 cells/mL and cultured in Iscove Dulbecco's medium (Biochrome KG) containing 10% human serum AB (Sigma Chemical Co.), 2-mmol/L L-glutamine (Biochrome KG), 100 µg/mL penicillin (Sigma Chemical Co.), 100 µg/mL streptomycin (Bristol-Myers Squibb S.r.l.),

6000 IU/mL human r-IL-2 (Chiron) at 37°C and 5% CO₂. Fresh medium containing r-IL-2 was added on day 3. After 6 days, cells were collected and used for the functional assays.

Monoclonal antibodies, conventional antisera, and reagents. The anti-HLA class I monoclonal antibodies (mAb) TP25.99 and the anti-HLA-A2/28 mAb CR11.351 were kindly provided by Dr. Soldano Ferrone (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY). The anti-HLA class I mAb W6/32 was purchased from the American Type Culture Collection. The anti-CD54 [intercellular adhesion molecule-1 (ICAM-1)] mAb 84H10 and the anti-CD58 (LFA-3) mAb were purchased from Becton Dickinson. The anti-gp100 mAb HMB-45 was purchased from Serotec. FITC-conjugated F(ab')₂ fragments of rabbit anti-mouse immunoglobulins (Ig) were purchased from DAKO. ChromePure mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc.

5-aza-CdR was purchased from Sigma Chemical Co.

Treatment of melanoma cells with 5-aza-CdR. Melanoma cells were pulsed with 1 µmol/L 5-aza-CdR every 12 h for 2 days as previously described (5). At the end of treatment, the medium was replaced with fresh culture medium without 5-aza-CdR, and after 48 h of incubation, cells were used for analyses.

Serologic assays. Indirect immunofluorescence (IIF) analysis was done as previously described (5). A sample was classified as positive when more than 10% of cells were stained with the relevant mAb. Mean value of fluorescence intensity obtained with isotype-matched mouse Ig was lower than 10 on all cell lines tested.

The intracellular expression of gp100 was evaluated on cells fixed and permeabilized with the Leucoperm kit (Serotec) according to the manufacturer's instructions.

Real-time quantitative reverse transcription-PCR. TaqMan quantitative reverse transcription-PCR (RT-PCR) reactions were done on the ABI prism 7000 Sequence Detection System (Applied Biosystems) as previously described (12) using primers, probes, and thermal cycle parameters previously identified for gp100 (13) and β-actin (14). Quantification of the β-actin cDNA was used to monitor cDNA input, and the number of gp100 cDNA molecules in each sample was normalized to the number of cDNA molecules of β-actin.

Cytotoxicity assay. Standard ⁵¹Cr-release assay was done in triplicate in 96-well round-bottom microplates (Costar). Briefly, 100 µL of ⁵¹Cr-labeled target cells (1×10^4 cells) were incubated with 100 µL of CTL at the effector/target (E/T) ratios of 25:1, 12:1, 6:1, 3:1, and 1:1 for 4 h at 37°C. Then, the supernatant (100 µL) was harvested from each well and counted in a gamma counter. The percentage of specific lysis was determined as previously described (15).

IFN-γ release assay. A fraction of untreated and 5-aza-CdR-treated Mel 275 melanoma cells used in one of the cytotoxic assays were cocultured with HLA-A2-restricted gp100-specific CTL (1×10^5 cells per well) at the E/T ratio of 1:1 in complete basal Iscove's medium and seeded in 96-well round-bottom microplates to a final volume of 200 µL per well for 24 h. Then, supernatants were harvested, and IFN-γ levels were determined using an IFN-γ ELISA kit (R&D Systems) according to the manufacturer's recommendations. Cells in culture medium alone were used as negative control.

IFN-γ ELISPOT assay. HLA-A2-restricted gp100-specific CTL were seeded with the target cells (1×10^4 cells) at the E/T ratios of 1:1 and 0.1:1 in triplicates at a final volume of 100 µL of medium in 96-well plates (Endogen Searchlight). Phytohemagglutinin was used as unspecific positive stimulator control at 10 µg/mL (Sigma Chemical Co.). Cells in culture medium alone were used as negative control. The plates were incubated at 37°C, 5% CO₂ for 24 h, and the frequency of gp100-specific IFN-γ-secreting cells was determined using an ELISPOT assay kit (Endogen), according to the manufacturer's instructions. Spots were evaluated with an Automated ELISPOT Reader System with ELIAnalyse V4.1 software (A.EL.VIS GmbH).

To evaluate the blocking effect of HLA-class I antigens and ICAM-1, 5-aza-CdR-treated Mel 275 melanoma cells were preincubated with 20 µg/mL of the anti-HLA class I mAb W6/36 or the anti-ICAM-1 mAb

Table 1. Real-time RT-PCR analysis of gp100 expression in melanoma cell lines treated with 5-aza-CdR

Cells	Ctrl	5-aza-CdR*	5-aza-CdR/Ctrl
Mel 40	$2.96 \times 10^{-1}^\dagger$	1.33×10^{-1}	0.4
Mel 116	1.57×10^{-1}	2.66×10^{-1}	1.7
Mel 158	3.50×10^{-5}	8.55×10^{-5}	2.4
Mel 275	4.89×10^{-1}	4.49×10^{-1}	0.9
Mel 281	5.84×10^{-2}	1.02×10^{-1}	1.7
Mel 531	2.36×10^{-1}	1.76×10^{-1}	0.7
Mel 592	2.55×10^{-2}	2.43×10^{-2}	1.0
Mel 603	2.12×10^{-3}	3.22×10^{-3}	1.5
Mel 610	1.44×10^{-1}	9.82×10^{-2}	0.7
Mel 611	2.06×10^{-1}	2.16×10^{-1}	1.0
Mel 640	3.95×10^{-1}	1.38×10^{-1}	0.3
P versus Ctrl		N.S.	

Abbreviations: Ctrl, control cells; NS, not significant.

*5-Aza-CdR-treated cells.

†Number of gp100 cDNA molecules/number of β-actin cDNA molecules.

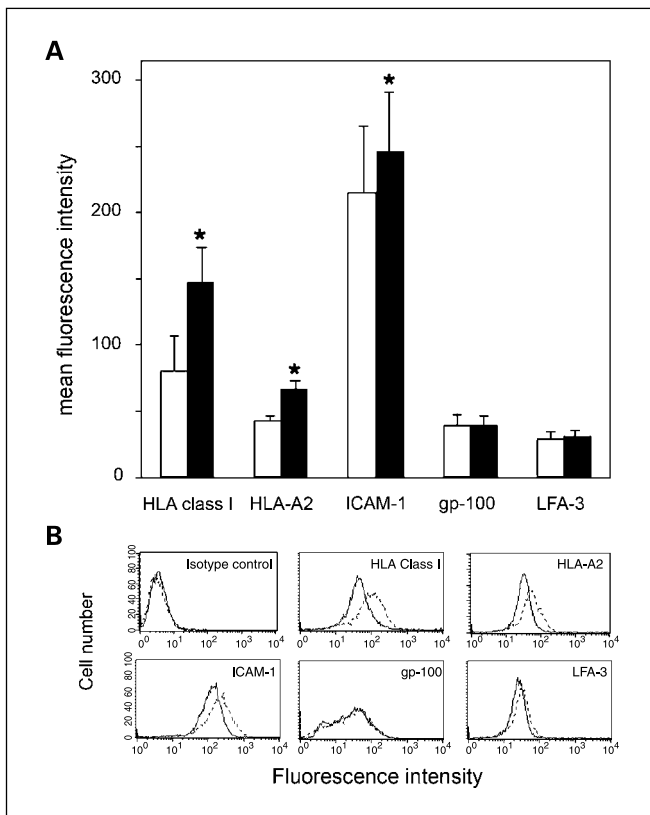


Fig. 1. Antigenic profile of 5-aza-CdR-treated Mel 275 melanoma cells. *A*, untreated (white columns) and 5-aza-CdR-treated (black columns) Mel 275 cells were sequentially incubated with the anti-HLA class I antigens mAb TP25.99, the anti-HLA-A2/28 mAb CR11.115, the anti-ICAM-1 mAb 84H10, the anti-gp100 mAb HMB-45, or the anti-LFA-3 mAb and with FITC-conjugated F(ab')₂ fragments of rabbit anti-mouse Ig. Cells were then analyzed by flow cytometry. Columns, mean values of mean fluorescence intensity obtained in three independent experiments; bars, SD. *, $P < 0.05$ versus control. *B*, histogram profile of each investigated antigen, stained with the specific mAb as described in (*A*), from one representative experiment are shown for untreated (—) and 5-aza-CdR-treated (---) Mel 275 cells.

84H10. After 30 min of incubation at 37°C, cells were washed and plated as described.

Statistical analysis. Data analyzed by Student's paired *t* test with $P < 0.05$ were considered statistically significant.

Results

gp100 expression in melanoma cell lines treated with 5-aza-CdR. Quantitative real-time reverse transcription-PCR (RT-PCR) assay showed a constitutively heterogeneous expression of gp100 among 11 human melanoma cell lines investigated; however, exposure to 5-aza-CdR did not significantly affect their baseline expression of gp100 (Table 1). In particular, the results of four independent experiments showed that the number of cDNA molecules of gp100 normalized to cDNA molecules of β -actin \pm SD were $4.2 \times 10^{-2} \pm 1.2$ and $4.3 \times 10^{-2} \pm 1.2$ in untreated and in 5-aza-CdR-treated Mel 275 melanoma cells, respectively.

Based on these findings, the HLA-A2-positive Mel 275, the HLA-A2-negative Mel 40 and Mel 158 melanoma cells were chosen to investigate the functional role of 5-aza-CdR-mediated up-regulation of HLA class I antigens and ICAM-1 expression in melanoma.

Immunophenotypic modulation of the antigenic profile of Mel 275 melanoma cells by 5-aza-CdR. Exposure to 5-aza-CdR significantly ($P < 0.05$) up-regulated the constitutive levels of expression of HLA class I antigens, HLA-A2 allospecificity, and ICAM-1 on Mel 275 melanoma cells (Fig. 1A) in three independent experiments. However, 5-aza-CdR treatment of Mel 275 melanoma cells did not significantly affect the basal levels of LFA-3 expression (Fig. 1A). Mean values of mean fluorescence intensity \pm SD for untreated and 5-aza-CdR-treated Mel 275 melanoma cells were 80.3 ± 26.7 and 147 ± 26.6 for HLA class I, 43.3 ± 2.8 and 67.3 ± 6.4 for HLA-A2, 214.6 ± 50 and 246.3 ± 44.5 for ICAM-1, and 28.6 ± 6 and 30.6 ± 5.5 for LFA-3.

Consistent with molecular data, IIF analysis confirmed the lack of modulation of gp100 expression on 5-aza-CdR-treated Mel 275 melanoma cells. In fact, mean values of mean fluorescence intensity \pm SD for gp100 were 39.4 ± 9.2 and 39 ± 7.1 on untreated and 5-aza-CdR-treated Mel 275 melanoma cells, respectively. Representative data are shown in Fig. 1B.

Susceptibility of 5-aza-CdR-treated Mel 275 melanoma cells to lysis by HLA-A2-restricted gp100-specific CTL. Cytotoxicity of HLA-A2-restricted gp100-specific CTL against untreated or 5-aza-CdR-treated HLA-A2-positive Mel 275 and HLA-A2-negative Mel 158 and Mel 40 melanoma cells was assessed at different *E/T* ratios. As shown in Fig. 2, the results of three independent experiments showed that levels of gp100-specific CTL-mediated lysis were significantly ($P < 0.05$) higher for Mel 275 treated cells compared with untreated cells at *E/T* ratios ranging from 25:1 to 6:1. In contrast, HLA-A2-negative Mel 158 melanoma cells (Fig. 2) and Mel 40 (data not shown) were resistant to the lysis by cytotoxic effector cells at all of the *E/T* ratios investigated. Furthermore, no cytotoxicity was observed when K562 cells were used as targets, indicating the absence of natural killer activity in the CTL culture used (Fig. 2).

Recognition of 5-aza-CdR-treated Mel 275 melanoma cells by HLA-A2-restricted gp100-specific CTL. To further evaluate the functional role of up-regulated levels of HLA class I antigens and ICAM-1, IFN- γ release and IFN- γ ELISPOT assays were done.

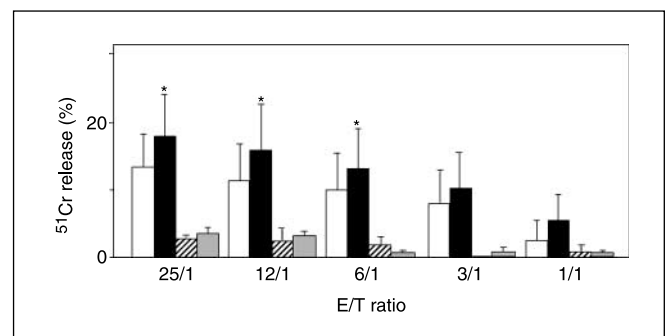


Fig. 2. Susceptibility of 5-aza-CdR-treated Mel 275 melanoma cells to lysis by gp100_(209–217)-specific CTL. Cytolytic activity of HLA-A2-restricted gp100-specific CTL was tested by a standard 4-h ⁵¹Cr release assay against Mel 275 melanoma cells, untreated (white columns) or 5-aza-CdR-treated (black columns), at effector/target (*E/T*) ratios of 25:1, 12:1, 6:1, 3:1, and 1:1. Mel 158 melanoma cells (striped columns) and K562 leukemia cells (gray columns) were used as control target cells.

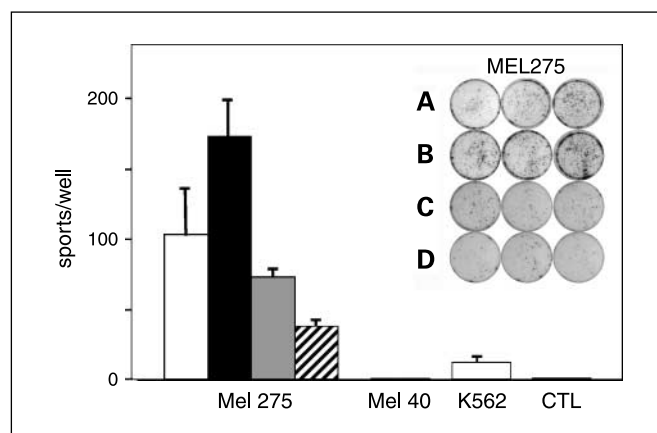


Fig. 3. IFN- γ -releasing gp100_(209–217)-specific CTL upon challenge with 5-aza-CdR-treated Mel 275 melanoma cells. Untreated (white columns) or 5-aza-CdR-treated (black column) Mel 275 melanoma cells, Mel 40 melanoma cells, and K562 leukemia cells were used as targets for HLA-A2-restricted gp100-specific CTL in ELISPOT assay at *E/T* ratio of 1:1. A fraction of 5-aza-CdR-treated Mel 275 cells has been preincubated with 20 μ g/mL of anti-HLA class I antigens mAb W6/32 (gray column) or anti-ICAM-1 mAb 84H10 (striped column). Columns, mean of spots per 10^4 cells of triplicate wells; bars, SE. Inset, images of triplicate wells for CTL stimulated with untreated (A), treated with 5-aza-CdR (B), treated with 5-aza-CdR and preincubated with anti-HLA class I antigens mAb W6/32 (C), or with anti-ICAM-1 mAb 84H10 (D) Mel 275 cells.

Exposure to 5-aza-CdR efficiently enhanced the recognition of Mel 275 melanoma cells by HLA-A2-restricted gp100-specific CTL. In particular, levels of IFN- γ released by HLA-A2-restricted gp100-specific CTL rose from 1,050 to 1,747 pg/mL when cytotoxic cells were challenged with untreated or 5-aza-CdR-treated Mel 275 melanoma cells, respectively. Instead, IFN- γ release by gp100-specific CTL was 37, 35, or 103 pg/mL when HLA-A2-negative Mel 158 and Mel 40 or K562 cells were used as stimulators and was 40 pg/mL in the absence of stimulators.

Consistent with the data reported above, ELISPOT assay showed that 5-aza-CdR-treated Mel 275 melanoma cells induced a higher number of IFN- γ -secreting CTL compared with untreated cells, at different *E/T* ratios investigated (Fig. 3). The number of IFN- γ -secreting T cells per well \pm SE detected in response to untreated and 5-aza-CdR-treated Mel 275 melanoma cells was 103 ± 33.3 and 172.7 ± 27.1 , respectively, at the *E/T* ratio of 1:1 (Fig. 3) and was 21.3 ± 2.7 and 39.7 ± 6.5 , respectively, at the *E/T* ratio of 0.1:1 (data not shown). This up-regulation was suppressed when the assay was carried out in the presence of anti-HLA class I antigens mAb W6/32 or the anti-ICAM-1 mAb 84H10 both at *E/T* ratio of 1:1 and 0.1:1 (data not shown).

By contrast, no IFN- γ -releasing CTL were observed against HLA-A2-negative Mel 40 melanoma cells or against K562 cells or in the absence of target cells (Fig. 3).

Discussion

Melanocyte differentiation antigens are consistently expressed in human cutaneous melanoma (16) and are immunogenic. In fact, circulating CTL and antibodies against distinct differentiation antigens are often detectable in melanoma patients (17, 18). Therefore, among known TAA, melanocyte differentiation antigens represent attractive candidates for active or adoptive immunotherapy of melanoma patients, and efforts

are ongoing to increase their efficacy as therapeutic targets (9, 19–23). Along this line, and based on growing and compelling evidences that strongly encourage the clinical use of 5-aza-CdR to design novel chemo-immunotherapeutic approaches in melanoma patients (6, 24), in this study, we investigated the potential of 5-aza-CdR to improve the clinical efficacy of melanocyte differentiation antigen-based T cell immunotherapy.

Expanding initial results obtained for the differentiation antigens MelanA/MART-1 and tyrosinase (25), 5-aza-CdR did not affect the constitutive expression of gp100 in a large panel of human melanoma cell lines, both at molecular and protein level. On the other hand, 5-aza-CdR significantly up-regulated the expression of HLA class I antigens on all melanoma cell lines investigated (Fig. 1, and data not shown). As compared with untreated cells, 5-aza-CdR-treated Mel 275 melanoma cells became significantly more susceptible to the cytotoxic activity of HLA-A2-restricted gp100-specific CTL and increased their release of IFN- γ . Supporting the functional activity of the up-regulation of HLA class I antigens expression by 5-aza-CdR in enhancing gp100-restricted CTL recognition of melanoma cells, treatment of Mel 275 cells with 5-aza-CdR also induced a greater number of gp100-specific CTL to release IFN- γ , and this phenomenon was reverted by blocking HLA class I antigens. Altogether, our present data obtained with gp100 clearly show that a significant proportion of the therapeutic potential of 5-aza-CdR relies on its ability to improve the targeting of melanoma cells by antigen-specific CTL through the up-regulation of the levels of expression of HLA class I antigens on neoplastic cells.

Targeting the immune system to different TAA currently represents a promising therapeutic option in melanoma, and different active or adoptive immunotherapeutic approaches are rapidly integrating into the comprehensive treatment of melanoma patients. However, their clinical effectiveness is still limited and clearly requires to be improved (26). In this context, the ability of 5-aza-CdR to up-regulate the expression of HLA class I antigens and its functional effect in increasing the recognition of melanoma cells by CTL specific for gp100 suggest for a broader clinical potential of 5-aza-CdR to improve the effectiveness of immunotherapeutic approaches against a broad range of known, unknown, and unique TAA in which expression is not affected by DNA methylation patterns.

Besides active immunotherapy, a renewed interest is currently focusing on adoptive immunotherapy of melanoma patients (27–29), and the therapeutic infusion of *ex vivo* generated T cells recognizing melanocyte differentiation antigens, CTA, or unique antigens has already been associated with clinical responses in melanoma (30–32). Therefore, the demonstration that 5-aza-CdR up-regulates the recognition of neoplastic cells by TAA-specific CTL provides a strong rationale for its clinical use also in the setting of adoptive immunotherapy of melanoma patients.

A down-regulated expression of HLA-class I antigens and allospecificities is frequently observed in melanoma (33, 34), as well as in other tumors (34), and it associates with an impaired CTL recognition of neoplastic cells, disease progression, and limited efficacy of immunotherapeutic approaches (34–36). In this scenario, the ability of 5-aza-CdR to up-regulate the expression of HLA class I antigens and of HLA class I allospecificities in melanoma has been investigated *in vivo*

and proven to be highly effective and long-lasting (6, 37). Based on these preliminary evidences, our present data further strengthen the idea that 5-aza-CdR can positively affect the recognition of melanoma cells expressing constitutively low levels of HLA class I antigens and allospecificities by circulating, vaccination-induced, or adoptively transferred TAA-specific CTL. Furthermore, even if 5-aza-CdR fails to revert allelic HLA class I loss on melanoma cells (38), it might compensate for these allelic defects by improving CTL recognition of immunogenic peptides presented in the context of other HLA class I allospecificities in which expression can be still up-regulated by 5-aza-CdR (5, 38).

In addition to HLA class I antigens, the up-regulation of ICAM-1 expression mediated by 5-aza-CdR was found to contribute in part to improve the recognition of melanoma cells by gp100-restricted CTL. This finding is consistent with the well-known functional role of ICAM-1 in favoring the interaction between melanoma cells and cytotoxic cells through the binding to its counter-receptor leukocyte-function-associated antigen-1 (10, 39). However, ICAM-1 has also been recently shown to recruit HLA class I antigens into rafts in the area of contact between T cells and target cells (40); therefore, the up-regulation of ICAM-1 induced by 5-aza-CdR might contribute to the more efficient CTL recognition of 5-aza-CdR-treated melanoma cells also through this additional mechanism.

Besides improving the recognition of melanoma cells by CTL directed against nonmethylation- and methylation-regulated TAA, upcoming evidences indicate that 5-aza-CdR might also potentiate the immunogenicity of neoplastic cells. In fact, immunization of BALB/c mice with 5-aza-CdR-treated human melanoma cells generated high titer circulating antibodies against the CTA NY-ESO-1 induced by 5-aza-CdR on vaccinating cells (6). Circulating antibodies against NY-ESO-1 have also been recently detected in patients with thoracic malignancies treated with 5-aza-CdR (41). Additionally, an increased immunogenicity of neoplastic cells mediated by 5-aza-CdR was also suggested to contribute to the progressing clinical response observed in patients with solid or hematologic malignancies after cessation of 5-aza-CdR administration (42, 43). These data and the functional results of this study suggest that 5-aza-CdR has a dual immunologic effect *in vivo* favoring neoplastic cells recognition by CTL on one side and improving the immunogenicity of neoplastic cells on the other.

Altogether, the results of this study and the evidence that the epigenetic immune remodeling induced by 5-aza-CdR is persistent (5, 37) further support the immune-regulatory properties of 5-aza-CdR and corroborate its clinical use to improve the effectiveness of immunotherapeutic approaches targeting melanocyte differentiation antigens, as well as additional nonmethylation-regulated therapeutic TAA.

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