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ORIGINAL ARTICLE

MAGE-A1, MAGE-A3, and NY-ESO-1 can be upregulated on neuroblastoma cells to facilitate cytotoxic T lymphocyte-mediated tumor cell killing

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Abstract Approximately half of patients with stage IV neuroblastoma are expected to relapse despite current therapy, and when this occurs, there is little likelihood of achieving a cure. Very few clinical trials have been conducted to determine whether cellular immune responses could be harnessed to fight this tumor, largely because potential tumor antigens for cytotoxic T lymphocytes (CTL) are limited. MAGE-A1, MAGE-A3, and NY-ESO-1 are cancer-testis (CT) antigens expressed on a number of malignant solid tumors, including neuroblastoma, but many tumor cell lines down-regulate the expression of CT antigens as well as major histocompatibility (MHC) antigens, precluding recognition by antigen-specific T cells. If expression of cancer antigens on neuroblastoma could be enhanced pharmacologically, CT antigen-specific immunotherapy could be considered for this tumor. We have demonstrated that the expression of MAGE-A1, MAGE-A3, and NY-ESO-1 can be upregulated on neuroblastoma cells following exposure to pharmacologic levels of the demethylating agent 5-aza-2′-deoxycytidine (decitabine, DAC). Expression of NY-ESO-1, MAGE-A1, or MAGE-A3 was induced in 10/10 neuroblastoma cell lines after 5 days of exposure to DAC. Culture of neuroblastoma cell lines with IFN-γ was also associated with an increased expression of either MHC Class I or II by cytofluorometry, as reported by other groups. MAGE-A1, MAGE-A3, and NY-ESO-1-specific CTL were cultured from volunteer donors by stimulating peripheral blood mononuclear cells with dendritic cells pulsed with overlapping peptide mixes derived from full-length proteins, and these CTL preferentially lysed HLA partially matched, DAC-treated neuroblastoma and glioblastoma cell lines. These studies show that demethylating chemotherapy can be combined with IFN-γ to increase the expression of CT antigens and MHC molecules on neuroblastoma cells, and pre-treatment with these agents makes tumor cell lines more susceptible to CTL-mediated killing. These data provide a basis to consider the use of demethylating chemotherapy in neuroblastoma patients, in conjunction with immune therapies that facilitate the expansion of CT antigen-specific CTL.

Keywords Cancer-testis antigens · Decitabine · Interferon-γ · Neuroblastoma · MHC molecules

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Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children and for those >1 year of age with advanced disease, the 3 year progression-free survival is only 30% [1]. For patients with relapsed disease, there is little that can be done with curative potential, making the development of novel therapies of much interest in this patient group. Current therapies include autologous stem cell transplantation and are associated with a moderate to severe degree of toxicity, precluding further intensification of chemotherapy. Therefore, alternate therapies such as cancer antigen vaccines or cellular immunotherapy are of interest not only for relapsed patients but also as an adjuvant to prevent disease recurrence. Limitations in the practical application of adoptive immunotherapy for NB include a lack of thorough understanding of which tumor

antigens can be reasonably targeted, as well as the mechanisms whereby tumor cells are able to escape immune surveillance. The latter includes down regulation of tumor antigens as well as MHC Class I and II molecules, preventing tumor cells from being recognized by cytotoxic T lymphocytes (CTL) [2]. Cancer-testis (CT) antigens are expressed by a number of malignant solid tumors and leukemias, as well as a subset of NB [3–5]. Some of the most immunogenic CT antigens include NY-ESO-1 and MAGE family proteins, and previous studies have demonstrated clinical and immunologic responses following CT antigen vaccines in cancer patients [6–12]. Nevertheless, the level of CT antigen expression can vary widely on tumors, and not all cells may express these proteins, complicating CT antigen-based immunotherapeutic approaches.

DNA methylation is an epigenetic mechanism used by cells to decrease the expression of several genes, including tumor suppressor genes [13, 14]. Decitabine (5-aza-2′-deoxycytidine, DAC) is a potent inhibitor of DNA methylation, and the doses associated with the demethylating action of this drug are much lower than those required for cytotoxicity [15–17]. There have been multiple in vitro studies demonstrating that exposure to demethylating agents can lead to expression of several CT antigens by tumor cell lines, including melanoma, malignant glioma, and leukemia cells, but there is no such data for NB [18–22]. Current challenges in applying cellular immunotherapy for malignant solid tumors such as NB include the identification of suitable tumor antigens as well as the problem of MHC Class I and II downregulation on the surface of tumor cells [23, 24]. Exposure to interferon-γ (IFN-γ) or DAC can be associated with increased or new onset expression of MHC Class I/II antigens on these tumors [18, 20]. Previous studies have shown that demethylating chemotherapy can facilitate the expression of CT antigens on tumor cells, but this has not been studied in NB [22]. If these antigens could also be upregulated on NB cells, then immunotherapy strategies such as vaccines or CTL infusions could be considered in high risk NB patients. We have demonstrated that DAC upregulates the expression of MAGE-A1, MAGE-A3, and NY-ESO-1 on the majority of NB cell lines, and treatment with IFN-γ increases MHC Class I expression. NB cells that are treated with these agents are more susceptible to killing by CT antigen-specific CTL-mediated killing.

Materials and methods

Treatment of tumor cell lines with DAC and IFN-γ

BE2C, NBL-S, Kelly, NGP, SHSY5Y, EB2M17, and IMR32 (Human NB cell lines, kindly provided by Dr. Rani E. George, Pediatric Hematology-Oncology, Children's Hospital of Boston) were grown in RPMI (GIBCO BRL). SKN-AS, SKN-SH, CHP134 (human NB cell lines purchased from American Type Culture Collection (ATCC), (Rockville, MD), and SKN-MC (human neuroepithelioma cell line, ATCC HB-10) were grown in Dulbecco Modified Eagle Medium (DMEM)(GIBCO BRL). T98G (human glioblastoma cell line; ATCC CRL-1690), A172 (human glioblastoma cell line; ATCC CRL-1620), U251 (Human glioma, kindly provided by Dr. Song Lee at Penn State Hershey Medical Center, Hershey, PA) and were grown in DMEM, and CCF-STTG1 (human astrocytic glioblastoma cell line; ATCC CRL-1718) cells were grown in RPMI 1640 (GIBCO BRL). All media were supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cells were counted and plated in 10-cm dishes 1 day prior to treatment, and then the media was removed and replaced with fresh media containing 1 μM of DAC (Sigma–Aldrich, St. Louis, MO) or 100 ng/ml human recombinant IFN-γ (R&D Systems, Minneapolis, MN). After incubation with DAC or IFN-γ for 5 days, the cells were harvested, counted, and then prepared for molecular and functional assays.

Reverse transcription polymerase chain reaction

Total RNA was prepared from 2 to 5 × 10^6 cells using the RNA-Bee-RNA Isolation reagent (TEL-TEST, Inc. Friendswood, TX) as per the kit instructions and was quantified by absorbance at 260 nm. A 1-μg aliquot of total RNA was converted to cDNA using the RETROscript (Ambion, Austin, TX), and PCR was performed on 1 μl of cDNA using Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Primers were synthesized using previously published sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [25], MAGE-A1 [26], MAGE-A3 [27], NY-ESO-1 [26]. After the PCR mixes were heated to 95°C for 10 min, 30 cycles of amplification were performed (1 cycle = 60 s denaturation at 94°C, 60 s annealing at the temperature as indicated below, 60 s extension at 72°C), and a final extension was completed at 72°C for 6 min. Annealing was 62°C for MAGE-A1 and NY-ESO-1, 70°C for MAGE-A3, and 55°C for GAPDH (25 cycles). Relative expression levels were determined by visualizing DNA bands on ethidium bromide-stained 1.5% agarose gels.

Western blotting

Cells were lysed with M-PER mammalian protein extraction reagent buffer (Pierce Biotechnology Inc., Rockford, IL) with proteinase inhibitors (Pierce Biotechnology Inc.,

Rockford, IL). Protein concentrations were determined according to the manufacturer's protocol using the BIO-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of whole cell lysates were separated on 10% SDS denaturing polyacrylamide gels and transferred onto nylon membrane (Millipore) at 30 V for 1 h at room temperature. Membranes were blocked in TBS containing 0.5% Tween 20 plus 5% nonfat dried milk for 1 h at room temperature and probed with primary antibodies at 4°C overnight. Blots were incubated with antibodies to MAGE A1 (MA545, Santa Cruz Biotechnology), MAGE A3 (mouse polyclonal, Abcam), NY-ESO-1 (monoclonal, E978, Invitrogen), and actin (Sigma), the latter as a loading control. Membranes were washed 3 times for 10 min each in TBS containing 0.5% Tween 20 and incubated with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Targeted proteins were visualized using an enhanced chemiluminescence detection system (Pierce Biotechnology Inc., Rockford, IL).

Flow cytometry

Flow cytometry was performed with a FACScan (BD Biosciences, San Jose, CA) for MHC Class I and Class II expression. MHC Class I and Class II expression levels were determined by staining with directly conjugated monoclonal antibodies (mAb): PE conjugated anti-human HLA-ABC mAb to detect MHC Class I and FITC conjugated anti-human HLA-DR, DP, DQ mAb (BD Biosciences, San Jose, CA) to detect MHC Class II.

Generation of dendritic cells for CTL stimulation

PBMC from normal volunteers were isolated by Ficoll-Paque (Amersham Biosciences) gradient centrifugation of 50 ml heparinized whole blood. Cells were seeded at 1×10^7 cells/2 ml/well into 6-well plates (Corning) in CellGenix™ DC medium. PBMC were incubated in a humidified incubator for 2 h at 37°C to allow plastic adherence. After 2 h of incubation, the nonadherent cells were removed and cryopreserved at −80°C, to be used later for cell isolation. The adherent blood monocytes were cultured 5 days in CellGenix™ DC medium supplemented with human recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF; 1,000 U/ml, Bayer, Seattle, WA) and interleukin 4 (IL-4; 10 ng/ml; R&D Systems, Minneapolis, MN). Immature DC were matured for 48 h in the presence of 10 ng/ml of TNF-α, 10 ng/ml of IL-1β, 10 ng/ml of IL-6 (R&D Systems, Minneapolis, MN), and 1 μg/ml of PGE2 (Sigma, St. Louis, MO).

CT antigen-specific T-cell cultures

To generate CT antigen-specific T cells, PBMC from healthy donors were stimulated with DC pulsed with MAGE-A1, MAGE-A3, and NY-ESO-1 overlapping peptide mixes, consisting of pooled, 11 amino acid overlapping 15mers derived from the full-length protein (JPT Peptide Technologies, Berlin, Germany). These cells were stimulated an additional two times (weekly) with DC pulsed with these peptide mixes. IL-7 and IL-15 (10 ng/ml; R & D Systems, Minneapolis, MN) were added to these cultures initially and replaced weekly, and IL-2 (50 units/ml, Novartis, East Hanover, NJ) was added every 3 days, starting at day 3.

To select antigen-specific T cells based on CD137 (human CD137 MicroBead Kit, Miltenyi Biotec Inc. Auburn, CA) production, we performed preliminary studies to determine the time at which CD137 reached its peak level of expression post-stimulation. CMV pp65 CTL were examined for CD137 expression, which was at its maximum from 18 to 24 h post-stimulation. Therefore, T cells that had already been stimulated twice with tumor antigen were re-exposed with DC pulsed peptide mixes for 24 h. After CD137 selection, activated T cells were further cultured for 14 days in a cocktail containing the anti-CD3 monoclonal antibody OKT3 (30 ng/ml; Ortho-Biotech, Raritan, NJ), irradiated (3 Gy) allogeneic feeder PBMC (1×10^6 cells/ml), irradiated (10 Gy) BLCL (0.1×10^6 cells/ml), and IL-2 (50 units/ml, Novartis, East Hanover, NJ). This cell culture was placed in a T25 flask, OKT3 was removed on day 5, and 50% of the culture medium (RPMI 1640 with 10% FBS) was replaced every 3 days or as needed with fresh medium and IL-2 [28, 29]. The tumor-specific function of expanded CTL was characterized in chromium release assays described below.

Chromium release assays

Cytotoxicity were determined by ^{51}Cr release assays using the following target cells: autologous B cell blasts (BB; used as a negative control), BB pulsed with the MAGE-A1, MAGE-A3, and NY-ESO-1 peptide mixes, as well as tumor cells before and after DAC and IFN-γ treatment. Targets were labeled overnight with ^{51}Cr (100 μCi/10^6 cells; Perkin-Elmer Life and Analytical Science), washed in PBS, and dispensed in triplicate into 96-well V-bottom plates (ICN) at 4×10^3 cells/well, as previously described [30]. CTL were added at a responder-to-target ratio (20:1), and after pelleting and incubation for 4 h, the supernatant was analyzed in a gamma counter. Spontaneous and total release for each target were used to calculate percent-specific release by the following formula: % specific release = (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm).

Results

Up-regulation of CT antigen expression on neuroblastoma cells by DAC

To determine whether the treatment of NB cell lines with DAC would alter the expression of MAGE-A1, MAGE-A3, and NY-ESO-1, ten NB cell lines were treated with 1 μM DAC, and RT-PCR was performed for MAGE-A1, MAGE-A3, and NY-ESO-1. Data presented in Fig. 1 demonstrated that most of these cell lines were initially negative for these antigens by RT-PCR, and after 5 days of exposure to DAC, all cell lines had either increased or new onset expression of at least one of these antigens. GAPDH (an endogenous control) was equally expressed on all cell lines before and after DAC treatment (data not shown). Similarly, four GBM cell lines were treated with 1 μM DAC, and all four had increased or new onset expression of one or more of these antigens (Fig. 2). Consistent with the observation that DAC induces expression of these tumor antigen genes at a transcriptional level, we also observed induction of protein expression by DAC-treated NB and GBM cell lines, which were used in cytotoxicity assays (Fig. 3). We assessed MHC Class I and Class II expression on NB cell lines after treatment with 1 μM DAC for 5 days by flow cytometry, and did not see any significant changes after DAC treatment (data not shown).

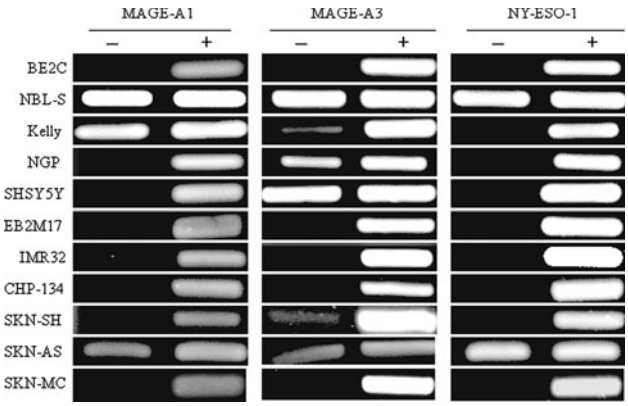

Fig. 1 RT-PCR for MAGE-A1, MAGE-A3, and NY-ESO-1 expression on neuroblastoma cell lines. Neuroblastoma cell lines were treated with 1 μM decitabine (DAC) for 5 days. − untreated; + DAC treated

The effect of the various doses of DAC and duration of treatment on MAGE-A1, MAGE-A3, and NY-ESO-1 expression

To select an optimal concentration of DAC which would result in upregulation of these antigens, one NB cell line was treated with varying concentrations of DAC for 5 days. RT-PCR analysis showed that the expression of these CT antigens was enhanced by increasing concentrations of DAC, which did not impact GAPDH gene expression (Fig. 4a). There was expression of MAGE-A1, MAGE-A3, and NY-ESO-1 after 5 days of exposure to 0.1 μM DAC, and this was increased using a concentration of 1 μM DAC. To determine the time point at which these antigens are expressed following the introduction of DAC, this NB cell line was exposed to 1 μM DAC, and expression of MAGE-A1, MAGE-A3, and NY-ESO-1 was determined at 6, 16, 24, 48, and 72 h post-exposure. Figure 4b shows that expression of NY-ESO-1, MAGE-A1, and MAGE-A3 was observed by RT-PCR at 24 h after DAC treatment. The expression of these antigens was best at 72 h compared to

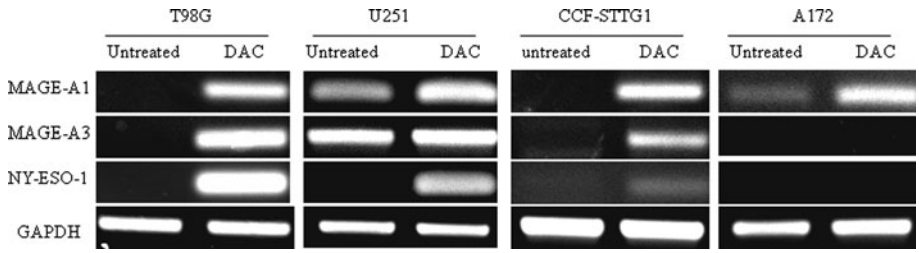

Fig. 2 RT-PCR for MAGE-A1, MAGE-A3, and NY-ESO-1 expression on GBM cell lines. GBM cells were treated with 1 μM DAC for 5 days

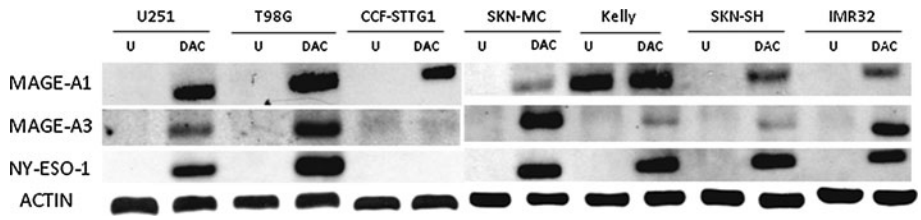

Fig. 3 MAGE-A1, MAGE-A3, and NY-ESO-1 protein expression were assessed by western blot assay after DAC treatment. Western blot for MAGE-A1, MAGE-A3, and NY-ESO-1 expression on glioblastoma multiforme and neuroblastoma cell lines following treatment with 1 μM decitabine (DAC). *U* untreated; *DAC* 1 μM DAC for 5 days

the other time points. GAPDH gene expression was not affected by the duration of treatment.

Enhancement of MHC Class I and Class II expression on NB cell lines after IFN-γ treatment

To confirm the effects of IFN-γ on the expression of MHC Class I and II molecules, we treated NB cell lines with 100 ng/ml of IFN-γ for 5 days, and MHC Class I and Class II expression in the untreated and IFN-γ-treated cells was assessed by flow cytometry using PE conjugated anti-human HLA-ABC mAb (to detect MHC Class I) and FITC conjugated anti-human HLA-DR, DP, DQ mAb (to detect MHC Class II). Figure 5 depicts flow cytometry results from NB cells prior to and after exposure to IFN-γ. Our studies show that MHC Class I was variably increased in several cell lines, and there was little change in MHC Class II expression in most cell lines.

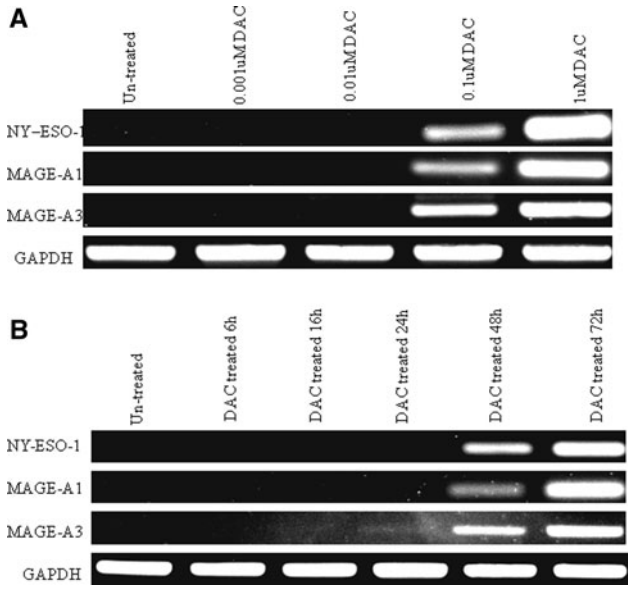

Fig. 4 Effect of different concentrations of DAC and treatment time on expression of NY-ESO-1, MAGE-A1, and MAGE-A3. **a** BE2C NB cell line was treated with different concentrations of DAC for 5 days. **b** BE2C NB cell line was treated with 1 μM DAC for 6, 16, 24, 48, and 72 h. Expression of NY-ESO-1, MAGE-A1, and MAGE-A3 was determined by RT-PCR

Generation of CT antigen-reactive T lymphocytes, and recognition of DAC-treated tumor cells

In order to culture MAGE-A1, MAGE-A3, and NY-ESO-1-specific CTL directed against a broad range of epitopes, peripheral blood mononuclear cells from healthy donors were stimulated with MAGE-A1, MAGE-A3, and NY-ESO-1 PepMix, selected for expression of the activation marker CD137, and then rapidly expanded with anti-CD3 and IL-2, as previously described [31]. Tumor antigen-reactive CTL were expanded from two healthy donors using this method. Figure 6 depicts antigen-specific cytotoxicity by these peptide mix stimulated CTL. The resulting CTL from donor 1 had antigen-specific cytotoxicity directed against all of these antigens (Fig. 6a), but CTL selected from donor 2 were only specific for MAGE-A1 (Fig. 6b). Donor 1 (HLA A2, 25; B18, 60; DR1; DR4) CTL had increased cytotoxicity against the HLA partially matched GBM cell lines T98G (HLA A2) and U251 (HLA A2, B18) and the partially matched NB cell line SKNSH (HLA B18) and SKNMC (HLA A25) following exposure to DAC and IFN-γ. Donor 2 (HLA A1,3; B7,8; Cw7; DR2; DR51) CTL, which only recognized MAGE-A1, had increased cytotoxicity against the partially matched NB cell lines Kelly (HLA A1, B8), SKNSH (HLA A1) and SKNMC (HLA A1, B8) after these lines had been treated with DAC and IFN-γ for 5 days (Fig. 6b). In contrast, untreated NB or GBM cells and HLA mismatched tumor cells treated with DAC were fully resistant to the lysis by antigen-reactive T lymphocytes. These data show that CTL stimulated with overlapping peptide mixes derived from full-length CT antigens can preferentially kill tumor cell lines that have had expression of MAGE-A1, MAGE-A3, and NY-ESO-1 upregulated.

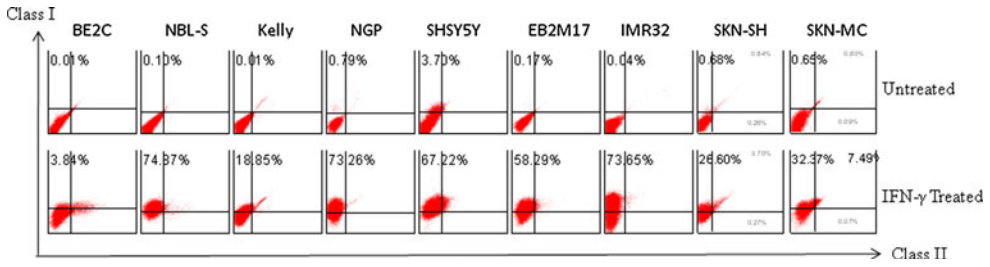

Fig. 5 Expression of MHC Class I and MHC Class II molecules on neuroblastoma cell lines upon treatment with IFN-γ. Neuroblastoma cell lines were incubated in presence or absence of 100 ng/ml human recombinant IFN-γ for 5 days. MHC Class I and Class II expression levels were determined by flow cytometry using PE conjugated anti-human HLA-ABC mAb to detect MHC Class I and FITC conjugated anti-human HLA-DR, DP, DQ mAb to detect MHC Class II

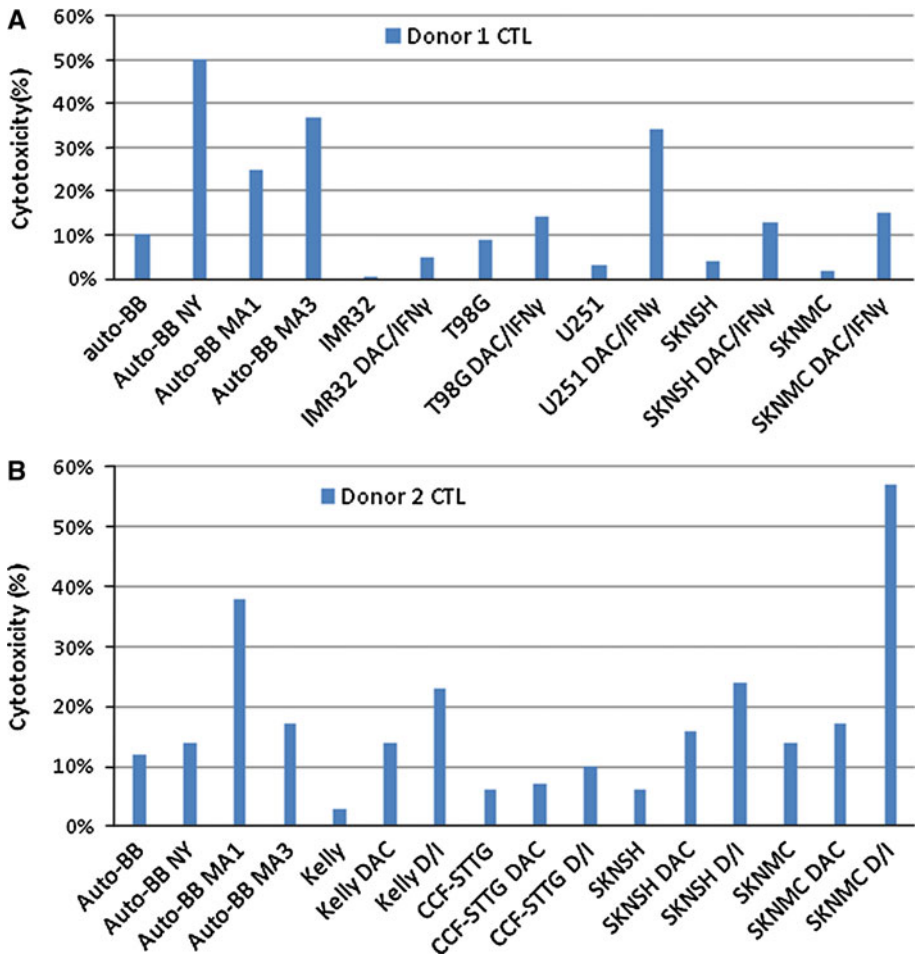

Fig. 6 Cytotoxicity assay. CTL derived from two normal donors (**a**, **b**) stimulated with autologous DC loaded with NY-ESO-1, MAGE-A1, and MAGE-A3 pooled peptides. CTL were incubated with the indicated target cells in a 4-h chromium release assay (target: responder ratio 1:20). Antigen-reactive CTL killed partially HLA-matched neuroblastoma and GBM cell lines treated with DAC and IFN-γ

Discussion

Epigenetic regulation has several effects on cancer cells, including the expression of tumor antigens, facilitating tumor cell apoptosis by methylation of tumor suppressor genes, and modulating caspase-8 activation [13, 14, 32]. There are several mechanisms by which tumor cells can escape immune surveillance, including the down regulation of tumor antigens and MHC Class I/II molecules [2, 23, 24]. Recently several groups have demonstrated the fact that the use of demethylating agents and histone deacetylase inhibitors can be used to increase the expression of CT antigens on tumor cell lines, but there is no data for NB or other tumors that primarily affect children [18–22]. Current therapy for stage IV NB includes the sequential use of high-dose chemotherapy, which cannot be further intensified. Therefore, further study is needed into the biologic feasibility to targeting CT antigens on NB cells by specific CTL. We have demonstrated that DAC and IFN-γ can have positive effects on the expression of CT antigens and MHC Class I/II molecules by NB cell lines. The majority of NB cells treated with DAC had either new onset or increased expression of MAGE-A1, MAGE-A3, or NY-ESO-1 as early as 24 h post-exposure. The fact that CT antigen-specific CTL have improved recognition/killing of DAC-treated tumor cell lines indicates that pharmacologic upregulation of specific antigens could be used prior to immunotherapy to maximize the likelihood of a clinical response.

One of the most significant challenges to the practical application of cellular immunotherapy is the identification of tumor antigens that are preferentially expressed on tumor cells. CT antigens are expressed on a limited number of tissues, including male germ line cells, placenta, melanoma, bladder, breast, prostate, non-small cell lung cancers, and NB [33, 34]. Three CT antigens that can be expressed on solid tumors include NY-ESO-1, MAGE-A1, and MAGE-A3 [3, 35, 36]. From previous studies, the frequency of NY-ESO-1 expression has been reported to range from 30 to 82% for NB, and IFN-γ producing NY-ESO-1-specific T cells have been detected from NB patients with NY-ESO-1 positive tumors [3, 37]. Screening NB for these antigens by RT-PCR has revealed that 44% are positive for MAGE-A1 and 21% for MAGE-A3, and immunohistochemical analysis showed a good correlation between protein and gene expression [3]. There is no data on the detection of MAGE-specific T cells in NB patients, but studies in adult patients

have demonstrated that MAGE-A1- and MAGE-A3-specific T cells were present and can be augmented following a vaccine, or by stimulating these T cells in culture [6–8]. There has also been a single report of the detection of IFN-γ producing NY-ESO-1-specific T cells in NB patients [37], as has been demonstrated in adult patients with NY-ESO-1 positive tumors. There have been several trials with MAGE-A1, MAGE-A3, and NY-ESO-1 vaccines in adult cancer patients with malignant solid tumors, using either whole tumor protein or individual epitopes. Clinical trials have been reported using DC-based vaccines as well as using whole tumor proteins or HLA-restricted, CT antigen epitopes, and several of these studies have demonstrated immune responses to MAGE-A1, MAGE-A3, and NY-ESO-1 post-vaccination [9–12, 38]. Some subjects have had regressions of metastatic lesions and improved survival following CT antigen vaccines, but many patients receiving immunotherapy experience disease progression [12, 38]. Therefore, strategies which facilitate the recognition of tumors by antigen-specific T cells are of interest in improving the clinical outcomes following immune-based therapies.

Some studies have shown that DAC can upregulate the expression of CT antigens and MHC Class I molecules on tumor cells, but data are lacking on whether CT antigens can be epigenetically upregulated on NB cells [18, 20]. Serrano et al. [20] demonstrated that MHC Class I antigens could be upregulated on DAC-treated melanoma cells, restoring MAGE-A1 antigen-specific killing of the DAC-treated cells. Teitz et al. [32] showed that epigenetic upregulation of the pro-apoptotic gene caspase-8 can also occur in NB as a result of demethylation with DAC, restoring capsase-8 expression and making these cell lines more susceptible to apoptosis. Fulda reported that DAC could be combined with IFN-γ to upregulate caspase-8, enhancing TRAIL-induced apoptosis in NB and medulloblastoma cell lines [39]. Our studies confirm previous reports showing that IFN-γ can induce expression of MHC Class I and Class II antigens on NB cells in vitro and in vivo [40, 41]. Our findings and previous studies demonstrated that MHC Class I was upregulated to a greater degree than MHC Class II, but some of the changes in MHC antigen expression were relatively small. IFN-γ has been used safely as an immunostimulant in numerous clinical situations, including cancer vaccines [42], and could be combined with a regimen that includes a demethylating agent such as DAC, to maximize antigen presentation.

Having demonstrated that NB cells upregulate CT antigens on a molecular as well as a protein level, we next utilized overlapping peptide pools derived from full-length MAGE-A1, MAGE-A3, and NY-ESO-1, so that the resulting CTL would be able to recognize CT epitopes in the context of multiple HLA-alleles. The degree of HLA matching and tumor antigen expression impact tumor cell recognition/killing by the CTL, and these factors are variably affected in tumor cell lines by pre-treating with DAC or IFN-γ. In the setting of using a panel of partially HLA matched tumor cell lines as we have done, it is difficult to ascertain precisely which HLA antigen represents the restricting element in terms of tumor cell killing. We used multiple tumor lines to permit cytotoxicity assays with targets that partially matched with the CTL donor. Donor 1 CTL had more cytotoxicity against U251 than IMR32, which could have been related to the fact that the U251 tumor line is matched for more HLA antigens with this donor than IMR32. While the U251 cell line had detectable tumor antigens by RT-PCR before DAC treatment, these antigens could not be detected by western blot before DAC, and were expressed at a protein level after DAC, which could be the reason for enhanced cytotoxicity. Although the Kelly cell line expressed MAGE-A1 before and after DAC treatment by both PCR and Western blot, MHC Class I expression was detectable only after IFN-γ treatment (from 0.01 to 18.85%), which could have been responsible for the increased cytotoxicity. Among all the targets tested with donor 2 CTL in this study, cytotoxicity against SKNMC was increased the most after exposure to both IFN-γ and DAC. It is not clear why some tumor cells become more sensitive to antigen-specific CTL than others after treatment with DAC or IFN-γ, and there are possibly other variables involved that could be making some cell lines more or less susceptible, requiring further investigation of these mechanisms.

The practical application of tumor antigen-specific cellular immunotherapy can be limited by the fact that these CTL exist at exceptionally low frequencies, precluding culture with methods typically used for memory CTL. Several authors have described MAGE-A1-, MAGE-A3-, and NY-ESO-1-specific epitopes that are restricted to certain HLA types [8, 9]. For practical purposes in clinical immunotherapy, stimulating T cells with individual epitopes restricts the patients that can potentially benefit from this strategy. Limitations in expanding CT antigen-specific CTL include having an advanced knowledge of all relevant CT antigen epitopes in the context of multiple HLA antigens. For this reason, we used a pooled mixture of overlapping peptides from full-length MAGE-A1, MAGE-A3, and NY-ESO-1. Wofl and group demonstrated that IFN-γ producing, antigen-specific T cells could be isolated by selecting cells expressing the activation marker CD137 [43]. Considering the low precursor frequencies of CT antigen-specific CTL, we decided to select these cells based on expression of CD137 and demonstrated that overlapping peptide mixes can be used to generate these cells. The resulting MAGE-A1-, MAGE-A3-, and NY-ESO-1-specific CTL preferentially lysed NB and GBM cells that have been exposed to DAC and IFN-γ. These data provide the proof of concept

that tumor cells can be made more susceptible to CTL killing by increasing the expression of CT antigens and MHC Class I/II molecules. This result is consistent with the findings that chemotherapy enhances tumor cell susceptibility to CTL-mediated killing in mice [44]. Further studies are needed to determine the mechanism of killing of tumor cells which have had the expression of CT antigens upregulated following exposure to demethylating chemotherapy.

Several clinical trials have been conducted using pharmacologic agents to epigenetically modify the expression of genes by tumor cells [17, 19]. Studies have shown that the clinical administration of doses of DAC in the range of 15–20 mg/m^2/day was associated with demethylation of the MAGE-A1 promoter [45]. In one series of 10 patients with refractory solid tumors, DAC was given at 2 mg/m^2/day for 7 days, with significant MAGE-A1 promoter hypomethylation by 14 days post-exposure in all subjects studied [45]. Genomic DNA methylation reverted to baseline levels by 28 days after initial exposure to DAC, indicating the potential need for cyclic administration of this drug to achieve sustained CT antigen expression. Blum and group reported global DNA hypomethylation at a DAC dose of 15 mg/m^2/day, with an average maximal plasma concentration of 1.3 μM, compatible with the concentrations used to treat the tumor cell lines were exposed to in this study. Further studies are needed to determine how long MAGE-A1, MAGE-A3, and NY-ESO-1 are expressed after the cessation of DAC and whether the same DAC dose can be used to restore CT antigen expression after several cycles. Since DAC has a low toxicity profile in this dose range, it could be considered to enhance immune responses to CT antigen vaccines or adoptively transferred antigen-specific CTL.

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