

Improving T-cell Therapy for Relapsed EBV-Negative Hodgkin Lymphoma by Targeting Upregulated MAGE-A4

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Abstract

Purpose: Patients with Hodgkin lymphoma (HL) relapsing after hematopoietic stem cell transplant have limited options for long-term cure. We have shown that infused cytotoxic T cells (CTL) targeting Epstein Barr virus (EBV)-derived proteins induced complete remissions in EBV⁺ HL patients. A limitation of this approach is that up to 70% of relapsed HL tumors are EBV-negative. For these patients, an alternative is to target the cancer/testis antigen MAGE-A4 present in EBV antigen-negative HL tumors. Furthermore, epigenetic modification by clinically available demethylating agents can enhance MAGE-A4 expression in previously MAGE-negative tumors.

Experimental Design: We explored the feasibility of combining adoptive T cell therapy with epigenetic modification of tumor antigen expression. We further characterized MAGE-A4-specific T-cell phenotype and function, and examined the effects of the epigenetic modifying drug decitabine on these T cells.

Results: Cytotoxic T cells were generated specifically recognizing MAGE-A4 expressed by autologous HL targets and tumor cell lines. Decitabine—previously shown to increase tumor antigen expression in HL—did not compromise MAGE-A4-specific T-cell phenotype and function. In patients treated with decitabine, expanded MAGE-A4-specific T cells had a broader antitumor T cell repertoire, consistent with increased antigen stimulation *in vivo*.

Conclusions: Adoptive transfer of MAGE-A4-specific T cells, combined with epigenetic modifying drugs to increase expression of the protein, may improve treatment of relapsed HL. *Clin Cancer Res*; 17(22); 7058–66. ©2011 AACR.

Introduction

Although the majority of patients with Hodgkin lymphoma (HL) respond favorably to conventional chemotherapy and radiotherapy, an appreciable number relapse (1). For patients relapsing after autologous and/or allogeneic hematopoietic stem cell transplantation (HSCT; ref. 2), the prognosis is especially poor. Moreover, the current success in HL treatment is tempered by the fact that many long-term survivors develop life-threatening complications, such as secondary malignancies and cardiac toxicities (3, 4).

An attractive option to reduce the toxicity of standard therapy is to use tumor-specific T cells as adoptive immunotherapy, to augment the host response against the tumor cells. About 30% of HL expresses Epstein Barr virus (EBV) antigens that are suitable targets for adoptive T-cell therapy. T cells specific for the EBV associated proteins LMP1 and LMP2 expand *in vivo* following infusion, infiltrate tumor sites, decrease viral load, and induce clinical remissions (5, 6). To develop adoptive immunotherapy for the majority of HL patients with relapsed/refractory EBV-negative tumors, however, nonviral tumor associated antigens must be targeted. Potential targets include cancer/testis antigens (CTA) which are particularly attractive because of their selective expression on tumor cells. MAGE-A4, an HL-associated CTA, is expressed only in malignant cells and in immune-privileged germ-line cells (7, 8). Like other members of the MAGE-A family of proteins, it is putatively required by tumors to mediate antiapoptotic functions by interacting with p53 (9). CTA, including MAGE-A4, are under epigenetic control and DNA methyltransferase and histone deacetylase (HDAC) inhibitors, which restore expression of apoptosis and antiproliferation genes, have shown promise in several malignancies (10–16). We and others have shown that a subset of EBV-negative HL express

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Translational Relevance

A subset of patients with Hodgkin lymphoma still relapses after conventional therapies and presents with a poor prognosis. Several new treatments are being investigated for this group, and some—such as T-cell immunotherapy—have shown great promise. Each therapy will, however, remain limited by inherent difficulties: in the case of Epstein Barr virus–specific T cells, the absence of the relevant antigen in most patients is a constraint on otherwise encouraging results. Choosing a different target antigen will inevitably present similar obstacles; a synergistic second therapy is thus needed. Epigenetic modifying drugs have been shown to upregulate tumor antigen expression. Exploring combinations of new therapies anticipated to have a better safety profile, while maintaining the multimodality approach (the hallmark of successful cancer therapy), should provide adequate alternatives to current regimens. This study validates such a therapy applicable to both autologous and allogeneic hematopoietic stem cell transplant recipients.

MAGE-A4, the only member of the MAGE family seen in this malignancy (Supplementary Fig. S1; ref. 8) and that MAGE-A4 expression is enhanced by the demethylating agent decitabine (10, 17). Furthermore, epigenetic therapy mediates immune recognition of tumors by upregulating the expression of tumor-specific antigens such as MAGE-A4 (10, 18, 19). Here we validated a combined immune and epigenetic therapy approach to treat relapsed HL. We hypothesized that epigenetic treatment would (i) upregulate MAGE-A4 expression in malignant cells, (ii) render tumor cells susceptible to the cytolytic effect of MAGE-A4–specific T cells without compromising T-cell function, and (iii) broaden the tumor-specific immune response *in vivo*. We show that combining decitabine with adoptive T-cell therapy is a feasible and potentially effective immunotherapeutic approach to treating relapsed HL.

Materials and Methods

Blood donors and tumor cell lines

Peripheral blood mononuclear cells (PBMC) used to generate dendritic cells (DC), CTL lines, and PHA blasts were obtained from healthy volunteers and patients with HL after obtaining informed consent on Baylor College of Medicine Institutional Review Board (IRB)-approved protocols. Umbilical cord blood units were obtained from the MD Anderson Cancer Cord Blood Bank. For *in vivo* immune monitoring studies, blood was collected from patients enrolled under MD Anderson Cancer Center IRB-approved protocols (# 2007-0536 and 2008-0769, NCT00543582 and NCT00866333, ClinicalTrials.gov database). The EBV-negative HL cell line L1236 was obtained from DSMZ.

Cells were maintained in RPMI 1640 with 10% FBS (Invitrogen) and 2 mmol/L L-glutamine (GlutaMAX; Gibco). The HLA type of L1236 was determined to authenticate this cell line, in accordance with recommendations of the NIH.

Immunohistochemistry

Patient samples were provided by the Texas Children's Hospital Pathology Department. Cells suspended in PBS were placed on glass slides by cytospin centrifugation (Shandon Cytospin Cyto centrifuge; Thermo Scientific) and immediately fixed by incubating for 15 minutes with 4% paraformaldehyde (BD Biosciences). Antigen retrieval was achieved by incubating slides in 0.3% Triton X-100 (Gibco) for 5 minutes and then Digest ALL1 (Zymed) for 10 minutes at 37°C. Endogenous peroxidase activity is blocked in 3% hydrogen peroxide. Immunohistochemistry was done using the Powervision+ kit (ImmunoVision Technologies) according to the manufacturer's instructions. For antigen detection, slides were first incubated in preblock/diluent for 30 minutes as provided in the kit and then incubated with anti-MAGE-A antibody–detecting MAGE-A4 and other members of the MAGE family of proteins (Abcam) diluted 1:50 in diluent for 1 hour at room temperature. The final step of detection was done using an anti-mouse/anti-rabbit HRP polymer provided in the kit and detected using DBA.

Generation of MAGE-A4–specific cytotoxic T cells

Monocyte-derived DCs presenting MAGE-A4 peptides were generated as previously described (20), with some modifications. Briefly, PBMCs were obtained from Ficoll gradient centrifugation of blood from donors. The same protocol was used for all sources of T cells (healthy donors, HL patients, and umbilical cord blood). CD14-positive cells were selected using magnetic cell sorting as described by the manufacturer (Miltenyi). Cells were then cultured in DC media (CellGenix supplemented with 2 mmol/L GlutaMAX TM-1; CellGenix; GlutaMAX; Invitrogen) with 800 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF; Sargramostim Leukine; Immunex Corp.) and 1,000 U/mL Interleukin (IL-4; R&D Systems) for 5 days. IL-4 and GM-CSF were replenished on day 3. DCs were matured on day 5 using a maturation cocktail consisting of 800 U/mL GM-CSF, 1,000 U/mL IL-4, 10 ng/mL IL-1 β , 100 ng/mL IL-6, 10 ng/mL TNF- α (R&D Systems), and 1 μ g/mL PGE2 (Sigma). On day 7, mature DCs were pulsed with an overlapping peptide library spanning the MAGE-A4 protein (JPT Technologies) for an hour. After incubation, DCs were used to stimulate the CD14-negative fraction at a responder: stimulator ratio of 10:1, along with the cytokines IL-7, IL-12, and IL-15 (at concentrations of 10 ng/mL each) in CTL media (50% RPMI 1640, 50% Click's media, 10% human AB serum, and 2 mmol/L GlutaMAX). Subsequent weekly stimulations were done using MAGE-A4–pulsed DCs as antigen-presenting cells (APC), again at responder: stimulator ratios of 10:1 with the addition of twice weekly feeds with the cytokines IL-7 and IL-2 (50 U/mL) during the second stimulation, and IL-2 or IL-15 (5 ng/mL) in

subsequent stimulations. In healthy donors, we carried out weekly stimulations for up to 4 weeks with MAGE-A4 antigen-pulsed DCs. After at least 2 stimulations, cells were harvested, counted, and their phenotype, specificity, and function assessed.

Enzyme-linked immunospot analysis

IFN γ enzyme-linked immunospot (ELISpot) analysis was used to evaluate the specificity of expanded MAGE-A4 T cells in response to peptides (Jerini AG) spanning the entire MAGE-A4 protein. Briefly, plates were coated with anti-IFN γ capture antibodies, and 1×10^5 cells were plated in each well. Peptide mixes or specific peptides were then added onto cells in duplicates or triplicates. Irrelevant peptides (Aspf16) and *Staphylococcus* enterotoxin B or phytohemagglutinin (PHA) were used as negative and positive controls, respectively. Their ability to elicit an IFN γ secretory response was then independently assessed by Zellnet Consulting and compared with input cell numbers to obtain the frequency of tumor-specific T cells.

Flow cytometry phenotyping

Expanded MAGE-A4-specific T cells were also assessed for surface expression of CD3, CD4, CD8, CD25, CD45RA, CD45RO, CCR7, CD27, CD28, CD69, and CD70 using fluorochrome-conjugated monoclonal antibodies against these proteins (BD Biosciences). T cells were harvested and washed with cold PBS ($1 \times$ PBS; Sigma) supplemented with 1% FBS (Invitrogen). They were spun down and antibodies were added to the pellets and allowed to bind at 4°C for 20 minutes in the dark. Cells were then washed twice with cold PBS with 1% FBS and acquired on a FACSCalibur flow cytometer. Data were analyzed using Cell Quest software (Becton Dickinson).

Chromium release assay

MAGE-A4 T cells were tested for selective killing of MAGE-A4 targets using a chromium release cytotoxicity assay. Briefly, ^{51}Cr -labeled target cells (autologous PHA blasts or LCL lines pulsed with the MAGE-A4 peptide mix) were cocultured with effector cells that have been serially diluted to produce the effector-to-target (E/T) ratios specified in the results. Target cells incubated in complete medium or 1% Triton X-100 (Sigma-Aldrich) were used to determine spontaneous and maximal ^{51}Cr release, respectively. After 4 to 6 hours, supernatants were harvested and radioactivity was measured on a gamma counter. Mean percentages of specific lysis of triplicate wells were calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$.

Incubation of T cells and tumor cells with decitabine

In vitro experiments with the demethylating agent decitabine (5'-aza-2'-deoxycytidine) were done by adding 1 $\mu\text{mol/L}$ of the drug to cultured T cells. For experiments with MAGE-A4-specific T cells, generated cells were cultured in the presence of the drug for 24 hours before assays (flow cytometry and IFN γ ELISpot) were done.

Evaluating the tumor-specific immune response in HL patients receiving decitabine

Patients with relapsed and refractory HL enrolled on MD Anderson protocol # 2007-0536 were treated with decitabine (75 mg/m 2) daily \times 5 days and oral MGCD-0103 therapy (85 mg fixed dose) for 3 consecutive weeks. Blood for tumor-specific immune analysis was collected predecitabine, 2 days after completion of decitabine and following completion of a cycle. Patients enrolled on MD Anderson protocol # 2008-0769 received entinostat starting at 10 mg and later increased to 15 mg orally self-administered every 2 weeks on 4 week cycles once weekly for 3 consecutive weeks. Blood for immune analysis was collected pretherapy and 1 week following treatment.

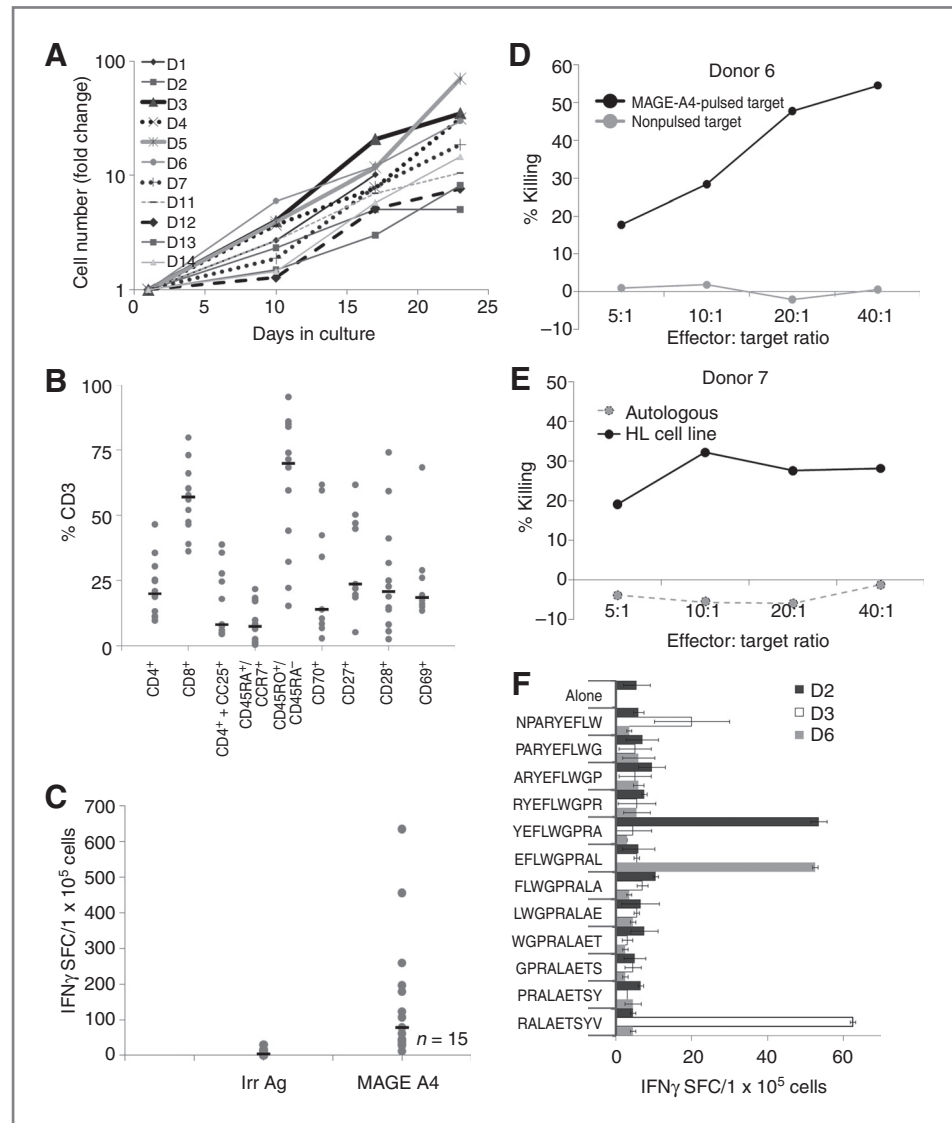
Results

MAGE-A4-specific T cells can be expanded from healthy adult donors for use in adoptive immunotherapy

To determine whether MAGE-A4-specific T cells can be successfully expanded from different donor sources, we generated allogeneic T cell lines from healthy adult donors and umbilical cord bloods and evaluated their phenotype and function *in vitro*. Healthy donor T cell lines, predominantly CD8 cytotoxic T cells (median 57%), and CD4 T helper cells (median 20%) expanded to sufficient numbers for clinical use (Fig. 1A and B). A median of 69% of T cells had a memory (CD45RA $^-$ CD45RO $^+$) phenotype, whereas a median of 7% had a naive phenotype (CD45RA $^+$ CCR7 $^+$). Median expression of activation molecules was 24% (CD27), 18% (CD69), and 14% (CD70; Fig. 1B). Specificity of the T cell lines was evaluated in IFN γ ELISpot assays. A median response of 78 IFN γ spot forming cells (SFC)/ 1×10^5 cells (mean 152; range: 12–635) was observed in response to MAGE-A4 compared with a median of 4 IFN γ SFC/ 1×10^5 cells (mean 4; range: 0–30) in response to irrelevant peptides (Fig. 1C). Both CD4 $^+$ and CD8 $^+$ T cells contributed to the IFN γ response (Supplementary Fig. S2). Furthermore, generated MAGE-A4 T cells killed MAGE-A4-pulsed autologous targets (PHA blasts), but not non-MAGE-A4 expressing autologous targets (Fig. 1D, Table 1) or allogeneic PHA blasts (data not shown). MAGE-A4 T cells also killed HL cell lines expressing MAGE-A4 matched in at least 1 HLA allele (Fig. 1E).

In contrast, it was only possible to expand 4 of 28 MAGE-A4-specific CTL lines from umbilical cord blood (median 81 IFN γ SFC/ 1×10^5 cells; mean 74, range: 20–115) for MAGE-A4 (versus median 6 IFN γ SFC; mean 12, range: 0–37 for irrelevant peptides; Supplementary Fig. S3). Using overlapping peptide pools, generated as previously described (21), we found that most MAGE-specific T cell lines mapped to 20mer peptide epitopes near the C terminus of the protein. This region contains the majority of previously published MAGE-A4 T-cell epitopes (22–29). Among several HLA A2 donors in our cohort, cytotoxic responses were restricted to the HLA A2 allele

Figure 1. Generation of MAGE-A4-specific T cells from healthy donors. A, expansion of MAGE-A4-specific T cells after at least 2 stimulations with MAGE-pulsed APCs. Fold change was calculated from weekly cell counts using the trypan blue exclusion method. B, phenotype of CD3+ cells after at least 2 stimulations was determined using flow cytometry of surface receptors listed on the X-axis. The median is shown in black bars. C, the specificity of MAGE-specific CTL lines generated from 15 donors as measured by IFN γ release (IFN γ ELISpot) in response to stimulation with MAGE-A4 pepmix. Results are expressed as SFC/1 \times 10 5 input cells. Control was IFN γ release in response to stimulation with irrelevant pepmix. The black bars show the median IFN γ secretion. CTL lines were able to kill MAGE-loaded autologous PHA blasts (D) and HLA A2-matched MAGE-positive HL cell line L1236. E, specific lysis was evaluated by standard Cr 51 release assay—E:T ratio of 40:1, 20:1, 10:1 and 5:1. F, further epitope mapping of the NPARYEFLWGPRALAETS γ V 20mer commonly seen in A2+ donors shows that 3 donors each recognize a unique and distinct 9mer epitope as shown using IFN γ ELISPOT assay. Results are expressed as SFC/1 \times 10 5 input cells \pm SD.



(Supplementary Fig. S4). We also consistently observed a response against the 20mer peptide—NPARYEFLWPRALAETS γ V (Table 2). Further characterization identified 3 novel HLA A2-restricted epitopes (YEFLWPRA, EFLWGPRAL, and RALAETS γ V) within this region (Fig. 1F).

MAGE-A4 T cells can be generated from HL patients

Although relapsed HL patients typically have low blood counts, T cells specific for MAGE-A4 can be expanded from HL patients. These cells phenotypically resembled T cell lines expanded from healthy donors (Fig. 2A). In T cells expanded from the peripheral blood of patients with HL ($n = 9$) a median 244 SFC/1 \times 10 5 cells (mean 296; range: 32–1,031) responded to MAGE-A4 peptides compared with a mean of 5 SFC/1 \times 10 5 cells (mean 1.5; range: 1–11) responding to irrelevant targets (Fig. 2B). We also mapped HL patient

responses to identified 20mer epitopes and generated these epitope-specific T cells (Table 3). In addition, specific killing of MAGE-A4-pulsed autologous targets (but not nonpulsed autologous targets) was seen in cytotoxicity assays (Fig. 2C).

Decitabine has minimal effects on MAGE-A4 T-cell phenotype and function

We studied the effects of decitabine *in vitro* on the MAGE-A4-specific T cells. Cell viability, measured by trypan blue exclusion assays, showed a slight decrease in cell numbers (approximately 80%, data not shown) following culture with decitabine. Importantly, however, treatment of the T cells with 1 μ mol/L decitabine for 24 hours did not affect surface marker expression (Fig. 3A) or IFN γ release by expanded T cells (Fig. 3B) in response to MAGE-A4 peptides.

Table 1. Cytolytic ability of 8 other donors shown by their ability to lyse MAGE-A4-pulsed autologous targets

Donor	% Killing-Irrelevant target	% Killing-MAGE-A4-pulsed target
2	12.70	40.35
3	7.22	19.44
4	1.1	21.5
5	8.00	40.30
8	3.8	24.7
9	9.3	24.13
10	7.10	20.4
13	0.6	10.36

NOTE: Specific lysis was evaluated by standard Cr⁵¹ release assay, shown is the E:T ratio at 20:1.

Patients with relapsed HL receiving epigenetic modifying agents have increased frequencies of MAGE-A4 T cells

To evaluate the *in vivo* effects of decitabine on tumor antigen-specific T cells, patient samples were obtained before and after treatment with epigenetic modifying drugs that included HDAC inhibitors with or without decitabine. We hypothesized that the tumor-specific T-cell immune response would show a broader epitope-specific T-cell

Table 2. Responses of healthy donor-derived T cells to MAGE-A4 mapped to 20mer regions of the protein

Donor	HLA type	Peptide recognized
1	A2, A24, B8, B65	SASEEEIWEELGVMGVYDGR
2	A2, A26, B15, B44	NPARYEFLWGPRLAETSYV
3	A2, B7, B40	NPARYEFLWGPRLAETSYV
4	A3, A24, B40, B44	NPARYEFLWGPRLAETSYV
5	A1, A11, B8, B49	ETSYVKVLEHVVRVNRVRI KVLEHVVRVNRVRIAYPSL
6	A2, B7, B40	NPARYEFLWGPRLAETSYV
7	A2, B60, B61	NPARYEFLWGPRLAETSYV
8	A3, A24, B8, B35	KVLEHVVRVNRVRIAYPSL
9	A1, A3, B8, B35	AYPSLAYPSLREAALEEEEE
11	—	IFPKTGLLIIVLGTIAMEGD
12	—	ELAHFLLRKYRAKELVTKAE
13	—	LEEVPAESAGPPQSPQGAS
14	—	SNKVDELAHFLLRKYRAKEL ELAHFLLRKYRAKELVTKAE LLRKYRAKELVTKAEMLERV RAKELVTKAEMLERVIKNYK
15	A3, A33, B14, B38	PRALAETSYVKVLEHVVRV

NOTE: Listed are the donors with their corresponding HLA types and 20mer regions recognized.

response or an increased cytokine response (signifying an increase in the frequency of antigen-specific T cells). In patients receiving a decitabine containing regimen with a clinical responses on PET scan, MAGE-specific T cells recognized a broader number of MAGE-A4 epitopes (Fig. 4A) and increased secretion of IFN γ in response to MAGE-A4 antigens (Fig. 4B) after decitabine treatment. In patients not receiving a decitabine containing regimen, IFN γ response was decreased (Fig. 4C and D). In other patients, no improvement in T-cell reactivity to MAGE-A4 antigen was observed (data not shown) following treatment with epigenetic modifying drugs, suggesting that although decitabine may modulate immune function, the presence of other agents may limit endogenous T-cell responses to tumor antigens. These results support combining adoptive T-cell therapy with decitabine to upregulate CTA expression in HL relapsing after HSCT.

Discussion

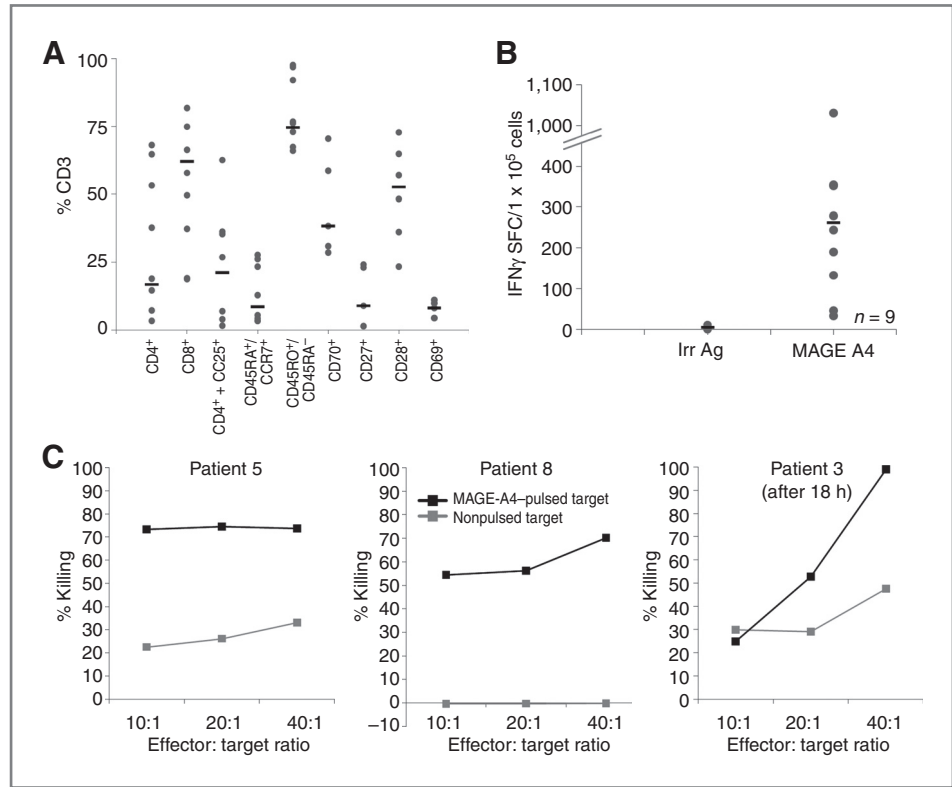
Previous observations by our group showed that T cells targeting antigens expressed on malignant cells in relapsed HL mediate clinical improvement (5), supporting the possibility that T cells recognizing CTAs target a subset of cells that are more resistant to traditional cytotoxic therapies (10). Epigenetic modifying drugs enhance tumor antigen expression and improve the cytotoxicity of antigen-specific T cells (10). On the basis of these findings, we validate here an approach to improve the immunotherapeutic potential of such adoptive T-cell therapy by combining it with decitabine. We successfully expanded MAGE-A4-specific CD8⁺ and CD4⁺ T cells from healthy donors and HL patients and showed that decitabine did not restrict specificity or function of polyclonal T-cell populations against MAGE-A4 targets *in vitro*.

In contrast to studies that have focused on single HLA-restricted epitopes to generate MAGE-A4-specific T cells (21, 22), the peptide mixture we used elicits a broad T-cell response. Using potent DCs as APCs permitted the expansion of antigen-specific T cells from HL patients for autologous use. We also evaluated the feasibility of combining decitabine with T-cell immunotherapy for relapsed HL and observed for the first time that tumor-specific T cells were largely unaffected by the presence of drug.

MAGE-A4 is an attractive target antigen because it is expressed by HL Reed Sternberg cells, subject to epigenetic regulation (ref. 7; Supplementary Fig. S1) and upregulated by DNA methyltransferase inhibitors such as decitabine (10, 23). Such MAGE-A4 upregulation by Reed Sternberg cells should enhance tumor-specific T-cell killing. Furthermore, MAGE-A proteins may reduce malignant potential by inhibiting the tumor suppressor p53 (9, 24). Finally, MAGE is expressed in a variety of malignancies (25–27), opening the possibility of extending this immunotherapeutic strategy to other hematologic malignancies and tumors.

The practical applicability of target antigens for immunotherapy depends on their immunogenicity and the ability to expand cells from as many donor sources as possible.

Figure 2. Generation of MAGE-A4 T cells from patients with HL. A, phenotype analysis of cells obtained using flow cytometry are shown and are similar to the phenotype of CTL derived from healthy donors. Black bars show the median percentage for each marker. B, specificity of the CTL was evaluated by IFN γ ELISpot assay. CTL responses to MAGE-A4 pepmix versus irrelevant pepmix are shown. Data from 9 HL patients are shown. IFN γ positive SFC/1 \times 10⁵ input cells are shown for each CTL line. The black bars show the median IFN γ secretion for each group. C, cytolytic activity of evaluable T cell lines generated from HL patients is evaluated using autologous targets pulsed with the MAGE-A4 peptide mix as targets in a chromium release assay.



HSCT is a common setting for adoptive transfer of either recipient or allogeneic donor T cells. Our MAGE-A4-specific T-cell expansion strategy is applicable for both donors and HL patients, even following immunosuppressive salvage high-dose chemotherapy and autologous HSCT and despite the potential immunomodulatory effect of Reed-Sternberg cells, which produce cytokines that skew T helper cells toward a Th2 phenotype and can mediate T-cell apoptosis (1, 28, 29).

Table 3. Responses of HL patient-derived T cells to MAGE-A4 mapped to 20mer regions of the protein

Patient	Peptide recognized
1	IKNYKRCFPVIFGKASESLK
2	NPARYEFLWGPRALAETSYV
3	ETSYVKVLEHVVRVNARVRI KVLEHVVRVNARVRIAYPSL
4	SASEEEIWEELGVMGVYDGR
5	QVPGSNPARYEFLWGPRALA NPARYEFLWGPRALAETSYV
6	IFGKASESLKMIFGIDVKEV SESLKMIFGIDVKEVDPTSN
7	VTKAEMLERVIKNYKRCFPV MLERVIKNYKRCFPVIFGKA
8	SASEEEIWEELGVMGVYDGR

Umbilical cord blood is an emerging source of donor cells for HSCT, particularly useful for underrepresented minorities and child recipients. Cord blood T cells have lower cytotoxic ability, as well as greater rates of activation-induced cell death (30, 31), and the generation of MAGE-A4 T cells from cord blood still presents a challenge for translation to the clinic. Although we successfully primed MAGE-specific T cells from cord blood, the procedure was not robust, with only 14% of the expanded CTL lines showing specificity for MAGE-A4. This contrasts with our ability to reliably expanded virus-specific T cells from umbilical cord blood (32, 33). The disparity may lie in the nature of the target antigen. Because CTAs such as MAGE-A4 are expressed in the placenta (34), the constant interaction with umbilical cord blood T cells in a highly tolerogenic environment may contribute to the lack of response to CTA.

The percentage of tumor cells expressing the target antigen can limit the success of immunotherapy. Because a single tumor target antigen is unlikely to be sufficient, some investigators have targeted multiple tumor antigens (35). An alternative and complementary strategy is to increase expression of target antigens *in vivo* using epigenetic modifying drugs (36, 37) which upregulate CTA expression (38, 39).

Epigenetic modifying drugs have synergistic effects on the immune system: enhanced T-cell responses occur in AML and myelodysplastic patients after treatment with decitabine (19), whereas a decitabine-induced increase in

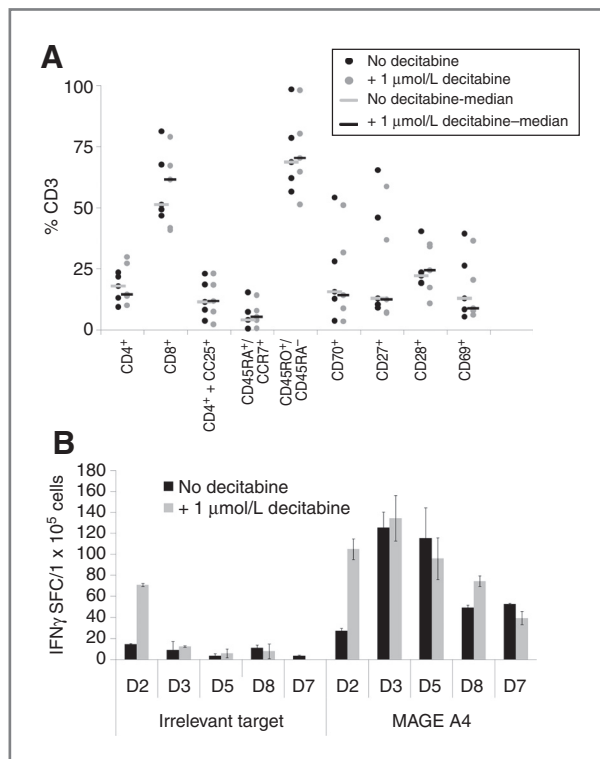


Figure 3. MAGE-specific T-cell phenotype and function are unaffected by decitabine. A, phenotype of the MAGE-specific T cells pre- and posttreatment with 5'-aza-2'-deoxycytidine is shown. B, T-cell secretion of IFN γ in response to MAGE-A4 peptides is shown pre- and postdecitabine treatment. Black bars indicate the IFN γ response in CTL lines incubated with decitabine (gray bars) versus without decitabine (black bars).

regulatory T cells (Treg) may prevent GVHD without affecting the graft versus tumor effect (40). Although the effects of demethylating agents on tumor cells have been extensively studied (11, 13), effects of these drugs on the T-cell effectors has not been explored. A murine transplant study suggests that T cells are actively inhibited and inactivated by these agents (41), but these observations may not be applicable to the adoptive transfer of *ex vivo* expanded antigen-specific T cells following epigenetic drug treatment. Our results show that epigenetic drugs in concentrations exceeding expected treatment levels does not adversely affect the phenotype and function of MAGE-A4-specific T cells *in vitro* and should not reduce efficacy of the adoptively transferred cells *in vivo*. Although we observed an inhibition of proliferation and the abrogation of antigen specificity at continued exposures to higher than 200 ng/mL (>1 μ mol/L) concentrations of decitabine, as well as cells exposed to longer term (>24 hours) culture with decitabine, such a situation would not occur *in vivo*. Pharmacokinetic studies of AML and MDS patients receiving decitabine describe maximum plasma concentrations of only 64 to 77 ng/mL (42). Moreover, we plan to administer T cells after decitabine treatment, circumventing the effect of prolonged exposure to the drug in the clinical setting.

We showed that patients with relapsed HL achieving clinical responses after decitabine also had enhanced MAGE-specific T-cell responses *in vivo*. However, although improved T-cell responses were seen following the administration of decitabine/HDAC inhibitor regimens, this effect was not seen in patients who received HDAC inhibitor therapy alone. A variety of reasons can be postulated for this discrepancy, including the more pleiotropic effects of

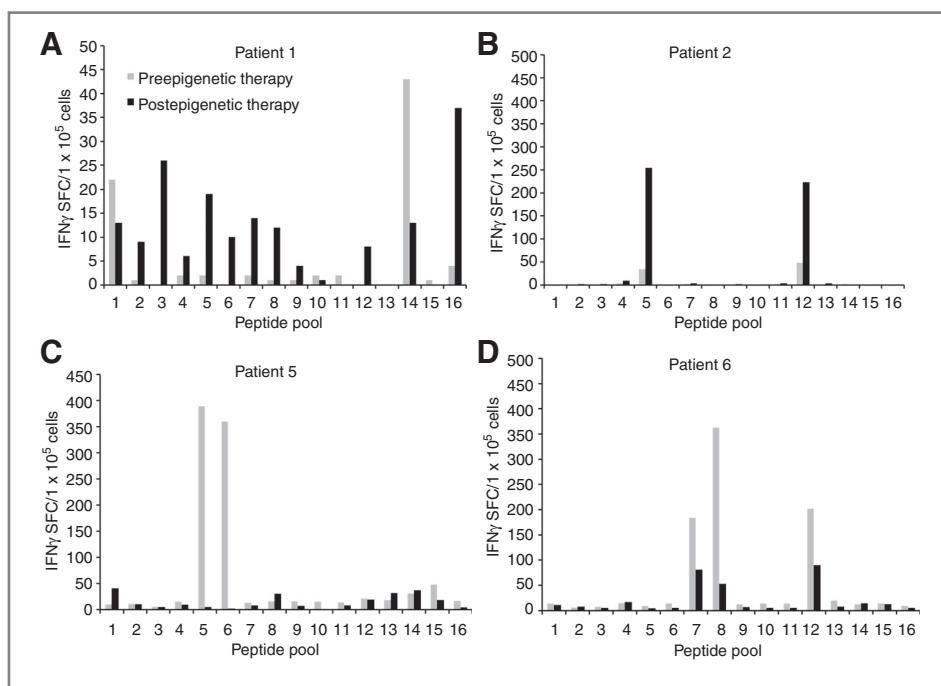


Figure 4. Increased frequency of MAGE-A4-specific T cells *in vivo* in patients receiving a regimen containing decitabine. T cells obtained from the peripheral blood of HL patients taken before and after treatment with an HDAC inhibitor with or without decitabine were expanded *in vitro*, and MAGE-specific responses were evaluated in an IFN γ ELISpot assay. Results are shown for patients who received decitabine containing regimens (A and B) versus those who did not (C and D).

HDAC inhibitors, which affect chromatin structure and interfere with the antigen-presenting machinery (43). Furthermore, the 2 classes of epigenetic modifying drugs target different genes in T cells, with one class effectively activating T cells while another suppresses them. The epigenetic modification of gene expression, while mediated by both DNA methyltransferase inhibitors and HDAC inhibitors, seems to have inherent gene restrictions through mechanisms that remain incompletely understood. It is likely that targets for DNA methyl transferase and HDAC inhibitors differ in T cells, although they mediate similar antitumor effects in malignant cells. DNA methyl transferase inhibitors, for example, seem to mediate a proinflammatory response by activating Th1 cells (44), whereas HDAC inhibitors result in opposite effects, thus limiting Th1 effector cell functions (45). In addition, Treg-induced immune suppression is differentially affected by these classes of epigenetic modifying drugs. Decitabine has been reported to limit FOXP3 expression (46), whereas HDAC inhibitors enhance FOXP3⁺ Treg-mediated suppressive activity (47).

One caveat to our findings is that epigenetic modification could have an impact on the autologous APC used in their *in vitro* expansion. We did not address the effect of epigenetic modification on APCs and few studies have explored this possibility. One study (48) suggests that tumor-infiltrating myeloid cells exposed to decitabine differentiate into mature MHC class II expressing DCs. Another study reports that antigen processing and presentation are unaffected by treatment with HDAC inhibitors (46). Nevertheless, to our knowledge, this is the first demonstration of the validity of using decitabine to enhance the tumor-specific T-cell immune response in patients with relapsed HL. Results support the initiation of larger studies to correlate enhanced tumor-specific immune responses with clinical response. Whether this effect is limited to MAGE-A4 or is also applicable to a host of other antigens (viral antigens and other tumor antigens) is still to be determined because this study

is limited by the small cell numbers we obtained from these heavily pretreated patients.

The lack of endogenous T cells in HL patients following chemotherapy (49) highlights the rationale for combining adoptive T-cell therapy with epigenetic modifying drugs. Although we successfully generated MAGE-A4-specific T cells from 9 HL patients, no response occurred in other patients tested (data not shown). PBMCs and DC numbers were reduced, and T cells from these patients failed to expand after several stimulations, consistent with the profound immune deficiency frequently seen in HL patients (49).

In conclusion, our results highlight the important synergistic role that adoptively transferred tumor-specific T cells could play with decitabine in the control of relapsed HL. Targeted immune-based treatment is a promising strategy for patients with HL to avoid the need for combination radiation and chemotherapy which carry the risk of unacceptable long term effects (4).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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