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Generation of Melanoma-Specific Cytotoxic T Lymphocytes by Dendritic Cells Transduced with a MART-1 Adenovirus[1]

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Dendritic cells (DC) are potent stimulators of primary T cell responses. In this study, we demonstrate that DC, genetically engineered to express the MART-1/Melan-A (MART-1) tumor-associated Ag, express MART-1 mRNA and protein, correctly process and present the HLA-A2.1-restricted immunodominant MART-1 peptide (MART-$1_{27–35}$), and serve as potent stimulators of MART-1-specific CTL in vitro. A replication-defective E1-deleted adenovirus (AdV) was constructed that expresses MART-1 (AdVMART1). Transduced DC produce full length MART-1 mRNA as well as MART-1 protein. AdVMART1 does not significantly down-regulate cell surface class I expression despite having an intact E3 region. Transduction of an HLA-A2-positive/MART-1-negative cell line with AdVMART1 renders these cells sensitive to lysis by CTL specific for the MART-$1_{27–35}$ immunodominant peptide. In addition, DC transduced with AdVMART1 stimulated MART-$1_{27–35}$-specific tumor-infiltrating lymphocytes to synthesize IFN-γ. Finally, AdVMART1-transduced DC were able to generate MART-$1_{27–35}$ peptide-specific, class I-restricted CTL in PBL cultures from normal donors. This study supports the use of tumor Ag-engineered DC in genetic immunotherapy. ***The Journal of Immunology,*** **1998, 161: 5607–5613.**

The identification of genes encoding cancer rejection Ags has allowed the development of new, rational strategies for the immunotherapy of human cancer. In malignant melanoma, these Ags were identified by tumor-infiltrating lymphocytes (TIL)[3] capable of recognizing shared MHC class I-restricted epitopes present on tumors from different patients with the same HLA type (1–3). The gene for the melanocyte/melanoma lineage Ag MART-1 was cloned by cDNA library transfection and TIL screening (4, 5). The proximal human MART-1 promoter has been characterized by us (6). In the context of HLA-A2, the immunodominant MART-$1_{27–35}$ peptide (AAGIGILTV) is recognized by MART-1 CTL (7–12).

Dendritic cells (DC) are the most potent APCs identified (13–16) and are capable of activating naive T cells. A potentially powerful strategy in cancer gene therapy involves the genetic engineering of DC with defined tumor Ag genes and their use as vaccines. We have recently described a murine model of genetic immunotherapy in which DC were transduced with an adenovirus vector expressing the human MART-1 gene (17). Vaccination of mice with murine DC transduced with MART-1 protected mice against a challenge of murine fibrosarcoma cells expressing the MART-1 gene.

Herein, we report the use of AdVMART1 to transduce human DC and generate MART-1-specific CTL in vitro from naive donor blood. DC transduced with AdVMART1 process and present the MART-$1_{27–35}$ immunodominant peptide. CTL generated from AdVMART1-transduced DC can recognize and lyse HLA-$A2^+$/MART-1^+ melanoma after only 7 days in culture.

Materials and Methods

Cell lines

All melanoma lines used (M201, M202, M207, M237, M238) were generated at UCLA, Los Angeles, CA, from surgical specimens, and two (M202 and M207) have been described previously (18). All melanoma lines were screened for HLA-A2 expression by flow cytometry and were subtyped by PCR and direct sequencing by the UCLA Tissue Typing Laboratory (P. Terasaki, director). MART-1 mRNA expression was assessed by RT-PCR, Northern blot analysis (6), or both. The HLA-$A2.1^+$, Ag processing-deficient T2 cells were provided by Peter Cresswell (Yale University School of Medicine, New Haven, CT). Saos-2 osteosarcoma cells were provided by Arnold Berk (UCLA); K562 erythroleukemia cells, the W6/32 and BB7.2 hybridomas, and HepG2 hepatoma cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). HepG2 and Saos-2 cells were tested for HLA-A2 by flow cytometry.

Adenoviruses

The empty adenovirus vector, AdVRR5, and those containing luciferase (*AdVLuc*) and β-galactosidase (*AdVLacZ*) reporter genes have been described previously and served as controls (18). Briefly, the AdVMART1 (17) contains the 400-bp MART-1 cDNA, originally cloned by RT-PCR and driven by the CMV promoter/enhancer in a pAC-CMVpLpA AdV type 5 backbone. The virus was prepared by recombination of this plasmid with pJM17, which contains the 35-kb AdV genome, deleted in the E1 region, in 293 cells which provide the E1 genes in *trans*. Recombinant viruses were released into the medium, purified by limiting dilution, and amplified on 293 cells. All viruses used have been purified on CsCl gradients as described (18).

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[3] Abbreviations used in this paper: TIL, tumor-infiltrating lymphocytes; DC, dendritic cell; MART-1, MART-1/Melan-A; AdV, adenovirus; AdVMART1, adenovirus encoding MART-1 cDNA; moi, multiplicity of infection; PSF, penicillin-streptomycin-Fungizone.

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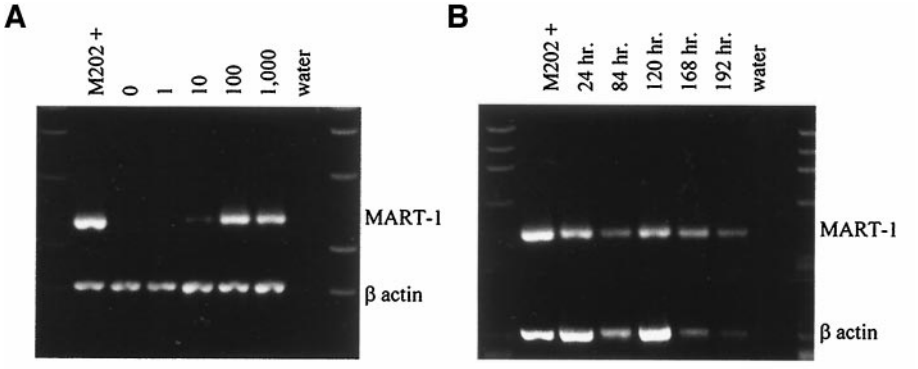

FIGURE 1. Dose-dependent synthesis of MART-1 mRNA and time course of mRNA expression. *A*, Human DC were transduced with AdVMART1 at various moi, and 48 h later mRNA was harvested and subjected to RT-PCR for MART-1 and β-actin. The lanes are as follows: M202 MART-1$^+$ melanoma-positive control, no AdV, AdVMART1 at moi = 1, moi = 10, moi = 100, moi = 1000, water (no RNA)-negative control. *B*, Time course of MART-1 mRNA expression in DC transduced with AdVMART1. DC were transduced at moi = 1000, and RNA was analyzed at different time points. The lanes are as follows: M202-positive control, AdVMART1 transduced DC 24 h posttransduction, 84 h posttransduction, 120 h (5 days), 168 h (7 days), 192 h (8 days), water-negative control. The β-actin RNA control for each sample is also shown.

Antibodies

The anti-MART-1 Ab used in immunohistochemistry is derived from clone A103, (Novacastra, Vector Laboratories, Burlingame, CA). Cells to be stained were either grown on coverslips or as cytospin preparations, fixed in 4% paraformaldehyde, and stained with the anti-MART-1 primary Ab. This was followed by an appropriate secondary Ab and peroxidase staining with the Vectastain Elite kit and reagent diaminobenzidine (Vector Laboratories), according to manufacturer's instructions. Counterstaining was performed with either Giemsa (Mediatech) or hematoxylin (Vector Laboratories), as the described in the figure legend. As a control to ensure primary Ab-specific binding, MART-1$^+$ melanoma cells were stained with every reagent except the primary Ab, and no color reaction was ever observed. The pan-anti-human class I Ab W6/32 and anti-HLA-A2 Ab BB7.2 were purified from hybridoma supernatant by Macrosep 30K column concentration (Filtron, Northborough, MA). The hybridoma cells were obtained from ATCC. Secondary goat anti-mouse FITC and preconjugated CD4 and CD8 Abs were obtained from Caltag (South San Francisco, CA). Anti-β_2-microglobulin used in some class I inhibition cytotoxicity assays was obtained from PharMingen (San Diego, CA).

RT-PCR

RT-PCR for MART-1 mRNA expression was performed as described previously (17). Human β-actin PCR was used for semiquantitation in PCR reactions. The primers were 5′-GGCATCGTGATGGACTCCG and 3′-GTCGGAAGGTGGACAGCGA. Primers were synthesized by Life Technologies (Gaithersburg, MD).

AdV transduction

Transduction of cell lines was performed in the following manner. Cells were removed from flasks (using trypsin for adherent cells), washed, and resuspended in 1 ml of RPMI 1640 (Life Technologies) with penicillin-streptomycin-Fungizone (PSF; Gemini Bioproducts, Calabasas, CA) and 2% FBS (Gemini). Virus was added at a multiplicity of infection (moi) of 10, and the cells were incubated at 37°C for 2 h. Excess virus was washed off, and the cells were resuspended in growth medium (RPMI 1640-PSF-10% FBS) and replated for 24–48 h until use.

DC were transduced after harvest from the 7-day culture with 2% human AB serum (Omega Scientific, Tarzana, CA)-RPMI-PSF at an moi of 1000 unless stated otherwise. After transduction, DC were rinsed and either left in a tube or plated in 5% autologous serum-containing medium (without addition of exogenous cytokines) for 24–48 h until use.

DC generation

DC were prepared as described (19) with some modifications. Peripheral blood was drawn from healthy volunteers by venous puncture, and lymphocytes were purified by Ficoll (Pharmacia, Piscataway, NJ) gradient separation. Mononuclear cells ($3–4 \times 10^7$) were plated in T-25 flasks (Costar, Cambridge, MA) in RPMI 1640-PSF-10% human AB serum (except for DC used in CTL generation, which were in 5% autologous serum instead of 10% AB) for 2 h at 37°C in a humidified CO_2 incubator. The nonadherent cells were removed by gentle rinsing with PBS, and the loosely adherent cells were cultured in medium with 800 U/ml granulocyte/macrophage colony-stimulating factor (Immunex, Seattle, WA) and 500 U/ml IL-4 (R&D systems, Minneapolis, MN) for 7 days. The nonadherent and loosely adherent cells were harvested by vigorous washing. These cells were generally 50% DC as assessed by morphology and phenotyping (19). To compare levels of class I molecules on the DC surface with and without AdV transduction, DC were transduced as described, and 48 h later they were harvested, washed, and stained for either total class I (W6/32 Ab) or HLA-A2 expression (BB7.2 Ab). Controls included untransduced DC. All DC populations were also stained with the secondary Ab to control for nonspecific binding. The DC were gated based on their large size and granular cytoplasm by forward and side scatter. Flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA.

TIL line generation

The CD8$^+$ MART-1$_{27–35}$-specific TIL line, CS TIL 8.0, was established from a metastatic melanoma nodule of an HLA-A2.1 patient (B.M., unpublished observations). CS TIL 8.0 was expanded in 50 U/ml rIL-2 and restimulated approximately every 10 days with an HLA-A2.1/MART-1$^+$ allogeneic melanoma line and an HLA-A2.1-allogeneic EBV-transformed lymphoblastoid cell line. CS TIL 8.0 is oligoclonal and exhibits MART-1$_{27–35}$ specificity.

Generation of peptide-specific CTL

Peptide-specific CTL were generated using a published protocol by Plebanski (20). Briefly, PBMC from HLA-A2.1 or A2.4 donors were pulsed with MART-1 peptide (AAGIGILTV, prepared by the peptide synthesis facility at UCLA, Dr. Joseph Reeve, director) at 50 μg/ml peptide, at 3×10^7 cells/ml serum-free RPMI, at 37°C for 90 min. The cells were rinsed and plated in wells of a 24-well plate at 3×10^6 cells/well in 10% autologous serum-RPMI-PSF with 10 ng/ml IL-7 (Biosource, Camarillo, CA) and 5 μg/ml keyhole limpet hemocyanin (Sigma, St. Louis, MO) (1.5 ml total volume). The nonadherent cells were restimulated weekly with fresh, autologous, peptide-pulsed and irradiated PBMC at a 1:1 ratio and supplemented with IL-2 (Hoffman-La Roche, Nutley, NJ) at 10 U/ml every 3–4 days.

CTL generation from AdV-transduced DC

DC (prepared as described above from HLA-A2.1 or A2.4 donors) were transduced with AdVMART1 at an moi of 1000 for 2 h. The transduced DC were irradiated and plated at $2–5 \times 10^5$ cells/well in a 24-well plate to serve as stimulators for CTL generation. Autologous nonadherent cells were depleted of CD4, CD14, CD19, and CD56$^+$ cells by magnetic bead depletion (Dynal, Lake Success, NY) to prepare CD8$^+$-enriched responder cells. The CD8$^+$ cells were plated with the AdVMART1-transduced DC at 2×10^6 cells/well, in 5% autologous medium plus IL-7 at 10 ng/ml to generate MART-1 CTL. These cultures were supplemented with IL-2 at 10 U/ml every 3–4 days. The CD8$^+$ CTL were restimulated weekly with fresh, autologous AdVMART1-transduced DC at a ratio of 1 DC to 5–10

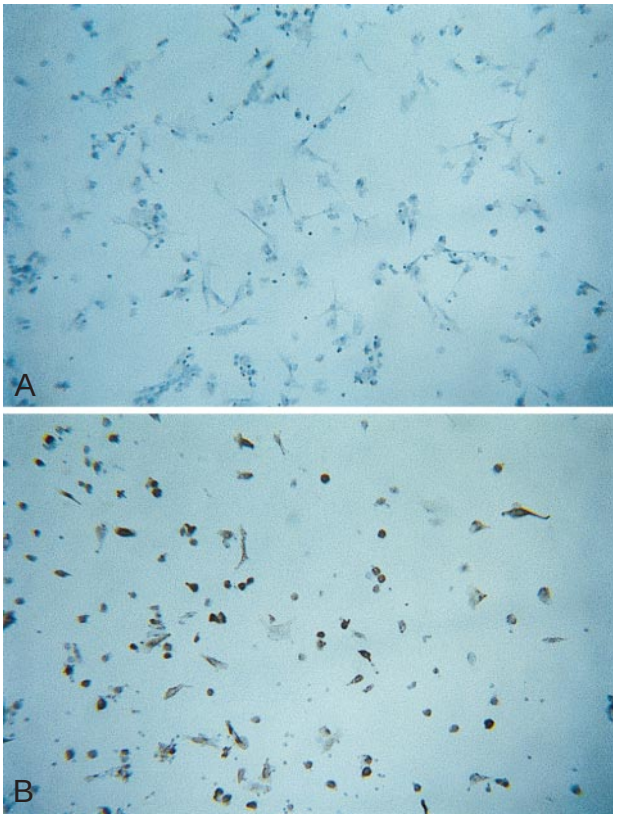

FIGURE 2. MART-1 protein expression in AdV transduced DC. DC were transduced with the empty AdV RR5 (*A*) or AdVMART1 (*B*), plated onto coverslips, and stained for MART-1 Ab 24 h posttransduction. Cells were counterstained with hematoxylin.

$CD8^+$ CTL. Most cultures were phenotyped for $CD4^+$ and $CD8^+$ cells on a weekly basis.

In the experiments presented in Fig. 5, CTL cultures were generated with PBL responder cells and both AdVMART1 and RR5 AdV-transduced autologous DC (DC:PBL ratio, 1:10) stimulator cells in the presence of 50 U/ml rIL-2.

^{51}Cr release and cytokine release

Cytotoxicity assays were performed as follows. Target cells were harvested, washed, counted (and T2 cells were peptide pulsed at 50 μg/ml), and chromated with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL), with shaking incubation at 37°C for 1.5-2 h. CTL were washed, counted, and diluted to desired concentrations in RPMI-10% AB and plated in triplicate wells at 100 μl/well in a round-bottom 96-well plate (Costar). Target cells were washed three times, diluted to 5×10^4 cells/ml, and plated with CTL at 100 μl/well. To control for nonspecific lysis by NK cells, a 10–50-fold excess of K562 cells was added to each target population (except "K562 alone" samples) before adding to CTL (21). For class I inhibition, 20–30 μl of either anti-β_2-microglobulin (PharMingen) or W6/32 Ab were added per well. The plates were spun briefly at 1000 rpm and incubated for 4 h. Supernatants (100 μl/well) were harvested and counted in a gamma counter. Triplicate wells were averaged and % specific lysis was calculated as [(sample − spontaneous release)/(maximum release − spontaneous release)]. For spontaneous release, targets were plated without CTL in 10% AB medium. For maximum release, targets were plated with 5% Nonidet P-40 (IGEPAL; Sigma) detergent.

For cytokine release studies, CTL were washed and plated at 1×10^5 cells/well in a flat-bottom 96-well plate (Costar) in 150 μl of RPMI/10% AB. Stimulator cells were harvested, washed, and plated at 1×10^5 cells per well in 100 μl. IL-2 was added at 300 U/ml, and the plates were incubated at 37°C. After 24 h, plates were spun briefly to pellet cells, and 100 μl of duplicate supernatants were assayed for TNF-α or IFN-γ release in an ELISA. Ab pairs and cytokine standards were purchased from PharMingen (anti-human TNF-α capture and biotinylated Abs are clones Mab1 and Mab11, respectively; anti-human IFN-γ capture and biotinylated detection Abs are clones NIB42 and 4S.B3, respectively), and the ELISA assays were performed according to the manufacturer's instructions. Plates were read at 450 nm in a Spectra ELISA plate reader (SLP Lab Instruments, Salzburg, Austria) within 30 min of assay completion.

Statistical analysis

SE values are presented as error bars in graphs or listed in the tables. Comparisons between experimental groups were performed by Student's *t* test to interpret the significance of differences observed, and *p* values are listed in the figure legends or table footnotes.

Results

DC transduction by AdVMART1

We have previously reported that AdV transduction is an efficient method for transgene expression in DC (19). Transduction of human DC with AdVMART1 results in synthesis of MART-1 mRNA. The amount of mRNA, as detected by semiquantitative RT-PCR, increases with increasing moi (Fig. 1*A*). We have previously determined that an moi of 1000 generates high levels of transgene expression in human DC (19) with minimal to no cytopathic effect. MART-1 mRNA expression in these transduced DC was detectable for the entire 8-day culture period (Fig. 1*B*).

AdVMART1 transduction of DC or a MART-1-negative cell line (the hepatoma cell line HepG2 or osteosarcoma cell line Saos-2) results in synthesis of MART-1 protein as determined by specific Ab staining (Fig. 2). The MART-1-positive M202 melanoma cell line has strong MART-1 staining (not shown), and untransduced DC and AdVRR5-transduced DC have only very faint background staining (not shown and Fig. 2*A*, respectively). DC

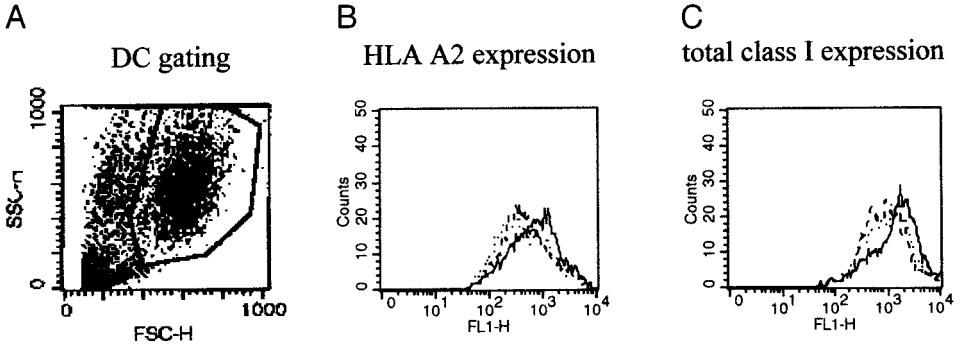

FIGURE 3. Cell surface class I expression by transduced DC as assessed by flow cytometry. *A*, Dot plot showing gating on large, granular DC for analysis of class I expression. *B*, HLA-A2 cell surface expression (antibody BB7.2) by untransduced DC (solid line), DC 48 h after AdVRR5 transduction at moi = 1,000 (dashed line), and DC 48 h after AdVMART1 transduction at moi = 1,000 (dotted line). *C*, Total class I expression (pan-class I Ab W6/32) by untransduced DC (solid line), DC 48 h after AdVRR5 transduction at moi = 1,000 (dashed line), and DC 48 h after AdVMART1 transduction at moi = 1,000 (dotted line) Data from a single donor are shown.

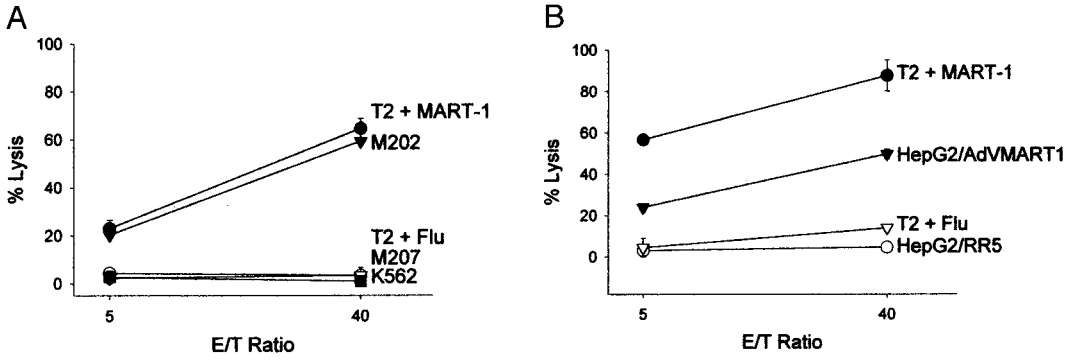

FIGURE 4. Cytotoxicity of MART-$1_{27–35}$ peptide-specific CTL. MART-$1_{27–35}$ peptide was used with HLA-A2 donor PBMC to generate CTL. *A*,, Specificity of the CTL for MART-$1_{27–35}$ is assessed with targets T2 + MART-$1_{27–35}$ vs T2 + Flu peptide. NK activity is assessed with K562 cells. Ability of the CTL to lyse melanoma cells in an MHC-restricted fashion is assessed with M202 HLA-A2.1$^+$/MART-1$^+$ melanoma cells vs M207 HLA-A2-/MART-1$^+$ melanoma cells. Differences in lysis between T2 + MART-1 and T2 + Flu are statistically significant (at E:T 40:1, $p = 0.01$; at 5:1, $p = 0.0003$), and differences between M202 and M207 lysis are also significant (at 40:1, $p = 0.002$; at 5:1, $p < 0.0001$). *B*, The ability of AdVMART1 transduction of MART-1-negative cells to sensitize targets to lysis was assessed. The specificity of the CTL was confirmed in this experiment with the T2+ MART-$1_{27–35}$ and T2 + Flu targets (at 5:1, $p < 0.0001$). HepG2 target cells were transduced with either AdVMART1 or AdVRR5 at moi = 10 and used 24 h later in the chromium release assay. Killing of HepG2/AdVMART1 compared with HepG2/AdVRR5 demonstrates the sensitization of targets by AdVMART1 transduction. The difference in lysis between HepG2/AdVMART1 and HepG2/RR5 is significant (at 40:1, $p = 0.001$; at 5:1, $p = 0.01$).

transduced with AdVMART1 show strong positive staining in >90% of the DC (Fig. 2*B*). Untransduced or AdVRR5-transduced HepG2 cells show only faint background staining, whereas AdVMART1-transduced HepG2 cells are strongly positive for MART-1 protein expression (data not shown).

Regulation of cell surface class I expression

The E3/19K gene product of AdV has been reported to down-regulate cell surface class I expression by affecting ER transport (22, 23). To assess any effect from E3-intact AdVMART1 transduction, HLA-A2$^+$ DC or HepG2 cells were transduced with AdVMART1 and assessed for class I (W6/32 Ab) and HLA-A2 (BB7.2 Ab) surface expression by flow cytometry. Large, granular DC were gated on as shown in the dot plot (Fig. 3*A*). There is only a minor decrease in total class I or HLA-A2 expression in DC transduced with AdVMART1 (15% decease in class I, average of two donors; 7% decrease in HLA-A2, average of two donors) (Fig. 3, *B* and *C*). Modulation of class I expression was also assessed in the HepG2 cell line to investigate any potential change after AdV transduction when these cells were used as cytotoxicity target cells. HepG2 showed a decrease in total class I (40%) or HLA-A2 (33%) after AdVMART1 transduction as well as a decrease after AdVRR5 transduction (59% decrease in class I; 30% decrease in HLA-A2). A control *AdVLacZ* transduction of HepG2 cells confirmed the percentage of cells transduced at the moi used (>95%, data not shown).

MART-$1_{27–35}$ peptide-specific CTL induction

MART-$1_{27–35}$ peptide pulsed PBMC were used to generate peptide-specific CTL in vitro from normal donors. After 3–4 wk of culture, these cells were tested for cytotoxic activity against a variety of targets. As shown in Fig. 4, these bulk cell populations (generally 30–40% CD8$^+$ by phenotypic analysis) demonstrate MART-$1_{27–35}$ peptide specificity by lysis of MART-1 pulsed T2 cells in a 4-h chromium release assay, but no lysis of Flu M1 peptide-pulsed T2. These cultures also show specific killing of the MART-1$^+$ M202 melanoma cells, indicating that MART-$1_{27–35}$-specific CTL recognize the endogenously processed and presented MART-1 epitope. The results of the cytotoxicity data are also reflected by cytokine release data (not shown). Both TNF-α and IFN-γ were released in increased amounts by CTL stimulated by T2 cells pulsed with the specific MART-1 peptide, not by the control Flu peptide-pulsed cells.

To confirm that AdVMART1 transduction of MART-1-negative, HLA-A2.1$^+$ cells would result in correct processing and presentation of MART-$1_{27–35}$ peptide, the HepG2 hepatoma cell line was used as a target in cytotoxicity assays. The experiment shown in Fig. 4*B* demonstrates that the transduction of HepG2 cells by AdVMART1 renders them sensitive to lysis by the MART-$1_{27–35}$ CTL, while transduction with AdVRR5 does not.

MART-$1_{27–35}$ TIL clone recognition of AdVMART1-transduced DC

A MART-$1_{27–35}$-specific melanoma TIL clone, generated from an HLA-A2.1 melanoma patient was used to further demonstrate that AdVMART1 transduction results in proper processing and presentation of the immunodominant MART-$1_{27–35}$ peptide. The TIL clone lysed MART-1 peptide-pulsed T2 cells and not control targets. The TIL line also secreted TNF-α upon recognition of MART-$1_{27–35}$ peptide-pulsed T2 cells and not a control peptide (MAGE 3)-pulsed T2 cells or T2 cells alone, confirming MART-$1_{27–35}$ peptide specificity (Table I). Normal HLA-A2.1 donor DC were transduced with AdVMART1 or RR5 and cocultured with the MART-$1_{27–35}$-specific TIL. The TIL line secreted IFN-γ and

Table I. *TIL specificity by cytotoxicity and cytokine release*[a]

	Cytotoxicity (% Specific Lysis)[b]			Cytokine Release (pg/ml)[c]
Stimulator	10:1	20:1	40:1	(TNFα)
T2 + MART-1	13 ± 2.6	21 ± 1.7	31 ± 2.1	125
T2 + MAGE-3	0	0	0	8
T2 only	0	0	0	16
Background				4

[a] MART-$1_{27–35}$ specificity of HLA A2.1 melanoma patient TIL was assessed by both cytotoxicity and TNFα cytokine release against T2 cells. The MART-$1_{27–35}$ peptide was compared with the control MAGE3 peptide and no peptide.

[b] Cytotoxicity was assessed in a standard 4-h ^{51}Cr release assay. The E:T ratios used are shown above each column. Data are presented as mean percentage of specific lysis of triplicate wells ± SEM.

[c] Cytokine release after TIL stimulation by T2 cells with and without peptide pulsing was assessed by ELISA. All samples were performed in duplicate.

Table II. *TIL recognition of AdVMART1-transduced cells*[a]

Stimulator	TNF-β Release (pg/ml)	IFN-γ Release (pg/ml)
T2 + MART-1	80	NT[b]
T2 + MAGE-3	32	NT
T2 only	32	NT
AdVMART1/DC	55	500
AdVRR5/DC	30	25
Background	36	55

[a] MART-1 specificity of melanoma patient TIL was confirmed by TNF-β release using T2 cells pulsed with peptides as stimulator cells. TIL recognition of AdVMART1-transduced DC was assessed by both TNF-β and IFN-γ release. All ELISA samples were performed in duplicate.
[b] NT, not tested.

TNF-β specifically in response to DC transduced with AdVMART1, and not DC transduced with the control AdVRR5 (Table II). This indicates that the AdVMART1-transduced cells process and present the same MART-$1_{27–35}$ peptide epitope present on the original melanoma from the patient from which the TIL line was derived.

AdVMART1-transduced DC generate melanoma-reactive CTL

DC were generated from healthy HLA-A2.1 (donor 1) and A2.4 donors (donor 2), transduced with AdVMART1, and used as APCs to generate MART-1-specific CTL from $CD8^+$ T cell-enriched PBMC. Specific killing of $A2^+$/MART-1^+ melanoma cells (M202) was detected in as little as 1 wk of culture (Table III). This cytotoxicity was increased with IFN-γ treatment of the targets and partially inhibited by anti-class I blocking Abs. The results of weekly assays over a 3-wk culture period from one donor and the first assay from a second donor are shown in Table III. Killing of either HLA-mismatched (A2-negative, M201, or M207) or $A2^+$/MART-1-negative melanoma cell lines (M237 or M238) was not observed. Four of four $A2^+$ normal PBMC donors could generate M202-killing CTL within 1 wk of culture with this DC/AdV-based culture system. Both HLA-A2.1 and A2.4 donors generated similar responses against the T2 cell (not shown) and M202 melanoma targets (Table III).

These AdVMART1 generated CTL also kill AdVMART1-transduced HepG2 but show only a low level of killing against HLA-$A2^+$/AdVRR5-transduced HepG2 (Table III). Therefore, any AdV Ag reactivity generated in these CTL cultures appears to be weak and does not dampen MART-1-specific reactivity.

To demonstrate MART-$1_{27–35}$ reactivity, cocultures were set up with total PBL from a normal donor and AdVMART1- or RR5-transduced autologous DC. When tested for cytotoxicity on day 7 (Fig. 5*A*), the cocultures with AdVMART1/DC generated MART-$1_{27–35}$ peptide-specific CTL. The AdVMART1/DC-stimulated effector cells also synthesized TNF-α upon specific recognition of MART-$1_{27–35}$ peptide (Fig. 5*B*). The RR5/DC-stimulated PBMC showed only background TNF-α secretion to the targets tested.

Lastly, to further assess AdV Ag reactivity in the transduced DC CTL cultures, RR5-transduced DC were cultured with $CD8^+$-enriched T cells. The RR5 AdV, which contains no insert in the plasmid backbone, stimulated cultures that did produce CTL reactive to RR5-transduced targets (Fig. 5*C*) compared with untransduced targets.

Discussion

Recent results from clinical immunotherapy trials have shown that vaccination with melanoma tumor Ag class I peptides in IFA (24, 25) or pulsed onto DC (26) can generate both immunologic and clinical responses. Another recent trial (27, 28) found that a vaccine of autologous HLA-A1 APCs pulsed with a MAGE-1 peptide resulted in increased MAGE-1-specific CTL in both the vaccination site and distant sites as well as in peripheral blood. These encouraging reports indicate the potential for antitumor responses directed against melanoma Ags. In this report, we have shown the utility of using a MART-1-expressing AdV to transduce human DC and generate MART-1-specific CTL in vitro.

DC have been shown to be efficient cells for in vitro CTL generation in several reports (10, 29–37). CTL specific for several melanoma Ags (MART-1, gp100, tyrosinase) have been generated utilizing peptide-pulsed DC (10, 38). In addition, use of DC has uncovered subdominant epitopes from gp100 that were not detected utilizing other APC (31). Infection of DC with influenza virus was used to generate Flu-reactive $CD8^+$ CTL in as few as 7 days (39). A MART-1-expressing retrovirus has been used to transduce $CD34^+$ hemopoietic progenitor cells (40) which, after differentiation into DC, could successfully generate anti-MART-1 CTL. Vaccinia viral vectors have also been used as DC transduction vectors with melanoma tumor Ags. A tyrosinase-vaccinia viral vector (41) was used to transduce melanoma patient APC; both $CD4^+$ and $CD8^+$ tyrosinase-reactive T cells were generated after

Table III. *AdVMART1-transduced DC stimulate CTL generation*[a]

Target	% Specific Lysis			
	Donor 1 wk 1	Wk 2	Wk 3	Donor 2 wk 1
M202 (MART-1^+A2^+)	8 ± 0.7	23 ± 1.0	20 ± 1.1	13 ± 0.6
M202 + IFN-γ	13 ± 0.8	30 ± 0.6*	23 ± 1.8	18 ± 0.4*
M202 + anti-class I Ab	3 ± 0.3*	18 ± 1.2*	16 ± 0.5*	9 ± 0.5*
M201 (MART-$1^+/A2^-$)	0	NT[d]	NT	0
M237[b], M238[c] (MART-$1^-/A2^+$)	5 ± 0.3	NT	0	5 ± 2.4
HepG2 + AdVMART1	30 ± 1.6	22 ± 0.3**	6 ± 0.2[e]	37 ± 1.4**
HepG2 + RR5	16 ± 5.8	16 ± 1.8	0 ± 1.2[e]	9 ± 3.1

[a] CTL from CD8-enriched responder cells from each of two donors were tested on a weekly basis for MART-1-specific, MHC class I-restricted cytotoxicity. Data represent lysis at E:T 25:1 and is presented as the mean percentage of specific lysis ± SEM. Significant increases in M202 lysis after IFN-γ treatment or decreases after class I Ab blocking compared with untreated M202 lysis are marked with an asterisk (*t* test, all differences $p < 0.05$). Significant increases in lysis of HepG2 cells transduced with AdVMART1 compared with HepG2 cells transduced with AdV RR5 are marked with two asterisks (*t* test, all differences $p < 0.02$).
[b] Used at wk 1.
[c] Used at wk 3.
[d] NT, not tested.
[e] Saos-2 transduced with AdVMART1 used at wk 3.

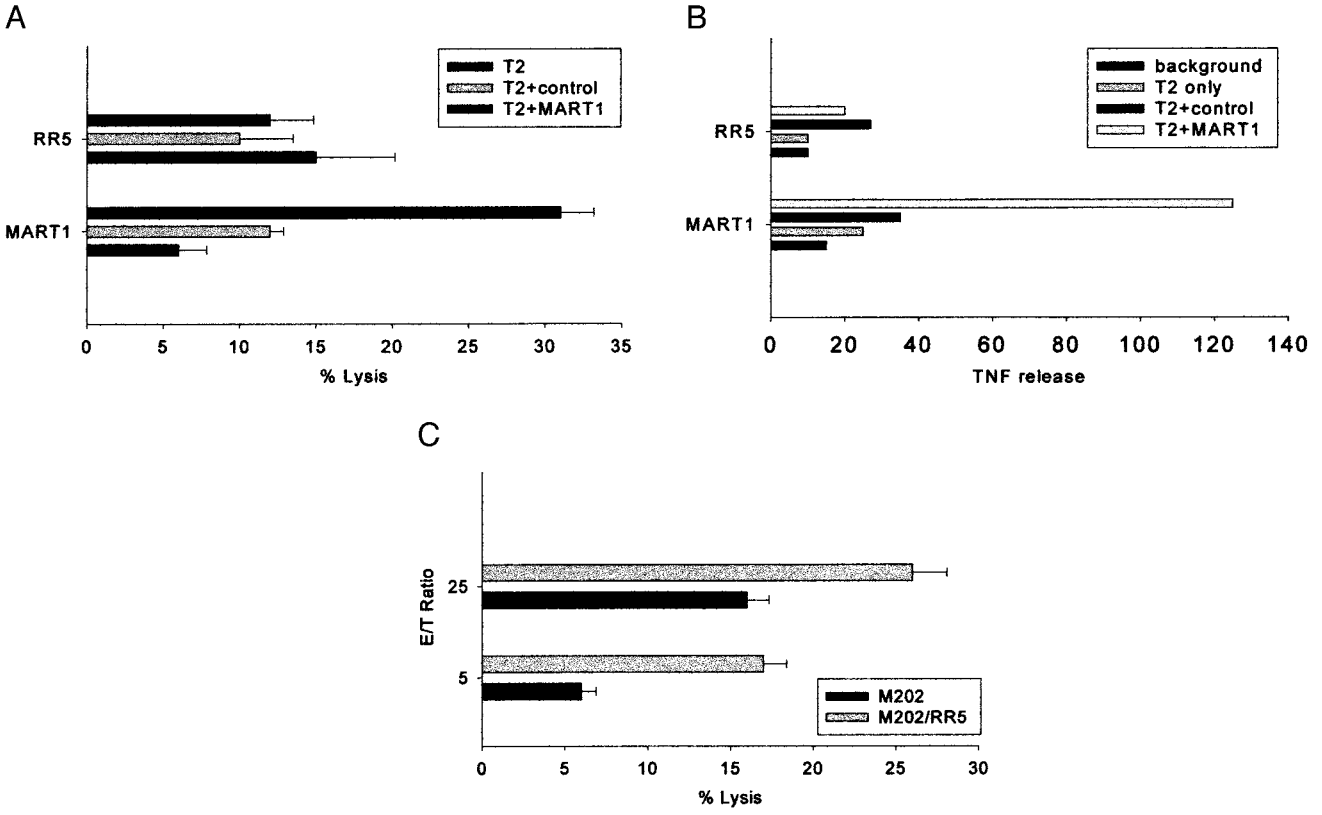

FIGURE 5. AdVMART1 and control AdV RR5-transduced DC generate CTL. Normal HLA-A2.1 donor DC were transduced with either AdVMART1 or empty control AdV RR5 and used to stimulate CTL from autologous PBMC (*A* and *B*) or $CD8^+$-enriched T cells (*C*). *A*, Cytotoxicity of CTL against T2 cell targets at E:T ratio of 10:1. The difference in lysis of T2 + MART-1 between AdVMART1 CTL and RR5 CTL is significant ($p = 0.05$). *B*, TNF release (pg/ml) by CTL upon recognition of T2 cell targets. *C*, Cytotoxicity of AdV RR5 CTL against AdV RR5-transduced vs untransduced M202 targets. Differences in lysis are significant at 25:1 ratio only ($p = 0.01$).

limiting dilution cloning. This viral vector was also used to successfully generate tyrosinase-specific CTL from two of four normal donors tested. A MART-1 vaccinia virus was used to transduce melanoma patient DC (42) and successfully generated $CD8^+$ CTL that recognized MART-1 targets in 6 of 6 patients within 7–14 days of culture. Likewise, a MART-1 expressing AdV was reported (43) that sensitized targets cells to MART-1-specific lysis. These reports support the continued investigation of virally encoded tumor Ags for the genetic engineering of DC. These viruses have in common the potential to express a variety of known and unknown tumor Ag epitopes on the surface of DC and without HLA restriction. This would also potentially reduce the problem of Ag loss variants that could evade the immune response generated (44, 45). In addition, processing and presentation of peptides through endogenous routes may be more efficient for cell surface display than exogenous loading of synthetic peptides (46).

An important finding of this work was the demonstration that MART-1-transduced DC generate CTL in vitro. We have not observed significant AdV Ag reactivity in the CTL cultures generated with AdVMART1-transduced DC, despite the high transduction efficiency with this virus (Fig. 2*B*). This may be due to the low levels of AdV gene transcription after removal of the E1 *trans*-activating region. There is, most likely, some low level of AdV protein synthesis, given that the CTL generated from the empty control AdV RR5 recognize AdV-transduced cells to a somewhat greater degree than nontransduced cells (Fig. 5*C*). As a potential improvement on this strategy, we are pursuing a third-generation, helper-dependent, adenoviral vector that will have all viral genes deleted (47, 48) to possibly improve the Ag-specific reactivity, eliminate the low level of class I expression down-regulation, and reduce viral gene immunogenicity.

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