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Dendritic Cells Loaded With MART-1 Peptide or Infected With Adenoviral Construct Are Functionally Equivalent in the Induction of Tumor-Specific Cytotoxic T Lymphocyte Responses in Patients With Melanoma

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Summary: Immunization with tumor-specific–associated antigen—pulsed dendritic cells has proved to be efficacious in various animal models and is being evaluated for the treatment of cancer in humans. Use of dendritic cells pulsed with specific peptides or transfected with tumor-associated antigen genes has been a focused area of investigation for inducing potent tumor and viral immune responses. In this study, the authors demonstrate transgene expression, including the lacZ and MART-1 genes, in dendritic cells infected with adenoviral constructs. These transiently transduced dendritic cells, derived from melanoma patients' monocytes cultured with granulocyte-macrophage colony-stimulating factor and interleukin-4, express the transgene and can stimulate patients' $CD8^+$ T cells to elicit an antitumor immune response comparable to dendritic cells loaded with a defined peptide. These cytotoxic T lymphocytes were able to recognize both known and unknown tumor-associated antigen epitopes and exhibited cytolytic activity against HLA-matched tumor cells expressing the antigen. The ability to induce tumor-specific cytotoxic T lymphocytes in vitro using gene-modified dendritic cells that transiently express tumor-associated antigens demonstrates the potential use of these antigen-presenting cells for developing in vivo cancer vaccines.
Key Words: Dendritic cells—Cytotoxic T lymphocytes—Tumor-associated antigen—Adenovirus—Gene expression.

Dendritic cells (DCs) play a central role in antigen processing and presentation to induce T-cell responses in vitro and in vivo (1). They possess a unique repertoire of cell surface molecules that include abundant levels of major histocompatibility complex (MHC), adhesion, and co-stimulatory molecules. These cells form a specialized system for initiating a primary immune response by activating naïve T cells in vivo and in vitro. Recent advances in generating functionally active DCs from peripheral blood monocytes make them an attractive candidate for cancer immunotherapy and gene therapy.

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In the last few years, research in cancer immunotherapy has centered on identifying and isolating tumor-associated antigens (TAA) recognized by cytolytic T cells (CTLs). These newly identified tumor antigens can be transferred into more efficient antigen-presenting cells (APC), such as DCs, to induce effective antigen-specific responses. Dendritic cells pulsed with antigens in the form of immunogenic proteins or synthetic peptides have been shown to induce antigen-specific CTLs in vitro and in vivo. These CTLs have been effective in eradicating established tumors in murine models (2–8) and patients with cancer who were treated in a clinical trial (9). Therefore, immunization with antigen-loaded DCs has the potential to become a powerful method for inducing anti-tumor immunity.

In humans, immunization with defined peptide anti-

gens is limited to a relatively small number of patients who express the appropriate class I MHC molecules for a particular peptide. In addition, these peptides represent a group of antigens specific for $CD8^+$ T cells. Recent studies have shown that differential presentation of tumor antigens by APC to T cells may be of critical importance for breaking tolerance and preventing anergy in tumor-specific immunity (10–13). Therefore, effective antigen presentation may be crucial for the induction of $CD4^+$ and $CD8^+$ T-cell–mediated tumor immune responses (14).

It has been known for some time, particularly for viral antigens, that APCs process endogenously synthesized proteins and present peptides in association with MHC class I molecules (15,16). In our studies, we have compared the induction of CTLs using the defined HLA-restricted peptide with the broader approach of expressing the TAA gene delivered by adenoviral vectors. We focused on expressing complete TAA proteins in DCs to allow the DCs to process and present both class I and II–restricted epitopes. Expression of a tumor antigen as an intact protein in DC eliminates the requirement for prior identification of the particular HLA-restricted peptides that can be presented by the APCs. Furthermore, expression of the entire antigen has the advantage of using the endogenous processing machinery of the DCs to deliver both helper and CTL epitopes to $CD4^+$ and $CD8^+$ T cells, respectively. This would facilitate generation of a broader CTL response, in contrast to the presentation of a single epitope by a peptide.

Recent advances have been made in the identification and cloning of the genes for TAA, such as MART-1 and Melan-A (17,18) and tyrosinase (19), which are widely distributed melanoma antigens. Expression of tumor antigens in DC requires an efficient vector system that can transfect primary cells. We chose to use adenoviral vectors that to infect DCs and express high levels of transgene product (20). In this study, we found that adeno MART-1 gene-modified DC is functionally comparable to peptide-loaded DCs in the induction of melanoma-specific CTLs from the peripheral blood lymphocytes of patients with melanoma.

We report here the generation, transduction, and phenotypic and functional analyses of DCs cultured from peripheral blood monocytes. Transduction of a full-length cDNA encoding the MART-1 antigen in an adenoviral vector led to the generation of melanoma tumor-specific, HLA-restricted CTLs from persons with melanoma. Furthermore, we show that gene-modified DCs induce functionally comparable tumor-specific CTLs as peptide-pulsed DCs.

MATERIALS AND METHODS

Preparation of Cells

Dendritic cells were generated from peripheral blood mononuclear cells isolated from healthy donor buffy coats by Ficoll-Hypaque density-gradient centrifugation. Peripheral blood mononuclear cells were stained with a purified hybridoma supernatant against HLA-A2 and examined by flow cytometric analysis. Samples with a fluorescent intensity beyond the first decade compared with the isotype control were considered HLA-A2 positive and used in this study. Dendritic cells were prepared using a modification of the procedure described by Romani et al. (21). Briefly, 1.5×10^8 peripheral blood mononuclear cells were allowed to adhere to T150 culture flasks for 2 hours at 37°C in RPMI 1640 with 10% fetal bovine serum. After incubation, the nonadherent cells were removed, washed, resuspended in freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide) and frozen for later use to generate T cells to be stimulated with DCs. The adherent cells were cultured for 6 or 7 days in 30 mL RPMI 1640 with 10% fetal bovine serum containing 800 units/mL recombinant human GM-CSF (Biosource International, Camarillo, CA, U.S.A.) and 500 units/mL recombinant human IL-4 (Biosource International).

Melanoma tumor cells were isolated from a tissue biopsy specimen. The tissue was minced and processed in a dounce homogenizer. Cells in suspension were collected and washed with Dulbecco's phosphate buffered saline (PBS). Cells were cultured in RPMI 1640 with 10% fetal bovine serum for 14 days before use in a cytotoxicity assay.

Phenotypic Analysis

Dendritic cells were harvested at various times during their culture in GM-CSF and IL-4 and also on the day after transfection. Cell surface markers were analyzed by immunostaining and flow cytometry. Approximately 5×10^5 cells were incubated for 25 minutes at 4°C with the following monoclonal antibodies: CD3, CD14, CD80, HLA-DR (Becton-Dickinson, San Jose, CA, U.S.A.), CD86 (Pharmingen, San Diego, CA, U.S.A.), and CD1a (Ortho Diagnostic Systems, Raritan, NJ, U.S.A.). The cells were washed and fixed with Dulbecco's PBS saline supplemented with 1% paraformaldehyde. Three-color flow cytometric analysis was performed using a FACScan flow cytometer (Becton-Dickinson). At the time of transfection, cultures were between 60% and

80% DCs as assessed by immunostaining for DC surface markers and flow cytometric analysis.

Adenoviral Constructs and Preparation

To construct the MART-1 gene encoding adenoviral vectors, we designed an expression cassette containing the cytomegalovirus immediate early promoter and SV40 late polyadenylation signals. The coding sequence for MART-1 was obtained by polymerase chain reaction using primers spanning the complete coding region. The entire expression cassette was transferred into pGY63 (22) to obtain recombination shuttle plasmids. This shuttle plasmid contains adenovirus 5 sequences 1-382 followed by an expression cassette and adenovirus sequence 3446-3968. These two regions undergo homologous recombination in *Escherichia coli* with E1- and E3-deleted adenoviral genome sequences in a pXL2689 derivative (22). After recombination, the transgene expression cassette is in the E1 region. The construct was digested with Pac I and transfected in 293 cells to obtain adenoviral prestocks. Adenoviral vectors were amplified in 293 cells and purified on cesium chloride density gradients.

Transduction of Dendritic Cells

Dendritic cells were harvested after 7 days in culture and counted, and 2.5×10^5 DCs in 500 μL AIM V media (Life Technologies, Gaithersberg, MD, USA) were distributed into a 24-well polystyrene tissue culture plate. Cells were transduced in suspension with adenoviral vectors containing lacZ or MART-1 gene at various multiplicity of infection (MOI = 50–200) at 37°C in 5% carbon dioxide for 2 hours. After the 2-hour incubation, additional culture medium was added to bring the final volume of each well to 1 ml and the cells were returned to culture. No additional cytokines were added to the cultures after transduction. The cells were harvested after 24 hours and assessed for gene expression or used in CTL stimulation experiments.

β-Galactosidase Staining With X-gal

Dendritic cells were stained for β-gal activity 24 hours after transduction. The cells were fixed by adding X-gal fixative solution (PBS, pH 7.4; 2% formaldehyde; 0.2% glutaraldehyde) to each well, incubated at room temperature for 5 minutes, and washed twice with PBS. The X-gal staining solution (PBS, pH 7.4; 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 2 mmol/L MgCl2, and 1 mg/mL X-gal) was added to the wells and incubated at 37°C for 6 to 24 hours. At the end of the incubation period, wells were washed multiple times with Dulbecco's PBS and left in Dulbecco's PBS until the wells were scored. Transduction efficiency was assessed by the percentage of blue cells viewed using a microscope.

Peptide-Loaded Dendritic Cells

Lyophilized MART-1 peptide (AAGIGILTV) was purchased from Endocrine Technologies (Newark, CA. U.S.A.), dissolved in DMSO at a concentration of 10 mg/mL, and stored at −20°C. Dendritic cells were stripped of antigen and loaded with MART-1 peptide as follows. The DCs were washed once in cold 0.9% NaCl with 1% bovine serum albumin, resuspended at 1×10^7 cells/mL in stripping buffer (0.13 mol/L L-ascorbic acid, 0.06 mol/L sodium phosphate monobasic [pH 3.0], 1% bovine serum albumin, and 3 μg/mL β2-microglobulin [Scripps Labs, San Diego, CA, U.S.A.]) containing 10 μg/mL MART-1 peptide and incubated for 2 minutes on ice. The cells were neutralized with five volumes of cold neutralizing buffer (0.15 mol/L sodium phosphate monobasic [pH 7.5], 1% bovine serum albumin, and 3 μg/ml β2-microglobulin) containing 10 μg/mL MART-1 peptide and recovered by centrifugation. Cells were resuspended in peptide solution (phosphate buffered saline-calcium magnesium free, 1% bovine serum albumin, 30 μg/mL DNAase, and 3 μg/mL β2-microglobulin) containing 40 μg/mL MART-1 peptide and incubated for 4 hours at room temperature. Cells were washed and irradiated with 30 Gy before being used to stimulate T cells.

Generation of Antigen-Specific Cytotoxic T Lymphocytes

Antigen-specific CTLs were generated using $CD8^+$ T cells obtained by adhering previously frozen autologous peripheral blood mononuclear cells on CD8 T25 MicroCELLector flasks (RPR Gencell, Santa Clara, CA. U.S.A.). Captured $CD8^+$ cells were stimulated with either DCs alone, DCs loaded with MART-1 peptide, or DCs transduced the previous day with the adeno-MART-1 virus. The DCs were added to $CD8^+$ cells at a stimulator to responder (S:R) ratio of 1:3. The cells were cultured in RPMI with 10% fetal bovine serum containing 10 ng/mL rIL-7 (R & D Systems, Minneapolis, MN. U.S.A.). $CD8^+$ cells cultured alone with rIL-7 served as controls. The CTL cultures were restimulated 10 to 12 days after the initial stimulation and weekly thereafter for a total of three or four restimulations at S:R ratios ranging from 1:3 to 1:15. rIL-7 was added twice a week throughout the culture period.

Cytotoxicity Assay

MART-1–specific cytolysis by CTLs was assessed using a standard 4-hour ^{51}Cr release cytotoxicity assay using 624Mel (HLA-A2+, MART-1+), MCF-7 (HLA-A2+, MART-1 negative), adenovirus MART-1 transduced MCF-7 (HLA-A2+, MART-1+), 397mel (HLA-A2-, MART-1+), and T2 cells (a processing defective cell line that expresses empty HLA-A2 molecules until it is stabilized with peptide) preincubated for 2 to 4 hours with MART-1 peptide at 40 μg/mL as tumor targets. Target cells were resuspended at 1×10^7 cells/mL in their appropriate culture medium, plated in six-well plates (2 mL/well), and incubated overnight with 100 μCi ^{51}Cr. After overnight incubation, targets were washed three times and mixed with effector cells at varying effector: target (E:T) ratios in round-bottom microtiter plates. After a 4-hour incubation, supernatants were harvested and the amount of ^{51}Cr released was measured by liquid scintillation counting. The percentage of specific cytotoxicity was calculated as [(cpm of test sample − cpm of spontaneous ^{51}Cr release)/(cpm of maximal ^{51}Cr release − cpm of spontaneous ^{51}Cr release)] × 100.

RESULTS

Tumor-Specific Cytotoxic T Lymphocyte Responses Using MART-1 Peptide-Pulsed Dendritic Cells

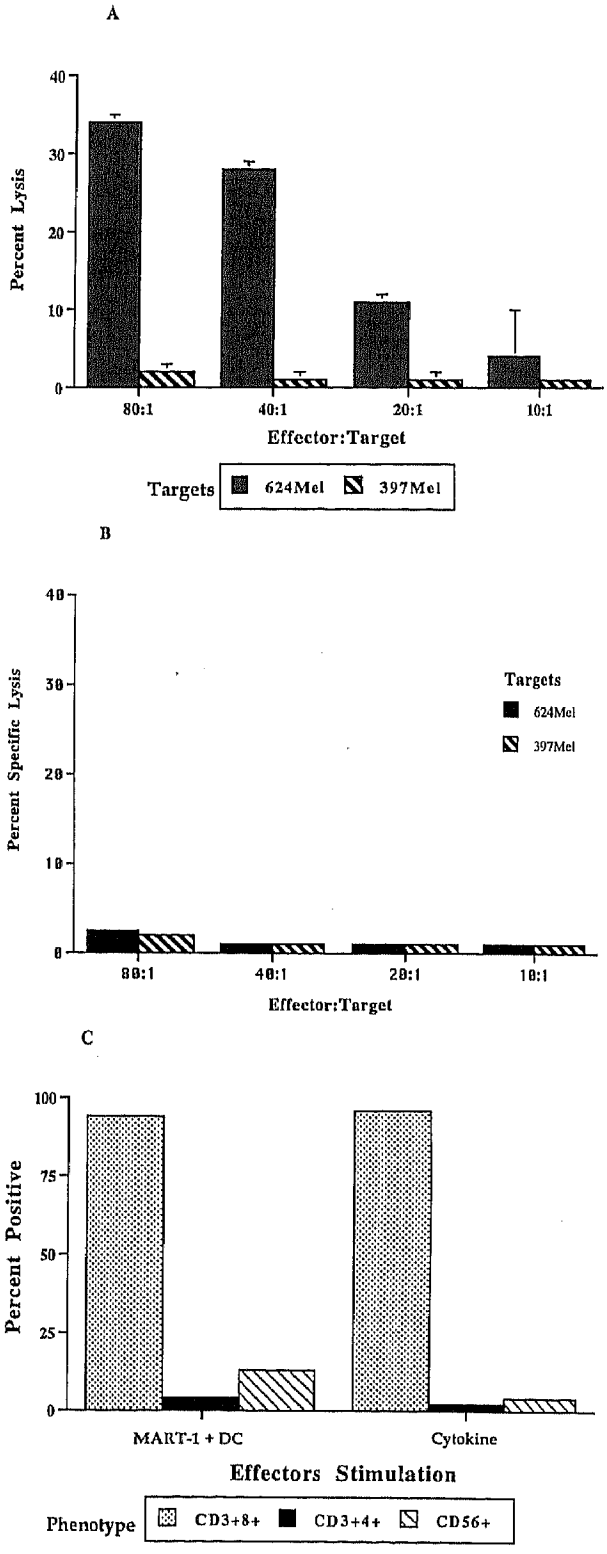
Previously, we showed that MART-1 peptide-pulsed DCs can elicit HLA-restricted, tumor-specific CTLs in healthy donors (23). We were interested in whether patients with melanoma would respond in a similar manner despite the T-cell anergy in vivo. Autologous DCs generated from GM-CSF– and IL-4–cultured adherent peripheral blood mononuclear cells were used as APCs to present MART-1 peptide to $CD8^+$ T cells. These cultured DCs were pulsed with defined MHC class I, HLA-A2–specific MART-1 peptide (AAGIGILTV), and cocultured with autologous peripheral blood T cells in the presence of IL-7. The T-cell cultures were restimulated twice with the peptide-pulsed DC-positive IL-7 before the effectors were tested for cytotoxicity using various target cells. The effectors that were stimulated with unpulsed DCs or cytokine alone were included as negative controls. The targets we selected for the cytotoxicity assay were 624Mel, a HLA-A2–positive, MART-1–positive human melanoma cell line, and 397Mel, an HLA-A2–negative but MART-1 antigen-positive melanoma tumor line. These cell lines were chosen to identify the antigen specificity and the HLA restriction of the T effector population.

As indicated in Figure 1A, the effector T cells stimulated with MART-1 peptide-pulsed DCs showed significantly greater cytotoxicity against the 624Mel cell line. Lytic activity was observed in an E:T ratio as low as 20:1. No cytotoxicity against the HLA-A2–negative cell line was observed. When the same autologous $CD8^+$ T cells were stimulated with cytokine alone in the absence of peptide-pulsed DCs, no measurable cytotoxicity was observed against 624Mel or 397Mel tumor cell lines (Fig. 1B). Similar unresponsiveness was observed when these T cells were stimulated with peptide-unpulsed DCs or phytohemagglutinin+IL-2 (data not shown). Because we did not detect any nonspecific killing of target cells, we determined the phenotype of the effector population at the time of the cytotoxicity assay. As indicated in Figure 1C, most of the T cells in both peptide-pulsed DC-stimulated and cytokine-stimulated T-cell cultures was of the $CD3^+$ $CD8^+$ T cells and not $CD4^+$ (helper T) or $CD56^+$ (natural killer) T cells. Interestingly, there was no increase in the $CD4^+$ or $CD56^+$ T-cell population in T-cell cultures stimulated with IL-7 alone.

Cytotoxic T Lymphocyte Responses Against a Tumor Cell Line and Autologous Tumor Cells

The next set of experiments compared the efficacy of the peptide-pulsed DC-stimulated T cells in response to both HLA-matched tumor cell line and autologous tumor. Peripheral blood lymphocytes from a second HLA-$A2^+$ melanoma was obtained and DCs were generated from cultured monocytes. These DCs were pulsed with the MART-1 peptide and used in combination with IL-7 to stimulate $CD8^+$ T cells. After 2 rounds of restimulation, these T cells were assessed for lytic activity against an HLA-$A2^+$ and MART-1^+ melanoma tumor line (624Mel), MART-1 peptide-pulsed T2 cells, and the primary cultured autologous tumor cells. T cells were cultured with IL-7 alone or with unpulsed DCs and served as negative controls.

As shown in Figure 2, a significant cytotoxic response was detected against the HLA-A2 and MART-1 antigen-positive target, 624Mel, when the $CD8^+$ T cells were stimulated with peptide-pulsed DCs. The response was titrated at E:T ratios of 60:1 down to 7.5:1. The response was slightly higher than observed with the previous patient described in Figure 1A. The lytic activity against the peptide-loaded T2 cells was slightly greater than the activity against 624Mel and was titratable across the various E:T ratios. The same peptide-pulsed, DC-stimulated effector T cells were assessed for their ability to lyse cultured autologous tumor cells. As shown in Figure 2, there was a significant level of cytotoxicity



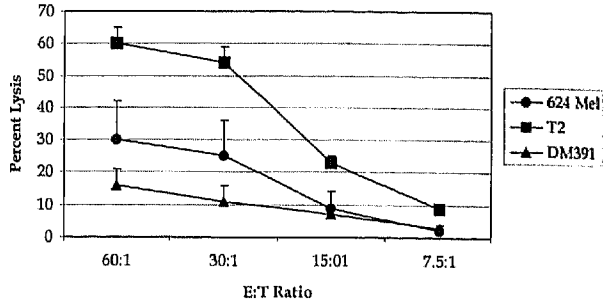


FIG. 2. Generation of MART-1–specific cytotoxicity from a second patient with HLA-A2–positive melanoma. The CD8 captured responders were stimulated with MART-1 peptide-pulsed DCs and cultured in 10 ng/ml IL-7. Cytotoxicity was assayed against 624Mel (A2+MART-1+) T2 cells pulsed with MART-1 peptide, and autologous melanoma tumor (MART-1+ A2+) targets after two rounds of restimulation with MART-1–pulsed DCs plus IL-7 at E:T ratios of 60:1, 30:1,15:1, and 7.5:1. The response on T2 cells pulsed with an irrelevant peptide has been subtracted from the response on T2 cells pulsed with MART-1 peptide. The response of cultures stimulated with unpulsed DCs plus IL-7 or IL-7 only was at background levels similar to that shown in Figure 1B. Mean values are represented (n = 3) with SDs designated by error bars.

against the autologous tumor cells. These tumor cells were positive for the MART-1 antigen and were HLA-$A2^+$. Although the response was lower (16%) than the response observed against 624Mel and T2 targets, the response of the peptide-stimulated T cells was greater when compared with T cells stimulated with the unpulsed DCs or IL-7 alone (data not shown). These peptide-pulsed, DC-stimulated $CD8^+$ T cells not only could lyse established or relevant peptide-pulsed T2 cells but they were also effective in recognizing and lysing autologous tumor cells. Furthermore, $CD8^+$ T cells stimulated with unpulsed DCs or cytokine alone (data not shown) did not show any lytic activity against any of the targets, which was observed in patient 1 described here.

Adenoviral-Mediated Transgene Expression in Cultured Dendritic Cells

Dendritic cells were generated from peripheral blood monocytes from two patients with melanoma and cul-

FIG. 1. The HLA-restricted cytotoxicity of MART-1 antigen-specific CTLs from a patient with HLA-A2 melanoma. The $CD8^+$ captured T-cell responders were stimulated with MART-1 peptide-pulsed DCs and cultured in 10 ng/mL IL-7. Cytotoxicity was assayed against 624Mel (A2+MART-1+) and 397Mel (A2-MART-1+) targets after two rounds of restimulation with MART-1–pulsed DCs plus IL-7 (**A**) or IL-7 only (**B**) at E:T ratios of 80:1, 40:1, 20:1, and 10:1. Mean values are represented (n = 3) with SD designated by error bars. The phenotype of MART-1 antigen-specific CTLs (**C**) from a patient with HLA-A2–positive melanoma. Phenotype was assessed at the time of the cytotoxicity assay by staining with antibodies to CD3, CD4, CD8, and CD56.

tured in the presence of GM-CSF and IL-4 for 7 days. At the end of the culture, the cells were infected with various MOI of adenoviral particles, as described in Materials and Methods. The infected cells were fixed and stained with X-gal after 24 hours. As depicted in Figure 3, infection with as low as 50 MOI produced positive lacZ gene expression in both donors. However, infection with 200 MOI produced the highest level of transgene expression. Use of as much as 500 MOI yielded only a minor increase in lacZ expression, but substantially increased adenoviral toxicity on DCs (data not shown). Thus, the 200 MOI concentration was selected for further studies.

Generation of MART-1 Antigen-Specific Cytotoxic T Lymphocytes Using Gene-Modified Dendritic Cells

Dendritic cells were generated from adherent monocytes from peripheral blood lymphocytes of a patient with melanoma and cultured for 7 days with GM-CSF and IL-4. At the end of the culture period, the cells were infected with an adenoviral vector carrying MART-1 cDNA. After 24 hours, the DCs were collected and an aliquot was mixed with autologous $CD8^+$ T cells. The first stimulation was performed with transfected DCs 1 day after transduction with subsequent weekly restimulations using the frozen transduced DCs for a total of three stimulations. Antigen-specific cytotoxicity was assessed 5 days after the last stimulation using 624Mel cells, and a MART-1–negative, HLA-A2–positive breast adenocarcinoma cell line, MCF-7, as targets. In addition, cytotoxic activity was assessed against MCF-7 transduced with MART-1 adenovirus or empty adenovirus.

Figures 4a and b show the results of the cytotoxicity assays with 624Mel and modified MCF-7 cells. The CTLs generated using MART-1 gene-modified DCs effectively killed the MART-1–positive, HLA-A2–positive 624Mel targets (Fig. 4a). Only a background level of

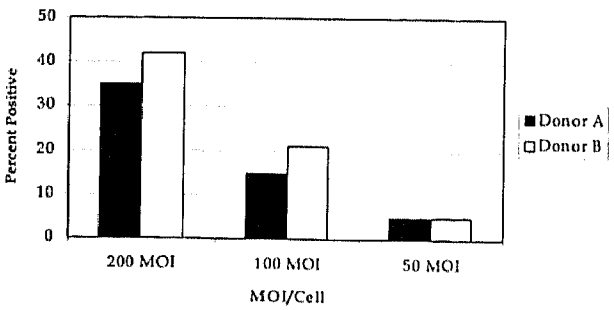


FIG. 3. β-galactosidase activity in DCs transduced with lacZ adenovirus. The DCs were infected with varying MOI and stained with X-gal 24 hours after infection. Transgene expression was observed starting with 50 MOI, with optimal expression at 200 MOI.

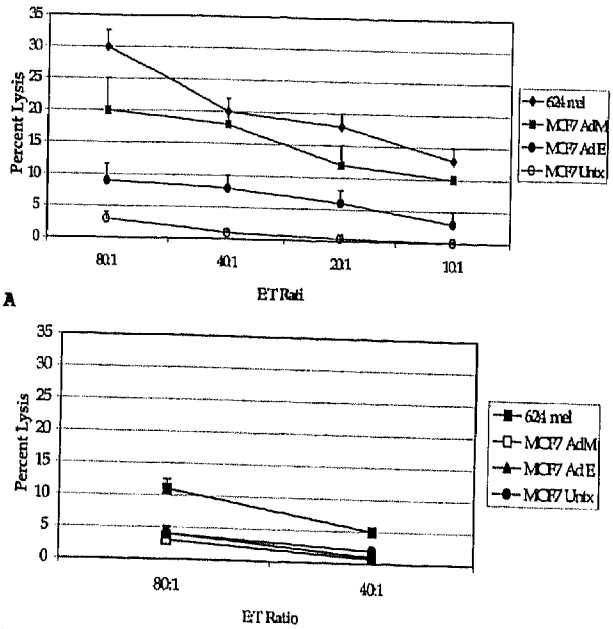


FIG. 4. Generation of MART-1–specific CTLs using adenovirus-transduced DCs. (A) T cells were stimulated with DCs infected with adenovirus containing the MART-1 gene or with empty adenovirus, and their cytotoxic activity was measured. (B) The CTLs stimulated with MART-1 gene-modified DCs exhibited antigen-specific lysis of 624Mel (A2+MART-1+) and MART-1 gene-modified MCF-7 (A2+MART-1+). Adenovirus-specific lysis was observed against MCF-7 transduced with empty adenovirus. Mean values are represented (n = 2) with SDs designated by error bars.

lysis was observed against the unmodified, MART-1–negative MCF-7 target cells. However, when MCF-7 cells were transduced to express MART-1, we observed a four- or fivefold increase in cytotoxicity, indicating that the CTLs were MART-1 antigen specific. Furthermore, 10% to 15% cytotoxicity was detected against adenovirus antigens as measured by the killing of MCF-7 transduced with a control adenovirus vector lacking the MART-1 transgene (Ad empty) (Fig. 4b). Nevertheless, there was a substantial increase in the cytotoxicity against the MART-1 expressing MCF7 when compared with either untransduced or empty adenovirus transduced MCF7 cells in three of five patients studied.

In contrast to the gene-modified, DC-stimulated T cells, the empty adenovirus transduced DC-stimulated T cells overall showed no response against any of the targets (Fig. 4b). Similar unresponsiveness was observed when the T cells were stimulated with IL-7 alone without antigen or DCs (data not shown).

DISCUSSION

Recent advances in cancer vaccine strategies include the activation of $CD8^+$ CTLs using tumor antigen pro-

cessed by professional APCs such as DCs (24–26). Antigen-loaded or transgene-expressing DCs have several advantages, including maximizing T-cell activation and reducing the possibility of anergy induction (1,27). With the cloning of tumor antigens and identification of specific epitopes recognized by T cells, it is possible to transfer these antigens into DCs to be presented exclusively to the immune system with multiple, and presumably optimal, immunostimulatory signals, avoiding potential autoimmunity. Dendritic cells genetically modified to present TAA may provide broad and important advantages over antigen peptide-pulsed DCs in presenting previously unknown $CD4^+$ and $CD8^+$ epitopes.

Recent reports on gene modification of DCs for antigen presentation have used retroviral transduction procedures and bone-marrow–derived DCs to stimulate CTLs in vitro. In addition, several investigators have reported that DCs can be infected with adenoviral vectors and these DC can stimulate naïve T cells using a model antigen such as β-gal in murine tumor models (28–30). Both partial and complete protection was demonstrated after intravenous administration of adenovirus-mediated, MART-1–engineered DCs in a murine melanoma model (31). Monocyte-derived GM-CSF and IL-4 cultured DCs have been shown to express transgenes by using retroviral and adenoviral transduction procedures and gene modification of DCs using plasmid DNA: cationic liposome complexes (23,32). However, no data have shown that transiently transduced human DCs can induce an antigen-specific CTL response in vitro in patients with melanoma. In this report, we found that human DCs can be gene modified using a transient adenovirus-mediated transduction system and that these DCs can induce T-cell activation in vitro and can generate tumor-specific immunity equivalent to that of specific peptide-pulsed DCs in patients with melanoma.

To identify the optimal experimental conditions for in vitro generation of antigen-specific CTLs in patients with melanoma using adenovirus-mediated gene-modified DCs, we first used a defined epitope peptide for the MART-1 antigen. We enrolled more than 10 patients for this study, and representative data from two patients are reported (Figs. 1A and 2). The response rate seen with patients with melanoma was much greater than with the healthy donors. Eight patients of 10 responded with high levels of CTL response. This observation may be the result of a higher number of readily reactivated antigen-specific CTL precursors present in persons with cancer compared with healthy donors (33) or of the high stimulation potency of the MART-1 antigen in HLA-A2 patients.

Previous studies have shown the generation of HLA-A2–restricted and peptide-specific CTLs using IL-2 for in vitro cultures (34). In our studies, replacing IL-2 with IL-7 more consistently produced a greater expansion of T cells in vitro. We also observed that peptide-specific CTLs not only lysed the appropriate peptide-loaded T2 targets but also lysed MART-1-positive melanoma tumor cells, indicating recognition of endogenously processed antigens. In one patient, the CTLs generated using peptide-pulsed DCs as APCs were able to recognize and lyse autologous tumor. Although the response against the melanoma cell lines were greater than the autologous tumor response, the CTLs were still able to recognize the endogenously processed low level of antigens on the autologous tumor. It is conceivable that the cultured established cell line targets express higher levels of antigen or MHC molecules, making them better targets for T cells compared with the freshly isolated autologous tumor cells. Furthermore, the increased CTL activity observed against the peptide-loaded T2 cells clearly indicates that the level of antigen expression and MHC expression alters the intensity of the response.

Adenovirus-mediated gene-modified DCs were similar to peptide-loaded DCs in their ability to activate T cells efficiently from patients with melanoma. High-level gene expression was achieved using adenoviral constructs (Fig. 3). There was an increase in lacZ gene expression with an increase in the MOI. However, infection with more than 200 MOI not only did not exponentially increase the level of gene expression but also increased the toxicity of the virus on the DCs. Human DCs were very sensitive to the adenoviral particles in contrast to murine DCs, which can tolerate an MOI up to 1,000 (28). This may be due to the greater availability of receptors for the adenovirus on human cells compared with murine cells.

We observed that adenovirus-transduced DCs not only induce transgene MART-1–specific CTLs but also induce, although at low levels, CTLs against the adenovirus (Fig. 4a). These MART-1–specific CTLs also can recognize endogenously processed MART-1 antigen on the 624Mel target cells and did not lyse the antigen-negative cell line, MCF-7, although this cell line is HLA-A2 positive. There was distinct cytolytic activity when MCF-7 was transduced to express either adenoviral proteins (Ad empty) or adenoviral MART-1 construct (Ad MART-1). Furthermore, higher levels of cytotoxicity were detected in response to Ad MART-1–transduced MCF7 compared with the Ad empty–transduced MCF7, indicating the presence of MART-1–specific T cells in addition to adenovirus-specific T cells. It was interesting to note that the Ad empty–transduced, DC-stimulated T cells did not display any increase in the levels of adeno-

viral response (Fig. 4b). One explanation for this low level of anti-adenovirus response or higher level of MART-1 response may be the greater number of CTL precursors for MART-1 in patients with melanoma than for adenovirus-specific T cells. Another explanation may be that the patients with melanoma are immunologically suppressed or anergized to exhibit any anamnestic response against the adenovirus. Nevertheless, adenovirus gene-modified DCs were able to generate MART-1–specific CTLs from patients with melanoma.

In samples from other patients, we observed CTLs that did not recognize the known HLA-A2–specific MART-1 peptide but did efficiently lyse the MART-1 antigen-positive melanoma cell line (data not shown). These data suggest that adenovirus-mediated MART-1 gene-modified DCs could process the whole protein and present unknown epitopes in addition to the known epitope for T-cell activation. These observations further support those of others (35) who have used vaccinia vector–transduced DCs that could induce tumor-specific T-cell responses independent of HLA phenotypes.

In conclusion, we found that gene-modified DCs are functionally comparable to peptide-loaded DCs in generating antigen-specific CTLs in patients with melanoma. The DCs can be gene modified by adenoviral transduction methods, and these gene-modified DCs can effectively process and present sufficient amounts of antigen to generate CTL responses specific to multiple epitopes in T-cell populations of patients with melanoma. These studies represent an important step in the evolution of novel and efficient immunotherapeutic approaches to the treatment of cancer using defined antigens.

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