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In Vitro Priming with Adenovirus/gp100 Antigen-Transduced Dendritic Cells Reveals the Epitope Specificity of HLA-A*0201-Restricted CD8\(^+\) T Cells in Patients with Melanoma

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Replication-deficient recombinant adenovirus (Ad) encoding human gp100 or MART-1 melanoma Ag was used to transduce human dendritic cells (DC) ex vivo as a model system for cancer vaccine therapy. A second generation E1/E4 region deleted Ad which harbors the CMV immediate-early promoter/enhancer and a unique E4-ORF6/pIX chimeric gene was employed as the backbone vector. We demonstrate that human monocyte-derived DC are permissive to Ad infection at multiplicity of infection between 100 and 500 and occurs independent of the coxsackie Ad receptor. Fluorescent-labeled Ad was used to assess the kinetics and distribution of viral vector within DC. Ad-transduced DC show peak transgene expression at 24–48 h and expression remains detectable for at least 7 days. DC transduced with replication-deficient Ad do not exhibit any unusual phenotypic characteristics or cytopathic effects. DC transduced with Ad2/gp100v2 can elicit tumor-specific CTL in vitro from patients bearing gp100\(^+\) metastatic melanoma. Using a panel of gp100-derived synthetic peptides, we show that Ad2/gp100v2-transduced DC elicit Ag-specific CTL that recognize only the G209 and G280 epitopes, both of which display relatively short half-lives (~7–8 h) on the surface of HLA-A*0201\(^+\) cells. Thus, patients with metastatic melanoma are not tolerant to gp100 Ag based on the detection of CD8\(^+\) T cells specific for multiple HLA-A*0201-restricted, gp100-derived epitopes. The Journal of Immunology, 2000, 164: 3402–3412.

Immature dendritic cells (iDC)\(^3\) are unique in their capacity to capture and process Ag by either MHC class I or class II pathways. Final maturation of iDC to mature DC (mDC) is prompted by various cytokines, ligation of CD40, or exposure to endotoxin (1–3). Up-regulation of key costimulatory molecules, adhesion molecules, as well as MHC class I and class II cell surface molecules accompanies final maturation of DC. mDC serve to present processed peptide Ags to CD4\(^+\) and CD8\(^+\) T lymphocytes (4, 5). The potential therapeutic use of DC for immunization against infectious agents, malignancy, and the induction of tolerance has been highlighted by Steinman (6). Preclinical tumor models provide striking evidence in support of the relative potency of DC in promoting tumor rejection. Several studies advance the notion that tumor Ag (or peptide)-loaded DC can elicit tumor-specific CD4\(^+\) and CD8\(^+\) T cells that cause tumor regression in mice (7–12). For example, peptide-pulsed DC administered weekly to mice bearing the syngeneic B16 murine melanoma provoke Ag-specific CD8\(^+\) T lymphocytes that mediate eradication of the tumor and confer long-term survival (13). Early clinical trials in patients with a variety of malignancies suggest that durable clinical responses are possible with DC immunization (14–16).

Gene transfer strategies have shown that replication-deficient Ad is well suited to serve as a vehicle for the ex vivo transduction of human DC (17–19). Distinguishing features of Ad vectors are their capacity to generate high titers necessary for clinical studies as well as their ability to infect nondividing cells. Recent developments in vector biology are built upon an appreciation that optimal virus production is influenced by distinct early region gene products while avoiding contamination by replication competent Ad (RCA) (20, 21). The version 2 Ad2 vector incorporates a unique E4 ORF-6/pIX chimeric element which reduces the generation of RCA via homologous recombination during propagation in the 293 helper cell line. The pIX capsid protein confers thermostability to the viral particle, ensuring production of high titer viral stocks essential for clinical studies. A well-recognized limitation of Ad vectors is that pre-existing Ad immunity can impair efficient in vivo transduction after systemic administration (22). A recent phase I clinical trial in patients with metastatic melanoma indicates that doses up to 10\(^{11}\) infectious units (IU) of Ad2 vectors can be safely administered; in addition, some individuals can be successfully immunized after systemic administration of Ad2 vectors encoding either MART-1 or gp100 Ags (23).

MART-1 and gp100 are melanocyte lineage-restricted, nonmutated Ags isolated through expression cloning of melanoma cDNA libraries (24). Expression of both MART-1 and gp100 is restricted...
to normal melanocytes, pigmented retinal epithelia, as well as primary cutaneous melanoma. Immunohistochemical analysis confirms gp100 expression in \(~75\%\) of distant metastases (25). Interestingly, only tumor-infiltrating lymphocytes specific for gp100, but not MART-1 Ag, demonstrate therapeutic efficacy when reinfused into patients (26). Initial studies identified five HLA-A*0201-restricted dominant epitopes (G154, G209, G280, G457, and G476) recognized by gp100-specific CD8\(^+\) T cells from patients with melanoma (26). Subsequent analysis showed that several subdominant epitopes of gp100 (G177 and G570) also exist and can elicit melanoma-reactive CTL after in vitro priming with DC (27). Several studies suggest that most circulating A*0201-restricted, gp100-specific T cells recognize the G280 epitope (28, 29).

We sought to examine the epitope specificity of gp100-specific, HLA-A*0201-restricted T cells upon in vitro priming with autologous DC transduced with Ad vector encoding gp100 melanoma Ag. Optimal conditions which permit \(>80\%\) transduction of DC in large scale suitable for clinical immunization protocols were established. Ad-transduced DC do not undergo any discernible maturation changes or apparent cytopathic effects (CPE). gp100 Ag-specific CTL obtained from three patients identifies the G209 and the G280 epitopes as dominant. Interestingly, none of the other higher affinity dominant (or subdominant) epitopes were recognized.

### Materials and Methods

#### Ad constructs

The first generation Ad2 vectors encoding the gp100 and the MART-1 human melanoma Ags have been described (30). The second generation vectors described herein, referred to as version 2 (v2) vectors, possess the same 5' CMV immediate-early promoter/enhancer and harbor a deletion in the E1 region. In addition, the entire E4 region is deleted except that a novel E4 ORF-6d/IF chimeric gene has been inserted within the E4 site and is transcribed in a right to left direction (21). The E2 and E3 regions remain intact. Viral titers of clinical grade vectors exceed 10\(^{11}\) IU/ml after purification by standard CsCl gradient ultracentrifugation. The particle/IU ratio of Ad2 vectors ranges between 3 and 12. No RCA are detectable using criteria described previously (20). Clinical grade Ad2 vector stocks are stored at \(-80^\circ\)C and used after only one freeze-thaw cycle.

#### Peptides

Synthetic peptides were purchased from Biosynthesis (Lewisville, TX) or manufactured at the Massachusetts General Hospital (MGH) biopolymer core facility (Charlestown, MA). Peptides were purified to \(>90\%\) by reversed-phase HPLC as confirmed by mass spectrometry. Peptides were dissolved in 10\% DMSO (v/v) at 2 mg/ml, sterile filtered through a 0.20 \(\mu\)M membrane (Schleicher & Schuell, Keene, NH) and stored at \(-80^\circ\)C.

#### T2 dissociation assay

T2 cells (174sCEM.T1 hybrid, CRL-1992) are TAP-deficient, HLA-A*0201 lymphoblastoid cells maintained in Iscove’s media (Life Technologies, Rockville, MD) with 10% FCS (31). Twenty-four hours before use, cells were allowed to log phase growth. T2 cells were washed three times with 50 ml PBS and resuspended in IMDM (serum free) at 5 \(\times 10^5\) cells/ml. After addition of 30 \(\mu\)M peptide plus 3 \(\mu\)g/ml human \(\beta_2\) microglobulin (Sigma, St. Louis, MO, or Calbiochem, La Jolla, CA), cells were kept at room temperature for 30 min. Cells were transferred to 24-well tissue culture trays and returned to 37°C in 5% CO\(_2\) overnight (\(\sim\)12 h). The next morning peptide-loaded T2 cells were washed twice in PBS, resuspended in Iscove’s media (serum free) containing 1 \(\mu\)g/ml brefeldin A (Epicentre Technologies, Madison, WI), and returned to the incubator. At the indicated time points, cells were removed, washed once, and stained with BB7.2 (HLA-A2-specific) mAb for 30 min on ice (32). Cells were washed in ice-cold staining buffer (PBS/1% BSA/0.2% sodium azide) and incubated with 1 \(\mu\)L secondary conjugate goat anti-mouse IgG-FITC (Caltag, South San Francisco, CA). T2 cells loaded either with the H-2 K\(^b\)-binding peptide (SIINFEKTL, OVA) or no peptide served as the negative control. Cells were analyzed on a FACSscan (Becton Dickinson, Mountain View, CA) with Lysis II software. A minimum of 10,000 events was analyzed for each sample. T2 cell death due to prolonged exposure to brefeldin A limits the reliability of this assay beyond a period of 24–30 h.

#### Cell collection and processing

Patients with stage IV malignant melanoma underwent leukopheresis at the MGH Blood Bank. Each patient had documented gp100-positive metastases by immunoperoxidase staining with the HMB-45 mAb and was HLA-A2 positive. The protocol was granted approval by the Dana-Farber Cancer Institute/MGH Institutional Review Board and patients provided written informed consent before study. Purified CD8\(^+\) T cells were obtained by negative selection of PBMC using a panel of mAb and magnetic beads. Briefly, PBMC were incubated with anti-CD4 (OKT4; American Type Culture Collection (ATCC), Manassas, VA), anti-HLA-DR (1213; ATCC), anti-CD11b (MY904; ATCC), anti-CD20 (1F5; ATCC), anti-CD14 (3C10; ATCC), and anti-CD56 (B159; PharMingen, San Diego, CA) at saturating concentrations for 60 min at 4°C. Cells were washed twice and then incubated with magnetic particles coated with goat anti-mouse IgG (PerSeptive Biosystems, Framingham, MA) for 2–4 h at 4°C. Cell separation was performed with a strong magnet. Cells were collected, washed twice, and placed in culture. Purified CD8\(^+\) T cells were \(>90\%\) positive for expression of CD3  CD8\(^+\) and \(<2\%\) positive for CD3  CD4\(^+\).

#### DC transduction

iDC were obtained from adherent PBMC fractions as described previously (4, 33). Briefly, adherent PBMC (obtained by a 2-h adherence step) were cultured in RPMI 1640, 10% AB\(^+\) serum, and penicillin/streptomycin with 100 ng/ml GM-CSF (Immunex, Seattle, WA) and 20 ng/ml IL-4 (Peprotech; Rocky Hill, NJ) for 6 days. Cells were harvested, washed twice in standard cell media, and resuspended in X-Vivo 15 (BioWhittaker, Walkersville, MD) at 10\(^7\)/ml. Cells were equilibrated to 37°C in a water bath for 20–30 min before transduction. Ad stocks were thawed on ice and added to the DC suspension at the indicated multiplicity of infection (moi). Cells were gently mixed by agitation and plated immediately back in the 37°C water bath. After a 20-min incubation, warm media (X-Vivo 15) containing GM-CSF and IL-4 were added to dilute the DC to a final concentration of 4 \(\times 10^7\)/ml. Transduced DC were transferred to low-adherence 6-well trays (Ultralonal 3471; Costar, Cambridge, MA) at 5 ml/well and maintained at 37°C in 5% CO\(_2\) for an additional 24 h. At the indicated time, DC were harvested, washed once in PBS, and used for analysis. For most studies, moi 300 was used to transduce DC. To obtain mDC, soluble trimeric human CD40 ligand (Immunex) at 1 \(\mu\)g/ml final concentration was added to DC on day 6 of culture. After 48 additional h, cells were harvested and analyzed by flow cytometry.

#### Blockade of Ad receptor or coreceptors

Cells (DC or melanoma, MGH-LH) at 10\(^7\)/ml were allowed to equilibrate in a 37°C water bath for 20 min. Three-fold serial dilutions of purified Ad2 fiber-knob protein (Genzyme, Cambridge, MA) (34) were prepared in serum-free media (X-Vivo 15) in a volume of 100 \(\mu\)l. Azide-free mAb specific for \(\alpha_2\) and \(\beta_3\) (Chemicon International, Temecula, CA) were fiber-knob conjugated at a final concentration of 1 \(\mu\)g/ml. Briefly, Ad virus particles were labeled with carbocyanide dye (Cy3; Amersham) and incubated with soluble trimeric human CD40 ligand (Immunex) at 1 \(\mu\)g/ml final concentration was added to DC on day 6 of culture. After 48 additional h, cells were harvested and analyzed by flow cytometry.

#### Fluorescent virus assays

Ad virus particles were labeled with carboxyamide dye (Cy3; Amersham, Arlington Heights, IL) as described previously (35). Briefly, 10\(^5\) virus particles/ml were incubated directly with DC and 100 particles/cell or to melanoma cells at 300 particles/cell. Cells were attached to poly-L-lysine-coated plates. Cy3-labeled Ad was added to cells and after a 2-min incubation period at 37°C, the cells were washed with PBS to remove residual virus. Fiber inhibition studies were performed by preincubation of cells with purified Ad2 fiber-knob protein (1 mg/ml) for 1 h before the addition of Cy3-labeled Ad. The addition of fiber protein was present during the entire incubation period. At the indicated time points, each culture was washed three times with PBS before fixation with 1% paraformaldehyde. The coverslips were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) to visualize the nuclei.
before mounting with Permount (Sigma). Fluorescence was visualized using the Olympus Provis AX70 microscope (New Hyde Park, NY) equipped with a Hamamatsu digital charge-coupled device camera (Middlesex, NJ) for capturing images.

**CTL generation**

Purified CD8+ T cells were cultured with autologous DC transduced with Ad2/gp100v2 at a ratio of 20:1 in RPMI 1640 (Life Technologies) plus 10% male AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, nonessential amino acids, and sodium pyruvate. A total of 5 × 10^6 CD8+ T cells with 2.5 × 10^5 transduced DC was cultured in a volume of 0.5 ml in 48-well tissue culture trays (Costar 3548) in the presence of IL-7 (Peprotech) 10 ng/ml and maintained at 37°C in 5% CO2 (27). Beginning on day 3, 50 U/ml IL-2 (Chiron Therapeutics, Emeryville, CA) was added to each well every 2–3 days. Every 7 days, responding T cells were harvested, transferred to new culture trays, and restimulated with fresh Ad2/gp100v2-transduced autologous DC. After the third restimulation, T cell lines were expanded in 24-well trays in a final volume of 1 ml medium/well. CTL were tested in cytolyis assays 5 days after each restimulation as described below.

**Cytolysis assays**

Cytolytic activity was measured using a standard 4-h 51Cr release assay. Briefly, T2 cells were labeled with 100 μCi Na2CrO4 (NEN, Boston, MA) at 1 × 10^6 cells/ml for 1 h at 37°C. Cells were washed three times, and then loaded with 5 μM peptide for 30 min before addition to wells containing effector cells. Melanoma cell lines were harvested by pipetting (avoiding the use of trypsin) and labeled with 50 μCi Na2CrO4 for 1 h at 37°C. When indicated, melanoma cell lines were washed three times and used as target cells when indicated. The melanoma cell lines DM13 (gp100^+^, A2.1^+^) and DM14 (gp100^+^, A2^+^) were kindly provided by T. Darrow (Duke University Medical Center, Durham, NC) (36). Assays were performed in 96-well round-bottom microtiter trays in a volume of 0.2 ml media with 1 × 10^6 target cells/well. Culture trays were centrifuged for 2 min at 25 × g before incubation at 37°C. After a 4-h period, each tray was centrifuged for 5 min at 200 × g, and 0.05 ml medium was transferred to a Lumiplate 96 tray (Packard, Meriden, CT) and placed in a gamma counter (TopCount; Packard). The percent specific lysis was calculated using the standard formula: % lysis = 100 × (experimental release – spontaneous release)/[maximal release – spontaneous release]. The spontaneous release is calculated from target cells incubated without effector cells, whereas the maximal release is calculated from target cells lysed with 1% Triton X-100. All determinations were performed in duplicate, and spontaneous release was usually 20–25% of the maximal release.

**Flow cytometry**

DC transduced with Ad2/CMVEGFP vector were harvested, washed once, and analyzed directly on the FL-1 channel (emission 530 nm peak fluorescence) after 488-nm excitation on a FACScan (Becton Dickinson) for capturing images.

To assess the susceptibility of iDC to transduction with Ad, the viral constructs used in this study were shown schematically in Fig. 1. iDC were obtained by culture of adherent PBMC in media containing 1% human AB serum plus GM-CSF and IL-4. After 6 days of culture, phenotypic characterization by flow cytometry yields a uniform population of DC that express high levels of HLA class II Ags, CD11c, CD86, and low levels of CD80 and CD83 (see below). Expression of CD14 is variable as determined with the My4 mAb but is always 10–50-fold lower than levels present on fresh PBMC. Expression of the CD3, CD19, and CD56 lineage markers are always negative. Using phenotypic and morphologic criteria established by other investigators (37), the DC obtained after 6 days of culture under these conditions are judged to be immature. Final yields of iDC obtained at day 6 are ~5–10% of the starting number of PBMC.

**Results**

**Human dendritic cells are susceptible to transduction with replication-deficient Ad**

The viral constructs used in this study are shown schematically in Fig. 1. iDC were obtained by culture of adherent PBMC in media containing 1% human AB serum plus GM-CSF and IL-4. After 6 days of culture, phenotypic characterization by flow cytometry yields a uniform population of DC that express high levels of HLA class II Ags, CD11c, CD86, and low levels of CD80 and CD83 (see below). Expression of CD14 is variable as determined with the My4 mAb but is always 10–50-fold lower than levels present on fresh PBMC. Expression of the CD3, CD19, and CD56 lineage markers are always negative. Using phenotypic and morphologic criteria established by other investigators (37), the DC obtained after 6 days of culture under these conditions are judged to be immature. Final yields of iDC obtained at day 6 are ~5–10% of the starting number of PBMC.

To assess the susceptibility of iDC to transduction with Ad, the Ad2/CMVEGFP vector was used as a reporter construct. Day 6 iDC were harvested, washed twice in serum-free media, and resuspended supplied by the manufacturer. MART-1-specific mAb (A103) was purchased from BioGenex Laboratories. The anti-HLA-DR mAb (L243) culture supernatant was used as a positive control.
in serum-free media containing GM-CSF and IL-4 at 10^7/ml. DC were exposed to serial 5-fold dilutions of Ad/CMVEGFP to achieve a range of moi from 4 to 2500. Cells were allowed to incubate for 20 min at 37°C. Additional prewarmed media were added to dilute the cells to a final concentration of 1 x 10^7/ml. DC were transferred to low-adherence tissue culture trays in 5-ml aliquots and recultured for an additional 48 h. As shown in Fig. 2A, iDC are susceptible to transduction with Ad vector in a dose-dependent manner. At 48 h postinfection, it appears that moi from 100 to 500 is optimal for the detection of EGFP as monitored by flow cytometry. For example, at moi 500, 93% of DC were positive for the expression of enhanced green fluorescent protein (EGFP) whereas uninfected cells were <1% positive. The mean fluorescence intensity (MFI) of EGFP expression was 6577 among DC transduced at moi 500; in contrast, control uninfected iDC exhibited a MFI of 25. We have consistently seen efficient EGFP expression (>80% positive cells) with iDC from >20 normal donors. We have noted that moi >500 typically results in a decrease in MFI with some CPE evident. For example, moi 2500 results in 82% positive cells at 48 h with an MFI of 3694.

To document the kinetics of transgene expression, DC were exposed to Ad2/CMVEGFP at moi 500 as described above, and, at various time points, cells were recovered and analyzed by flow cytometry. EGFP expression was evident as early as 14 h postinfection and was sustained for periods up to 7 days (data not shown). As shown in Fig. 2B, detection of EGFP was optimal between 24 and 48 h postinfection. In the representative experiment, EGFP was undetectable at 2 h, but was clearly evident by 14 h postinfection. At 24 h, 95% of DC were expressing EGFP with an MFI of 1807; at the 48-h time point, 98% of DC were judged positive with an MFI of 1319. By 168 h postinfection, only 77% of cells (MFI 342) were positive (data not shown). At each time point measured, uninfected DC population cultured under identical conditions served as a negative control. The MFI of each control DC population was always <10. In Fig. 2C, representative fluorescence histograms show the time course of EGFP levels in human DC. Similar results have been obtained in our laboratories using Ad2/CMVβ-gal vectors to infect human DC by detection with the fluorescein di-β-D-galactopyranoside (FDG) substrate using flow cytometry (data not shown).

Ad transduction of human DC is independent of the coxsackie Ad receptor

Since human DC are susceptible to infection with Ad only at relatively high moi (Fig. 2), we sought to determine whether the coxsackie Ad receptor (CAR) pathway is intact (38). Using the RmC8 mAb, which has specificity for the human CAR, it appears that human DC (n = 7 donors) are deficient in the expression of CAR as monitored by flow cytometry (Fig. 3A). As a positive control, the melanoma cell line (MGH-LH) expresses significant levels of CAR and is highly susceptible to Ad virus infection. For example, at moi 3, ~95% of the melanoma cells are transduced with Ad2/CMVEGFP, whereas human DC require moi >300 to achieve this level of transgene expression (data not shown). Both human DC and the melanoma line express detectable amounts of the Ad coreceptors αvβ3 and αvβ5 as determined by flow cytometry, suggesting that integrins are not limiting Ad transduction of DC.

To further assess the role of CAR in Ad transduction, we used purified Ad2 fiber-knob protein to block CAR on susceptible target cells before addition of Ad2/CMVEGFP. Addition of fiber-knob protein can block Ad transduction (moi 30) of the CAR-positive melanoma in a dose-dependent manner (Fig. 3B). In contrast, CAR did not block the transduction of human DC at moi 300. In fact, we have reproducibly seen modest enhancement in Ad transduction of DC with the addition of Ad2 fiber-knob protein. One possible explanation for this finding would be the presence of a small contaminant in the fiber-knob preparation that activates the endocytic compartment of DC to facilitate uptake by Ad vector. To assess the contribution of integrin-mediated internalization, Ab-mediated blockade of αvβ3 and αvβ5 was performed using a pool of mAb specific for both integrins. Ad transduction of both cell types was partially blocked by the anti-integrin mAbs (Fig. 3B). The MFI of transduced DC was reduced 57% in the presence of anti-integrin mAb (control mAb reduced by 6%), whereas the MFI of transduced MGH-LH was reduced 48% by anti-integrin mAb (control mAb reduced by 16%). This observation suggests a role for αvβ3 and αvβ5 integrins in Ad vector gene transfer in human monocyte-derived iDC.

Localization of fluorescent-labeled Ad virions in transduced human DC

To confirm the above observations, Cy3-labeled Ad particles were used to infect CAR-negative iDC and the CAR-positive human melanoma line. Fluorescent photomicrographs of representative fields illustrate the binding, uptake, and subcellular localization of Ad after incubation at 37°C for either 2, 15, or 90 min. After the indicated incubation period, the cells were gently washed to remove residual unbound Ad, counterstained with DAPI, and immediately processed for microscopic inspection. Fig. 4 shows the kinetics of distribution of viral vector in each cell type after incubation with Cy3-labeled Ad. A 2-min incubation (denoted as
0 min) is sufficient for Ad binding and internalization by the melanoma cell line. In contrast, little fluorescence is evident either on or within the cytoplasm of DC after the 2-min incubation. When Cy3-labeled Ad was allowed to remain on DC for longer periods (15 or 90 min), uptake and perinuclear localization of Ad vector was clearly visible. Preincubation of cells with Ad2 fiber-knob protein was performed to assess CAR-mediated transduction of Cy3-labeled Ad particles. As expected, transduction of the human melanoma cell line was substantially decreased with prior incubation of Ad2 fiber-knob protein at all time points studied. In contrast, fiber-knob protein had no discernible effect on Cy3-Ad uptake by and localization within DC after a 15- or 90-min incubation period. Thus, these data suggest that the dwell time of Ad vector particles is critical for the efficient transduction of DC and perhaps other CAR-negative cells.

Transduction of human DC with Ad vectors encoding the melanoma Ags gp100 and MART-1

To assess the potential use of Ad-transduced DC for cancer vaccine therapy, we monitored the expression of two major lineage-restricted melanocyte Ags in DC after ex vivo transduction with Ad2/gp100v2 and Ad2/MART-1v2. DC were transduced with each vector at moi 300 as described above and were cultured in GM-CSF and IL-4 containing media for an additional 24 – 48 h. To assess the feasibility of large-scale transduction required for clinical studies, we utilized 10 – 10^6 DC. In large-scale experiments performed under the conditions detailed above, transduction efficiency of >80% was routinely measured as assessed by immunoperoxidase detection of single-cell suspensions using mAb directed to gp100 or MART-1. In a representative experiment shown in Fig. 5, DC were transduced with replication-deficient recombinant Ad at moi 300. Forty-eight hours later, single-cell suspensions of DC were processed for immunoperoxidase staining. More than 95% of Ad2/MART-1v2-transduced DC were judged positive for MART-1 expression by detection with A103 mAb. Ad2/gp100v2-transduced DC scored 92% positive when stained with the HMB-45 mAb. In contrast, CD40 ligand-treated DC were stained positive using the L243 mAb; in contrast, staining with either anti-gp100 or anti-MART-1 mAb was judged to be nonreactive.

Adenoviral transduction does not alter the DC phenotype

To assess the possible effect of replication-defective Ad on DC maturation, we studied the expression of various cell surface markers on Ad-transduced and nontransduced DC cultured in parallel. DC obtained after 6 days of culture were harvested and split into three groups. DC were either transduced with Ad2/gp100v2, exposed to soluble trimeric human CD40 ligand (nontransduced), or left untreated. The CD40 ligand-treated DC served as a positive control to assess the mDC phenotype. All three groups were recultured in parallel under identical conditions in X-Vivo 15 serum-free media containing GM-CSF and IL-4. As shown in Fig. 6, day 6 DC have the phenotype characteristic of iDC: HLA class I, HLA-DR, CD11c^+^, CD14^−^, CD80^+^, CD83^−^, CD86^−^.

### FIGURE 3.

Ad transduction of human DC is CAR independent. A, Human iDC, mDC, and a melanoma cell line (MGH-LH) were stained with mAb for CAR, αβ3, αβ5, and MHC class I (W6/32) was analyzed by single-color flow cytometry (indicated by the dark histogram). An isotype, non-binding primary Ab is included as a negative control (indicated by thick line open histogram). This is representative of seven normal donors evaluated for CAR expression. B, Fiber-knob protein and anti-integrin mAb (αβ3 and αβ5) blockade of Ad transduction for DC and melanoma cells. Target cells were preincubated with either fiber-knob protein (at the indicated concentrations) or anti-integrin mAbs (at 100 μg/ml) for 30 min as described in Materials and Methods. Cells were then exposed to Ad2/CMVEGFP at moi 300 (DC) or moi 30 (MGH-LH) for 30 min at 37°C, washed once, and recultured for an additional 24 h. The MFI (mean ± SD of triplicates) of the entire population is shown for each sample. The MFI for untransduced (uninfected) melanoma cells and DC was 4.0 and 5.1, respectively. Uninf, uninfected; iso., isotype control mAb; int., anti-integrin mAb.
promote terminal maturation as assessed by the expression levels of the cell surface markers studied. In Fig. 6, the MFI of HLA-DR expression on uninfected day 8 DC (MFI, 1042) is not significantly different when compared with Ad2/gp100v2-transduced DC (MFI, 1059); in contrast, CD40 ligand-treated DC express greater amounts of HLA-DR cell surface Ag (MFI, 1336) consistent with terminal maturation. We have consistently noted (n = 8 experiments) that Ad-transduced DC retain the phenotypic markers (and relative levels) present on uninfected DC. We conclude that Ad infection with replication-deficient virus does not adversely affect HLA class I Ag expression nor the levels of costimulatory molecules or adhesion molecules such as CD54 and CD58 (data not shown). Moreover, Ad-transduced DC remain viable for periods up to 7 days in culture as monitored by exclusion of propidium iodide and detection of esterase activity after loading with the cell permeable calcein AM ester (data not shown).

**FIGURE 4.** Dynamic tracking of fluorescent Ad in human DC and melanoma cells. Cy3-labeled Ad was added to the CAR-positive human melanoma cell line (MGH-LH) or iDC. Cy3-labeled Ad was allowed to remain in contact with cells at 37°C for the indicated time (2, 15, and 90 min) before rinsing with PBS. Cells are stained with DAPI and then processed for fluorescence microscopy. The images are false color enhanced to show the DAPI-stained nuclei (blue) and the Cy3-labeled virus (red). Preincubation of target cells with Ad2 fiber-knob protein (1 μg/ml) is indicated. Photomicrographs are shown at ×400 magnification.

**gp100-Specific HLA-A*0201-restricted CTL are specific for the G209 and G280 epitopes**

To evaluate the immunogenicity of Ad-transduced DC, purified CD8+ T lymphocytes from patients with melanoma were restimulated in vitro with autologous DC transduced with Ad2/gp100v2. The three patients studied in detail were HLA-A*0201-positive and had stage IV disease with documented gp100-positive metastasis. Responding T cells of each patient were restimulated weekly using autologous DC expressing gp100 and expanded in the presence of IL-2. To assess for reactivity to gp100 Ag, effector T cells were tested in a standard 51Cr release assay. Specific killing of the gp100-positive melanoma cell line DM13 (HLA-A2.1, -31, -B13, -18) was noted using effector cells from all three patients. In contrast, the gp100-negative melanoma cell line DM14 (HLA-A11, -28, -B5, -8, -Cw2, -4) was not lysed (Fig. 7A). Effector T cells obtained under these conditions do not exhibit significant lysis of K562 cells and are Ag specific as assessed by cold target inhibition assays (Fig. 7B). Specifically, cold DM13, but neither DM14 nor K562, can compete for lysis of 51Cr-labeled DM13 melanoma cells. Addition of an HLA class I-specific mAb (W6/32) completely blocks recognition of melanoma cell line DM13 by CTL from all three patients (data not shown). To assess the epitope specificity of effector cells from each donor, T2 cells pulsed individually with a panel of peptides from gp100 were used as target cells (Fig. 7C). Interestingly, only the G209 and G280 peptides were recognized from among the entire panel of seven dominant and subdominant epitopes. CTL from patients 057-02 and 057-03 lysed T2 targets pulsed with either the G209 or the G280 peptide. Patient 057-01 CTL killed only T2 targets pulsed with the G280 epitope. Furthermore, the remaining HLA-A*0201-restricted epitopes (G154, G177, G457, G476, G570) were not recognized by CTL from any patient studied.
HLA-A*0201/G209 and HLA-A*0201/G280 cell surface complexes have short half-lives

Most dominant epitopes exhibit high affinity for MHC class I molecules (39). Previous studies using a competition assay with a radiolabeled high-affinity HLA-A*0201-restricted peptide have established a rank order of binding affinity for the gp100 epitopes (26, 27). The G154 and G476 peptides are classified as high-affinity (IC50 , 50 nM) epitopes, whereas the remaining five peptides were of intermediate affinity (IC50 , 50 –500 nM) for HLA-A*0201. We chose to investigate this issue using a live cell assay with the TAP-deficient HLA-A*0201 T2 lymphoblastoid cell line to measure the dissociation rate of A*0201/β2-microglobulin/peptide complexes. T2 cells were loaded with individual peptides in the presence of exogenous human β2-microglobulin (and in the absence of FCS). After overnight culture at 37°C, the loaded T2 cells were washed vigorously and recultured at 37°C in serum-free media containing brefeldin A to block transport of newly synthesized class I molecules to the cell surface. At each time point, T2 cells were removed, washed, and stained with the BB7.2 mAb to quantitate folded forms of HLA-A*0201 cell surface molecules. The ΔMFI obtained at each time point (ΔMFI = MFI with peptide − MFI with no peptide) is depicted in Fig. 8 for each gp100 epitope as well as a reference hepatitis B virus (HBV) core 18-27 (C18) epitope previously shown to have high binding affinity for HLA-A*0201 (40). Linear regression analysis was used to obtain the measured half-life (t1/2) at 37°C of each peptide when complexed with HLA-A*0201. The rank order of peptides from gp100 as determined by this analysis corresponds closely to that seen by others using different methods (Table I): G154 (t1/2 = 24.7 h) > G177 (13.9 h) > G570 (11.2 h) > G209 (8.25 h) > G280 (7.0 h), > G457 (6.35 h). We were unable to accurately assess the G476 peptide because of poor solubility; however, it has been characterized previously as the second most avid binder to HLA-A*0201.

The reference high-affinity HBV core C18 peptide has a half-life of 22.8 h.

It appears that gp100-specific CTL elicited from patients with metastatic melanoma retain specificity for only two of the seven epitopes restricted to HLA-A*0201. We have been unable to detect CTL specific to gp100 epitopes other than G209 or G280, at least in the melanoma patients studied to date. Interestingly, G209 and G280 rank as fifth and sixth and have been previously characterized as having intermediate affinity for HLA-A*0201 (26, 27).

Discussion

Convincing evidence from numerous laboratories shows that Ag-loaded DC can stimulate tumor-specific immunity capable of eradicating established tumors in experimental models (10, 13, 41). Animals cured of existing tumors by DC vaccination are immune
to subsequent challenge consistent with the development of a durable Ag-specific memory response. In this report, we outline a strategy using replication-deficient recombinant Ad vectors encoding melanoma Ags gp100 or MART-1 to transduce human DC ex vivo to optimize the generation of Ag-specific T cells directed against melanoma. We provide evidence to demonstrate the feasibility of this approach, including the generation of gp100-specific HLA class I-restricted CTL that retain specificity for melanoma cells.

Several previous reports (17–19) show that human DC are susceptible to Ad transduction ex vivo. Results from our study provide additional information about optimization of Ad transduction of human DC. First, using Ad2 viral stocks with a particle:IU ratio 3–12:1 there appears to be no absolute requirement for liposomes to enhance transduction (data not shown). Second, it appears that Ad-transduced DC exhibit no unusual phenotypic changes or evidence to suggest differentiation to the mature state. This is of potential interest since iDC have unique chemokine receptor and cytokine/chemokine profiles which contribute to their homing properties (42). In addition, iDC are very efficient at capturing and processing Ags; in contrast, mDC are less efficient at Ag capture so timing would be critical for proper loading of Ag ex vivo before readministration (43, 44). Third, we provide formal documentation of dose response and time course of transgene expression in human DC transduced with Ad vector. Human DC transduced with the second-generation Ad2 vectors described herein at moi 100–500 are viable and show no evidence of CPE. However, careful titration of Ad stocks is essential since moi >500 cause CPE of DC and result in suboptimal Ag expression. One possible explanation for the CPE is the toxicity of the penton (pIII) protein evident at higher particle inoculations. Our finding that human DC are devoid of CAR provides one explanation for the higher moi requirement for iDC transduction. The blocking experiment using anti-integrin mAbs supports the notion that \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \) act as coreceptors (45) for Ad infection of human DC. We emphasize that, unlike vaccinia virus (46), replication-deficient Ad vectors are not cytopathic for human DC, ensuring Ag presentation beyond 24 h after transduction.

We were surprised to find that iDC are susceptible to transduction by subgroup C (Ad2) Ad vectors, despite their CAR-negative phenotype. Several studies have clearly demonstrated the requirement for CAR expression in Ad vector transduction of multiple cell types (35, 47, 48). For example, the basolateral localization of CAR by respiratory epithelium probably limits transduction when Ad vector is administered in aerosolized or aqueous form in the lumen (49). In a second study, a panel of 14 human melanoma cell lines were surveyed for susceptibility to Ad-mediated transduction; expression of CAR, but neither \( \alpha v \beta 3 \) or \( \alpha v \beta 5 \) integrins, correlated with transgene expression among the cell lines tested (50). Elegant studies by Leopold et al. (35) using Cy3-labeled Ad clearly show the temporal-spatial distribution of Ad vector on CAR-positive epithelial A549 cells. Ad internalization in A549 cells occurs with a \( t_{1/2} \) of 2.5 min and, by 60 min postinfection, >80% virus is localized to the nucleus. In agreement with this report, we find that transduction of the CAR-positive melanoma line is sensitive to inhibition by Ad fiber-knob protein. In addition,
we show that CAR-negative DC can be efficiently transduced under conditions that promote virus cell contact under conditions of high cell density in serum-free media for 20 min at 37°C. It appears that the α, β, and αβ integrins do play a role in viral uptake by DC based on inhibition studies with mAb; however, despite integrin blockade, we still observed significant Ad transduction. A distinguishing feature of iDC is the constitutive high rate of macropinocytosis (43, 51). We postulate that macropinocytosis of Ad vector allows internalization and delivery to an endosomal compartment before cytoplasmic transport. If macropinocytosis is the primary mechanism underlying CAR-independent transduction, then DC would appear to have a selective advantage for infection over most other cell types that lack the high-affinity receptor.

Tolerance to self nonmutated Ags is a critical issue when designing optimal vaccine formulations for certain malignancies such as melanoma (52). Virtually all candidate melanoma-specific tumor Ags under clinical study are self nonmutated proteins with restricted patterns of tissue expression and include Mage-1/3, tyrosinase, TRP-1/2 as well as MART-1 and gp100 (24). Using tumor-infiltrating lymphocytes as an enriched source of tumor-specific CD8+ T cells expanded in IL-2, Kawakami et al. (26) identified five dominant HLA-A*0201-restricted peptides (G154, G209, G280, G457, and G476) within gp100. Using peptide-pulsed DC, Tsai et al. (27) identified the G177 and G570 epitopes as subdominant based on primary in vitro stimulation of normal donor CD8+ T cells that were shown to specifically kill HLA-A*0201+ melanoma lines. Thus, for the gp100 Ag, there are seven known epitopes restricted to HLA-A*0201 that are potential candidates for inclusion into a subunit vaccine formulation. We chose to use Ad-transduced DC to expand gp100-reactive CD8+ T cells in vitro from patients with metastatic melanoma known to harbor gp100+ metastases. We were surprised to discover that only two epitopes, G209 and G280, are dominant. Interestingly, the G209 and G280 epitopes are among the lower affinity peptides for HLA-A*0201. Since we were unable to detect CD8+ T cells specific for the remaining higher affinity (G154, G177, G476, and G570) peptides, this suggests either deletion or perhaps anergy as the mechanism for tolerance. Furthermore, G457 has the weakest binding to HLA-A*0201 as measured in the competition assay and the T2 dissociation assay; we found no measurable G457-specific response in any of the patients studied. We conclude that, from among the panel of seven epitopes identified for HLA-A*0201, G209 and G280 represent good candidates for further study. Moreover, clinical studies in patients with metastatic melanoma provide clear evidence that both G209 and G280 are immunogenic in vivo and can elicit melanoma-reactive CTL (53).

Based on the results presented in this report, a phase I/II clinical trial utilizing Ad-transduced DC encoding gp100 and MART-1 is underway to assess the safety, immunogenicity, and efficacy in

Table I. Rank order of HLA-A*0201 restricted gp100 melanoma Ag epitopes as determined by various methods

<table>
<thead>
<tr>
<th>Epitope</th>
<th>This Study, t1/2, h</th>
<th>Tsai et al., IC50, nM</th>
<th>Parker et al., t1/2, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>G154</td>
<td>24.7 (1)</td>
<td>11 (1)</td>
<td>315 (2)</td>
</tr>
<tr>
<td>G476</td>
<td>ND</td>
<td>13 (2)</td>
<td>2194 (1)</td>
</tr>
<tr>
<td>G177</td>
<td>13.9 (2)</td>
<td>52 (3)</td>
<td>185 (4)</td>
</tr>
<tr>
<td>G570</td>
<td>11.2 (3)</td>
<td>79 (4)</td>
<td>285 (3)</td>
</tr>
<tr>
<td>G209</td>
<td>8.3 (4)</td>
<td>83 (5)</td>
<td>3.8 (6)</td>
</tr>
<tr>
<td>G280</td>
<td>7.0 (5)</td>
<td>94 (6)</td>
<td>1.4 (7)</td>
</tr>
<tr>
<td>G457</td>
<td>6.3 (6)</td>
<td>500 (7)</td>
<td>14 (5)</td>
</tr>
</tbody>
</table>

* A rank order of the seven dominant and subdominant epitopes using three different methods reveals general agreement. This study refers to the T2 cell dissociation assay which measures the t1/2 based on detection of peptide-loaded HLA-A*0201 molecules on live cells. Tsai et al. (27) refer to the competition assay based on a radiolabeled reference high-affinity standard such as the HBV core 18-27 peptide and is expressed as the IC50 of experimental peptide. Parker et al. (56) refer to the NIH computer algorithm based on the rate of β2-microglobulin dissociation from peptide-loaded/class I molecules using values that were determined experimentally for other reference peptides. ND, not determined. The number in parentheses refers to the rank of each peptide in order of decreasing affinity as determined by the indicated method.
patients with metastatic melanoma. Calculation of precursor frequencies of peptide-specific CTL should allow one to further assess the hierarchy of dominant and subdominant epitopes encoded by gp100. Several recent reports confirm the relative potency of DC as an adjuvant capable of breaking tolerance to self Ags (12, 54, 55) and provide a rationale for clinical trials with DC which utilize self nonmutated Ags such as gp100 and MART-1.

We detail the use of replication-deficient recombinant Ad to transduce human DC ex vivo. We provide evidence that gene transfer to human DC is efficient at moi 100–500 of Ad2 vector, and that transduced DC do not exhibit any phenotypic or matura
tional changes. Ad infection of human DC occurs independently of CAR, but integrins expressed by DC appear to participate in viral uptake. Tracking studies with fluorescent-labeled Ad document the rapid internalization and distribution of virus within DC. Using autologous DC transduced with Ad2/gp100v2 to stimulate and expand gp100-reactive T cells from patients with melanoma, we show that the G209 and G280 (HLA-A*0201-restricted) epitopes are dominant. We conclude that melanoma patients who harbor gp100 metastases are not tolerant to gp100 based on the detection of melanoma-reactive CTL.

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References


