Human dendritic cells adenovirally-engineered to express three defined tumor antigens promote broad adaptive and innate immunity

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Dendritic cell (DC) immunotherapy has shown a promising ability to promote anti-tumor immunity in vitro and in vivo. Many trials have tested single epitopes and single antigens to activate single T cell specificities, and often CD8+ T cells only. We previously found that determinant spreading and breadth of antitumor immunity correlates with improved clinical response. Therefore, to promote activation and expansion of polyclonal, multiple antigen-specific CD8+ T cells, as well as provide cognate help from antigen-specific CD4+ T cells, we have created an adenovirus encoding three full length melanoma tumor antigens (tyrosinase, MART-1 and MAGE-A6, "AdVTMM"). We previously showed that adenovirus (AdV)-mediated antigen engineering of human DC is superior to peptide pulsing for T cell activation, and has positive biological effects on the DC, allowing for efficient activation of not only antigen-specific CD8+ and CD4+ T cells, but also NK cells. Here we describe the cloning and testing of "AdVTMM2," an E1/E3-deleted AdV encoding the three melanoma antigens. This novel three-antigen virus expresses mRNA and protein for all antigens, and AdVTMM-transduced DC activate both CD8+ and CD4+ T cells which recognize melanoma tumor cells more efficiently than single antigen AdV. Addition of physiological levels of interferon-α (IFNα) further amplifies melanoma antigen-specific T cell activation. NK cells are also activated, and show cytotoxic activity. Vaccination with multi-antigen engineered DC may provide for superior adaptive and innate immunity and ultimately, improved antitumor responses.

Introduction

There are 70,230 new cases of invasive melanoma and 8,790 deaths from melanoma in the US estimated for 2011 (www.cancer.org). The incidence of melanoma has risen dramatically in the last several decades, 6% annually through the 1970s and it is now 3% per year. Recent clinical studies have demonstrated that immunotherapy can significantly impact this disease.[1,2] Our focus has been on immunization with well-characterized shared antigens, which we and others have found can lead to complete objective clinical responses in a minority of patients in Phase I/II clinical trials,[3-8] and which allow for careful immunological analysis of tumor responses.

DC are the key physiological stimulators of naïve and primed cells.[9-11] We demonstrated successful genetic engineering of DC with recombinant adenovirus (AdV/DC), and its superiority to traditional physical methods of transfection, such as $CaPO_4$ and lipids.[12] Tumor antigen-engineered DC are capable of processing and presenting peptide epitopes in the context of both MHC Class I and II[13-15] for at least ten days, while peptide-pulsed DC present at 50% maximal level by day 2 post-pulsing.[16] Many comparisons of exogenous peptide pulsing and tumor antigen transfection have been performed, supporting the superiority of DC transfection with full length tumor antigen genes for optimal T-cell activation.[17-20] We have found broad and potent activation of multiple CD8+ T-cell specificities by AdV/ DC[21] as well as strong type 1 cytokine production by CD4+ T cells activated by AdV/DC.[22] DC-based genetic immunotherapy strategies have been characterized in several preclinical models systems[23-30] and some have been tested clinically.[31-34]

AdV transduction also has a positive biological impact on human DC function. AdV/DC become more mature phenotypically (increased CD83, CD86, HLA-DR) and have decreased secretion of IL-10 and increased IL-12p70.[35-37] AdV transduction has also been shown to result in increased expression of IFNα,

IFNβ, IFNγ, IL-1β, TNF, IL-8, IL-15 and IL-6 by DC[37,38] as well as antigen processing machinery components TAP-1, TAP-2 and ERp57.[37] More recently, we have found that AdV/DC can secrete a number of chemokines, including CXCL8 (IL-8) and CXCL10 (IP-10), which promote NK cell migration[39] and subsequent activation of both CD56^{high} and CD56^{low}/CD16^{high} subsets of NK cells via transmembrane TNF and trans-presented IL-15.[40] In the first clinical trial in which AdV/DC were administered to melanoma patients (GM-CSF+IL-4 DC transduced with both AdVMART-1 and AdVgp100), one of 17 evaluable patients had a complete response.[32] We tested an AdVMART-1/DC vaccine in a Phase I/II trial with metastatic melanoma patients. These DC stimulated MART-1 specific CD8^{+} and CD4^{+} Type 1 T-cell responses, induced clinical responses, and also induced NK cell activation in vivo.[41]

In order to increase the immunologic strength of DC-based vaccines, we have investigated several potential improvements: (1) engineering the DC with multiple defined tumor antigens to activate more diverse CD8^{+} T-cell clones; (2) providing broad cognate CD4^{+} T cell help; (3) using a maturation cocktail for DC which functions well with AdV transduction; (4) activating innate immunity (NK cell) and (5) further boosting DC-primed immunity with IFNα. Here, we present the preclinical data supporting this novel approach for AdVTMM2/DC-based immunotherapy of melanoma.

Results

AdVTMM1: Cloning and testing. To promote broad immunity to multiple, defined antigens, we created a single adenovirus encoding three full length melanoma antigens which include multiple MHC class I and II epitopes. We initially subcloned the individual cDNAs into a single CMV-driven expression cassette, in which the three cDNAs were separated by two iterations of an IRES sequence. This configuration of three cDNAs was shown to be superior to related configurations.[42] The cassette was cloned into the AdV shuttle plasmid and sequenced to confirm the integrity of the transgenes (data not shown). This shuttle plasmid was then recombined into a virus and a seed amplification of AdVTMM1 was produced (**Fig. S1**). This seed virus was purified, titered and tested for RNA and protein production from each of the antigen cDNAs in the cassette. RT-PCR and protein gel blotting demonstrated that the expected mRNAs and proteins were produced (data not shown).

AdVTMM1 T cell responses. We tested AdVTMM1 transduced DC for the ability to activate CD8^{+} and CD4^{+} T cells specific to all three of these antigens. This was tested in blood from healthy donors. Both CD8^{+} and CD4^{+} T cells expanded to each antigen as demonstrated by recognition of targets pulsed with immunodominant HLA-A2.1-restricted or HLA-DR4-restricted peptide epitopes (**Fig. 1A; Table S1**). T cells specific to AdV hexon peptides were also detected, particularly among CD4^{+} T cells. These data indicate that expression of three different antigens in DC did not lead to interference, or dominance of any particular antigen, as no antigens were immunodominant among the healthy donors tested for responses. Importantly, these polyclonal, melanoma antigen-specific T cells also recognized antigen expressing, HLA-matched Mel526 melanoma cells. The melanoma-specific cells in AdVTMM1/DC-stimulated cultures were at a frequency higher than that achieved by single antigen vector-transduced DC (**Fig. 1B**). HLA-A2^{+}/HLA-DR4^{+} donors expanded both CD8^{+} and CD4^{+} T cells which recognized the HLA-A2^{+}/HLA-DR4^{+} melanoma cells, indicating that these Th1 CD4+ T cells also contribute to melanoma-specific cytokine production.

Systemic administration of IFNα is an approved adjuvant treatment for melanoma with pleiotropic effects on immunity. IFNα was tested specifically because of in vivo clinical data suggesting that high dose IFNα treatment may boost levels of antigen-specific T cells activated by vaccines.[43] We show that the frequency of melanoma-specific cells expanded in vitro was further augmented by including physiological levels (1,000 U/ml) of IFNα in the culture (**Fig. 1C**). Any potential effect of IFNα on the tumor cells was excluded as IFNα was only added to the AdV/DC/T cell cultures during expansion. Together, these data indicate that an AdV encoding three antigens can activate a higher frequency of melanoma-specific CD8^{+} and CD4^{+} T cells than single antigen AdV/DC. They also indicated that there was no significant competition between antigens, as specific T cells to each antigen were activated, and the presence of IFNγ/IL-2 secreting AdV hexon-specific CD4^{+} T cells did not preclude melanoma recognition. Finally, that addition of IFNα allowed for greater T cell expansion.

Instability of AdVTMM1. While testing antigen specific T cell expansion, we performed plaque purifications on AdVTMM1 to derive a stable clone for clinical translation. Individual plaques derived from AdVTMM1 no longer produced three mRNAs by RT-PCR. From viral DNA-based PCR and sequence analysis, we found that the CMV promoter and at least part of the 5' most cDNA (tyrosinase) were deleted, indicating that the genetic structure was not stable. We surmised that this was most likely due to the duplicated IRES sequences, separated by only the 350 bp MART-1 cDNA. Therefore, we recloned the virus. We subcloned the MAGE-A6 cDNA into an RSV-driven AdV E3 region shuttle plasmid, and the tyrosinase-IRES-MART-1 cassette was recloned together into the same CMV-driven Ad E1 region

Figure 1 (See opposite page). T cell activation by AdVTMM1 transduced DC. (A) AdVTMM1/DC T cell specificity. Healthy donor T cells were activated with AdVTMM1-transduced DC, expanding CD8^{+} and CD4^{+} T cells specific for each of the three encoded antigens, as tested by HLA-restricted peptides, in the IFNγ and IL-2 ELISPOT assays. (B) AdVTMM1/DC T cell recognition of antigen-expressing melanoma cells. Seven of nine individual cultures stimulated with AdVTMM/DC recognized Mel526 by IFNγ ELISPOT at a superior frequency than AdV-single antigen/DC, from three different healthy donors. Five of the seven positive cultures are shown. (C) AdVTMM1/DC^{+/-} IFNα. Five of five AdVTMM1/DC cultures testing addition of IFNα showed superior melanoma recognition by ELISPOT, compared with AdVTMM alone. Paired cultures expanded at the same time from the same donor are shown together (B and C).
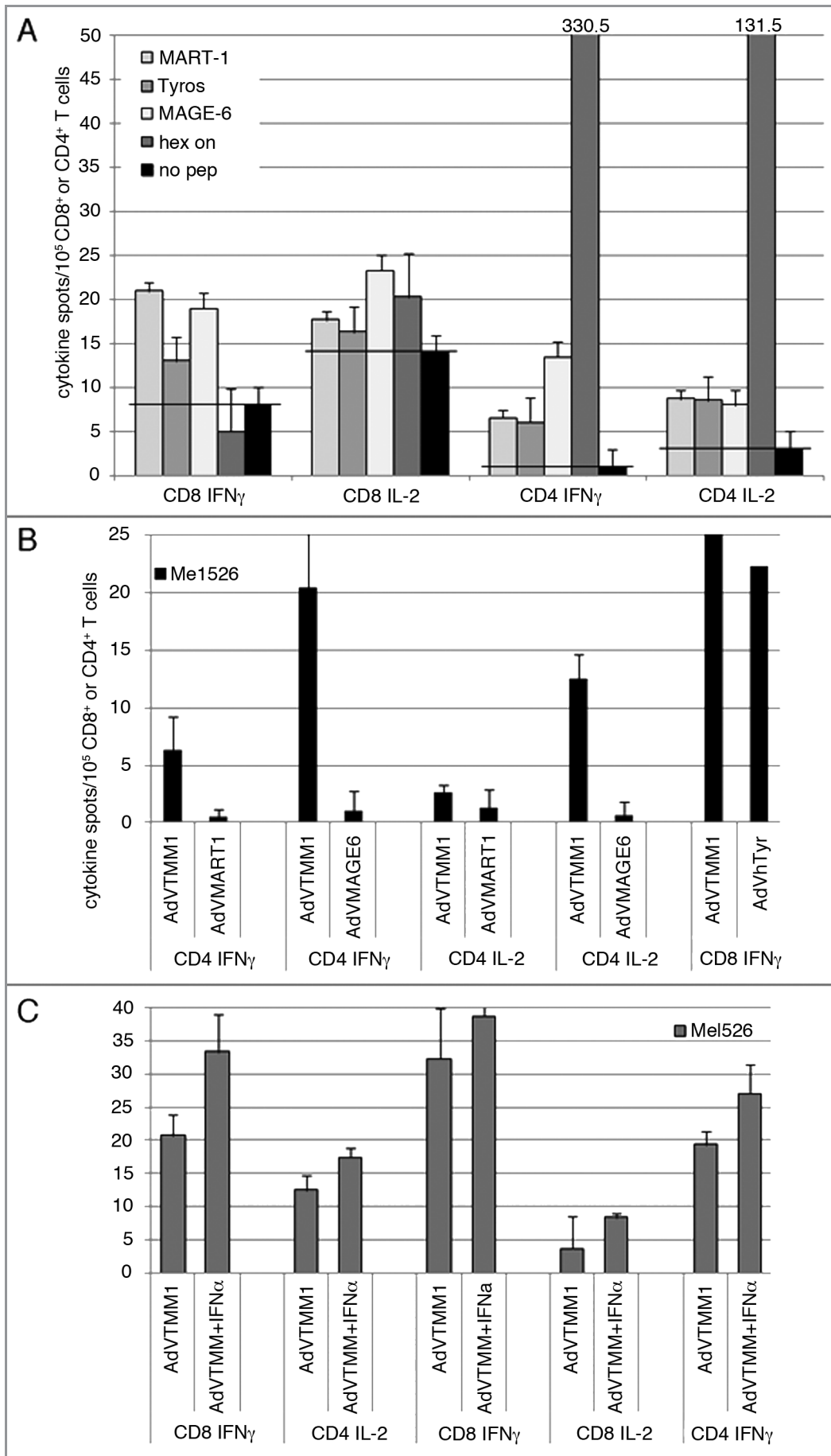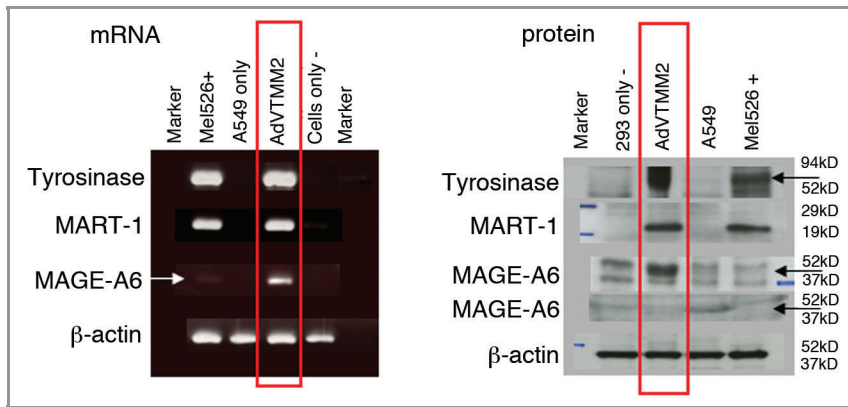
Figure 2. The AdVTMM2 cloned virus produces mRNA and protein for all three antigens. mRNA expression in AdVTMM2 was tested in 293 cells which were transduced with AdVTMM2 or left untransduced (negative control). Antigen expressing Mel526 cells served as a positive control for tyrosinase and MART-1. A549 served as a positive control for MAGE-A6. RNA was isolated and underwent RT-PCR to detect mRNA for each antigen. The same groups were tested for protein by protein gel blot, with B-actin as a loading control. Two tests with the 5B57 antibody for MAGE-A6 are shown.

shuttle plasmid (**Fig. S1**), and these were recombined sequentially to create AdVTMM2.

AdVTMM2 expression analysis. AdVTMM2 was tested for both mRNA by RT-PCR and protein expression by protein gel blot for all three antigens at multiple stages: initial lysate (**Fig. S2A**), each of two rounds of plaque purification, and subsequent large scale amplification and purification (**Fig. 2**). MAGE-A6 was the most difficult to clearly detect, despite using five different antibody clones. This may be due in part to cross-reactivity among the large and highly homologous MAGE-A gene family members.[44] The three antigen proteins are expressed at different levels in 293 cells, with tyrosinase at 1.2–3.1-fold higher than MAGE-A6, and MART-1 1.1 to 2.1-fold higher than MAGE-A6. This is expected, due to the two different promoters used (CMV and RSV) and relative positions of the two genes in the bicistronic cassette (tyrosinase 5' to MART-1), where the 5' gene is often more efficiently translated.[45] In DC, the transgene proteins can be detected by intracellular staining, with expression levels increasing with increased MOI (**Fig. S3**).

AdVTMM2 T cell responses. We next stimulated T cells with human DC transduced with AdVTMM2, to determine whether all three antigen specificities of both CD4+ and CD8+ T cells could be activated by this new viral clone. IFNγ and IL-2 producing CD8+ and CD4+ T cells specific to peptides derived from all three antigens were expanded, indicating successful T-cell activation (**Fig. 3A and B**). For CD4+ T cell response determination, for the donors who are not HLA-DR4+, we determine the antigen specificity by using single antigen AdV-transduced targets. These are compared with control AdVLacZ-transduced targets, (the difference representing the antigen-specific response) and untransduced targets (the large difference representing the antigen-specific response plus the AdV virus-specific response). An example of such data are shown in **Figure 3C**, in which CD4+ T cells were expanded to each of the encoded antigens. As with AdVTMM1, with AdVTMM2 we have not observed any evidence for antigen immunodominance, despite the relative differences in protein expression level in transduced cells. Among the four donors tested with AdVTMM2 in vitro, we successfully expanded CD8+ or CD4+ T cell responses to all three antigens in all 4 donors. CD8+ and CD4+ T cells producing IFNγ were most common, followed by IL-2-producing CD4+ T cells. Multiplex analysis of cell culture supernatants identified CXCL10 (IP-10)

and CXCL8 (IL-8) as the highest concentration analytes. We detected lesser amounts of IFNγ and low levels of IL-10, while IL-12p70, IFNα and VEGF were below detection (data not shown).

Pre-maturation of AdVTMM2/DC. Fully mature DC have been demonstrated to have superior T cell activation properties, which is due in part to increased costimulation, increased IL-12p70 production, and greater stability. We and others found that AdV transduction alone partially matures DC, and previously investigated several maturation cocktails after AdV transduction.[35,36] While DC phenotype indicated increased maturation, the T cell stimulatory activity was not improved over AdV transduction alone. We subsequently tested IFNγ + LPS as a cocktail before and after AdVLacZ transduction[37] and observed very different DC phenotype and cytokine and chemokine production profiles which were dependent upon the timing of maturation relative to AdV transduction. Here, we compared AdVTMM2/DC with IFNγ + LPS pre-matured AdVTMM2 for the frequency of antigen-specific T cell activation. Pre-maturation proved to be a more reliable stimulus for expansion of antigen-specific T cells to all three antigens, and resulted in at least similar or often increased frequency of those T cells by IFNγ and IL-2 ELISPOT (**Fig. 4**). The pre-matured AdVTMM2/DC also show increased levels of CD80, CD86, CD83 and CCR7, as well as improved viability compared with AdVTMM2/DC (data not shown).

NK cell activation. We have recently found that AdVLacZ/DC activate both major subsets of NK cells via transmembrane TNF and trans-presented IL-15.[40] AdV/DC also secrete a number of chemokines, including CXCL8 and CXCL10, which are chemotactic in vitro and in vivo for NK cells.[39] In addition, circulating CD69+ and CD25+ NK cells were identified in melanoma patients immunized with AdVMART-1/DC suggesting an enhanced level of NK cell activation.[41] These NK-DC interactions may prove critical to shape the adaptive immune response. In order to confirm that this effect is not transgene-dependant, we tested NK cells isolated from AdVTMM1/DC and AdVTMM2/DC cultures for activation. We found that these cultures contained activated CD25+/CD69+ NK cells, both CD56hi/CD16- and CD56+/CD16+ subsets. These activated NK cells were capable of killing the LAK target Daudi cells (**Fig. 5**), compared with the NK cells from the unstimulated PBMC. Therefore, the AdVTMM/DC activate NK cells, as expected.
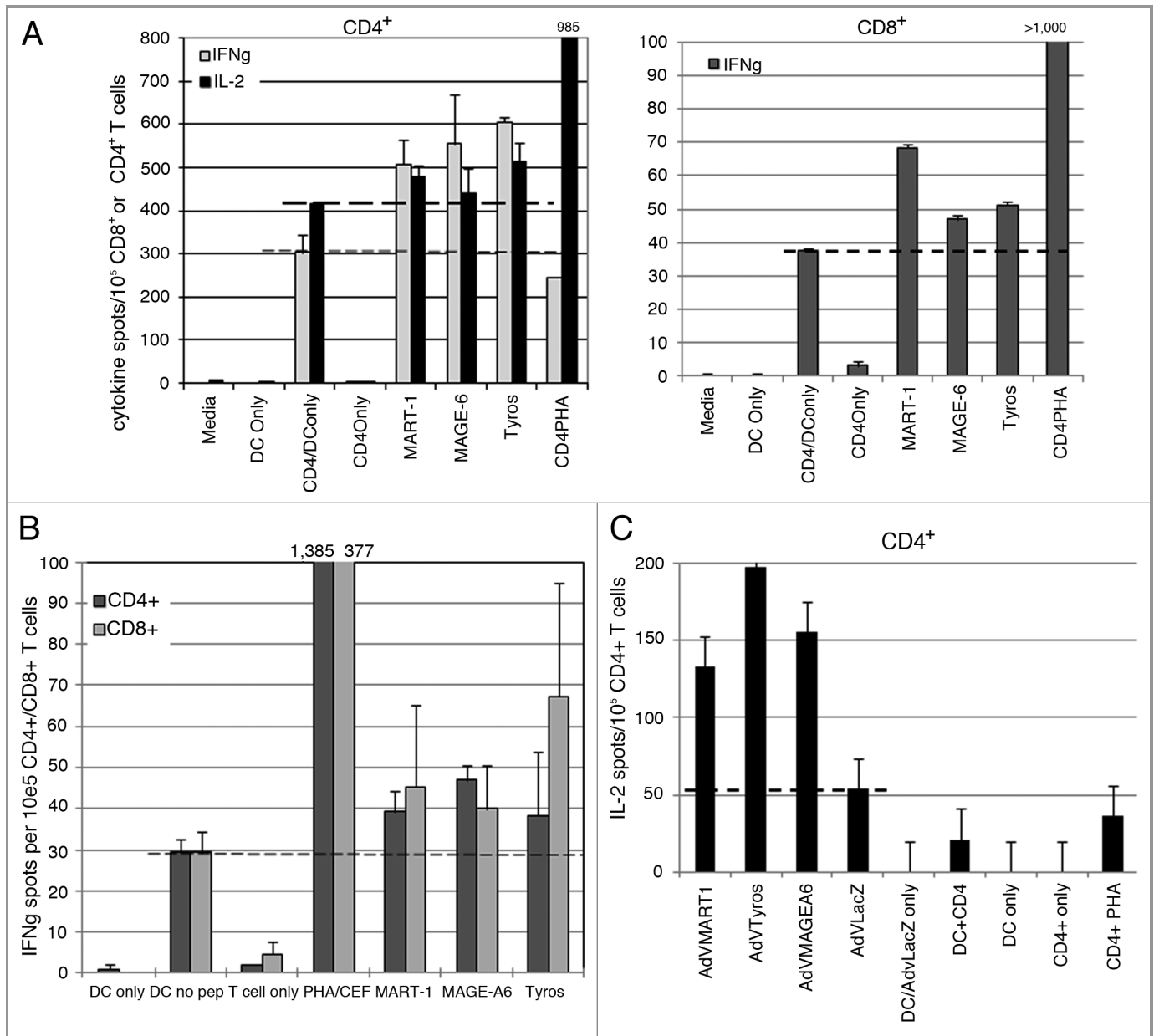
Figure 3. AdVTMM2 transduced DC activate CD8+ and CD4+ T cells specific to all three antigens. Antigen specificity was tested with T cells from four different healthy donors which were activated with AdVTMM2-transduced DC. The cultures expanded CD8+ and CD4+ T cells specific for each of the three encoded antigens, as tested by HLA-A2 and DR4-restricted peptides (**Table S1**), in IFNγ and IL-2 ELISPOTS (A and B) in five different experiments. In the case of HLA-DR4-negative donors, total CD4+ T cell responses to each antigen were determined by testing recognition of autologous DC transduced with single antigen AdV and control AdVLacZ (C).

In summary, these data indicate that the structure of AdVTMM2 is stable, that this virus directs expression of the three encoded antigens, and that DC transduced with three full length antigens can activate and expand CD8+ and CD4+ T cells which produce IFNγ and IL-2 upon recognition of all three antigens. These polyclonal, multiple antigen-specific T cells recognize antigen-expressing melanoma cells in a manner superior to single antigen engineered DC. These effects are further amplified by IFNα at physiologically achievable levels. These data also support translation of this strategy to a clinical trial in which

melanoma patients are vaccinated with IFNγ/LPS pre-matured AdVTMM2/DC to promote adaptive antigen-specific CD8+ and CD4+ T cell responses as well as innate NK cell activation, in conjunction with high dose IFNα to further amplify these effects.

Discussion

In this report, we describe the preclinical development of an adenovirus expressing three full length melanoma tumor associated antigens and its ability to stimulate multiple antigen-specific, type 1
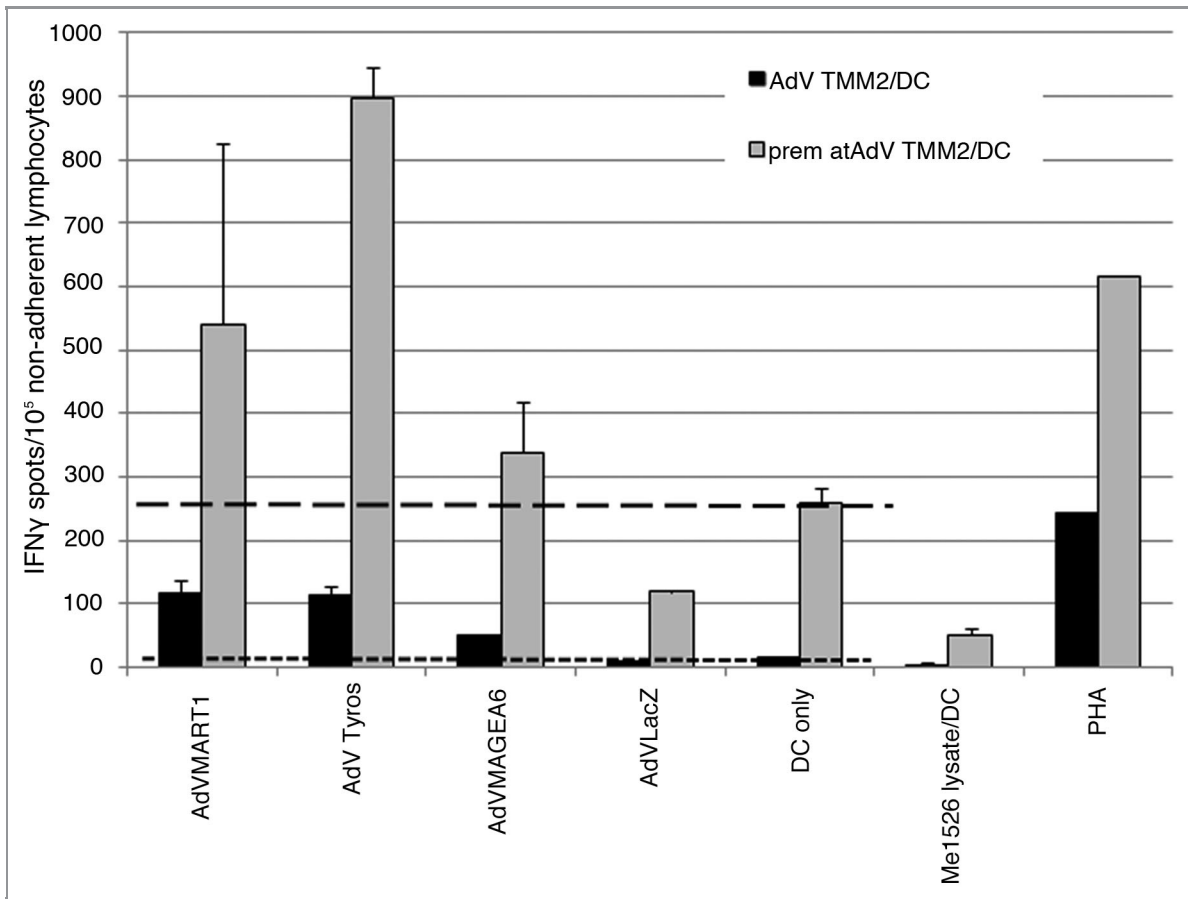
Figure 4. Maturation of DC prior to AdVTMM2 transduction enhances immunogenicity. AdVTMM2/DC stimulated cultures were tested with and without DC pretreatment with IFNγ + LPS. After three weekly stimulations, the total frequency of antigen-specific lymphocytes (without T cell subset purification) producing IFNγ was tested in two donors. Reactivity to autologous DC loaded with Mel526 lysate was also compared.
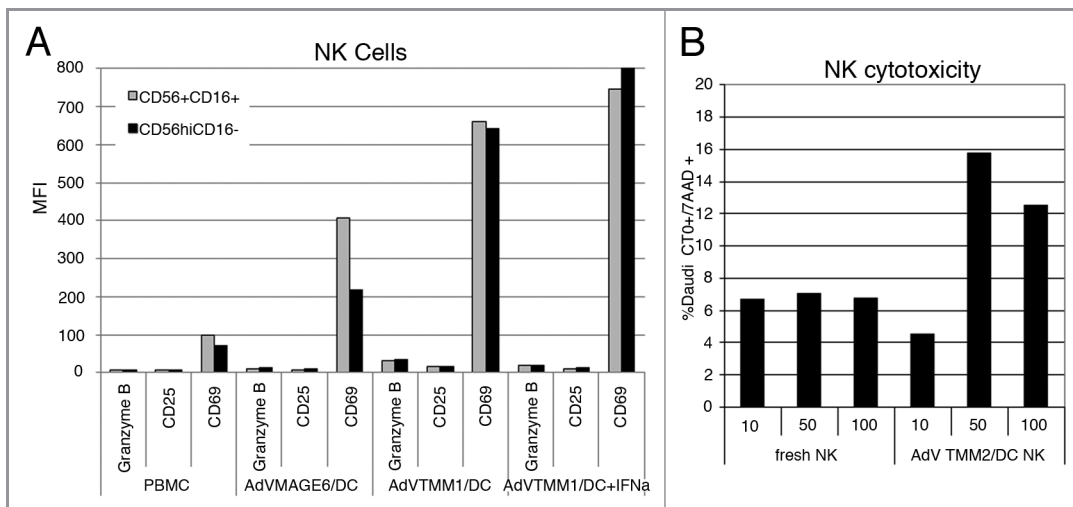
Figure 5. AdVTMM/DC activate NK cells. Phenotype and cytotoxicity of NK cells from two different donors was tested by flow cytometry. (A) CD56^{hi}/CD16^{-} and CD56^{+}/CD16^{+} NK cells from unstimulated PBMC and from cultures stimulated with AdVMAGE A-6/DC, AdVTMM1/DC and AdVTMM1/DC + IFNα were stained for surface CD25 and CD69 and intracellularly stained for granzyme B content. AdVTMM2/DC cultures also contained > 95% CD69^{+} NK cells (not shown). (B) The ability to upregulate 7-AAD on the LAK target Daudi (labeled with CTO) was compared between unstimulated NK cells and NK cells isolated from AdVTMM2/DC cultures. Three different ratios of NK cells to Daudi targets were tested (10:1, 50:1 and 100:1).

T cells, as well as activate NK cells when used to transduce DC. The rationale for development of this virus is based on the immunological responses of our previous melanoma immunotherapy clinical trials in which immunization with a single MART-1$_{27–35}$ peptide on DC[4,7] or an AdV encoding one full length (118aa) antigen (MART-1)[41] all led to successful immunization of patients, with activation and expansion of MART-1 specific T cells. However, we observed that clinical response and improved survival were correlated with determinant spreading and antigenic breadth, indicating that immune responses to multiple antigens is critical. This may relate to the importance of in vivo cross-presentation after tumor lysis or to overcoming antigen loss variants. Therefore, in order to induce antigenically broad immune responses from the vaccine administered, we created a virus encoding these three different antigens.

The antigens included in the AdV were chosen based on our experience with MART-1 and data in the literature suggesting immunogenicity and ability to induce clinical response. The MART-1 protein has been hypothesized to be localized to the melanosome.[46,47] For HLA-A2.1 allele, the immunodominant peptide (MART-1$_{27–35}$) has been identified, although other peptides, including MART-1$_{53–62}$ may be processed and presented.[48] The immunodominant MART-1$_{27–35}$ peptide has been shown to bind other subtypes of the A2 superfamily (A2.2, 2.4, 2.5, 2.6, 2.9 and A69.1) with similar affinity to that of HLA-A2.[49] The MHC class II-restricted DR4 epitope was also described.[50] This antigen has been associated with the induction of vitiligo, a positive prognostic factor for patients with melanoma.[4,33,51-54]

Tyrosinase is also a melanoma lineage antigen, and is part of the melanin biosynthesis pathway. It is expressed by 70% of melanoma cell lines and almost all melanoma tumors.[55] It is recognized by TIL infiltrating melanoma lesions and associated with clinical response.[56] It is also associated with human vitiligo[52,53] although that is controversial.[57,58] MAGE-A6 is a tumor-specific "cancer-testes" antigen, part of the large family of MAGE genes. MAGE-A6 T cells were found to be expanded in a regressing tumor lesion, and stably expressed in patient tumor.[59] Work from the Storkus group found that Type 1 CD4$^+$ T-cell responses (IFNγ) to this antigen are associated with lower tumor burden while Type 2 (IL-5) T-cell responses are associated with high tumor burden.[60] MAGE-A genes may also play a tumor-promoting function.[61] Lineage antigens MART-1 and tyrosinase are expressed in >93% of tumors regardless of stage, and several MAGE-A family members increased expression with increased tumor stage. These data support targeting these widely expressed shared antigens and reduces concern that they may be commonly lost with progression.

There are other strategies for induction of multiple antigen-specific responses. One is with synthetic peptides (which are commonly used and inexpensive), but peptide binding affinity and binding half-life are a concern, as well as restricting trial enrollment to patients whose HLA alleles correspond to defined epitopes. This is a particular concern with MHC class II alleles which are more polymorphic and have fewer epitopes defined. Another is loading DC with tumor-derived DNA, mRNA or protein, however, the uncharacterized nature of these approaches makes immune monitoring for vaccine-induced responses and those acquired spontaneously via cross-presentation and subsequent determinant spreading impossible to detect. Our construct did express the antigens at different levels. This could potentially impact T cell responses resulting from DC transduction with this virus. There are many reports indicating that antigen must be present at a sufficiently high level for processing and presentation of peptide epitopes at a level sufficient for T-cell activation, but not at such a high level that responses are inhibited, or develop in a tolerogenic or regulatory way.[62,63] Due to the lack of immunodominance, Type 1 immunity and minimal IL-10 we observed, the antigen expression levels from AdVTMM2 are within acceptable limits. One could also use three single antigen AdV vectors for DC transduction, but the enormous cost of GMP production and testing of each vector for clinical use is prohibitive.

Cancer vaccines may benefit from being combined with other immune stimulants. IFNα has pleiotropic effects on many hematopoietic and tumor cell types. We hypothesize that systemic delivery of IFNα may act to prolong the expansion of CD8$^+$ T cells activated by the vaccine,[64] as well as promote cross-priming.[65,66] Our in vitro data (**Fig. 1C**) supports this positive effect. There is only one report of administration of IFNα following genetic vaccination regimen,[43] in which four of seven patients demonstrated increased CD8$^+$ T cell responses (tetramer) specific for the previous gp100 vaccine, and two patients with active measurable tumor exhibited objective antitumor responses. We hypothesize that patients receiving systemic IFNα after vaccination will have a significant boost in vaccine-stimulated T cell responses compared with directly post-DC vaccinations.

NK cells have been demonstrated to play a critical role in immune surveillance of cancer,[67] and their ability to cross-talk with DC is well described.[68,69] We recently showed that AdV/DC secrete CXCL8 and CXCL10, which recruit both major NK cell subsets toward the AdV/DC,[39] and, that once in contact, the AdV/DC activate NK cell Th1 functions via transmembrane TNF and transpresented IL-15.[40] These early, innate immune activation events may be of critical importance in shaping a strong Th1/Tc1 adaptive immune response. Here, we confirmed that, like AdVLacZ/DC, AdVTMM/DC are also capable of NK cell activation and promotion of lytic function.

In summary, our data support clinical testing of AdVTMM2/DC for broad activation of polyclonal, Type 1 CD8$^+$ and CD4$^+$ T cells, as well as NK cells, which, particularly when "boosted" by systemic IFNα, may promote strong melanoma antitumor immunity in vivo. This study is now planned as part of the Melanoma and Skin Cancer Program at the University of Pittsburgh Cancer Institute. The use of such defined antigens allows immunologic monitoring to detect spontaneous immunity, vaccine-stimulated immunity and determinant spreading to other defined antigens (like gp100 or NY-ESO-1), which will shed light on the immunologic mechanism of clinical response.

Materials and Methods

Cell lines. The human CML cell line K562 stably transfected with HLA-A*0201 (K562/A2.1) was generously provided by

Wolfgang Herr (Mainz, Germany) and the HLA-A2⁺/AFP-expressing HCC cell line HepG2 was obtained from ATCC (HB-8065). Mel526 is a human melanoma cell line which is HLA-A2⁺/HLA-DR4⁺ and which expresses MART-1/Melan-A, tyrosinase and MAGE-A6. NK and LAK activities were tested against K562 erythroleukemia and DAUDI lymphoma cell lines (ATCC, CCL-243, CCL-213), respectively. The cell lines were cultured in Advanced RPMI 1640 medium (12633-012) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (15140-163) and 1% L-glutamine (25030164, all reagents from Invitrogen), in a humidified 37°C incubator under 5% CO_2 tension.

Antibodies and flow cytometry. DC phenotype and maturation were examined using fluorochrome-conjugated antibodies against the following cell surface markers: CD80, CD83 (BD PharMingen), HLA-A,B,C (BioLegend), HLA-DR and CD86 (Beckman Coulter). NK cell phenotype and activation were investigated using: CD8⁺, CD16, CD3 (Beckman Coulter), CD56 and CD69 (BD PharMingen). Transgene expression was confirmed by protein gel blot using several different primary antibodies to MAGE-A6 [ab38495, Abcam; sc33233, sc33234 and sc20034, Santa Cruz; 5B57 (a gift from W. Storkus)], Tyrosinase (ab55902) and MART-1 (ab3168) (Abcam), and secondary antibodies (ab672, SC-2314 from Abcam and Santa Cruz Biotechnology, respectively). Intracellular staining for antigen expression in DC was performed using primary antibodies to MAGE-A6 (Santa Cruz, 20034), Tyrosinase (Santa Cruz, 20035) and MART-1 (Santa Cruz, 20032) and PE-labeled secondary antibody (Dako, R0480). Briefly, transduced cells were fixed for 20 min. in 4% w/v paraformaldehyde/PBS, washed in FACS buffer (PBS/0.2% BSA/0.02% NaN3), blocked for 30 min. with goat serum, and permeabilized (eBioscience, 00–8333) according to manufacturer's instructions. Cells were analyzed using a CyAn flow cytometer (Dako) and Summitt v.4.3 software.

Peptides. Peptides were synthesized at the University of Pittsburgh Peptide Synthesis Facility using standard f-moc technology. Stock solutions were prepared in DMSO (10 mg/mL) and were kept at -80°C until use. The following peptides were used (also shown in **Table S1**): HLA-A2-restricted: MART-1$_{27-35}$ AAGIGILTV, Tyrosinase$_{1-9}$ MLLAVLYCL, MAGE-A6$_{271-279}$ FLWGPRALV and AdV hexon$_{711-721}$ TFYLNHTFKK. HLA-DR4-restricted: MART-1$_{51-73}$ RNGYRALMDKSLHVGTQCALTRR, Tyrosinase$_{448-462}$ DYSYLQDSDPDSFQD, MAGE-A6$_{121-144}$ LLKYRAREPVTKAEMLGSVVGNWQ and MAGE-A6$_{246-263}$ LTQYFVQENYLEYRQVPG and the AdV promiscuous hexon peptides: $_{571-600}$ NLLLLPGSYTYEWNFRKDVNMVLQSSLGND, $_{856-885}$ VDSITQKKFLCDRTLWRIPFSSNFMSMGAL and $_{901-930}$ LDMTFEVDPMDEPTLLYV LFEVFDVVRVHR.

Cloning of AdVTMM1 and AdVTMM2 and sequencing. AdVTMM1 is an Ad5 E1/E3 deleted adenovirus with the cDNAs for tyrosinase, MART-1 and MAGE-A6 cloned into a single expression cassette, with two copies of an Internal Ribosome Entry Site (IRES) sequence, driven by the CMV promoter and ending in the SV40 poly A sequence, subcloned into the E1

region. The IRES sequence, obtained from pIRES (Clontech Laboratories, Inc., 631605), is derived from the encephalomyocarditis virus (ECMV).

The adenoviral vector "AdVTMM2" is driven by a single CMV promoter and a single RSV promoter, contains the same three cDNAs (Tyrosinase, MART-1, and MAGE-A6). These cDNAs are in two expression cassettes, one CMV-driven, with Tyrosinase and MART-1 joined by an IRES. The second cassette is an RSV-driven MAGE-A6 cDNA.

The shuttle vector construct was built in several sequential subcloning steps. First, the Tyrosinase coding sequence was subcloned from pEF6huTyros (a gift from V.H. Engelhard, University of Virginia), into the pShuttle2 vector (Clontech Laboratories, Inc., 631513). The sequence of this cDNA was confirmed to match that of human Tyrosinase, GenBank accession #BC027179. The MART-1 cDNA was then sub-cloned from pRcCMV-MART-1[29] into pIRES. The MART-1 cDNA was confirmed to match that of human MART-1, GenBank accession #NM_005511. The IRES-MART-1 fragment was then ligated into pShuttleHuTyros. The Tyrosinase-IRES-MART-1 cassette was then subcloned into the Viraquest shuttle plasmid VQAd5CMV. Lastly, the MAGE-A6 cDNA was amplified from pBAD-Topo-MAGE-A6 (a gift from W.J. Storkus, University of Pittsburgh[70] by PCR). The cDNA was amplified using gene-specific primers with added SalI and NotI sites to facilitate subcloning into pIRES. The MAGE-A6 cDNA was confirmed to be human MAGE-A6, GenBank accession #BC067731. For AdVTMM1, The 1639 bp fragment XhoI-IRES-MAGE6-NotI was ligated into pShuttle2huTyr-IRES-MART1. This cassette was subcloned into the Viraquest shuttle plasmid VQAd5CMV, and recombined into an intial virus lysate, which underwent mRNA and protein expression testing.

For AdVTMM2, the fragment XhoI-IRES-MAGE6-NotI was ligated into VQAd5RSV shuttle plasmid. The VQAd5RSV-MAGEA6 shuttle plasmid and the VQAd5CMV-Tyros.-IRES-MART-1 shuttle plasmid were sent to Viraquest for sequential recombination into the AdV. A lysate was sent to the Butterfield lab to confirm mRNA and protein expression of all three melanoma antigens. After this mRNA and protein expression confirmation, this initial lysate construct was sequenced to confirm the accuracy of the insert sequences. The lysate was then plaque purified, and all tested clones expressed all three mRNAs (RT-PCR). One plaque was amplified and underwent a second round of plaque purification, and tested second-round plaques again expressed mRNA for all three antigens. Protein expression from two plaques was tested, and one clone was selected for amplification, purification, titering and further testing.

Virus amplification, purification and titer. Infected 293 cells (ATCC, CRL-1573) are freeze/thawed and the cleared supernatant is layered onto a CsCl step gradient (1.2 g/mL/1.4 g/mL) and centrifuged at 35,000 rpm 1.5 h. The virus band is dialyzed (Pierce Slide-a-lyzer 10K, Tris/MgCl2/glycerol), and concentrated (Millipore, UFC205024PL) in formulation buffer (2.5% glycerol (w/v), 25 mM NaCl and 20 mM TRIS-HCl, pH 8.0; Clontech), aliquoted and titered (Adeno-X Rapid Titer Kit, Clontech, TakaraBio, 632250).

RT-PCR. Total RNA was isolated (Qiagen Kit.; 74104, Qiagen) and quantified by UV spectrophotometry. RT-PCR was performed using Random Primers, 11034731001 and Superscript™ III Reverse Transcriptase, 18080–044, Ampli Taq Gold polymerase, EN-387. Sequenced-specific primers were as follows:

Tyrosinase, Forward: 5'-ATT-CCA-TAT-TGG-GAC-TGG-CGG-GAT-3'/Tyrosinase

Reverse: 5'-CAA-TGG-GTG-CAT-TGG-CTT-CTG-GAT-3'

MART-1, Forward: 5' – TGC-AGA-TAT-CCA-TCA-CAC-TGG- 3'

MART-1, Reverse: 5' – GGA-GGG-GCA-AAC-AAC-AGA T – 3'

MAGE-A6, Forward: 5'- AGT-AGG-AAG-GTG-GCC-AAG-TTG-GTT-3'

MAGE-A6, Reverse: 5'- TAT-TGG-GTG-AGC-TTC-TTG-GGA-3'

β-Actin, Forward: 5'-GGC-ATC-GTG-ATG-GAC-TCC-G-3' Reverse: 5'-GCT-GGA-AGG-TGG-ACA-GCG-A-3'

All primers were prepared by Integrated DNA Technologies, Inc. PCR reactions for β-Actin, Tyrosinase and MART-1 were 35 cycles, and for MAGE-A6, 37 cycles were used.

Protein gel blotting. Proteins were isolated in lysate buffer and samples containing equal amounts of protein were boiled in the loading buffer and separated by SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 h at RT, and incubated the primary antibodies at the optimal concentrations overnight at 4°C. After 3 washes in phosphate-buffered saline with 0.2% Tween 20, a horseradish peroxidase-conjugated secondary antibody was applied, and the blot was developed with enhanced chemical reagents (Amersham Biosciences). Quantitation was performed by scanning protein gel blot films (Epson Perfection 4490 Photo) and analyzing the band intensity (Image J 1.44 P). The intensity for each transgene was normalized to the β-Actin control for that sample. The data reported is averaged from five different protein gel blot experiments.

Isolation of peripheral blood mononuclear cells (PBMC). Peripheral blood was obtained from six different HLA-A2⁺/HLA-DR4$^{+/-}$ normal donors with their written consent, under an IRB-approved protocol (UPCI 04-001). PBMC were separated from blood using Ficoll-Hypaque gradient centrifugation (Cellgro; Mediatech, Inc.). Buffy coats from healthy donors were obtained from the Central Blood Bank (Pittsburgh, PA).

Generation of dendritic cells (DC) and DC culture. DC were prepared as described[12] with some modifications. Mononuclear cells (7–8 × 10⁷) were plated in T-75 flasks (Costar, CLS430641) in RPMI 1640/PSF/5–10% human AB serum 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 GM-CSF (Sargramostim; Amgen) and 500 U/mL IL-4 (Schering Plough) for 6–7 d. The DC were harvested by vigorous washing.

Selection of CD8⁺ and CD4⁺ lymphocytes. Before assays, the CD8⁺ (130-045-201) and CD4⁺ T cells (130-096-533) were sequentially isolated by positive and negative MACS, respectively with the use of immunomagnetic beads following the manufacturer's instructions (Miltenyi Biotech). Resulting cell populations were > 95% CD8⁺-positive according to FACS analysis.

CTL Generation from Peptide-pulsed and AdV-transduced DC. Peptide-specific CTL were generated as follows:[13,29] DC were transduced with AdV at a multiplicity of infection of 1000 for 2 h at 37°C in serum-free AIM V. The DC were centrifuged and counted, then plated in wells (1–5 × 10⁵ DC/well) of a 24-well plate with autologous non-adherent PBMC in 5–10% AB serum/RPMI/antibiotics with 10 ng/mL IL-7 (Biosource, PHC0076) for 1 week, and supplemented with IL-2 (Hoffman-La Roche) at 30 U/mL every 2–4 d. After one week culture, the nonadherent cells were counted and restimulated with fresh transduced DC. After two restimulations, the CTL were harvested and the CD8⁺ and CD4⁺ cells were isolated and tested.

ELISPOT assays. Multiscreen HA plates (Millipore, MAHAS4510) were coated with 4–10 ug/mL of monoclonal Ab anti-human IFNγ (1-D1K, MabTech, 3420-3) or anti-IL-2 (MabTech IL2-1, 3440-2), in PBS overnight at 4°C. After blocking the plates with PBS/10% FBS or RPMI/10% AB (2 h, RT), CD8⁺ or CD4⁺ T cells were plated at 10⁵ cells/well in duplicate wells. K562/A2.1 cells (0.5–1 × 10⁵ well) were pulsed with different peptides, rinsed and plated. Control wells contained T cells (alone or with 10–100 ug/mL PHA), T cells with unloaded APC ("no peptide") or negative antigen pulsed APC. Cells were removed and captured cytokine was detected by biotinylated mAb anti-hIFNγ or anti-IL-2 (MabTech, 3420-6, 3440-6) at 2 µg/mL in PBS/0.5% BSA. After washing, Avidin Peroxidase Complex (1/100; Vectastain Elite Kit, PK-6100) was added, and peroxidase staining was performed with 3-amino-9-ethyl-carbazole (AEC, Sigma, AEC101) and stopped by rinsing the plates under tap water. Spot numbers were automatically determined with the ImmunoSpot imaging system from Cellular Technology, Ltd. To calculate the number of responding T cells, the mean numbers of spots with APC alone were subtracted from mean spot numbers induced by antigen-loaded APC.

Cell tracker orange (CTO)-based flow cytometry cytotoxicity assay. The assay was performed as previously described with some modification.[40] Briefly, Daudi (target cells) were labeled with CTO. CTO⁺DC and NK (effector) cells were co-incubated at various ratios at 37°C for 3 h. Cells were stained with 7-AAD and cytotoxicity was quantified by flow cytometry. The percentages of cytotoxic activity was calculated using the following equation: % specific cell death = (% 7-AAD⁺CTO⁺Daudi⁺NK cells - % spontaneous 7-AAD⁺CTO⁺Daudi)/(100-% spontaneous 7-AAD⁺CTO⁺Daudi) × 100%.

Supplemental Material

Supplemental materials may be found here: http://www.landesbioscience.com/journals/oncoimmunology/article/18628/

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