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Research Paper

Expression of Melanoma-Associated Antigens in Human Dendritic Cells Pulsed with an Interleukin-2 Gene Encoded Vaccinia Melanoma Oncolysate (rIL-2VMO)

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KEY WORDS

dendritic cells (DCs), melanoma-associated antigens (MAAs), vaccinia melanoma oncolysate (VMO) vaccine, recombinant vaccinia virus expressing the human interleukin-2 insert (rIL-2VV), active specific immunotherapy (ASI)

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ABSTRACT

Dendritic cells (DCs) possess the unique abilities to initiate a primary immune response and to present antigens to naïve T lymphocytes. Recently, there has been a rapidly growing interest in the use of DCs in active specific immunotherapy (ASI) for the treatment of patients with cancer. In the present study, we determined the ability of DCs to express Melanoma-Associated Antigens (MAAs) from a polyvalent Melanoma Vaccine (DC-MelVac; Patent #11221/5) developed in our facility. The vaccine consists of a recombinant IL-2 gene-encoded vaccinia melanoma oncolysate (rIL-2VMO) derived from an established human melanoma cell line. Our results show that r-IL2VMO-pulsed DCs express MAAs presented by the Mel-2 melanoma cell line oncolysate used in this study. We believe that these promising results will prove useful as an active specific immunotherapeutic agent for patients with Stage III melanoma.

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INTRODUCTION

Dendritic cells (DCs), known as the most potent of all antigen-presenting cells, have been shown to profoundly affect both the quality and intensity of the immune response. Following their stimulation by antigens, DCs undergo a process of maturation in which these cells lose their antigen-capturing capabilities and migrate to secondary lymphoid areas where they present antigens to T cells and secrete cytokines such as IL-12 and IFN-α.[1,2] Mature DCs possess a characteristic cell morphology in which large numbers of membrane folds, commonly known as dendrites, bend, retract, and reextend in an effort to capture antigen.[3] The ability of mature DCs to effectively initiate active specific immunity has made them attractive as potential adjuvants in the treatment of certain immunogenic malignancies, such as melanoma,[4] and renal cell carcinoma.[5]

Our study focuses on the use of DCs and immunotherapy in the treatment of melanoma. The refractory nature of melanoma to adjuvant therapy has launched a search for novel therapeutic options. Currently there are approximately 27 ongoing studies, listed with the FDA, evaluating the clinical efficacy of DC-related immunotherapy for the treatment of melanoma. In this study, we isolated human peripheral blood mononuclear cells (PBMCs), cultured immature DCs by growing these cells in media containing GM-CSF and IL-4, and pulsed these with a polyvalent, recombinant IL-2 gene-encoded vaccinia melanoma oncloysate (rIL-2VMO) preparation. The oncolysate used was composed of nine melanoma-associated antigens (MAAs) from one of our laboratory's unique cell lines. We assessed the DC maturation process and more importantly determined the antigen presentation capability of these cells with the possibility of using these rIL-2VMO-pulsed DCs as a component of a melanoma-based vaccine, which has been approved by the FDA for use in Phase I human clinical trials.

MATERIALS AND METHODS

Blood sample. The test sample was acquired from the Saint Vincent's Hospital —New York Blood Bank—Department of Laboratories. The blood sample (500cc) was acquired from a healthy donor and placed in BD Vacutainer ACD Solution A tubes (Becton-Dickinson, Franklin Lakes, NJ).

Peripheral blood mononuclear cells (PBMCs) isolation. Whole blood was mixed with equal volumes of Hanks Balanced Salt Solution (1X), HBSS (Invitrogen, Grand Island, NY) and placed in 50 ml conical tubes containing 30 ml of the blood-HBSS mixture. Ten ml of filtered Ficoll Plaque™ Plus (Amersham Biosciences, Piscataway, NJ) was pipetted at the

bottom of each conical tube below the blood level and centrifuged at 1600 rpm for 30 minutes. The buffycoat, containing the PBMC's from each tube, was collected and pooled together in a new conical tube. The resuspended cells were mixed with equal volumes of HBSS and centrifuged again at 1,400 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in incomplete X-VIVO 15™ media (Biowhittaker™ Cambrex Bio Science, Walkersville, MD) and centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended once more in incomplete X-VIVO media.

Trypan blue exclusion testing/viability testing of isolated PBMCs. Standard Trypan Blue exclusion testing/viability testing was performed on 20 µl of the sample. The unstained (live/viable) and stained (dead/non-viable) cells were both counted to determine viability.

In vitro generation of DCs. The isolated PBMC's were placed in 0.2 µl Vented Blue Plug Seal Cap Tissue Culture Treated BD Falcon flasks (Becton-Dickinson, Franklin Lakes, NJ) and incubated at 37°C + 5% CO_2 for 2 hours. After 2 hours, the floating cells were aspirated and centrifuged at 1,200 rpm for 5 minutes. These nonadherent cells were then counted and resuspended in complete X-VIVO media containing 10% Dimethyl Sulfoxide, DMSO (American Type Culture Collection, Manassas, VA) and stored in liquid nitrogen for future use. The cells that remained adherent to the flasks after 2 hours of incubation were gently washed 7 times with HBSS and then replenished with complete X-VIVO media composed of: 1µg/ml of IL-4 (PeproTech Inc., Rocky Hill, NJ) + 500 µg/ml GM-CSF (Sargramostim Leukine Liquid, Berlex, Montville, NJ) + 2 µg/ml Gentamycin solution (Invitrogen, Carlsbad, CA). These cells were incubated at 37°C + 5% CO_2 for 7 days. During days 2–3, 20 ml of complete X-VIVO media was added to the flasks to enhance DC proliferation.

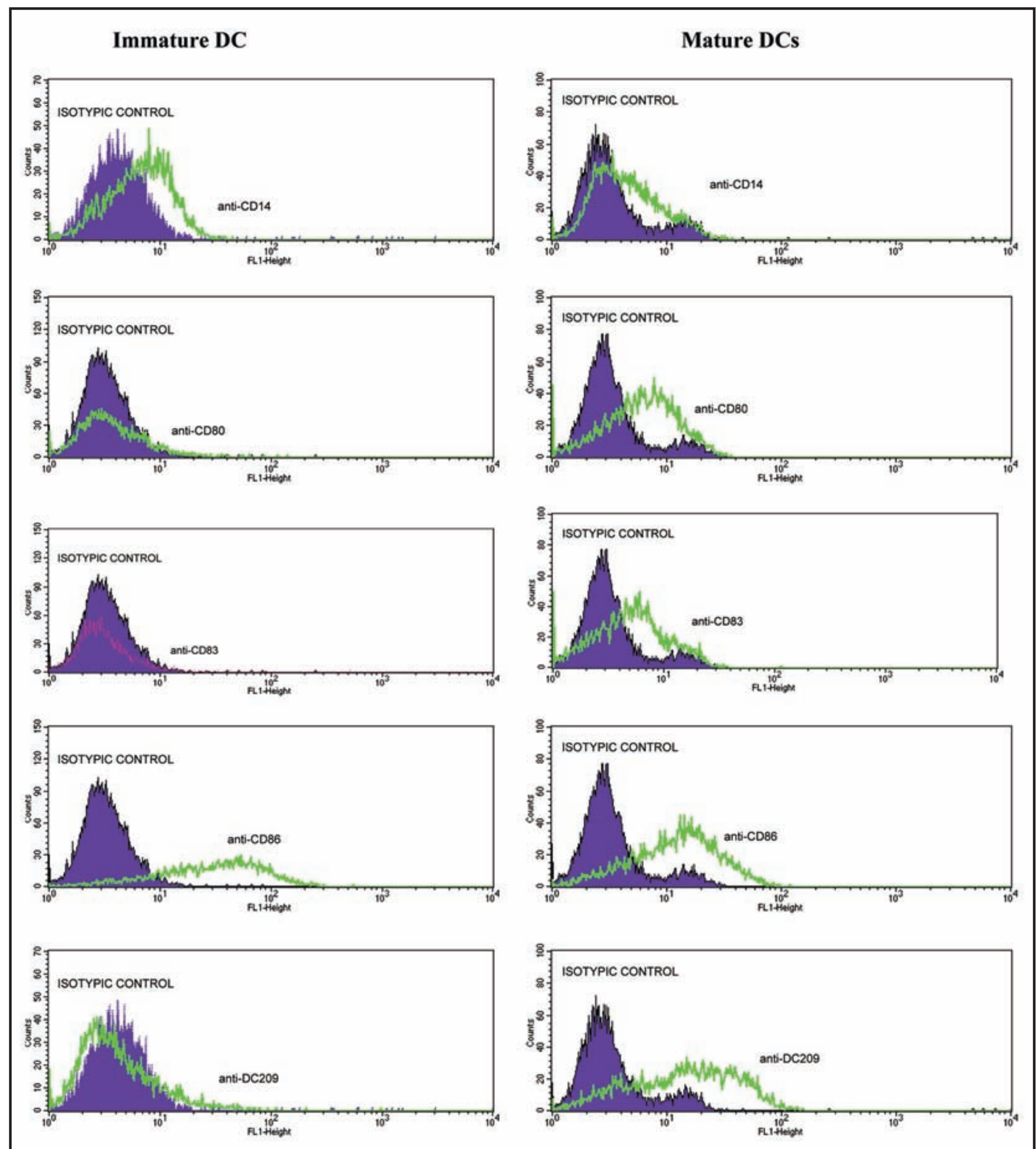

Figure 1. Expression of maturation markers on immature (non-pulsed), and mature (pulsed) DCs using FACS analysis.

Sterility testing. Sterility testing was performed 48 to 72 hours prior to the final harvest using the Sterility Test Method described in 21 CFR 610.12. Positive control bacteria included *Bacillus subtilis*, *Clostridium sporogenes*, *Candida albicans*, *Micrococcus luteus* a.k.a. *Kocuria rhizophila* and *Bacteroides vulgatus*.

Mycoplasma Testing. A PCR-based mycoplasma test (Mycoplasma Detection Kit, Version 2.0, American Type Culture Collection, Manassas, VA) was used on the cultured DCs in this experiment.

rIL-2VMO vaccine preparation. The vaccinia melanoma oncolysate composed of rIL-2VV and the Mel-2 melanoma cell line was prepared utilizing the methodology developed in our facility, which has been approved by the US FDA for Phase I Clinical Trials. Fifty million melanoma cells were resuspended in 1.0 ml of AIM-V serum free lymphocyte medium (Gibco Invitrogen Corporation, Carlsbad, CA). The suspension was cocultured with rIL-2VV at a ratio of 10 melanoma cells to 1 plaque forming unit (PFU) of rIL-2VV, and incubated at 37°C for four hours. The sample was then centrifuged at 1,200 rpm for 10 minutes, and the pellet containing the infected melanoma cells was resuspended in 1ml of PBS. The supernatant containing the unbound virus was collected and centrifuged at 4°C for 1 hour at 32,000 rpm, and this pellet was resuspended in 1ml of PBS. The resuspended melanoma cells transfected with rIL-2VV were then mechanically lysed probe sonication. The cell lysate was then centrifuged at 800 rpm for 10 minutes and the resulting pellet containing the cell nuclei was discarded. The vaccinia virus was inactivated with ultraviolet radiation, made into aliquots, and stored at -70°C until use.

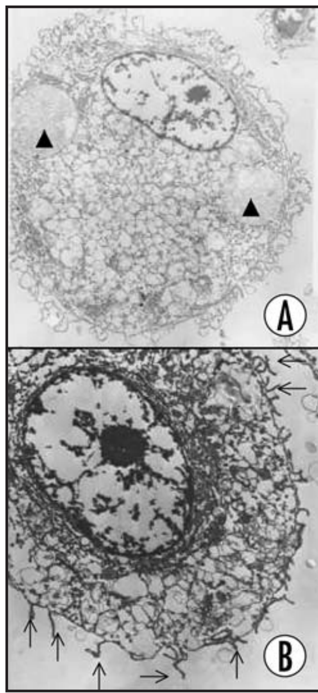

Figure 2. Transmission Electron Microscopy (TEM). Non-pulsed/immature DCs have large vacuoles (▲) as part of the process of phagocytosis, 11,000x (A). Pulsed, mature DCs have numerous dendrites (↑) for antigen presentation, 15,000x (B).

DC pulsing with VMO. The DCs were harvested on the 7th day by gently pipetting the cells and the inner walls of the flasks. This was followed by centrifugation of the cell/media suspension at 1,200 rpm for 5 minutes. The supernatant was discarded and the DC pellet was resuspended in HBSS and counted. The resuspended pellet was then centrifuged again at 1,200 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 200 μl of sterile PBS and mixed with 1.0 ml of the rIL-2VMO dosage (2 mg). This DC-rIL-2VMO was incubated for 4 hours at 37°C. The oncolysate used to pulse DC with does not contain an active virus, therefore no infection of DC is expected.

Maturation marker assay of pulsed DCs and melanoma-associated antigen (MAA) uptake assay. All DCs were treated with 1X BD FACS permeabilizing solution (Becton Dickinson Biosciences, Franklin Lakes, NJ), and were then analyzed by flow cytometry using FACScan (Becton Dickinson Bioscisnces, Franklin Lakes, NJ). The mouse anti-human mAbs were purchased from Becton Dickinson Biosciences, Franklin Lakes, NJ, and included: R-PE labeled CD 11c, and FITC-labeled CD14, CD80, CD83, CD86, CD-209. The isotype control anti-mouse mAbs, also from Becton Dickinson Biosciences, included FITC-labeled IgG_1, IgG_{2a}, IgG_{2b}, IgG_3. The ratio of DCs to mAb was 500,000 DCs to 10 μl mAb.

Expression of MAAs by flow cytometry using FITC-labeled antibodies. The total DC harvest was pooled and divided into two groups, namely: (1) the DCs treated with the rIL-2VMO (2 mg) = pulsed DCs; and (2) non-pulsed (negative control = not treated with rIL-2VMO). After pulsing, these cells were further divided and labeled with commercially available mAb markers against MAAs following the manufacturer's recommendations. The tested MAAs were: GD_3, High MW Proteoglycan/ Mel-2, HLA-DR1a, Mr 130,000 glycoprotein/Mel-4, Gp75 (75kD)/TRP-1/Mel-5, Mart-1 Fusion protein (Signet, Dedham, MA), Transferrin receptor (Becton Dickinson Biosciences, Dedham, MA), p97/Nuclear Transport Factor (Affinity Bioscience, Golden, CO), and Melanoma Peptide Ag-1 (Biocare Medical, Concord, CA). Anti-mouse antibodies to the same MAAs as listed above were used as our negative control. Of note, several melanoma antigens were tested. The antigens chosen represented uptake into the cell.

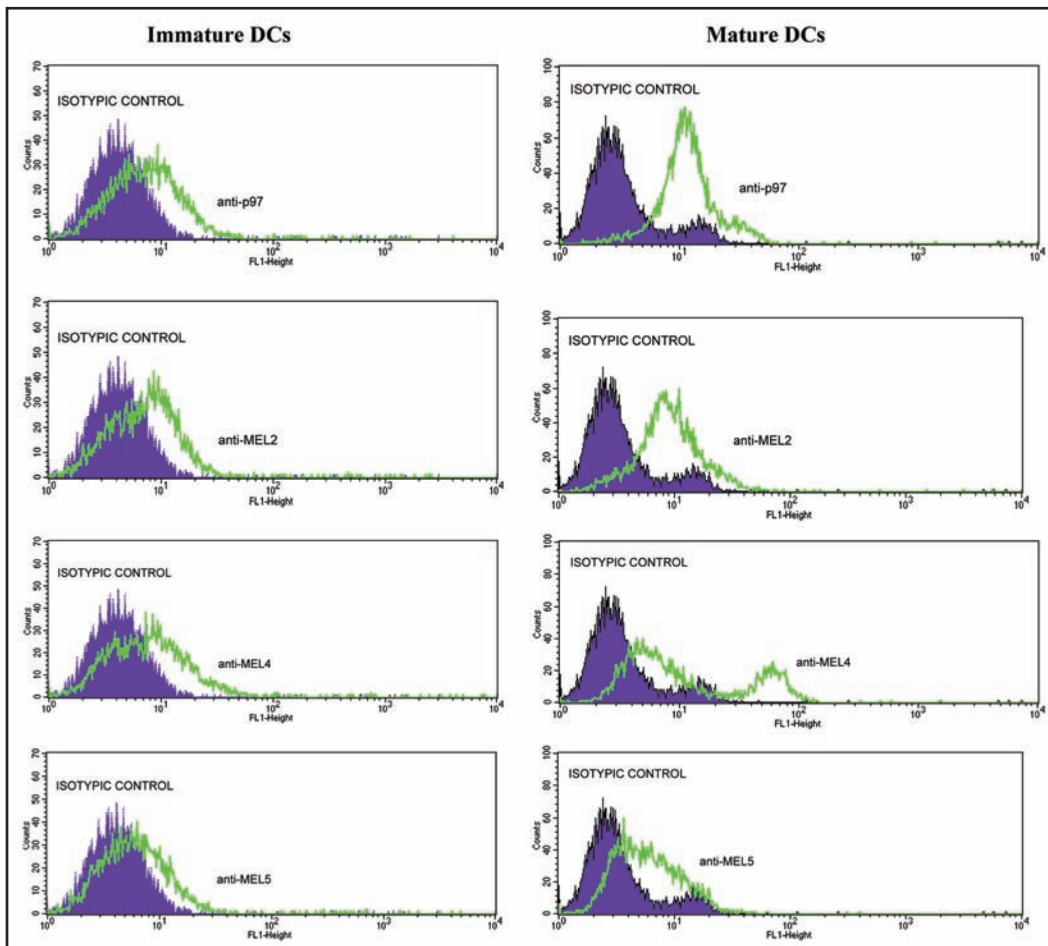

Figure 3. Expression of MAAs by DCs after pulsing with rIL-2VMO. Rightward shifts were seen for p97, Mel-2 (HMWP), Mel-4 (Mr 130,000), Mel-5 (gp75), MAGE-1 and GD3.

RESULTS AND DISCUSSION

In the past two decades, the identification of numerous MAAs has led investigators to test various immunotherapeutic treatments against this fatal disease.[6,7] One of the most studied methods of tumor immunotherapy involves the in vitro manipulation of DCs. In this experiment, we investigated the possibility of using human DCs pulsed with our laboratory's polyvalent melanoma vaccine (DC-IL-2VMO) for the possible treatment of Stage III melanoma.

There are several unique components to our melanoma vaccine. Vaccinia vaccine virus modifies membrane-associated tumor antigens and enhances the immunogenicity of cancers by reexpressing these antigens.[8] In addition, vaccinia vaccine virus enhances the expression of antigen heat-shock proteins[9,10] and nonspecifically helps the induction of tumor-specific cytotoxic T lymphocytes.[11] Moreover, its safety has been well established in the vaccination of millions of people against smallpox.

The vaccinia vaccine virus used in this experiment has been modified so that its genome encodes for IL-2. The modified vaccinia vaccine virus (rIL-2VV) provides continuous secretion of low-dose IL-2, thereby potentially avoiding the organ toxicities, such as capillary leak syndrome,[12] associated with higher doses of the cytokine. It has been confirmed in murine models that rIL-2VV replicates inside the host and produces IL-2, thereby increasing lymphocyte activity.[13] In addition, mice receiving rIL-2VV had a significantly reduced tumor burden and increased survival when compared to mice receiving vaccinia oncolysate alone.[13,14]

In this study, we successfully demonstrated that human DCs cultured from PBMCs and subsequently grown in media containing GMCSF and IL-4 can process and effectively present several MAAs in vitro after being pulsed with rIL2-VMO. The maturation process of the DCs, which occurred as a result of pulsing these cells with rIL-2VMO, was illustrated by the expression of several characteristic surface markers of mature DCs (Fig. 1), as well as by microscopy. Nonpulsed, immature DCs retained their large vacuoles as part of the cell machinery necessary for antigen uptake and processing (Fig. 2A), while mature DCs lost their vacuoles and folded their cell membranes into multiple dendrites for optimal antigen presentation capacity (Fig. 2B).

Using commercially available mAb markers, we tested our laboratory's unique cell line for the expression of several MAAs. We then confirmed that our cultured DCs expressed these antigens after being pulsed with the rIL-2VMO (Fig. 3). The DC expression of these well-established tumor antigens is important when considering the possible therapeutic implications of this vaccine as an active specific immunotherapeutic agent against melanoma. Our results showed a positive shift in immunofluorescence for p97, HMWP (Mel-2), Mr130,000 (Mel-4), gp75 (Mel-5), MAGE-1 and GD3. The remaining three MAAs tested, namely HLA-DR, CD71 and MART-1, were not expressed as strongly by the pulsed DCs, although they were in fact present on the surface of these cells, according to our FACS analysis.

In summary, the present study successfully shows that pulsing DCs with rIL-2VMO induces these cells to process and effectively express all of the MAAs in our cell line. Experiments that are presently underway in our laboratory involve the in vitro assessment of effector cell activation by DC-rIL-2VMO. These studies include T-cell cytokine release, T cell proliferation, and T cell cytotoxicity against the Mel-2 cell line. These experiments are scheduled to be completed prior to the initiation of our FDA-approved Phase I clinical trials in patients with high-risk, stage III melanoma.

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