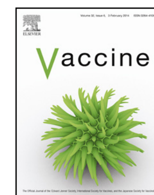
Vaccine 32 (2014) 938–943

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Efficient lysis of epithelial ovarian cancer cells by MAGE-A3-induced cytotoxic T lymphocytes using rAAV-6 capsid mutant vector

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ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form
11 December 2013
Accepted 18 December 2013
Available online 6 January 2014

Keywords:
Epithelial ovarian cancer
MAGE-A3
Adeno-associated Virus 6
Capsid tyrosine mutant
Dendritic cells
Cytotoxic T lymphocytes

ABSTRACT

MAGE-A3 is highly expressed in epithelial ovarian cancer (EOC), making it a promising candidate for immunotherapy. We investigated whether dendritic cells (DCs) transduced with a rAAV-6 capsid mutant vector Y445F could elicit effective MAGE-A3-specific anti-tumor cytotoxic T lymphocyte (CTL) responses *in vitro*. MAGE-A3 was cloned and rAAV-6-MAGE-A3 purified, followed by proviral genome detection using real-time PCR. Immunofluorescence detection of rAAV-6-Y445F-MAGE-A3-transduced DCs demonstrated 60% transduction efficiency. Fluorescent in situ hybridization analysis confirmed chromosomal integration of rAAV vectors. Flow cytometric analysis of transduced DCs showed unaltered expression of critical monocyte-derived surface molecules with retention of allo-stimulatory activity. Co-culture of autologous T lymphocytes with MAGE-A3-expressing DCs produced CTLs that secreted IFN-γ, and efficiently killed MAGE-A3+ EOC cells. This form of rAAV-based DC immunotherapy, either alone or more likely in combination with other immune-enhancing protocols, may prove useful in the clinical setting for management of EOC.

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1. Introduction

Epithelial ovarian cancer (EOC) remains the leading cause of mortality from gynecologic malignancies, with an estimated 22,240 new cases and 14,030 deaths in the US in 2013 [1]. The majority of cases are International Federation of Gynaecological Oncologists (FIGO) stage III or IV at the time of diagnosis, with long-term survival of less than 20% [2]. Although combination chemotherapy in addition to surgical tumor debulking often achieves an initial complete clinical response, tumor relapse due to chemoresistance still remains a major problem. This underscores the need to develop effective strategies to eradicate drug-resistant tumor cells while minimizing toxic side effects to normal tissue that can become dose-limiting. In this regard, immunotherapy enables the targeting of tumor-specific antigens (TSAs) while sparing normal tissue.

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that endocytose exogenous antigens (Ags) [3] and have the ability to provide signals required for T cell activation, making them ideally-suited for cancer immunotherapy [4]. A variety of approaches have been successfully used to deliver TSA to DCs, including loading of peptide mixtures, recombinant protein, mRNA, and direct fusion with tumor cells [5–7]. While each of these approaches has its advantages and shortcomings, the most effective mechanism for generating lasting anti-tumor immunity in cancer patients is still an ongoing area of research [7].

Efficient intracellular expression of TSA is needed for better presentation of tumor peptides to HLA class I molecules [8]. In this regard, bioengineered rAAV are promising vectors because of their excellent safety profile, including a modest frequency of integration, minimal generation of immune responses, and lack of association with human disease [9], and represent another approach to effectively present TSA intracellularly to DCs. Along these lines, rAAV-2 vectors have been shown to efficiently

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http://dx.doi.org/10.1016/j.vaccine.2013.12.049

transduce DCs, leading to effective generation of CTLs [10–12]. Further, rAAV-2 pseudotyped virus with an AAV-6 capsid (rAAV2/6) exhibited a high degree of tropism for DCs [13], resulting in enhanced generation of cytotoxic T lymphocytes (CTLs) [14,15].

MAGE-A3 is a cancer testes (C/T) Ag restricted to tumor cells and immune-privileged gonadal germ cells. It has attracted particular attention as a potential target for immunotherapy since it is more highly expressed in advanced cancer stages and its presence is associated with a poorer prognosis [16]. MAGE-A3 expression has been observed in various EOC cell lines [17] and is positively correlated with disease status in a large number of EOC specimens [18].

As extension of the above observations, the goal of the present study was to clone MAGE-A3 in rAAV-2; produce recombinant virus in rAAV2/6 Y445F mutant vector; demonstrate enhanced expression of MAGE-A3 in DCs; and finally, show targeted killing of EOC with MAGE-A3 CTLs. Our results indicate that DCs can elicit effective antitumor responses against MAGE-A3-expressing EOC cell lines, and provide a basis for potential translation to the clinical arena.

2. Materials and methods

2.1. Reagents, antibodies, and cell culture

Unless otherwise specified, reagents were obtained from Sigma Chemical Co. (St. Louis, MO). HLA-DR, CD3, CD19, CD40, CD80, CD83, and CD86 antibodies were purchased from R&D Systems (Minneapolis, MN). MAGE-A3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Benzonase was purchased from Invitrogen (Grand Island, NY). NIH:OVCAR-3 (HLA-A2) and OV-90 (HLA-A2) cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA) and were propagated in RPMI with 20% FBS. MDAH-2774 (HLA-A3) cell line was also obtained from ATCC and propagated in McCoy's 5A medium supplemented with 10% FBS. SKOV-3/Luc (HLA-A3) and ES-2/GFP (HLA-A3) cell lines were obtained from Cell Biolabs (San Diego, CA) and propagated in DMEM with 20% FBS. All media were supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Cloning and purification of rAAV vector

The expression vector pET 32a was obtained from Novagen (Madison, WI) and AAV-MCS vector was acquired from Agilent Technologies Inc. (Santa Clara, CA). Single-stranded AAV-6 and mutant AAV-6-Y445F plasmids were generated and AAV vector production was carried out as previously described [19]. In brief, a triple transfection method using the AAV-293 cell line was employed. Cells were harvested at 72 h after transfection, freeze-thawed twice, treated with benzonase, and purified by iodixanol gradient ultracentrifugation. Fractions from ultracentrifugation were further subjected to anion exchange Q Sepharose chromatography (GE Healthcare, Buckinghamshire, UK). AAV titers were determined by quantitative DNA slot blot using a non-radioactive method with a MAGE-A3 DNA probe. Probe preparation was done with an ECL direct nucleic acid labeling detection system (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

2.3. Electrophoresis and Western blotting

Total protein was resolved on 4–12% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The detection of MAGE-A3 protein in five EOC cell lines (NIH:OVCAR-3, OV-90, SKOV-3/Luc, MDAH-2774 and ES-2/GFP) was performed at 1:10,000 dilution and protein bands visualized by chemiluminescence assay. Three percent nonfat milk powder dissolved in PBST was used as a blocking agent. Secondary antibodies conjugated to HRPO (Santa Cruz Biotechnology, Santa Cruz, CA) were used along with enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia, Uppsala, Sweden) to view the protein bands.

2.4. Generation of DCs and rAAV transduction

Monocyte-derived DCs were generated as previously described [20]. In brief, peripheral blood mononuclear cells were collected from normal individuals after informed consent, subjected to Ficoll gradient centrifugation, and adhered to plastic for 1 h T lymphocytes were purified by negative selection from the non-adherent cell fraction using immunomagnetic beads coated with anti-CD19, anti-CD33, anti-CD56, and anti-CD14 antibodies. The plastic adherent cells were cultured in AIM V with GM-CSF (100 ng/ml) and IL-4 (1000 IU/ml). rAAV transductions were carried out on days 2, 3, and 4 at 2×10^5 vector genomes/cell. DC maturation was induced by the addition of LPS (5 μg/ml) on day 6. We performed fluorescent *in situ* hybridization (FISH) analysis to evaluate the AAV genome in transduced DCs as described earlier with minor modifications [11,21].

2.5. Real-time PCR

Vector titers were assessed using the ABI Biosystems 7700. Viral DNA samples were prepared by digesting the cell lysate with proteinase K for 2 h at 37 °C, followed by inactivation of the enzyme by boiling for 5 min. Viral titers were determined by comparing the amplification curve to the standard curve using MAGE-A3 specific primers and probes: Forward: 5′-CAT CGA GCT GAT GGA AGT GGA-3′; Reverse: 5′-CAG GCA GGT GGC AAA GAT GT-3′; and Probe: 5′-CCC ATC GGC CAC TTG-3′.

2.6. Immunofluorescence microscopy

Intracellular staining of EOC cell lines for MAGE-A3 and staining of rAAV-6-Y445F-MAGE-A3-transduced DCs were conducted as previously described [11,20,22]. Slides were incubated with MAGE-A3 mouse anti-human antibody at room temperature for 1 h followed by two washes with PBS (pH 7.2) containing 0.05% IGPAL CA-630 (NP40; Sigma, St. Louis, MO). The cells were further incubated with Alexa Fluor 594 rabbit anti-mouse IgG, washed, fixed, and analyzed using a confocal microscope.

2.7. Generation of MAGE-A3–specific CTLs

MAGE-A3-specific CTL generation was performed according to our earlier protocol with minor modifications [23]. Briefly, control and transduced DCs were irradiated with 2500 cGy before co-culturing with autologous T lymphocytes. Cultures were fed every third day with 50% fresh media (AIM V and 20 IU/ml IL-2) and re-stimulated weekly with irradiated autologous DCs for 3 weeks to generate CTLs.

2.8. Flow cytometry

Cells were collected during the logarithmic phase, incubated at 37 °C for 24 h, and then stained with antibodies to HLA-DR, CD-3-PE, CD19PE, CD40-PE, CD83-FITC, CD80-PE, and CD86-FITC (R&D Systems, Minneapolis, MN) as previously described [20]. Isotype-specific negative controls were used in all experiments.

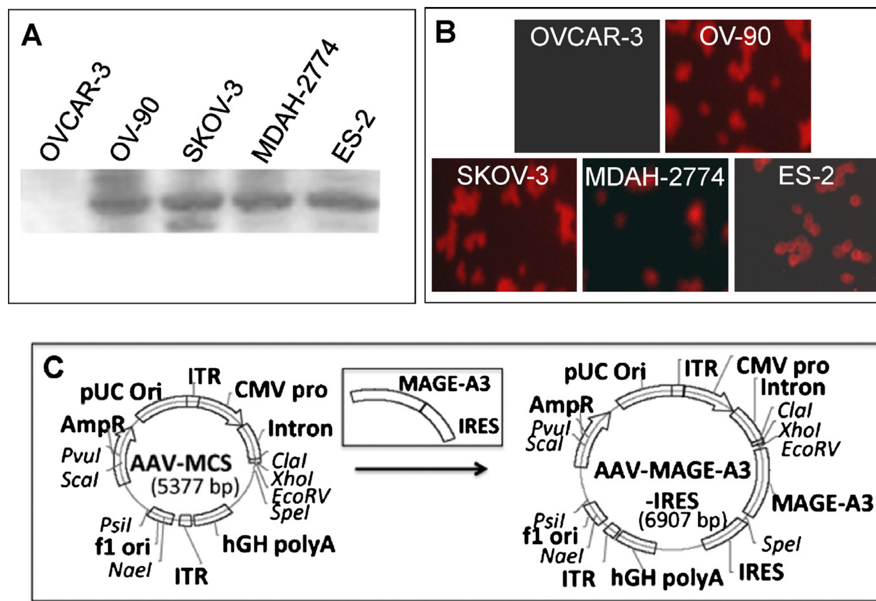

Fig. 1. Identification of MAGE-A3 in EOC cell lines and construction of AAV-MAGE-A3-IRES. (A) Total protein extracts from five EOC cell lines were resolved on 4–12% polyacrylamide SDS gels and transferred on to polyvinylidene fluoride membrane and probed with MAGE-A3 antibodies. β-Actin was used as a loading control (data not shown). (B) Immunofluorescence staining with MAGE-A3 antibody shows strong expression of the protein in four of five EOC cell lines tested (magnification 20×). (C) Two-step generation of AAV-MAGE-A3-IRES: excision from pET 32-IRES vector using EcoRV and BglII; and ligating into AAV-MCS.

2.9. *Mixed lymphocyte reaction (MLR) assays*

Allogeneic T lymphocytes from mismatched donors were co-cultured with either control or rAAV-transduced DCs in triplicate, flat-bottom 96-well microplates according to our established protocol [20]. The plates were incubated at 37 °C in a humidified chamber with 5% CO_2, and cell proliferation was measured using the CCK-8 Cell Proliferation Kit (Dojindo, Gaithersburg, MD) according to manufacturer's instructions.

2.10. *Cytokine levels*

Detection of cytokines in culture medium was determined by standard sandwich enzyme-linked immunosorbent assay as described previously [24].

3. Results

3.1. *Detection of MAGE-A3 in EOC cell lines and construction of the rAAV MAGE-A3-IRES vector*

We observed the presence of MAGE-A3 in four of the five EOC cell lines tested by both Western blot analysis (Fig. 1A) and immunofluorescence (Fig. 1B), with the NIH:OVCAR-3 cell line negative in both cases. PCR-amplified MAGE-A3 from human testis was sub-cloned into pET 32-IRES. MAGE-A3-IRES was then excised from pET-32-MAGE-A3-IRES using EcoRV and BglII and ligated into AAV-MCS to generate AAV-MAGE-A3-IRES (Fig. 1C).

3.2. *Transduced DCs show integrated provirus and high-level transgene expression*

High-titer rAAV-6-MAGE-A3 and mutant rAAV-6-Y445F-MAGE-A3 viral particles were purified with triple-plasmid transfection followed by iodixanol density gradient centrifugation [25]. DCs were then transduced with these particles and examined for MAGE-A3 expression. Proviral genome was detected by real-time PCR (Fig. 2A) with rAAV-6-Y445F-MAGE-A3 demonstrating the highest expression. The primer/probe combination was highly specific for MAGE-A3 and did not react with other viruses tested. Multiple exposures were needed to achieve high transduction rates. Cells were counter-stained with DAPI to visualize the nucleus along with expression of MAGE-A3 (Fig. 2B), indicating approximately 60% transduction efficiency. rAAV transduction did not alter cell viability, which remained >90%, nor cell yield (data not shown). FISH analysis indicated chromosomal integration of rAAV vectors in transduced DCs (Fig. 2C).

3.3. *Functional activity and phenotype of DCs is not affected by rAAV transduction*

To determine whether rAAV-6-Y445F-MAGE-A3 transduction changed the surface Ag expression of DCs, we analyzed several unique DC markers by flow cytometry. The persistent, high-level expression of HLA-DR, CD80, CD83, CD40, and CD86, together with lack of CD3 and CD19 expression, is consistent with a mature DC phenotype and indicates that transduction did not interfere with expression of these critical monocyte-derived surface molecules (Fig. 3A). In order to assess functional activity, we tested the allo-stimulatory capacity of DCs following rAAV-6-Y445F-MAGE-A3 transduction in the MLR assay. Both transduced and control DCs demonstrated similar degrees of cell proliferation (Fig. 3B).

3.4. *MAGE-A3-specific CTLs kill EOC cells and secrete IFN-γ*

To confirm the utility of MAGE-A3 as an immunotherapy target for EOC, Ag-specific CTL were generated via repeated stimulation of purified autologous T lymphocytes with rAAV-6-MAGE-A3-Y445F-transduced DCs from HLA-$A2^+$ donor. These CTLs efficiently killed MAGE-A3+ DCs as well as MAGE-A3+ OV-90 cells, but not MAGE-A3-negative NIH:OVCAR-3 cells (Fig. 4A). Further, MAGE-A3-specific CTLs secreted IFN-γ, with significantly lower amounts of IL-4, IL-10, IL-12, and TNF-α in the culture supernatant (Fig. 4B), a signature cytokine profile for CTLs that are critical for tumor eradication.

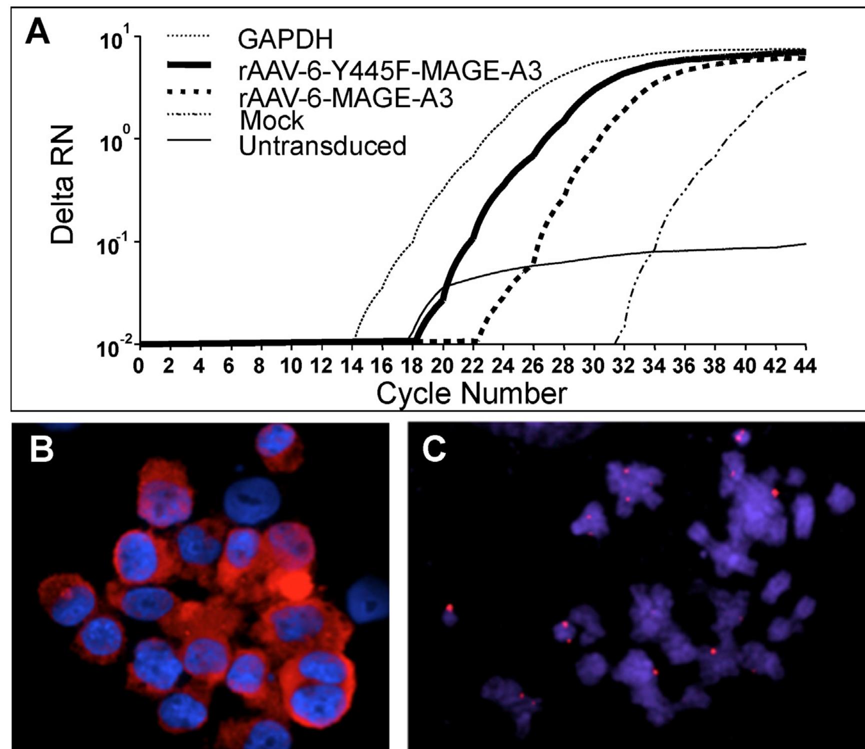

Fig. 2. rAAV-6-MAGE-A3 and mutant rAAV-6-Y445F-MAGE-A3 vector production and DC transduction. (A) The real-time PCR curves show quantification of MAGE-A3 in both rAAV-6-MAGE-A3 and rAAV-6-Y445F-MAGE-A3. GADPH was used as a positive internal control. (B) Immature DCs were transduced with rAAV-6-Y445F-MAGE-A3 virus and then stained with MAGE-A3 antibody followed by visualization with Alexa 660 (magnification 100×). (C) FISH analysis of metaphase chromosomes confirming integration of rAAV vectors. Chromosomes are stained blue with DAPI and orange dots indicate integrated rAAV provirus.

4. Discussion

Rejection of tumor cells via anti-tumor immunity is well established in ovarian cancer [26], illustrated by the correlation between presence of tumor-infiltrating lymphocytes and patient survival [27]. However, the propensity of cancer cells to ultimately evade immune detection by suppressing immunologic functions may ultimately tip the balance toward tumor growth and acceptance. In this regard, our DC-based immunotherapy vaccine aims to augment host immune responses in order to promote tumor rejection with negligible adverse effects. Further, effective cancer immunotherapy relies on intracellular TSA delivery

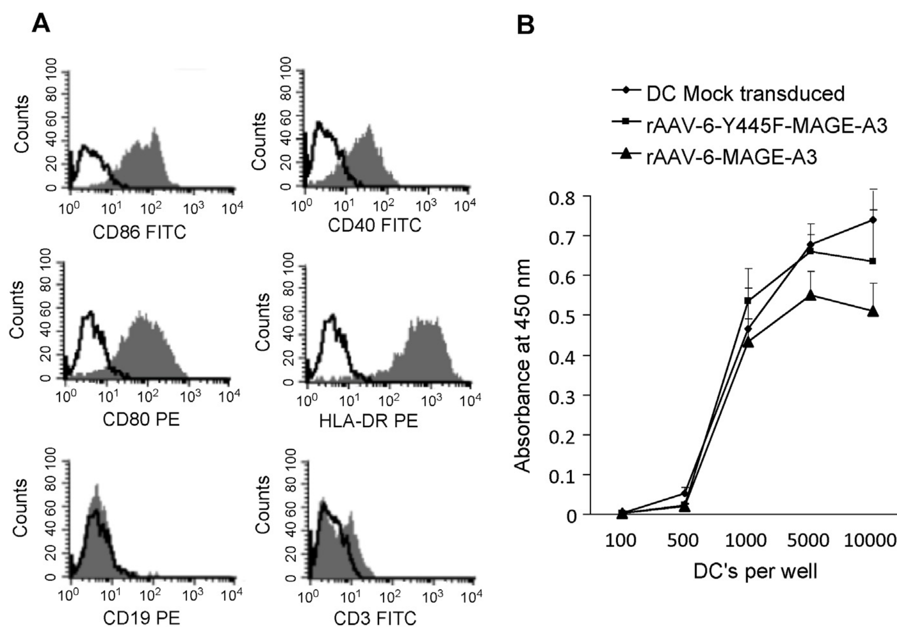

Fig. 3. DC immunophenotype and allo-stimulatory activity: (A) Flow cytometric analysis of rAAV-6-Y445F-MAGE-A3-treated DCs. Percentage indicates the level of expression of the respective DC surface markers when compared with isotype control (left peak). The shaded area indicates staining for the marker of interest. (B) rAAV-transduced DCs elicit equipotent allogeneic T lymphocyte proliferation when compared with mock-transduced DCs in MLR assay. Data is representative of three separate experiments.

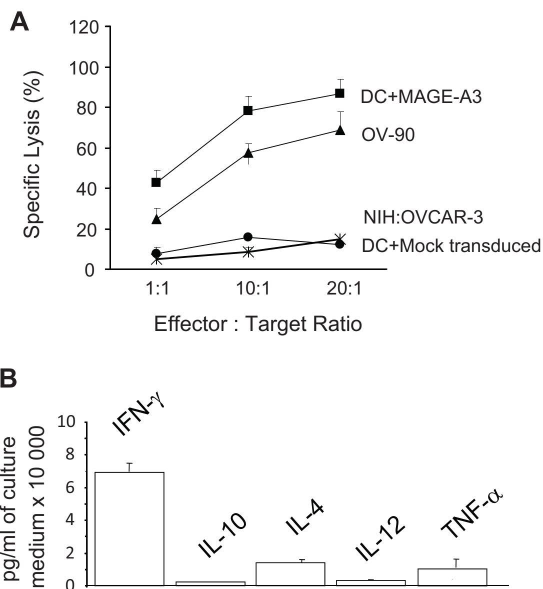

Fig. 4. Cytotoxicity and secreted cytokine profile of MAGE-A3-specific CTLs: (A) Lysis of viral-transduced DCs and two EOC cell lines by MAGE-A3-specific CTLs. (B) Cytokine secretion profile of MAGE-A3-specific CTLs. Data is representative of three separate experiments.

to strongly elicit HLA class I responses and produce tumor cell lysis.

MAGE-A3 has been shown to induce both humoral and cell-mediated immune responses [28], and has been successfully used as an immunotherapy target in melanoma and non-small cell lung cancer [29,30]. Studies examining the expression pattern of C/T Ags in EOC cell lines demonstrated high levels of MAGE-A3 due to aberrant demethylation [31]. Moreover, the presence of MAGE-A3 tumor-infiltrating lymphocytes in EOC patients [26] validates its use as an immunotherapy target in our study. Along these lines, four out of five EOC cell lines we investigated showed high levels of MAGE-A3. In addition, the DNA hypomethylating agent 5-aza-2′-deoxycytidine has been shown to increase the expression of MAGE-A3 in patients with multiple myeloma [32,33], suggesting that a MAGE-A3-based vaccine may even be useful in EOC patients with low-level Ag expression (e.g. NIH:OVCAR-3 cells) when combined with this drug. Finally, use of the full-length MAGE-A3 gene circumvents the need to define HLA class I allele binding before vaccination and increases the number of epitopes recognized by CTLs when compared with peptide-pulsed DCs.

Tumor lysates, mRNA, or recombinant proteins have been successfully used for *ex-vivo* DC pulsing [5–7]. Viral vectors represent another attractive choice for expressing TSA in DCs since they enhance cytosolic bio-availability of tumor peptides to HLA class I molecules. More specifically, bioengineered rAAV vectors are preferred for immuno-gene therapy due their lack of pathogenicity, modest frequency of integration into the host genome, ability to establish long-term transgene expression in non-dividing cells, and weak induction of innate immune responses when compared with other viral-based vector systems [9,34]. These vectors have yielded promising results in an increasing number of DC immunotherapy trials [35]. Of the various AAV serotypes shown to transduce DCs, we chose AAV-6 for gene modification because it has the highest tropism to these cells [13,36]. We then used a Y445F capsid mutant of AAV-6 [25] to prevent intracellular degradation, producing 60% DC transduction efficiency. Similarly, while studying the transduction efficiency of hematopoietic stem cells, Song et al. [37] demonstrated significant enhancement in transgene expression with AAV-6-Y445F when compared with AAV-6.

It has been shown in both DCs and other cell types that double-stranded, self-complementary rAAV vectors achieve earlier and higher transgene expression levels with fewer exposures than conventional, single-stranded particles because they bypass second-strand DNA synthesis [12]. We were able to demonstrate significant transduction efficiency with single-stranded rAAV vectors by using multiple exposures of DC-tropic rAAV-6 in combination with a capsid mutation that avoided intracellular degradation. We are currently cloning MAGE-A3 in double-stranded AAV to further enhance transgene expression.

It is well established that success with immunotherapy is inversely correlated with tumor burden and the associated immunosuppressive environment created by the cancer itself [38]. Patients with early disease may benefit from direct injection of recombinant viral particles, while treatment of later-stage disease may require cell-based therapy since the host immune system may not support generation of optimally-functioning DCs. Along these lines, Xu et al. [39] were able to reduce tumor burden in a murine model following direct intramuscular injection of rAAV-2, presumably due to local cross-priming of DCs. In this regard, our work with rAAV-6 is potentially suitable for both direct injection and adoptive immunotherapy approaches.rAAV-based immunotherapy may be further enhanced by adding other treatment modalities which also augment anti-tumor immune responses. Jensen et al. [40] identified various DC maturation cocktails that break the tolerance often induced by T_{reg} cells. In this regard, combining rAAV transduction with activation of toll-like receptors 7 and 9 has been shown to enhance CTL responses to Trp2 Ag in mouse melanoma [35]. In order to generate effective EOC cell killing *in vivo*, it may be necessary to combine our approach with activation of toll-like receptors and/or suppression of T_{reg} cells [35,40,41].

5. Conclusions

In summary, we demonstrated effective transduction of DCs with a rAAV-6 capsid mutant incorporating full-length, endogenously-expressed MAGE-A3 without affecting cell functionality. We achieved potent cell-mediated immune responses and tumor cell lysis. This form of rAAV-based DC immunotherapy, either alone or more likely in combination with other immune-enhancing protocols, may prove useful in the clinical setting for management of EOC.

Conflict of interest statement

The authors declare no conflict of interest.

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