

Recovery of HLA-A2 and Beta2-microglobulin Expression in Tumor Cells Using Viral Vectors

Carretero FJ¹, Del Campo A^{1,2}, Zinchenko S², Garrido F^{1,2} and Aptsiauri N^{1,2*}

¹Department of Biochemistry, Molecular Biology III and Immunology, University of Granada Medical School, Granada, Spain

²Department of Clinical Analysis, Virgen de las Nieves University Hospital, Institute of Biosanitary Research (ibs.Granada), Granada, Spain

Abstract

Background: Tumor elimination and the success of cancer immunotherapy depend on the proper expression of HLA class I complex (HLA-I) required for the presentation of tumor-associated peptides to cytotoxic T-lymphocytes. Tumors escape immune attack by losing HLA-I expression, often due to irreversible genetic/chromosomal alterations, including mutations in beta2-microglobulin (B2M) or lack of HLA-A2 allele due to a haplotype loss. The introduction of these genes and re-expression of the missing HLA-I specificity on the tumor cell surface is an attractive strategy to induce tumor rejection by T-lymphocytes.

Methods: Using genomic HLA-I typing and gene sequencing we determined HLA-I phenotypes and alterations in different human tumor cell lines previously characterized in our laboratory. We used adeno- and adeno-associated viruses to reconstitute/up-regulate HLA-A2 or/and B2M expression in these cells *in vitro*. Using flow cytometry and immunocytochemistry we evaluated levels and patterns of HLA-I expression in these cells.

Results: We have defined altered HLA-I genotypes in various human tumor cell lines. Ad5 and, to a lesser degree, AAV2 vectors were efficient in a replacement of mutated B2M, recovery of lost endogenous HLA-A2 allele, and *de novo* expression of an additional HLA-A2 allele in tumor cells naturally lacking HLA-A2. Ad5-mediated co-transfection of both HLA-A2 and B2M demonstrated that the *de novo* expressed proteins associate to form a detectable HLA-I/B2M complex on the cell surface.

Conclusion: Using gene therapy, it is possible to recover normal B2M and HLA-A2 gene expression caused by structural "hard" alteration and to induce co-expression of both genes in cells naturally lacking HLA-A2 allele. In addition, we demonstrated that transfected tumor cells are able to express seven HLA-I alleles. The recovery of the missing HLA-I molecules in tumor cells using adeno and adeno-associated viruses can be a useful strategy to circumvent cancer immune escape and increase tumor rejection.

Keywords: HLA class I antigens; Beta2-microglobulin; Tumor immunology; Gene therapy; Oncology; Viral vectors; Cancer immune escape; Cancer immunotherapy

Introduction

Cancer immunotherapy recently started to benefit from novel approaches such as "immune checkpoint" inhibitors, adoptive T-cell transfer, or vaccines aimed at guiding the immune system specifically toward tumor associated antigens. Despite the significant advances in this field, consistent and long-lasting responses to cancer vaccines remain vague [1,2], likely owing to different mechanisms of cancer immune escape. In particular, the inability of the immune system to eliminate malignant cells may be due to the loss of tumor human leukocyte antigen class I (HLA-I) expression and lack of presentation of tumor-associated peptides to cytotoxic T lymphocytes (CTL) [3-5]. Hence, there is an urgent need to develop novel approaches to induce upregulation of tumor HLA-I in order to overcome immune evasion, stimulate tumor rejection and increase clinical efficacy of cancer immunotherapy [6].

HLA-I molecules are comprised of a highly polymorphic heavy chain (HC) (HLA-A, -B, and -C locus variants, each represented by different allelic versions) non-covalently associated with a non-polymorphic subunit called beta-2-microglobulin (B2M), which is common for heavy chains and has an important role in stabilization of the cell surface HLA-I complex and in the formation of an antigenic peptide binding groove. This way, tumor-associated peptides are recognized by T-cell receptors and tumor cells are targeted for elimination. Importantly, specific tumor-associated peptides are presented by restricted HLA-I

alleles. Hence, the lack of HLA-I molecules frequently detected in various types of malignancy compromises the cytotoxicity of T-lymphocytes and provides a route for immune escape [7]. Activation of the CTL-mediated tumor rejection may be achieved by recovering or increasing HLA-I surface expression on malignant cells using different methods depending on the cause of altered HLA-I expression. These alterations can range from total loss to selective locus or allelic deficiencies with two main types of underlying molecular defects: 1) reversible regulatory or 'soft' alterations leading to downregulation of HLA-I and/or antigen presentation machinery molecules; and 2) irreversible structural or 'hard' alterations caused by mutational events and genomic aberrations, such as loss of heterozygosity (LOH) in HC and B2M regions of chromosomes 6 and 15, respectively [8-14]. Loss of expression of one particular allele, HLA-A2, has been reported in different types of cancer [15-17] with important clinical implications,

*Corresponding author: Natalia Aptsiauri, Department of Clinical Analysis, Virgen de las Nieves University Hospital, Institute of Biosanitary Research (ibs.Granada), Granada, Spain, Tel: 216 71 562 083; E-mail: napsiauri@ugr.es

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since HLA-A2 has one of the widest peptide repertoires among human class I molecules [18,19]. Moreover, many of cancer vaccines and immunotherapies use HLA-A2-specific tumor peptides to stimulate HLA-A2-restricted CTL responses in patients positive for genomic HLA-A2 allele [20]. Thus, loss of this particular allele in a tumor could compromise the efficacy of immunotherapy. Recuperation of HLA-A2 expression using gene transfer methods might improve the clinical outcome of the treatment by recovery of peptide-presenting ability of tumor cells and recruiting pre-existing CTLs specific for HLA-A2-restricted peptides.

Tumor cells with structural defects may escape immune recognition even after immunomodulatory treatment can become a major threat to anti-cancer immunity. These defects and can only be corrected by the transfer of the healthy gene into tumor cells. With the recent developments in tumor immunology and biotechnology, cancer gene therapy in combination with immunotherapy and chemotherapy has become a promising approach for therapeutic intervention. Our group has previously reported a recovery of HLA-I cell surface expression in B2M-deficient tumor cells by replication-deficient adenovirus carrying B2M gene. We have also demonstrated that this type of cellular gene therapy increases tumor cell immunogenicity and elimination by CTLs in HLA-matched fashion without affecting antigen presentation [21-23]. It is also important to choose an appropriate vector with highest gene transfer capability and low vector immunogenicity and toxicity. Here we describe particular HLA and B2M gene alterations in different tumor cell lines. We used viral vectors (Ad5 and AAVs of different serotypes) and evaluated their efficacy in transducing different types of human tumor cells with HLA-A2 alone or together with B2M gene.

Material and Methods

Cell lines

In this study, we used four groups of human tumor cell lines for virus-mediated recovery/upregulation of HLA-A2 and/or B2M (based on the baseline expression status of these molecules) (Table 1). All melanoma cell lines, except for Ando-2, were obtained from the European Searchable Tumour Cell Line Data Base (ESTDAB) [24] and have been previously genotyped for HLA-I and characterized for HLA phenotype [25]. Ando-2 was kindly provided by Dr P. Coulie (Université Catholique de Louvain (UCL), Brussels, Belgium) and is characterized by the loss of HLA-A2 expression due to HLA-I haplotype loss [26]. Prostate cancer cell lines DU145 and PC3, as well as bladder cancer cell lines T24, WIL and RT112, were obtained from American Type Culture Collection (ATCC). OPCN3, OPCN1 and OPCT1 cell lines derived from patients with prostate cancer were obtained from a collaborative European project ENACT (European Network for the identification and validation of Antigens and biomarkers in Cancer and their application in clinical Tumor immunology, LSHC-CT-2004-503306). All melanoma cells and prostate cells DU145 and PC3 were grown in RPMI-1640 medium (Biochrom KG) supplemented with 10% FCS, 2% glutamine (Biochrom KG) and 1% penicillin/streptomycin (Biochrom KG) at 37°C in a humidified atmosphere with 5% CO₂. Prostate cancer cells OPCN3, OPCN1 and OPCT1 were cultured in keratinocyte medium with a growth supplement (GIBCO, Life Technologies). Bladder cancer cells were cultured in Dulbecco media supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine and 1% penicillin/streptomycin.

Cell line	HLA-I genomic typing			Origin
	Locus A	Locus B	Locus C	
Cell line negative for HLA-A2 due to HLA-I haplotype loss				
Ando-2*	3201	4001	0304	Melanoma
Cell lines negative for genomic HLA-A2				
PC3	2402/0101	1302/5501	0102/0602	Prostate
DU145	0301/3303	5001/5701	0602/0602	Prostate
OPCN1	0101/2301	3701/4001	0304/0602	Prostate
OPCT1	0101/2301	3701/4001	0304/0602	Prostate
WIL**	6802	1503	1203	Bladder
T24**	0101	1801	0501	Bladder
RT112**	2601	270305	0102	Bladder
E130**	1101	4001	030401	Melanoma
Cell lines negative for genomic HLA-A2 allele with total HLA-I loss caused by B2M alterations				
E038	0101/2902	3501/4403	040101	Melanoma
E109	0101/2501	0801/1801	070101/120402	Melanoma
OPCN3	0101/2501	5701/5701	0602/0602	Prostate
Cell lines positive for genomic HLA-A2				
E050	0201/2601	1402/3801	0802/1203	Melanoma
E102	0201/0301	1801/4001	0304/070101	Melanoma
E120	0101/0201	0702/0801	0701/0702	Melanoma
E013	0101/0201	0801/15010101	070101/0304	Melanoma
E019	0101/0201	0801/0702	0701/0702	Melanoma
E025	0101/0201	0801/15010101	0304/070101	Melanoma

*Ando-2 cells have loss of HLA haplotype caused by loss of heterozygosity in chromosome 6, as confirmed by the analysis of autologous PBMC; **homozygote or a probable loss of heterozygosity, not confirmed due to the absence of autologous material

Table 1: Genomic HLA-I typing in the studied human cancer cell lines.

HLA genomic typing

Genomic HLA typing of all cell lines was performed using Dynal RELI^{SSO} Typing Trays and Dynal RELI^{SSO} Strip Detection Reagent kit, following the manufacturer's instructions. Table 1 summarizes HLA typing of all the studied cell lines.

Viral vectors

Two replication-deficient adenoviral vectors (serotype 5 (Ad5)) carrying GFP and B2M gene under the control of cytomegalovirus (CMV) (AdCMV-GFP and AdCMV-B2M) were produced in our laboratory as described earlier [21]. Same method with minor modifications was used to construct Ad5 coding for human HLA-A*0201 (AdCMV-HLA-A2) gene, which was initially amplified from pClnx-A2 plasmid (ATCC) using specific primers for human HLA-A*0201 (forward primer: 5'-AAGCTTGCCACCATGGCCGTCATGGCGCCCCGAA-3'; reverse primer: 5'-AATTGGATCCCCTACAGGTGGGGTCTTTC-3') with restriction sites HindIII and BamHI in 5' and 3' end, respectively. KOZAK sequence was added to facilitate the recognition of the initiation sequence. Adeno-associated vectors of various serotypes, AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9, carrying GFP gene under the control of CMV promoter were kindly provided by our collaborators at the University of Florida Gene Therapy Center, Gainesville, Florida, USA. Recombinant AAV2 carrying HLA-A*0201 gene (AAV2-HLA-A2) under the control of CMV promoter was constructed using method described previously [27].

Antibodies

The following anti-HLA class I monoclonal antibodies (mAb) were used as previously described [25]: W6/32 that recognizes cell surface complex of HLA-I heavy chain/ B2M, L-368 directed against free B2M, and CR11 specifically recognizes HLA-A2 (and A28) [28]. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Sigma) antibody was used as a secondary Ab.

Cell infection *in vitro*

The purified virus was added either directly to the cell culture flasks in a 2% FCS supplemented media or was first used to infect cells in a small volume (200 μ l) for 2 hrs at 37°C before transferring to a bigger flask. Viral vectors were used at following doses or multiplicity of infection (MOI): 200, 500 or 1000 MOI for AAVs, and 20, 50, 100, 200 MOI for Ad5. Comparable transduction effect was observed at 100 MOI for AdCMV-GFP and 1000 MOI in case of AAVs. 100% of cells infected with AdCMV-GFP at 200 MOI were GFP-positive. However, cytopathic effect prevailed limiting the possibility of increasing the virus dose. Therefore, two optimal viral doses with maximum efficacy were selected for cell infection: 100 MOI for Ad5 and 1000 MOI for AAV2. In some experiments tumor cells were simultaneously infected with two adenoviral vectors, AdCMV-HLA-A2 and AdCMVHLA-B2M. The expression of the transgenes was determined by flow cytometry using specific antibodies. To examine the time course of transgene expression, cells were harvested on days 3, 6, 10, 15, 20, 25, 30 and 35 after infection, and HLA-I complex and HLA-A2 expression was evaluated by flow cytometry using w6/32 and CR11 mAb.

Flow cytometry

The expression of GFP was evaluated directly by FACS. The cell surface expression of HLA class I complex, and of HLA-A2 and B2M molecules was evaluated by indirect immunofluorescence using specific antibodies as described earlier. Results were presented as representative fluorescence plots or as mean fluorescence intensity (MFI) bars or as

percentage of positive cells. Data were analyzed using Flowing Software (University of Turku, Finland).

Immunocytochemistry

Immunocytochemical staining of tumor cells grown on glass slides before or 48 hours after infection with AdCMV-HLA-A2 or AAV2-HLA-A2 was performed using mAb CR11 recognizing HLA-A2, followed by incubation with HRP-conjugated secondary antibodies and visualization of the immunolabeling using Novolink Polymer detection system (Leica, Newcastle, UK) according to manufacturer's manual.

Statistical analysis

We used ANOVA and Bonferroni data analysis method with a significance threshold of $p < 0.05$ to evaluate possible differences in HLA-A2 expression levels between control and infected cells. SPSS v17.0 software was used for all statistical analyses.

Results

Comparative analysis of the transduction efficacy of adenoviral and adeno-associated viral vectors driving GFP expression in human tumor cells

In order to optimize viral vector application and choose a vector with the best transduction efficacy we compared adenovirus and six AAV serotype variants (AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9) driving GFP expression by infecting one melanoma, two prostate, and two bladder cancer cell using different virus doses. GFP expression after infection of PC3 cells was dose-dependent, as shown in Figure 1a, with highest transgene expression at 1000 MOI (in case of AAV2). We discovered that AAV serotype 2 has the highest transduction efficiency of GFP gene among the studies AAV serotypes 1, 2, 5, 6, 8 and 9. However, Ad5 demonstrated similar results at a ten times lower MOI (100) indicating superior transduction efficacy of adenoviral vector. Infection with AAV1 and AAV9 serotypes induced GFP expression in around 10% of cells. Similar dose-dependent pattern was obtained in all cell lines (data not shown). Furthermore, we compared the transduction efficacy of all the studied vectors at the maximum optimal dose (100 MOI for Ad5 and 1000 MOI for AAV) in different cell types and found that the highest transduction rate in most of the cell lines, except for RT112, was achieved using adeno-GFP. Among adeno-associated-viral vectors AAV2-GFP showed the best results with varying efficacy depending on the cell line. AAV serotypes 1, 5, 6, 8, and 9 showed none or very little ability to induce GFP expression in the studied cells (Figure 1b). These differences were more evident when we compared the MFI values (data not shown).

Both Ad5 and AAV2 are efficient in the recovery of HLA-A2 allele in HLA-A2-deficient cells

Based on the results described above, we have selected the most effective viral vectors for HLA-A2 transfer into tumor cells: replication-deficient Ad5 and AAV2. We constructed both vectors carrying HLA-A2 gene and compared their efficacy to induce HLA-A2 transgene expression in different tumor cell lines. Using these viral vectors, we successfully recovered HLA-A2 expression in melanoma cell line Ando-2 which has been characterized to lack endogenous HLA-A2 due to a haplotype loss caused by loss of heterozygosity at chromosome 6 (Table 1) [26]. Figure 2 depicts recovery of HLA-A2 expression after infection with AdCMV-HLA-A2 and AAV2-HLA-A2 as demonstrated by FACS (Figure 2a) and by immunocytochemistry (Figure 2b).

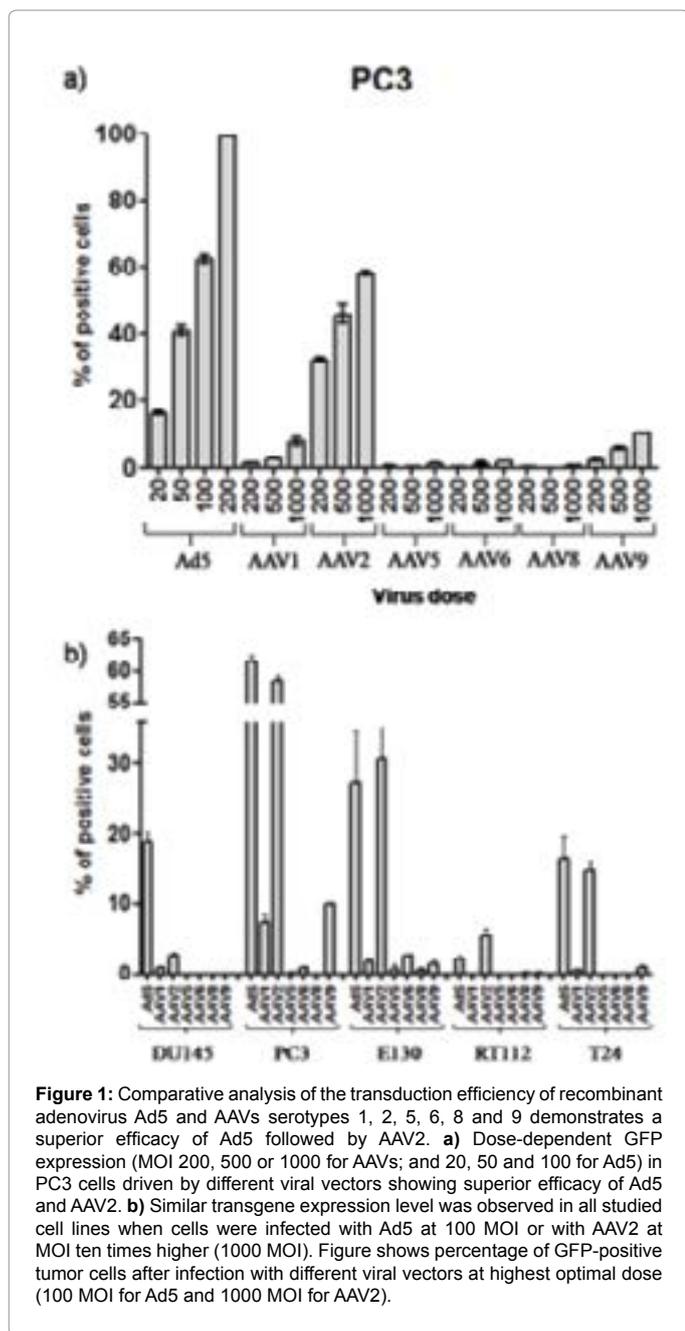
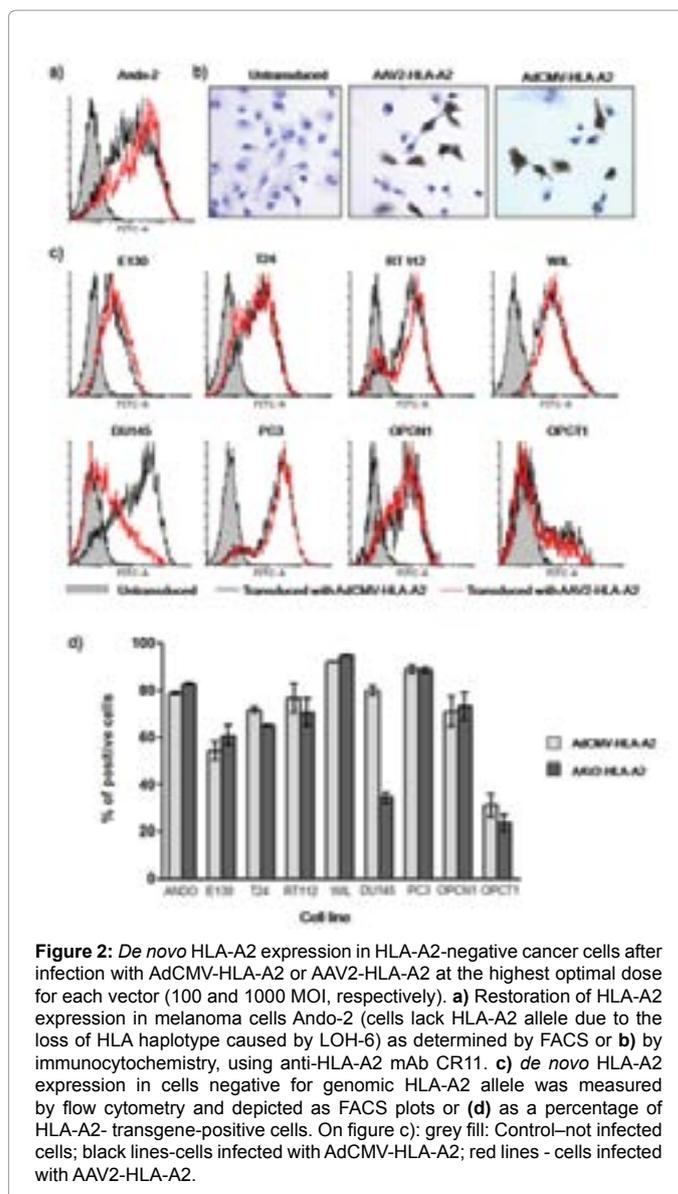


Figure 1: Comparative analysis of the transduction efficiency of recombinant adenovirus Ad5 and AAVs serotypes 1, 2, 5, 6, 8 and 9 demonstrates a superior efficacy of Ad5 followed by AAV2. **a)** Dose-dependent GFP expression (MOI 200, 500 or 1000 for AAVs; and 20, 50 and 100 for Ad5) in PC3 cells driven by different viral vectors showing superior efficacy of Ad5 and AAV2. **b)** Similar transgene expression level was observed in all studied cell lines when cells were infected with Ad5 at 100 MOI or with AAV2 at MOI ten times higher (1000 MOI). Figure shows percentage of GFP-positive tumor cells after infection with different viral vectors at highest optimal dose (100 MOI for Ad5 and 1000 MOI for AAV2).

De novo expression of HLA-A2 allele in tumor cells negative for endogenous genomic HLA-A2

Infection with AdCMV-HLA-A2 or AAV2-HLA-A2 induced expression of HLA-A2 allele in various types of human tumor cells (melanoma, bladder, and prostate cancer cells) naturally lacking HLA-A2 based on HLA class I genotyping results (Table 1). HLA-A2 expression was measured by flow cytometry 48 hours after infection, confirming a successful introduction of HLA-A2 allele *de novo* in addition to the existing ones (Figure 2c). Comparable results were also obtained when we analyzed the percentage of the transduced cells almost in all cell lines (Figure 2d). Importantly, the level of transgene expression was comparable only when MOI of AAV2 was ten time higher than that of Ad5.



Kinetics of *de novo* HLA-A2 expression induced by AdCMV-HLA-A2 or AAV2-HLA-A2

Seven cell lines negative for endogenous HLA-A2 were infected with AdCMV-HLA-A2 and AAV2-HLA-A2 and transgene HLA-A2 expression was analyzed by FACS on days 3, 6, 10, 15, 20, 25, 30 and 35 after infection (Supplementary Figure 1) demonstrating overall similar expression level and kinetics for both types of vectors. Most cell lines had maximum HLA-A2 expression on day 6, decreasing gradually until day 15-25. Only WIL retained more than 50% of HLA-A2-positive cells until day 30 (Supplementary Figure 1). In DU145, RT112 and T24 cells with similar time-curves for both vectors types, the adenovirus induced transgene expression in a higher percentage of infected cells during the first days. On the contrary, Ando-2 and PC3 cells showed higher percentage of HLA-A2 expressing cells when infected with the AAV2.

Virus-mediated upregulation of HLA-A2 expression on tumor cells positive for genomic HLA-A2 allele

We also analyzed how HLA-A2 upregulation after viral transduction

depends on the pre-existing baseline level of endogenous HLA-A2 expression in HLA-A2-positive cells. We used melanoma cell lines positive for HLA-A2 according to HLA-I genotyping [25]. Based on the level of HLA-A2 expression measured by FACS, we selected three cell lines with high and three cell lines with low baseline HLA-A2 expression to infect with AdCMV-HLA-A2 or AAV2-HLA-A2. As shown in Figure 3, in low-HLA-A2 expressing cells (E050, E102 and E120) virus-mediated upregulation of HLA-A2 is much more evident and statistically significant than in cells with higher baseline HLA-A2 level, with AAV2-HLA-A2 demonstrating superior transduction efficacy than adenovirus in two out of three cell lines. On the contrary, viral vectors showed different efficacy to upregulate HLA-A2 in cells with the high endogenous baseline HLA-A2: almost none or very little efficacy in E-025 and E-013 cells (Figure 3), and significant increase in transgene expression in E-019 cells, especially when adenoviral vector was used (Figure 3). MFI fold-change increment of HLA-A2 expression after infection of the cells is also depicted in this figure.

Restoration of cell surface HLA class I allele in B2M-deficient tumor cells negative for genomic HLA-A2 after co-infection with AdCMV-HLA-A2 and AdCMV-B2M

To confirm the capacity of *de novo* expressed HLA-A2 heavy chain to form a complex with B2M on the cell surface, we co-infected with both AdCMV-HLA-A2 and AdCMV-B2M three tumor cell lines (E038, E109, and OPCN3) all B2M-deficient and with HLA genotype naturally lacking HLA-A2 allele. As shown in Figure 4, all cell lines are negative for total HLA-I, B2M, and HLA-A2 in baseline conditions (Figure 4a). Cells infected with AdCMV-B2M, as expected, recover HLA-I expression (Figure 4b). Infection with AdCMV-HLA-A2

renders these cells HLA-I negative (Figure 4c) since the pre-existing lack of B2M prevents formation of cell surface HLA-I complex with newly expressed HLA-A2 protein. Only after co-transfection with both vectors, cell surface HLA-HLA-I/B2M complex was recovered and HLA-A2 expression was detected by flow cytometry using specific antibodies CR11 (Figure 4d), suggesting that simultaneous use of both adenoviral vectors led to an association of newly expressed HLA-A2 and B2M molecules to form a cell surface HLA-I complex.

Discussion

Tumor cell surface HLA-I complex is required for the presentation of tumor-associated antigenic peptides to CTLs. Tumor cells escape immune attack by losing HLA-I expression. HLA-I abnormalities in tumor cells, with a frequency up to 90% in some types of tumor, have been well documented and are caused by distinct molecular mechanisms [3,4,7]. Accumulating experimental and clinical evidence suggests that the nature of HLA-I alterations predetermines cancer progression and clinical response to different types of immunotherapy [8]. Some HLA-I

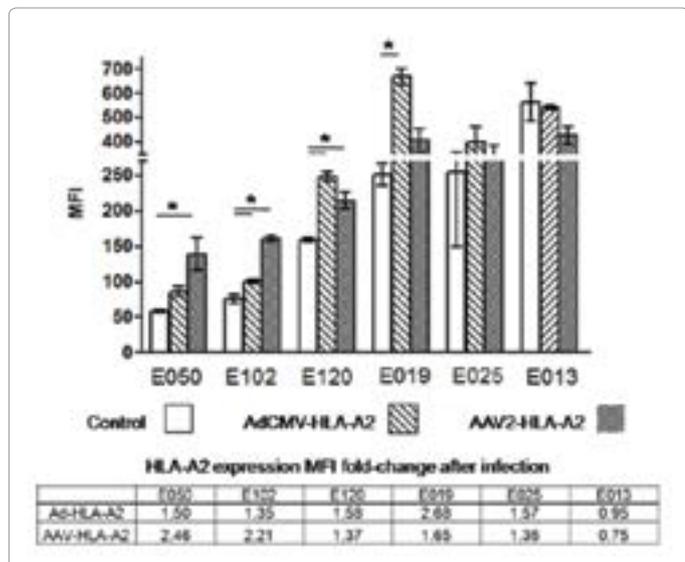


Figure 3: Virus-mediated upregulation of HLA-A2 expression on tumor cells positive for genomic HLA-A2 allele. In cell lines (E050, E102 and E120) with low HLA-A2 expression in baseline conditions (control) virus-mediated upregulation of HLA-A2 is greater than in high HLA-A2 expressing cells, as measured by FACS. Statistically significant differences were found in E050 after transduction with AAV2-HLA-A2 ($p=0,030$), E102 after transduction with AAV2-HLA-A2 ($p=0,001$) and AdCMV-HLA-A2 ($p=0,037$), and E120 after transduction with AAV2-HLA-A2 ($p=0,020$) and AdCMV-HLA-A2 ($p=0,005$). In cells with high endogenous baseline HLA-A2, the transduction efficacy varied, i.e. almost no transduction of E-013 cells, very little efficacy in E-025 cells, and statically significant higher level of transgene expression in E-019 cells, especially when adenoviral vector was used ($p=0,004$). The table summarizes the vector-induced fold-change in HLA-A2 expression on cells with low and high baseline HLA-A2 expression.

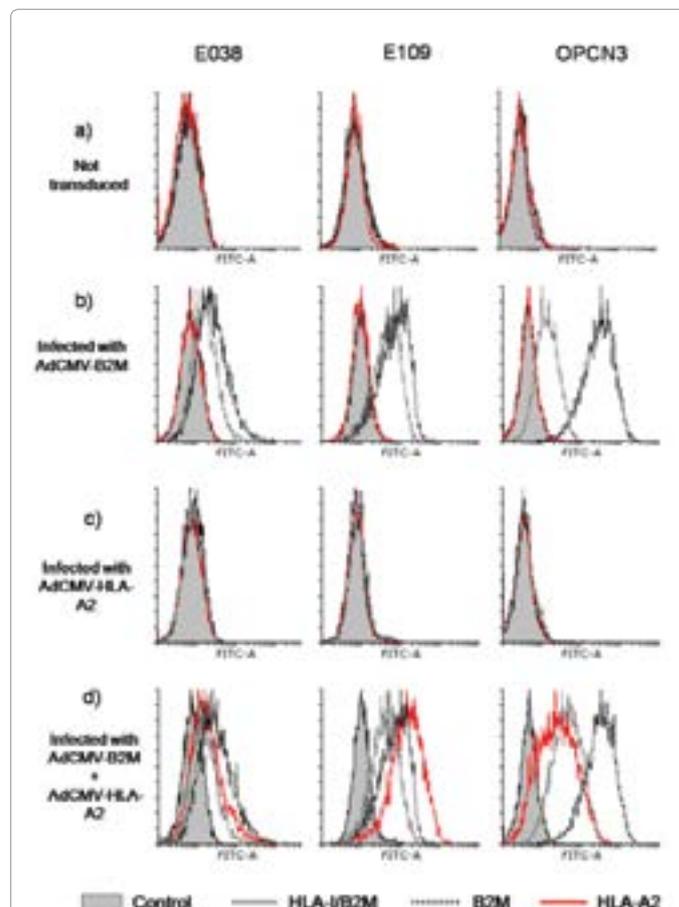
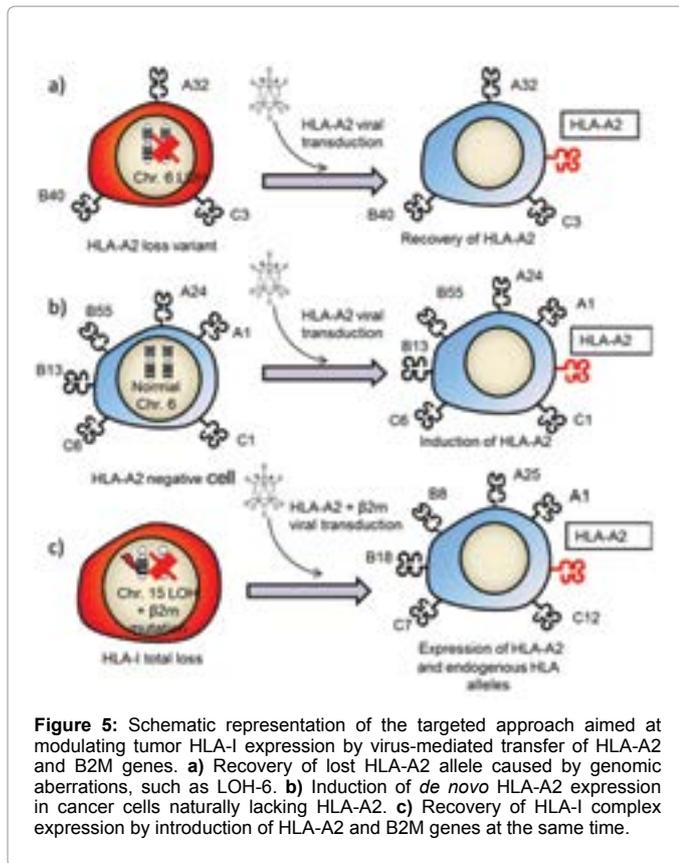


Figure 4: Recovery of tumor cell surface HLA-I expression by co-infection of B2M-deficient tumor cells negative for genomic HLA-A2 allele with two adenoviral vectors AdCMV-HLA-A2 and AdCMV-B2M. **a)** FACS plots demonstrating absence of HLA-A2 allele (CR-11 mAb), of B2M (L-368 mAb), and of cell surface HLA-I/B2M complex (w6/32 mAb) in baseline conditions. **b)** AdCMV-B2M-mediated recovery of B2M and HLA-I/B2M complex expression. **c)** Infection with AdCMV-HLA-A2 did not render cells positive for HLA-I/B2M, **d)** Co-infection with both vectors induced HLA-A2 and recovered HLA-I/B2M complex expression. Grey fill - control with only secondary antibody; red line-HLA-A2; grey line-HLA-I/B2M complex; and dotted line-B2M.



alterations can be reversed by cytokines (“soft” lesions) leading to regression of tumor lesion. There is an accumulating evidence indicating that different types of immunotherapy (including BCG, IL-2, IFN- α , autologous tumor vaccine, etc.) activate tumor microenvironment for cytokine production and induce HLA-I upregulation on tumor cells harboring “soft” HLA lesions [29]. In contrast, tumor cells with permanent HLA-I losses due to genetic defects (“hard” lesions) may fail to increase HLA-I expression in response to immunotherapy and eventually progress. Therefore, immunotherapy, in addition to curative anti-cancer effects, may promote immunoselection of tumor variants with irreversible structural aberrations in HLA-I molecules, creating mixed responses and resistance to therapy [30]. To circumvent this problem, development of a strategy to restore HLA-I expression on malignant cells is essential.

It is believed that the loss or decreased expression of only one HLA allele could be sufficient to promote tumor immune escape with a negative effect on the clinical efficacy of immunotherapy, because the presentation of many tumor-associated antigenic peptides is restricted to specific alleles. HLA-I heavy chain is highly polymorphic and some HLA alleles appear to be able to bind more epitopes than other ones. One particular allele, HLA-A2, is the most common HLA-I allele in Caucasian population with widest tumor-associated peptide repertoire, and its absence, as a part of commonly seen HLA-I haplotype loss, has been described in association with cancer progression and resistance to immunotherapy. In addition, it plays an important role in tumor rejection since the affinity to a large number of known cancer-associated epitopes recognized by CTLs is by far the highest for HLA-A*0201 allele [19]. Selective loss of HLA-A2 has been described in transformed cells [12], in some reports it had a negative impact on the presentation of

NY-ESO-1 peptide used for cancer immunotherapy [31,32]. Total loss of HLA class I expression is frequently caused by mutations/deletions in one copy of the B2M gene and loss of another copy in chromosome 15 due to LOH [14]. Loss of specific alleles is usually caused by LOH in chromosome 6p21 that generates HLA haplotype loss in various human tumors with high incidence [11,31].

In this study, we describe an experimental approach to recover tumor HLA-I loss using gene transfer by different recombinant viral vectors. Infection of cells with Ad5 or AAV2 coding for HLA-A2 gene resulted in restoration of HLA-A2 expression in tumor cells deficient for this allele due to a chromosomal loss. It also increased the expression of endogenous genomic HLA-A2 in cells with low baseline HLA-A2 level. In cells, naturally lacking HLA-A2 allele this viral transduction induced *de novo* expression of a new HLA-A2 allele in addition to the existing six HLA alleles. In addition, tumor cell co-transfection with Ad5 coding for HLA-A2 and B2M genes demonstrated that the *de novo* expressed proteins can form a detectable HLA-I/B2M complex on the tumor cell surface.

In earlier experimental systems, introduction of MHC class I genes into class I negative tumor cells led to higher immunogenicity and decreased tumorigenicity [33]. In some reports B2M transfection led to recovery of MHC class I expression and escape from natural killer cells [34], other publications describe the reversal of metastatic phenotype in murine carcinoma cells after transfection with syngeneic H-2 gene [33].

Advances in cancer gene therapy involving viral vectors coding for a wide range of therapeutic genes has offered novel tools for boosting anti-cancer immunity. Both adenoviral and adeno-associated vectors are widely used for transfer of a wide range of therapeutic genes (CD40L, p53, APM genes, different cytokines, etc.) aimed at boosting anti-cancer immunity both in experimental models and in clinical trials [35,36]. Most commonly used adenoviral vectors of type 5 serotype (Ad5) offer a number of advantages including a broad cellular tropism, efficient gene transfer, persistence of gene expression, low toxicity, and they do not integrate in host DNA [36]. On the other hand, Ad5 induce high immunological response by capsid proteins and hepatotoxic effects when administered systemically. We have reported previously that adenovirus-mediated recovery of B2M expression in tumor cells with genetic alterations in B2M gene recovers normal cell surface HLA class I expression and recognition by CTL [21,22].

Adeno-associated viruses (AAV) have emerged as promising vectors due to low pathogenicity, immunogenicity and toxicity, although they have small packaging capacity (up to 4.5 kb) [37] and lesser transfection efficacy than adenovirus. To date, AAV serotype 2 (AAV2) is the most commonly used AAV vector already used in clinical trials. Tumor cell *in vitro* transduction with different AAV serotypes of a variety of human cells has been reported, but there is still much controversy with reference to the transduction efficiency of AAV vectors in human malignant cells [38-40]. However, there are certain problems associated with viral vectors for cancer gene therapy. Among them for AAVs, the highly prevalent pre-existing humoral immunity to AAVs (especially to AAV2) and the AAV vector-induced neutralizing antibodies after gene therapy are two main obstacles that negatively impact the efficiency of gene delivery in cancer patients who need to receive repeated doses [41].

Here we report that AAV2 among other studied AAV serotypes has the highest GFP transduction efficiency in different human tumor cell lines, although, in general, inferior to adenovirus. The efficacy of Ad5 to drive a *de novo* expression of HLA-A2 allele was higher than that of AAV2. Using both these vectors, we achieved a recovery of HLA-A2

expression on A2-deficient Ando-2 melanoma cells, and an increase in HLA-A2 expression even in tumor cells already positive for endogenous genomic HLA-A2 allele. Importantly, a simultaneous transfer of both B2M and HLA-A2 genes into B2M-deficient/HLA-A2 negative tumor cells using Ad5 vector recovered cell surface HLA-I expression.

Increased HLA-I expression and tumor cell immunogenicity can be also achieved by the introduction of viral vectors encoding the peptide transporter TAP into APM-deficient tumor cells [42]. Other strategies to counteract tumor HLA-I deficiencies have been suggested, including the development of a genetic vaccine based on introduction of a membrane-attached B2M [43] or search for CTLs that are capable of recognizing HLA-I-low tumors [44]. Very promising approach involves the use of genetically modified T-cells that express chimeric antigen receptors (CARs) [45], in which case, unlike for TCR recognition, the antigen does not need to be processed and presented by HLA-I.

Given the impact of structural HLA-I alterations in the tumor resistance to immunotherapy, correction of antigen presentation in these tumor types using gene transfer methods may increase the efficacy of treatment. Recovery of HLA-I expression on tumor cells restores HLA-I-restricted, antigen-specific cytotoxic anti-tumoral CTL responses. Importantly, upregulation of HLA-I expression could lead to the presentation of a variety of previously hidden tumor specific peptides, which subsequently activate a pre-existing T-cell pool. Similar events were previously reported in clinical trials using peptide-based immunotherapy and were defined as 'epitope spreading' [46].

Here we describe a restoration/upregulation of HLA-A2 surface expression in different tumor cell lines by using a replication-deficient AdV5 or AAV2 vector that carry HLA-A2 gene. In addition, introduction of both HLA-A2 and B2M genes leads to an efficient association of both molecules into cell surface HLA/B2M complex, which gives an option of correcting simultaneously multiple defect causing HLA-I loss. Figure 5 summarizes different targeted approaches to recover optimal HLA-I expression on tumor cells.

Conclusion

This study provides information on optimization of the viral delivery system for future gene therapy studies targeting human cancer cells. Our results indicate that viral vectors carrying HLA-I and B2M genes represent a useful tool for recovering tumor HLA-I expression and can be used to increase rejection of tumors harboring structural genetic defects responsible for HLA-I loss. Tumor HLA-I upregulation may enable cancer biologists to better understand how to specifically target CTL-mediated immune responses and further improve therapeutic efficacy of cancer immunotherapy.

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