Universidad de Granada

Beta-2-microglobulin gene transfer in HLA class I deficient tumor cells using recombinant adenovirus

ANA BELÉN DEL CAMPO ALONSO

GRANADA, 2014

Editor: Editorial de la Universidad de Granada
Autor: Ana Belén del Campo Alonso
D.L.: GR 1845-2014
ISBN: 978-84-9083-028-4

Unidad Gestión Clínica de Laboratorio Hospital Virgen de las Nieves.

Departamento de Bioquímica, Biología Molecular III e Inmunología.

Universidad de Granada

Programa Oficial de Posgrado en Inmunología (P42.56.1)

Norma Reguladora RD 56/2005

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El doctorando Ana Belén del Campo Alonso y los directores de la tesis Federico Garrido Torres-Puchol, Gloria González-Aseguinolaza y Natalia Aptsiauri, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, a 29 de Noviembre de 2013.

Director/es de la Tesis

Fdo.: Federico Garrido Torres-Puchol

Doctorando

Fdo.: Ana Belén del Campo Alonso

Fdo.: Gloria González-Aseguinolaza

Fdo.: Natalia Aptsiauri

El trabajo de esta tesis doctoral ha sido dado a conocer mediante la publicación de dos artículos científicos, una revisión, además de un artículo recientemente envíado para su publicación, y la exposición de varias comunicaciones en congresos internacionales y nacionales.

1. Publicaciones:
   - del Campo AB, Carretero J, Muñoz JA, Zinchenko S, Ruiz-Cabello F, González-Aseguinolaza G, Garrido F, Aptsiauri N. **Adenovirus expressing β2m enhances antitumor immunity by increasing T-cell recognition without affecting the antigen-presentation machinery.** Submitted 2014.

   - del Campo AB, Kyte JA, Carretero J, Zinchencko S, Méndez R, González-Aseguinolaza G, Ruiz-Cabello F, Aamdal S, Gaudernack G, Garrido F, Aptsiauri N. **Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma.** International Journal of Cancer. 2014;134(1):102-13. doi: 10.1002/ijc.28338.

   - del Campo AB, Carretero J, Aptsiauri N, Garrido F. **Targeting HLA class I expression to increase tumor immunogenicity.** Tissue Antigens. 2012 ;79(3):147-54. doi: 10.1111/j.1399-0039.2011.01831.x.

   - del Campo AB, Aptsiauri N, Méndez R, Zinchenko S, Vales A, Paschen A, Ward S, Ruiz-Cabello F, González-Aseguinolaza G, Garrido F. **Efficient recovery of HLA class I expression in human tumor cells after beta2-microglobulin gene transfer using adenoviral vector: implications for cancer immunotherapy.** Scandinavian Journal of Immunology. 2009; 70, 125–135. doi: 10.1111/j.1365-3083.2009.02276.x.

2. Congresos Internacionales:
   - Targeting tumor HLA expression to increase the immunogenicity of cancer cells. ESGCT and SETGyC Collaborative Congress. Sociedad Española de Terapia Génica y Celular (SETGyC) and European Society of Gene and Cell Therapy (ESGCT). 25-38 October 2013, Madrid, Spain.

   - Analysis of the molecular mechanisms underlying HLA class I altered expression in human prostate cancer and in prostatic hyperplasia. EFI 25th EFI European Immunogenetics and Histocompatibility Conference Prague, Czech Republic 011 Tissue Antigens Volume 77, Issue 5, pages 370–513, May 2011.

   - The modulation of the antigen presentation machinery in human beta2m-deficient melanoma cell lines by adenoviral vectors. Second international

conference. Cancer immunotherapy &Immunomonitoring (CITIM). Budapest, Hungary, 2011.

- HLA class I and II antigen expression in human bladder cancer cell lines. Second international conference. Cancer immunotherapy &Immunomonitoring (CITIM). Budapest, Hungary, 2011.

- Analysis of HLA class I expression in metastatic lesions obtained from a melanoma patient before and after treatment with tumor-mRNA-transfected DCs: immunoselection of cells with beta2-microglobulin gene alterations. 24th European Immunogenetics and Histocompatibility (EFI) Conference & 17th Italian Society for Immunogenetics and Transplantation Biology Meeting. May 15-18, Florence Italy. 2010. Tissue Antigens. Volume 75, Issue 5, May 2010, Pages: 466–643.

- Immunoselection of HLA-class I-negative tumor cells with beta-2-microglobulin gene alterations in a melanoma patient undergoing cancer immunotherapy. Tenth International Conference on Progress in Vaccination Against Cancer. PIVAC-10, Cambridge, United Kingdom, 2010.

- The adenoviral vector AdCMVb2m restores the MHC class I antigens expression and T-cell recognition in a melanoma cell line carrying a novel mutation in beta 2-microglobulin gene. Tenth International Conference on Progress in Vaccination Against Cancer. PIVAC-10, Cambridge, United Kingdom, 2010.

- HLA class I and II antigen expression profiles in human prostate cancer cell lines and tumors. Tenth International Conference on Progress in Vaccination Against Cancer. PIVAC-10, Cambridge, United Kingdom, 2010.

- Succesful recovery of beta 2 microglobulin/HLA class I expression in vitro and in human xenograft tumor model using adenoviral vector. 23rd EFI conference, Ulm, Germany 9-12 Mayo 2009. Tissue Antigens. Vol 73, Mayo 2009: 513.

- Total HLA class I loss in a human melanoma cell line: identification of a mutation in beta 2-microglobulin gene and its correction using adenoviral vector (AdCMVbeta2m). 23rd EFi conference Ulm, Germany 9-12 Mayo 2009. Tissue Antigens. Vol 73, Mayo 2009: 513-514.

- Efficient recovery of HLA class I expression in human tumor cells after beta-2 microglobulin gene transfer using adenoviral vector: implications for cancer immunotherapy. 2nd European Congress of Immunology. Septiembre 13-16, 2009. Berlin, Germany. European Journal of Immunology. Vol 39 No S1-544 September 2009.
- Restoration of MHC class I antigens using adenoviral vector AdCMVb2m in one melanoma cell line with a novel mutation in beta 2 microglobulin gene.

First international conference. Cancer immunotherapy &Immunomonitoring (CITIM). Kiev, Ukraine, 2009.

- Functional analysis of the efficient recovery of HLA class I expression in human tumor cells alter beta-2 microglobulin gene transfer using adenoviral vector. Ninth International Conference on Progress in Vaccination Against Cancer. PIVAC 9. Sofia, Bulgary. 2009.

3. Congresos Nacionales.
   - Escape inmunológico de células con alteraciones irreversibles en el gen de la beta 2-microglobulina: Análisis de la expresión HLA de clase I en lesiones metastáticas obtenidas de un paciente de melanoma antes y después del tratamiento con DCs transfectadas con mRNA tumoral. XXXV Congreso de la Sociedad Española de Inmunología. Junio 23 al 26. San Sebastian 2010. Inmunología. Vol. 29 Supl. 1 Junio 2010.

   - Adenovirus-Mediated Restoration of Normal HLA class I expresión on Human Tumor Cells: Implications for Cancer Immunotherapy. V Congreso de la SETyC (Sociedad Española de Terapia Génica y Celular). Granada 30 de Septiembre al 2 de Octubre 2009. Human Gene Therapy. 20 (9)1061-1062.

4. Otras Publicaciones Relacionadas
   - Mendez R, Aptsiauri N, Del Campo A, Maleno I, Cabrera T, Ruiz-Cabello F, Garrido F, Garcia-Lora A. HLA and melanoma: multiple alterations in HLA class I and II expression in human melanoma cell lines from ESTDAB cell bank. Cancer Immunol Immunother. 2009 Sep;58(9):1507-15.

   - Méndez R, Rodríguez T, Del Campo A, Monge E, Maleno I, Aptsiauri N, Jiménez P, Pedrinaci S, Pawelec G, Ruiz-Cabello F, Garrido F. Characterization of HLA class I altered phenotypes in a panel of human melanoma cell lines. Cancer Immunol Immunother. 2008 May;57(5):719-29.

   - Rodríguez T, Méndez R, Del Campo A, Jiménez P, Aptsiauri N, Garrido F, Ruiz-Cabello F. Distinct mechanisms of loss of IFN-gamma mediated HLA class I inducibility in two melanoma cell lines. BMC Cancer. 2007 Feb 23;7:34.

   - Rodríguez T, Méndez R, Del Campo A, Aptsiauri N, Martín J, Orozco G, Pawelec G, Schadendorf D, Ruiz-Cabello F, Garrido F. Patterns of constitutive and IFN-gamma inducible expression of HLA class II molecules in human melanoma cell lines. Immunogenetics. 2007 Feb ;59(2):123-33.

   - Méndez R, Ruiz-Cabello F, Rodríguez T, Del Campo A, Paschen A, Schadendorf D, Garrido F Identification of different tumor escape mechanisms in several metastases from a melanoma patient undergoing immunotherapy. Cancer Immunol Immunother. 2007 Jan;56(1):88-94. Epub 2006 Apr 19.

La doctoranda Ana Belén del Campo Alonso, y los directores de la tesis Dra. Natalia Aptsiauri, Dra. Gloria González-Aseguinolaza, y Dr. Federico Garrido Torres-Puchol, declaran que la tesis doctoral con el título de "Beta-2-microglobulin gene transfer in HLA class I deficient tumor cells using recombinant adenovirus", se presenta como una agrupación de publicaciones que la doctoranda Ana Belén del Campo Alonso consiguió durante su formación como estudiante pre-doctoral.

A continuación se refieren dichas publicaciones, detallando el Factor de Impacto de las revistas científicas en las que fueron publicadas, según el Instituto para la Información Científica:

- International Journal of Cancer. 2014 Jan 1;134(1):102-13. doi: 10.1002/ijc.28338.2013. **Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma**. Del Campo AB, Kyte JA, Carretero J, Zinchencko S, Méndez R, González-Aseguinolaza G, Ruiz-Cabello F, Aamdal S, Gaudernack G, Garrido F, Aptsiauri N. **Impact Factor: 6.198.**
- Tissue Antigens 2012 Mar;79(3):147-54. doi: 10.1111/j.1399-0039.2011.01831.x. **Targeting HLA class I expression to increase tumor immunogenicity**. del Campo AB, Carretero J, Aptsiauri N, Garrido F. **Impact Factor: 2,753.**
- Scandinavian Journal of Immunology, 2009; 70, 125–135. doi: 10.1111/j.1365-3083.2009.02276.x. **Efficient recovery of HLA class I expression in human tumor cells after beta2-microglobulin gene transfer using adenoviral vector: implications for cancer immunotherapy**. del Campo AB, Aptsiauri N, Méndez R, Zinchenko S, Vales A, Paschen A, Ward S, Ruiz-Cabello F, González-Aseguinolaza G, Garrido F. **Impact Factor: 2,199.**

En Granada, a 29 de Noviembre de 2013

Firmado:

Doctoranda:

Ana Belén del Campo Alonso

Director de la Tesis:

Natalia Aptsiauri

Director de la Tesis:

Gloria González-Aseguinolaza

Director de la Tesis:

Federico Garrido Torres-Puchol

This work has been possible thanks to European Comission [Fifth Framework Program: European Searchable Tumor Cell Line Database Proyect (QLRI-CT-2001-01325) and Sixth Framework Program: Proyecto Europeo ENACT (European Network for the identification and validation of Antigens and biomarkers in Cancer and their application in clinical Tumor immunology (OJ2004/C158, 518234)] and the Institute of Health Carlos III (Plan Nacional I+D+I 2008-2011: CM08/00252; PI-0386/2008**;** RD06/0020/0030; 04/00245; FIS-PI 081265). I am also grateful to the Andalusian Council of Innovation, Science and Technology from Regional Government of Andalusia- Junta de Andalucía (Proyecto de Investigación de Excelencia CTS-3952**,** CVI-4740 y RD 06/0020), the Andalusian Health Service (IP 0382/2009) and the Andalusian Plan for Research, Development and Innovation (Group CTS 143).

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1. Chapter 1. Abstract

Antitumor immune response and the success of cancer immunotherapy depend on the proper recognition of the tumor HLA class I complex (HLA class I heavy chain/ β2-microglobulin/peptide) by cytotoxic T-cells. It has become apparent that lack of tumor rejection is the result of immune selection and escape of tumor cells that develop low immunogenic phenotype. One of the central mechanisms of immune evasion is associated with the outgrowth of HLA class I-negative tumor cells. Altered expression of tumor HLA class I molecules is a frequent event described in almost all types of cancer and often is associated with metastatic dissemination, poor prognosis and resistance to immunotherapy. In some cases, up to 90% of tumor cells have HLA class I altered phenotypes. The molecular mechanisms underlying these alterations vary and can occur at any step required for HLA synthesis, assembly, transport or expression on cell surface. These defects can occur at the genetic, epigenetic, transcriptional and posttranscriptional levels and represent either regulatory abnormalities, which can be recovered with cytokine treatment, or structural alterations, including genetic/chromosomal defects. Both reversible/regulatory and irreversible/structural defects of HLA class I have been described in a variety of human tumor cell lines, in primary tumors and metastatic lesions, and in animal models of cancer. However, the structural defects may have more serious implications on T-cell-mediated tumor rejection and, ultimately, on the outcome of cancer immunotherapy.

The prevalence of structural genetic HLA defects in tumor cells has been proposed as a key factor that determines the inability of T lymphocytes to destroy malignant cells during immunotherapy. Therefore, such cells and tumors should be considered for gene therapy to restore normal expression of damaged HLA genes.

In this study we provide evidence supporting the accumulation of HLA class I loss in metastatic melanoma and immune escape of HLA-negative tumor cells. We also describe the construction and characterization of an adenoviral vector coding for β2m microglobulin that effectively restores tumor HLA class I expression.

Currently, only limited data are available on the changes in the tumor HLA expression during natural cancer progression, metastatic dissemination and in the course of cancer immunotherapy. This is due to the difficulties in obtaining successive tumor

samples from the same patient during cancer progression and in the course of immunotherapy, what constitutes a major shortcoming in the monitoring of the tumor HLA class I alterations during metastatic growth and in response to immunotherapy.

In this study, we have been able to illustrate the immune escape of HLA class I negative tumor cells and chronological sequence of appearance of β2m gene mutation in successive lesions obtained from a patient with metastatic melanoma resistant to DC vaccination. We observed a gradual decrease in HLA expression in consecutive lesions with few β2m-negative nodules in a primary tumor, followed by immune selection and outgrowth of β2m-negative melanoma cells that emerge in a tumor lesion at later stages of the disease. We detected in the β2m-negative nodules an early onset of β2m loss due to a combination of a novel mutation at codon 67 of exon 2 of the β2m gene, and loss of the second allele by LOH at chromosome 15. It is likely that tumor cells with HLA class I alterations in this patient first escaped from the immune attack during natural metastatic progression and that HLA-negative immune escape variants were further immunoselected during the vaccination, which might explain the failure of the therapy as the gradual decrease in HLA expression correlated with decrease T-cell infiltration ($CD8^+$).

Thus, strategies to overcome the lack of HLA class I expression require consideration and targeting of β2m as an attractive option to recover HLA class I expression in β2m-negative tumor cells in patients with metastatic progression or recurrent tumors. For this purpose, we have constructed a replication-deficient adenoviral vector carrying human β2m gene (AdCMVβ2m) and characterized its efficacy to recover HLA class I expression using different human cancer cell lines with structural defects in β2m.

We show that *in vitro* transduced tumor cells become sensitive to lysis by peptide-stimulated HLA-restricted T-cells, and recover the ability to induce peptide-specific IFNγ secretion by T cells in a HLA-restricted manner without compromising the antigen processing and presentation.

In *in vivo* experiments using human tumour xenograft model, the intratumoral injection of AdCMVβ2m also led to restoration of normal HLA class I expression.

Our data support the clinical application of such vector in patients with metastatic cancer with structural defects in β2m gene and/or LOH in chromosome 15, as in such patients, the immunotherapy might lead to generation of metastatic lesions with irreversible defects in HLA class I expression that would not respond to therapy and eventually would progress.

Our findings emphasize the importance of carefully defining the molecular mechanisms responsible for a particular altered HLA class I phenotype to design specific ways to restore in situ normal tumor HLA class I expression. Therefore, the optimization of the existing immunotherapy approaches will greatly benefit from the characterization of the HLA class I alterations in primary tumors and from the analysis of the correlation of impaired HLA expression with metastatic progression.

1. CHAPTER 1. RESUMEN

La resupuesta immune antitumoral y el éxito de la inmunoterapia contra el cáncer dependen del reconocimiento adecuado de la expresión tumoral del complejo HLA de clase I (cadena pesada HLA de clase I/β2-microglobulina/péptido) por las células T citotóxicas. Se ha evidenciado que la falta de rechazo tumoral es el resultado de la selección inmune y el escape de las células tumorales que desarrollan fenotipos poco inmunogénicos. Uno de los mecanismos centrales de evasión inmune está asociado con la diseminación de células tumorales HLA de clase I negativas. La expresión alterada en el tumor de moléculas HLA de clase I es un evento frecuente descrito en casi todos los tipos de cáncer y a menudo está asociado con la diseminación metastática, mal pronóstico y resistencia a la inmunoterapia. En algunos casos, hasta el 90% de células tumorales poseen un fenotipo alterado de HLA de clase I. Los mecanismos moleculares subyacentes a estas alteraciones varían, y pueden tener lugar en cualquier paso requerido en la síntesis de HLA, ensamblaje, transporte o expresión en la superficie celular. Estos defectos pueden ocurrir a nivel genético, epigenético, transcripcional y post-transcripcional, y representan tanto alteraciones regulatorias, que puden ser revertidos mediante tratamiento con citoquinas, como irregularidades estructurales, incluyendo defectos genéticos/cromosómicos. Los defectos de HLA de clase I reversibles/regulatorios e irreversibles/estructurales han sido descritos en una gran variedad de líneas celulares tumorales humanas, en tumores primarios, lesiones metastáticos, y en modelos animales de cáncer. Sin embargo, los defectos estructurales pueden tener implicaciones más severas en el rechazo tumoral mediado por células T y, a la larga, en el resultado de la inmunoterapia del cáncer.

La prevalencia de defectos genéticos estructurales de HLA en las células tumorales se ha propuesto como un factor clave que determina la incapacidad de los linfocitos T para destruir las células malignas durante la inmunoterapia. Por lo tanto, se debe considerar en dichas células y tumores el uso de terapia génica para restaurar la expresión normal de los genes HLA dañados.

En este studio, proporcionamos evidencias que corroboran la acumulación de la pérdida de HLA de clase I en melanoma metastático y el escape inmune de células

tumorales HLA-negativas. También describimos la construcción y caracterización de un vector adenoviral que codifica el gen de beta-2-microglobulina (β2m) y restaura efectivamente la expresión tumoral de HLA de clase I.

Actualmente, sólo hay escasos datos disponibles sobre los cambios en la expresión tumoral de HLA durante la progresión natural del cáncer, diseminación metastática y en el curso de la inmunoterapia tumoral. Esto es debido a las dificultades en la obtención de muestras tumorales sucesivas procedentes de un mismo paciente durante la progresión tumoral y el tratamiento de inmunoterapia, lo que constituye una deficiencia importante en la monitorización de las alteraciones tumorales de HLA de clase I durante el crecimiento metastático y la respuesta a la inmunoterapia.

En este studio, hemos sido capaces de ilustrar el escape immune de células tumorales HLA de clase I negativas y la secuencia cronológica de aparición de la mutación del gen β2m en lesiones sucesivas obtenidas de un paciente con melanoma metastático resistente a la vacunación de células dendríticas. Observamos una disminución gradual en la expresión de HLA en lesiones consecutivas con pocos nódulos β2m-negativos en un tumor primario, seguido por la selección inmune y diseminación de células de melanoma β2m-negativas que emergen en una lesión tumoral en fases posteriores de la enfermedad. Detectamos en los nódulos β2m-negativos el establecimiento temprano de la pérdida de β2m debido a la combinación de una nueva mutación en el gen β2m, en el codon 67 del exón 2, y la pérdida del segundo alelo por pérdida de heterozigosidad del cromosoma 15. Es probable que las células tumorales con alteraciones de HLA de clase I en este paciente escapen primero el ataque inmunológico durante la progresión natural metastática, y que las variantes de escape inmune HLA-negativas fueran posteriormente inmunoseleccionadas durante la vacunación, lo que podría explicar el fallo de la terapia, así como la disminución gradual de la expresión de HLA correlacionada con la disminución de la infiltración de células T (CD^+).

Así, se requiere considerar estrategias que superen la falta de expression de HLA de clase I y se focalicen en β2m como una opción atractiva para recuperar la expresión de HLA de clase I en células tumorales β2m-negativas en pacientes con progresión metastática o tumores recurrentes. Con este propósito, hemos construído un vector

adenoviral deficiente para su replicación portando el gen humano de β2m (AdCMVβ2m) y caracterizado su eficacia para recuperar la expresión de HLA de clase I usando diversas líneas celulares tumorales humanas con defectos estructurales en β2m.

Mostramos que células tumorales transducidas *in vitro* se vuelven sensibles a la lisis por células T HLA-restringidas y estimuladas por péptido, y recuperan la habilidad de inducir la secreción péptido-específica de IFNγ por células T de forma HLA-restringida sin comprometer la presentación y procesamiento antigénico.

En experimentos *in vivo* usando modelos xenográficos de tumores humanos, la inyección intratumoral de AdCMVβ2m conduce también a la restauración de la expresión normal de HLA de clase I.

Nuestros datos apoyan la aplicación clínica de dicho vector en pacientes con tumores metastáticos con defectos estructurales en el gen de β2m y/o pérdida de heterocigosidad del cromosoma 15, ya que en dichos pacientes, la inmunoterapia podría conducir a la generación de lesiones metastáticos con defectos irreversibles en la expresión de HLA de clase I que no responderían a la terapia y progresarían eventualmente.

Nuestros resultados enfatizan la importancia de definer cuidadosamente los mecanismos moleculares responsables de un fenotipo concreto de HLA de clase I alterado para diseñar formas específicas para recuperar *in situ* la expresión tumoral normal de HLA de clase I. Por lo tanto, la optimización de los protocolos de inmunoterapia existentes se beneficiará ampliamente de la caracterización de las alteraciones de HLA de clase I en los tumores primarios y del análisis de la correlación de la expresión alterada con la progresión metastática.

2. Chapter 2. General introduction

2.1. IMMUNE RECOGNITION OF CANCER

In Europe during 2012 there were an estimated 3.45 million new cases of cancer (excluding non-melanoma skin cancer) and 1.75 million deaths from cancer, among which the types of cancer with most frequent lethal outcome are lung (353,000 deaths), colorectal (215,000), breast (131,000) and stomach (107,000), while the most frequent cancers are breast (464,000 cases), followed by colorectal (447,000), prostate (417,000) and lung cancer (410,000) (Ferlay *et al*, 2013).

Already in 1909, Paul Ehrlich proposed that the incidence of cancer would have been much greater if not for the vigilance of the immune defense system in identifying and eliminating nascent tumor cells (Ehrlich, 1909). Years later, Burnet and Thomas proposed in the "cancer immunosurveillance" hypothesis that immune cells identify and eradicate transformed cells (Burnet, 1970; Burnet, 1957; Burnet, 1964; Thomas, 1959). Although some data corroborated this hypothesis (Klein, 1976; Old & Boyse, 1964), it was rejected until the 1990s, when improved mouse models of immunodeficiency showed that tumors formed in the absence of an intact immune system are, as a group, more immunogenic than tumors that arise in immunocompetent hosts (Engel *et al*, 1997; Kaplan *et al*, 1998; Shankaran *et al*, 2001). Since then, the use of different mouse models has demonstrated that numerous immune effector cells and pathways are important for the suppression of tumor development (Swann & Smyth, 2007; Vesely *et al*, 2011).

A fundamental point of cancer immunosurveillance is that cancer cells express antigens to be differentiated from their non-transformed counterparts. Their existence was first demonstrated in mice (Old & Boyse, 1964) and later in humans (van der Bruggen *et al*, 1991). Since then, many tumor associated antigens (TAA) have been identified (Boon *et al*, 1994) and classified into several groups recognized by cytotoxic T lymphocytes (CTL) on human tumors (Boon & van der Bruggen, 1996; Novellino *et al*, 2005; Vigneron *et al*, 2013).

HLA molecules play a crucial role in the interaction of tumor cells with the host immune system as they present TAAs to CTLs and regulate the cytolytic activity of

natural killer (NK) cells. There are two classes of HLA molecules: HLA class I molecules (HLA-I) that present endogenously synthesized antigens (e.g., tumor associated antigens) to $CD8^+$ cytotoxic T lymphocytes (CTLs), and HLA class II molecules that present exogenously derived proteins (e.g., bacterial products) to $CD4^+$ helper T lymphocytes (Benacerraf, 1981). Both HLA class I and class II antigens are key molecules for antigen presentation and activation of antitumor immune response (Cresswell *et al*, 2005; Jensen, 2007).

CTLs can recognize the TAA by T-cell receptors (TCR) only in a complex with the self-HLA molecule. When this recognition occurs in conjunction with costimulatory molecules, the CTLs can become activated and lead to apoptosis of TAA-expressing cancer cells. NK cells through their killer inhibitory receptor molecules recognize HLA-I negative, and, when they engage their ligand, NK cell-mediated lysis is inhibited.

With accumulation of new knowledge on tumor-host interaction, the theory of "cancer immunesurveillance" evolved into a concept of "cancer immunoediting", stressing the dual host-protective and tumor-sculpting actions of immunity on developing tumors (Dunn *et al*, 2002). Cancer immunoediting hypothesis establishes three dynamic phases (Schreiber *et al*, 2011), the first of which, elimination, corresponds to the concept of immunosurveillance, in which innate and adaptive immunity detect the presence of a developing tumor and destroy it before it becomes clinically apparent (Smyth *et al*, 2006; Vesely *et al*, 2011). In instances in which tumor cell destruction is complete, the elimination phase represents an endpoint of cancer immunoediting. However, in other cases, a percentage of transformed cells persist in a period of latency, named the "equilibrium phase", in which a continuous sculpting of tumour cells leads to the immune selection of those with reduced immunogenicity which are better suited for survival in the immuno-competent host and become resistant to immune effector cells (Koebel *et al*, 2007; Teng *et al*, 2008). In the third phase, "immune escape", some of the tumor cell variants emerged from the equilibrium phase develop the capacity to grow in an immunologically intact environment (Restifo *et al*, 2002). Therefore, the escape phase represents the failure of the immune system to eliminate or to control transformed cells; moreover, the immune system contributes to

tumor progression by selecting more aggressive tumor variants, suppressing the antitumor immune response, or promoting tumor cell proliferation (Dunn *et al*, 2004b). In the third phase, escape, some of the tumor cell variants emerged from the equilibrium phase develop the capacity to grow in an immunologically intact environment (Restifo *et al*, 2002). Therefore, the escape phase represents the failure of the immune system to eliminate or to control transformed cells; moreover, the immune system contributes to tumor progression by selecting more aggressive tumor variants, suppressing the antitumor immune response, or promoting tumor cell proliferation (Dunn *et al*, 2004b).

2.2. TUMOR IMMUNOESCAPE MECHANISMS

There is a multitude of mechanisms by which tumors can evade immune responses what makes essential a better understanding of the manifold interactions between tumors and the immune system to improve current immunotherapy strategies. Many studies have documented that tumor escape can be a direct consequence of alterations occurring in tumor cells affecting different levels of the immune system (Dunn *et al*, 2002; Dunn *et al*, 2004a; Dunn *et al*, 2004b; Khong & Restifo, 2002; Marincola *et al*, 2000). Thus, by generating the appropriate environment, tumors can skew the immune response in such a way that their growth, rather than their elimination, is favored. These tumor immunoescape mechanisms can be categorized generally as cell-autonomous modifications at the level of the tumor cell that directly evade immune detection and destruction, or modifications in immune cells effected by tumor cells to generate an immunosuppressive network, as summarized in Table 1.

MODIFICATIONS AT THE LEVEL OF THE TUMOR CELL	
Loss of tumor antigen expression	(Berset *et al*, 2001; Jager *et al*, 1996; Khong *et al*, 2004).
Defects in antigen-processing and presentation pathways: -Loss or downregulation of HLA class I molecules and/or β2m. -Loss or downregulation of APM molecules: TAP1, LMP2, LMP7, and tapasin	(Aptsiauri *et al*, 2007; Garrido *et al*, 2010a; Marincola *et al*, 2000; Meissner *et al*, 2005; Restifo *et al*, 1996; Seliger *et al*, 2001).

Resistance of tumor cells to IFN-γ or IFN-α/β: mutation or epigenetic silencing of genes encoding the IFN-γ receptor signaling components (IFNGR1, IFNGR2, JAK1, JAK2, and STAT1)	(Dunn *et al*, 2005; Kaplan *et al*, 1998; Respa *et al*, 2011; Rodriguez *et al*, 2007b).
Mechanisms that provide tumors with the ability to escape immune destruction -Upregulating inhibitors of apoptosis (Bcl-XL, FLIP) -Expressing inhibitory cell surface molecules that directly kill cytotoxic T cells (PD-L1, FasL) -Release of pro-apoptotic factors [TRAIL receptor, DR5, and Fas B7-H1 (PD-L1)] that inhibit local antitumor T cell responses	(Catlett-Falcone *et al*, 1999; Dong *et al*, 2002; Hinz *et al*, 2000; Kataoka *et al*, 1998; Schreiber *et al*, 2011; Takahashi *et al*, 2006; Whiteside, 2002; Zou *et al*, 2007).
TUMOR-INDUCED MODIFICATIONS IN IMMUNE CELLS	
Tumor-derived immunosuppressive factors that inhibit effector immune cell functions: -Transforming growth factor-β (TGF-β), IL-10 -Vascular endothelial growth factor (VEGF) -Metabolic enzymes such as indoleamine 2,3 dioxygenase (IDO) and arginase -Gangliosides, soluble MICA -Factors that recruit regulatory cells: IL-4, IL-13, GM-CSF, IL-1β, VEGF, or PGE2 Colony-stimulating factors, IL-1β, VEGF, or PGE2 that increase an accumulation of MDSCs	(Aruga *et al*, 1997; Gabrilovich *et al*, 1998; Herber *et al*; Khong & Restifo, 2002; Uyttenhove *et al*, 2003; Villablanca *et al*; Wrzesinski *et al*, 2007; Zitvogel *et al*, 2006).
Mechanisms that prevent tumor cell recognition by NK cells or CTLs: -Loss of ligands for NK cell effector molecules (as NKG2D) -Secretion of soluble ligands (as NKG2D) -Tumor cell expression of HLA-E or HLA-G -Lack of expression of co-stimulatory molecules on malignant cells Signaling defects through TCR-decreased expression of CD3ζ or tyrosine kinases	(Aoe *et al*, 1995; Fruci *et al*, 2013; Groh *et al*, 2002; Pietra *et al*, 2013; Swann & Smyth, 2007).
The accumulation of regulatory cells decrease antitumor response through: -Release of immunosuppressive cytokines, including IL-10 and TGF-β -Altering the nutrient content of the microenvironment -Inhibit effectors T cells through expression of CTLA-4 and PD-L1 IL-2 consumption	(Frey & Monu, 2006; Sakaguchi *et al*, 2001; Terabe & Berzofsky, 2004; Vesely *et al*, 2011; Zitvogel & Kroemer, 2012).
Recruitment and polarization of MDSCs from myeloid precursors that can block T cell function by expressing TGF-β, ARG1, iNOS, and IDO.	(Frey & Monu, 2006; Umansky&Sevko 2013b; Vesely *et al*, 2011).

Table 1. Various types of cancer immune escape mechanisms. Modified from (Aptsiauri *et al*, 2013) .

2.3. HLA CLASS I MOLECULES

2.3.1. HLA class I genes and molecules

Human Leukocyte Antigen (HLA) molecules are named this way because of their first discovery through antigenic differences among white blood cells from different individuals (Dausset, 1958). Their homologues in mice are called Major Histocompatibility Complex (MHC) after their discovery in transplantation studies (Gorer, 1936; Gorer, 1937; Snell, 1952). HLA-I molecules are glycoproteins found on the surface of virtually all nucleated cells, composed of two noncovalently associated polypeptide subunits (Cresswell *et al*, 1973): a polymorphic chain of 45 kDa (heavy chain or α chain) and a non-polymorphic protein of 12 kDa, called β2-microglobulin (β2m) (light chain or β chain) (Grey *et al*, 1973).

The genes encoding heavy chain are located on the short arm of chromosome 6 in humans (6p21.3) (Breuning *et al*, 1977) and are divided into three regions (Figure 1): class I, II, and III (Le Bouteiller, 1994). Class I molecules comprise the highly polymorphic classical human leukocyte antigens (HLA)-A, -B, and –C (class Ia), and the nonclassical E, F, G, and H (class Ib) (Bjorkman *et al*, 1987). Each gene encode an α-chain of a class I molecule. The extracellular region of the heavy chain shapes the peptide binding groove where the 8-10 amino acid antigenic peptide processed from endogenously degraded proteins is bound.

The light chain, β2m, is a member of the immunoglobulin gene superfamily (Peterson *et al*, 1972) and its gene is located on chromosome 15 (15q21-q22.2) in humans (Goodfellow *et al*, 1975). The complete mRNA of β2m consists of 945 nucleotides, excluding the poly(A) tail, and 4 exons that encode a protein of 119 amino acids in length. The sequence similarity between mouse and human in coding regions is about 72% (the same as the amino acid similarity, 70%) (Gussow *et al*, 1987).

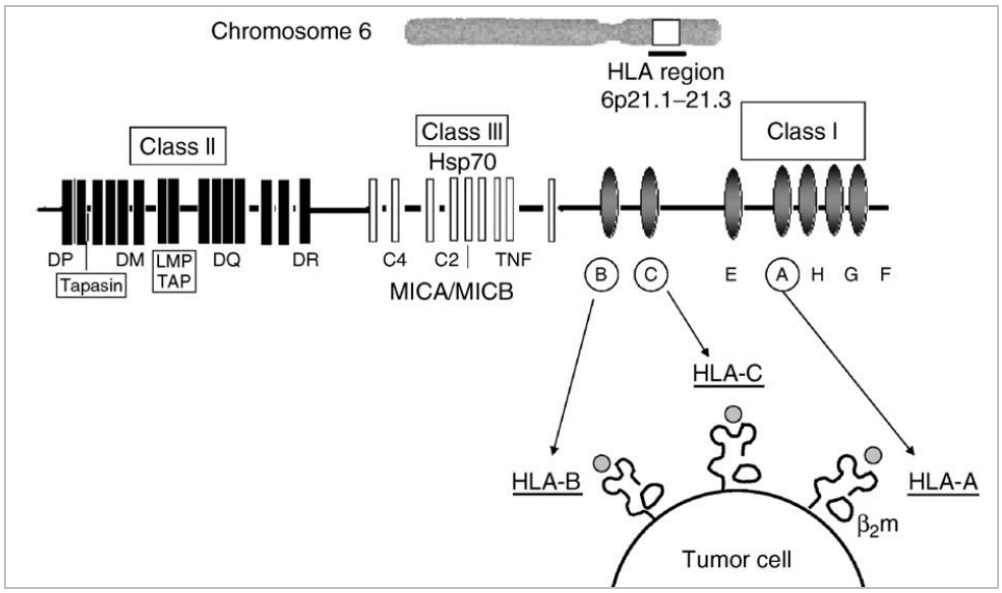


Figure 1. Gene map of the HLA region in chromosome 6p. It includes genes encoding class I (heavy chain), class II, and class III molecules. HLA class I region contains genes of the classical HLA class I molecules (HLA-A, -B, and -C). The HLA class II region comprises HLA class II molecules and also has genes for proteasome subunits (LMP2/7) and TAP1/2 subunits. The class III region contains many genes encoding proteins that regulate the immune response and complement proteins. Genes encoding nonclassical HLA-I molecules MICA and MICB are also located in this region. Figure reproduced from (Aptsiauri *et al*, 2007).

2.3.2. HLA class I antigen processing, transport, and assembly.

The proper HLA-I surface antigen expression necessary for the presentation of self/non-self antigens to $CD8^+$ CTLs (Vyas *et al*, 2008), is mediated by different molecular processes and involves a number of distinct molecules (Cresswell *et al*, 2005) (Figure 2). Shortly after or during synthesis in the endoplasmic reticulum (ER), heavy chain is bound by and with the thiol oxidoreductase ERp57 and calnexin (David *et al*, 1993; Degen & Williams, 1991), a chaperone which stabilizes and protect it from degradation (Williams, 1995). Upon binding of β2m, calnexin is released and replaced by calreticulin (Sadasivan *et al*, 1995) creating a multimeric complex called the peptide loading complex (PLC), which enhances the supply of peptides into the ER, and facilitates their binding onto HLA-I molecules. Seven proteins conform its structure:

two peptide transporters associated with antigen processing (TAP1 and TAP2), which translocate class I peptide ligands from the cytosol into the ER (Androlewicz *et al*, 1993; Neefjes *et al*, 1993), the class I-specific accessory molecule tapasin, the chaperone calreticulin (Sadasivan *et al*, 1996), ERp57 (Morrice & Powis, 1998), and the heavy chain/β2m dimmer.

The majority of antigenic peptides are derived from intracellular proteins that are degraded via the multicatalytic proteasome complex. The peptides are translocated, via TAP1 and TAP2 subunits, from the cytosol into the lumen of the ER and, if necessary, they are trimmed by ER associated aminopeptidases (ERAAP or ERAP1) to 8–10 amino acids (Bukur *et al*, 2012). The activity of the pathway could be modified by interferon IFN-γ generating the immunoproteasome, that contains novel active subunits as the low molecular weight proteins LMP2 and LMP7 (Gaczynska *et al*, 1993).

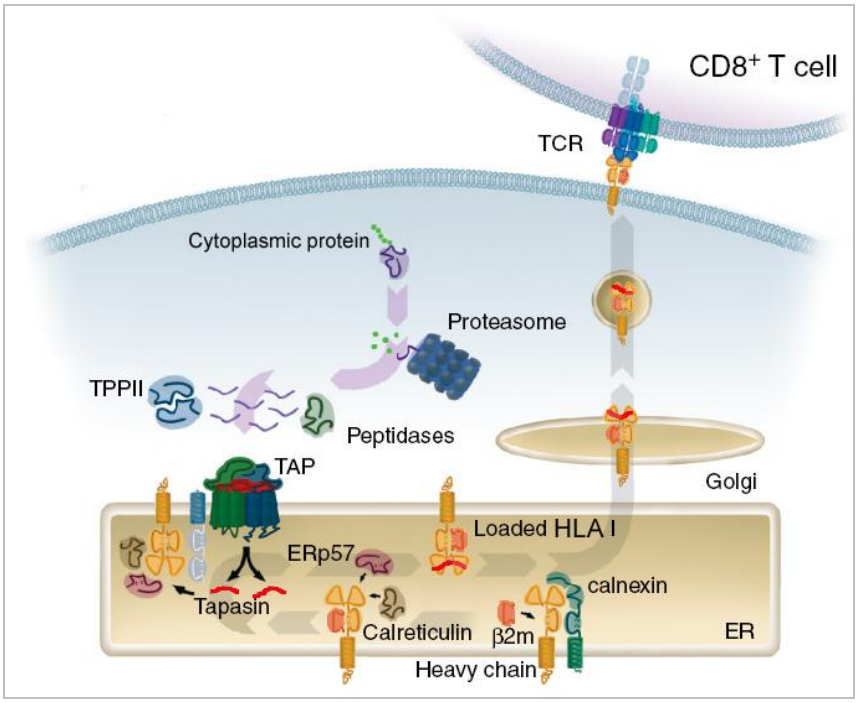


Figure 2. HLA class I antigen processing and presentation to CTL. Ubiquitinated proteins are degraded by the proteasome (or immunoproteasome) and peptidases into peptides which are translocated into the endoplasmic reticulum (ER) by TAP1 and TAP2. In the ER peptides are further trimmed by ER aminopeptidases and bind to HLA-I-β2m complex with the help of the

chaperone tapasin. The HLA class I-β2m complex formation in the ER is done with chaperons ERp57, calnexin, and calreticulin. The stable HLA-I- β2m complex is subsequently transported via the Golgi system to the cell surface, where peptides are presented for recognition by the cytotoxic $CD8^+$ cells via TCR. Figure modified from (Groothuis et al, 2005).

β2m has been demonstrated to be also a chaperone-like molecule for HLA-I folding. The association of heavy and light chain is a pre-requisite for cell-surface expression of the complex (Ploegh *et al*, 1979; Rein *et al*, 1987) and in the absence of β2m, most HLA-I molecules are not expressed efficiently on the cell surface (Seong *et al*, 1988; Zijlstra *et al*, 1990). Once peptides are bound, stable trimmers of heavy chain/β2m/peptide are released from the loading complex and exported to the cell surface via the trans-Golgi and displayed on the surface.

2.3.3. HLA class I alterations.

Alterations in tumor HLA class I expression can occur at any step of protein expression, transcriptional regulation, antigen processing, assembly or transport to the cell surface. In any case it results in the loss or downregulation of HLA class I molecules on tumor cell surface leading to the loss of TAA presentation to the CTLs, which could lead to tumor progression.

It has been known for many years that tumours exhibit altered expression of HLA-I molecules (Festenstein, 1987; Hellstrom, 1960; Klein *et al*, 1960). Changes in HLA profiles were described even before the role of HLA molecules in antigen presentation and in cancer immune escape was characterized (Garrido *et al*, 1976). The production and characterization of monoclonal antibodies against HLA and other cell surface antigens made it possible to analyze HLA expression in human tumor cell lines leading to the significant finding that human tumors also displayed altered HLA phenotypes (Festenstein & Schmidt, 1981; Pellegrino *et al*, 1977; Pollack *et al*, 1980). Altered HLA class I expression is extensively documented in primary tumors and metastases (Aptsiauri *et al*, 2007; Bukur *et al*, 2012; Garrido *et al*, 1993; Garrido *et al*, 1997;

Hicklin *et al*, 1999; Khong & Restifo, 2002) and it has been described that HLA-I loss occur when the tumor became invasive, and in tumors of a more aggressive histological type (Garrido et al, 1993; Petersen et al, 1993; Torres et al, 1993). The presence of altered HLA-I phenotypes in primary tumours of distinct histological origin has been described with different frequency: 90.2% in prostate carcinoma (unpublished data), 90% in cervical cancer (Koopman et al, 2000), 88.5% in breast carcinoma (Cabrera et al, 1996), 77% in laryngeal (Maleno et al, 2002) and bladder tumours (Maleno et al, 2006), and 74% in colorectal tumours (Maleno *et al*, 2004a).

Loss or downregulation of HLA class I antigens in tumor cells represents an important cancer immune escape mechanism (Drake *et al*, 2006; Garrido *et al*, 1997; Marincola *et al*, 2000) and deficient HLA-I surface expression has been reported to have clinical relevance, since in some types of cancer is associated with advanced tumor grade, stage, progression and a reduced patients' survival (Carretero *et al*, 2008; Garrido *et al*, 2011; Kasajima *et al*, 2010; Reinis, 2010). Alterations in HLA-I expression have been correlated with worth prognosis in head and neck squamous cell carcinoma (Bandoh *et al*, 2010), breast cancer (de Kruijf *et al*, 2010a; Morabito *et al*, 2009) ovarian cancer (Shehata *et al*, 2009), colorectal cancer (Kloor *et al*, 2010), renal cell carcinoma (Kitamura *et al*, 2007), medulloblastoma (Smith *et al*, 2010), and bladder cancer (Homma *et al*, 2009). However the prognostic value of HLA-I antigen expression phenotype or its association with patient outcome remains controversial, since in some cancers, like breast carcinoma and non-small-cell lung cancer lacks HLA-I expression has been associated with good prognosis (Madjd *et al*, 2005; Ramnath *et al*, 2006). Some studies have failed to show a correlation between HLA expression and patient prognosis (Chang *et al*, 2003; Marincola *et al*, 2000; Powell *et al*, 2012).

The analysis of HLA-I alterations have lead to their classification according to the cell surface expression pattern and mode of expression (Garrido *et al*, 1997). Seven major altered HLA-I phenotypes have been described in different tumor tissues (Garcia-Lora *et al*, 2003a; Garrido & Algarra, 2001) (Figure 3): phenotype I (HLA-I total loss or downregulation); phenotype II (HLA haplotype loss); phenotype III (HLA A, B, or C locus downregulation); phenotype IV (HLA allelic loss); phenotype V (compound

phenotype); phenotype VI (resistance to stimulation with interferons); phenotype VII (a downregulation of classical HLA A– B–C molecules coincides with the appearance of HLA-E molecules). Among HLA alterations, phenotype I is a common altered phenotypes accounting for 11-25% of the cases (Rodriguez *et al*, 2007a; Aptsiauri et al, 2013).

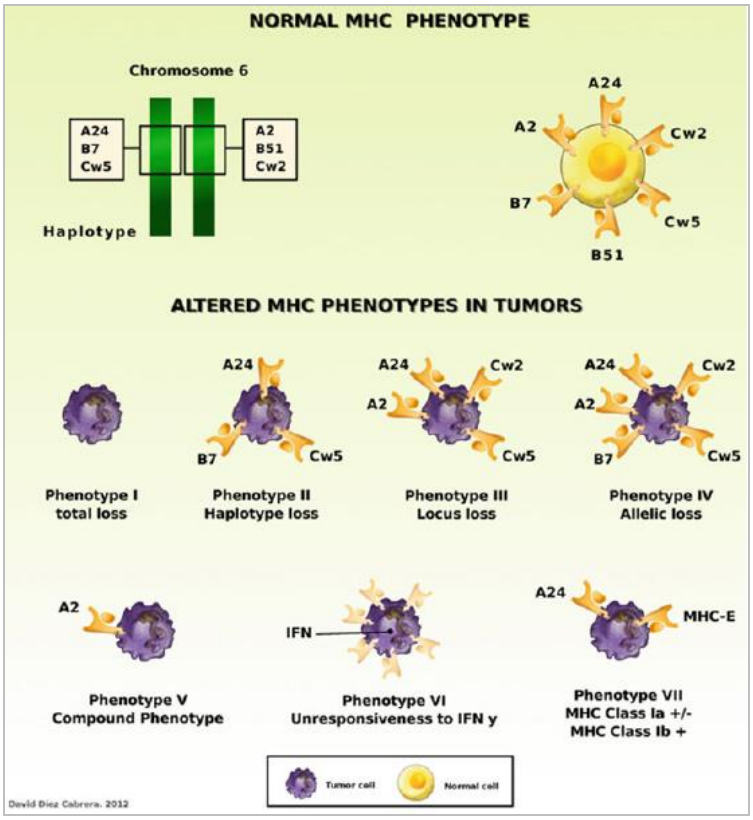


Figure 3. Altered HLA-I phenotypes found in human tumors. HLA-I–positive normal and tumor cells express six class I alleles (two HLA-A, two HLA-B, and two HLA-C). Figure reproduced from (Aptsiauri *et al*, 2013).

Figure 4 demonstrates a frequency of different tumor HLA class I phenotypes reported in melanoma, melanoma cell lines (74-78%, 69% and 67% of HLA alterations respectively) and other types of cancer, including the phenotype I (total loss of HLA class I expression) frequently caused by mutations and genomic alterations in β2m gene, representing the main focus of our study.

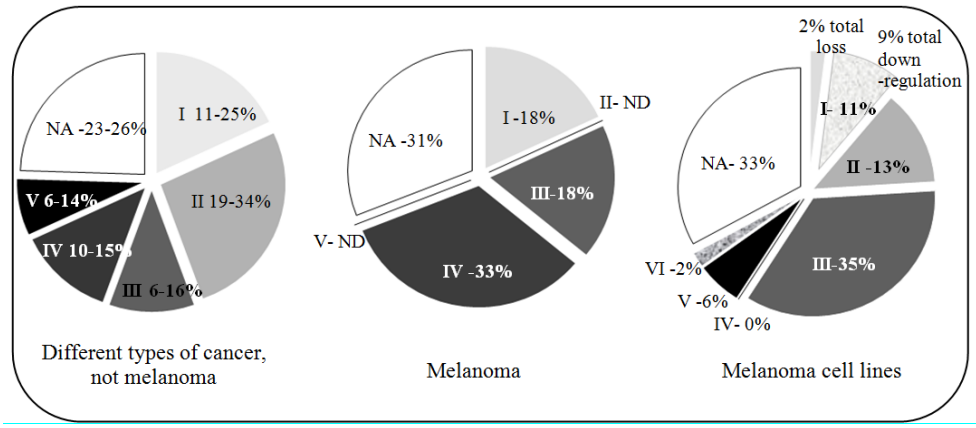


Figure 4. Distribution of HLA class I phenotypes among different types of human cancer, including melanoma and 91 human melanoma cell lines obtained from the European searchable tumour cell line database (http://www.ebi.ac.uk/ipd/estdab). Total loss or downregulation of HLA class I (Phenotype I); haplotype loss (Phenotype II); locus downregulation (Phenotype III); allelic loss (Phenotype IV), compound phenotype (Phenotype V); resistance to stimulation with IFNγ (Phenotype VI). Modified from (Aptsiauri *et al*, 2013; Mendez *et al*, 2009).

2.3.4. Molecular mechanisms underlying total loss of HLA class I

Phenotype I is characterized by the absence of any HLA-I antigen expression in tumor cells. The incidence of Phenotype I ranges from 16 to 80% in various types of tumors with the highest frequency in breast and prostate carcinoma and the lowest frequency demonstrated in renal cell carcinoma (Chang et *al*, 2005a). The exception represent liver carcinoma, which is practically negative for classical HLA class I antigens, but acquires the expression of these antigens during malignant transformation (Fukusato et *al*, 1986; Kurokohchi et *al*, 1996), and lymphoma, in which HLA where abnormalities have been detected with low frequency (Amiot et *al*, 1998; Drenou et *al*, 2002).

Two different subgroups constitute phenotype I: total loss of HLA-I or total downregulation.

a. Total loss of HLA-I: This phenotype has been described in bladder carcinoma (25%) (Cabrera *et al*, 2003b; Maleno *et al*, 2006), colorectal carcinoma (18%)(Cabrera

et al, 1998), melanoma 18% (Kageshita *et al*, 2005), laryngeal carcinoma (11%)(Cabrera *et al*, 2000; Maleno *et al*, 2002) and cervical cancer 10% (Koopman et al, 2000).

This phenotype is frequently associated with the loss of β2m due to structural irreversible alterations in the corresponding gene. Commonly, loss of both copies of the gene is necessary to produce the total loss of HLA-I in malignant cells (Ferrone & Marincola, 1995; Seliger *et al*, 2002), a loss of heterozygocity (LOH) of chromosome 15 (LOH-15) causing a loss of one copy of β2m gene (D'Urso *et al*, 1991; Hicklin *et al*, 1998; Jordanova *et al*, 2003; Paschen *et al*, 2003), and mutation in the other copy of β2m gene (Hicklin *et al*, 1998; Perez *et al*, 1999; Wang *et al*, 1993). Interestingly, the coincidence of mutations in both β2m genes has been reported in colorectal carcinoma (Bernal *et al*, 2011), renal cell carcinoma (Hsieh *et al*, 2009), in B-cell lymphoma cell lines (Pasqualucci *et al*, 2011), and in DLD-1 cell line (Bicknell *et al*, 1994; Gattoni-Celli *et al*, 1992), but not in melanoma.

- LOH in chromosome 15:

Loss of β2m due to LOH-15 can be caused by interstitial deletion because of a mitotic recombination event (Natrajan et al, 2003) during intra- or inter-chromosomal rearrangements or could result from the complete loss of one parental chromosome 15 (Paschen et al, 2006).

This alteration is more frequent than mutations in β2m gene in melanoma cell lines, although only few studies have analyzed the b2m region of chromosome 15 (15q21) (Feenstra et al, 1999; Maleno et al, 2011; Natrajan et al, 2003). According to recent publications, the frequency of LOH-15q21 in primary tumors vary: 44% of bladder carcinoma (Maleno et al, 2011), 41% of laryngeal carcinoma (Maleno et al, 2002), 35% of colorectal carcinoma, 29% of sporadic breast cancer (McEvoy et al, 2002), 12% of breast cancer (Morabito et al, 2009), 16% of melanoma, and 7%of studied renal cell carcinoma (Maleno et al, 2011) lose a copy of β2m gene via LOH. LOH-15q21 is also described in primary tumors with normal HLA or low expression of HLA-I (Paschen et al, 2006).

- Mutations in β2m gene:

β2m gene mutations have been detected in 29% of diffuse large B cell lymphomas (Challa-Malladi et al, 2011), in colon carcinoma (21%) and melanoma (15%), but rarely in other types of cancer (<5%), such as head and neck squamous cell carcinoma, renal cell carcinoma or cervical carcinoma (Fernandez et al, 2000; Jimenez et al, 2000; Marincola et al, 2000; Seliger et al, 2006).

Most of the mutations identified inhibit their translation without affecting their transcription (Seliger et al, 2002). Recent publication from our group summarized these mutations described in tumor samples and tumor cell lines (Bernal et al, 2012) (Figure 5):

- Microdeletions, mainly localized within the hotspot in exon 1, described in colon cancer and melanoma, in lymphoma cell lines (Challa-Malladi *et al*, 2011; Jordanova *et al*, 2003), in renal carcinoma (Hsieh *et al*, 2009), and in cervical cancer (Koopman *et al*, 2000).
- Insertions described in colorectal cancer (CRC) and B cell lymphoma (Challa-Malladi *et al*, 2011; Pasqualucci *et al*, 2011), with localization in repetitive nucleotide motifs of the β2m gene.
- Single nucleotide substitutions that are distributed randomly along exons E1 and E2 are described mainly in melanoma and colon cancer, and it has been also described in lymphoma cell line Daudi (Challa-Malladi *et al*, 2011; Rosa *et al*, 1983) and in two lung cancer cell lines (Baba et al, 2007; Chen et al, 1996b). Three types of substitutions have been described: a) nonsense mutations, very frequent in CRCs and melanoma, can generate a premature stop codon resulting in a truncated β2m protein or in a silent mutation described in CRC that does not affect the HLA class I expression;
b) missense mutations are frequent in B-cell lymphoma (Challa-Malladi et al, 2011), when amino acid substitutions inhibit mRNA translation [at the start codon (ATG) in 5 out of 10 missense mutations found in exon 1, described in Burkitt lymphoma cell line Daudi (Rosa et al, 1983)]. They can also cause a loss of the disulfide bond formation in the β2m protein leading to its degradation by the proteasome, or results in a production of abnormal β2m variants inefficiently processed limiting the number of HLA-I cell

surface molecules (Chang et al, 2006); c) modifications of the splicing sites introducing a premature stop codon.

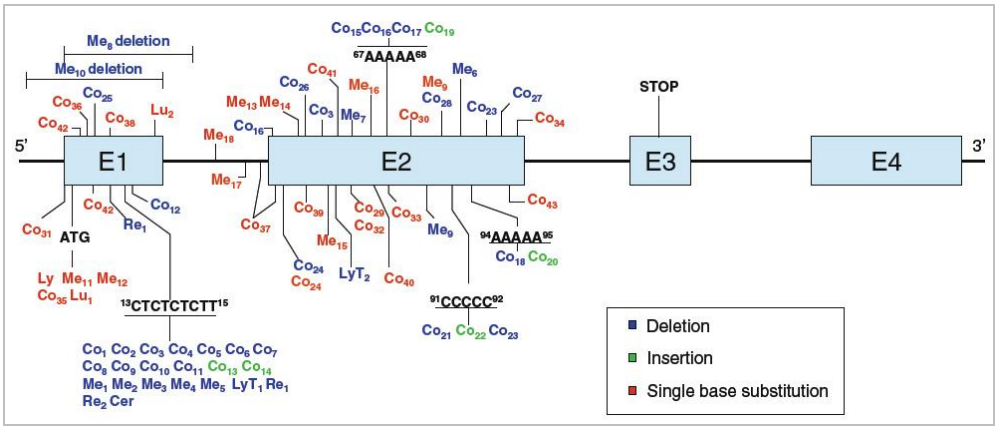


Figure 5. β2m gene alterations in human tumors. Summary of the different β2m gene mutations found in tumor samples and cell lines including melanoma (Me, n = 18), colon cancer (Co, n = 43), Daudi lymphoma (Ly, one case), lung cancer (Lu, 2 cases), sarcomatoid renal carcinoma (Re, 2 cases), cervical cancer (Cer, one case), and testicular diffuse large B cell lymphoma (LyT, 2 cases). The type of mutation is indicated by color according to the legend. Reproduced from (Bernal *et al*, 2012).

β2m mutations have been described in melanoma and in microsatellite instable (Cabrera et al, 2003a), but not in microsatellite stable colorectal and gastric cancers (Bukur et al, 2012). The reason is that β2m gene contains a repetitive $(CT)_4$ sequence in exons1 and two repetitive $(A)_5$ sequences in exon 2. The CT repeat region in exon 1 has been proposed as a mutation "hotspot" (Perez et al, 1999) within β2m gene, since the CT deletion has been identified in more than 75% of the studied tumor cells with total HLA-I antigen loss (Chang et al, 2005b). This possibility is supported by the higher susceptibility of such nucleotide elements to defective DNA repair mechanisms and by the association found between the lack of functional β2m and the presence of a mutator phenotype in colon carcinomas (Browning et al, 1996). However, these β2m mutations do not appear to be closely linked to a defective mismatch repair phenotype in

melanoma cells because microsatellite instability is not frequently observed (Jimenez et al, 2000).

Some β2m mutations have been studied and characterized in both tumor cell lines and the tumors from which they derive (Baba *et al*, 2007; Chang *et al*, 2006; Hicklin *et al*, 1998; Hsieh *et al*, 2009; Wang *et al*, 1993) ensuring that the alterations do not appear *in vitro* in cell culture. Some data describe β2m mutations (Paschen *et al*, 2003) in primary melanoma indicating that evasion strategies can develop early on cancer progression. Many of the reported β2m gene lesions are described in tumor without correlation with the type of therapeutic intervention patient received (Browning *et al*, 1996; D'Urso *et al*, 1991; Paschen *et al*, 2006), or in patients with recurrent metastases (Benitez *et al*, 1998; Chang *et al*, 2006; Hicklin *et al*, 1998; Mendez *et al*, 2001), while other studies try to correlated β2m alterations with response to immunotherapy (Restifo *et al*, 1996). Unfortunately, there are only few reports demonstrating the emergence and immune escape of β2m-negative tumor cells during metastatic cancer progression and in response to immunotherapy in the same patient.

b. Total downregulation: Sometimes tumor cells show downregulation or decreased level of expression of all the expressed HLA loci and alleles, without total or selective locus/allelic losses. This happens due to distinct mechanisms:

- Altered binding of regulatory factors to heavy chain gene enhancer elements (Blanchet *et al*, 1992; Henseling *et al*, 1990).
- Defects in transcriptional regulation of all HLA-I genes (Blanchet *et al*, 1991; Lenardo *et al*, 1989; Ruiz-Cabello *et al*, 1991).
- Defects in the peptide loading of HLA-I antigens due to abnormalities in different components of the APM (Seliger *et al*, 2000): down- regulation of TAP1/2 and LMP2/7 (Cabrera *et al*, 2003a; Meissner *et al*, 2005; Restifo *et al*, 1993); LMP2, LMP7, and TAP1 down-regulation (Meissner *et al*, 2005); LMP7 downregulation (Yoon *et al*, 2000); impaired expression of immunoproteasome subunits (Cabrera *et al*, 2003a; Miyagi et al, 2003) and tapasin (Cabrera *et al*, 2005); downregulation of calnexine (Ritz et al, 2001); coordinated downregulation of several APM components together with

heavy chain downregulation (Ritz *et al*, 2001; Romero *et al*, 2005); or structural defects in TAP2 gene (Chen *et al*, 1996a; Seliger *et al*, 2001).

- Epigenetic mechanisms; abnormal methylation of heavy chain promoters (Nie *et al*, 2001; Serrano *et al*, 2011; Serrano *et al*, 2001), APM components (Hasim *et al*, 2012; Nie *et al*, 2001; Serrano *et al*, 2011), tumor antigens (Sigalotti *et al*, 2004), or histone deacetylation of APM components (Khan *et al*, 2008).

All known HLA class I alterations can occur at genetic, transcriptional, posttranscriptional and epigenetic levels (Garcia-Lora *et al*, 2003a; Garrido & Algarra, 2001; Marincola *et al*, 2000), representing either reversible regulatory defects, or irreversible structural defects (Garrido *et al*, 2010b). The reversible HLA class I deficiencies usually involve alterations of the HLA class I-restricted antigen presentation machinery on transcriptional level that can be repaired *in vitro* by cytokines, such as IFN-gamma (Martini *et al*, 2010; Seliger *et al*, 2000) or even *in vivo* with interleukin 2 and/or interferon (Bleumer *et al*, 2003; Figlin, 1999; Yagoda *et al*, 1995). In addition, alterations caused by epigenetic events, can be reversed with pharmacologic agents that induce DNA hypomethylation or inhibit histone deacetylation (Fonsatti *et al*, 2007; Fratta *et al*, 2013; Serrano *et al*, 2001; Sigalotti *et al*, 2005).

However, irreversible structural defects caused by mutations or chromosomal losses in genes encoding heavy chain and β2-microglobulin can be corrected only by replacement of the defective gene with the wild type gene. HLA-negative cells without such gene replacement will not be recognized by cytotoxic T-cells, will escape immune surveillance and disseminate. More importantly, immunotherapy may fail, since it is not likely to induce HLA upregulation in tumor cells with altered HLA-I genes.

2.3.5. Association of β2m defects with cancer progression, metastatic dissemination and clinical prognosis

Emergence of new more profound HLA class I alterations is known to be associated with cancer progression. This is evidenced from experimental mouse cancer

models by our group (Garcia-Lora *et al*, 2001; Garcia-Lora *et al*, 2003b), and some studies that have shown accumulation of HLA alterations in advanced stages of disease, compared to lesions with normal expression in early stages of the disease (Cordon-Cardo *et al*, 1991; Cromme *et al*, 1994; van Driel *et al*, 1996). Studies in melanoma (Ferrone & Marincola, 1995; Kageshita *et al*, 2005; Marincola *et al*, 2000), oesophageal squamous cell carcinoma (Mizukami et al, 2008), prostate cancer (Blades et al, 1995), and breast cancer (Redondo et al, 2003) showed a higher frequency of alterations in metastatic lesions than in primary tumours, supporting the idea of a clinical significance of HLA alterations in disease progression. Alterations in β2m expression or the presence of LOH-15 have been associated with development of metastasis in renal cell carcinoma (Romero *et al*, 2006) and colorectal cancer (Wick *et al*, 1996). In breast cancer, the incidence of LOH at 15q21.1 region in relapses is higher than in primary tumor (Rhiem *et al*, 2003). Similar correlation was described in primary tumors and metastases in head and neck cancer (Poetsch & Kleist, 2006).

β2m mutations reported in different lesions from patients with no history of chemotherapy or immunotherapy (Browning *et al*, 1996; D'Urso *et al*, 1991; Paschen *et al*, 2006; Wang *et al*, 1993) reflect that tumor cells acquire mechanism to escape from tumor specific-HLA restricted immune attack during natural tumor progression (Garcia-Lora *et al*, 2001). For example, in a long-term survival patient with metastatic melanoma without immunotherapy (Yamshchikov *et al*, 2005), a β2m mutation was found among other immune escape mechanism. Our group reported that the incidence of LOH-15 is increased in post-immunotherapy progressing melanoma metastases (Cabrera *et al*, 2007; Carretero *et al*, 2008) and in recurrent bladder tumors after BCG immunotherapy (Carretero *et al*, 2011).

Low levels of β2m mRNA in colon cancer or lack of β2m mRNA in glioblastoma is an independent prognostic marker of poor patient survival (Blum et al, 2008). Similarly, β2m downregulation is associated with poor prognosis in ovarian (Andersso*n et al*, 2012; Ha*n et al*, 2008; Leffer*s et al*, 2009; Rolland *et al*, 2007), colorectal (Shrout et al, 2008), clear cell renal cell carcinoma (Yua*n et al*, 2013), while in esophageal (Tanaka et al, 2011) and colon cancer it has been correlated with disease recurrence

(Blum et al, 2008). Likewise, β2m loss is associated with poor prognosis in laryngeal squamous cell carcinoma (Ogino *et al*, 2006).

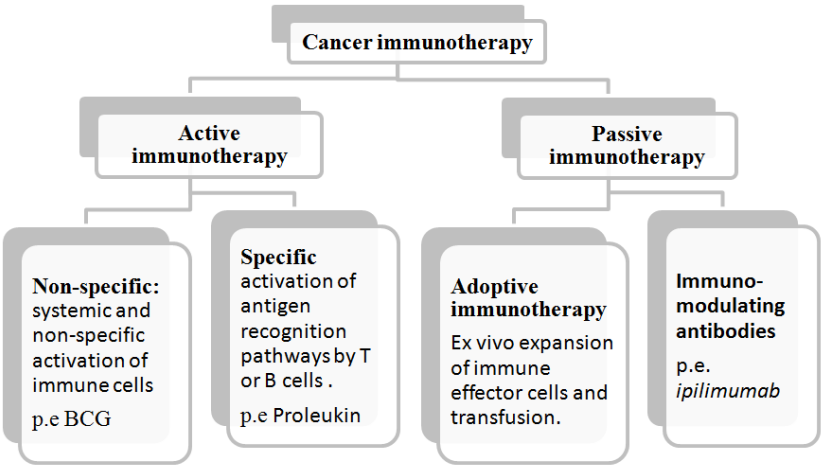
However, these results are in conflict with the data published by other groups who report improved overall survival when β2m is downregulated in breast cancer (Morabito et al, 2009). In MSI-H (microsatellite instable-high) colon cancer β2m mutations have been associated with a favorable clinical course (Koelzer et al, 2012) and a complete absence of disease relapse or tumor-related death events (Tikidzhieva et al, 2012).

2.4. CANCER IMMUNOTHERAPY

Current classical approach to treat cancer includes surgery, radiotherapy, and chemotherapy. A considerably novel treatment option represents cancer immunotherapy, or a manipulation of the immune response to elicit clearance of cancer, based on the premise that cancer cells express TAA that can be recognized by antibodies or T-cells.

Hanahan and Winberg proposed six capabilities shared in common, by most if not all, types of human tumors providing a framework for understanding the remarkable diversity of cancer (Hanahan & Weinberg, 2000). Years later, the hallmark of "evading immune surveillance" was suggested (Kroemer & Pouyssegur, 2008), recognizing that cancer cells should acquire the skills of evading immune recognition and suppressing immune reactivity on their way to giving rise to tumors (Cavallo *et al*, 2011). Thereby, the development of novel cancer immunotherapy approach to treat neoplastic diseases becomes crucial.

Currently, immunotherapy strategies are based on two major approaches: active and passive immunotherapy (Buonaguro *et al*, 2011).



2.4.1. Active immunotherapy

a) Non-specific immunotherapy

One of the first strategies to enhance immune responses to cancer was the administration of adjuvants directly into solid tumors to stimulate inflammation and recruit immune effector cells. Already two centuries ago, William B. Coley reported the first successful immunotherapy (Coley, 1891). This approach is still commonly used for treating superficial bladder carcinomas with BCG (Kresowik & Griffith, 2009).

Cytokines used to enhance presentation of tumor antigens are currently being used in clinical trials, including interleukines that were the first tested and are currently evaluated as vaccine or vaccine adjuvants (Yaddanapudi et al, 2013). IL-2 (Beyer, 2012) is extensively used for metastatic renal cell cancer and melanoma, with an example of Aldesleukin-Proleukin, an FDA-approved therapy. Also the effects of GM-CSF and IL-12 are being investigated in cancer clinical trials (Dougan & Dranoff, 2009); and IFN-α is used for treatment of kidney cancer and melanoma, representing the only agent approved in the USA for adjuvant use in high risk melanoma patients.

b) Specific immunotherapy

Specific cancer immunotherapy uses tumor-associated antigens from various sources, including whole autologous or allogeneic tumor cells, lysates of tumor cells,

defined proteins, specific peptide epitopes or mRNA/DNA encoding for relevant antigens.

Among the specific epitopes, melanoma-associated antigen MAGE-3 generated the first observed clinical response to peptide-based immunotherapy in melanoma patients (Marchand *et al*, 1995), and a modified gp100 peptide is used in patients with metastatic melanoma (Schwartzentruber et al, 2011). A DNA allovaccine using HLA-B7 DNA (Senovilla et al, 2013) either alone (Nabel et al, 1996; Rini et al, 1999) or in the complex with β2m DNA (*Allovectin-7*®) has been tested in patients with metastatic melanoma (Chowdhery & Gonzalez, 2011; Soares & Lutzky, 2010).

However, the most promising approach is based on the use of dendritic cells as nature's adjuvants (Banchereau & Palucka, 2005; Galluzzi *et al*, 2012) as Sipuleucel-T [*Provenge*® (Kantoff *et al*, 2010) containing the antigen prostatic acid phosphatase and GM-CSF] approved by the U.S. Food and Drug Administration (FDA) for some prostate cancer. Other group of vaccines are the first explicitly prophylactic cancer vaccine *Gardasil*® and *Cervarix*®, approved by the FDA designed to prevent cervical cancer induced by HPV (type 16 and 18)(Mariani & Venuti, 2010).

2.4.2. Passive immunotherapy

a) Adoptive immunotherapy

It is based on the selection and manipulation *ex vivo* of patient's specific anti-tumor T lymphocytes and their reinfusion after *in vitro* expansion (Morgan *et al*, 2006; Rosenberg, 2011). This approach has been applied largely for melanoma, together with IL-2 and chemo-radiotherapy (Dudley *et al*, 2008). Another population of T-cells used in adoptive transfer are tumor-infiltrating lymphocytes (TILs) which are selected for T-cell receptor (TCR) specificity, expanded with TAAs or used with engineered T cells (Curran *et al*, 2012; Sadelain *et al*, 2003).

b) Immunomodulating antibodies

Antibody-based therapy right now is more effective than other immunotherapy approaches leading in some cases to a complete regression of tumors. Different

antibodies are currently approved or being tested: *rituximab*, against anti-CD20 (McLaughlin *et al*, 1998); *trastuzumab* targeting human epidermal growth factor receptor 2 (HER2) (Brenner & Adams, 1999); *bevacizumab* against vascular endothelial growth factor, VEGF (Ferrara *et al*, 2004); *cetuximab* inhibiting epidermal growth factor receptor, EGFR (Wong, 2005); and immunostimulatory antibodies as *ipilimumab* that targeting CTLA-4, (Margolin, 2012).

2.4.3. Resistance to immunotherapy

Although many new protocols of cancer immunotherapy lead to an increase in the presence of tumor-specific T lymphocytes and/or partial responses in patients with certain malignancies, they have not yet delivered clear clinical benefits, such as significant induction of tumor regression or increased disease-free survival (Rosenberg, 2004). In this decade only a small percentage of patients experienced tumor regression with current protocols of T-cell based cancer immunotherapy: 3.7% with peptide vaccines, 4.2% with dendritic cell vaccines, 0.9% with recombinant viral vaccines, 2% with tumor cell vaccines, and 6.7% with DNA plasmid vaccines) (Klebanoff et al, 2011), meaning that there has not been a significant improvement in the efficacy of early phase therapeutic cancer vaccine trials. In more recent clinical trials with more advanced phase it also has been demonstrated that the efficacy of anti-tumor immunotherapies has been modest compared to their theoretical and previous early phase promise. For example, just recently, in 2013, GlaxoSmithKline announced that a Phase III randomized, blinded, placebo-controlled trial of the MAGE-A3 cancer immunotherapeutic showed that the study did not meet its first co-primary endpoint as it did not significantly extend disease-free survival when compared to placebo in the MAGE-A3 positive population (http://www.gsk.com/media/press-releases/2013/the-investigational-mage-a3-antigen-specific-cancer-immunotherap.html).

Therefore, understanding of the possible causes of such poor clinical outcome has become very important for improvement of the existing cancer treatment modalities. Figure 6 summarizes the possible mechanism of the failure of cancer immunotherapy.

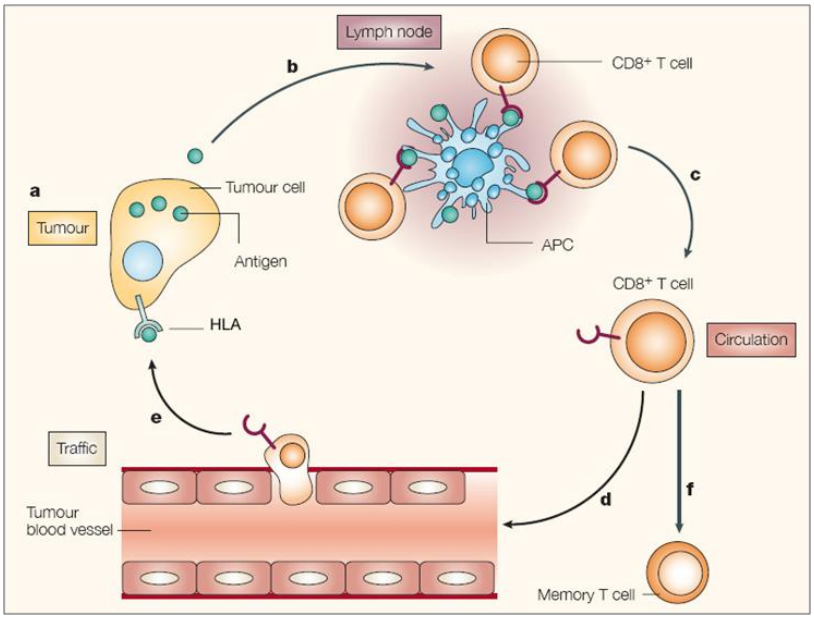


Figure 6. The six steps necessary for an effective antitumour CD8+ T-cell response. Effective destruction of tumours by antigen-specific CD8+ T cells is a multistep process. Each of these six steps is required and can be modulated by a range of factors: tumour antigens must be present (a); these antigens must reach/load antigen-presenting cells (APCs) in the draining lymph node (b); specific T cells must respond by proliferation (c); the circulating T cells must enter the tumour (d); once in the tumour the T cells must be able to overcome local immune-suppressive molecules to recognize and kill targets (e); memory cells should be generated (f). Cancer immunotherapy can fail at any of these steps. Reproduced from (Lake & Robinson, 2005).

One of the possible explanations of the pitfalls of peptide-based immunotherapy could be the absence of HLA class I on tumor cells preventing the expected increase in the T-cell recognition of TAA expressing malignant cells.

Figure 7 shows the different types of T-cell based cancer immunotherapy approaches used in clinical settings aimed at increasing antitumor immune response that would lead at the end to HLA-restricted tumor cell recognition by CTLs and consequent elimination. However, if tumor cells have lost normal HLA class I expression, they may escape T-cell recognition and grow. Therefore, the commonly observed HLA class I defects in tumors constitute a potential problem for T cell-based immunotherapy and their correction in these tumor types might result in much higher success rates (Lampen & van Hall, 2011).

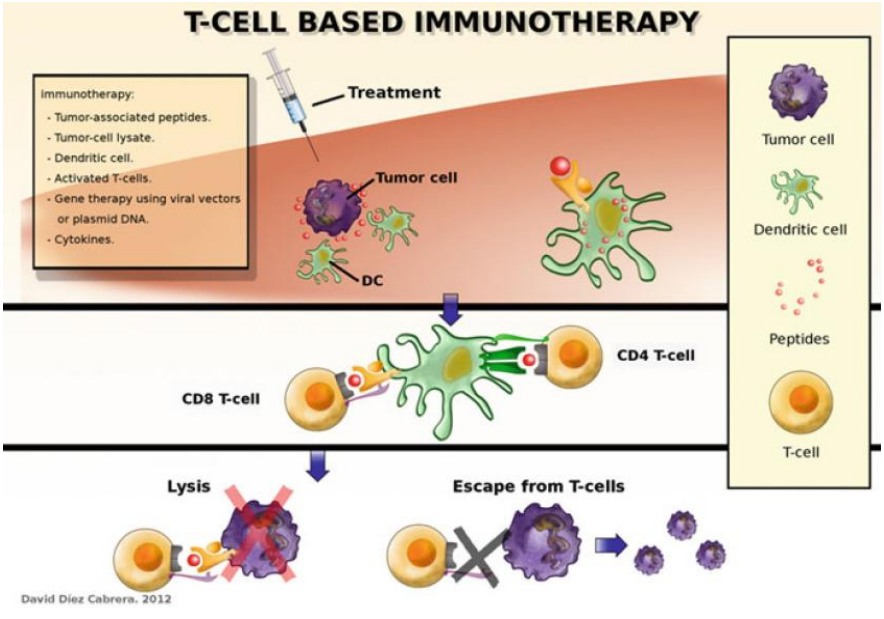


Figure 7. Possible outcomes of cancer immunotherapy. The existing protocols of cancer immunotherapy are aimed at increasing the recognition of tumor cells by CD4 and CD8 T lymphocytes leading to tumor cell elimination by cytotoxic CD8 T cells. This recognition requires presentation of a tumor-associated peptide in a complex with HLA class I molecule to T cells. Therefore, absence or low expression of HLA molecules may diminish T-cell based antitumor immunity. Reproduced from (Aptsiauri et al, 2013).

Altered HLA-I expression could be determinant in the poor outcome of immunotherapy (Ahmad *et al*, 2004; Geldmacher *et al*, 2011; Morgan *et al*, 2006; Pawelec, 2004; Rosenberg & Dudley, 2004) as indicates the increased frequency of HLA-I abnormalities in recurrent cancers from patients treated with T cell-based immunotherapy (Mendez *et al*, 2007; Neller *et al*, 2008). Our group has recently published that in melanoma patients treated with cytokines and autologous tumor vaccine showed more profound HLA class I alterations progressing melanoma on post-therapy progressing lesions (Carretero et al, 2008). Similarly, in patients with bladder carcinoma, recurrent tumors that appeared after BCG therapy had a higher percentage of LOH in chromosome 15 and increased incidence of HLA class I-altered expression (Carretero et al, 2011).

On the basis of the evidence obtained from experimental mouse cancer models and metastatic human tumors, the structural defects underlying HLA class I loss may have profound implications onT-cell-mediated tumor rejection and ultimately on the outcome of cancer immunotherapy what makes essential to develop strategies to overcome this obstacle. We hypothesized that gene therapy could be a suitable option to recover normal expression of HLA class I genes on tumor cells harboring structural HLA class I alterations.

In addition, other strategies are being explored to restore the TAA recognition by CTLs. For example the group leaded by T.Van Hall (University of Leiden, The Netherlands) suggests a novel approach to overcome the frequent escape of HLA-class I-negative tumor variants from protective immunity through the exploitation of "T cell epitopes associated with impaired peptide processing" (TEIPP) (Chambers *et al*, 2007; Oliveira CC & van Hall T 2013; Van Hall et al, 2006). These peptides derived from commonly expressed self-proteins that are selectively presented by HLA class I molecules on tumors with antigen-processing defects, such as TAP deficiencies. The group reported that a subset of conventional CTL is capable of eradicating these HLA-negative tumor immune-escape variants and demonstrated that TEIPP epitopes act as true tumor rejection antigens prevent the outgrowth of TAP-deficient tumor cells Van Hall et al, 2006 They also demonstrated that vaccination with TEIPP peptide-presenting DC resulted in a $CD8^+$ T cell–dependent protection against tumor immune-escape variants (Chambers *et al*, 2007)

Other strategies that circumvent the HLA class I loss and antigen recognition from major histocompatibility complex restriction is the use of chimeric antigen receptors (CARs) (Sadelain et al, 2009) that usually combine the antigen binding site of a monoclonal antibody with the signal activating receptor of a T cell. By arming effector lymphocytes (such as T cells and natural killer cells) with such chimeric receptors, the engineered cell is redirected with a predefined specificity to any desired target antigen, in a non-HLA restricted manner (Eshhar Z, 2008). Currently a great diversity of tumor associated antigens is being targeted in preclinical models (Ramos CA & Dotti P, 2011; Turtle et al, 2012).

2.5. CANCER GENE THERAPY

Nowadays, gene therapy can be broadly defined as an intracellular delivery of genomic material into specific cells to generate a therapeutic effect by correcting an existing abnormality or providing the cells with a new function (Stone, 2010). Almost 1900 clinical trials using gene transfer methods have been registered since the first human gene therapy trial (Ginn *et al*, 2013).

Cancer is the disease most frequently targeted by gene therapy in clinical trials (64%-72% of them are focused on cancer treatment) (Ginn *et al*, 2013; NIH, 2013) and fifteen categories of cancer gene therapy trials are described in the NIH database *(*NIH, *2013)*, of those, 72% are focused on the genetic modulation of the immune response (*in vitro or in vivo*) (Figure 8).

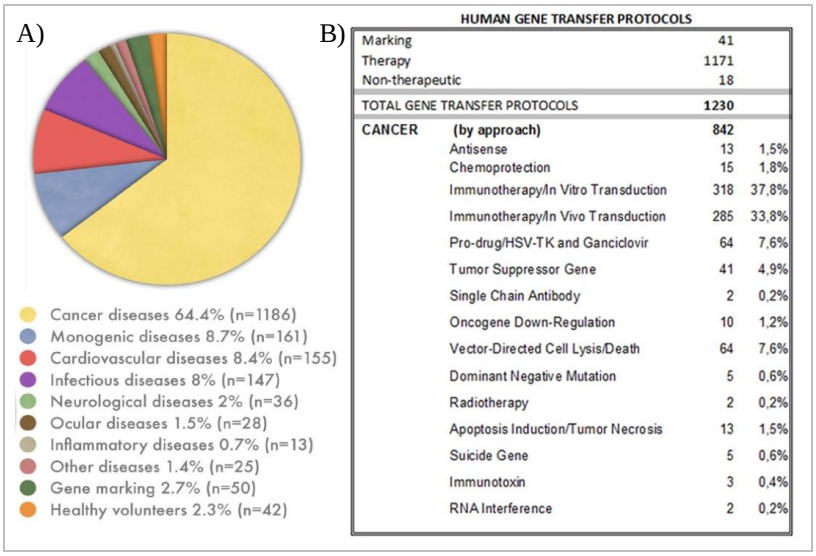
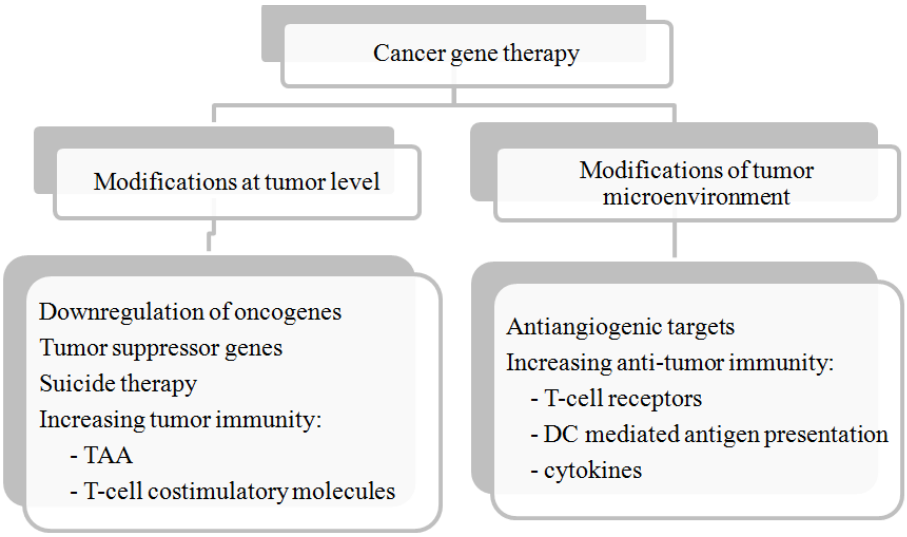


Figure 8. Gene therapy application to treat different diseases (Ginn *et al*, 2013) (A) and human gene transfer protocols applied to treat cancer (B). The total number of human gene transfer protocols are shown together with the number of protocols applied for cancer treatment. Cancer gene transfer protocols are classified according to the approach used in the protocol. Modified from: Database of the National Institute of Health. Last updated August 2013. http://oba.od.nih.gov/rdna/adverse_event_oba.html .

Cancer gene therapy can be categorized depending on the target, including the tumor cell itself or tumor microenvironment, cytokines, angiogenic factors, etc.



2.5.1. Gene therapy approaches targeting tumor cells

a) <u>Down-regulation of oncogenes</u>

Different viral vectors targeting oncogenes have been designed, among them *Rexin-G*, a retroviral vector that interferes with the cyclin G1 gene, (Gordon *et al*, 2004; Gordon & Hall, 2005). Antisense oligonucleotides (ASO) targeted against bcl-2 mRNA (*Genasense*) have been tested in melanoma cells (Jansen *et al*, 2000; listed, 2007).

b) <u>Expression of tumor suppressor genes</u>

A number of tumor suppressor genes have been inserted to block tumor growth. The first gene therapy product approved by China is based on p53 transfer using *Gendicine*®, an adenovirus carrying p53 gene (Wilson, 2005). p53 expression has been also tested for melanoma treatment (Dummer *et al*, 2000; Tazawa *et al*, 2013).

c) <u>Expression of suicide genes</u>

Herpes simplex type-1 thymidine kinase/ganciclovir (HSV-TK/GCV) and cytosine deaminase/5-fluorocytosin (CD/5FC) are the best characterized systems (Altaner, 2008). This approach has been used in a phase I/II study of retrovirus-mediated HSV-1TK suicide gene therapy for metastatic melanoma (Klatzmann *et al*, 1998).

d) Increase tumor immunity

– tumor antigens: different viral vectors encoding distinct tumor antigens as CEA (Conry *et al*, 1999), oncofetal antigen 5T4 (named *TroVax*)(Kim et al, 2010), MUC1 plus IL-2 (Quoix et al, 2011), or PSA (Lubaroff *et al*, 2009).

– T cell costimulatory molecules: some viral vectors have been designed to deliver as B7.1(CD80) (Kaufman *et al*, 2005), or three T-cell co-stimulatory molecules in the TRICOM system, encoding B7-1, ICAM-1 and LFA-3, for different types of cancer (Madan *et al*, 2012).

2.5.2. Modifications of tumor microenvironment

a) Anti-angiogenic gene therapy

Negative regulators of angiogenesis, such as angiostatin, endostatin (Li *et al*, 2008), vasostatin, modulators of vascular endothelial growth factor activity (sFLT1), and chemokines such as CXCL10 (Yang *et al*, 2009), are investigated in tumor therapy (Persano et al, 2007).

b) Increasing the anti-tumor immunity

- Cytokines (Hernandez-Alcoceba et al, 2013): IFN-γ used in a retroviral vector for advanced melanoma (Nemunaitis et al, 1999); GM-CSF gene is used in GVAX (Nemunaitis, 2005) and the oncolytic virus OncoVex (Senzer et al, 2009) used in melanoma (Amgen, 2013; Kaufman & Bines, 2010); TNFα is used in TNFerade an adenoviral vector (Chang et al, 2012; Herman et al, 2013); IL-12 tested in preclinical models using oncolytic adenovirus (Bortolanza et al, 2009) or other adenoviral vectors (Barajas et al, 2001), with cooperative effects with radiotherapy or chemo-therapy (Fujita et al, 2007; Gonzalez-Aparicio et al, 2010).

- T cell modification therapy: T cells can be modified to express tumor specific TCR genes as showed by Rosenberg´s group when a retroviral vector expressing the α and β chains of an anti-MART-1 TCR achieved complete tumor regression (Morgan et al, 2006); others TAA specific TCR tested are CEA for colorectal carcinoma, or NY-ESO-1 in melanoma (Park et al, 2011).

- DC-based cancer gene therapy: use of DC transduced with adenoviral vectors encoding interleukins as IL-23 (Hu et al, 2006) and IL-12 (Mazzolini et al, 2005; Melero et al, 1999), or CD40L (Tomihara et al, 2008), or CD transfected with autologous tumor-mRNA in malignant melanoma and prostate cancer (Kyte & Gaudernack, 2006), or RNA-pulsed encoding different TAAs (Galluzzi et al, 2012).

2.6. CHOOSING THE GENE TRANSFER STRATEGY: ADENOVIRAL VECTORS

Noticeably, more than two thirds of current ongoing clinical studies are based on the use of viral vectors for gene administration (Figure 9) (Ginn *et al*, 2013).

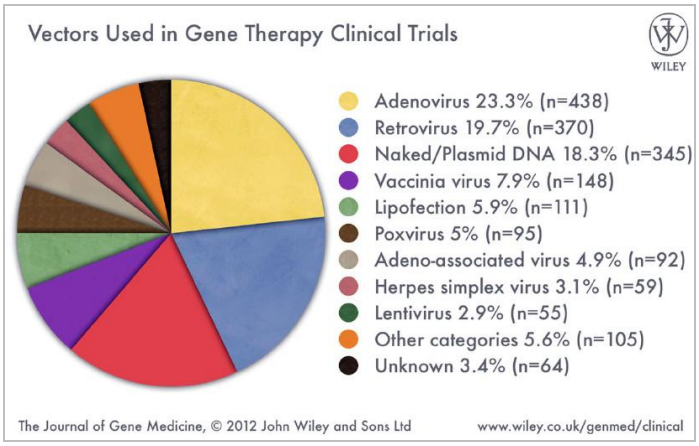


Figure 9. Vectors used in gene therapy clinical trials. Reproduced from (Ginn et al, 2013).

Although retroviruses were the earliest viral vectors used for *in vivo* gene transfer and are still being extensively used for *ex vivo* approaches, most current protocols for transfer of tumor suppressor, anti-angiogenic, suicide, and pro-apoptotic genes; and for tumor vaccination approaches and cytokine gene transfer utilize adenoviral vectors (Wilson, 2002; Liu et al, 2010; Kaufmann &Nettelbeck 2012).

We have selected an adenoviral vector as the method to restore HLA class I expression because this vector is considered to be the most effective vector in gene delivery (Cevher *et al*, 2012) and they have several biologic characteristics that can

make it effective for cancer gene therapy: (a) a broad host/ cell range, (b) high levels of gene expression, (c) the ability to infect both dividing and nondividing cells (Seth, 2000), and (d) low risk of insertional mutagenesis (Jager & Ehrhardt, 2007) because the adenoviral genome remains mainly episomal within the nucleus. Additionally, adenoviral vectors production reaches high titers (up to 10^{12} pfu/ml) allowing the generation of the amount of viral vector needed for the clinical use. Their DNA genome and high fidelity DNA polymerase confers relative genomic stability in comparison to RNA viruses. Finally, this vector allows fitting the needed transgene due to its fairly large packaging capacity (up to 35 kb) in some vectors. Table 2 shows the characteristics of adenoviral vector while compare them with the different vectors mainly used in gene therapy.

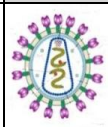
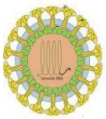
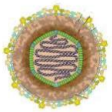
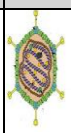
		Retrovirus Lentivirus	**Alphavirus**	**Vaccinia virus**	**Herpes virus**	**Adeno-Associated virus**	**Adenovirus**
Particle characteristics							
	Genome	ssRNA (+)	ssRNA (+)	dsDNA	dsDNA	ssDNA	dsDNA
	Coat	Enveloped	Enveloped	Enveloped	Enveloped	Naked	Naked
	Virion diameter	80-130 nm	60-70 nm	300-400nm	150-200nm	18-26 nm	70-110 nm
	Genome size	3-9 kb	12 kb	130-280kb	120-200kb	5 kb	30-39 kb
Gene therapy properties	Infection/ tropism	Dividing cells*	Dividing and non dividing cells	Dividing and non dividing cells	Dividing and non dividing cells	Dividing and non dividing cells	Dividing and non dividing cells
	Host genome interaction	Integrating	Non-integrating	Non-integrating	Non-integrating	Non-integrating*	Non-integrating
	Transgene expression	Long lasting	Transient	Transient	Potential long lasting	Potential long lasting	Transient
	Packaging capacity	8 kb	7,5 kb	25 kb	>30 kb	4,5 kb	7,5 kb

Table 2. A comparison of different viral vectors in use for gene therapy: overview of their advantages and disadvantages.* Wild type Adeno-associated viruses are able to integrate with low frequency into chromosome 19. Lentiviruses also infect non-dividing cells.

2.6.1. Adenoviruses

There are 51 serotypes of human adenoviruses (Ad) classified originally on the basis of their ability to be neutralized by specific animal antisera. Human serotypes are divided into six subgroups (A–F; B is subdivided into B1 and B2) (Russell, 2005), with Ad serotypes 5 (Ad5) and 2 (Ad2) of group C being the most frequently used in cancer gene therapy.

The linear genome flanked by two origins for DNA replication (ITRs) has eight units for RNA polymerase II mediated transcription. The genome is composed of various transcriptional regions which include five early (E1A, E1B, E2, E3, and E4), four intermediate (IVa2, IX, VAI, and VAII), and one late transcriptional units (L1 through L5) (Shenk, 1996).

After entry into the nucleus, genes from the early region 1 (E1a and E1b) are quickly transcribed and during the early phase of viral replication, four noncontiguous regions of the genome are expressed (E1 to E4) (Vorburger & Hunt, 2002).

Adenoviruses enter the target cells through receptor-mediated endocytosis: attachment of Ad5 and Ad2 to a susceptible cell is mediated by high-affinity binding of the Ad fiber knob to the primary receptor [coxsackievirus and Ad receptor (CAR)], followed by a secondary interaction of the penton base with integrins, resulting in virus internalization into the cell (Bergelson *et al*, 1997; Wickham *et al*, 1993). As CAR is expressed in a variety of normal tissues it contributes to promiscuous Ad tropism and lack of specific targeting. Inside the cell, they lose their protein coat, and transfer DNA into the nucleus, where it is transcribed. This DNA does not integrate into the host genome, remaining in the nucleus as an episomal element and thus, its effects are transient (Figure 10).

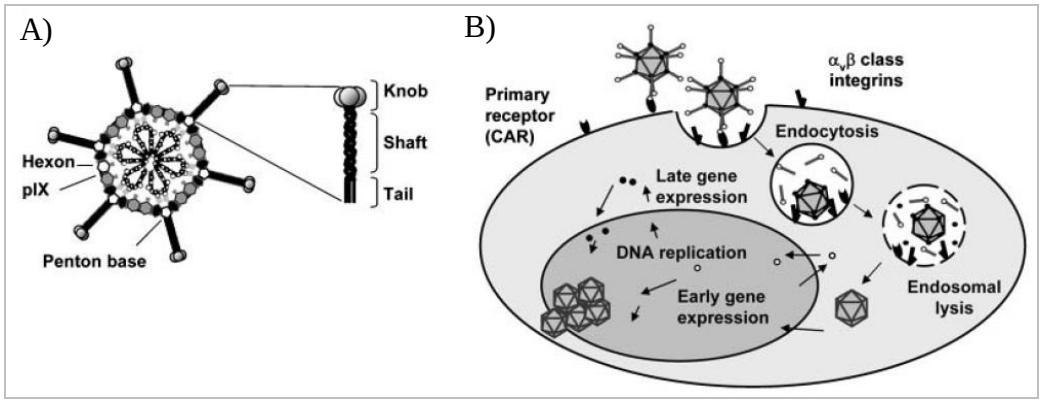


Figure 10. A) The adenovirus particle. Major structural components of wild-type Ad5 are shown. The capsid encompasses an approximately 36 kb double-stranded DNA genome. B) The adenoviral infection pathway. Cell entry is initiated by high-affinity binding of the fiber knob domain to its primary receptor, CAR. CAR-binding is followed by endocytosis mediated by penton base RGD interaction with cellular α&β integrins. After endosomal lysis, viral DNA is transported to the nucleus through a microtubule-mediated process, and viral genes or transgenes are expressed. Reproduced from (Kanerva & Hemminki, 2005).

Viral particle assembly in the nucleus starts about 8 h after infection and results in the production of 10^4–10^5 progeny particles per cell, which are released from cell as mature particles 30–40 h post-infection by cell lysis.

The genes encoded in regions E1 and E3 have a special interest due to their ability to interact with antigen processing machinery. The E1A unit located next to the left terminal ITR is transcribed first and, with the help of cellular factors, activates transcription of the other viral genes and its deletion renders the virus replication-defective. E3 proteins subvert the host immune response and allow persistence of infected cells (Bennett *et al*, 1999). E1A and E3-19K protein allow the virus to escape immunosurveillance. E1A plays a role in immunoevasion by inhibiting the activity of STAT1, which is needed for activation of interferon-responsive genes (Chatterjee-Kishore *et al*, 2000). E1A also downregulates TAP1 and TAP2 at the mRNA and protein level, depending on the individual cell type.

2.6.2. Adenoviral vectors

At least three regions of the viral genome can accept insertions or substitutions of DNA to generate a helper independent virus: a region in E1, a region in E3, and a short region between E4 and the end of the genome (Hitt & Graham, 2000; Imperiale & Kochanek, 2004). Four groups of adenoviral vectors have been developed so far (Figure 11).

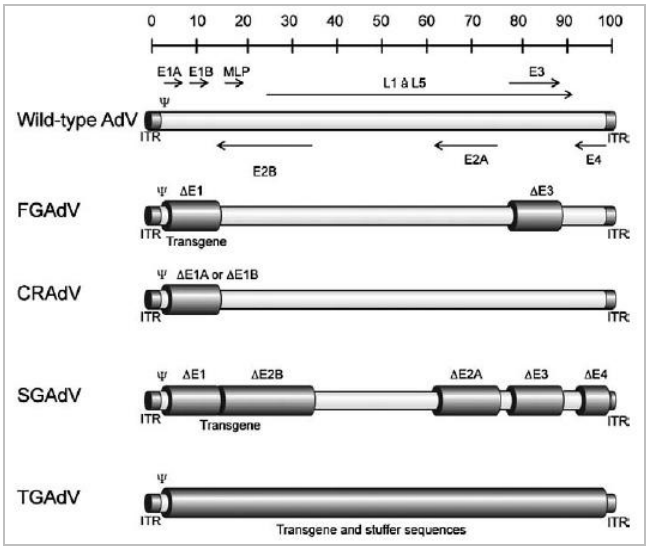


Figure 11. Schematic representation of wild-type AdV genome and different generations of AdV. Genomes are divided into 100 map units (28 to 38 kb). E1 to E4: early transcript units, L1 to L5: late transcript units, ITR: inverted terminal repeats, MLP: major late promoter, ψ: packaging signal, FGAdV: first generation adenoviral vectors, CRAdV: conditionally replicative AdV, SGAdV: second generation AdV, TGAdV: third generation AdV. Reproduced from (Dormond *et al*, 2009).

The first generation of adenoviral vectors has deleted E1 and/or E3 regions, which severely impaired their ability to replicate. One of the approaches to construct E1 deleted adenovirus is homologous recombination (Hardy *et al*, 1997): a purified adenoviral genome where the E1 and/or E3 regions is deleted, is cotransfected with a shuttle vector that contains the left-handed ITR, the E1a enhancer, the encapsidation signal, the cytomegalovirus promoter, a multicloning site for insertion of the gene of interest, and the SV40 poly(A) signal followed by sequences from the adenoviral

genome located 3´of the E1 domain. These replication-impaired adenoviral vectors must be grown on cell lines that complement the missing E1 functions, such as 293, an embryonic kidney cell line whose genome carries the adenoviral E1 region (Graham *et al*, 1977).

While deletion of the E1 region to make room for the therapeutic transgene impairs viral replication, deletion of the E3 region provides additional free space. Adenoviral vectors that have deletions in the E1 and E3 regions can carry 7000-8000 base pairs of genetic material (Bett *et al*, 1994).

Conditionally replicative (CRAdV) adenoviruses employed in cancer treatment (Everts & van der Poel, 2005) have mutated either the E1A or E1B region, which confers the capacity to replicate in human tumor cells that harbor a defective retinoblastoma tumor suppressor functions or p53.

Today's second generation adenoviral vectors have deletions of various E1, E2, and E4 genes, because viral proteins encoded by these DNA sequences were shown to induce most of the host immune response. They are less immunogenic than the first generation vectors and provide a larger capacity for transgene insertion. These new vector constructs have decreased toxicity and result in prolonged gene expression in vivo (Schiedner et al, 1998). The major problem of these vectors concerns the isolation of a complementary cell line (Dormond et al, 2009).

Third generation Ad vectors, also called "high capacity", "gutless" or "helper-dependent" vectors contain only ITR repeats and a packaging signal and can accommodate up to 37 kb of foreign DNA (Hardy et al, 1997; Lieber et al, 1999) but have to be complemented for replication.

However, the use of adenoviral vectors has some disadvantages. Ad vector administration into a naïve host produces transgene expression that generally peaks within days of injection after which expression is eliminated within two weeks due to CTL responses against Ad, Ad-infected cells, and/or transgene epitopes (Yang *et al*, 1994). It occurs because, even in the absence of E1 gene products, there is low-level transcription of the remaining viral genes, resulting in early innate host cytokine

transcription followed by antigen-dependent immune responses (Hartman *et al*, 2008; Russell, 2009). This immune response was a major drawback of early adenoviral gene therapy and might well have contributed to the decrease of gene expression in a number of adenovirus gene-transfer studies in patients. Another limitation of recombinant adenoviruses is the difficulty in obtaining efficient gene transfer upon a second administration of virus due to formation of neutralizing antibodies (Bangari & Mittal, 2006). Several strategies have been tested to reduce adenovirus-mediated inflammatory reaction without losing transduction efficiency as administration of anti-inflammatory or immunosuppression agents (Fontanellas *et al*, 2010; Seregin & Amalfitano, 2010), and modification of the route of administration (Bessis *et al*, 2004; Crettaz *et al*, 2006).

2.6.3. Adenoviral gene therapy

Soon after Ad isolation in 1953 (Rowe *et al*, 1953) its anti-tumor potential was evident from the fact that tumor regression was observed in clinical cases of cervical carcinoma following Ad inoculation (Huebner *et al*, 1956). During the last decades Ad vectors have evolved as an efficient tool for cancer treatment and more importantly, Ad therapeutic applications have also been demonstrated to be safe in several clinical trials (Douglas, 2007; Wu *et al*, 2001).

Different adenoviral vectors have been designed and tested in clinical trials rendering promising results, as showed by the approval of two vectors (in China) for cancer treatment: *Gendicine® ADVEXIN* (Merritt *et al*, 2001; Shimada *et al*, 2006; Swisher *et al*, 2003) and the promising results of conditionally replicative vectors as *ONYX-015* (Lamont *et al*, 2000; Nemunaitis *et al*, 1999; Nemunaitis *et al*, 2001), *Oncorine®* (Xia *et al*, 2004), the p16/Rb pathway selective oncolytic adenovirus (Fueyo *et al*, 2000; Page *et al*, 2007; Pesonen *et al*, 2012a), or *ICOVIR-5* (Cascallo et al, 2007).

Adenoviral vectors have also been developed with the purpose to boost anticancer immunity in melanoma delivering different cytokines, including IFNγ (ClinicalTrials.gov Identifier: NCT01082887), hIL-12 (ClinicalTrials.gov Identifier: NCT01397708 and NCT00815607), or IL-12 (Cao *et al*, 2013). Also different TAA

have been transduced using adenoviral vectors with encouraging results, as MAGE-1 in melanoma (Reed *et al*, 1997), or MART-1 (Butterfield *et al*, 1998; Zhai *et al*, 1996) used in phase I/II clinical trial with metastatic melanoma patients (Butterfield et al, 2008). Recently a recombinant adenovirus coding a truncated form of survivin, especially in combination with IL-2 has shown complete tumor rejection in melanoma (Zhang *et al*, 2012).

Costimulatory molecules have been also tested using adenoviral vectors as the co-expression of B7–1 with cytokines like GM-CSF used in an oncolytic virus that showed for the first time antitumoral immunity in human cancer patients (Cerullo *et al*, 2010; Kanerva *et al*, 2013), or IL-12 in immunocompetent melanoma mice models (Choi et al, 2006; Lee et al, 2006). Transduction of CD40 ligand to orthotopic bladder cancer model has elicited strong anti-tumor immunity and suppressed tumor growth (Loskog *et al*, 2004; Loskog *et al*, 2005; Malmstrom *et al*, 2010), while a CD40L-encoding oncolytic adenovirus stimulated beneficial immunologic responses in patients with progressing advanced solid tumors (Diaconu *et al*, 2012; Pesonen *et al*, 2012b).

2.7. MELANOMA

Melanoma is a neoplasia originated by malignant transformation of melanocytes and is characterized by rapidly growing incidence and mortality rates, by metastatic dissemination at early stages of tumor development and resistance to current therapies. Although accounting for only 4% of cases of all cutaneous malignancies, melanoma accounts for more than 75% of all deaths from skin cancer (Levine & Shapiro, 2012).

Surgical excision remains the standard of care for the treatment of primary melanomas. Until recently, chemotherapy with dazacarbazine was the therapeutic standard in patients with unresactable metastatic melanoma. Immunotherapy has been included into cancer therapy modalities in the last decades, including IFN-α, GM-CSF, (Garbe et al, 2011; Wolchok, 2012) and the recently approved for metastatic melanoma *ipilimumab* (anti-CTLA-4) (Hodi *et al*, 2010), BRAF kinase inhibitor *vemurafenib* (Chapman et al, 2011) and pegylated interferon for high-risk resected melanoma

(Herndon *et al*, 2012). However, the impact of conventional systemic therapy for metastatic melanoma is minimal, with best response rates for conventional therapy nearing only 30% and cure rates well below 10%; dacarbazine shows an approximately 20% objective response rate for metastatic melanoma and complete response rates of 5% (Serrone et al, 2000). Neither IFN-α2b nor interleukin-2 (aldesleukin, Proleukin) have shown an antitumor response rates higher than 20% (Atkins et al, 1999; Davar et al, 2012).

However, the various immunization protocols used in clinical trials led to only limited clinical improvement with disease progression in the majority of the patients (Lee *et al*, 2013; Rosenberg *et al*, 2004). Circulating T cells capable of recognizing tumor antigens can be induced in cancer patients by peptide-based immunotherapy, but the efforts to achieve regression of established tumors or a clear positive clinical response in the treatment of metastatic disease has been also below expectations (Godelaine *et al*, 2003; Rosenberg *et al*, 2005). On the other hand, some patients with metastatic melanoma show mixed responses to treatment with some lesions that undergo dramatic responses to therapy, even complete regression, while other lesions in the same patient continue to progress, or in some cases, new lesions develop, indicating the emergence of drug resistant clones. As melanoma progresses from primary to metastatic disease, the tumor acquires additional genetic and biologic properties that support tumor growth, invasion, and metastasis (Somasundaram et al, 2012).

As the immunotherapies to treat melanoma are aimed to activate anti-tumor immune recognition mechanisms, increase cytokine production and activate HLA-restricted tumor cell recognition and elimination by CTLs, the commonly observed HLA-I defects in tumors constitute a potential problem for T cell-based immunotherapy. Therefore, there is critical need for novel therapeutic strategies, such as gene therapy, with a suitable target molecule of key importance as those that lead to HLA class I upregulation on tumour cells which might improve outcomes in immunotherapy-based treatments.

3. Chapter 3. Overlay and Objetives

OVERLAY

Restoration of tumor cell HLA class I expression is very desirable as HLA class I loss is a common mechanism by which tumors evade immune surveillance. Recovery of HLA class I expression might therefore correct the inability of $CD8^+$ T cells to recognize tumor cells, as class I expression has been positively correlated with T-cell infiltration on melanoma among other malignancies. As β2m defects caused by mutations and/or LOH in chromosome 15 are one of the mechanisms implicated in HLA class I alterations, β2m defects might underlie the immune resistance that develops in some tumors following immunotherapy treatment.

Therefore, development of clinically applicable methods of gene therapy to recover HLA class I expression due to β2m defects may represent a promising approach to improve peptide-based immunotherapy in some subgroups of cancer patients.

GENERAL OBJETIVE

Identification of β2m genetic alterations in cancer cells during the course of metastatic melanoma progression and experimental correction of HLA class I expression in β2m-negative tumor cells using adenoviral vector.

SPECIFIC OBJETIVES

1. Analysis of HLA class I expression and analysis of the underlying molecular mechanisms implicated in the loss of HLA class I expression in several successive metastatic melanoma lesions during disease progression and in a cell line derived from a metastatic lesion obtained after immunotherapy with DC.
2. Construction of a non-replicating adenovirus expressing human β2m gene to recover normal human leucocyte antigen (HLA) class I expression in β2m-null cancer cells.
3. Assessment of the functional ability of the adenovirus-mediated restored HLA class I complex to induce anti-tumor activity of T-cells.
4. Characterization of the impact of the β2m-encoding adenovirus on tumor cell antigen presentation machinery, proliferation and apoptosis.

4. Chapter 4. Material and Methods and Results

IJC
International Journal of Cancer

Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma

Ana B. del Campo[1,2], Jon Amund Kyte[3], Javier Carretero[1,2], Svitlana Zinchenko[1], Rosa Méndez[1] Gloria González-Aseguinolaza[4], Francisco Ruiz-Cabello[1,2], Steinar Aamdal[5], Gustav Gaudernack[6] Federico Garrido[1,2] and Natalia Aptsiauri[1]

1 Department of Clinical Analysis and Immunology, University Hospital Virgen de las Nieves, Granada, Spain
2 Department of Biochemistry, Molecular Biology III and Immunology, University of Granada Medical School, Granada, Spain
3 Department of Oncology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway
4 Division of Hepatology and Gene Therapy, Center for Applied Medical Research (CIMA), University of Navarra Medical School, Pamplona, Spain
5 Department of Clinical Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway
6 Department of Immunology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

Cancer cells escape T-cell-mediated destruction by losing human leukocyte antigen (HLA) class I expression via various mechanisms, including loss of beta2-microglobulin (β2m). Our study illustrates the immune escape of HLA class I-negative tumor cells and chronological sequence of appearance of tumor β2m gene mutation in successive lesions obtained from a patient with metastatic melanoma. We observed a gradual decrease in HLA expression in consecutive lesions with few HLA-negative nodules in the primary tumor and the emergence of a totally negative lesion at later stages of the disease. We detected loss of β2m in β2m-negative nests of the primary tumor caused by a combination of two alterations: (i) a mutation (G to T substitution) in codon 67 in exon 2 of β2m gene, producing a stop codon and (ii) loss of the second gene copy by loss of heterozygosity (LOH) in chromosome 15. The same β2m mutation was found in a homogeneously β2m-negative metastasis 10 months later and in a cell line established from a biopsy of a postvaccination lymph node. Microsatellite analysis revealed the presence of LOH in chromosomes 6 and 15 in tumor samples, showing an accumulation of chromosomal loss at specific short tandem repeats in successive metastases during disease progression. HLA loss correlated with decreased tumor $CD8^+$ T-cell infiltration. Early incidence of β2m defects can cause an immune selection and expansion of highly aggressive melanoma clones with irreversible genetic defects causing total loss of HLA class I expression and should be taken into consideration as a therapeutic target in the development of cancer immunotherapy protocols.

Key words: tumor HLA class I loss, beta2-microglobulin mutation, immune escape, metastatic melanoma
Additional Supporting Information may be found in the online version of this article.
Conflict of interest: Nothing to report
Grant sponsor: ISCIII; Grant numbers: Miguel Servet CP04=00254/ I3 SNS contract to NA, and Rio Hortega grant CM08=00252 to AdelC; Grant sponsor: Servicio de Salud (SAS), Junta de Andalusia, Spain;Grant number: PI 09=0382 to FG; Grant sponsor: Fondo de Investigaciones Sanitarias (FIS); Grant numbers: PI 11=01022, PI 08=1265 and RETIC RD 06=0020 to FG; Grant sponsor: Consejeria de Ciencia, Innovacion y Empresa, Junta de Andalucýa, Proyectos Excelencia; Grant numbers: CTS-695, CTS-3952; CVI-4740; Grant sponsor: European Community (ENACT Project); Grant numbers: LSHC-CT-2004-503306, OJ 2004=c158, 18234
DOI: 10.1002/ijc.28338
History: Received 29 Mar 2013; Accepted 31 Mar 2013; Online 20 Jun 2013
Correspondence to: Natalia Aptsiauri, MD, PhD, Department of Clinical Analysis and Immunology, Virgen de las Nieves University Hospital, Avda. Fuerzas Armadas 2, 18014 Granada, Spain, Tel.: +34-958-020320, Fax: 134-958-283147, e-mail: naptsiauri@hotmail.com or natalia.aptsiauri.exts @juntadeandalucia.es

What's new?

In order for immunotherapy to work, tumor cells must first present tumor-associated peptides on their surface via the HLA class I complex. In this chronological study of melanoma progression, the authors suggest that early loss of the beta2-microglobulin (β2m) component of the HLA class I complex allows some tumor cells to evade the immune system. This, in turn, gives these cells a selective advantage for growth, metastasis, and resistance to immunotherapy. β2m/HLA class I expression may thus provide both a biomarker and a therapeutic target in the development of cancer immunotherapy protocols.

It is known that the immune system is able to mount a specific T-cell-mediated immune response against solid tumors. However, activated tumor-specific T-cells are frequently unable to reject the tumor, leading to cancer progression. This is attributable to the immune escape and expansion of cells with low immunogenicity and high metastatic capacity. Antitumor immune response and the success of cancer immunotherapy depend on the recognition of the human leukocyte antigen (HLA) class I complex (heavy chain/beta2-microglobulin (β2m)/tumor peptide) by cytotoxic T-cells (CTLs).[1–3] Therefore, the frequently observed defects in tumor HLA class I expression lead to cancer immune escape and metastatic progression.[3–7] Some HLA alterations can be recovered after stimulation with cytokines or immunotherapy (so-called "soft" lesions), whereas tumor cells with irreversible structural defects ("hard" lesions) may escape immune recognition and become a major threat to innate immunity.[8] It has been demonstrated that in melanoma patients undergoing immunotherapy, the lack of response to immunotherapy and generation of progressing metastases appears to be associated with immune selection of HLA-negative tumor cell variants with irreversible defects.[9,10] Hence, to select an appropriate immunotherapy protocol, it is important to know whether the nature of the tumor HLA class I defect is regulatory or structural.

Moreover, it was recently demonstrated that tumor HLA class I antigen expression in melanoma is an important component of the "immunological constant of rejection," in which similar molecular pathways lead to CTL-mediated tumor rejection, allograft rejection, graft-versus-host disease or the development of an autoimmune disease.[11]

There is compelling evidence that β2m deficiencies create immune escape phenotypes in different types of cancer, including melanoma.[12,13] Two genetic events, a mutation of one copy of the β2m gene and the loss of the other copy, i.e., loss of heterozygosity (LOH), have been found to underlie β2m loss in malignant cells,[14,15] although the exact chronological sequence of the two events has not been fully elucidated. The negative impact of this phenomenon on the clinical course of the disease is indicated by the correlation between HLA class I abnormalities in tumor lesions and a poor clinical outcome in various types of malignant diseases.[16,17]

HLA alterations have been previously reported in tumor tissues and/or cancer cell lines obtained from different patients at a particular time point during cancer development.[12,13,18–21] Most of these studies did not follow the evolution of β2m genetic alterations during metastatic progression. There have been only few reports describing a sequential immune escape mechanisms associated with HLA class I loss, when both tumor samples and tumor-derived cell lines obtained from the same patient have been analyzed; most of these publications describe genetic defects underlying HLA class I altered expression only in cell lines derived from metastases.[19,22,23]

In our study, we were able to track the fate of a β2m gene mutation and LOH in chromosome 15 in several successive metastatic melanoma lesions and in a cell line derived from a lymph node biopsy obtained after immunotherapy with dendritic cells (DCs) transfected with autologous tumor mRNA. We detected a β2m mutation in microdissected β2m-negative areas of a heterogeneous primary tumor and the same

mutation was also found almost a year later in a metastasis with homogeneously negative β2m expression phenotype. This observation suggests that β2m loss could be an early event in the tumor progression, leading to the immune escape of HLA class I-negative tumor cells.

Material and Methods

Tumor tissue samples and cells

Tumor samples and the melanoma cell line DNR-DC-M010 established from a postvaccination lymph node fine needle biopsy were obtained from a 72-year-old patient with metastatic melanoma (received as part of European collaborative project ENACT). This patient was treated in Norwegian Radium Hospital using DCs transfected with autologous tumor mRNA. 24 In October 2001, a primary tumor was surgically removed (Sample 1, biopsy 13872) and a second lesion (Sample 2, biopsy 17130—several blocks) was removed 2 months later. In June 2002, several metastases from lymph nodes at both axillae and a cutaneous lesion were surgically removed (Sample 3, biopsy 8755—several blocks and Sample 4, biopsy 9168—several blocks). In October 2002, a metastasis in the axilla sinister (Sample 5, biopsy 17790) was used to isolate the tumor mRNA and to develop a DC vaccine that was administered in December 2002. After vaccination, in January 2003, the DNR-DC-M010 cell line was established from a fine needle biopsy of the right inguinal lymph node (Fig. 6). The patient died in February 2003. Supporting Information Table S1 summarizes the sampling time points with clinical and pathological characteristics of tumor specimens and the cell line obtained from the patient. After detection of a β2m mutation in the cell line, we retrospectively analyzed 20 tumor samples from six paraffin-embedded biopsies obtained from this patient at different time points during the disease course. The cell line was grown in RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies, Karlsruhe, Germany), 2% glutamine (Biochrom KG) and 1% penicillin/ streptomycin (Biochrom KG) at 37°C in a humidified atmosphere with 5% CO2. Autologous PBMCs (A*0205, 3201; B*5001, 5101; Cw*0602, 1502) were used for LOH analysis and genome sequencing.

Monoclonal antibodies

The following anti-HLA class I antibodies were used: mAb W6/32, which recognizes a cell surface complex of β2m with HLA heavy chain; L-368—anti-human β2m mAb; HC-10— mAb against free HLA heavy chain and anti-pan-HLA class I mAb EMR8-5 (for paraffin-embedded tumor samples), which recognizes a common HLA-A,B,C heavy-chain sequence[25] (MBL, Naka-ku, Nagoya, Japan); antibodies against TAP-1, TAP-2, tapasin, LMP-2, LMP-7, calnexin, calreticulin and ERP-57 were purchased from Abcam (UK) and used for flow cytometry and immunocytochemistry. Antibodies to CD3, CD4, CD8 and CD56 markers for immunohistochemical detection of tumor-infiltrating lymphocytes (TILs) were purchased from Master Diagnostica (Granada, Spain). The mAbs against human NK ligands including anti-ULBP-1- Fluorescein monoclonal antibody (for intracellular staining), anti-human ULBP-2-phycoerythrin antibody and the human ULBP-3 antibody were purchased from R&D systems (Minneapolis, MN), and anti-MICA-MICB (clone 2C10) from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma, St Louis, MO) was used as a secondary antibody for flow cytometry.

Immunohistochemistry

Immunocytochemical analysis of the DNR-DC-M010 cell line was performed on cytospin glass slides as previously described.[26] After deparaffinization of 4- to 6-μm-thick sections from formalin-fixed paraffin-embedded tissue blocks and antigen retrieval (heat, EDTA or citrate buffer, depending on the antibody) slides were rehydrated and used for antibody staining with the biotin–streptavidin system (supersensitive Multilink-HRP/DAB kit, BioGenex).

DATE	EVENT	BIOPSY NUMBER	LOCATION	PATHOLOGY REPORT	OBSERVATIONS
Sample 1 October 2001	Lesion surgically removed	13872 (1 block)	Cutis (dorsum)	Primary tumor	
Sample 2 December 2001	Lesion surgically removed	17130 (6 blocks)	Cutis (dorsum)	Second primary tumor or metastasis	
Sample 3 June 2002	Lesion surgically removed	8755 (4 blocks)	Lymph node (axilla dexter)	Melanoma in 3/5 lymph nodes. Metastasis	
Sample 4 June 2002	Lesion surgically removed	9168 (blocks # 1-5)	Lymph node (axilla sinister)	Melanoma in 4/9 lymph nodes. Metastasis	
Sample 4 June 2002	Lesion surgically removed	9168 (blocks # 6-8)	Cutis (dorsum)	Metastasis	
Sample 5 October 2002	Lesion surgically removed	17790 (1 block)	Lymph node (axilla sinister)	Metastasis	mRNA from this tumor was used in the DC vaccine
December 2002	DC vaccine administration				DC vaccine with autologous tumor mRNA from 17790 biopsy.
Cell line January 2003	Fine needle biopsy		Right inguinal lymph node		Origin of the melanoma cell line DNR-DC-M010
February 2003	Death of the patient				

Supplementary Table 1S: Sampling time points with clinical and pathological characteristics of tumor specimens and a cell line obtained from patient with metastatic melanoma.

RNA isolation and PCR

Total RNA was extracted from cultured melanoma cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and used for reverse-transcriptase PCR analysis with the iSriptTMcDNA Synthesis Kit (Bio-Rad). Primers used for PCR amplification of β2m cDNA were as follows: forward 5´-GGGCATTCCTGAAGCTGACA-3´ and backward: 5´-GGTTGCTCCACAGGTAGCTCTA-3´ with 618 bp of predicted PCR product size. The primers used for HLA-A, HLA-B and HLA-C heavy-chain amplification have been described previously,[27] and their predicted PCR product sizes are 197, 140 and 151 bp, respectively.

Genomic DNA extraction and sequencing of β2m gene

DNA extraction from cells was performed using DNA Midi Kit (Qiagen, Valencia, CA). Immunolabeled paraffin sections were microdissected using a laser micromanipulator (PALM Microlaser Technologies GmbH, Bernried, Germany). Microdissected fragments were collected in PALM Adhesive Caps and were used for DNA extraction using the QIAampVR DNR FFPE Tissue Kit (QIAGEN, Hilden, Germany).

The β2m cDNA from the cell line was sequenced using the same primers as in the PCR analysis. The amplification of β2m gene from the microdissected tumor nests of paraffin-embedded samples was performed on genomic DNA with Illustra PuRe- Taq Ready-To-Go™ PCR Beads (GE Healthcare Europe, Barcelona, Spain) using the following forward primers: 5´-CGATATTCCTCAGGTACTCC-3´and 5´-GGTGAATTCAGTGTAGTACAAG-3´, and with one backward primer: 5´-ACACAACTTTCAGCAG-CTTAC-3´. The predicted PCR product sizes are 311 and 114 bp, respectively. Sequencing was performed with the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK) using Centri-Sep Columns (Applied Biosystems), ABI 3130x/Genetic Analyzer and SequencingAnalysis v5.2 software (Applied Biosystems).

Microsatellite analysis

DNA obtained from the cell line, from microdissected tumor and from the patient's PBMCs was studied using eight short tandem repeats (STRs; D6S291, D6S273, C.1.2.C, C.1.2.5, D6S265, D6S105, D6S276 and D6S311) mapping the HLA region in chromosome 6. The β2m studies used five STR markers that flanked the gene in chromosome 15 (DS15209, DS15126, DS15146, DS151028 and DS15153). The 5´ end of one primer of each primer set was tagged with a fluorescent label (Applied Biosystems). Details were previously described[28] and STR markers for chromosome 15 are summarized in Supporting Information Table S2. Microsatellite instability was determined by using the ABI 3130x/Genetic Analyzer with 16 capillaries and GeneMapper v4.0 software (Applied Biosystems). LOH was assigned when the signal of one allele in the tumor sample was reduced by more than 25% in comparison to the PBMCs or to tissue stroma.

STR	Location	Size	Alleles	%Het *	Sequence
D15S146	15q15.1	217-229	7	68	Fw:GGAAGCCTGACTTTAATCCG-3´ Bw:ATGTCTGTTCAGATCCTTTGC-3´
D15S1028	15q21.1	171-187	9	82	Fw:TGTCCTGAAATTCCCAAC-3´ Bw:GAACTGTGCTCTGTGCTC-3´
D15S126	15q21.1	188-218	11	82	Fw:GTGAGCCAAGATGGCACTAC-3´ Bw:GCCAGCAATAATGGGAAGTT-3´
D15S209	15q21.3	198-208	10	78	Fw:AAACATAGTGCTCTGGAGGC-3´ Bw:GGGCTAACAACAGTGTCTGC-3´
D15S153	15q22.31	194-226	12	86	Fw:ÁGTACCTGAAAGGGTGGG Bw:GATCAGTGTAGGCTCCAAA-3´

* Expected frequency of heterozygosity in the population

Supplementary Table 2: Microsatellite markers flanking the β2m gene in chromosome 15 used for LOH analysis.

Flow cytometry

FACS analysis using a panel of specific antibodies directed against HLA class I antigens, APM components and NK ligands was performed on a BD FACS Canto Apparatus (Becton Dickinson, Franklin Lakes, NJ) in baseline conditions and after 48-hr incubation with IFN-g (800 U/ml). Secondary FITC-labeled antibodies were used. Results were presented as representative fluorescence plots and expressed as relative mean fluorescence intensity.

Restoration of tumor HLA class I expression by adenoviral-mediated β2m gene transfer

The purified recombinant adenovirus carrying human β2m (AdCMVβ2m) under the control of cytomegalovirus (CMV) promoter[26] was added to the cells in a 2% FCS supplemented medium at MOI 2,5 for 24–48 hr and the recovery of β2m expression and restoration of normal surface HLA class I expression was confirmed by FACS, immunocytochemistry and in ELISPOT and IFN-γ production assays. In control experiments, cells were infected with a similar adenoviral vector carrying GFP or Luciferase gene (AdCMVGFP and AdCMVLuc, respectively).

IFN-gamma production and chromium release assays for CTL analysis

Donor HLA-A2 PBMCs were primed with HLA-A2-restricted influenza A virus matrix 58–66 peptide [GILGFVFTL, a gift from Dr. Esther Larrea (CIMA, Pamplona, Spain)]. PBMCs (10^5 cells per well) were cocultured in 96-well round-bottom plates with untreated DNR-DC-M010 melanoma cells ($5x10^4$ cells per well) or with cells manipulated in one of the following ways: pulsed with flu peptide, transduced with AdCMVβ2m, peptide-pulsed and transduced with AdCMVβ2m, transduced with a control AdCMVLuc vector or peptide-pulsed and transduced with AdCMVLuc. After 48 hr of coculture, T-cells producing IFN-γ were counted using an ELISPOT kit (BD-Pharmingen, San Diego, CA). Melanoma cell line ESTDAB-064 (ESTDAB melanoma cell collection; http://www.ebi.ac.uk/ipd/estdab/) with normal HLA class I expression was used as control. Tumor cell lysis was measured by incubating the effector T-cells with 3000 $Na_{251}CrO_4$-labeled target cells at different tumor-effector cell ratios. Supernatants were harvested 4 hr later and the percentage of specific lysis was calculated according to the formula: [(cpm experimental x cpm spontaneous)/(cpm maximum x cpm spontaneous)]x100, where spontaneous lysis corresponds to target cells incubated in the absence of effector cells, and maximum lysis is obtained by incubating target cells with 5% Triton X-100.

Statistical analysis

Fisher's exact test was used to analyze the data. Statistical significance was established at $p < 0.05$. The statistical analysis was performed using SPSS 10.0 software (SPSS, Chicago, IL).

Results

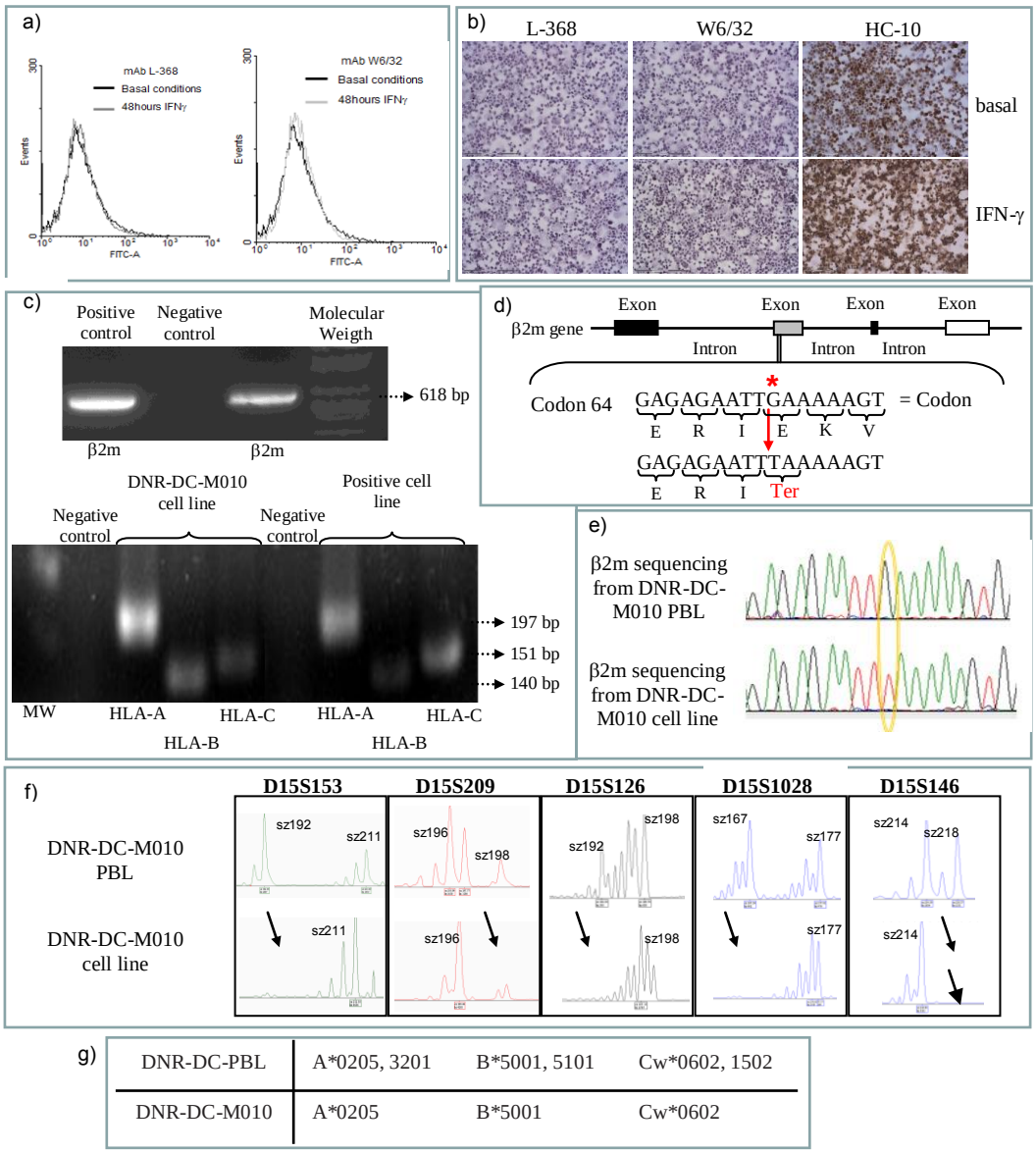
Total loss of HLA class I expression in DNR-DC-M010 cell line

Melanoma cell line DNR-DC-M010 (M010) was established from a fine needle biopsy of a postvaccination lymph node metastasis. FACS and immunocytochemical analysis of cells stained with W6/32 and L-368 antibodies showed a total absence of cell surface expression of HLA class I complex and β2m, both under baseline conditions and after incubation with IFN-γ (Figs. 1a and 1b). In contrast, intracellular labeling of free HLA class I heavy chain (HC-10 antibody) was positive (Fig. 1b). Intracellular immunostaining of antigen presentation machinery (APM) components, including LMP2/7, TAP1/2, tapasin, calreticulin, ERp57 and calnexin, was positive (data not shown). These results and the lack of HLA upregulation by IFN-γ suggest that the loss of HLA expression in this cell line is caused by the loss of β2m. We further confirmed this hypothesis by recovery of HLA class I expression after adenoviral-mediated β2m gene transfer and by molecular analysis of the β2m gene.

β2m gene alterations

We analyzed the PCR products of the β2m and of HLA heavy chain A, B and C genes in melanoma

cell line DNRDC- M010 and in control melanoma cell line with normal HLA class I expression. Figure 1c demonstrates that β2m and HLA-A, -B and -C genes were transcribed correctly, with a predicted amplicon size similar to that of the control cell line. Nucleotide sequencing of the open reading frame of β2m gene was performed and compared with the sequence of that gene in patients' PBMCs. We detected a point mutation at codon 67 in exon 2, where guanine was substituted for thymine (G to T), leading to generation of an early stop codon (Figs. 1d and 1e). The short version of β2m protein cannot form a functional complex with HLA heavy chain because of the absence of the key amino acids necessary for binding with the heavy chain.



g)

DNR-DC-PBL	A*0205, 3201	B*5001, 5101	Cw*0602, 1502
DNR-DC-M010	A*0205	B*5001	Cw*0602

Figure 1. Negative HLA class I surface expression on DNR-DC-M010 cells analyzed by flow cytometry using the anti-β2m antibody L-368 (a, left FACS image) and the anti-HLA/β2m complex antibody W6/32 (b, right FACS image). Immunofluorescence was measured either under baseline conditions (black lines) or after 48 hr of incubation with IFN-γ (gray lines). Immunocytochemistry of the cells (b) confirmed the loss of both HLA class I complex (W6/32) and β2m (L-368) labeling; however, immunostaining with antibody HC-10 recognizing free HLA heavy chains was positive. (c) β2m, HLA-A, -B and -C mRNA expression was analyzed by RT-PCR using specific primers, and positive amplification of all the genes with the predicted amplicon size was observed. (d) β2m gene was sequenced in the cell line and a mutation was found. The sequence from codon 64 to 69 of the β2m gene in exon 2 is shown in the schematic diagram. Asterisk indicates the location of the G-to-T transversion mutation (codon 67) creating a premature stop codon. (e) Sequencing histograms illustrating the G-to-T substitution. Sequencing of the β2m gene from cDNA isolated from autologous patient PBMCs was used as a control. (f) Analysis of the heterozygosity of chromosomes 15 and 6 in melanoma cell line DNR-DC-M010 was performed using microsatellite analysis on DNA from melanoma cells and compared to DNA obtained from the patient's autologous PBMC. Scans from the results of PCR analysis of five microsatellite markers on chromosome 15 reveal a pattern of allelic loss. Arrows indicate the loss of heterozygosity at five markers. (g) Results obtained from genomic typing of chromosome 6 in DNR-DC-M010 cells compared to autologous PBMC cDNA indicate LOH at chromosome 6.

LOH in chromosomes 15 and 6 in melanoma cell line

Microsatellite analysis of the melanoma cell line revealed LOH at chromosome 15 for the five markers, showing a single allele at STR sites D15S1028 and D15S146, which flank β2m gene, and also in STR D15S153, D15S126 and D15S209, whereas PBMC control showed retention of heterozygosity with two bands at the studied sites (Fig. 1f). HLA typing was performed using a low-resolution genomic sequence-specific oligonucleotide analysis, and LOH in chromosome 6 was found (HLA typing results for melanoma cell line DNR-DCM010 were A*0205, B*5001 and Cw*0602) (Fig. 1g); it was verified by microsatellite analysis of chromosome 6, which detected LOH in six of eight STR (the remaining two were noninformative), whereas the autologous PBMC retained heterozygosity.

Adenovirus-mediated reconstitution of HLA class I expression and its functional activity

A nonreplicating adenovirus (AdCMVβ2m) expressing the wild-type human β2m gene was constructed as previously described.[26] Melanoma cells transduced with this adenovirus recovered HLA class I expression, as demonstrated by FACS (Fig. 2a) and by immunocytochemical analysis (data not shown) using antibodies against β2m (L-368) and HLA class I complex (W6/32). The functional restoration of the HLA class I complex was confirmed by analyzing the ability of virally transduced tumor cells to stimulate IFN-γ production by autologous and HLA-matched T-cells and to induce antitumor cytotoxic activity in an HLA-A2-restricted manner. IFNγ secretion by HLA-matched T-cells was measured by ELISPOT after 48 hr of coculture with the original β2m–negative melanoma cells or AdCMVβ2m-infected melanoma cells pulsed with HLA-A2-restricted virus matrix 58–66 peptide (GILGFVFTL). Figure 2b shows a significant elevation of ELISPOT reactivity to the peptide in melanoma cells infected with AdCMVβ2m virus. In the ^{51}Cr release assay, the percentage of T-cell lysis of peptide-pulsed melanoma cells transduced with AdCMVβ2m virus was higher than that of melanoma cells transduced with the control vector carrying luciferase gene (AdCMVLuc) (Fig. 2c). Immunohistochemical analysis of tumor HLA class I expression We analyzed HLA class I expression in paraffin-embedded primary tumor and four consecutive metastases surgically removed from the patient at different time points during disease progression before the administration of the DC vaccine. Positive labeling for HLA-A, -B, -C heavy chain (EMR 8-5 antibody) and β2m (L-368 antibody) was detected in the primary tumor.

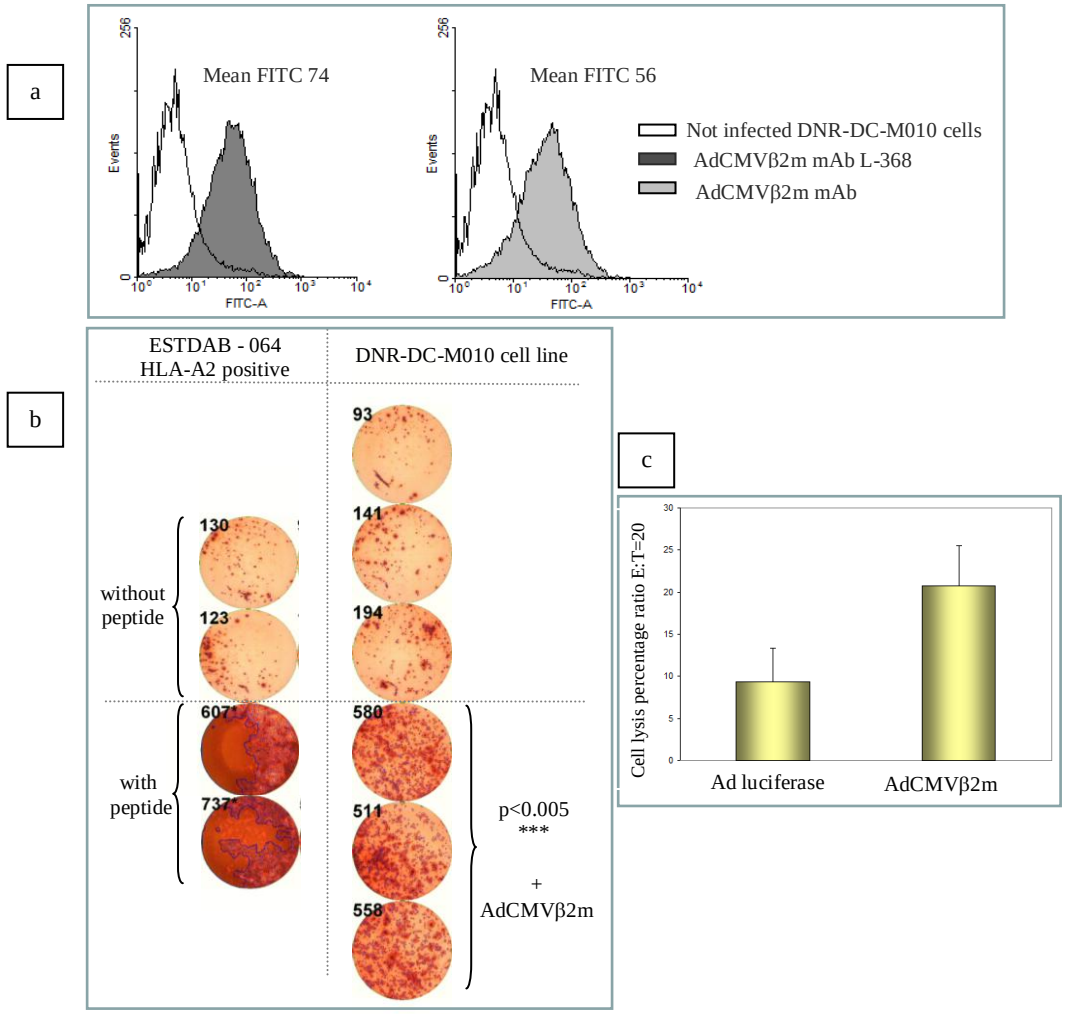


Figure 2. (a) Restoration of surface HLA class I expression and function on DNR-DC-M010 cell line 48 hr after infection with AdCMVβ2m virus, as demonstrated by flow cytometry using L-368 (black filled histograms) and W6/32 antibodies (gray histograms). (b) Increase in IFNγ production (ELISPOT) by HLA-A2+ donor T-cells primed with influenza A peptide after coculture with M010 cells infected with AdCMVβ2m virus. HLA-A2+ melanoma cells with normal HLA class I expression were used as a positive control. (c) The percentage of specific T-cell-mediated lysis (calculated by radioactive ^{51}Cr release) of M010 cells transduced with β2m gene was higher than the lysis of luciferase-transfected control cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

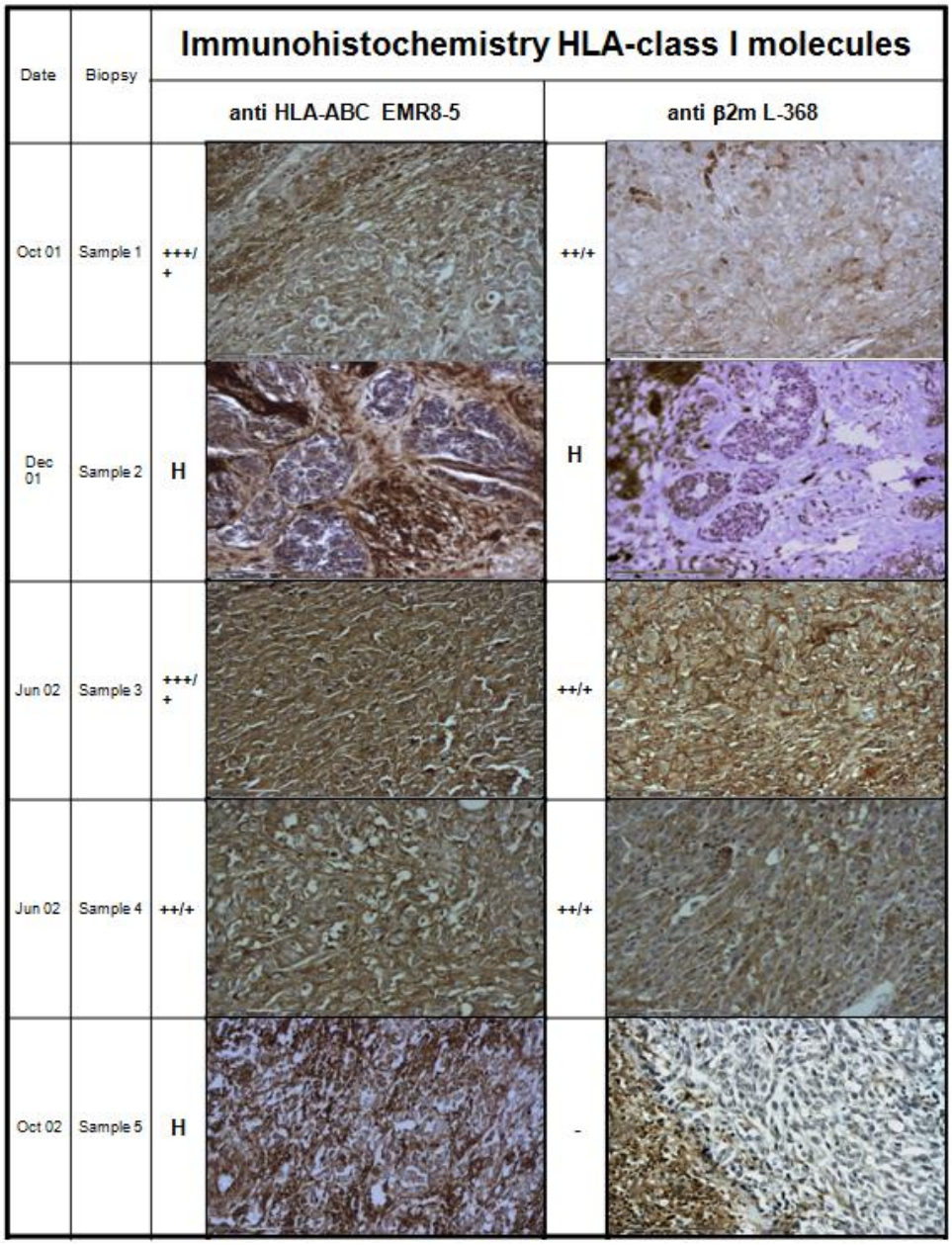


Figure 3. Immunohistochemical analysis of HLA class I expression in paraffin-embedded tumor samples with antibodies that recognize free HLA-A, B, C heavy chain (EMR 8-5) and β2m (L-368). Samples 1, 3 and 4 are positive for HLA expression. Sample 2 has a heterogeneous (H) pattern of HLA-ABC and β2m expression. The last sample obtained during metastatic progression (Sample 5) is heterogeneous for HLA expression and homogeneously negative for β2m.

(Sample 1) and metastatic lesions (Samples 3 and 4) (Fig. 3). The second metastatic nodule (Sample 2) was heterogeneous (Figs. 2 and 3a), with tumor nests positive or negative for both heavy chain and β2m. Sample 5 also showed a heterogeneous immunolabeling of heavy chain, but all the tumor areas were negative for β2m. The same β2m mutation causing total HLA class I loss detected in DNRDC- M010 cell line derived from a postvaccination lesion was found in microdissected β2m-negative tumor nests from Sample 2 (Fig. 4a) and Sample 5, the latter of which was used to isolate mRNA for DC vaccine.

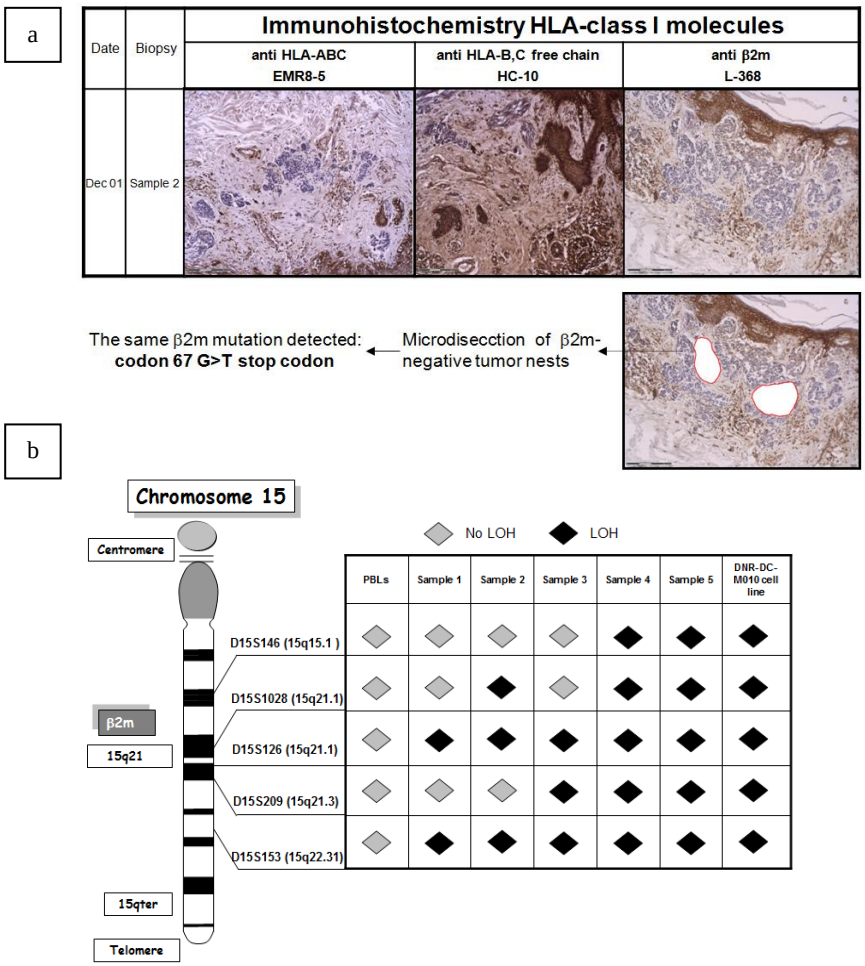


Figure 4. (a) A heterogeneous pattern of HLA heavy chain (EMR 8-5 and HC-10 antibody) and β2m (L-368 antibody) immunolabeling in tumor Sample 2. Microdissection was performed to isolate β2m-negative nodules; the mutation detected in the β2m gene was identical to the one found in the cell line DNR-DC-M010 (G-to-T substitution in codon 67). (b) Loss of a second copy of β2m gene was confirmed by a microsatellite analysis, which revealed the presence of LOH in four of five tumor samples and showed an accumulation of chromosomal loss at specific STRs on chromosome 15 during metastatic progression.

Thus, the β2m gene mutation first detected in the second consecutive lesion with a heterogeneous HLA phenotype, reappeared again in another HLA-negative lesion used for the vaccine preparation.

LOH in chromosomes 15 and 6 in a primary tumor and in consecutive melanoma metastasis

We analyzed microdissected tumor specimens for LOH at chromosomes 15 and 6. LOH for chromosome 6 was detected in all studied samples. LOH at chromosome 15 was detected for all five specific markers in Samples 4 and 5 (Fig. 4b). Sample 3 exhibited LOH for three markers, two of which (D15S126 and D15S209) flank the β2m region. Tumor Sample 2 exhibited LOH for three markers, one of which (D15S1269) was located close to the β2m region, whereas the other two of the five markers retained heterozygosity. Figure 4b demonstrates an accumulation of chromosomal loss at specific STRs at chromosome 15 during metastatic progression. Finally, Figure 6 summarizes the evolution of β2m gene alterations in recurrent metastases during the melanoma progression, showing a growth and dissemination of β2m-negative cells.

Immunohistochemical analysis of tumor infiltration by lymphocytes

Tumor infiltration with $CD8^+$ T-cells was correlated with a high expression of HLA class I antigens, while the infiltration of $CD56^+$ NK cells was low in all studied lesions. Figure 5a depicts that infiltration with $CD3^+$ and $CD8^+$ lymphocytes is stronger in β2m -positive samples (Samples 3 and 4) than in β2m-negative tumor nests (Biopsies 2 and 5), where they were only observed on the periphery of the tumor node.These results demonstrate that CTLs have a better ability to penetrate HLA-positive tumors. In addition, tumor infiltration with $CD56^+$ NK cells was practically negative in all samples.

Expression of activating NK ligands on DNR-DC-M010 melanoma cells

To evaluate the potential ability of NK cells to eliminate HLA class I-negative melanoma cells, we analyzed the expression of several activating NK ligands on DNR-DC-M010 cells using FACS analysis. We found that the expression of MICA/B and ULBP-1, -2 and -3 was positive on the studied melanoma cells (Fig. 5b).

Discussion

Our study illustrates the evolution of HLA loss in successive metastases caused by a structural β2m gene defect in a melanoma patient with an aggressive clinical course and resistance to DC vaccination. Immunohistochemical and mutational analyses revealed a chronological sequence of HLA class I alterations in successive metastatic lesions, with an early onset of tumor β2m loss in heterogeneous primary tumor (Figs. 3 and 4a) followed by immune selection and outgrowth of β2m-negative melanoma cells. We detected a β2m gene mutation (G to T substitution in the codon 67 in exon 2 that generates a stop codon) and LOH in chromosome 15 in β2m-negative tumor nests microdissected from an early lesion with heterogeneous HLA expression pattern (Fig. 4a). The same β2m mutation was found in a late metastatic lesion with homogeneously β2m-negative immunophenotype (source of vaccine preparation) at 10 months of the disease and in HLA class I-negative melanoma cell line established 3 months later from a fine needle biopsy of a postvaccination lymph node (Figs. 1 and 6). The mutation found in this melanoma cell line is different from previously published β2m mutations in melanoma, colon cancer or lymphoma cells, although it is located in the previously described mutational "hot-spot" area of the β2m gene on chromosome 15.[14,23,29] Adenovirus-mediated reconstitution of β2m expression recovered total HLA class I expression, suggesting that β2m loss was the primary cause of HLA loss. In addition, β2m transduced melanoma cells became sensitive to lysis by peptide-stimulated HLA-A2-restricted T-cells and recovered the ability to induce peptide-specific IFN-γ secretion by T cells in an HLA-restricted manner (Fig. 2). IFN-γ production was induced by both influenza virus peptide (Fig. 2) and Melan-1/Mart-A peptide, for which this cell line is positive (data not shown).

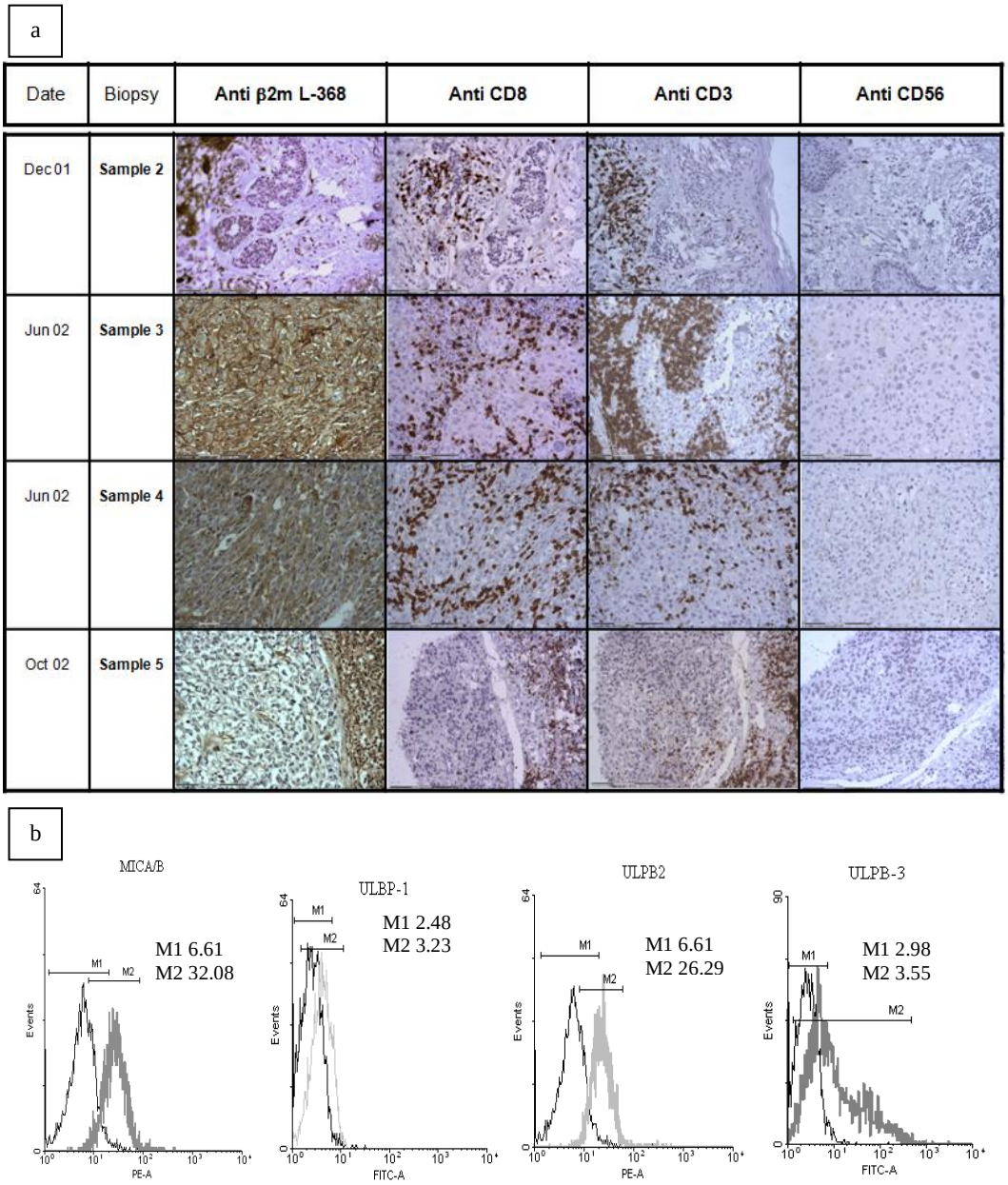


Figure 5. (a) Immunohistochemical analysis of β2m expression and tumor infiltration by T-cells and NK cells in paraffin-embedded tumor samples with antibodies that recognize β2m (L-368), $CD8^+$, $CD3^+$ and $CD56^+$ markers. Tumor infiltration with $CD3^+$ and $CD8^+$ T-cells correlates with positive β2m immunostaining and has mostly peritumoral localization; almost negative labeling for CD56 indicates very low incidence or absence of NK cells in the tumor samples. (b) Positive expression of NK ligands (MICA/B, ULBP1, 2 and 3) in DNR-DC-M010 cells.

Microsatellite analysis revealed LOH in chromosomes 6 and 15 with a gradual accumulation of chromosomal loss at specific STRs in successive metastases (Figs. 1, 4b and 6). LOH for chromosome 15 was detected in four of five studied cancer samples coinciding with the mutation in β2m gene. LOH-15 is frequently detected even in HLA-positive tumor cells, suggesting that LOH in chromosome 15 may be an early event in malignant transformation. Damage of a single β2m allele by LOH on chromosome 15 may be sufficient to generate a tumor cell precommitted to escape, and our group previously reported that the percentage of LOH in chromosome 15 is elevated in different types of malignancy.[15]

Altered expression of HLA class I molecules can also be caused by a transcriptional downregulation of APM proteins.[7,30] In our study, the tumor samples and cell line were positive for APM expression at both protein and transcriptional levels (data not shown).

Early tumor β2m loss during natural tumor development allows malignant cells to evade T-cell recognition. In our study, we observed a high degree of infiltration with $CD3^+$ and $CD8^+$ T-cells in HLA-positive tumors (mostly peritumoral localization) and low numbers of these TILs in HLAnegative nodules (Fig. 5a). The failure to halt tumor growth and dissemination is likely due to the inability of HLAnegative tumor cells to present tumor-associated peptides to T-cells. However, loss of tumor HLA class I expression is anticipated to make malignant cells susceptible to NK cell attack. However, in our study, we found that tumor infiltration with $CD56^+$ NK cells was very low (Fig. 5a). At the same time, DNR-DC-M010 cells were positive for the expression of activating NK ligands MICA/B and ULBP-1/2/3 (Fig. 5b). Thus, NK cells could have eliminated these melanoma cells but failed to migrate into the tumor, possibly attributable to suppressive factors in the tumor microenvironment. In addition, interaction between activating and inhibiting signals in NK cells frequently leads to the failure of natural cytotoxic reactions against malignant cells. This is supported by a number of reports indicating low NK cell activity in solid tumors.

However, on the basis of the sequencing and LOH data, we can see that the loss of HLA class I expression owing to structural alteration in the β2m gene is an early event; it can be detected at the very beginning of the disease and accumulates during metastatic progression, giving tumor escape variants an advantage for growing and disseminating. It is supported by the early onset of LOH in chromosome 15 and by the emergence of β2m mutation. The fact that we found the same β2m mutation in a late-stage lesion homogeneously negative for β2m and in a postvaccination cell line strongly suggests that these cells are escape variants that eventually contributed to the patient's death.

The studied cell line with total loss of HLA class I expression due to β2m mutation was derived from a post-therapy lesion, suggesting that these cells have been exposed to an additional immunotherapy-induced T-cell-selective pressure. It is likely that the tumor cells with HLA class I alterations in this patient first escaped from the immune attack during natural metastatic progression and that HLA-negative immune escape variants were further immunoselected during the vaccination, which might explain the course of HLA expression on tumor cells and the failure of the therapy.

The clinical efficacy of peptide-based and T-cell-based immunotherapy depends on proper copresentation of tumor associated peptides by HLA class I complex. Immunotherapy usually leads to upregulation of HLA expression unless the tumor cells harbor structural genetic defects, e.g., β2m mutation. [31] In the studied case, the patient was treated with an individualized melanoma vaccine based on the transfection of DC with autologous tumor mRNA. DC loaded with complete tumor mRNA may generate an immune response against a broad repertoire of antigens. This therapy stimulates antitumor T-cell responses by presentation of tumor antigens by DC, not by tumor cells, helping to override the loss of antigen presentation ability of tumor cells associated with HLA loss. Although the metastasis from which tumor mRNA was isolated already harbored a β2m

mutation, it would not have compromised the antigen presentation ability of the DC, especially that the HLA class I expression on the patients' DC was normal (data not shown).

We cannot make a conclusion that the resistance to immunotherapy with DC vaccination in this patient was primarily due to tumor β2m loss, and we cannot exclude a possibility that β2m-positive and β2m-negative cells coexist until the end and that the vaccination was ineffective for other reasons.

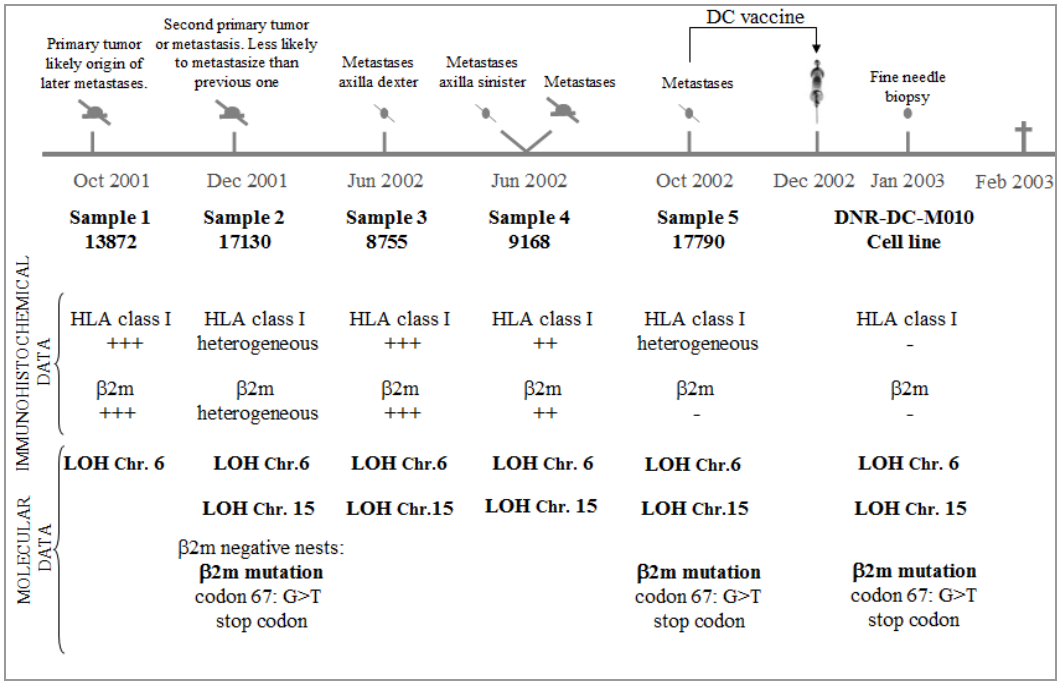


Figure 6. Clinical evolution of metastatic melanoma showing the early appearance of β2m mutation in a heterogeneous primary tumor (Sample 2); the same mutation was detected in a homogeneously β2m-negative metastatic lymph node 10 months later (Sample 5) and in the cell line derived from a postvaccination lymph node. LOH in chromosome 15 was detected in all lesions except for the first tumor specimen. The intensity of HLA class I-positive immunolabeling (+++,++) was determined by using antibodies that recognize β2m and HLAA, B,C heavy chain.

There is an accumulating body of evidence suggesting that primary melanoma tumors are heterogeneous, with different molecular mechanisms generating different subsets of cancer cells with distinct metastatic capacity, resulting in distinct clinical courses and variations in the response to therapy.[32] Hence, the identification of genetic variants and the characterization of molecular mechanisms underlying the development of aggressive phenotypes could contribute to a better understanding of cancer immune escape. This could be useful to identify novel targets for melanoma treatment and to select the most effective therapy for different melanoma subsets. Our data demonstrate that immune evasion strategies can develop before the clinical application of immunotherapy. Moreover, immunological tumor analysis before treatment may provide a rationale for excluding patients who are unlikely to respond to immunotherapy. The considerable differences in clinical responses among melanoma patients receiving ipilimumab or adoptive TIL therapy have highlighted the need for novel cancer biomarkers.

In tumor cells with β2m gene defects, HLA class I expression can only be recovered by the introduction of wild-type β2m gene. In our study, adenovirus-mediated transfer of β2m gene to melanoma cells restored HLA class I antigen expression and recognition by peptide-specific HLA-A2-restricted cytotoxic T lymphocytes. Loss of a normal HLA class I phenotype is a pivotal strategy for tumor cells to circumvent an effective immune response and is associated with tumor progression in cancer patients. Our observations support the importance of developing strategies for the restoration of tumor HLA class I-positive phenotype. In particular, development of clinically applicable methods of gene therapy to recover HLA class I expression may represent a promising approach to improve peptide-based immunotherapy in some subgroups of cancer patients.

Acknowledgements

The authors thank Dr. Isabel Maleno and Anabel Rodriguez (University Hospital Virgen de las Nieves) for the help with microsatellite analysis and for immunohistochemistry technical assistance. They are also thankful to Drs. Aasmund Berner, Else Marit Suso (The Norwegian Radium Hospital) and Jan Kristian Godoey (Fredrikstad Hospital) for their cooperation. Dr. Natalia Aptsiauri was supported by ISCIII and FPS contracts (Miguel Servet CP04/00254 and I3 SNS) and by grants from the Servicio de Salud (SAS), Junta de Andalusia, Spain (PI 09/0382). Ana B. del Campo was supported by ISCIII Rio Hortega contract CM08=00252. This study was also supported by Spanish grants to Federico Garrido from the Fondo de Investigaciones Sanitarias (FIS) (PI 11/01022; PI 08/1265 and RETIC RD 06/0020), and from the Consejería de Ciencia, Innovación y Empresa, Junta de Andalucía, Proyectos Excelencia CTS-695, CTS-3952, CVI-4740; and also by grants from the European Community (ENACT project (European Network for the identification and validation of antigens and biomarkers in cancer and their application in clinical tumour immunology, LSHC-CT-2004-503306) and OJ 2004/c158,18234 project

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Efficient Recovery of HLA Class I Expression in Human Tumor Cells After Beta2-Microglobulin Gene Transfer Using Adenoviral Vector: I mplications for Cancer Immunotherapy.

A.B.del Campo*, N.Aptsiauri*, R.Méndez*, S.Zinchenko*, A´.Vales[ϕ], A.Paschen[¥], S.Ward[§], F. Ruiz-Cabello*, G.González-Aseguinolaza[ϕ]& F.Garrido[*¶].

*Departamento de Análisis Clínicos Hospital Universitario Virgen de las Nieves, Granada. [ϕ]Laboratory of GeneTherapy of Viral Hepatitis Division of Hepatology and Gene Therapy, Center for Applied Medical Research (CIMA), ClínicaUniversitaria/School of Medicine University of Navarra,Pamplona,Spain; [¥]Skin Cancer Unit of the German Cancer Research Center,Heidelberg,Germany;[§]OnyvaxLtd, St George's University of London, London, UK; and [¶] Departamento de Bioquímica,Biología Molecular III e Inmunología, Facultadde Medicina,Universidad de Granada, Granada, Spain.

Received 4 March 2009; Accepted in revised form 4 May 2009.

Correspondence to:Dr F. Garrido,Departamento de Análisis Clínicos, Hospital Universitario Virgen de las Nieves, Avd. Fuerzas Armadas, 2, 18014 Granada, Spain.

E-mail: federico.garrido.sspa@juntadeandalucia.es

Abstract

Here we report a successful use of a non-replicating adenovirus expressing the wild-type human β2m gene in recovery of normal human leucocyte antigen (HLA) class I expression in β2m-null cancer cells. Total loss of HLA class I expression in these cell lines is caused by a mutation in β2m gene and a loss, of heterozygosity in chromosome 15 carrying another copy of that gene. Normal HLA class I expression on the tumour cell surface is critical for the successful outcome of cancer immunotherapy as T cells can only recognize tumour-derived peptides in a complex with self-HLA class I molecules. In this report we characterize the newly generated adenoviral vector AdCMV β2m and demonstrate an efficient β2m gene transfer in tumour cell lines of different, histological origin, including melanoma, prostate and colorectal carcinoma. The β2m re-expression lasted for an extended period of time both *in vitro* and *in vivo* in human tumour xenograft transplants. We propose that in a subset of cancer patients with structural defect in β2m gene or chromosome 15, the adenoviral-mediated recovery (or even increase) of HLA class I expression on tumour cells in combination with vaccination or adoptive T-cell therapy can provide a complementary approach to improve the clinical efficacy of cancer immunotherapy.

Introduction

Defects in human leucocyte antigen (HLA) class I expression with different underlying molecular mechanisms have been documented extensively in a large variety of primary tumours and metastatic lesions, and have important implications for cancer immunotherapy [1–4]. The existing protocols of peptide-based cancer immunotherapy that are aimed at augmenting tumour cell immunogenicity and cytotoxic T lymphocyte (CTL) responses also depend on HLA class I expression on cancer cells [5]. So far, most immunization therapies induce a stimulation of anti-tumour cellular responses, scarcely associated with notable clinical improvement [6]. It is believed that an immune selection during tumour progression and additional selective pressure of immunotherapy favours the outgrowth of various types tumour escape

variants, many of which are characterIzed by loss or downregulation of HLA class I mole cules [7–12]. In this context, we have recently reported that cancer immunotherapy induces HLA upregulation in some metastatic lesions that ultimately regress but not in the metastases that have a tendency to progress [7, 13].

Low expression or total absence of HLA class I molecules in some cases is caused by irreversible alterations including mutations in β2m gene, and loss of heterozygosity (LOH) in chromosome 15 [14, 15]. In particular, there have been various reports suggesting that mutations in the β2m gene in melanoma contribute to the poor clinical response in peptide-based immunotherapy and to disease recurrence [16, 17]. In patients undergoing immunotherapy the lack of response to the treatment and generation of progressing metastases seems to be associated with immune selection of tumour cells variants with low HLA class I expression associated with the irreversible defects [18]. Hence, recovery of normal class I expression in patients with metastatic cancer presenting β2m mutations, LOH in chromosome 15, HLA haplotype loss, or other structural genetic HLA defects using clinically applicable methods of gene transfer becomes essential. We constructed an adenoviral vector carrying wild-type human β2m gene in order to restore the antigenicity of tumour cells with total loss of HLA class I molecules due to β2m mutations and LOH in chromosome 15, and characterize the efficacy of this vector *in vitro* using different human cancer cell lines and *in vivo* in human tumours grown from these cell lines in immunocompromised mice

Materials and methods

Cell lines. The human embryonic kidney (HEK293) cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Table 1 Cell lines with beta 2-microglobulin gene defects

Cell line	Alterations in β2m gene	Reference
ESTDAB-109 melanoma	Microdeletion of 498 bp including exon l and part of its flanking up stream regions Macrodeletion of the entire copy of second β2m gene. LOH chromosome 15	[15]
ESTDAB-038 melanoma	T →A substitution start codon deleted LOH chromosome 15	[17]
DNR-DC-MOlO-mcl melanoma	G →T substitution in exon 2: stop codon LOH chromosome 15	Unpublished[1]
Ma-Mel-86 b melanoma	Genomic deletion in exon l LOH chromosome 15	[20]
Ma-Mel-128 b melanoma	2 bp deletion in exon l	Unpublished[2]
OPCN3 prostate	Deletion in the β2m gene LOH in chromosome 15	Unpublished[3]
DLD-1 colon	C →A substitution in exon 2 G →T substitution in last codon intron I: no stop codon	[21]
DAUDI lymphoma	G →C substitution at the start codon Rearrangement in chromosome 15	[22]

LOH, loss of heterozygosity.

[1] Kindly provided by Dr G Gaudernak, Norwegian Radium Hospital, Oslo, Norway. [2] Skin Cancer Unit, German Cancer Research Center, Heidelberg and Universily Medical Center, Mannheim, Germany. [3] Onyvax Ltd, St George's University of London, London, UK.

The 116 cell line that has an expression cassette driving a Cre recombinase gene with a N-terminal nuclear localization signal stably integrated into HEK293 cells [19]was kindly provided by Dr Phillip Ng (Baylor College, Houston, TX, USA).

The following five melanoma cell lines (Table 1) with characterized defects in β2m gene used in our experiments were UKRV-Mel-2 [15] (synonym ESTDAB-109; from the ESTDAB collection http://www.ebi.ac.uk/ipd/ estdab), LB1622 [19] (synonym ESTDAB-038); DNRDC-M010-mcl (synonym M010); Ma-Mel-86b [20] and Ma-Mel-128b. OPCN-3 is a cell lines derived from a patient with prostate cancer. A colorectal adenocarcinoma DLD-1 [21] and a Burkitt lymphoma cell line DAUDI [22] were obtained from the ATCC Melanoma cell line ESTDAB-058 has a normal expression of HLA class I without any defects in β2m gene.

Culture conditions. The 116 and HEK293 cell lines were cultured in Minimal Essential Medium and Dulbecco media, respectively, supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mML-glutamine and 1% penicillin ⁄streptomycin. Prostate cells were cultured in keratinocyte medium with a growth supplement (Gibco, Paisley, UK; Invitrogen, Carlsbad, CA, USA). Melanoma, colorectal carcinoma and lymphoma cell lines were grown in RPMI-1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% FCS, 2% glutamine (Biochrom KG) and 1% penicillin ⁄streptomycin (Biochrom KG) at 37 C in a humidified atmosphere with 5% CO2

Antibodies. Monoclonal antibody (mAb) W6 ⁄32 that recognizes HLA class I cell surface complex of β2m and HLA heavy chains [23] and anti- β2m mAb L-368 were a kind gift from Dr Bodmer (Tissue Antigen Laboratory, Imperial Cancer Research Fund Laboratories, London, UK) In some experiments a rabbit polyclonal anti-human β2m (Abcam,UK) conjugated goat anti-mouse (BioSigma, St Louis, antibody (Ab) was used. Fluorescein isothiocyanate MO, USA) Ab was used as a secondary Ab for low cytometry, horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ab (Bio-Rad, Richmond, CA, USA) was employed for immunochemistry and alkaline phosphatase (AP)-conjugated Ab for western blotting.

Construction of recombinant adenoviral vectors. As shown in Fig. 1, recombinant adenovirus carrying human β2m (AdCMV β2m) under the control of cytomegalovirus (CMV) promoter was constructed by homologous recombination between Ψ5 (as a donor virus to supply the viral backbone) and pAdlox β2m (a shuttle plasmid with a single loxP site carrying the β2m gene) using the Cre-lox recombination system [24]. Total cellular RNA from peripheral blood mononuclear cells from a healthy donor was isolated with Ultraspec (Biotecx Laboratories, Houston, TX, USA) and amplified by reverse transcription–polymerase chain reaction (RT-PCR) using specific primers for human β2m (forward primer:5′-AAGCTTGCCACCATGTCTCGCTCC-3′; reverse primer :5′-GGATCCTGCGGCATCT TCAAACCTCCATG-3′) including the sites for restriction enzymes HindIII and BamHI. The 600-bp fragment corresponding to β2m cDNA was cloned into pCR4-TOPO plasmid generating TOPO β2m (TOPO TA cloning Kit; Invitrogen, Carlsbad, CA, USA). The HindIII ⁄BamHI β2m fragment excised from TOPO- β2m was ligated into HindIII ⁄BamHI-digested alkaline phosphatase-treated pAdlox.

AdCMV β2m construction. New pAdlox β2m, linearized with SfiI, was cotransfected along with Ψ5 DNA (Ψ5is an E1–E3 deleted version of Ad5-containing loxP sites flanking the packaging site) into 116 cells using jet Pei™, (Redox Lab. S.L., Malaga, Spain) After development of complete cytopathic effect (7 or 8 days), the cell lysate was passaged three times in 116 cells to eliminate the Ψ5 virus contamination. An AdCMV β2m single clone was expanded to produce a large-scale concentrated stock in HEK293 cells, purified on CsCl gradient, desalted using PD-10 columns (Amersham Biosciences, Uppsala, Sweden) and stored in Tris–HCl 0.1 M and 10% glycerol at 80° C). Virus titres were determined by plaque assay in HEK293 cells based on the visual detection of infected cells stained with the mouse anti-adenovirus monoclonal antibody blend (Chemicon Int., Temecula, CA, USA) and the titre was expressed as plaque-forming units Concentrated stocks typically had titres in the range of 10^8– 10^{10} PFU⁄ml. Adenoviral vectors containing green

fluorescent protein (GFP) and Luciferase reporter genes under the control of CMV promoter (AdCMVGFP and AdCMVLuc) were produced similarly [25].

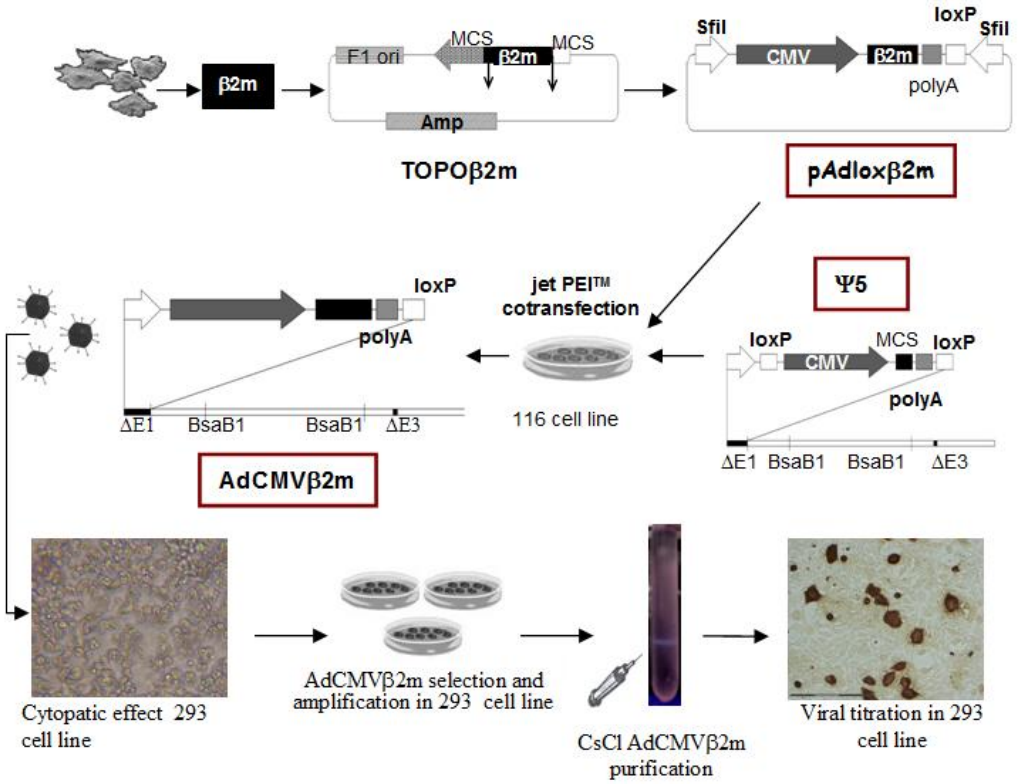

Figure1. Schematic presentation of the construction of the adenoviral vector AdCMV β2m using Ψ5 and pAdlox as shuttle vectors. β2m was cloned into the TOPO vector and subcloned into pAdlox and recombination of pAdlox β2m and Ψ5 was realized by Cre recombinase via loxP sites in 116 cell line creating AdCMV β2m which has an intact packaging site and carries the recombinant gene β2m. Several rounds of growth are necessary to amplify the virus in HEK293 cell line. The new virus is purified on a CsCl gradient, desalted on PD10 columns and titrated with adenovirus monoclonal antibody.

*Detection of HLA class I recovery in infected tumour cells. Cell infection.*The purified virus was added directly to the cells in a 2% FCS supplemented media at various multiplicity of infection (MOI), ranging from 25 to 80, depending on the cell line. To examine the time course of β2m expression cells were harvested every day during 18 days and HLA class I surface expression was evaluated by flow cytometry. In some experiments, cells were re-infected with the virus for a second time.

Flow cytometry. The cell surface expression of HLA class I molecules was evaluated by indirect immunofluorescence using various antibodies as described earlier [14]. Results were presented as representative fluorescence plots.

Immunocytochemistry and fluorescent image analysis. Untreated tumour cells and virus-infected cells were centrifuged on glass slides using cytospin (Sigma Centrifuge 3K18C; Sartorius BBI Systems GmbH, formerly; B. Braun Biotech International, Melsungen, Germany). Acetone-fixed cells on the slides were immunostained with antibodies against β2m and HLA class I complexes. Samples were stained using the biotin–streptavidin immunoperoxidase technique (supersensitive Multilink-HRP /DAB; BioGenex,

San Ramon, CA, USA). The slides were counterstained with Meyer's haematoxylin; as a negative control in each experiment, the specific antibody was replaced in one sample with a non-immune serum. The samples were analysed using a Zeiss Palm Microlaser Systems microscope (Carl Zeiss Microimaging GmbH, Gottingen, Germany) under 40Xmagnification. Expression of GFP protein in cells infected with Ad-CMVGFP vector and centrifuged on glass slides was visualized with a fluorescent microscope Nikon Eclipse E400 (Izasa, Barcelona, Spain) under 100·magnification.

Protein extraction and western blotting. Protein extract was obtained from untreated or AdCMV β2m infected (MOI 25–80) tumour cells (1 to 5 $\cdot 10^6$ cells) collected 72 h after infection using ristocetin-induced platelet agglutination buffer (Sigma) with protease inhibitors (Sigma). Proteins were separated by 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis according to Laemmli and transferred to a nitrocellulose membrane. Membranes were incubated overnight with polyclonal rabbit anti- β2m antibodies (diluted 1:2000), 1 h with AP-conjugated secondary antibody (Bio-Rad), and developed with the AP-Conjugate Substrate Kit (Bio-Rad). β2m positive melanoma cell line ESTDAB-058 was used as a control. Anti-alpha-tubulin antibodies (Sigma) were used to confirm that similar amounts of proteins were loaded from each extract.

RNA isolation, reverse transcription and quantitative real time-PCR. Total RNA was extracted from culture cell lines using the RNeasyMini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with the iSript^{TM}cDNA Synthesis Kit (Bio-Rad) and analysed for β2m gene expression by quantitative real-time PCR. To control for variations in amounts of mRNA, the glucose-6-phosphate dehydrogenase (G6PDH) gene was amplified as well. All reactions were performed in a LightCycler instrument (Roche Diagnostics, Manheim, Germany) using the primers provided in the Housekeeping Gene Set Kit (Roche Diagnostics). The final expression levels of target genes were given relative to the expression levels of G6PDH.

In vivo adenovirus-mediated gene transfer and biolumines-cence imaging using human tumour xenograft model. Six-to nine-week-old male nude /nude mice were obtained from Charles River Labs (Barcelona, Spain). Cells in concentration of 5 $\cdot 10^6$ in a volume about 50–100 llin phosphate-buffered saline (PBS) were injected subcutaneously into a neck area. After 2–3 weeks, when the tumours reached about 7–10 mm in diameter, different doses of recombinant adenoviruses (AdCMVb2m at 10^9 PFU; AdCMVLuc or AdCMVGFP at 10^8 or 10^{10} PFU) diluted in PBS were injected into tumours in a volume of 50μl. Seven days after virus administration each tumour was excised, part of the tissue was used for protein and RNA extraction for western blot and RT-PCR analysis, another part of each tumour was embedded into optimal cutting compound and frozen in liquid nitrogen for further immunohistochemical examination. Expression of HLA class I in tumours after adenoviral β2m gene transfer was evaluated by western blotting and immunohistochemistry using W6⁄32 and L-368 antibodies. In control experiments invivoimaging of luciferase luminescence was performed. For invivoimaging and quantification of light emission, mice were anesthetized with a mixture of Xylacine and Ketamine and 150 mg⁄kg D-luciferin (100μl of a 30.3 mg⁄ml solution dissolved in phosphate-buffered saline) was injected intraperitoneally. Ten minutes later, animals were placed in the dark chamber for light acquisition. Typically, a circular region of interest measuring 3 cm in diameter was defined in the tumour area of mice, and quantification of light emission was performed in photons⁄second. Time exposure ranged from 1 s to 5 min depending on light intensity.

Results

Adenovirus-mediated recovery of HLA class I expression in tumour cell lines

We generated a new recombinant adenovirus, AdCMV β2m , encoding the human β2m gene, for gene transfer into different HLA class I negative tumour cells. All the studied tumour cell lines, except for DAUDI, demonstrated positive HLA class I cell surface expression 72 h after the

administration of the AdCMV β2m vector, as demonstrated by flow cytometry using anti- β2m and anti-HLA-ABC antibodies (Fig. 2). Different cell lines required infection at different moi to achieve maximum efficiency of β2m re-expression. An MOI of 25 was an optimal infection dose for the prostate cell line OPCN3, for the colon cell line DLD-1 and for the melanoma cell line ESTDAB-109. Melanoma cell lines ESTDAB-038 and Ma-Mel-128b required higher MOI of 60. An MOI of 80 was optimal for recovery of β2m expression on melanoma cell line Ma-Mel-86b. Therefore, even between cell lines of the same histological type there were differences in the effectiveness of the β2m gene transfer. For instance, infection of the cell line ESTDAB-109 required less amount of virus to reach high levels of HLA class I re-expression and the positive expression lasted longer when compared with another melanoma cell line ESTDAB-038.

In control experiments cells were infected with Ad-CMVGFP and the expression of green fluorescent protein (GFP) was assessed both by fluorescence-activated cell sorter analysis (data not shown) and by immunocytology with fluorescence microscope (Fig. 3.1). GFP expression was strongly positive in all transfected cell lines.

Immunocytochemistry analysis of cells infected and centrifuged onto glass slides also demonstrated a recovery of both β2m and HLA class I expression (Fig. 3.2). To corroborate the β2m protein expression we analysed lysates from non-infected and AdCMV β2m -infected cells by western blotting. The expression of β2m protein was recovered after infection of the cells with AdCMV β2m at the indicated MOI (Fig. 4A).

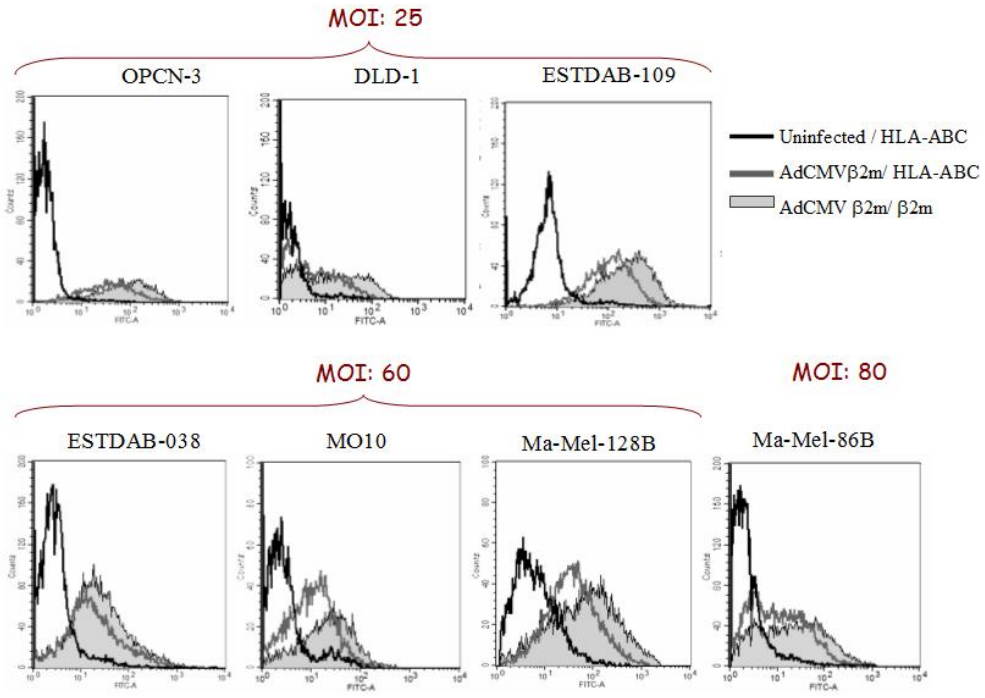

Figure 2. Surface re-expression of human leucocyte antigen (HLA) class I on cell lines infected with AdCMV β2m. Different HLA class I negative tumour cells, characterized by a deficiency in β2m expression, were infected with AdCMV β2m at various MOI optimal for each cell line. After 72 h cells were analysed by flow cytometry with antibodies against β2m/HLA class I complexes (W6⁄32) and against human β2m (L-368). Black lines represent uninfected control cells labelled with the W6⁄32 antibody. Grey line represents AdCMV β2m infected cells labelled with the W6⁄32 antibody. Solid grey histogram represents AdCMV β2m infected cells stained with L-368 antibody. MOI, multiplicty of infection.

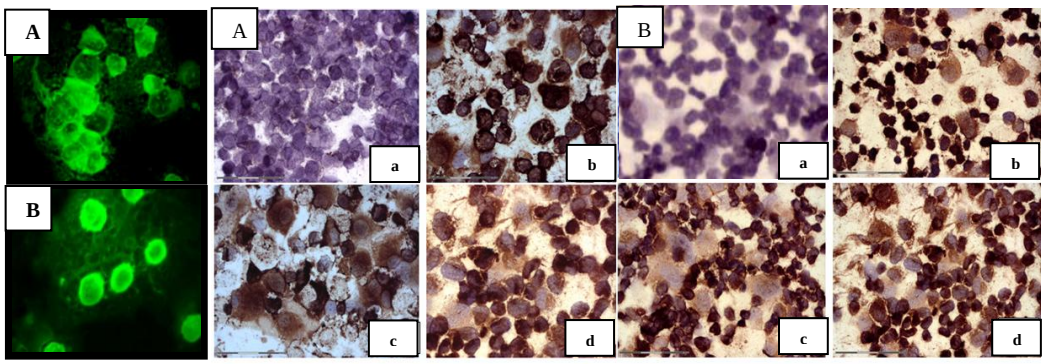

Figure 3. Expression of the recombinant proteins after infection with the viral vectors AdCMVGFP and AdCMVβ2m. (1) Expression of green fluorescent protein in cell lines after infection with AdCMVGFP and cytospin: (A) M010 melanoma cell line (B) OPCN3 cell line. (2) Immunocytochemical analysis of β2m expression in two melanoma cell lines ESTDAB-109 (A) and M010 (B) 72 h after infection with AdCMVβ2m. (a) Uninfected cells and (b) infected cells were immunostained with anti-β2m antibody L-368. (c) Infected cells immunostained with W6⁄32 antibody that recognizes the β2m heavy chain complex and (d) infected cells immunostained with polyclonal antibody against human β2m.

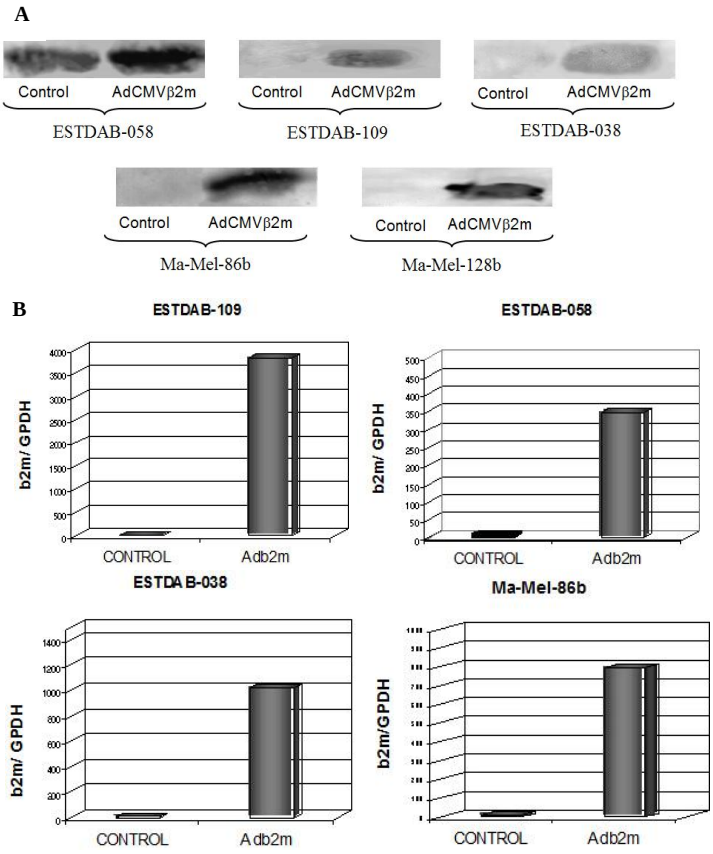

Figure 4 (A) Western blot analysis showing an AdCMVβ2m-mediated increased expression of β2m in infected cells. Tumor cells were harvested 72 h after the infection for protein extraction. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and immunostained with anti β2m antibodies. The melanoma cell line ESTDAB-058 with normal β2m expression was used as a positive control. (B) Increased transcription of β2m in the AdCMVβ2m infected cell lines determined by quantitative real time PCR. G6PDH, glucose-6-phosphate dehydrogenase.

Quantitative real-time PCR

PCR was performed to study β2m mRNA expression patterns in normal and AdCMVβ2m infected cells. In all tumour cell lines analysed, infection with the AdCMVβ2m vector led to increase in the level of β2m mRNA. Representative data in Fig. 4B demonstrate increased median levels of β2m mRNA in four melanoma cell lines, 3 days after infections with the AdCMVβ2m virus.

Time course of cell infection with AdCMVβ2m

All the studied cell lines transduced with AdCMVβ2m vector (except for DAUDI lymphoma cell line) showed positive β2m protein expression for up to 20 days. Figure 5 shows two representative time course experiments following β2m expression in the melanoma cell line ESTDAB-109 and the prostate cell line OPCN-3. The highest HLA class I expression level was detected 72 h after the infection with almost 90% of cells being the tumour cells with AdCMVβ2m for a second time positive for HLA class I surface expression as assessed by with same moi increases once again the level of HLA flow cytometry. We also discovered that re-infection of class I expression (data not shown).

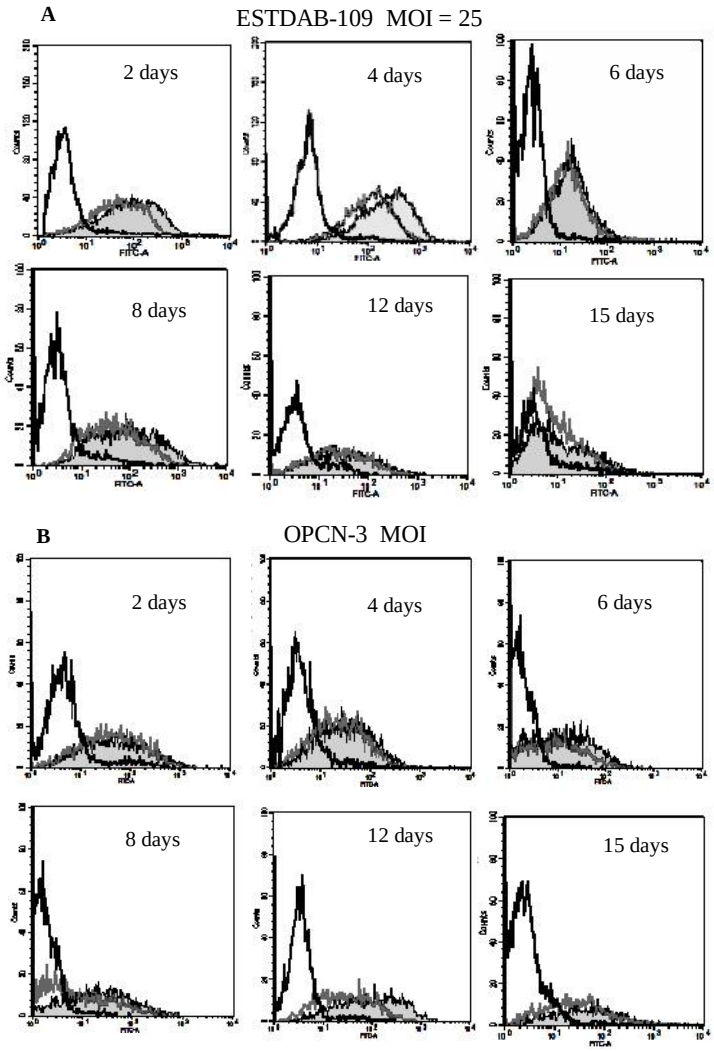

Figure 5. Adenovirus-mediated surface re-expression of β2m lasts up to 15 days. Melanoma cells ESTAB-109 (A) and the prostate cell line OPCN3 (B) were infected with AdCMVβ2m. Re-expression of the β2m protein upon infection was analysed by flow cytometry over time with antibodies against the β2m/human leucocyte antigen class I complex (W6/32) and the human β2m molecule (L-368). Black lines represent uninfected cells labelled with the W6/32 antibody. Grey line represents AdCMVβ2m infected cells labelled with W6/32 antibody. Solid grey histograms represent AdCMVβ2m infected cells stained with L-368 antibody. MOI, multiplicty of infection.

Recovery of HLA class I expression in xenografted human tumour cells by AdCMVβ2m

To check the effectiveness of the new adenoviral vector AdCMVβ2m in restoration of the HLA class I expression in an invivosystem we used a human-mouse xenograft model and the GFP-and luciferase-carrying vectors as a control. Luciferase-based assays have been widely used to study changes in gene expression intensity invitroand invivo. The intensity of light is proportional to the amount of luciferase expressed in each individual cell, and the number of cells in which the gene has been transferred. Two different doses of AdCMV-luciferase (10^8 or 10^9 PFU) were injected into Ma-Mel-86b tumours. A cooled charge-coupled device camera attached to a light-tight chamber was used to detect the intensity and location of light emission. Luciferase expression was measured at 2, 5 and 7 days after adenovirus injection. As shown in Fig. 6A, luciferase could be clearly detected in the tumour area. The quantitative analysis of luciferase expression showed that maximum luciferase expression was reached 5 days after adenovirus administration (Fig. 6B) and at that time point there was a direct correlation between the dose of virus and luciferase expression level. HLA class I negative tumours injected with a dose of 10^9 PFU of the AdCMVβ2m vector recovered positive expression of β2m and HLA class I complexes as demonstrated by immunohistochemical analysis (Fig. 6C) and western blotting (Fig. 6D). Tissue analysis showed that GFP was positively expressed in adeno-GFP-transfected tumours.

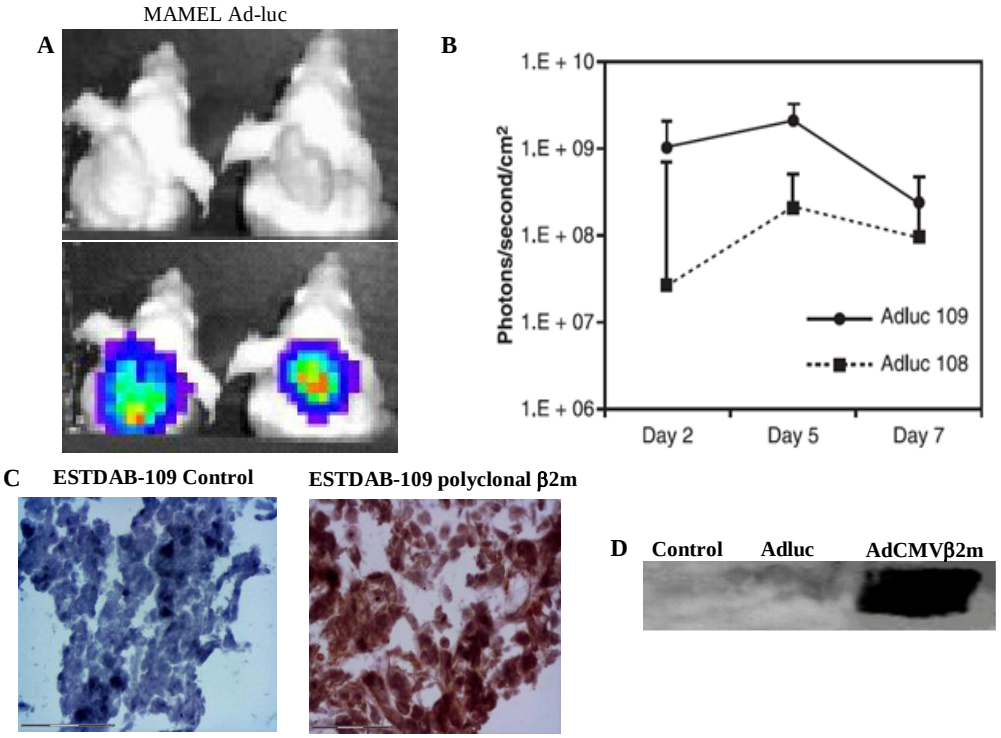

Figure 6. *In vivo* luciferase and β2m gene transfer by adenoviral vectors. Human tumour cells were transplanted into nude/nude mice. Viral vectors were injected into tumours of 5–8 mm in diameters. (A) In vivo imaging of luciferase expression in human xenograft tumours 5 days after injection of adenoviral vector AdCMVluc in a dose of $1 \cdot 10^9$ PFU. (B) Time-and dose-dependent intensity of bioluminescence of the luciferase in human tumour xenografts. (C and D) Positive re-expression of β2m protein in the tumours transfected with AdCMVβ2m vector demonstrated by immunohistochemistry (C) and western blotting (D) using anti-β2m antibody.

Discussion

Human leucocyte antigen class I abnormalities in tumour cells, with a frequency up to 90% in some types of tumour, have been well documented and were caused by distinct molecular mechanisms [4, 26]. Total loss of HLA class I expression was frequently caused by mutations/deletions in one copy of the β2m gene and loss of another copy in chromosome 15 due to LOH [27]. These alterations are irreversible in nature and require restoration of a wild-type functional gene. LOH in chromosome 15 has been described in various types of tumour, including melanoma and head and neck cancer [28, 29], and has been reported to correlate with the risk of metastatic progression [30]. Analysing primary tumours of various histological types we found that LOH in chromosome 15 could be detected in 40% of colon and laryngeal carcinomas [31] and in 50% of bladder carcinomas (unpublished data). Interestingly, we have found that LOH in chromosome 15 in tumours occurs more frequently than mutations in β2m gene and can be found even in some melanoma cells with normal or low expression of HLA class I [28]. Thus, LOH in chromosome 15 may be unnoticed if tumour cells have 'normal' HLA class I pattern and can represent one of the early class I alterations in malignant cells leading to generation of new tumour escape variants. Recently, we have reported LOH in chromosome 15 in HLA-class I deficient metastatic melanoma lesions in patients with poor clinical response to immunotherapy [7]. We observed that melanoma patients undergoing various types of immunotherapy had a tendency to develop progressing metastasis with HLA class I alterations ('hard lesions') [13]. We believed that tumour cells with reversible defects ('soft lesions') responded to therapy by upregulation of HLA class I expression and regress, while tumour cells with structural irreversible defects (hard lesions) demonstrated resistance to immunostimulation, failed to upregulate HLA class I antigens and eventually progressed. Lack of HLA upregulation in this case is likely to be associated with underlying structural alterations. Therefore, immunotherapy may lead to immunoselection of tumour variants with irreversible structural defects in HLA class I molecules, and these cells become the most dangerous enemy both for innate tumour immunity and for immunotherapy. In clinical trials with melanoma patients the lack of response to the treatment with melanoma antigen-encoding gene (MAGE) peptides was correlated with the presence of β2m gene mutations and the loss of HLA class I surface expression in the tumoural cell [17]. Total loss of HLA class I expression due to β2m alterations seems to be associated with the failure of the therapy and cancer progression in another report [15]. Thus, targeting β2m is an attractive option to recover normal HLA class I expression in these cases.

In various experimental systems introduction of the major histocompatibility complex (MHC) class I molecules into class I negative tumour cells led to higher immunogenicity and decreased tumourigenicity of these cells [32–34]. In some reports β2m transfection led to recovery of MHC class I expression and escape from natural killer cells [35], other publications describe the reversal of metastatic phenotype in murine carcinoma cells after transfection with syngeneic H2-Kb gene [36]. In most recent publications transient or stable transfection of tumour cell lines with β2m gene using cDNA transfer, retroviral vectors or other types of gene transfer methods were used to prove that *in vitro* restoration of β2m led to HLA class I re-expression and recognition of tumour cells by specific cytotoxic T lymphocytes [15, 16]. All these data indicate that restoration of β2m and HLA class I expression deserve more investigation and a possibility to bring it closer to the clinical trials as an alternative approach to improve the efficacy of T-cell based cancer vaccines.

In this study we used adenoviral vector carrying human β2m gene to recover normal expression of cell surface HLA class I complex on class I negative/β2m deficient human tumour cells originated from different types of cancer. Adenovirus-based vector are commonly used as a gene delivery vehicles in both basic research and in vaccine development in clinical gene therapy applications. They have very efficient nuclear entry and low pathogenicity for humans [37]. In our experiments the level of positive expression of the transferred gene was close to 100% as assessed by flow cytometry and immunocytochemistry. Our results showed that infection of human tumour cell lines of distinct histological origin with replication-

deficient adenoviral vector carrying human β2m gene resulted in high expression of β2m protein. In the majority of the studied cell lines this expression was dose-dependent on vector and persisted for 15 days indicating that the high β2m protein expression is not toxic to the cells. The expression of the HLA class I complex was positive on the cells surface of the infected tumour cells indicating that the de novo expressed protein is able to form a complex with the HLA class I heavy chain and can be transported to the cell surface.

In our experiments, the degree and the duration of the recovery of normal class I expression after adenoviral β2m gene transfer varied between cell lines. Melanoma, colon carcinoma and prostate cells were more prone for the gene transfer with this type of vector, while the lymphoma cell line DAUDI was resistant to it. This resistance of DAUDI cells to AdCMVβ2m infection coincided with previous reports describing resistance of lymphoma cells to most of the gene transfer methods and abnormalities with antigen processing machinery complex in this cell line [38]. Some published reports indicated that adenovirus by itself was able to decrease the HLA expression in various cell types [39]. However, we used E1–E3 deleted low immunogenic viral vector which in melanoma cells with normal HLA class I expression did not change class I expression phenotype after infection with the control vector AdCMVGFP or empty vector Ψ5 (data not shown).

In *in vivo* experiments using human tumour xenograft model, the intratumoural injection of β2m carrying vector also led to restoration of normal HLA class I expression. In bioluminescent imaging of the tumour inoculated with adeno-luciferase vector we found that maximum luciferase expression was reached 5 days after adenovirus administration and the intensity was dose dependent on virus.

In summary, in this report we describe a new replication-deficient adenoviral vector carrying human β2m gene, which is effective in restoration of HLA class I expression in various types of β2m negative human tumour cells. This is a step to come closer to a possibility of clinical application of such vectors in patients with metastatic cancer with structural defects in β2m gene and/or LOH in chromosome 15. In such patients the immunotherapy might lead to generation of metastatic lesions with irreversible defects in the HLA class I antigens and that would not respond to therapy and eventually progress.

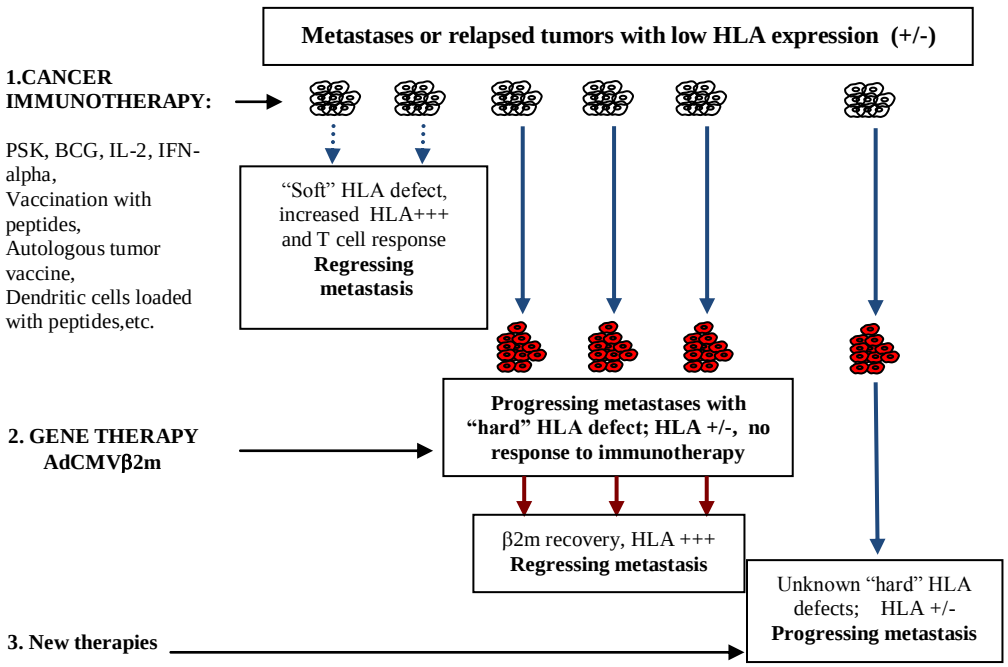

Figure 7. Schematic presentation of the possible clinical application of the adenoviral vector AdCMVβ2m in addition to immunotherapy. Immunotherapy of metastatic cancer as a result of immune selection produces mixed responses: regression of some malignant lesions and progression of others. The therapy leads to modification of tumour microenvironment and release of immunostimulating cytokines that upregulate HLA class I expression in metastases with reversible ('soft') HLA alterations leading to tumour regression. In the lesions with irreversible structural defects ('hard' lesions) the level of HLA class I remained unchanged due to the resistance to cytokine treatment leading to progression of such metastases. Low expression of HLA class I in some progressing metastases is due to mutations in β2m gene and LOH in chromosome 15. Administration of adenoviral vector aimed to restore normal β2m and HLA class I expression is expected to enhance the response to immunotherapy subsequent regression of the metastasis. Some of the metastases that still progress obviously have other molecular mechanisms underlying poor response to the therapy, which need to be investigated further.

Local administration of β2m-adenoviral vector into such metastatic lesions or into relapsed tumours might help to boost tumour cell immunogenicity and recognition by T-cells, prevent malignant progression and improve the response to immunotherapy (Fig. 7).

The use of adenoviral vectors is preferable as an intratumoural injection, rather than systemic to limit possible toxicity and antiviral immune reactivity. It is possible to use β2m-adenovirus in primary melanoma lesions and⁄or lymph node metastases in patients undergoing immunotherapy with confirmed structural defects in chromosome 15⁄β2m gene. Patients with bladder carcinoma could also benefit from intravesicular administration of β2m adenovirus in case of local tumour relapse after Bacille Calmette-Guerin therapy, as we have an evidence demonstrating that relapsed tumours have increased number of HLA class I alterations than primary ones (unpublished data). This strategy may provide an additional tool for future clinical application in order to improve cancer immunotherapy.

Acknowledgment

We would like to acknowledge Dr Teresa Cabrera and her PhD students, Rafael Carretero and Pablo Saenz-Lopez, for their help with immunohistological analysis of human xenograft tumour. This work was partially supported by grants from the Fondo de Investigaciones Sanitarias (FIS, PI-04 ⁄1405), Red Genomica del Cancer (RETICRD 06 ⁄020), Consejeria de Salud Junta de Andalucia, Proyecto de Investigacion de Excelenica (CTS-3952) and Plan Andaluz de Investigación (PAI, Group CTS-143) in Spain; from the European Searchable Tumour Cell Line Database (ESTDAB project,contract No. QLRI-CT2001-01325; http://www.ebi.ac.uk/estdab from the European Network for the identification and validation of antigens and biomarkers in cancer and their application in clinical tumour immunology (ENACT) project (European community LSHC-CT-2004-503306) and from the Cancer Immunotherapy project (European community OJ 2004 ⁄c158,18234). The financial support of Kureha Corporation, Pharmaceutical Division, Tokyo, Japan, is also gratefully aknowledged.

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Adenovirus expressing β2m enhances antitumor immunity by increasing T-cell recognition without affecting the antigen-presentation machinery

del Campo AB[1,2], Carretero J[1,2], Muñoz JA, Zinchenko S[1], Ruiz-Cabello F[1], Kyte JA[4], González-Aseguinolaza G[4], Garrido F[1,2], Aptsiauri N[1]

1 Department of Clinical Analysis and Immunology, University Hospital Virgen de las Nieves, Granada, Spain
2 Department of Biochemistry, Molecular Biology III and Immunology, University of Granada Medical School, Granada, Spain
3Research Assistance Unit. Microscopy and Cytometry Unit. University Hospital San Cecilio, Granada, Spain.
4 Department of Oncology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway
5Division of Hepatology and Gene Therapy, Center for Applied Medical Research (CIMA), University of Navarra Medical School, Pamplona, Spain

Abstract

Optimal tumor cell surface expression of HLA class I molecules is essential for the presentation of tumor-associated peptides to T-lymphocytes. However, a hallmark of many types of tumor is the loss or downregulation of HLA class I expression associated with ineffective tumor antigen presentation to T-cells. Frequently, HLA loss can be caused by structural alterations in genes coding for HLA class I complex, including the light chain β2-microglobulin. Its best characterized function is to interacts and stabilize HLA heavy chain leading to a formation of antigen-biding cleft recognized by TRC on CD8+ T cells. Our previous study demonstrated that alterations in the β2m gene are frequently associated with cancer immune escape leading to metastatic progression and resistance to immunotherapy. These types of defects require genetic transfer strategies to recover normal expression of HLA genes. Here we characterize a replication-deficient adenoviral vector carrying human β2m gene, which is efficient in recovering proper tumor cell surface HLA class I expression in β2m-negative tumor cells without compromising the antigen presentation machinery. Tumor cells transduced with β2m induced strong activation of T-cells in a peptide-specific HLA-restricted manner. Gene therapy using recombinant adenoviral vectors encoding HLA genes increases tumor antigen presentation and represents a powerful tool for modulation of tumor cells immunogenicity by restoration of missing or altered HLA genes. It should be considered as part of cancer treatment in combination with immunotherapy.

INTRODUCTION

Various mechanisms contribute to the failure of the immune control of tumor growth and enable malignant tumors to evade immune surveillance (Aptsiauri *et al*, 2007); hence, currently there is a great effort to develop effective vaccination therapies in order to boost the T cell response and induce tumor regression in cancer patients (Shashidharamurthy *et al*, 2011). Cytotoxic T lymphocytes (CTL) directed against tumor-associated peptides presented by HLA-class-I complex consisting of a heavy chain and β2m as a light chain constitute powerful effectors of the immune system against tumors.

However, tumor cells often lose HLA class I antigens limiting the efficacy of cancer immunotherapy aimed at activation of the immune system to recognize malignant cells and initiate tumor rejection.

Impaired tumor HLA class I expression can be due to alterations at any stage of HLA class I component production, assembly or transport to the cell surface. The expression of HLA class I molecules at the cell surface depends upon the appropriate assembly of three major components, a heavy chain, β2-microglobulin and a peptide in the endoplasmic reticulum (ER). The antigenic peptide epitopes are generated in the cytosol by degradation of endogenously synthesised protein, primarily by the proteasome complex. The peptides generated are transported from the cytosol into the ER by the ATP-dependent

transporter associated with antigen processing (TAP). Within the ER, peptides are further trimmed by aminopeptidases. The binding of peptides by class I molecules is thought to occur within a multimolecular compound termed the peptide loading complex (PLC), which consists of a TAP heterodimer, tapasin, calreticulin, calnexin and ERp57. While the chaperone calreticulin and the thiol-oxidoreductase ERp57 promote appropriate folding and disulphide bond formation, tapasin promotes the stabilization of the peptide loading complex and aids in the appropriate selection of peptides. Following peptide binding, HLA-peptide complexes are released from the PLC and shuttled via the Golgi apparatus to the cell surface where they are recognized by CTLs, which triggers an immune response (Elliott, 2006; Koch & Tampe, 2006).
The expression of many of the components of the antigen presentation machinery is inducible by IFN-γ. Therefore, upregulation of the tumor HLA class I expression as a result of immunostimulation by IFN-γ is, at least partially, responsible for the clinical efficacy of the therapy. However, if the molecular alteration underlying HLA loss is irreversible and caused by genetic defects in genes coding for class I molecules, the immunotherapy might fail.

Our recent studies shown that the resistance to immunotherapy and generation of progressing metastases seems to be correlated with HLA class I altered expression and is a consequence of immune escape and dissemination of HLA-negative tumor cells. Recently, we reported that progressing metastases or recurrent tumors after immunotherapy have increased percentage of HLA class I alterations with profound structural defects in HLA genes (Aptsiauri *et al*, 2008; Garrido *et al*, 2010).
Therefore, recovery of normal tumor HLA class I expression in patients with local cancer relapse or metastatic progression with underlying genetic defects becomes essential. This approach has been employed previously in *in vitro* studies to demonstrate the importance of HLA class I molecules in specific tumor lysis by CTL and NK cells (Tanaka *et al*, 1985; Wallich *et al*, 1985), although without using adenoviral vectors.

For transient transduction of target cells, adenoviral vector systems possess the highest efficacy (Cevher *et al*, 2012) and they have been used in 23% of the registered clinical trials of gene therapy for different indications, including for boosting anticancer immunity (Ginn et al, 2013; Sharma et al, 2009). These vectors have several biologic characteristics which make them effective for cancer gene therapy: a broad host/ cell range, high levels of transgene expression, and episomal localization with almost no integration into tumor cell genome which prevents increasing genetic instability and activation of oncogene-expression. In addition, adenoviral vectors can be produced in large titers (up to 10^{12} pfu/ml) with amounts sufficient for clinical use.

However, some components of the HLA class I assembly pathway have been found to be inhibited by numerous viral proteins (Hansen & Bouvier, 2009; Hewitt, 2003) in a virus counterattack strategy against the cellular immune response with the goal of freely replicate in infected cells. Adenoviruses are able to specifically down-regulate the cell surface expression of HLA class I antigens (Paabo *et al*, 1989) via two distinct mechanisms both involving a gene product of the E3 region, the E3/19K protein. The adenoviral protein E3/19K binds (Beier *et al*, 1994; Flomenberg *et al*, 1994) and down-regulates cell surface HLA class I expression by direct retention of HLA heavy chain in the ER (Andersson *et al*, 1985; Burgert & Kvist, 1985; Cox *et al*, 1990) through a endoplasmic reticulum retention signal that contributes to intracellular sequestration of HLA class I molecules (Sester *et al*, 2013)(Sester *et al*, 2013). At the same time E3/19K also binds to TAP, resulting in the blockage of the association of TAP with tapasin, preventing class I/TAP association (Bennett *et al*, 1999; Petersen *et al*, 2003).
Furthermore, viral proteins have been also reported to modulate (either positively or negatively, either in a direct or indirect fashion) the apoptotic response of host cells to infection. Following the infection with human adenoviruses, cells exhibit an apoptotic response mediated by the expression of the viral E1A protein (Kirshenbaum, 2001) and E4orf6, encoded by the adenoviral gene

E6. On the other hand, E1B-19K blocks host cell apoptosis, thereby sustaining viral replication. Various proteins encoded by the E3 transcription unit, E3-10.4K, E3-14.5K and E3-6.7K exert antiapoptotic and immune-modulatory functions, thereby protecting infected cells from cytotoxic T cells and lethal cytokines. (Galluzzi *et al*, 2008).
Although we are using an adenoviral vector lacking the E1 and E3 regions, we can not exclude the existence of other targets for virus-mediated inhibition of HLA expression which has not been sufficiently investigated yet or an effect due to the presence of the traces of replication competent adenovirus in a recombinant adenovirus preparation.
We also analysed how transduction with adenoviral vector carrying β2m gene influences tumor cell proliferation and apoptosis.

We have constructed a replication-deficient adenoviral vector carrying human β2m gene and demonstrated its effectiveness in restoration of HLA class I expression both *in vitro*, in various types of β2m-negative human tumor cells, and *in vivo*, in human tumour xenograft transplants (del Campo *et al*, 2009). Here, we further characterize the vector and confirm the functional restoration of the HLA class I complex by analyzing the ability to induce tumor-antigen specific activation of cytotoxic T lymphocytes. Furthermore, for cancer gene therapy application it is critical to ensure that antigen processing machinery is not inhibited by the viral vectors used to increase tumor immunogenicity and upregulate HLA class I expression. We examined how the β2m coding adenoviral vector influences antigen presentation machinery components, tumor cell proliferation and apoptosis.

MATERIAL AND METHODS:

Cells lines and antibodies

Human cancer cell lines, including melanoma (ESTDAB-109, ESTDAB-038, MaMel-86b, MaMel-128b, DNR-DC-M010), colorectal adenocarcinoma DLD-1, Burkitt lymphoma cell line Daudi, and prostate cancer cell line OPCN-3 were previously described (del Campo *et al*, 2009). Prostate cancer cell line PC-3 and DU-145 were purchased from ATCC. ESTDAB-007, ESTDAB-038 and ESTDAB-109 melanoma cell lines were obtained from the European Searchable Tumor Cell Line Data Base (ESTDAB project, contract no.QLRI-CT-2001-01325) (Pawelec & Marsh, 2006).MaMel-86b and MaMel-128 were kindly provided by Dr Annette Paschen (University Hospital Essen, Essen, Germany) and DNR-DC-M010 melanoma cell line (received as part of European collaborative project ENACT) kindly provided by Dr Gustav Gaudernack (Institute for Surgical Research, Universtiy of Oslo, Oslo, Norway).HLA class I expression was studied with the following monoclonal antibodies (mAbs): W6/32 (anti-HLA-ABC), L-368 (anti-β2m) previously described (del Campo *et al*, 2009) and HC-10 (anti-HLA-BC free heavy chain; Stam et al. 1986). Rabbit polyclonal anti-human β2m (Abcam,UK) was used in some assays. Antigen Processing Machinery (APM) components were studied with the following mAbs: anti-calnexin and anti-calreticulin (PIERCE), anti-tapasine, anti-ERP57, Proteasome 20S LMP-2 and LMP-7 (Abcam, UK), and anti TAP-2 (MBL, Japan). Rabbit polyclonal Ab anti-TAP-1 (Abcam, UK). FACS analysis of Melan-Mart-1 antigen was studied using anti-Melan A/MART-1, A103 (Chen *et al*, 1996).
Secondary Abs: Fluorescein isothiocyanate-conjugated goat anti-mouse (Sigma, St Louis, USA) Ab was used for flow cytometry; horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Abs (Bio-Rad, Richmond, USA) were employed for immunochemistry; Alexa Fluor 488 goat anti-mouse Ab and Alexa Fluor 555 goat anti-rabbit (Invitrogen, Life Technologies, UK) were used for confocal analysis.

Adenoviral vectors

The construction and production of the adenoviral vector carrying human β2m gene (AdCMVβ2m) and the adenoviral vector carrying GFP gene (AdCMVGFP) were previously described (del Campo *et al*, 2009). The empty viral vector phi5 (Ψ5) was used as a transduction control for some cell line assays.

Adenoviral infection of tumor cells Cell lines were infected at different multiplicity of infection (MOI) depending on the cell line. MOI=7 for DLD-1, ESTDAB-109, OPCN-3 and MaMel-128; MOI= 15 for ESTDAB-038, DNR-DC-M010 and Ma-Mel86b and MOI =30 for DAUDI with AdCMVβ2m in a 2% FCS supplemented media, and β2m protein expression was determined 48 hr after infection using flow cytometry. In some assays, cell lines were incubated for 3, 6, 12 or 24 hours with AdCMVβ2m and after that, culture media was replaced for a fresh media and the cells were left in culture for up to 48 hours in total before testing β2m expression.

To analyze a dose dependent effect in β2m ELISA, proliferation and apoptosis assays, cell lines were infected with MOIs 10,15 or 20 times higher than the optimal dose (minimum MOI necessary to achieve maximum recovery of β2m expression measured by FACS).

Flow cytometry and Immunocytochemistry

Cell surface expression of HLA class I molecules was analyzed by flow cytometry as previously described (del Campo *et al*, 2009). Briefly, cells were incubated with primary antibodies W6/32 or L-368, washed, labelled with secondary FITC-conjugated antibodies for 30min at 4ºC and analyzed on BD FACS Canto flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Immunocytochemical staining of tumor cells on glass slides (cytospin) was performed as previously described (del Campo *et al*, 2009) using primary antibodies directed against different APM proteins, followed by incubation with HRP-conjugated secondary antibodies. Cells seeded in 6 well-plates were immunostainned after 48 hours of AdCMVβ2m transduction. Cells were washed with PBS and fixed in ice-cold methanol-acetone (1:1) for 10 min and incubated overnight with anti-β2m L-368 in blocking solution at 4°C in a humidified chamber. After 3×10-min wash with PBS-0.1% BSA samples were incubated with secondary antibody HRP-conjugated secondary antibody as described for cytospin.

Confocal microscopy: Cells were seeded in 8-well slides (Nunc Lab-Tek Chamber Slide™ system, Sigma Aldrich, St. Louis, USA), incubated overnight and transduced with AdCMVβ2m or left uninfected. 24 hours later culture media was replaced by fresh media and cells were left in culture for another 24 hours. Cells were fixed in ice-cold methanol-acetone (1:1) for 10 min and incubated overnight with anti-β2m L-368, anti-β2m/heavy chain W6/32 or anti-heavy chain HC-10 monoclonal antibodies in blocking solution at 4°C in a humidified chamber. After 3×10-min wash with PBS-0.1% BSA samples were incubated with 1:1000 Alexa Fluor 488 (green) in blocking solution for 3 hours and later washed 3×10-min with PBS-0.1% BSA Nuclear counterstaining with propidium iodide (red) was performed after removal of excess of secondary antibody and slides were mounted with fluorescence mounting media (DAKO). In some experiments two primary antibodies were used to co-localize HLA proteins with various AMP components, including calnexin, calreticulin, tapasine, and ERP57; Alexa Fluor 488 (green) or Alexa Fluor 555 (red) were used as secondary antibodies. In negative control primary antibodies were omitted in control wells. Immunostaining was visualized with a Leica Spectral confocal microscope.

DNA and RNA isolation, reverse transcription, and quantitative real time-PCR

DNA from melanoma cell lines was isolated using FlexiGene DNA Kit (Qiagen, Hilden, Germany). Primers used for Melan-Mart-1 analysis were: Fw 5´-CACGGCCACTCTTACACCAC-3´ and Bw: 5´- GGAGCATTGGGAACCACAGG-3 with a predicted PCR product size of 254bp. Total RNA was extracted from cultured tumor cells under basal conditions, stimulated by IFN-γ, or transduced with Ψ5 or AdCMVβ2m using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and utilized for reverse-transcription-PCR using iSriptTMcDNA Synthesis Kit (Bio-Rad). The products of reverse transcription were analyzed by quantitative real-time PCR for the expression of various target genes (HLA-A, HLA-B, HLA-C, β2m, TAP-1, TAP-2, LMP-2, LMP-7 and tapasin). To control for variations in amounts of mRNA, the

glucose-6-phosphate dehydrogenase (G6PDH) and β-actin genes were amplified as well. All PCR reactions were performed in a Light Cycler instrument using the LC-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Manheim, Germany). The primer sequences used in this study, amplicon size and annealing temperatures have been described earlier (del Campo *et al*, 2014).

T-cell stimulation assay

Peptide-specific T-cells against HLA-restricted peptides MELAN-A/MART (26-35, EAAGIGILTV, HLA-A*02 restricted) and flu protein epitopes (58-66, GILGFVTL, HLA-A*02 restricted; and 44-52, CTELKLSDY, HLA-A*01 restricted) (a kind gift from Dr. Cecile Gouttefangeas, Univ. of Tuebingen, Germany) were generated from PBMC isolated from buffy coats of naive donor peripheral blood supplied by the Regional Blood Transfusion Centre of Granada. Autologous PBMC for melanoma cell line DNR-DC-M010 (del Campo *et al*, 2014) were received as part of European collaborative project ENACT. PBMCs were isolated by density gradient centrifugation on Histopaque and incubated overnight in tissue culture flasks at 37°C to separate adherent from non-adherent T-cell enriched cells (purity of CD3+ was higher than 85%). Adherent cells were further stimulated with 2 ng/ml IL-4 (R&D Systems, UK) and 3 ng/ml of GM-CSF (R&D Systems, UK) for induction of DC. T-cells were expanded by stimulation with 1 ug/ml of IL-2 (Proleukin, Novartis Pharmaceuticals, Basel, Switzerland) and 10 ng/ml of IL-7 (R&D Systems, UK). T cells were stimulated two-three times (5-7- days apart) with either autologous irradiated DCs or with irradiated HLA-A*0201-matched HLA-positive tumor cells (stimulating cells) in the presence of a specific peptide (1 μg/ml). After that the peptide-specific responder T-cell were seeded into 96-well plates with tumor cells in a ratio of (10:1) in the presence or absence of a corresponding peptide. T-cell stimulation was measured as IFN-γ release (pg/ml) by T-cell after 48 hours of incubation with β2m-negative melanoma cells or cells transfected with AdCMVβ2m (or AdCMVGFP in control experiments). Transfected cells were also checked for β2m (HLA class I) recuperation by FACS. Control melanoma cell lines ESTDAB-007 constitutively expressing HLA-A*0201, were left untransfected. Tumor cells were pulsed with corresponding specific peptide for two hours before incubation with responder T-cells in a volume of 200μl culture medium/well. 48 hours later standard capture ELISA (BD Biosciences, San Diego, CA, USA) was used to measure IFN-γ in the supernatants of each well. Level of IFN-γ secretion by T cells in response to PHA (Sigma Aldrich, St. Louis, USA) stimulation was used as a positive control.

β2m ELISA and proliferation assay.

The levels of secreted β2m in culture supernatants after AdCMVβ2m transduction were determined by a commercial Human β-2 Microglobulin ELISA Kit (Alpha Diagnostic International, Texas, USA). Briefly, cells were cultured with media alone or infected with AdCMVβ2m at previously described MOIs. Culture supernatants were collected 48 hours later and the amount of β2m protein was determined according to the manufacturer's protocol.

The Methylthiazol Tetrazolium (MTT) assay was used to measure the impact of soluble β2m protein on the proliferation of prostate cancer cells (PC-3 and DU-145 cell lines). Briefly, cells were plated into 96-well plates in culture medium with known concentration of β2m and containing 10%FBS. After 24 hours of incubation, the media was replaced by the supernatants of AdCMVβ2m- or Ψ5-infected tumor cells and left in culture for 72 hours. fter the incubation period, the assay was performed according to manufacturer's manual and the absorbance of formazan-containing cell culture medium was measured in each well using a ELISA microplate reader (at A570 nm) (Biotek, Power-Wave XS).

Analysis of apoptosis: PE-AnnexinV / 7-AAD staining.

FACS analysis with Annexin-V (PE-Annexin-V) and 7-amino-actinomycin D (7AAD) staining was used for the determination of cell apoptosis following transduction with AdCMVβ2m or with

control virus Ψ5 at optimal MOI (PE-Annexin-V positive: early apoptosis; 7-AAD: late apoptosis/ necrotic) following the manufacturer's protocol (PE Annexin V Apoptosis Detection Kit, BD Biosciences, USA). Briefly, cells were washed twice in PBS and suspended in Anexin binding buffer. Staining mixture of 5 µl PE-Annexin-V and 5 µl 7-AAD was added to 100 µl of cell suspension (10^6 cells⁄ml). Cells were incubated in the dark for 15 min at room temperature and then analyzed on BD FACS Canto flow cytometer. Anti-β2m antibody L368 was used to confirm the recovery of β2m expression after viral transduction.

RESULTS

We have previously described that adenoviral vector coding for human β2m gene (AdCMVβ2m) was able to successfully recover HLA class I expression on different types of tumor cells in vitro and in vivo (del Campo *et al*, 2009). Here we further characterize the vector-mediated transgene expression, describe how the virus affects tumor antigen presentation machinery (APM), and how it influences the proliferation and apoptosis of cancer cells.

β2m transgene expression analysis

Time-dependent analysis of the transgene expression was done by FACS and it revealed that 63% of the melanoma cells were positive for β2m 3 hours after infection, while after 6 hours post-infection the percentage of β2m-positive cells increased to 85% and at 12 hours post-infection it reached 93% (Figure 1). Longer incubation periods did not change significantly neither the percentage of transduced cells nor transgene expression levels indicated by MFI (.These results slightly varied among tumor cells of distinct histological type (data not shown). Interestingly, the positive β2m expression on tumor cells was maintained even after freezing at -80C for two days and thawing the infected cells as demonstrated by FACS using anti-HLA complex mAb W6/32 (data not shown).

β2m re-expression of AdCMVβ2m-infected melanoma cells analyzed by FACS (Figure 2a) was confirmed by immunocytochemestry using anti-β2m mAb L-368. Figure 2b depicts different patterns of β2m immunolocalization in transduced cells ranging from epinuclear transgene expression (i) to cytoplasmic and membrane immunolabeling of various intensity (ii and iii).

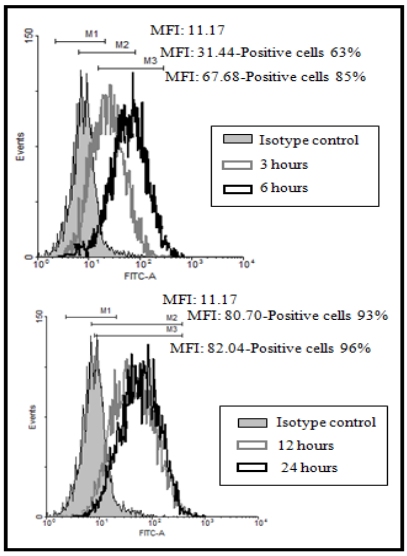

Figure 1: Adenovirus-mediated time-dependent recovery of β2m expression on DNR-DC-M010 melanoma cells. Cell were infected with the virus and left in the culture for various periods of time (3, 6, 12, and 24 hours); after that the culture media was replaced for a fresh virus-free one and the cells were left in culture for up to 48 hours in total. Recovery of β2m expression was detected 3 hours after infection (upper panel), reached its maximum level 12 hours after of infection and remained unchanged at 24 hours after viral transduction (lower histogram).

In addition, using confocal microscopy we demonstrated more detailed intracellular distribution, assembly with the HLA heavy chain and trafficking to the cell surface. **Figure 2c** demostrates β2m-negative melanoma cells (left upper image) with positive heavy chain labelling (upper right) that recover normal β2m (lower left image) after infection with AdCMVβ2m. These cells did not show any alteration in heavy chain expression after adenoviral transduction. Proper positive labelling with W6/32 mAb (lower right image), which recognize HLA class I complex on the cell surface suggest s proper HLA class I assembly and transport to the cell surface. Co-localization of the green fluorescent signal corresponding to the heavy chain molecules (HC-10 mAb, green) and polyclonal anti-β2m protein (red) was found as expected in the Golgi area of the cytoplasm of transduced cells (**Figure 2d).** Double staining with W6/32 mAb (green) and β2m recombinant protein (red) (**Figure 2d**) indirectly confirmed the expected proper assembly with the heavy chain assisted by APM molecules, ER localization and transport to the cell surface.

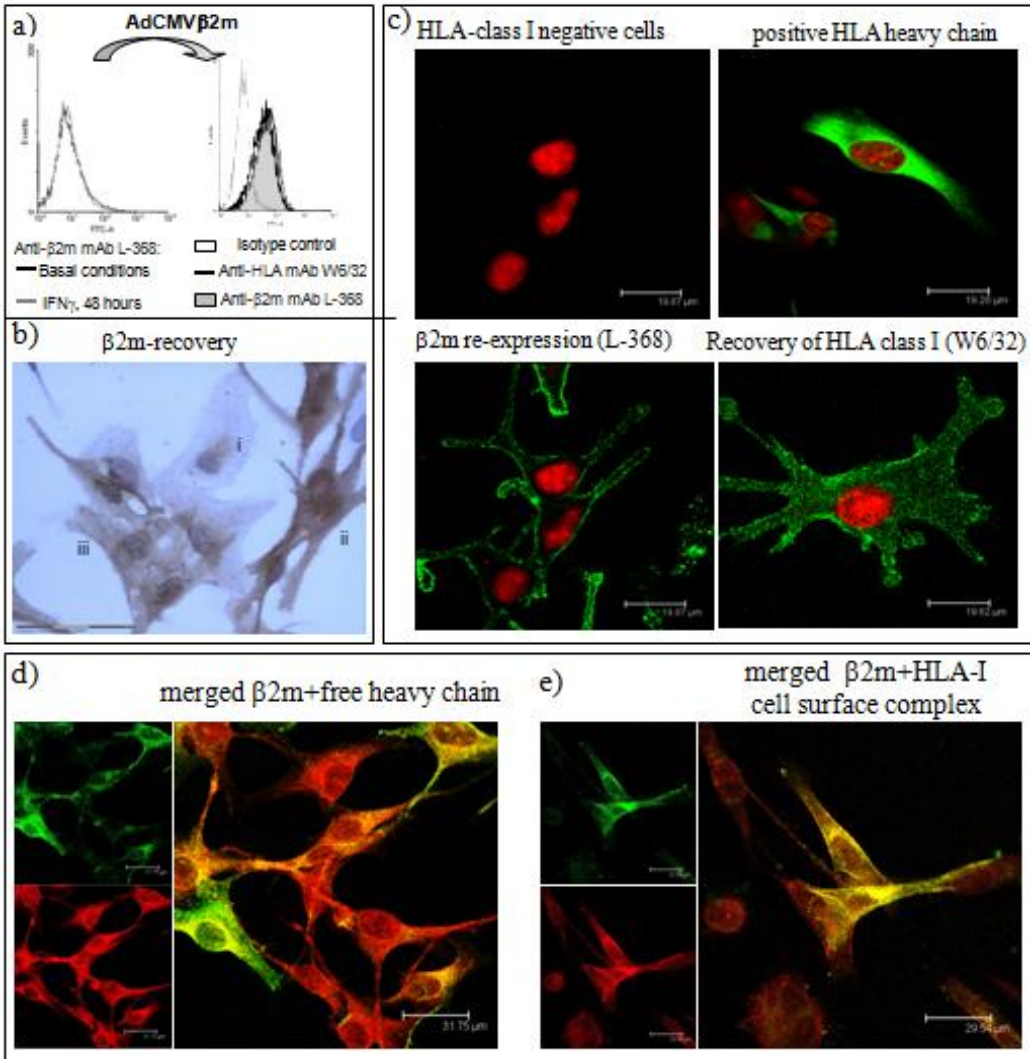

Figure 2. (a) Flow cytometry, immunocytochemistry and immunofluorescence demonstrating recuperation of β2m and HLA class I complex expression on DNR-DC-M010 melanoma cells after transduction with AdCMVβ2m virus. (a) Flow cytometry demostrates total loss of tumor HLA class I expression resistant to IFN-γ treatment and recuperation of the expression after infection with the adenovirus. (b) Positive immunolabeling of β2m protein in AdCMVβ2m transduced tumor cells (L-368 antibody) showing different immunolabeling patterns of transgene expression: weak epinuclear (ER) expression (i), strong complete cellular labeling (ii), cells with intermediate expression pattern (iii). (c) Confocal images of re-expression of β2m, HLA class I heavy chain and β2m/HLA class I complex on melanoma cells infected with the virus and labeled with various mAb: W6/32 against β2m/heavy chain complex (green), L-368 against β2m (green), and anti-free-heavy chain HC-10 (green). Propidium iodate was used for nuclear labeling (red). (d) In some experiments a co-localization of newly expressed β2m protein with the heavy chain (HC) was analyzed by merging the images of a polyclonal anti-β2m antibody (red) targeting β2m, and mAbs HC-10 and W6/32(both green).

Impact of the adenovirus-mediated β2m recovery on the expression of HLA-A, B-, C-loci

It is also important to examine how adenovirus-mediated upregulation of β2m expression affects tumor HLA heavy chain locus expression since different tumor-specific peptides have different binding affinity and antigen presentation specificity via various HLA-ABC loci. We measured the transcriptional levels of the HLA-A, B or C loci by quantitative PCR in melanoma cells DNR-DC-M010 and ESTDAB-109 3 days after infection with the adenovirus. In control experiments, cells were transduced with Ψ5 or incubated 48 hours with IFNγ to upregulate the expression of the loci. **Figure 3** shows that both Ψ5 and AdCMVβ2m increase locus B transcription in the studied melanoma cell lines. Locus C expression remains unaltered in DNR-DC-M010 cells, but is higher in ESTDAB-109 cells after infection. HLA-A transcription is lower in DNR-DC-M010 cells transfected with AdCMVβ2m, but not with Ψ5, while in ESTDAB-109 cells locus A expression remained unchanged independently on the type of the virus used for infection. These results show that transfection of melanoma cells with AdCMVβ2m viral vector does not have a dramatic affect on HLA locus expression pattern, although there is a variation between tumor cell lines originated from different patients. Importantly, the slight changes caused by β2m gene transfer did not affect the expression of the loci on a protein level and the interaction with β2m, since we achieved a recovery of functional cell surface expression of the HLA class I complex.

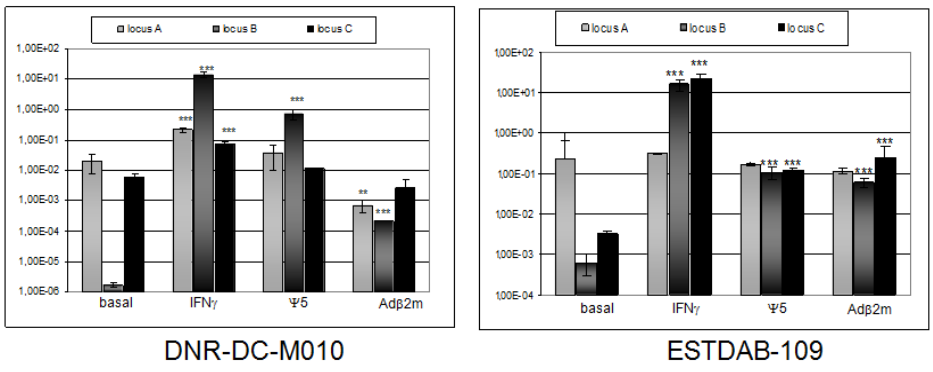

Figure 3. Transcriptional levels of HLA-A, B-, and C- loci under basal conditions and after treatment with IFN-g, Ψ5 or AdCMVβ2m in (a) DNR-DC-M010 melanoma cells and (b) ESTDAB-109 melanoma cells. *** p<0.005; ** p<0.05

Effect of AdCMVβ2m on APM

The cell-surface expression of class I major histocompatibility HLA molecules involves the participation of specialized proteins known as Antigen Processing Machinery components. In order to investigate the impact of adenoviral transduction on the APM proteins in tumor cells, we studied the transcription and expression levels of tapasin, calnexin, calreticulin and ERP57 (HLA class I antigen folding and stabilizing chaperones), and of the IFN-γ inducible proteasomal subunits LMP-2. Transcription of LMP-2, LMP-7, TAP-1, TAP-2, and β2m was measured in DNR-DC-M010 melanoma cells before and three days after AdCMVβ2m transduction. As control, cells were transduced with control virus Ψ5 or incubated 48 hours with IFNγ. As expected, AdCMVβ2m vector but not Ψ5 increases β2m transcription. Also IFNγ increases the transcription as expected as the defect in β2m in the cell lines is at translational level. Infection with Ψ5 and AdCMVβ2m decreases transcription of LMP2, LMP7, TAP1 and TAP2, and increases the level of tapasin. However, we observed different patterns of APM changes in six tumor cell lines (five melanomas and one colon adenocarcinoma) in response to the infection with adenovirus. **Figure 4b** shows an example of the AdCMVβ2m-induced modulation of LMP-7 expression among the studied cell lines. Furthermore, we examined LMP-7 expression in DNR-DC-M010 cells before and after AdCMVβ2m transduction by immunocytochemistry and did not find any dramatic difference in the intensity of the immunolabeling (**Figure 4c**).

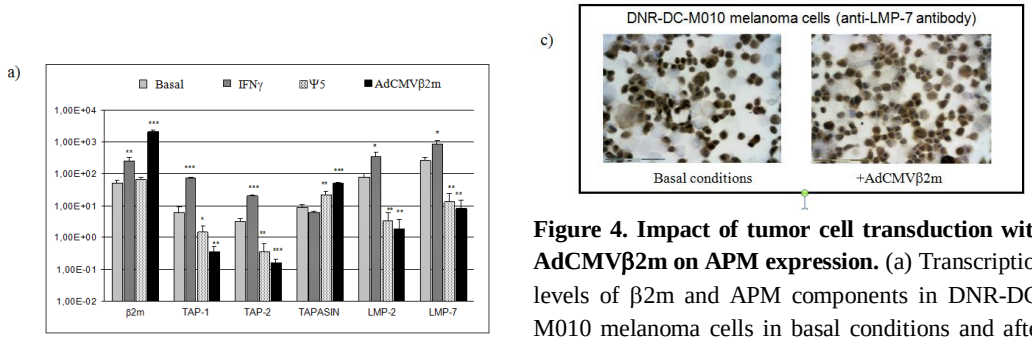

Figure 4. Impact of tumor cell transduction with AdCMVβ2m on APM expression. (a) Transcription levels of β2m and APM components in DNR-DC-M010 melanoma cells in basal conditions and after treatment with AdCMVβ2m, Ψ5 or IFN-γ. (b) LMP-7 transcription before and after AdCMVβ2m transduction in four melanoma cell lines and one colon adenocarcinoma cell line DLD-1. p<0,005 except for MaMel128 cell;data are normalized against the expression of housekeeping gene G6PDH; (c) Immunocytochemical staining of LMP-7 in DNR-DC-M010 melanoma cells is similar in basal condition and after infection with AdCMVβ2m.

We analyzed by confocal immunofluorescence microscopy the cellular co-localization of the newly produced β2m and several molecules of the APM complex, including tapasin, calnexin, calreticulin, and ERp57. **Figure 5** depicts a co-localization of the newly synthesised β2m protein with each of the studied APM molecules in the epinuclear area within the endoplasmic reticulum. These results showed that adenovirus-mediated modification of APM transcription varies among the studied tumor cell lines. Although in some cases transduced cells show transcriptional downregulation if some APM

molecules, it does not affect the protein expression allowing a proper interaction with β2m and HLA class I heavy chain, and correct transport from ER to the cell surface via Golgi apparatus.

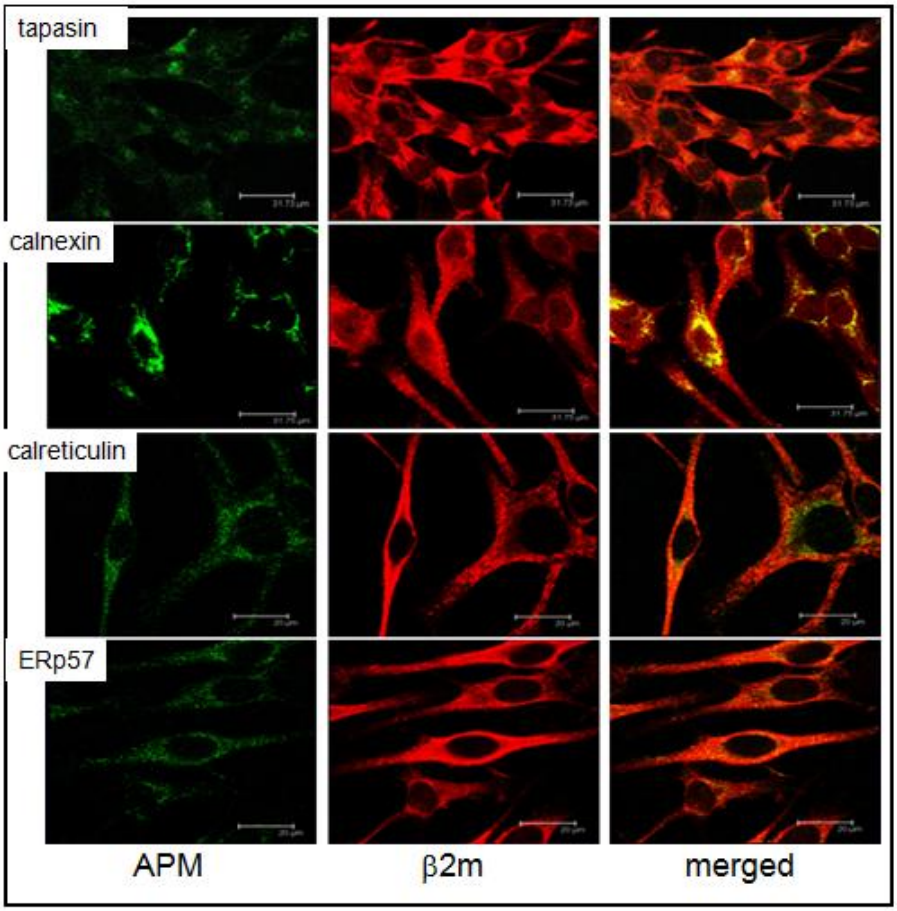

Figure 5. Immunohistochemical localization of APM components DNR-DC-M010 melanoma cells 48 hours after transduction with AdCMVβ2m. Confocal images of intracellular localization of fluorescence-labeled APM components in infected cells (green, left panel); β2m labeling is red (middle). The right panel has merged images demonstrating a co-localization of the APM and β2m proteins (in yellow).

Functional activity of re-expressed HLA class I complex

To confirm that the restoration of HLA class I expression on β2m-negative cells was able to induce cellular immune responses mediated by cytotoxic T lymphocytes, we tested the ability of AdCMVβ2m-transduced DNR-DC-M010 melanoma cells to induce IFN-γ secretion by melan/MART-1-specific HLA-matched T-cells. Melanoma cell lines DNR-DC-M010 (does not express HLA class I due to a mutation in β2m gene)(del Campo et al, 2014) and ESTDAB-007 (HLA-A*0201-positive) were used in these assays. First, using PCR and FACS analysis with A103 antibody we confirmed that both cells lines express melanoma peptide Melan/MART-1 (**Figure 6a and 6b).** HLA-matched Melan/Mart-1-specific T-cells were co-cultured Melan/Mart-1-pulsed-DCs as feeder cells and DNR-DC-M010 cells infected with AdCMVβ2m or with control adenovirus Ψ5 and 48 hours later IFN-γ in collected supernatants was measured by ELISA. As shown in **Figure 6c** IFN-γ production was detected at a

significantly higher level (4-fold) in the supernatants when target cells were transduced with AdCMVβ2m in the presence of Melan/Mart-1 peptide, as opposed to the same target without the addition of the peptide or untransduced target ($p < 0.005$), which remained at background level as indicated by a negative control (T cells alone). PHA-stimulated T cells were used as positive control. **Figure 6d** shows that IFN-γ secretion was more than 4-fold higher when naive donor T cells (A*0201) stimulated with Melan/Mart-1-pulsed irradiated ESTDAB-007 melanoma cells (also A*0201 and Melan/Mart-1 positive) were incubated with AdCMVβ2m-transduced DNR-DC-M010 cells in the presence of Melan/Mart-1 peptide as compared to non-transduced cells ($p<0.005$) or cells transduced with control virus coding for GFP (AdCMVGFP). IFN-γ production was also observed when HLA-A*02-restricted flu peptide (GILGFVTL epitope, 58-66) was used, but not in the presence of HLA-A*01 restricted flu epitope (data not shown). Melanoma cells ESTDAB-007 constitutively expressing HLA-A*0201 were used as a positive control for IFN-γ production by HLA-matched T-cells. PHA stimulated donor T cells were used as a control for non-specific positive IFN-γ production.

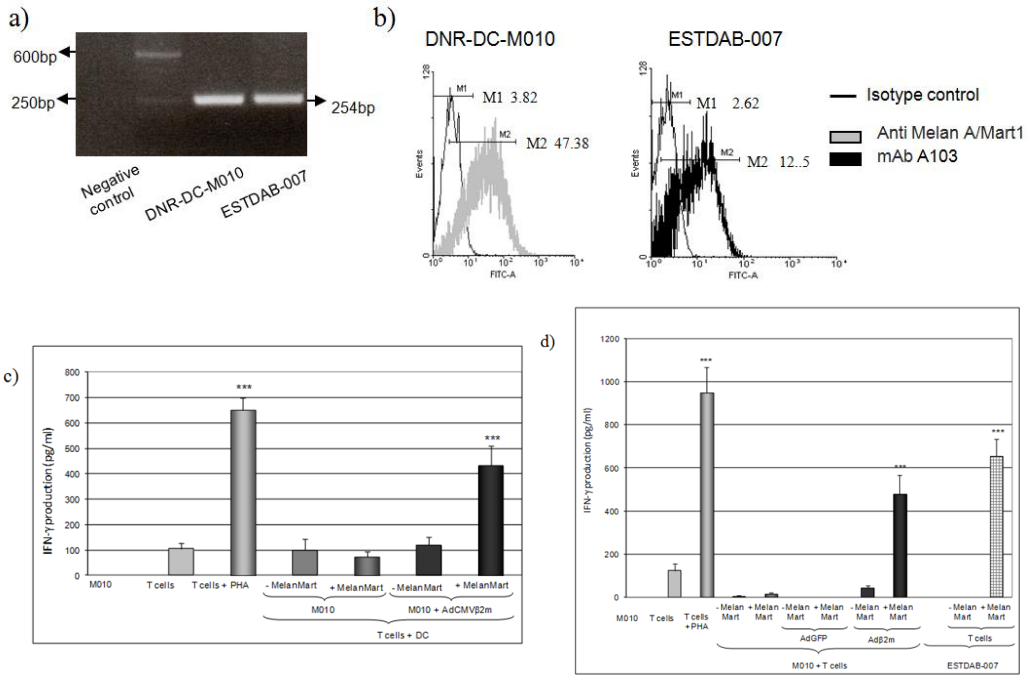

Figure 6. Recovery of the ability of melanoma cells DNR-DC-M010 transduced by AdCMVβ2m virus to induce IFN-γ production by T-cells. Positive expression of Melan A/MART-1 peptide on both melanoma cell lines demonstrated by PCR (a) and by flow cytometry (b) using anti-Melan A/Mart-1 mAb A103. (c) IFN-γ production by the autologous T cells stimulated with autologous Melan/Mart-1-pulsed-DCs in the presence of HLA-negative or HLA-positive transduced melanoma DNR-DC-M010 cells. IFN-γ was detected at a significantly higher level (4-fold) in the supernatants of the effector/target co-culture when target cells were transduced with AdCMVβ2m in the presence of Melan/Mart-1 peptide (1mg/ml), as opposed to the same target without the addition of the peptide or untransduced target ($p < 0.005$), which remained at background level as indicated by a negative control (T cells alone). PHA-stimulated T cells were used as positive control. Assay was performed in triplicate. (d) 4-fold increase in INF-γ secretion when healthy donor T cells (A*0201) stimulated with Melan/Mart-1-pulsed irradiated ESTDAB-007 melanoma cells (A*0201) were incubated with AdCMVβ2m-transduced DNR-DC-M010 cells in the presence of

Melan/Mart-1 peptide (1μg/mL) as compared to non-transduced cells ($p<0.005$) or cells transduced with virus coding for GFP (AdCMVGFP). Similar positive production of IFN-g was observed using another HLA-A*02-restricted flu peptide (GILGFVTL), but not in the presence of HLA-A*01 restricted irrelevant flu epitope (data not shown). Melanoma cells ESTDAB-007 constitutively expressing HLA-A*0201 were used as a positive control for IFN-g production by HLA-matched T-cells. PHA stimulated donor T cells were used as a control for non-specific positive IFN-γ production. Assays were performed in triplicate in three independent assays.

Measurement of secreted β2m protein in the supernatants of AdCMVβ2m transduced cells and its affect on tumor cell proliferation

We have demonstrated above that β2m protein produced by adenovirus-mediated gene transfer is properly expressed on tumor cell surface in a complex with HLA heavy chain. However, it is expected that in case of β2m overproduction the excess of the newly synthesized β2m will not have enough free heavy chains to form HLA class I complex with peptides and will be secreted into the extracellular space.We wanted to test whether in our experiments the newly expressed β2m protein sheds off the cell surface and potentially can increase tumor cell proliferation. We analysed the level of β2m protein in the supernatants of melanoma cells transduced with AdCMVβ2m and used these samples to incubate with non-transduced prostate and bladder HLA-positive cancer cells. After 72 hours of incubation cell proliferation was measured by MTT test and by direct counting of the number of trypan blue negative cells using a haemocytometer. First we measured the β2m protein levels in culture media from different cell lines after 72 hours of transduction with AdCMVβ2m at their correspondent MOIs (see del Campo et al., 2009). As it is shown in **Figure 7a**, the β2m protein level as determined by ELISA at basal conditions was undetectable (as expected because of the lack of β2m protein in the cells). After adenoviral transduction, there was an increase in β2m protein level reaching levels ranging from 50 to 200 ng/ml depending on the cell line. In all the five studied melanoma cell lines the β2m protein secretion level was above 100ng/ml, reaching the maximum level of 200 ng/ml in the supernatants of transduced ESTAB-109, DNR-DC-M010 and MaMel-128 cells. Comparable numbers were obtained for colorectal adenocarcinoma DLD-1 and for the Burkkit lymphoma DAUDI, although this last one did not recover cell surface β2m expression, but secreted soluble β2m protein. Prostate cell line OPCN-3 showed a minimal production of secreted β2m protein. The concentration of the secreted soluble β2m protein was dose-dependent (**Figure 7B**). Under these conditions, increase of MOI in 10 times lead to the augmented secretion of β2m around 1μg/ml, while infection with 20x MOI increased this concentration to 2μg/ml (for ESTDAB-109 cell line).

The dose-dependent effect of secreted β2m on cell proliferation was examined in cultured prostate cell lines DU-145 and PC-3 using MTT assay As shown in **Figure 8a**, the addition of supernatants from β2m transduced melanoma cell lines (with different MOI) did not change the proliferation rate of prostate cell lines, although a slight decrease in DU-145 proliferation was observed (**Figure 8b**), although direct cell counts using a haemocytometer did not show any significant changes in tumor cell proliferation in the presence of β2m-rich supernatant.

Analysis of apoptosis in tumour cell lines transduced with AdCMVβ2m

Flow cytometry analysis with phycoerythrin-conjugated annexin-V and 7-Amino-Actinomycin D (7AAD) was used to examine the effect of AdCMVβ2m infection on tumor cell apoptosis at different two MOIs: 15(optimal dose) and 225. Melanoma cells were transduced with adenovirus vector and 48 hours later cells were stained with PE-Annexin-V and 7AAD. Simultaneous staining with PE-Annexin-V and 7AAD non-vital dye makes it possible to distinguish between intact cells (stained negative for both annexin V and 7AAD), cells in early apoptosis (stained positive for PE-Annexin-V and negative for 7AAD), and in late apoptosis or after cell death (stained positive for both and annexin V and 7AAD). β2m recovery on transduced

cells was detected as a positive staining for L-368 mAb using the FITC channel. The percentage of apoptotic cells that bound PE-annexin-V but excluded 7-AAD was determined in the β2m cell population, to estimate fraction of β2m expressing cells (transduced cells) in early stages of apoptosis.
In control culture, 89.4% of DNR-DC-M010 cells were viable, 2,3% were in early apoptosis, and 4,8% were in the late or final stages of apoptosis (**Figure 9a1**). In this cell line the β2m labelling was negative as expected. 87.4% of the transduced cells at MOI of 15 were viable, 3% were in early apoptosis, 5.7% were in the late or final stages of apoptosis, and 72.1% expressed β2m as indicated by FITC labelling with L-368 mAb (**Figure 9a2**). In cells infected with MOI of 225, the percentage of β2m positive cells increased to 86.5% but the percentage of apoptotic cells was the same as with lower MOI (**Figure 9a3**). Similar results were obtained in control experiments when cells were infected with Ψ5 (**Figure 9b**).

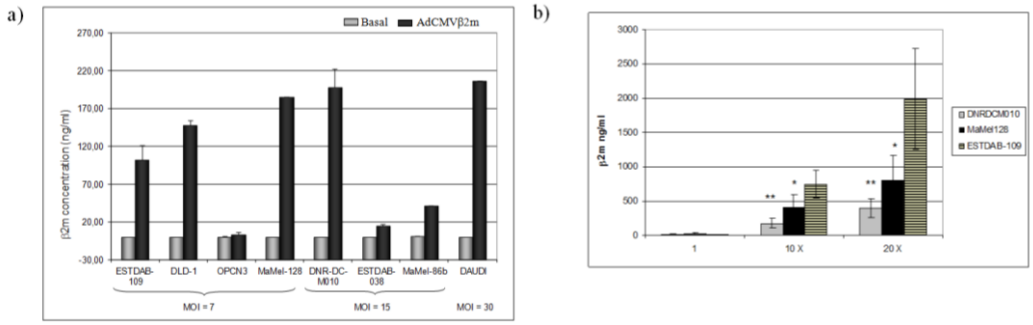

Figure 7. ELISA measurement of β2m secretion in the supernatants of different tumor cell lines 3 days after transduction with AdCMVβ2m. a) β2m in HLA-class I -negative cells transduced at the MOI that restores HLA class I expression. b) dose-dependent (optimal MOI (1x), times 10, and times 20 of the optimal MOI) secretion of β2m protein in the supernatants of three melanoma cell lines. $p<0,005$ except for the indicated with * $p<0,5$ or ** $p>0,05$.

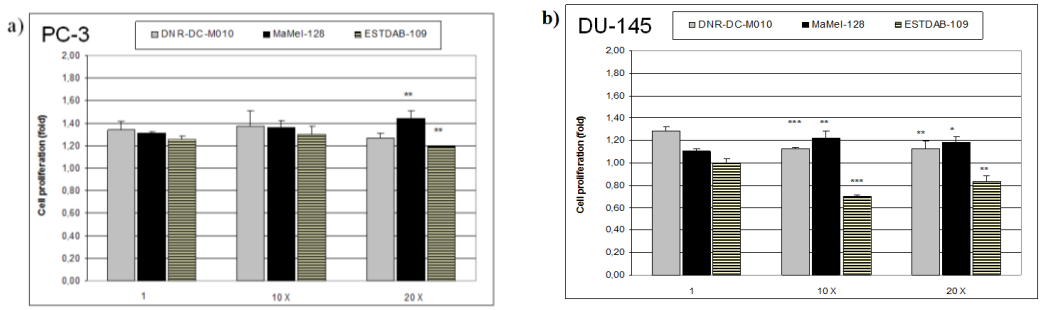

Figure 8. Proliferation of prostate cells PC-3 (a) and DU-145 (b) measured using MTT assay after incubation with β2m-containing supernatants of AdCMVβ2m transduced melanoma cells. Prostate cells were incubated for 72 hours with supernatants from melanoma cells infected with the virus at different MOI (optimal MOI, x10 and x20) and with control supernatants from non-transduced cells (cell proliferation was normalized against control proliferation value).

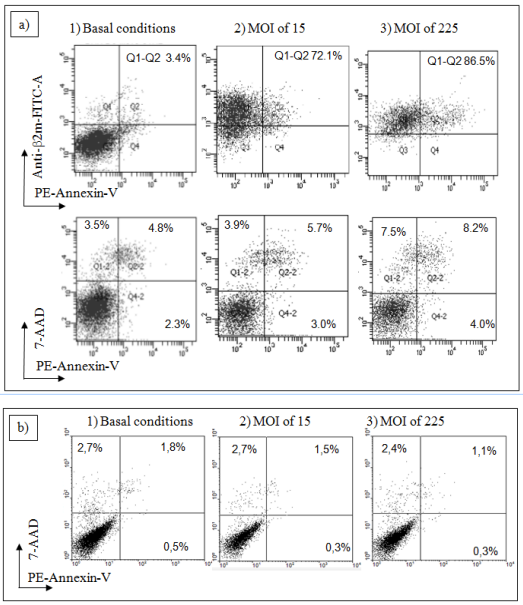

Figure 9 Transduction of DNR-DC-M010 melanoma cells with AdCMVβ2m (a) or with control virus Ψ5 (b) does not affect cell apoptosis as measured by flow cytometry in basal conditions (1), 48 hours after transduction with MOI=15 (2), or with MOI 15 times higher (3). Figure (a) upper panel depicts three plots with FITC-A-labeled anti-β2m mAb (L-368) versus PE-annexin-V showing increase in the number of β2m-positive cells after adenoviral infection. Lower panel on figure (a) and figure (b) demonstrates that there are no change in the apoptosis of the infected cells: plots with 7-AAD versus PE-annexin-V labeling reflecting the percentage of viable cells in Q2-3 quadrant, late apoptotic cells in the Q1-2 (7-AAD positive only), apoptotic cells in Q2-2 quadrant (both annexin-V and 7-AAD positive), and early apoptotic cells in Q4-2 quadrant (annexin-V positive only).

DISCUSSION

The lack of tumor rejection is associated with multiple cancer immune escape mechanisms, including the loss or low expression of tumor HLA class I molecules. There is accumulating evidence suggesting that immunotherapy is effective in eliminating HLA positive tumor cells, while cells with loss or downregulation of HLA antigens escape therapy-induced immune attack and produce new distant tumor lesions. Therefore, corrections of antigen presentation in tumor cells might result in much higher success rates of cancer treatment.

We have previously demonstrated that adenovirus coding for β2m HLA constitutes a promising approach to restore normal HLA class I expression in β2m-negative tumor cells of different histological type (del Campo et al, 2009). β2m forms a complex with heavy chain of HLA class I molecules and it has an important role in tumor immunity. In this study we extended the characterization of the viral vector and demonstrated that re-expression of β2m leads to up-regulation of functional HLA class I complex on tumor cell surface which is able to stimulate specific T cell immunity against malignant cells without compromising the antigen processing machinery function. We have demonstrated that tumor cells infected with the β2m-coding replication-deficient adenovirus (AdCMVβ2m) recover normal HLA class I surface expression as measured by indirect fluorescence with mAb directed against β2m or HLA class I cell surface complex (del Campo et al, 2009). Here we demonstrate that this positive expression could be detected three hours after the infection and the percentage of infected cells and the expression levels was gradually increasing with the time of incubation reaching the plato at 12 hours after infection (Fig.1). In addition, newly formed HLA complex remained stable after freezing and thawing the transduced cells, a method used by some authors to ensure the stability of transgene expression (Gülen *et al*, 2012; Vatakis *et al*, 2011).

Expression of the functional HLA class I complex on the cell surface requires coordinated participation of multiple proteins, which are part of APM, including TAP1/2, tapasin, calnexin, calreticulin, LMP2/7, and Erp57. They process HLA antigens in the endoplasmic reticulum (ER), control correct folding of the heavy chain, its association with β2m, and correct transport of this complex to the cell surface via Goldgi apparatus. The proper assembly in the ER and later transport to the cell surface are critical for successful antigen presentation, determines the efficiency of peptide

loading and T-cell stimulation. Immunocytochemistry and confocal analysis confirmed positive HLA class I expression on transduced tumor cells. In addition, intracellular co-localization of the re-expressed β2m, HLA heavy chain and APM molecules in the ER area indirectly verified the expected proper binding of the transgene and the heavy chain assisted by APM molecules, as well as transport of the complex to the cell surface.

Adenoviruses have been widely studied and used for gene therapy because their high transduction efficiency and their lack of integration into the host genome avoiding an increase in genomic instability that is a critical characteristic for cancer therapy. Therefore, adenoviral vectors coding for tumor-associated antigenic peptides have been generated and used to increase tumor cell immunogenicity in the past. However, detailed knowledge on their mechanism of action for anti-tumor efficacy is lacking. In particular, there is not much information regarding the effect of recombinant adenoviral vectors on the antigen presention machinery and HLA expression in tumor cells. There are several important issues that have to be addressed to make this type of gene delivery vehicle appropriate for HLA class I recuperation. This includes analysis of the possible influence of adenovirus on the HLA class I/heavy chain/β2m/peptide complex assembly and transport to the cell surface supported by the components of the antigen processing machinery (APM).

There is an accumulating evidence suggesting that the change in the expression of heavy and light chains of the HLA class I complex, as well as APM components happens in coordinated fashion (Romero *et al*, 2005) although the exact mechanism is not known. Therefore, we examined how re-expression of β2m using adenoviral vector affects the mRNA levels of the HLA-A, B and C loci, as modulation of HLA heavy chains expression could potentially affect peptide presentation, their affinity and specificity of locus-specific peptide binding to HLA class I complex. The analysis of HLA-ABC locus transcription after β2m recovery showed a common locus B upregulation and various patterns of locus A and C changes in different cell (Fig 3). This finding is in acordance with previous despription of diferential expression of HLA class I loci after β2m plasmid transfection of HLA-I deficient melanoma cell lines (Chang *et al*, 2005), a phenotype that appears to be caused by abnormalities in regulatory mechanisms, since it can be recovered by IFN-γ.. Morover, it has been also described that the three loci can be expressed at different levels in different types of tumor cells (Johnson, 2000; Versteeg *et al*, 1989), and in normal tissues (Garcia-Ruano et al, 2010), probably due to differences in the expression of regulatory factors that control HLA–ABC constitutive expression affecting the promoter region of the HLA heavy chain, (Griffioen *et al*, 2000; Johnson, 2003). Therefore, we can not exclude appearance of aditional HLA class I alterations in the cells after adenoviral-mediated recuperation of β2m expression.

There is a controversial data on how adenoviruses with different serotypes affect antigen processing and presentation. Some authors indicate that adenoviruses inhibit the intracellular transport of class I antigens and dramatically reduce their cell-surface expression, (Paabo et al, 1986) Other reports show that Ad5 but no Ad12-transformed cells show variable but significant expression of class I H-2 antigens on mouse cells (Eager *et al*, 1985), even increased levels of MHC class I expression (Nielsch *et al*, 1991) or the same level as untransformed Ad5 cells. (Vasavada *et al*, 1986). There have been different reports on the adenoviral-mediated modulation of the APM molecules in antigen-presenting cells, including tumor cells and DCs. Cells transformed by Ad5 but not Ad12 expressed either higher (Mey-Tal *et al*, 1997; Rotem-Yehudar *et al*, 1994) or normal (Mey-Tal *et al*, 1997; Rotem-Yehudar *et al*, 1996) levels of TAP1/TAP2 and LMP2/LMP7 mRNAs; data from murine tumors injected with AdGFP vector show no alteration of the equivalent TAP1/TAP2 and LMP2/LMP7 mRNAs (Putzer *et al*, 2002).

Adenoviral vectors have been used to restore TAP1 expression in tumor cells and tumor-specific immune response was stronger while other APM molecules remained unchanges (Lou *et al*, 2007; Seliger *et al*, 2006). Virus-mediated transfer of calreticulin gene also increases specific anti-tumor immunity without modification of other APM components (Gomez-Gutierrez *et al*, 2007).

Adenoviral vectors are frequently used to transfer tumor associated genes or tumor mRNA into DC in preparation of cancer vaccines. A recent publication demonstrated that recombinant adenoviruses can increase ERp57, TAP-1 and TAP-2 levels in transduced DCs (Vujanovic *et al*, 2009).

E3/19K gene product is the adenoviral protein widely implicated in the modulation of HLA class I expression; it was the first adenoviral protein shown to down-regulate the class I antigens (Andersson *et al*, 1985) by direct retention of HLA heavy chains in the ER (Beier *et al*, 1994; Burgert & Kvist, 1985) through the α1- and α2-domains (peptide-binding groove) of HLA class I molecules (Fu & Bouvier, 2011). E3/19K protein binds TAP (Bennett et al, 1999) interfering with peptide loading, and as a result, blocks the association of TAP with tapasin and the transport process (Gruhler & Fruh, 2000; Petersen *et al*, 2003). However, some studies demonstrate that adenoviral vectors constructed without elimination of E3/19K do not always induce reduction of surface MHC class I expression in tumor cells (Zhai *et al*, 1996).

In our case, AdCMVβ2m is replication-deficient and lacks E3/19K gene. Thus, virus-mediated APM downregulation is not expected in tumor cells transduced with this virus.

On the other hand, other mechanisms might negatively affect antigen presentation by the viral vector. Therefore, we examined APM component expression in virally transduced tumor cells by quantitative PCR, immunocytochemical and confocal microscopy. All the results demonstrate that the adenoviral vector upregulates β2m and the cell surface HLA class I complex expression and does not inhibit antigen presentation, since the mRNA levels of the APM components as well as their protein expression is unchanged after viral infection. In addition, confocal microscopy data suggests that the intracellular trafficking and assembly of the β2m and HLA heavy chain with the support of AMP molecules takes place as expected, similar to other publications (Bedard *et al*, 2005; Everett & Edidin, 2007; Mery *et al*, 1996; Tasdemir *et al*, 2008)

The proper expression of the cell surface HLA class I complex results in the presentation of the tumor-associated peptide to the cytotoxic T-cells and their activation. We observed that IFN-γ secretion by T-cells primed with β2m-negative melanoma cells was recovered after adenoviral transfer of wild type β2m and recovery of normal HLA class I expression. This stimulation of T-cells by melanoma cells pulsed with melanoma-specific or flu peptide was observed only after transfer of wild type β2m gene and recovery of HLA class I cell surface expression. Therefore, the recovery of HLA class I expression mediated by adenoviral β2m transfer induces stimulation of T-cells, allowing the proper recognition of tumor-associated antigenic peptides by HLA class I-restricted CD8+ T cells, a fundamental event for the detection and destruction of malignant cells.

The efficacy of gene therapy also depends how the transgene affects target cells, how it influences cell viability and proliferation. We have demonstrated above that β2m protein produced by adenovirus-mediated gene transfer is properly expressed on tumor cell surface in a complex with HLA heavy chain. However, it is expected that in case of β2m overproduction the excess of the newly synthesized β2m will not have enough free heavy chains to form HLA class I complex with peptides and will be secreted into the extracellular space.

We found different levels of soluble β2m in the culture supernatant of AdCMVβ2m transduced tumor cells, ranging from 50 to 200 ng/ml at optimal MOI and reaching 2μg/ml with 20x MOI. Physiological serum β2m concentrations varies from 1 to 3μg/ml in physiological conditions and it increases up to 10-20μg/ml in disease conditions (Rowley *et al*, 1995). Prostate cell line showed a minimal production of soluble β2m protein while, while Burkkit lymphoma cell line DAUDI that did not recover cell surface β2m expression (del Campo et al, 2009), secreted soluble β2m protein. It could be explained by the data recently published suggesting that this cell line does not require β2m for cell surface expression of HLA heavy chains and peptide presentation (Martayan *et al*, 2009).

Free β2m is found in body fluids under physiological conditions as a result of shedding and extracellular release, or cell death in cancer or other diseases Elevated levels of serum β2m correlate with poor prognosis in renal diseases and are present in hematological malignancies, including lymphomas, leukemias and multiple myeloma (ref). This observation suggests an important, yet unidentified,

role of this protein in these malignancies. In addition to the roles in immunity, the level of β2m is associated with proliferation, apoptosis and metastasis in several cancer types. Some studies have shown that increased concentration of soluble β2m protein is linked to increased tumor growth in various types of malignancy, including breast, lung and renal cancer (Rasmuson *et al*, 1996). Some publications demonstrate an evidence that β2m acts as a signalling and growth-promoting factor for prostate cancer and cancer-associated bone metastasis (Huang *et al*, 2006; Nomura *et al*, 2006). However, there is an evidence that β2m secreted in sera is modified by proteolytic cleavage (Shemesh & Ehrlich, 1993) and this modified form would exert other biological functions compared to β2m when bound to cells.

Since our in vitro experiments demonstrated that most of the studied cell lines after AdCMVβ2m transduction not only recover cell surface expression of β2m/heavy chain complex, but also produce free soluble form of β2m protein, we decide to analyse how it affects tumor cell proliferation and apoptosis. We measured proliferation of prostate tumor cells DU-145 incubated with β2m-rich supernatants collected after transduction of melanoma cells with various doses of the AdCMVβ2m and did not see any increase in the proliferation measured by MTT method or by direct cell counting, although in some cases MTT reading were slightly reduces in the presence of the β2m transduced melanoma cell supernatants.

In addition, viral proteins are capable to modulate the apoptotic response of host cells. Following the infection with human adenoviruses, cells exhibit an apoptotic response mediated by the expression of the viral E1A protein (Kirshenbaum, 2001) and adenoviral gene E6 products. On the other hand, E1B-19K blocks host cell apoptosis, and various proteins encoded by the E3 transcription unit, exert antiapoptotic (Galluzzi *et al*, 2008). However, in this study neither replication-deficient adenovirus coding for β2m nor control Ψ5 virus induced apoptosis in infected tumor cells.

In summary, our data indicate that replication-deficient adenoviral vector coding for human b2m gene that had been generated to upregulate tumor HLA class I expression, is suitable suitable for gene therapy of cancer since it effectively induces specific anti tumor T cell activity without inhibition of antigen processing and presentation or stimulation of tumor cell proliferation, or apoptosis.

The AdCMVβ2m would be suitable for patients with metastatic cancer with structural defects in β2m gene, where local administration of the vector into such metastatic lesions or into relapsed tumours might help to boost tumour cell immunogenicity and recognition by T-cells, preventing malignant progression and improving the response to immunotherapy.

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TISSUE ANTIGENS
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Review article Tissue Antigens ISSN0001-2815

Targeting HLA class I expression to increase tumor immunogenicity

A. B. del Campo[1], J. Carretero[1,2], N. Aptsiauri[1] & F. Garrido[1,2]

1 Servicio de Análisis Clínicos, Hospital Universitario Virgen de las Nieves, Granada, Spain 2 Departmento de Bioquímica, Biología Molecular 3 e Inmunología, Facultad de Medicina, Universidad de Granada, Granada, Spain

Key words: cancer; gene therapy; human leukocyte antigen class I; immune escape.

Correspondence:

Federico Garrido
Departamento de Análisis Clínicos
Hospital Universitario Virgen de las Nieves
Avd. Fuerzas Armadas 2
18014 Granada Spain.
Tel: +34 958 020319
Fax: +34 958 020069
e-mail: federico.garrido.sspa@juntadeandalucia.es

and

Natalia Aptsiauri
Departamento de Análisis Clínicos
Hospital Universitario Virgen de las Nieves
Avd. Fuerzas Armadas 2
18014 Granada Spain
Tel: +34 958 020320
Fax: +34 958 020120
e-mail: natalia.aptsiauri.exts@juntadeandalucia.es

Abstract

The dynamic interaction between the host immune system and growing cancer has been of central interest to the field of tumor immunology over the past years. Recognition of tumor-associated antigens (TAA) by self-HLA (human leukocyte antigen) class I-restricted CD8+ T cells is a main feature in the detection and destruction of malignant cells. The discovery and molecular characterization of TAA has changed the field of cancer treatment and introduced a new era of cancer immunotherapy aimed at increasing tumor immunogenicity and T-cell-mediated anti-tumorimmunity. Unfortunately, while these new protocols of cancer immunotherapy are mediating induction of tumor-specific T lymphocytes in patients with certain malignancies, they have not yet delivered substantial clinical benefits, such as induction of tumor regression or increased disease-free survival. It has become apparent that lack of tumor rejection is the result of immune selection and escape by tumor cells that develop low immunogenic phenotypes. Substantial experimental data support the existence of a variety of different mechanisms involved in the tumor escape phase, including loss or downregulation of HLA class I antigens. These alterations could be caused by regulatory (‘soft’) or by structural/irreversible (‘hard’) defects. On the basis of the evidence obtained from experimental mouse cancer models and metastatic human tumors, the structural defects underlying HLA class I loss may have profound implications onT-cell-mediated tumor rejection and ultimately on the outcome of cancer immunotherapy. Strategies to overcome this obstacle, including gene therapy to recover normal expression of HLA class I genes, require consideration. In this review, we outline the importance of monitoring and correction of HLA class I alterations during cancer development and immunotherapy.

Introduction

Despite the significant advances in our understanding of anti-tumorimmunity and considerable efforts to enhance immune reactivity against malignancies, consistent and long-lasting responses to cancer vaccines remain vague and the overall clinical efficacy of cancer immunotherapy is still below expectations (1,2). Understanding the possible causes of such poor clinical outcomes has become very important for the improvement of existing cancer treatment modalities. Failure of cancer immunotherapy to mount strong immune responses and tumor rejection could be associated with various mechanisms of tumor immune escape, including expression of immunosuppressive factor such as Fas ligand, increased production of vascular endothelial growth factor, tumor growth factor-β, or interleukin-10(IL-10), loss of tumor-specific antigens, lack of costimulatory signals and/or adhesion molecules, induction of 2,3-indoleamine dioxygenase, and deficiencies in the dendritic cell (DC) function or in the signal transduction path-way of CD8+ cytotoxic T cells [cytotoxic T lymphocytes - (CTL)](3–5). In particular, the inability of the immune system to recognize malignant cells may be due to the loss of tumor human leukocyte antigen (HLA) class I expression and lack of presentation of tumor-associated peptides to T cells(6,7). Hence, the critical role of HLA class I antigens in the success of T-cell-based immunotherapy has led to a growing interest in investigating the expression and function of these molecules in metastatic cancer progression and response to immunotherapy.

Our laboratory has been analyzing the molecular mechanism of HLA class I-altered expression in cancer patients undergoing immunotherapy and our recent data suggest that this type of treatment frequently leads to immune selection of malignant cells with irreversible structural alterations in HLA class I genes and development of progressing malignant lesions resistant to therapy (8–11). In this review, we will summarize these observations, and discuss current perspectives of the combination of immunotherapy with gene therapy that offer new approaches for improving the clinical effect of immunotherapy.

Identification of TAA recognized by CTL

A significant progress in understanding the mechanism of anti-tumor immunity has been achieved since the identification and molecular characterization of tumor-associated antigens (TAA)(12). Cancer cells are genetically unstable and can overexpress cellular proteins or express protein that are usually developmentally regulated and are ectopically expressed during malignant transformation or viral infection. These abnormally expressed TAA, when presented to cytotoxic T cells as peptides in conjunction with major histocompatibility complex (MHC) molecules on malignant cell surface, initiate a chain of events leading to tumor cells destruction and eradication, or prevention, of cancer. Today the classical concept of immune surveillance against tumors has evolved into a modern concept of tumor recognition and elimination, based on a significant amount of molecular data identifying the nature of TAA and the role of MHC class I molecules in T-cell-mediated immunity (13). Over the years, scientists have identified and characterized various families of TAAs recognized by CTL on human tumors (14,15), including:1) immunogenic proteins that are encoded by normal cellular genes, i.e. genes normally expressed only in male germ cells in testis (‘cancer/testis’genes), such as melanoma antigen-encoding (MAGE) gene family and NY-ESO-1/LAGE-1 genes; 2) antigens that are strictly tumor specific, the result of point mutations or gene rearrangements during the oncogenesis, e.g.cyclin-dependent kinase 4 (CDK4) and β-catenin in melanoma; 3) tissue-specific differentiation antigens, which are expressed during some stages of differentiation in normal cells of the cell lineage from which the tumor developed, e.g.proteins involved in pathways of melanin production, tyrosinase, gp100, Melan-A/MART-1,or oncoprotein HER-2/neu (c-Erb-2), which is a receptor tyrosine kinase homologous to the epidermal growth factor receptor and is overexpressed in many adenocarcinomas, including breast and ovarian cancers; 4) molecules that display abnormal posttranslational modifications, such as under glycosylated

mucin(MUC-1) which is expressed by a number of tumors, including pancreatic cancers; and 5) proteins encoded by viral oncogenes, such as human papilloma type 16 virus proteins, E6 and E7, expressed in cervical carcinoma.

Low efficacy of current cancer immunotherapy protocols: induction of T-cell immunity without tumor rejection

Cancer immunotherapy is designed to activate the immune system for mounting a rejection strength response against tumor cells by modification of the host–tumor relationship. Early attempts at immunotherapy, such as Coley'stoxins or BCG, were undertaken to induce a strong inflammatory response leading to tumor reduction, but without an understanding of the processes mediating the effects. More recent therapeutics using cytokines, including IL-2 and interferon (IFN), were focused on activation and development of immune response via the activation of T-cell-mediated anti-tumor reactivity (16). Subsequent attempts to enhance the immune response essentially focused on a vaccine basis, trying to induce a specific response against the tumor. More sophisticated tumor-specific types of immunotherapy were developed after the discovery of TAA and were aimed at boosting specificT-cell response and induce tumor regression. Tumor peptides alone,or with other immunostimulatory agents, are widely used in cancer treatment to enhance anti-tumor immune response. Current immunotherapeutic approaches vary with respect to target specificities, effector mechanisms, mode of administration, and prospects for translation into clinical practice. Currently, various types of immunotherapy are used in treatment of cancer, including cytokines adjuvants, active vaccination with TAA peptides, passive vaccination with specific lymphocytes against TAAs, adoptive transfer of natural or gene modified T cells, monoclonal antibodies, and DNA transfer methods (17). Some of the approaches explore the use of irradiation inactivated whole-cell vaccines derived from both autologous and allogeneic tumors and genetically modified versions of such cellular vaccines. DC vaccines seek to improve the presentation of TAAs to naïve T lymphocytes (18). Among new technologies used in pre-clinical and clinical studies are gene therapy approaches using various types of gene transfer methods and modulating the expression of genes essential for tumor rejection (19).

Numerous experimental animal models of cancer immunotherapy have yielded promising results, however, the results of clinical trials have not been as exciting as expected (20). Although initially advocated as being more specific for cancer and more promising compared to conventional therapies, such as chemotherapy, radiotherapy, and surgery, it is becoming increasingly clear that many immunotherapies do not induce dramatic tumor regression or increase disease-free survival. Tumor-specific response essentially depends on the presence of functionally active CTL responsible for tumor regression or elimination. Many current clinical trials involve regular monitoring of the presence and activity of tumor-infiltrating lymphocytes. These studies have shown increasing presence of tumor-infiltrating T lymphocytes and tumor-specific activated T cells in peripheral blood, although their presence and activa-tion status have not indicated exceptional tumor protection in a large percentage of the patients (21–25). Therefore, although general immune activation directed against the tumor antigens contained within the cancer vaccine has been documented in most cases, reduction in tumor load has not been frequently observed, and tumor progression and metastasis usually takes place, possibly following a slightly extended period of remission. Hence, after nearly 20 years of 'specific immunotherapy' there has been no major breakthrough in the treatment of cancer by these means, and there is no clear understanding of the missing key factors.

Tumor escape and altered HLA class I expression: structural and regulatory defects

The lack of concordance between immunological and clinical responses in cancer immunotherapy trials is thought to result from the presence of tumor-driven immune suppres-sion and loss of tumor immunogenicity that allows the tumor to escape, something that has not been adequately targeted by current therapies (26–28). These

various immune escape mechanisms include the generation of tumor cell variants that no longer express HLA class I complex and no longer present antigenic peptides to T cells. Through the natural selection process, the host leads to the selective enrichment of clones of highly aggressive neoplastically transformed cells. Specific activation of the immune system in such cases only leads to lysis of the cells expressing the particular TAAs in the context of the particular HLA subclass and the necessary costimulatory molecules. However, the most dangerous clones of tumor cells lack these features and thus a cancer vaccine is of little use. Currently much work in tumor immunology and oncology has focused on targeting 'tumor escape phase' to increase tumor immunogenicity and on developing new therapeutic modalities.

The revival of the cancer immunosurveillance theory, along with the disappointing clinical results obtained in T-cell-based immunotherapy trials conducted in patients with cancer, has reemphasized the pathogensis of immune escape mechanisms, and the clinical course of the disease. As a result, immunologists have been focusing their investigations on the identification and molecular characterization of the multiple mechanisms by which tumor cells evade immune recognition and destruction, including low immunogenicity of TAAs, downregulation of MHC molecules, the lack of adequate costimulatory molecule expression, and secretion of immuno inhibitory cytokines. However, there is always the possibility of defective antigen presentation which could result in tolerance induction to the antigens contained within the vaccine, and subsequent rapid tumor progression. Changes in HLA class I antigen expression by tumor cells should be at the center of attention of tumor immunologists and clinical oncologists. The frequency of tumor HLA loss and underlying molecular mechanisms have been well documented for different types of malignancy (29). The highly polymorphic HLA class I molecules serve an important integral role in adaptive immunity by presenting peptides to cytotoxic T cells. HLA class I complex consists of a membrane-bound heavy chain (gene located on chromosome 6), non-covalently linked β2-microglobulin (β2m; encoded in chromosome15) and a tumor-associated peptide. HLA class I downregulation helps tumor cells evade the classical T-cell-dependent immune responses but simultaneously imposes natural killer (NK) cell-mediated surveillance stimulated by the 'missing self' signals. However, the delicate balance between the expression of activating and inhibitory ligands regulating NK cell activity is sometimes not effective in eliminating HLA-negative cancer cells.

From experimental work, it is clear that the malignant behavior of a tumor cell may depend on the level of MHC class I expression and that the loss of MHC class I antigens in tumors may be a factor in escape from immune surveillance (30). T cells have the capability to destroy tumor cells, and at the same time T-cell activity might select for the outgrowth of tumor cells that are no longer targets of host immune effectors, due to regulatory and mutational events affecting genes involved in HLA expression and antigen processing and presentation. Many human tumors express low amounts of HLA class I molecules relative to the normal cells from which they are derived. Various types of alterations in the HLA class I phenotype can be detected frequently in tumor tissues and cell lines. Abnormal expression of HLA class I molecules in malignant cells is a frequent event that ranges from total loss of all class I molecules to partial loss of HLA-specific haplotype or allele. Different mechanisms underlie these alterations and might require different therapeutic approaches. A complete characterization of molecular defects may suggest strategies for the selection and follow-up of patients undergoing T-cell-based immunotherapy. Moreover, a precise identification of the mechanism leading to HLA class I defects in cancer patients will help to develop new personalized patient-tailored treatment protocols.

Two types of tumor HLA class I alterations are known: 1) caused by reversible or 'soft' regulatory defects leading to the coordinated downregulation of genes encoding, HLA class I complex, and components of the antigen processing and presenting machinery; and 2) structural or 'hard' irreversible alterations caused by mutational events and chromosomal abnormalities, affecting the HLA heavy chain and β2m genes (31–34).

The reversible 'soft' HLA class I deficiencies involve all levels of the MHC class I-restricted antigen presentation machinery and they can be

corrected *in vitro* by IFN-γ or other cytokines. This type of HLA class I defects are usually caused by abnormalities in the gene regulation of HLA class I heavy chain genes, β2m genes, and the components of the antigen-processing machinery (APM)(35). Such abnormal MHC class I phenotypes show low mRNA levels of specific genes (heavy chain, β2m, and APM) that seem to be coordinately downregulated. Epigenetic events associated with tumor development and with cancer progression have been found to underlie changes in HLA antigen and APM components. Unlike genetic alterations, epigenetic modifications can, in some cases be reversed *in vitro* with pharmacologic agents that induce DNA hypomethylation or inhibit histone deacetylation. Among the 'hard' lesions, the loss of heterozygosity (LOH) of chromosome 6p21 is an important mechanism that generates HLA haplotype loss in various human tumors with high incidence. Mutations in β2m gene and loss of another gene copy due to LOH in chromosome 15 are responsible for the irreversible total loss of HLA class I expression, and it has been described in various types of malignancy, both in cell lines and in tumor tissues (36–48) (Table1).

Table1. Incidence of β2m gene mutations and of the LOH in chromosome 15 of human tumors

Tumor type	β2m mutations	% LOH of chromosome 15
Colorectal carcinoma	+++ (37–39)	40% (34)
Laryngeal carcinoma	−	41% (34)
Renal cell carcinoma	+ (40)	9% (34)
Bladder carcinoma	+ (41)	51% (34)
Melanoma	+++ (31, 32, 42–44)	20% (34)
Prostate cancer	+ (unpublished observations)	25% (unpublished observations)
Breast cancer	−	50% (unpublished observations) 29% (45)
Lung carcinoma	+ (46, 47)	−
Cervical carcinoma	+ (48)	−

+,rare; +++, frequent; −, no data available on incidence; β2m, β2-microglobulin; LOH, loss of heterozygosity.

LOH in chromosome 15 may be unnoticed as tumor cells might have a 'normal' HLA class I pattern and could represent one of the early events in malignant cells leading to precommitted tumors becoming MHC escape variants (34). LOH in chromosome 15 in tumors is found more frequently than mutations in β2m gene. A mutation hot spot located in the CT repeat region of exon 1 of the β2m gene has been proposed, reflecting an increased genetic instability in this region in malignant cells (42,43).

Coexistence of distinct mutations in the two β2m genes is a rare event and has been reported in colorectal carcinoma and a sarcomatoid renal carcinoma (40). It is evident that any alteration in the expression of any of the HLA class I molecules can affect both T and NK cell-mediated immunity, with a negative effect on the tumorigenic phenotype, metastatic capacity, and resistance to immunotherapy in various types of cancer.

Selection of HLA class I-deficient cancer cells during immunotherapy

Frequently, active immunization can induce enhancement of anticancer T cells, but most often

they are unable to induce tumor regression. This phenomenon remains poorly understood and data derived from *in vivo* human observations are rare. Cases with tumor recurrence or repeated metastatic progression after immunotherapy may provide a better understanding of the possible ways to improve the efficacy of immunotherapy. Difficulties in obtaining progressing and regressing lesions from the same patient during immunotherapy have always been a major short coming in the analysis of the metastatic growth. However, we have been able to obtain and analyze HLA class I expression in such metastatic lesions.

Our recent studies have shown a strong correlation between HLA class I-altered expression and tumor progression. Moreover, we have observed that progressing metastases or recurrent tumors after immunotherapy have an increased percentage of HLA class I alterations. We discovered that, in a melanoma patient undergoing immunotherapy with autolgous tumor vaccine, several existing subcutaneous metastases responded in different ways to the therapy. Some of the lesions progressed and some showed considerable regression. Importantly, the progressors showed low HLA class I expression and higher frequency of LOH in chromosomes 6 and 15 in addition to a locus B downregulation (8).

In another melanoma patient undergoing immunotherapy, five lesions were obtained after INF-α-2b treatment and five after autologous vaccination plus BCG (M-VAX). Eight metastases were regressing after immunotherapy while two were progressing. The eight regressing metastases showed high level of HLA class I expression, whereas the two progressing lesions had low levels, as measured by real-time polymerase chain reaction and immunohistological techniques (9). These results indicate a strong association between HLA class I expression and progression or regression of the metastatic lesions. In this study, a comparative gene array analysis of the progressing and regressing lesions experiments showed that the significantly upregulated genes in regressing metastases are associated with HLA expression and IFN-γ signaling pathways. Similarly, in patients with bladder carcinoma, recurrent tumors that appeared after BCG therapy had a higher percentage of LOH in chromosome 15 and increased incidence of HLA class I-altered expression (10).

The possible chain of events leading to resistance to therapy and metastatic progression in the studied cases is illustrated in Figure 1. Primary tumor consists of a heterogeneous tumor cell population with various levels of HLA class I expression. During natural tumor development, cells with high class I expression (HLA+++) are eliminated by CTLs. Immunotherapy upregulates HLA expression on HLA+/− tumor cells with reversible alterations, butHLA-negative cells with underlying structural defects will not respond to therapy and will outgrow, producing progressing lesions. Presumably, immunotherapy changes th tumor microenvironment and creates an additional immune selection pressure on tumor cells. Therapy-induced immunoselection will promote the growth of tumor cells with pre-existing irreversible HLA class I lesions, as well as the proliferation of cells with new additional HLA defects as part of the oncoprogression.

Our data also suggest that the level of HLA class I expression is an important parameter of tumor immune escape that needs to be monitored in cancer patients. In addition, under-standing the molecular mechanisms causing these alterations may help to select patients for alternative treatment in order to enhance the efficacy of clinical response to immunotherapy. If the defect is reversible or ‘soft’, HLA downregulation can be restored by cytokine treatment and patients may therefore benefit from T-cell-based immunotherapy. However, tumors with irreversible HLA class I loss due to structural defects, such as mutations or LOH in chromosome 6 or 15, have a low possibility of benefiting from this therapeutic approach. They should be considered for gene therapy to restore normal expression of damaged genes. These observations only further underscore the multivariate and dynamic nature of the immune system and the many ways in which tumor cells modulate themselves and their surroundings to escape immunesurveillance. Therefore, in order to treat metastatic tumors, new directions must be taken to improve current immunotherapeutic strategies.

Therapeutic manipulation of HLA class I expression in tumor cells

Successful immunotherapeutic and vaccination protocols should be optimized against tumors with distinct cell surface expression of the HLA class I molecules.

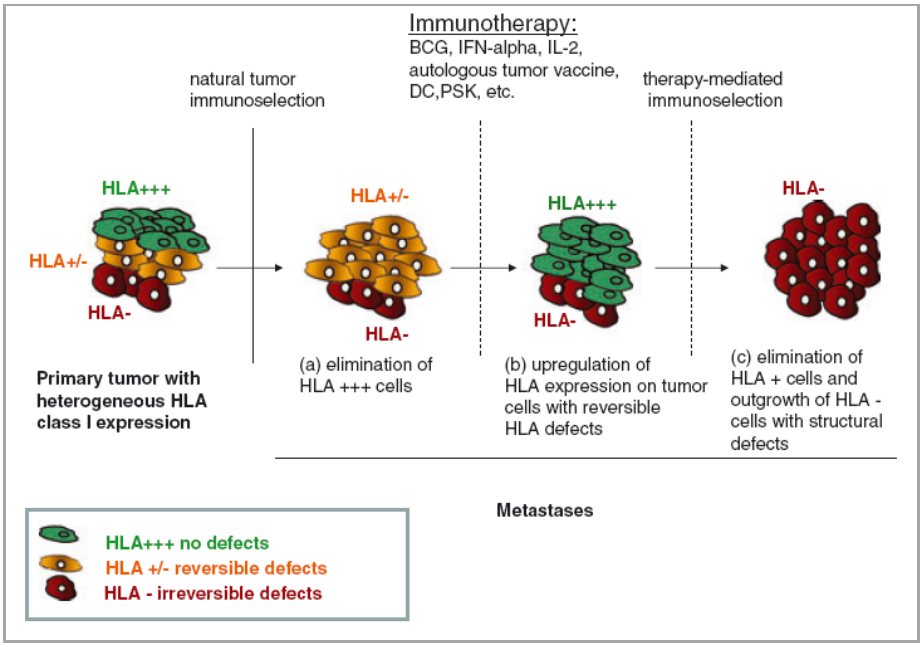

Figure 1. Schematic illustration of cancer natural progression and immunotherapy-mediated immunoselection of human leukocyte antigen (HLA) class I-negative tumor cells with irreversible 'hard' alterations. During natural cancer progression, tumor cells with high HLA class I expression (HLA+++) are eliminated by the immune system, while HLA negative (HLA−) cells and cells with low class I expression (HLA+/−) proliferate. Any type of immunotherapy normally changes the local tumor microenvironment, leading to increased production of pro-inflammatory cytokines/chemokines, to upregulation of tumor HLA class I surface expression, and to attraction of activated cytotoxic T cells. Hence, the fate of cancer growth in response to therapy will depend on the molecular mechanism underlying tumor HLA class I defects; namely, if the defect is reversible or 'soft', then immunotherapy will upregulate HLA class I expression followed by recognition and elimination of these cells by the activated tumor-infiltrating T cells. In contrast, if the defects are structural or 'hard', immunotherapy will not change the tumor HLA class I profile and these cells will remain unrecognized by T cells escaping from immune recognition and progressing into dangerous metastases resistant to treatment.

Attempts are in progress to revert the defects in tumor MHC class I surface expression by introducing the different elements of the antigen presentation pathway. Such studies will not only provide improved understanding of the mechanisms of tumor escape, but will also suggest strategies to repair cellular defects in cancer patients having impaired expression of MHC class I antigens.

Recovery of normal HLA class I expression in patients with local cancer relapses or metastatic progression with β2m mutations, LOH in chromosomes 6 and 15, or other structural genetic HLA defects is essential. To achieve this goal, it is necessary to develop methods to restore normal MHC class I expression in tumor cells with structural MHC class I defects. This can only be

achieved if a wild-type MHC class I heavy chain or β2m genes are delivered to the tumor lesion.

Recent developments in tumor immunology and biotechnology have made cancer gene therapy a promising approach for therapeutic intervention of cancer. Various strategies have been employed, including vaccination with tumor cells engineered to express immunostimulatory molecules, vaccination with recombinant viral vectors encoding tumor antigens, vaccination with dendritic cells expressing tumor antigens or tumor-derived RNA (18), naked DNA vaccines, and intratumoral injection of vectors encoding cytokines or major histocompatibility molecules. Central to all these therapies is the development of efficient vectors for gene therapy. Viral vectors are efficient natural gene delivery systems and are one of the obvious choices for cancer gene therapy. By far, adenovirus-mediated gene therapy is one of the most promising approaches, as confirmed by studies relating to animal tumor models and clinical trials. Immunostimulating gene therapy with adenoviral vectors expressing CD40ligand (AdCD40L) efficiently eradicates experimental bladder cancer and this vector has also been successfully used for a local transfer of the human CD40L gene into the bladder of patients with urinary bladder carcinoma (49).

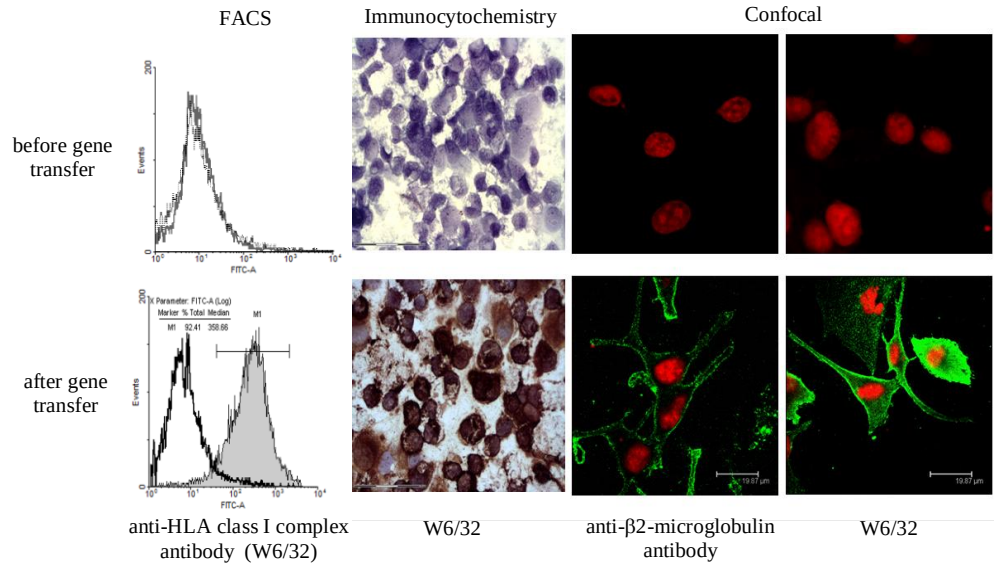

Figure 2. Recovery of human leukocyte antigen (HLA) class I expression by HLA-negative melanoma cells harboring a β2-microglobulin (β2m) mutation and LOH of chromosome 15 after adenoviral-vector (AdCMVβ2m) mediated β2m gene transfer. Upregulation of HLA class I expression is confirmed by flow cytometry (FACS) and immunocytochemistry using the monocolonal antibody (mab) W6/32 that recognizes the cell surface HLA class I W6/32 complex, and by confocal microscopy using an anti-β2m mab and W6/32.

In vitro manipulation with β2m gene and with other genes involved in MHC class I complex expression has generated evidence that restoration of normal MHC class I reexpression is important for the tumor cell recognition and elimination by CD8+ T cells. Recovery of MHC class I expression has been employed previously by various investigators to show the importance of class I molecules in specific tumor lysis by CTL and NK cells (50). In various experimental systems, introduction of the MHC class I molecules into class I-negative tumor cells led to higher immunogenicity and decreased tumorigenicity of these cells (51,52). In spite of highly encouraging mouse experiments in the mid 1980s, which clearly showed that restoration of MHC class I molecules can induce tumor rejection and inhibition of metastasis, no similar application has been developed in clinical cancer therapy. In

our laboratory, we made a replication-deficient adenoviral vector with human β2m gene to recover HLA class I expression in various HLA class I-negative cancer cell lines with a double knock out of the two β2m gene copies (53). Figure 2 shows restoration of HLA class I expression on melanoma cells after infection with the AdCMVβ2m vector measured by flow cytometry, and immunocytochemistry using antibodies against HLA class I complex and anti-β2m antibodies. From the confocal images it is evident that β2m protein is properly expressed on the tumor cells surface confirming its proper transport to the cell surface and binding to HLA heavy chain. The functional ability of the newly expressed HLA class I complex to present peptides to T cells in HLA-restricted manner was confirmed in ELISPOT and CTL assays. In addition, *in vivo* experiments using human tumor xenograft model, theintratumoral injection of β2m-carrying vector led to the restoration of normal HLA class I expression. We showed that our replication-deficient adenoviral vector carrying human β2m gene is effective in restoration of HLA class I expression in various types of β2m-negative human tumor cells. This is a step closer to the possibility of the clinical application of such vectors in patients not responding to immunotherapy with tumors harboring structural 'hard' defects responsible for HLA class I loss. We believe that a combination of chemotherapy, immunotherapy, and gene therapy aimed at recovering normal HLA class I expression can provide a successful approach to improve the clinical efficacy of cancer treatment. Such a combined therapy may have some advantages in combating well-established tumors and metastatic cancer.

Conclusions

Advances in our understanding of the relationship between the immune system and tumor cells has given rise to new prospects for immunological treatment and the prevention of cancer. Clinical trials report so far only limited efficacy of cancer immunotherapy. As T-cell recognition of malignant cells depends on tumor antigen presentation, in the absence of a suitable HLA-peptide target on the cell surface, peptide-based immunotherapy is unlikely to improve the anti-tumor CTL response. We favor the idea that the primary tumor is composed of tumor cells with a large diversity in HLA class I expression which therefore present tumor antigens with a different efficiency. Analysis of the correlation o impaired HLA expression with metastatic progression is important for the improvement of existing protocols of cancer immunotherapy. The two types of HLA defects classified as 'soft' (reversible/ regulatory) and 'hard'(irreversible /structural) may have profound implications on T-cell-mediated rejection of tumor cells in primary or metastatic lesions and ultimately on the outcome of the cancerimmunotherapy. Importantly, immunotherapy induced local cytokine production will increase tumor HLA class I levels in 'soft' lesions leading to their regression, while resistance to cytokines in tumor cells with 'hard' defects will allow the progression of metastases. Therefore irreversible los of tumor HLA class I expression represents a serious problem for immunotherapy, because it may lead to resistance to immunomodulatory therapy and generation of dangerous HLA class I-negative tumor escape variants. Recovery of tumor HLA expression using gene therapy methodology is a promising future perspective that could lead to the enhancement of tumor immunogenicity and hence the efficacy of immunotherapeutic modalities.

Acknowledgments

This work was supported by grants from the Fondo de Investigaciones Sanitarias (FIS), Red Genomica del Cancer (RETICRD06/0020), Plan Andaluz de Investigacion (GroupCTS143), Consejeria Andaluza de Salud (SAS,PI09/0382), and Proyecto de Excelencia de Consejeria de Innovacion(CTS03952 and CVI4740).

Conflict of Interests

The authors have declared no conflicting interests.

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5. Chapter 5. Discussion

Cancer escape T-cell-mediated destruction by losing HLA class I expression via various mechanisms, including loss of beta-2-microglubulin (β2m) expression. Better understanding of the mechanisms of HLA class I loss in tumor immune escape and of the resistance to immunotherapy is critical for the improvement of current cancer treatment protocols.

In the present work we demonstrated a chronological sequence of appearance of tumor β2m gene mutation in successive metastatic lesions obtained from a patient with melanoma. We observed a gradual decrease in HLA expression in consecutive lesions with few HLA-negative nodules in the primary tumor and the emergence of a totally negative lesion at later stages of the disease. We detected loss of β2m in β2m-negative nests of the primary tumor caused by a combination of two alterations: 1) a mutation (G to T substitution) in codon 67 in exon 2 of β2m gene, producing a premature stop codon; 2) loss of the second gene copy by loss of heterozygosity (LOH) in chromosome 15. The same β2m mutation was found in a homogenously β2m-negative metastasis many months later and in a cell line established from a biopsy of a post-vaccination lymph node. We also observed an accumulation of chromosomal loss at specific short tandem repeats (STRs) in chromosomes 6 and 15 in successive metastases during disease progression.

HLA loss correlated with decreased tumor $CD8^+$ T-cell infiltration. This work provides a novel insight into the evolution of HLA class I alterations in cancer cells. We believe that early incidence of β2m defects can cause an immune selection and expansion of HLA-negative highly aggressive melanoma clones resistant to immunotherapy. β2m/HLA class I expression should be taken into consideration as a therapeutic target in the development of cancer treatment strategies, including novel gene therapy strategies for the restoration of tumor HLA class I expression. Therapies directed at the re-expression of HLA class I antigens might improve outcomes in immune-therapy-based treatments.

We have constructed a replication-deficient adenoviral vector coding for β2m gene and demonstrated its efficacy in restoration HLA class I expression on different types of cancer cells *in vitro* and in xenogenic tumors *in vivo* (del Campo et al, 2009; del Campo et al, 2014 submitted). We also demonstrated that the newly expressed β2m protein forms a functional complex with HLA chain on the surface of tumor cells without compromising the antigen presentation machinery and it leads to tumor cell recognition by peptide-specific HLA-restricted cytotoxic T lymphocytes (del Campo et al, 2014a; del Campo et al, 2014 submitted).

Gene transfer aimed at recovery normal HLA class I expression could be beneficial for patients with metastatic cancer harbouring structural defects in β2m gene, where local administration of the vector into such metastatic lesions or into relapsed tumours might help to boost tumour cell immunogenicity and recognition by T-cells, preventing malignant progression and improving the immune response.

1. Loss of tumor HLA class I expression caused by structural defects in the β2m gene.

There is little information on the different patterns of HLA class I alteration in metastases and its correlation with cancer progression (Cabrera et al, 2007; Carretero et al, 2008). This is partially due to the difficulties in obtaining samples from sequential metastatic lesions from the same patient during immunotherapy, especially if the lesions are regressing. HLA alterations caused by β2m defects have been previously reported in tumor tissues and/or cancer cell lines obtained from different patients at a particular time point during cancer development (Baba *et al*, 2007; Bicknell *et al*, 1994; Cabrera *et al*, 2003a; Feenstra *et al*, 1999; Fernandez *et al*, 2000; Paschen *et al*, 2006; Perez *et al*, 1999; Wang *et al*, 1993). Most of these studies did not follow the evolution of β2m genetic alterations during metastatic progression (Real et al, 1998). There are only few reports describing a sequential immune escape mechanisms associated with HLA class I loss, when both tumor samples and tumor-derived cell lines obtained from the same patient have been analyzed. In addition, most of these publications describe genetic defects underlying HLA-I loss only in cell lines derived from metastases. In one study,

in addition to the distinct mutations detected in the β2m gene in three melanoma cell lines derived from metastases obtained from different patients, the authors discovered that a cell line from a primary tumor had the same mutation that the one found in a cell line derived from a metastatic lesion. However, only cell lines were available for analysis and the status of second copy of the β2m gene was not investigated (Hicklin et al, 1998).

Other reports examine both β2m alleles, but only in cell lines derived from a primary tumor (Hsieh *et al*, 2009). Some studies describe a sequential immune escape mechanisms analyzing both tumor samples and tumor-derived cell lines, but with the detailed analysis of genetic defects causing the lack of HLA class I done only in cell lines (Chang *et al*, 2005b)(Mendez *et al*, 2007; Mendez *et al*, 2001; Paschen *et al*, 2003).

Few studies examine the expression of both β2m and HLA class I complex, including alterations in the β2m gene, in the same patient during the course of cancer progression. Two successive melanoma lesions removed from a patient at two time points, and the cell lines derived from each of them. They detected a β2m mutation and a LOH-15 causing in the second metastatic melanoma and in the corresponding cell line (Chang *et al*, 2006; Yamshchikov *et al*, 2005). Our group, years ago, analyzed the lack of HLA class I expression in metastases and in a cell line derived from a melanoma patient treated with peptide immunotherapy (Benitez *et al*, 1998) and β2m mutation and the LOH-15 were detected in both these samples, while a late metastases also negative for HLA class I was not examined for LOH.

In the present study, we were able to track the fate of a β2m gene mutation and LOH in chromosome 15 in several successive metastatic melanoma lesions and in a cell line derived from a lymph node biopsy obtained after immunotherapy with dendritic cells (DC) transfected with autologous tumor-mRNA (del Campo *et al*, 2014a). We investigated HLA expression in different tumor samples obtained from a 72-year-old patient with metastatic melanoma and an aggressive clinical course. This patient has been enrolled at the Norwegian Radium Hospital in a clinical trial using autologous tumor mRNA transduced DC-vaccine. This patient did not show any immune response

to the vaccine and developed a progressive disease leading to his death two months after the vaccination (Kyte et al, 2006). We have had a chance to analyze 5 successive melanoma lesions (three of them in several tissue blocks), and one cell line, DNR-DC-M010, established from a postvaccination lymph node fine needle biopsy. We have described and characterized the molecular mechanism causing the loss HLA class I expression in DNR-DC-M010 cell line, and tracked the origin of this loss in the successive malignant lesions.

Immunohistochemical and mutational analyses revealed an early onset of tumor β2m loss in heterogeneous primary tumor followed by immune selection and outgrowth of β2m-negative melanoma cells. We detected a novel β2m gene mutation (G to T substitution in the codon 67 in exon 2 that generates a stop codon) and LOH in chromosome 15 causing loss of β2m expression in several tumor nests microdissected from an early lesion with heterogeneous HLA expression pattern. The same β2m mutation was found in a late metastatic lesion developed almost a year later (source of vaccine preparation), which was already homogeneously β2m-negative (Figure 12), and in HLA-class I-negative melanoma cell line established three months later from a fine needle biopsy of a post-vaccination lymph node.

This observation suggests that β2m loss could be an early event in the tumor progression leading to the immune escape of HLA class I-negative tumor cells.

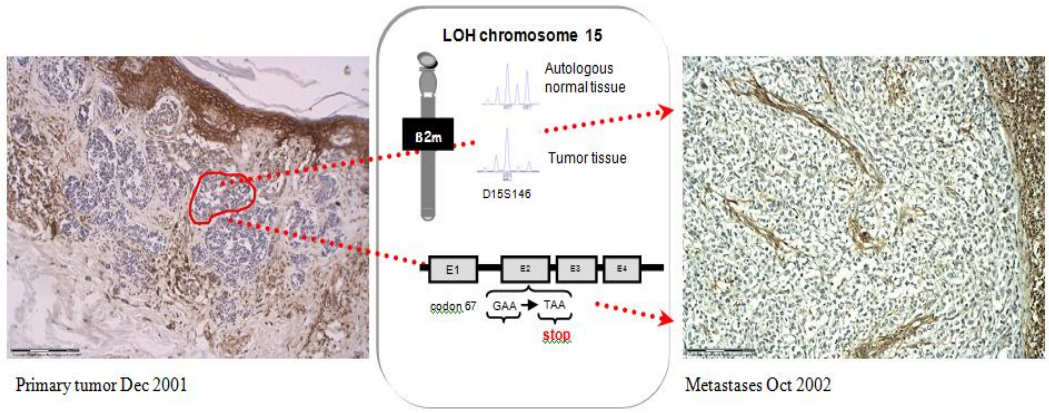

Figure 12. Alterations found in primary tumor and last metastases obtained from DNR-DC-M010 melanoma patient. Microdissection of β2m negative nests in primary tumor allowed to

identify a mutation at codon 67 that generated a truncated β2m protein, and a LOH at chromosome 15. Both β2m defects were present in the last metastases from the patient.

To our knowledge, the β2m mutation discovered in this study differs from the ones described in melanoma, colon cancer, or lymphoma cells previously (Perez et al, 1999; Chan, 2005). It results in a production of a nonfunctional truncated version of β2m protein not detectable intracellularly or on the cell surface with any of the available anti-β2m antibodies. This protein lacks one of the cysteins, Cys-80, located after the premature stop codon. Since the disulfide bridge between Cys-25 and Cys-80 is essential for maintaining the proper β2m structure (Bjorkman et al, 1987), the short β2m cannot properly fold. In addition, the absence of the two amino acids, Asp-98 and Met-99, which are fundamental for the interaction of β2m protein with HLA heavy chain (three contacts established through Asp-98 and 2 contacts through Met-99) (Tysoe-Calnon *et al*, 1991), disrupts the binding of the truncated β2m to the HLA heavy chain. Figure 13 shows the location of these amino acids in the β2m structure and how their loss could negatively affect the interaction of the truncated β2m with HLA-A2 heavy chain.

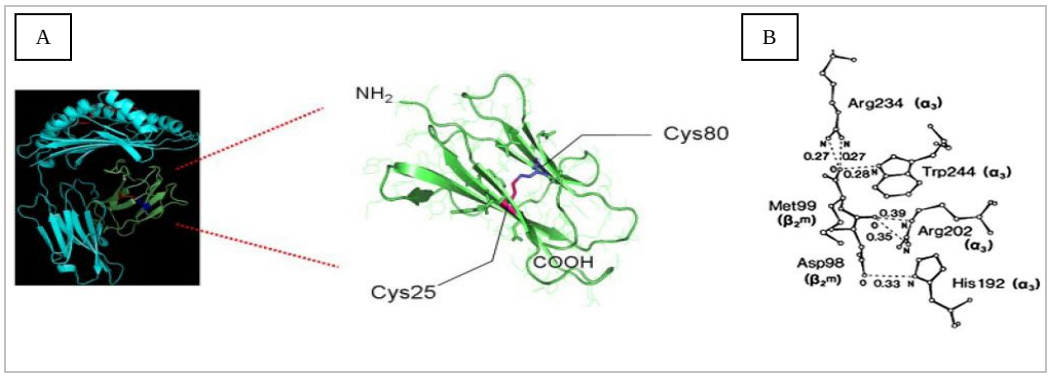

Figure 13. Location of the β2m mutation generating a premature stop codon detected in DNR-DC-M010 melanoma cell line. A) Three-dimensional structure of the HLA-A2-β2m-HIVpeptide complex with highlighted β2m disulfide bond between Cys-25 and Cys-80, which is altered in the studied cell line. B) Schematic representation of the biochemical interaction between β2m and HLA-A2 heavy chain, which is disrupted due to the β2m mutation: Asp-98

and Met-99 (β2m) with His-192, Arg-202, Arg-234 and Trp-244 (α3 domain from A2.1 heavy chain). Modified from (Chang *et al*, 2006; Tysoe-Calnon *et al*, 1991).

The detected β2m defect is caused by a substitution and represents a nonsense mutation, a frequent type of alteration in β2m gene. It usually originates from nucleotide substitutions or deletions of one or several base pairs, that disrupts translation of β2m (Bernal et al, 2012). Nonsense mutations generating a premature stop codon and a truncated β2m protein have been previously reported in different types of cancer: renal cell carcinoma (Hsieh *et al*, 2009), colorectal carcinoma cell line DLD-1 (Bicknell *et al*, 1994) where a C→A transversion generates a stop codon at exon 2, in B-cell lymphomas (Challa-Malladi *et al*, 2011), lung cancer (Baba *et al*, 2007), and in or melanoma cell lines (Hicklin *et al*, 1998; Wang *et al*, 1993).

Many of the reported β2m mutations inhibit its translation without affecting its transcription (Seliger et al, 2002), while other mutations are silent, such as transitions described in melanoma cell line SKMEL-33 (Wang *et al*, 1993), or in some B-lymphoma cell lines (Challa-Malladi *et al*, 2011) rendering normal expression of β2m proteins. However, there is only one described example of β2m structural abnormality (a Cys to Trp substitution at position 25, one of the disulfide bridge-forming Cys) that causes HLA class I downregulation, but not a total loss (Chang et al, 2006).

The majority of β2m mutations are nucleotide microdeletions or substitutions in which their relevance on HLA class I cell surface expression depend on the type of amino acid exchanged. Deletions in the CT repeat region in exon 1 proposed as a mutational "hot spot" in the β2m gene have been found in melanoma (Chang *et al*, 2005b; Hicklin *et al*, 1998; Perez *et al*, 1999) and colorectal carcinoma (Bicknell *et al*, 1994; Cabrera *et al*, 2003a; Gattoni-Celli *et al*, 1992; Kloor *et al*, 2007). In colon cancer and B-lymphoma (Bernal *et al*, 2012; Challa-Malladi *et al*, 2011; Jordanova *et al*, 2003; Pasqualucci *et al*, 2011) deletions and insertions have been detected throughout exon 1 and exon 2, whereas in melanoma only deletions have been described in the mutation "hot spot" of exon 1 (CT repeat sequence), and exon 2; insertions have been described in the intron I as a result of a mutation in the donor splicing site (Paschen *et al*, 2006).

Microdeletions have been described in melanoma cell lines (del Campo *et al*, 2009), in one of which a mutations changes the splicing site and causes a frameshift resulting in a 11bp and 14bp deletions, respectively (Hicklin *et al*, 1998). In colorectal carcinoma cell line DLD-1 (Bicknell *et al*, 1994) used in our study (del Campo *et al*, 2009; del Campo *et al*, 2014 submitted), a missense G→T transversion affects the first intron splicing sites causing a deletion of exon 2 and resulting in a truncated protein. In our study we used two cell lines, Burkitt lymphoma cell line Daudi (Rosa *et al*, 1983) and melanoma cell line ESTDAB-038 [or LB1622-MEL (Benitez *et al*, 1998)], in which nucleotide substitutions at the start codon prevent the translation of β2m protein.

Few studies describe large deletions within the β2m gene abolishing the transcription. Such deletions have been reported in melanoma cell line FO-1 (D'Urso *et al*, 1991), and in two melanoma cells lines used in our study (del Campo *et al*, 2009; del Campo *et al*, 2014a), including ESTDAB-109 [or UKRV-Mel-2b (Paschen *et al*, 2003), where 498 bp including exon l are deleted], and Ma-Mel-86b (Schwinn *et al*, 2009). Another large 500bp deletion was found in a prostate cell line OPCN-3 (unpublished data).

2. LOH on chromosome 15, an early event in cancer progression generating tumor cells precomitted to escape

Discovery of the same β2m mutation in the primary lesion, in late metastasis and the cell line derived from another late metastatic lesion, supports the hypothesis that the lack of β2m in melanoma may be an early event in the progression to the malignant phenotype providing an immune escape for HLA class I deficient cells (Hicklin *et al*, 1998; Paschen *et al*, 2003). However, alterations in one copy of the β2m gene are not sufficient for the observed total loss of β2m expression. LOH analysis of the studied malignant lesions and cell line using microsatellite markers revealed LOH in chromosomes 6 and 15 practically in all samples, including the cell line, with a gradual accumulation of chromosomal loss at specific STRs in successive metastases.

Although it is apparent that the total loss of β2m expression is the result of alterations affecting both copies of the gene (Paschen *et al*, 2003), it is not well established which of them, mutations or LOH-15, is the initiating event of the β2m loss during cancer progression (Chang et al, 2005a). In our study LOH-15 was detected in four out of five studied cancer samples coinciding with the mutation in β2m gene. The second lesion (possibly another primary tumor according to the pathologist) was still positive for HLA class I expression, but already had lost one copy of β2m gene due to LOH-15 (del Campo *et al*, 2014a). Similar results have been previously reported in melanoma, in head and neck squamous cell carcinoma, and other types of cancer (Carretero *et al*, 2008; Maleno *et al*, 2011; Feenstra et al, 1999).

While the early appearance of LOH at chromosome 6 during metastatic progression has been previously reported in different types of malignancy (Mendez *et al*, 2001; Maleno *et al*, 2011; Maleno *et al*, 2004b; Maleno *et al*, 2006), less information is available regarding LOH-15. Some reports suggest that LOH-15 is a late event in cancer progression (Hoglund *et al*, 2004; Koene *et al*, 2004), however, our group previously reported that the percentage of LOH-15 is elevated in different types of malignancy (Maleno *et al*, 2011) and it can be detected even in HLA-positive primary tumors (Benitez *et al*, 1998; Carretero et al, 2011; Maleno *et al*, 2011; Paschen *et al*, 2006). However, in some studies, the techniques used for the analysis of tumor samples did not allow a separation of the tumor tissue from stroma, therefore, authors could make only an assumption and the nature of cells with β2m mutation and /or LOH-15 could not be completely confirmed. The tumor microdisection methodology used in the present study (del Campo *et al*, 2014a) allowed us to perform β2m sequencing and microsatellite analysis in β2m-negative tumor nests isolated from HLA-positive stroma avoiding this issue (Ramal *et al*, 2000).

Hence, LOH-15 can represent one of the early HLA class I alterations in malignant cells leading to generation of new tumour escape variants. Damage of a single β2m allele by LOH-15 may be sufficient to generate a tumor cell pre-committed to escape.

Consecutive mutagenesis would generate a mutation in the second β2m copy leading to a total HLA class I loss, like we observed in our melanoma case.

Tumor type	% Phenotype I, Total loss of HLA class I	β2m mutations	% LOH chromosome 15
Colorectal carcinoma	18% (1)	+++ (Bicknell *et al*, 1994) (Cabrera *et al*, 2003a) (Kloor *et al*, 2007)	35% (7), 25%(8)
Laryngeal carcinoma	11% (2)	−	41% (7)
Renal cell carcinoma	−	+ (Hsieh *et al*, 2009)	7% (7)
Bladder carcinoma	25% (3)	+ (Nouri *et al*, 1992)	44% (7),39%(9)
Bladder cancer cell lines	0%(4)	−	17% (4)
Prostate cancer	50% (4)	+ (4)	27% (4)
Breast cancer	52%	−	50% (4) 29% (10)
Lung carcinoma	−	+ (Chen *et al*, 1996b) (Baba *et al*, 2007)	−
Cervical carcinoma	19% (Mehta *et al*, 2008)	+ (Koopman *et al*, 2000)	−
B-cell lymphoma	75%(Challa-Malladi *et al*, 2011)	+++ (Pasqualucci *et al*, 2011) (Challa-Malladi *et al*, 2011)	25% (Challa-Malladi *et al*, 2011)
Melanoma	18% (5)	+++ (Garrido *et al*, 2010b) (Paschen *et al*, 2006) (Perez *et al*, 1999) (Chang *et al*, 2005b) (Benitez *et al*, 1998)	16% (7)
Melanoma cell lines	11% (6)		29%

Table 3. Incidence of β2m gene mutations and LOH in chromosome 15 in human tumors. +, rare; +++, frequent; −, no data available on incidence. Modified from (del Campo *et al*, 2012).

1.(Garrido *et al*, 1997)
2.(Maleno *et al*, 2002)
3.(Romero *et al*, 2005)
4.Unpublished data
5.(Kageshita *et al*, 2005)
6.(Mendez *et al*, 2008)
7.(Maleno et al, 2011)
8.(Park *et al*, 2000)
9.(Natrajan *et al*, 2003)
10.(McEvoy *et al*, 2002)

Table 3 summarized the percentages of total HLA class I loss described in human tumors and cell lines, the percentages of LOH in chromosome 15 and the β2m mutations reported in these types of malignancies. It shows that LOH-15 in tumours occurs more frequently than mutations in β2m gene, and can be found even in some cells with positive expression of HLA class I as previously mentioned with the risk of being unnoticed. It has also been reported, that expression of some HLA alleles requires higher levels of β2m and, therefore, could be more sensitive to the loss of one copy of the β2m gene (Bicknell et al, 1994). Nevertheless, no correlation was found between LOH-15 and the intensity of HLA class I expression as analyzed by immunohistochemistry (Feenstra *et al*, 1999; Maleno *et al*, 2011; Maleno *et al*, 2002; Yeung *et al*, 2013).

3. Implications of HLA class I loss in cancer immune escape and resistance to immunotherapy

The mutation found in the primary tumor heterogeneous for β2m expression was also detected in a late-stage lesion homogeneously negative for β2m. This strongly suggests that these cells are escape variants from the immune attack during natural metastatic progression. On the other hand, the HLA-negative cell line DNR-DC-M010 established from a post-vaccination lesion, which harbors the same β2m mutation and LOH-15, have been exposed to an additional immunotherapy-induced T-cell-selective pressure, which might explain the course of tumor HLA expression and the failure of the therapy. Immunotherapy usually leads to upregulation of HLA expression unless the tumor cells harbor structural genetic defects (del Campo *et al*, 2012), e.g. β2m mutation like in our case. The immunomodulatory treatment used for this patient was based on DC vaccine loaded with autologous tumor mRNA that is expected to stimulate antitumor T-cell responses against a broad repertoire of antigens presented by DC, helping to override the loss of antigen presentation ability of tumor cells associated with HLA loss. HLA class I expression on the patients' DC was normal, therefore, the β2m mutation harbored by the metastasis, which was the source of tumor mRNA, would not

have compromised the antigen presentation ability of the DC. We cannot make a definitive conclusion that the resistance to immunotherapy with DC vaccination in this patient was primarily due to tumor β2m loss. We also cannot exclude the possibility that β2m-positive and negative cells coexist until the end and that the vaccination was ineffective for other reasons. However, our group has reported that in patients undergoing immunotherapy, poor clinical response to immunotherapy and generation of progressing metastases appears to be associated with immune selection of HLA-negative tumor cells variants with irreversible defects (Cabrera et al, 2007; Carretero et al, 2008; Carretero et al, 2012) including LOH-15 in HLA class I deficient metastatic melanoma lesions.

Based on our results, the loss of HLA class I expression in the studied melanoma lesions would impede presentation of tumor associated peptides to T-cells, and, therefore, tumor may grow and disseminate as a consequence of the early tumor β2m loss. This escape from T-cells is reflected by the analysis of TILs in melanoma tissue samples, which showed a high degree of infiltration with $CD3^+$ and $CD8^+$ T-cells in HLA-positive tumors and low lymphocyte infiltration in HLA-negative nodules. This, may be caused by the inefficient recruitment of T-cell to HLA-I defective tumor cells. T-cell infiltration has been identified as an important prognostic factor in primary melanoma (Azimi *et al*, 2012; Clemente *et al*, 1996; Taylor *et al*, 2007) and other primary solid tumors (Mahmoud et al, 2011; Piersma et al, 2007). In addition, our group previously described a dramatic difference in the number of infiltrating cells between progressing metastatic lesions with low number of infiltrating cells, and regressing lesions high number of infiltrating T-cells (Carretero *et al*, 2012). Increased presence of TILs in HLA class I positive metastases was reported by other groups (Ryschich *et al*, 2005; Yeung *et al*, 2013). In a study of the association between tumor infiltration and patient survival in melanoma, it has been demonstrated that diffuse intratumoral T-cell infiltration has a strong correlation with better survival. Meanwhile, intermediate survival has been associated with predominantly perivascular, but not intratumoral infiltration of melanoma, possibly because of the lack of stromal molecules that provide homing of T-cells within the tumor (Erdag *et al*, 2012). Moreover, immunosuppressive

tumor environment could also explain the lack of intratumoral infiltration of HLA class I-negative tumors in our study.

The reason why antitumor T cells in cancer patients are not able to mediate the regression of established tumors is poorly understood. Active immunization can induce T cell activation and tumor infiltration, but these lymphocytes most often are unable to induce tumor regression in a large percentage of patients (Anichini *et al*, 2004; Boon & van der Bruggen, 1996; Godelaine *et al*, 2003; Harlin *et al*, 2006; Kruit *et al*, 2005; Rosenberg *et al*, 2005; Wang *et al*, 2001). However, some clinical trials showed that adoptive T cell transfer can induce CTL-mediated elimination of metastatic lesions in melanoma patients (Dudley *et al*, 2008).

Loss of tumor HLA class I expression is anticipated to make malignant cells susceptible to NK cell attack (Ljunggren & Karre, 1990). However, in the present study we found that tumor infiltration with $CD56^+$ NK cells was very low. At the same time, DNR-DC-M010 cells were positive for the expression of activating NK ligands MICA/B and ULBP-1/2/3. Thus, NK cells could have eliminated these melanoma cells but failed to migrate into the tumor, possibly attributable to suppressive factors in the tumor microenvironment (Chang *et al*, 2005b; Fruci et al, 2013; Schwinn *et al*, 2009; Stojanovic *et al*, 2013; Umansky & Sevko, 2012) or due to the lack of cell trafficking signals (Stojanovic *et al*, 2013). In addition, interaction between activating and inhibiting signals in NK cells frequently leads to the failure of natural cytotoxic reactions against malignant cells. This is supported by a number of reports indicating low NK cell activity in solid tumors (Baba *et al*, 2007; Carrega *et al*, 2008; Le Maux Chansac *et al*, 2005; Restifo *et al*, 1996).

According to the published data, the appearance of tumor lesions with different β2m alterations after immunotherapy suggests their contribution to the poor clinical response and to disease recurrence. Among them are publications that describe two melanoma cell lines used in the present study (del Campo *et al*, 2009; del Campo *et al*, 2014 submitted): ESTDAB-109 (Paschen *et al*, 2003), and ESTDAB-038 (Benitez et al, 1998). Melanoma cell line ESTDAB-109, obtained from a patient after IFN-α

immunotherapy, lacks HLA class I expression due to a β2m mutation and a LOH-15 (Paschen *et al*, 2003). The same alteration was detected in a tumor lesion obtained prior to immunotherapy, which could be responsible for the failure of the immunotherapy. Similarly, in the other study, the resistance to peptide-based immunotherapy (MAGE peptide) in two melanoma patients was correlated with the loss of HLA-I expression caused by β2m mutations and LOH-15 detected in two melanoma cell lines established from these patients and in tumor samples from where they derived (Benitez et al, 1998).

An initial positive clinical response to therapy before the appearance of β2m alterations has been reported in some cases, indicating the acquisition of this immune escape mechanism after the immunotherapy. In another report five cell lines were established from metastatic melanoma lesions from five different patients after immunotherapy, and all of them showed distinct β2m mutations detected after T-cell based immunotherapy (TILs, IL-2 and TNF-α)(Restifo et al, 1996). Tumor samples obtained from three of these patients before the therapy were β2m-positive, but the post-treatment melanoma cell lines as well as the tumor lesions from where they were established lack of β2m protein. Characterization of the β2m loss in these cell lines showed CT deletions in 3 out of the 5 samples (Chang *et al*, 2005b). This CT deletion has been previously described in literature in 1 out of 5 samples not treated with T-cell based therapy (Hicklin et al, 1998) suggesting the emergence of a preferential β2m gene mutation hot spot (delCT) associated to T cell-based immunotherapy in melanoma cells.

β2m mutations could also account for the lack of immune recognition of a melanoma cell line by HLA-restricted autologous TIL clones and helps explaining the recurrence and progression of tumor after adoptive transfer therapy (Khong *et al*, 2004). In this study a β2m mutation is described in melanoma cell line derived from a recurrent subcutaneous tumor from a patient, who experienced a dramatic response to immunotherapy, but later died of recurrent disease. Pre-treatment tumor samples were positive for HLA class I expression, showing a situation that may become more prevalent as immunotherapy-based treatments for cancer become more effective: the destruction of immunosensitive tumor cells but the eventual progression of immunoresistant tumor variants.

As it has been previously demonstrated by our group, cancer immunotherapy induces HLA upregulation in some metastatic lesions which ultimately regress, but not in the metastases that have a tendency to progress (Aptsiauri *et al*, 2008; Cabrera *et al*, 2007). This lack of response to immunotherapy and generation of progressing metastases appears to be associated with immune selection of HLA-negative tumor cell variants with irreversible defects (Carretero *et al*, 2008; Carretero *et al*, 2012), including alterations in β2m gene and LOH at chromosome 15. These results also suggest that an additional selective pressure may be exerted during T-cell-based immunotherapy, favoring the outgrowth of irreversible HLA class I-deficient tumor cells harboring β2m mutations, a major reason to develop a methodology to recover its expression before immunotherapy.

Finally, in the present study, we also investigated whether alterations of the APM component may have contributed to the loss of tumor HLA class I expression, since transcriptional downregulation of APM proteins have been described in cancer (Romero *et al*, 2005; Seliger *et al*, 2000). As a result, we found that the studied melanoma cell line was positive for APM expression, both at protein and transcriptional levels. In addition, transduction with the AdCMVβ2m vector leaded to the recovery of the β2m expression confirming the proper functionality of the antigen processing and presentation machinery.

4. β2m and HLA class I expression as biomarkers for monitoring cancer progression and response to immunotherapy

Due to the development of different immunotherapies, there is a need to identify immunological and genetic biomarkers to provide additional and much more detailed information for the prognostic classification of malignancies (Basil *et al*, 2006). Numerous serologic (Utikal et al, 2007), immunohistochemical and genetic markers of malignant melanoma associated with prognosis (Griewank et al, 2013; Ugurel *et al*, 2009) are proposed or currently used in the clinic. In addition, soluble HLA forms,

including ssoluble HLA-E and HLA-G (Cao *et al*, 2011; Silva *et al*, 2011) have been proposed as biomarkers for melanoma (Westhoff *et al*, 1998).

Despite the importance of HLA for the success of immunotherapy, the impact of HLA class I defects on tumor resistance to immunotherapy is largely unknown, as the majority of ongoing cancer immunotherapy clinical trials do not include tumor HLA class I expression analysis. However, we have been able to trace the accumulation of HLA class I alterations along the clinical course of metastatic melanoma, and due to the association of these alterations with immunotherapy failure and cancer progression, we believe that the analysis of the HLA class I status in the biopsies obtained from patients during the follow up of their disease should be considered, using flow cytometry or immunohistochemistry as we did in the current study (del Campo et al, 2014a).

Altered HLA class I expression has been correlated with prognosis as mentioned before, and its evaluation could be a potential prognostic marker allowing a detailed characterization of the patient prior or during the treatment course. Some publications supports our proposal regarding the analysis of HLA class I expression in the tumor, as it might help to choose an appropriate treatment protocol and monitor clinical response to cancer immunotherapy. It has been proposed that HLA class I is a useful biomarker with an important role in the pathogenesis of cancer, as it can identify a group of patients who may benefit from immunotherapeutic strategies such as cytokine or vaccine therapy, while tumors with weak HLA class I expression would not benefit from immune intervention (Simpson et al, 2010). HLA monitoring has been proposed in ovarian cancer, since altered expression of HLA heavy chain/β2m had been sown to correlate with reduced survival and improved prognosis (Rolland et al, 2007). Similarly, tumor HLA expression has been suggested to be used as a biomarker of patient enrollment in immunotherapy protocols. The survival advantage of patients with platinum-resistant tumours expressing high levels of HLA class I suggests that immunotherapy may be of use in these ovarian cancers resistant to standard chemotherapy (Shehata et al, 2009).

Immunohistochemical staining for HLA heavy chains was found to be useful as a prognostic marker and for selection of adjuvant therapy for stage III and IV esophageal cancer patients (Tanaka et al, 2011), as its downregulation is associated with a poor

prognosis. Evaluation of tumor HLA class I expression could be added to other independent prognostic factors to help further stratify breast cancer patients for adjuvant therapy (Madjd et al, 2005) or chemotherapy (de Kruijf *et al*, 2010b). In colorectal cancer high HLA class I expression is associated with improved disease-specific survival (Watson et al, 2006). Moreover, as β2m mutations have been described as potential contributors to the favorable outcome of MSI-H colon cancer patients, Kloor and colleagues proposed the evaluation of β2m as potential prognostic marker in future clinical trials (Tikidzhieva *et al*, 2012). The analysis of β2m expression levels has been proposed as a useful tool for identifying colorectal carcinoma patients with lymph node metastasis and/or poor survival (Shrout et al, 2008) since a decrease in β2m expression is associated with poor prognosis and metastatic progression.

There is an accumulating body of evidence suggesting that primary melanoma tumors are heterogeneous (Garrido *et al*, 2010b) and when it progresses to metastatic dissemination, tumor cells acquire new properties that support tumor growth, invasion, metastasis, and resistance to therapy (Somasundaram *et al*, 2012). Identification of genetic variants and the characterization of molecular mechanisms underlying the development of aggressive phenotypes could contribute to a better understanding of cancer immune escape, as represented in Figure 14.

Our findings highlight the importance of carefully defining the molecular mechanisms responsible for a particular altered HLA-I phenotype in order to choose immunotherapy treatment or design specific ways to restore normal tumor HLA-I expression, especially in tumors with HLA loss caused by irreversible genetic alterations. However, the majority of ongoing cancer immunotherapy clinical trials do not include tumor HLA class I expression analysis before or during the treatment, although we have shown that its knowledge is essential in order to identify patients as potentially eligible for peptide immunotherapy because of the positive correlation between HLA-I loss and metastasis progression.

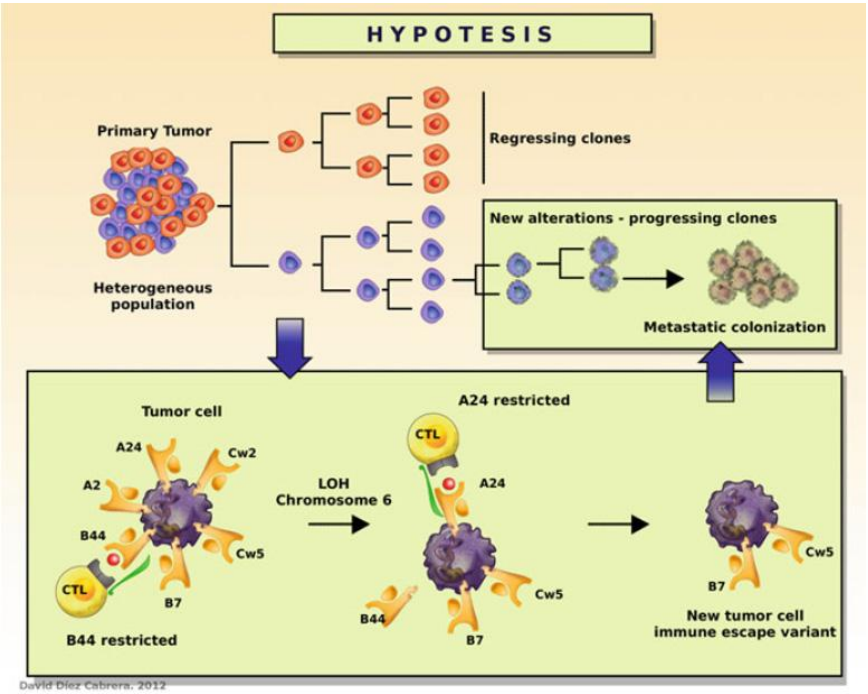

Figure 14. HLA class I mediated immunoselection of tumor escape variants during cancer progression. Primary tumors consist of heterogeneous populations of cells that give rise to different cell clones undergoing immune selection. The combination of somatic evolution of genetically unstable tumor cells and immune selection during cancer development leads to the generation of tumor variants that have better survival properties. This selective pressure will lead to the expansion of new populations of cells capable of evading different immune responses. In this way, tumor cell with normal HLA class I expression are subjected to T-cell cytotoxic response restricted to an HLA class I allele (e.g., B44 restricted CTL reactivity) and destroyed, but new HLA-negative clones appear due to additional alterations caused by LOH in chromosome 6 as HLA haplotype loss and generation of B44-negative cell clones. These newly emerged clones are positive for HLA-A24 and CTL response is now A24-restricted leading to elimination of HLA-A24 positive malignant cells, but new tumor escape variants appear. Reproduced from (Aptsiauri et al, 2013).

We believe that the correlation between HLA expression and clinical outcome cannot be clearly defined without identification of the exact type of tumor HLA defects in each patient, which would predict the ability of CTLs to recognize tumor-associated peptides. As we have shown that immune evasion strategies can develop before the

clinical application of immunotherapy (del Campo et al, 2014a), it would be useful to analyze the HLA expression to select the most effective therapy for different melanoma subsets. Its importance could be observed for example in a clinical trial that analyzes the immune responses associated to multiple tumor-associated peptides used for vaccination in renal cancer, although they do not check previously the expression of the HLA presenting molecule (Walter et al, 2012). We believe, that pre-selection of patients with tumors expressing the specific HLA might have improved the outcome of the therapy. In fact, this study was continued with a previous selection of HLA positive patients suggesting that the systematic use of T-cell response monitoring and cellular biomarkers may constitute a valuable strategy to optimize immunotherapeutic regimens (Walter et al, 2013).

Therefore, immunological and molecular analysis of tumors before treatment may provide a rationale for excluding patients who are unlikely to respond to immunotherapy, and may help to select the more successful treatment for a specific patient as shown in Figure 15.

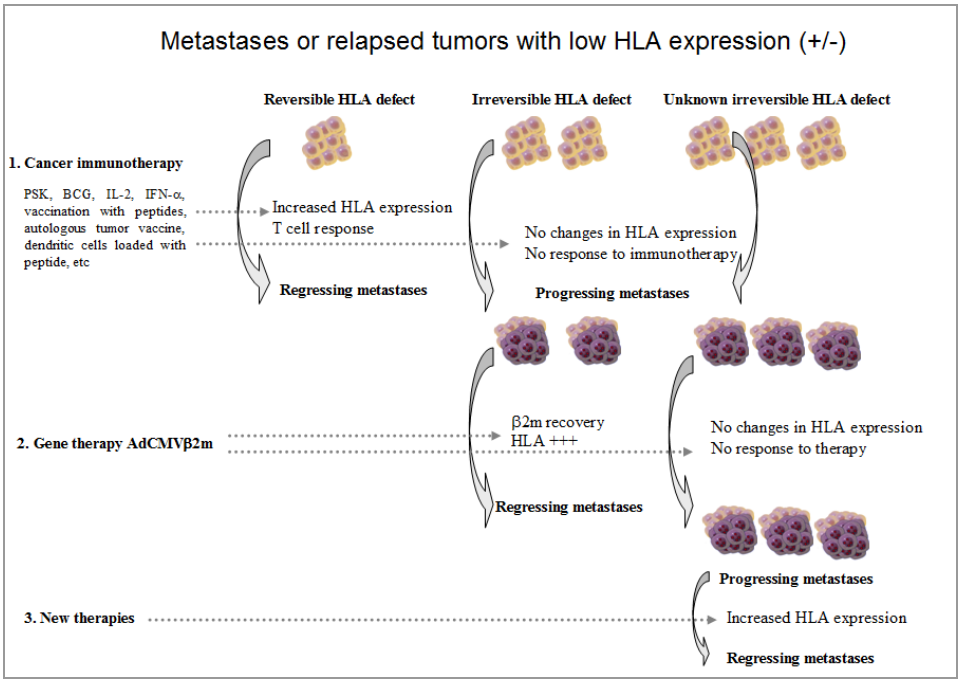

Figure 15. Schematic presentation of the possible clinical application of the adenoviral vector AdCMVβ2m in addition to immunotherapy. Immunotherapy of metastatic cancer as a

result of immune selection produces mixed responses: regression of some malignant lesions and progression of others. The therapy leads to modification of tumour microenvironment and release of immunostimulating cytokines that upregulate HLA class I expression in metastases with reversible ('soft') HLA alterations leading to tumour regression. In the lesions with irreversible structural defects ('hard' lesions) the level of HLA class I remained unchanged due to the resistance to cytokine treatment leading to progression of such metastases. Low expression of HLA class I in some progressing metastases is due to mutations in β2m gene and LOH in chromosome 15. Administration of adenoviral vector aimed to restore normal β2m and HLA class I expression is expected to enhance the response to immunotherapy subsequent regression of the metastasis. Some of the metastases that still progress obviously have other molecular mechanisms underlying poor response to the therapy, which need to be investigated further.

5. Construction of adenoviral vector coding for human β2m for recovery of HLA class I expression in human tumor cells

The rational to develop gene therapy approach aimed at the recuperation of HLA class I expression on tumor cells is based on the key role of HLA in anti-tumor immunity. Tumors lose HLA class I expression with high frequency, providing an escape mechanism from T-cell mediated cancer recognition and elimination. In many types of malignancy, altered HLA class I expression has been associated with cancer progression and metastatic dissemination, as well as with poor clinical prognosis. Finally, the lack of response to immunotherapy in spite of the presence of tumor infiltrating T-lymphocytes seems to be associated with immune selection of HLA-deficient tumor cell variants with irreversible defects, such as mutations in the β2m gene and LOH in chromosome 15. These genetic defects can only be corrected by replacement of the defective gene. Therefore, it seems to be important to develop a novel therapeutic strategy, which could be clinically applied in a subset of cancer patients with structural defect in β2m gene, with the purpose to restore (or increase) HLA class I expression on tumour cells.

HLA class I re-expression on melanoma biopsies has been positively correlated with T-cell infiltration, a more favorable disease stage and longer survival (Doukas & Rolland, 2012). Therefore, restoration of HLA class I in HLA class I negative tumor

cells might correct the inability of $CD8^+$ T cells to recognize those cells and avoid the tumor escape mechanism that allows that primary tumor or metastatic lesion progress improving the poor outcome of T-cell based immunotherapy protocols.

Recovery of HLA class I expression has been employed previously by various investigators to demonstrate the importance of class I molecules in specific tumor lysis by CTL and NK cells. The earliest reports in the 1980s were based on mouse models with known MHC defects. Transfection of MHC heavy chains genes into murine cell lines or tumors with reduced or absent H-2K decreased the tumorigenicity of these cells inhibiting tumor growth or formation of metastases, (Hui et al, 1984; Hui et al, 1989; Wallich et al, 1985), led to higher immunogenicity (Hammerling et al, 1986; Mandelboim et al, 1995) and reversal of metastatic phenotype (Gelber et al, 1989; Mandelboim et al, 1992; Plaksin et al, 1988).

Transient (D'Urso et al, 1991; Paschen et al, 2003; Wang et al, 1993) or stable expression (Chang *et al*, 2005b) of β2m gene in tumour cell lines using plasmid DNA transfer (Paschen et al, 2006; Paschen et al, 2003; Restifo et al, 1996), retroviral vectors (Baba et al, 2007), lentiviral vectors (Challa-Malladi et al, 2011) or vaccinia virus (Restifo et al, 1996; O´Neil et al, 1993) have proved that in vitro restoration of β2m led to HLA class I re-expression and recognition of tumour cells by specific cytotoxic T lymphocytes. In some reports β2m transfection led to recovery of HLA class I expression and escape from natural killer cells (Baba et al, 2007; Glas et al, 1992; Ljunggren et al, 1990). Transfer of murine β2m into human cells also recuperated HLA class I expression, but with lower efficacy than in case of human β2m gene recovery (Maio et al, 1991). Other reports describe the transfer of HLA-A2 restricted peptide epitope linked to the N terminus of β2m together with a specific heavy chain using a retroviral vector to tumor cells which were subsequently recognized and killed by appropriate CTL clones (Tafuro *et al*, 2001). These data prove that β2m transfer into β2m-deficient tumor cells restores HLA class I expression. However, most of the previous experimental gene transfer methods are not very good for cancer therapy, since the used viral vectors (retroviral and lentiviral vectors) integrate into the host genome increasing genomic instability and limiting their application for cancer treatment. The

direct transfer of DNA-liposome complexes of the HLA-B7 gene into HLA-B7-negative patients with advanced melanoma reported encouraging results as it estimulated local antitumor immune responses that facilitated the generation of effector cells for immunotherapy of cancer (Nabel et al, 1996). Also aadenoviral transduction of MHC heavy chain has been tested by other authors showing tumor regression in murine models (Campbell et al, 2000).

A great variety of studies support the use of adenoviral vector as a suitable tool to induce the immune response against tumors and ddifferent adenoviral vectors have been developed with the purpose to boost anticancer immunity in melanoma delivering different cytokines, TAAs or costimulatory molecules as we described previously.

We have constructed a replication-deficient adenoviral vector coding for human β2m gene under CMV promoter (AdCMVβ2m) using Cre-Lox recombination method (Hardy et al, 1997). In this type of adenovirus two regions are deleted; elimination of E1 region renders a replicant deficient vector, while deletion of the E3 region generates more space for the transgene and reduces vector immunogenicity.

We tested the capacity of our vector carrying β2m gene to transduce tumor cell lines of different histological origin, including melanoma, colorectal carcinoma, prostate cancer, and a Burkitt lymphoma. AdCMVβ2m successfully recovered β2m expression in all tumor cell lines (at different MOIs), except for lymphoma cell line DAUDI. Previosuly, DAUDI cells have been transfected with plasmids carrying either the murine or human β2m gene (Rothenfusser et al, 2002; Seong et al, 1988) supporting the capacity of the cells to restore HLA class I expression after β2m recovery. However, lymphoma cells have also been demonstrated to be resistant to most of the currently available gene transfer methods (Meunier-Durmort et al, 1997; Prince et al, 1998) showing only 14% of transfection efficiency for B lymphocytes (Prince et al, 1998). More detailed analysis is needed to elucidate the lack of HLA re-expression on DAUDI cells after viral infection, although it could be explained by impared binding of the recombinant β2m to HLA heavy chain, since this cell line had been previously characterized by a presence of a functional cell surface HLA class I in the absence of

β2m (Martayan *et al*, 2009). Presence of high amounts of free β2m in the culture supernatant of DAUDI cells transduced with AdCMVβ2m prove this possibility.

For all the cell lines that recovered β2m expression after adenoviral infection, 3 hours of infection followed by 48 hour incubation with fresh media was sufficient for positive transgene expression as measured by FACS. However, the percentage of positive cells was different in different cell lines, reaching the maximum percentage of transduced cells and transgene expression levels 12 hours after infection. FACS analysis showed almost 90% of cells positive were for β2m and HLA class I surface expression after 72 hours of AdCMVβ2m infection with the first viral stocks (del Campo et al, 2009). Later on we achieved higher transduction efficiencies decreasing the incubation time to 48 hours and lower MOIs (del Campo et al, 2014 submitted) as observed in figure 14.

Recovery of β2m and HLA class I complex expression was confirmed using different techniques, including immunocytochemistry with different antibodies, confocal microscopy, western blot, FACS and quantitative PCR.

The recovered HLA class I and β2m expression lasted for up to 15 days in all cell lines transduced with AdCMVβ2m as expected for a transient adenoviral infection, meanwhile it confirms that high β2m protein expression was not toxic to the cells, since the cells survived this period after transduction (del Campo et al, 2009). We also demonstrated that a second dose of the adenoviral vector can recover HLA/β2m level of expression that decreases during incubation cell culture (Figure 15).

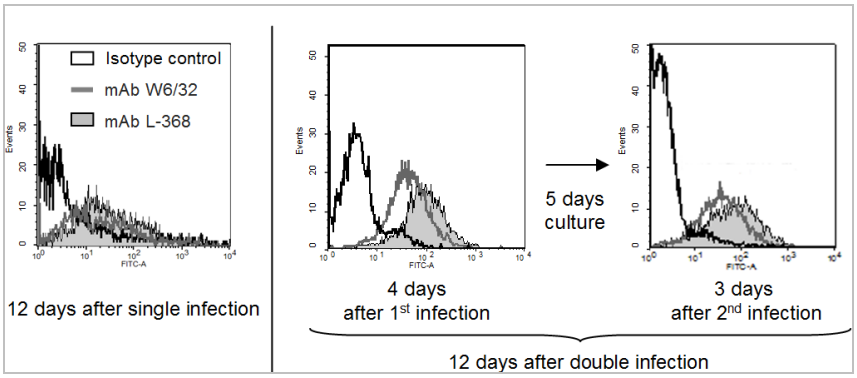

Figure 15. Reinfection of DNR-DC-M010 melanoma cells with adenoviral vector (AdCMVβ2m) increases cell surface expression of HLA class I and β2m. Left histogram represent the expression level after 12 days of a single dose infection, while the right histogram demonstrates expression after three doses of the vector administered at different time points.

Once we proved *in vitro* that adenoviral β2m infection restores HLA class I, we moved to an *in vivo* system to test our vector. Unfortunately, it is difficult to develop a syngenic mouse model of HLA-deficient cancer to test our viral vector, since there is no mouse tumor cell line or cancer animal model with characterized β2m defects, except for murine lymphoma (Glas *et al*, 1992; Parnes & Seidman, 1982). Thus, we used a xenogenic tumor model, in which we grow subcutaneously human β2m-deficient tumor cell lines in immunocompromised nude mice and inject the adenovirus intratumorally. Currently, human tumor xenografts are widely used to analyse antitumor efficacy of cancer therapy in a preclinical setting, as they reproduce the histology and metastatic pattern of most human tumors and can be used to molecularly dissect the metastatic process and to evaluate *in vivo* tumor response to therapy (Feuerer *et al*, 2001; Langdon, 2012)(Rofstad, 1994). We first tested the capacity of adenoviral vectors to transduce tumor cells using an adenovirus expressing luciferases as a reporter gene. In vivo bioluminescent imaging of the tumour inoculated with adeno-luciferase vector (10^8 and 10^9 PFU) showed that maximum luciferase expression was reached 5 days after adenovirus administration and the intensity was dose-dependent.

Next we confirmed that intratumoral injection of AdCMVβ2m (10^9 PFU) recovered tumor HLA class I expression in vivo. The transgene expression in the

xenogenic tumors injected with the vector was analysed by immunohistochemical analysis (Figure 17) and western blotting (del Campo et al, 2009). In addition, melanoma cells derived from transduced tumors retained positive β2m and cell surface HLA class I expression as measured by FACs and depicted on Figure 16.

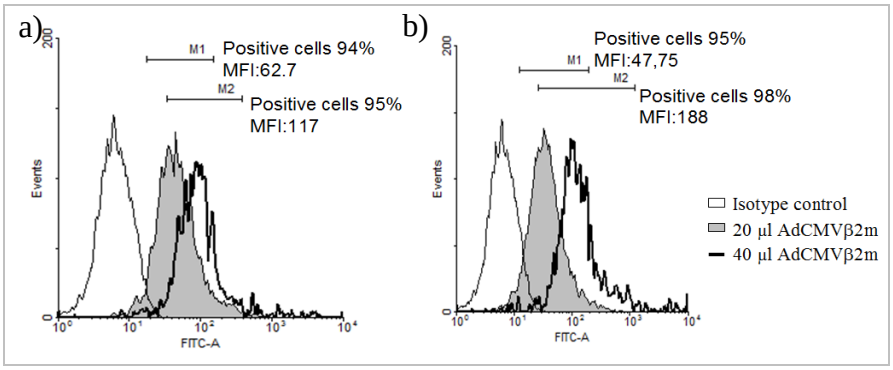

Figure 16. Positive re-expression of β2m protein in the tumours transfected with AdCMVβ2m vector demonstrated by immunohistochemistry using mAb anti-β2m/heavy chain complex (W6/32) (a) and mAb anti-β2m (L-368) (b). Human β2m-deficient tumour cells (MaMel-86b) were transplanted subcutaneously into nude/nude mice and AdCMVβ2m vectors was injected into tumours of 5–8 mm in diameters in two doses of 20 and 40µl (10^9 PFU).

However one of the major limitations of adenoviral vectors in gene therapy is their immunogenicity. Nevertheless, it can become beneficial in cancer therapy, since it implies an adjuvant effect or a non-specific activation of the immune system. The innate immune reactions to the capsids of adenoviral vectors induce activation and/or recruitment of Kupffer cells, endothelial cells, neutrophils and splenic macrophages and dendritic cells (DCs) (Thacker *et al*, 2009), and even non-immune cells transduced by adenoviral vector via binding to CAR and αv integrins, also respond to transduction by increasing the production of inflammatory chemokines and cytokines, such as IL-6, IL-12, IL-10, TNF-α, and IFN-γ (Liu & Muruve, 2003). In the same context, CTLs induced after adenoviral infection are capable of eliminating transduced cells expressing adenoviral genes or transgene peptides, which could help in mounting an effective response against HLA class I negative tumor where the lack of infiltration of T cells or NK cells is quite common (Khare et al, 2011).

Other major limitation of adenoviral vectors for clinical application is the inflammatory and hepatotoxic immune response associated with systemic administration of high doses of virus. González-Aseguinolaza and colleagues analyzed the effect of adenoviral vector administration route on the inflammatory immune response and liver transgene expression and showed that intra-tumoral injection into liver resulted in a lower inflammatory response and a higher transgene expression as compared to intravenous injection (Crettaz *et al*, 2006). We used intratumoral injection of the vector as an administration route as it has been shown to provide an important safety advantage over intravenous injection because the vector infects predominantly the cells of the injected tumor (Hu *et al*, 1999). In fact, intratumoral injection in cancer treatment has been shown to be well tolerated and to reduce the spread of the virus (Gerolami *et al*, 2000; Sangro *et al*, 2004). By using intratumoral injection we also reduce of virus neutralization by antibodies that can decrease transduction efficacy (Khare *et al*, 2011).

Intratumoral administration is the preferred route of virus administration in different ongoing clinical trials that use adenoviral vectors delivering immunostimulatory genes for treating melanoma or bladder cancer (ClinicalTrials.gov Identifiers: NCT00815607, NCT01082887 NCT01397708, NCT01455259, NCT00005057 or NCT01082887). Both tumor types are the potential targets of our proposed therapy because they could be easily accessed and treated *in situ*. Importantly both in melanoma and in bladder cancer resistance to immunotherapy has been correlated with β2m genetic alterations (Carretero *et al*, 2011), and there are clinically relevant mouse models available mimicking human situation for both melanoma (Kato *et al*, 1998; Umansky & Sevko, 2013) and bladder carcinoma (Kasman & Voelkel-Johnson, 2013; Malmstrom *et al*, 2010) that could be used to analyzed the effect of the adenoviral vector at least in cell lines harboring LOH in chromosome 15.

6. Functional activity of the re-expressed HLA class I complex in transduced tumor cells

To evaluate the functional integrity of HLA class I expressed on cell surface different groups tested the ability of HLA-positive tumor cells to induce cytokine release by HLA class I–restricted/peptide-specific CTL and for their susceptibility to lysis by these CTLs (Hicklin *et al*, 1998; Paschen *et al*, 2003), meanwhile others uses the susceptibility of HLA-negative cancer cells to NK cell-mediated lysis (Maio et *al*, 1991; Ljunggren et al, 1990) or both (Baba et *al*, 2007). In the present study, we confirmed that the restoration of HLA class I expression on β2m-negative cells was able to induce cellular immune responses mediated by CTLs. AdCMVβ2m-transduced DNR-DC-M010 melanoma cells stimulated IFN-γ secretion by autologous and HLA-matched T-cells induced by both influenza virus peptide and a melanoma specific peptide Melan-1/Mart-A, for which the cell line is positive. We did not see IFN-γ production using non HLA-matched T-cells, or using cell lines transduced with control adenoviral vector carrying GFP, corroborating the specificity of T-cell activation (del Campo et al, 2014a; del Campo et al, 2014 submitted).

We also showed that HLA-matched donors T-cells primed with influenza peptide are able to lyse peptide-pulsed melanoma cells transduced with AdCMVβ2m virus in a higher degree, as the same cells transduce with luciferase-coding virus. Therefore, the recovery of β2m expression mediated by AdCMVβ2m induces stimulation of T-cells that properly recognize tumor-associated antigenic peptides presented by the restored HLA class I, a fundamental event for the detection and destruction of malignant cells.

Since the melanoma cell line used in this study has been established from a lesion in advanced disease stage (del Campo et al, 2014a), it appears that these cells have acquired mechanisms to evade CTL attack by means of loss of HLA-class I expression due to alterations in β2m gene in this patient making of AdCMVβ2m vector a suitable tool to restore the sensitivity to CTL attack.

Figure 17 summarizes the *in vitro* and *in vivo* assays used to confirm functional recovery of β2m/HLA class I expression on tumor cells transduced with AdCMVβ2m.

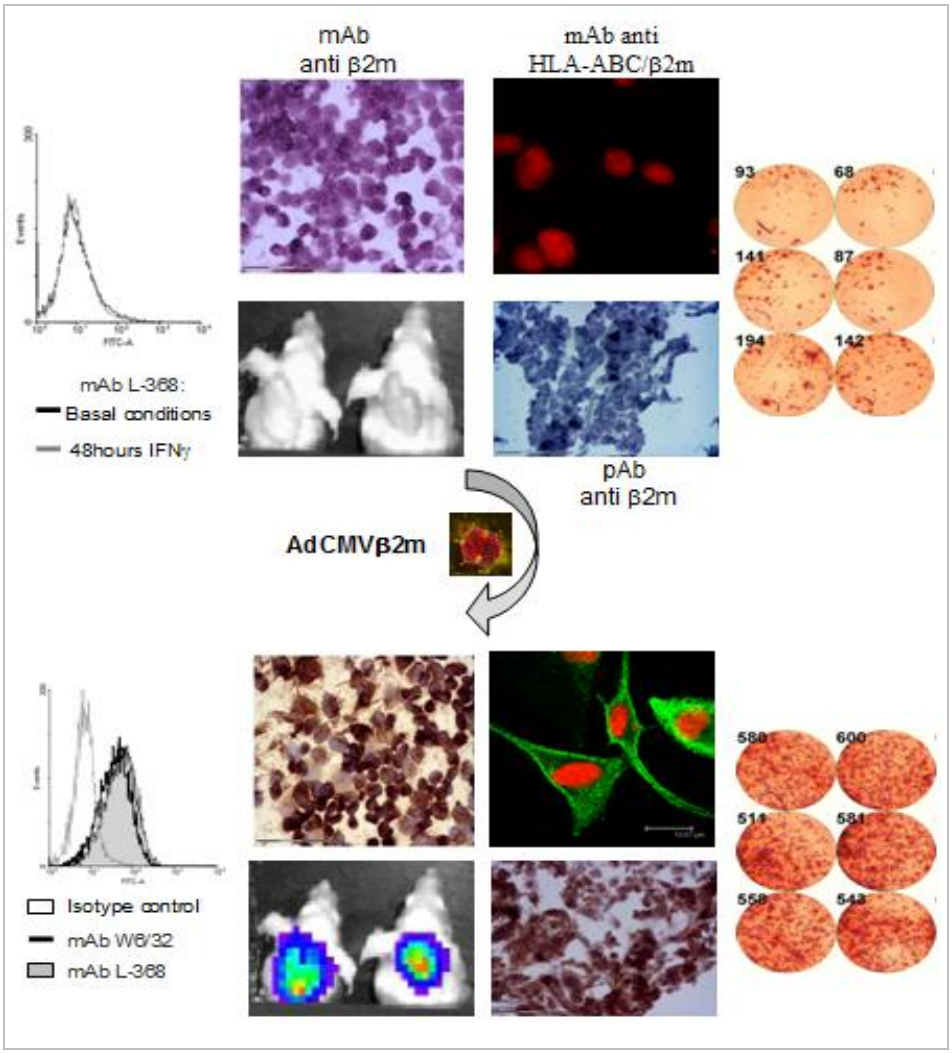

Figure 17. Adenoviral-mediated β2m recovery allows the expression of a functional HLA class I complex on tumor cells. β2m-deficient cells (as tested by FACS, immunocytochemistry and confocal analysis) recover β2m/HLA-I expression after adenoviral transduction inducing the activation of peptide-stimulated HLA-restricted T-cells as assayed by ELISPOT. In addition, HLA class I negative tumor xenografts are efficiently transduced either by a reporter vector (AdLuc) or by AdCMVβ2m.

7. Changes in antigen presentation machinery and tumor cells biology in cells transduced with AdCMVβ2m

Although adenoviral vectors coding for tumor-associated antigenic peptides have been generated and used to increase tumor cell immunogenicity, detailed knowledge on their mechanism of action for anti-tumor efficacy is lacking. In particular, there is not much information regarding the effect of recombinant adenoviral vectors on the HLA expression and antigen presentation machinery in tumor cells. There are several important issues that have to be addressed to make of this type of gene delivery an appropriate vehicle for HLA class I recuperation. This includes analysis of the possible influence of adenovirus on the HLA class I/heavy chain/ β2m/peptide complex assembly and transport to the cell surface supported by the components of the antigen processing machinery (APM).

Some authors report that adenoviruses inhibit the intracellular transport of class I antigens and dramatically reduce their cell surface expression (Paabo *et al*, 1986; Paabo *et al*, 1989), while others indicate that cells transformed by Ad5 show the same (Vasavada *et al*, 1986) or and even higher levels of HLA class I expression (Eager *et al*, 1985;Nielsch *et al*, 1991).

Different components of the HLA class I assembly pathway have been found to be inhibited by numerous viral proteins (Hansen & Bouvier, 2009; Hewitt, 2003), a virus counterattack strategy against the cellular immune response with the goal of preventing the presentation of viral peptides to CTLs to freely replicate in infected cells. The adenoviral protein E3/19K was first shown to down-regulate cell surface MHC class I expression (Flomenberg *et al*, 1994) by direct retention of MHC heavy chains in the ER (Andersson *et al*, 1985; Burgert & Kvist, 1985; Cox *et al*, 1990) through the α1- and α2-domains (peptide-binding groove) of HLA class I molecules (Fu & Bouvier, 2011) that contributes to intracellular sequestration of HLA class I molecules (Sester *et al*, 2013). Later, it was demonstrated that E3/19K also binds to TAP interfering with peptide loading by blocking the association of TAP with tapasin and the transport process (Gruhler & Fruh, 2000; Petersen *et al*, 2003) and by preventing the association of HLA class I with TAP1 (Bennett *et al*, 1999; Petersen *et al*, 2003). Recently, it was found that other proteins may play a part in MHC class I trafficking, such as amyloid precursor-

like protein 2 (APLP2) (Morris *et al*, 2003; Morris *et al*, 2004) that inhibits MHC class I surface expression in an association that appears to be regulated by E3/19K (Sester *et al*, 2000).

Rosenberg´s group used a first generation E1-deleted adenovirus and observed in only 3 of 10 infected tumour cell lines a notable reduction in expression of HLA-A2 and -B7 (Zhai *et al*, 1996), HLA alleles known to bind E3/19K with relatively high affinity (Beier et al., 1994). In another study, adenovirus with some E1 regions eliminated was used (Reed *et al*, 1997) to transduce 8 human melanoma cell lines and none of them showed a significant decrease in surface expression of HLA class I; at the same time they stimulated cytotoxicity of MART1 and gp100-specific TILs. However, since we are using an adenoviral vector to restore HLA class I expression, we have investigated if the adenovirus transduction with our vector has any effects on the antigen presentation process in tumor cells. Therefore, we examined APM component expression in virally transduced tumor cells by quantitative PCR, immunocytochemical analysis and confocal microscopy.

Regarding HLA class I expression, all our results demonstrate that AdCMVβ2m upregulates β2m and restores cell surface HLA class I complex expression while antigen presentation is not inhibited since it results in the presentation of the tumor-associated peptide to the cytotoxic T-cells (del Campo et al, 2014a; del Campo et al, 2014 submitted). We examined how re-expression of β2m using adenoviral vector affects the transcription of HLA-A, B and C loci, as modulation of HLA heavy chain expression could potentially affect peptide presentation, their affinity and specificity of locus-specific peptide binding to HLA class I complex. The analysis of HLA-A, B and C loci transcription after β2m recovery showed that locus B expression is upregulated, while the pattern of locus A and C expression varies depending on the cell line. These results are in accordance with previous description of differential expression of HLA class I loci after β2m plasmid transfer into HLA-I deficient melanoma cell lines (Chang *et al*, 2005b), a phenotype that appears to be caused by abnormalities in regulatory mechanisms, since IFN-γ treatment induces an upregulation of the different locus in all the cells lines. Moreover, it has been also described that the three loci can be expressed

at different levels in different tumor cell types (Johnson, 2000; Versteeg *et al*, 1989), or normal tissues of different histotype (Garcia-Ruano et al, 2010). This effect is probably due to the differences in the expression of regulatory factors, such as the presence of locus-specific residues throughout the entire promoter region of the HLA heavy chains, which suggests that the various HLA class I loci are differentially regulated (Griffioen *et al*, 2000; Johnson, 2003).

There have been different reports on the adenoviral-mediated modulation of the APM molecules in antigen-presenting cells, including tumor cells and DCs. Cells transformed by Ad5 expressed either normal (Mey-Tal *et al*, 1997; Rotem-Yehudar *et al*, 1996) or higher levels (Mey-Tal *et al*, 1997; Rotem-Yehudar *et al*, 1994) of TAP1/TAP2 and LMP2/LMP7 mRNAs; data from murine tumors injected with AdGFP vector show no alteration of the equivalent TAP1/TAP2 and LMP2/LMP7 mRNAs (Putzer *et al*, 2002). Other studies have checked the correct expression and function of some APM components after their transduction with an adenoviral vectors: TAP1 re-expression increased MHC class I antigen surface expression (Chen et al, 1996a) and susceptibility to antigen-specific CTLs (Lou et al, 2008), while calreticulin transduction allowed the induction of a strong specific immune response (Gomez-Gutierrez et al, 2007), indicating the proper function of the antigen presentation machinery after adenoviral infection. In addition, an increase in ERp57, TAP-1 and TAP-2 levels has been reported in adenovirally transduced DC. (Vujanovic *et al*, 2009). Therefore, currently published information regarding the impact of adenoviral infection on APM molecules is controversial.

Our results from transcriptional studies of APM components and confocal microscopy data indicate that the intracellular trafficking and assembly of the β2m and HLA heavy chain with the support of AMP molecules takes place as expected, similar to other publications (Bedard *et al*, 2005; Everett & Edidin, 2007; Mery *et al*, 1996; Tao *et al*, 2008; Tasdemir *et al*, 2008), despite certain changes in APM component transcription after infection, which varied depending on the studied tumor cell lines (del Campo et al, 2014 submitted).

The efficacy of gene therapy also depends on how the transgene affects target cells, how it influences cell viability and proliferation. We have demonstrated that β2m protein produced by adenovirus-mediated gene transfer is properly expressed on tumor cell surface in a complex with HLA heavy chain. However, it is expected that in case of β2m overproduction the excess of the newly synthesized β2m will not have enough free heavy chains to form HLA class I complex with peptides and will be secreted into the extracellular space.

We found different levels of soluble β2m in the culture supernatant of AdCMVβ2m transduced tumor cells, ranging from 50 to 200 ng/ml at optimal MOI and reaching 2μg/ml with an MOI 20-fold higher. Physiological serum β2m concentrations in humans varies from 1 to 3 μg/ml in physiological conditions and increases up to 10-20μg/ml in disease conditions (Rowley *et al*, 1995). In this study, the prostate cell line showed a minimal production of soluble β2m protein, while Burkkit lymphoma cell line DAUDI that did not recover cell surface β2m expression (del Campo et al, 2009), secreted soluble β2m protein. It could be explained by the data recently published suggesting that this cell line does not require β2m for cell surface expression of HLA heavy chains and peptide presentation (Martayan *et al*, 2009). Therefore, absence of HLA class I recuperation in AdCMVβ2m-transduced DAUDI cells can be explained by the possibility that the newly expressed β2m is not able to bind properly HLA heavy chain and is produced in a free soluble form.

Free β2m is found in body fluids under physiological conditions as a result of shedding and extracellular release, or cell death in cancer or other diseases. Elevated levels of serum β2m correlate with poor prognosis in renal diseases and are present in hematological malignancies, including lymphomas, leukemias and multiple myeloma. This observation suggests an important, yet unidentified, role of this protein in these malignancies. In addition to the roles in immunity, the level of β2m is associated with proliferation, apoptosis and metastasis in several cancer types. Some studies have shown that increased concentration of soluble β2m protein is linked to increased tumor growth in various types of malignancy, including breast, lung and renal cancer

(Rasmuson *et al*, 1996). Some publications demonstrate an evidence that β2m acts as a signalling and growth-promoting factor for prostate cancer and cancer-associated bone metastasis (Huang *et al*, 2006; Nomura *et al*, 2006). However, there is an evidence that β2m secreted in sera is modified by proteolytic cleavage (Shemesh & Ehrlich, 1993) and this modified form would exert other biological functions.

Since our in vitro experiments demonstrated that most of the studied cell lines after AdCMVβ2m transduction not only recover cell surface expression of β2m/heavy chain complex, but also produce free soluble form of β2m protein, we decide to analyse how it affects tumor cell proliferation and apoptosis. We measured proliferation of prostate tumor cells DU-145 incubated with β2m-rich supernatants collected after transduction of melanoma cells with various doses of the AdCMVβ2m and did not see any increase in the proliferation measured by MTT method or by direct cell counting, although in some cases MTT reading were slightly reduces in the presence of the β2m transduced melanoma cell supernatants.

In addition, viral proteins are capable to modulate the apoptotic response of host cells. Following the infection with human adenoviruses, cells exhibit an apoptotic response mediated by the expression of the viral E1A protein (Kirshenbaum, 2001) and adenoviral gene E6 products. On the other hand, E1B-19K blocks host cell apoptosis, and various proteins encoded by the E3 transcription unit, exert anti apoptotic (Galluzzi *et al*, 2008). However, in this study neither replication-deficient adenovirus coding for β2m nor control Ψ5 virus induced apoptosis in infected tumor cells, although this effect would even be beneficial in our context as it would eliminate the tumor cells.

6. Chapter 6. Conclusions

1. Early incidence of structural β2m defects in melanoma provides an immune escape route and leads to expansion of HLA-negative melanoma clones resistant to immunotherapy.

2. Damage of a single β2m allele by LOH on chromosome 15 may be sufficient to generate a tumor cell precomitted to escape.

3. β2m/HLA class I expression may thus provide both a biomarker and a therapeutic target in the development of new cancer immunotherapy protocols.

4. A new replication-deficient adenoviral vector carrying human β2m gene has been developed, AdCMVβ2m. It is effective in restoration of HLA class I expression in various types of β2m negative human tumour cells both *in vivo* and *in vitro*.

5. AdCMVβ2m proved to be suitable for restoration of HLA class I expression. It induces tumor specific T-cell activation without compromising antigen processing and presentation.

6. We propose that, the adenoviral-mediated recovery (or even increase) of HLA class I expression on tumour cells in combination with vaccination or adoptive T-cell therapy can provide a complementary approach in a subset of cancer patients with structural defect in β2m gene or chromosome 15 to improve the clinical efficacy of cancer immunotherapy.

1. La incidencia temprana en melanoma de defectos estructurales de β2m proporciona una ruta de escape inmune y conduce a la expansión de clones de HLA negativos resistentes a la inmunoterapia.

2. El daño de un único alelo de β2m mediante LOH del cromosoma 15 puede ser suficiente para generar una célula tumoral predeterminada al escape.

3. La expression β2m/HLA de clase I puede constituir tanto un biomarcador como una diana terapéutica en el desarrollo de nuevos protocolos de inmunoterapia del cáncer.

4. Un nuevo vector adenoviral deficiente para su replicación que porta el gen humano de β2m se ha desarrollado, AdCMVβ2m. Es efectivo en la restauración de la expression de HLA de clase I en varios tipos de células tumorales humanas β2m negativas tanto *in vivo* como *in vitro.*

5. AdCMVβ2m se ha visto que es apropiado para la restauración de la expresión de HLA de clase I. Induce la activación tumor-específica de células T sin comprometer el procesamiento y presentación antigénica.

6. Proponemos que, la recuperación (o incluso el incremento) de la expression de HLA de clase I mediada por adenovirus en células tumorales en combinación con vacunación o terapia adoptiva de células T puede proporcionar un estrategia complementaria en un subtipo de pacientes de cáncer con defectos estructurales en el gen β2m o en el cromosoma 15, para mejorar la eficicacia clínica de la inmunoterapia del cáncer.

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ABBREVIATIONS USED IN THE TEXT

HLA	Human Leukocyte Antigen
β2m	β2-microglobulin
DC	Dendritic cells
LOH	Loss of heterozigosity
AdCMVβ2m	Adenoviral vector coding for human β2m gene under citomegalovirus promoter
IFN	Interferon
TAA	Tumor associated antigens
CTL	Cytotoxic T lymphocytes
NK	Natural killer cells
TCR	T-cell receptors
MHC	Major Histocompatibility Complex
TAP1 /TAP2	Transporters associated with antigen processing
LMP2/LMP7	Low molecular weight proteins
ER	Endoplasmic reticulum
MSI-H	Microsatellite instable-high
CRC	Colorectal cancer
APM	Antigen processing machinery
BCG	Bacillus Calmette and Guérin
FDA	Food and Drug Administration from United States of America
IL	Interleukin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
TNF	Tumor necrosis factor
MAGE	Melanoma-associated antigen
TILs	Tumor-infiltrating lymphocytes
TEIPP	T cell epitopes associated with impaired peptide processing
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CEA	Carcinoembryonic antigen
MUC1	Mucin 1
ICAM-1	Intercellular Adhesion Molecule 1
LFA-3	Lymphocyte function-associated antigen 3
MART-1	Melanoma antigen recognized by T-cells 1
ITR	Inverted terminal repeats
Ad	Adenovirus
CAR	Coxsackievirus and adenovirus receptor
CRAdV	Conditionally replicative adenoviruses
STR	Short tandem repeat
MICA/B	MHC class I polypeptide-related sequence A/B
ULBP	UL16 binding proteins
FACS	Fluorescence Activated Cell Sorting
MOIs	Multiplicity of infection
GFP	Green fluoresecent protein

Una persona es tan grande como los sueños que sueña,
Como el amor que siente,
Como los valores que aprecia,
Y como la alegría que comparte.
Una persona es tan grande como las ideas que piensa,
Como la verdad que dice,
Y como la ayuda que imparte.
Tan grande como el destino que busca y como la vida que vive.