Tumor escape after immunotherapy: Implication of HLA class I expression in melanoma and bladder tumors

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National Institutes of Health

Tesis Doctoral

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Granada 2011

Editor: Editorial de la Universidad de Granada
Autor: Rafael Carretero Coca
D.L.: GR 2424-2011
ISBN: 978-84-694-2943-3

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CERTIFICA: Que el presente trabajo de investigación titulado: TUMOR ESCAPE AFTER IMMUNOTHERAPY: IMPLICATION OF HLA CLASS I EXPRESSION IN MELANOMA AND BLADDER TUMORS, ha sido realizado bajo mi dirección por Rafael Carretero Coca, para optar al título de doctor en Biología.

Y para que conste se firma el presente certificado en Granada a 1 de Marzo del 2011

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Jose Manuel Cózar Olmo

Este trabajo está dedicado especialmente
a mis padres,
a mis hermanos
y a mi Alex

"If you know the enemy and know yourself, you need not fear the result of a hundred battles.
If you know yourself but not the enemy, for every victory gained you will also suffer a defeat.
If you know neither the enemy nor yourself, you will succumb in every battle"**.**

Sun Tzu. ***The Art of War***

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Chapter 1.

Abstract/Resumen

1.1. Abstract

It has been demonstrated that the immune system can recognize tumor cells as "non-self" elements. This knowledge has prompted the development of different immunotherapies aimed at the passive or active activation of the immune system to eliminate transformed cells. However, cancer immunotherapy clinical trials have shown only partial success, with a large group of patients showing no response to therapy.

Various studies have demonstrated molecular abnormalities in tumor cells that allow them to escape the immune response. One of the major escape mechanisms is alteration in human leukocyte antigen (HLA) class I expression. These alterations could be responsible for the tumor growth in patients with a functional immune system.

In this thesis, we studied different metastases obtained from two melanoma patients showing mixed response after immunotherapy as well as bladder tumors from patients treated with Bacillus Calmette-Guerin (BCG). The first melanoma patient developed several metastases, including 3 progressing and 3 regressing lesions that we obtained and analyzed after autologous vaccination plus BCG (M-VAX). Out of several secondary metastases in the second melanoma patient, we studied 4 regressing and 1 progressing lesions obtained after interferon α2b, and 4 regressing and 1 progressing lesions obtained after M-VAX. All metastases showed HLA class I alterations. However, the progressing metastases developed additional and more profound defects in HLA expression.

All metastases from the first melanoma patient presented with LOH in chromosome 6. In addition, progressing metastases showed loss of HLA-B, a weak expression of HLA class I, and LOH in chromosome 15. All metastases from the second melanoma patient had LOH in chromosome 6 and 15 and loss of surface expression of HLA-B. Progressing metastases showed additional defects in the HLA system.

Comparative study of the genome expression pattern in mixed melanoma responders to therapy allowed us to isolate genes differentially expressed in regressing and progressing lesions, with the majority of them being implicated in regulation of the immune response. Upregulation of antigen presentation and immune rejection pathways, including HLA-A, B and C, antigen processing machinery (APM), interferon regulatory factor 1 (IRF-1), signal transducers and activators of transcription 1 (STAT-1), allograft inflammatory factor (AIF-1), granzymes, etc., were found in regressing metastases. These data suggest that regressing tumors are under an acute immune rejection response. The molecular pathways of tumor rejection in our case are similar to those described during allograft rejection, autoimmune disease, graft-versus-host disease and pathogen clearance.

We also studied HLA class I expression in18 bladder cancer patients: 13 of these underwent BCG therapy, with eight relapse-free patients and five patients with relapse after

the treatment; and 5 mitomycin-treated patients were used as controls.

Post-BCG relapsed bladder tumors had haplotype and/or loci loss before the treatment. In contrast, four relapse-free patients showed no alterations, two showed heterogeneous expression, and two had HLA-B locus loss. We found a higher percentage of structural alterations in lesions with recurrence after BCG: 80% and 60% of tumors showed loss of heterozygosity (LOH) in chromosomes 6 and 15, whereas only 25% of relapse-free patients had LOH in either chromosome.

Bladder tumor relapses showed additional alterations in HLA class I expression in comparison to the primary tumors. Three relapses developed total HLA class I loss and the other two showed an additional loss of HLA-A locus. Mitomycin-treated patients conserve the same HLA class I pattern in primary and relapsed tumor.

Our results show that tumors with additional, irreversible alterations in HLA class I can escape the immune system despite immunotherapy activation. We propose that tumors with reversible “soft” alterations will upregulate antigen presentation after treatment, leading to recognition and elimination by T cells. In contrast, transformed cells bearing irreversible “hard” alterations will not show any response after treatment and will continue growing. Analysis of HLA class I alterations in tumor cells is a key factor to select the appropriate immunotherapy.

1.2. Resumen

En los últimos años se ha demostrado que el sistema inmunitario es capaz de reconocer a las células tumorales a pesar de ser elementos "propios" de nuestro organismo. Estos datos han promovido la aparición de distintos tipos de inmunoterapias encaminadas a la activación pasiva o activa del sistema inmunitario para promover la eliminación de los tumores. Sin embargo, los distintos ensayos clínicos realizados solo consiguen la regresión tumoral en una parte de los pacientes, existiendo un gran porcentaje de pacientes que no responden al tratamiento.

Existen numerosos estudios que describen la presencia de distintas alteraciones moleculares en las células tumorales que les permiten no ser eliminadas por el sistema inmunitario. Una de las alteraciones que aparece con mayor frecuencia es la falta de expresión de las moléculas HLA de clase I. Existe la opinión de que dichas alteraciones son las responsables de que los tumores puedan crecer en personas con un sistema inmunitario funcional.

En esta tesis hemos estudiado metástasis 2 pacientes de melanoma con respuesta mixta tras inmunoterapia y en tumores vesicales de pacientes sometidos a BCG. Se obtuvieron 3 metástasis de melanoma en progresión y 3 en regresión del paciente 1 tras un tratamiento con células tumorales autólogas más BCG (M-VAX). Del paciente 2 se obtuvieron 5 metástasis de melanoma en regresión y una en progresión tras inmunoterapia con interferón α2b y otras 5 regresoras y 1 progresora tras M-VAX. A pesar de que todas las metástasis muestran alguna alteración en HLA, las metástasis progresoras contrastan con las regresoras por su baja expresión. En el primer paciente todas las metástasis presentan LOH en la región del gen de la cadena pesada del cromosoma 6. La diferencia es que las lesiones en progresión son negativas para el locus B y tienen una expresión residual de cadena pesada, mientras que las regresoras tienen una alta expresión de los loci A y B. Además las progresoras presentan una LOH en la región de la β2m del cromosoma 15, alteración que no está presente en la lesiones en regresión. En el segundo paciente todas las metástasis tienen LOH en los cromosomas 6 y 15 y una expresión negativa del locus B. Sin embargo las metástasis en regresión tienen una expresión alta de cadena pesada mientras que las regresoras solo tienen una expresión residual.

También hemos analizado el patrón transcriptómico de las metástasis de melanoma, encontrando que las diferencias de expresión entre las metástasis en progresión y en regresión son debidas en su mayoría a genes de la respuesta inmunitaria. Las lesiones regresoras tienen activadas las vías de presentación antigénica y del rechazo inmunitario, con genes como HLA-A, B y C, genes de la maquinaria de procesamiento antigénico (APM), Factor regulador

del interferón (IRF-1), transductor de señales y activador de la trascripción 1 (STAT-1), factor de inflamación en injertos (AIF-1) granzimas… Estos datos demuestran que los tumores en regresión tienen están sufriendo un rechazo por parte del sistema inmunitario. Este rechazo es el mismo que se da durante el rechazo agudo de trasplantes, en las enfermedades autoinmune, en el síndrome de injerto contra huésped, y en la eliminación de patógenos virales. Por lo tanto los tumores pueden ser reconocidos y eliminados de manera efectiva por el sistema inmunitario.

Así mismo se han estudiado 18 pacientes con cáncer urothelial de vejiga. 13 de los pacientes fueron tratados con BCG, 8 de los cuales no desarrollaron recidiva tras el tratamiento mientras que en 5 el tumor volvió a aparecer. También se estudiaron 5 pacientes con recidiva tras mitomicina como controles sin tratamiento inmunoterápico.

Para dilucidar la razón de la distinta respuesta a la inmunoterapia hemos estudiado el patrón de expresión de HLA de clase I y las alteraciones moleculares que afectan a dicho patrón. Hemos encontrado que los tumores vesicales que recidivan tras BCG tienen más alterada la expresión de HLA de clase I. Todos los pacientes con recidiva tras BCG presentaban pérdida de haplotipo o de locus antes del tratamiento. En cambio en 4 de los pacientes libres de recidiva no se encontró ninguna alteración, 2 presentaba una expresión heterogénea y 2 mostraban perdida de locus B. También se encontró un porcentaje mucho mayor de alteraciones estructurales; un 80% y 60% de los pacientes con recidiva tiene perdida de heterocigosidad (LOH) en cromosoma 6 y 15 respectivamente frente a solo un 25% de los que no recidivan presentan LOH en alguno de los 2 cromosomas.

Además las recidivas de tumores vesicales tras BCG tienen alteraciones adicionales en HLA de clase I respecto a los tumores obtenidos antes del tratamiento. 3 de los pacientes pierden totalmente la expresión y los otros 2 pierden el locus A, además uno de los pacientes presenta una nueva LOH en el cromosoma 15. Este fenómeno no ocurre cuando el tratamiento es mitomicina, un citoestático sin acciones inmunitarias.

Los experimentos realizados muestran como los tumores con mayores alteraciones pueden seguir escapando del sistema inmunitario a pesar de la activación por la inmunoterapia. Proponemos que aquellos tumores con lesiones reversibles o “blandas” recuperan la expresión gracias al tratamiento y por consiguiente, son reconocidas como extrañas y rechazadas por el sistema inmunitario. Sin embargo, las células tumorales con lesiones irreversibles o “duras” no responden al tratamiento y pueden seguir creciendo. Creemos que el análisis de las alteraciones de HLA de Clase I es de vital importancia para determinar el tipo de tratamiento que debe seguir el paciente.

Chapter 2.

General Introduction

2.1. Melanoma and bladder cancer

2.1.2 Melanoma

The World Health Organization (WHO) defines melanoma as a cancer that begins in melanocytes. It may start in a nevus, but can also begin in other pigmented tissues, such as in the eye or in the intestines.

Melanoma is typically located in the epidermis and occasionally in the dermis. Clark and coworkers (Clark et al. 1984) defined six lesional steps of tumor progression from the neoplastic system that affects the human melanocyte: 1) acquired melanocytic nevus, 2) melanocytic nevus with melanocytic hyperplasia, 3) melanocytic nevus with aberrant differentiation and melanocytic nuclear atypia 4) primary melanoma (radial growth phase) 5) secondary melanoma (vertical growth phase) and 6) metastatic melanoma. Melanoma can also be grouped according to the American Joint Committee on Cancer (AJCC) recommendations (AJCC staging manual 2010).

In contrast to others skin cancers such as basal cell cancer (BCC) and squamous cell cancers (SCC) which are rarely fatal, melanoma features rapid progression and high mortality. Melanoma represents only a 1.5-7% of all types of skin cancer, but it causes 65% of skin cancer-related deaths (Jemal et al. 2002). In 2009 an estimated 68720 patients were diagnosed with melanoma, and 8650 died of this disease within the US (Jemal et al. 2009). Melanoma is likely to become an increasingly important public health issue (Jemal et al. 2001; Parkin et al. 2005). From 1950 to 2000 an increase of 619% for annual incidence of this disease and 165% for annual deaths could be observed. This increase is higher than for any other cancer (Tsao et al. 2004). Incidence varies by latitude and altitude, with regions closer to the equator and higher in altitude generally having higher rates of skin cancer (Tucker and Goldstein 2003).

Melanoma risk factors have been described during the past several decades. These factors are grouped into host and environmental factors. Environmental factors mainly include sun exposure (Tucker et al. 2009), solarium use usage (Gallagher et al. 2005; IARC 2007), and redox-active metals (Meyskens et al. 2008). Host factors include number of nevi, complexion, tanning ability, extent of freckling (Tucker and Goldstein 2003; Gandini et al. 2005; Olsen et al. 2009; Chang et al. 2009), family history of melanoma (Rutter et al. 2004; Gandini et al. 2005) and gene mutations (Goldstein et al. 2006; Hocker and Tsao 2007; Smalley et al. 2009; Goldstein et al. 2007; Raimondi et al. 2008).

2.1.2 Bladder urothelial cancer

The World Health Organization (WHO) defines bladder cancer as the ones form in any of the different tissues of the bladder. Most bladder cancers are transitional cell carcinomas (97%); this cancer begins in cells that normally make up the inner lining of the bladder. Other types include squamous cell carcinoma (cancer that begins in thin, flat cells) and adenocarcinoma (cancer that begins in cells that make and release mucus and other fluids). The cells that form squamous cell carcinoma and adenocarcinoma develop in the inner lining of the bladder.

Transitional cell carcinomas can be grouped depending on the differentiation grade and tumor infiltration of the bladder by following the AJCC recommendations (AJCC staging manual 2010).

Bladder cancer is considered to be the fourth most common cancer worldwide and the ninth frequent cause of death from cancer in men (Jacobs et al. 2010). During 2010, 70530 new cases of bladder cancer will be diagnosed and 14680 patients will die in the United States. The median ages of patients presenting cancer are 72 in men and 74 in women. The disease affects a higher percentage of men than women (ratio 3:1) (Horner et al. 2009).

The risk factors associated with bladder cancer have been described based on data obtained from the clinical observation, epidemiologic and experimental investigation, and genetic analysis of bladder carcinogenesis. Environmental factors include Cigarette smoking (Brennan et al. 2000; American Cancer Society 2010), chronic irritation of the urinary tract caused by infection, catheters and bladder stones (Mostafa et al. 1999; Shokeir 2004; Golijanin et al. 2006), many chemicals including aniline dyes and cyclophosphamide (Delclos et al. 2008; Nilsson et al. 2008). In contrast, an increased fluid intake may reduce the risk of bladder cancer (Michaud et al. 1999; Leppert et al. 2006). There is no strong epidemiologic evidence for a hereditary cause of bladder cancers (Golijanin et al. 2006). However, different studies have found a correlation between the risk of bladder cancer and genetic polymorphisms in detoxification (Green et al. 2000; Marcus et al. 2000; Wu 2005; Garcia-Closas et al. 2005; Choudhury et al. 2008) and cytokine genes (Ahirwar et al. 2008 and 2009; Liao et al. 2010).

2.2. Immune recognition of cancer

The idea of the role of the immune system in the repression of cancer was first proposed by Paul Ehrlich in 1909 (Ehrlich 1909). However, this idea had to wait 50 years to be sustained due to the development of the immunology field. The hypothesis of tumor immunosurveillance was formally proposed by Burnet and Thomas (Burnet 1957; Thomas 1959, 1964 and 1970) and was corroborated by animal experiments in which mice were immunized against syngeneic transplant of tumors (Old 1964; Klein 1966). Following, several studies were conducted to test the prevalence of tumor development in immunocompromised mice (Grant 1965 and Miller; Nishizuma et al. 1965; Trainin 1967; Bursteinand and Law 1971; Kaplan 1971; Sanford et al. 1973; Stutman 1975). These studies only found a higher rate of tumors induced by virus and lymphomas, failing to prove or disprove the hypothesis. In addition experiments using athymic mice (Nude mice) didn't reveal significant correlation between immunodepression and tumorogenesis (Rygaard and Povlsen 1974; Stutman 1974; Outzen et al. 1975). These results led to an abandonment of the immunosurveillance hypothesis.

Further studies demonstrated that nude mice still have a low population of αβ T cells, a normal population of γδ T cells and a higher population of Natural Killer (NK) cells (Maleckar and Sherman 1987; Hayday 2000; Pardoll 2003). In the nineties, the immunosurveillance hypothesis was resurrected by the knowledge and development of new murine models. Engel and coworkers described how combined immune deficiency mutation (SCID) mice develop more tumors than controls (Engel et al. 1997). Dighe and coworkers demonstrated that fibrosarcomas grow faster in mice treated with antibodies against interferon (INF) γ (Dighe et al. 1994). Similar results were found in mice with mutations in genes encoding interferon γ receptor or signal transducer and activator of transcription 1 (STAT1) (Kaplan et al. 1998). Mice without perforin, a component of cytolitic granules of cytotoxic T cells, are also more susceptible to tumor development (Street et al. 2001). The development of mice carrying a genetic mutation which impairs lymphocyte receptor expression also revealed a higher susceptibility of these mice to develop tumors (Shankaran et al. 2001). Based on these studies a protective effect of elements of immune system against cancer was verified.

Proving that the hypothesis is also true for humans was likewise difficult. Epidemiology studies shows that immunedeficient patients have a higher cancer incidence

(Penn 1999), however most of these tumors have a viral etiology, like Epstein-Barr virus, herpesvirus or papilloma virus, impairing the direct effect of the immune system on tumor development. However other epidemiological studies as well show a higher incidence of non-viral tumors, like melanoma, non-Kaposi sarcoma, colon, lung, bladder, kidney and endocrine tumors (Sheil 1986; Birkeland et al. 1995; Penn et al. 1995ab y 1996; Dunn et al. 2004; Trofe et al. 2004; Kauffman 2006). In addition the T cell infiltration into the tumor and an immune transcription profile are good prognosis factors for patient survival (Naito et al. 1998; Pages et al. 2005).

Analyses of tumor recognition mediated by the immune system led to the discovery of tumor specific antigens (TSA) (Mumberg et al. 1996) and tumor associated antigens (TAAs) (van der Bruggen et al 1991). TSA are originated by mutation and are present only on tumor cells, TAAs can also be expressed in normal cells. Both of them can be recognized by T cells via HLA presentation. New TAAs are continuously been described (Paschen et al. 2004; Olsen et al. 2010) and can be grouped in five categories (Van den Eynde and Brichard 1995; Boon and van der Bruggen 1996; Rosenberg 1999; Old 2003; Simpson et al 2005; Antonia et al. 2006; Cloosen et al. 2007; Schietinger et al. 2008; Bioley et al. 2009; van der Bruggen et al. 2009):

- Differentiation antigens as Melan-A/MART-1 or gp-100.
- Mutations, like abnormal p53.
- Over expressed antigens like HER-2/neu.
- Germ line antigens like MAGE.
- Viral antigens like HTLV-1 p40x (Koenig et al.1993), LMP2 from EBV (Li et al. 2007) and HPV-16 E7 (Yin et al. 2009)

The mechanisms inducing cancer rejection in humans remain to be defined. It is suggested that the immune response against tumors starts with the activation of the innate immune response during tissue alterations and invasion by tumoral cells (Carmeliet and Jain 2000; Vicari and Caux 2002). Innate immune cells can detect “danger signals” released during this process and migrate to the tumor (Matzinger 1994). Subsequently, cytokines such as interleukin (IL)-1, 15, tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), INFs…are released as well as cytotoxic molecules that directly

affect tumoral growth (Dunn et al. 2004; Smyth et al. 2005). Immature dendritic cells (DCs) reach the tumor acquire TAAs (Li et al. 2002; Srivastava 2002). Mature DCs then migrate to the lymph nodes and present the TAAs to Th cells (Sallusto et al. 2000). Th cells in turn promote and active specific Tc cells against TAA expressing cells (Huang 1994).

2.3. Cancer Immunotherapy

The involvement of the immune system in tumor recognition and rejection has prompted the development of immune based cancer treatment. The goal of tumor immunotherapy is provision of either active or passive immunity against malignancies by harnessing the immune system to target tumors (King et al. 2008). Tumor immunotherapy can generally be classified as **A**) passive (or adaptive), consisting of administration of cells or antibodies ex vivo, and **B**) active, represented by vaccines, aimed at eliciting a specific immune response against TAAs.

2.3.1 Passive immunotherapy

Monoclonal and chimerical antibodies featuring tumor cell specificity have been approved and used against different cancers (King et al. 2008). Monoclonal antibodies exert their effects via mechanisms which include triggering apoptosis, activating cellular cytotoxicity, blockade of growth factor receptors and the activation of complement. Clinical success was achieved with passively acquired monoclonal antibodies directed against a number of TAAs targets (Slamon et al. 2001; Ferrara et al. 2004; Quezada et al. 2006; Pescovitz 2006; Hudis 2007; Kurai et al. 2007; Ferris et al. 2010). This class of cancer therapeutics continued to grow rapidly, with a notably toxic side effect profile than conventional chemotherapy and radiotherapy.

Adoptive cell therapy is a new passive immunotherapy based in the ex vivo selection of antitumor cells. The most frequently administered one is adoptive T cell therapy. Antigen-specific T cells are isolated and expanded in vitro, and finally transferred into patients (Rosenberg and Dudley 2009; Morgan et al. 2010; Hershkovitz et al. 2010; Brenner et al. 2010). This type of treatment was also carried out with T γδ, DCs, NKs and artificial antigen-presenting cells (Gomes et al. 2010; O´Neill 2010; Turtle et al. 2010; Geller et al 2011).

2.3.2 Active immunotherapy

The first description of successful cancer immunotherapy came in 1890 by William Coley. He inoculated *Streptococcus pyogenes* in patients within operable tumors with limited success. Once he incorporated *Serratia marcescens* to improve the toxicity, he successfully treated sarcoma with this mixture composed of heat-killed *S. pyogenes* and *S. marcescens*,

which became known as ‘‘Coley’s Toxins’’ (Coley 1991). Unspecific stimulators of the immune system are still used for cancer treatment. At the present time, Bacillus Calmette-Guerin (BCG), imiquimod, aluminum-based salts and a squalene-oil-water emulsion are approved for clinical use (Ebensen and Guzman 2008; Karve et al. 2008). These molecules can locally or systemically induce the immune response (Böhle 2003) and are used as vaccine adjuvant like M-VAX therapy, which consist in autologous tumor cells with dinitrophenyl (DNP) mixed with bacilli Calmette-Guerin (BCG) as an immunological adjuvant (Berd 2004). These molecules can also be used alone, like imiquimod as topic therapy against basal and squamous cell carcinoma (Love et al. 2009) or the standard use of BCG in pT1G3 bladder cancer (Demkow et al. 2008; Babjuk et al. 2009).

Cytokine therapy is based on the administration of different molecules with the aim of activating the immune system against cancer cells. There is a broad spectrum of cytokines used for cancer elimination in clinical trials either individually or in combination; among them GM-CSF, IL-12, IL-7, TNF-alpha, IL-15, IL-18, IL-21, IL-23, IL-27, IL-21 (Heaton 1993; di Carlo et al. 2007; Weiss et al. 2007; Jinushi et al. 2009; Yoshimoto et al. 2009). Moreover a group of cytokines has already been approved by the FDA (Food and Drug Agency) for clinical use. Interferon-α-2b is approved for patients with high-risk locally advanced melanoma (Garbe et al. 2008). It has demonstrated a significant survival benefit versus placebo-treated patients (Kirkwood et al. 1996). IL-2 has been extensively used for metastatic renal cell cancer and melanoma (Wang et al. 2008). Further granulocyte colony-stimulating factor (G-CSF) has been used as standardized cancer treatment (Repp et al. 1995).

Different tumor vaccines have been broadly used in cancer treatment. There exist two major groups of cancer vaccines; vaccines against oncogenic virus and vaccines against tumoral antigens.

Vaccines against viruses enhance the immune response versus pathogens inductor of cancer like Human papillomavirus (HPV) in cervical cancer (Stanley 2010; Mariani and Venuti 2010).

Tumor antigen vaccines are characterized by an administration of TAAs in order to break tumor tolerance (figure 1) (Coulie et al. 2001; Marchand et al. 2001). Different tumor vaccines are using different ways to improve the stimulation of the immune system against tumoral cells: autologous whole tumor cell vaccines, allogenic vaccines derived from whole tumor cells, peptide vaccines, vaccination with plasmid DNA and viral vectors encoding tumor antigens, ganglioside vaccines as well as dendritic cell vaccination (Terando et al. 2007; Vergati 2010; Shashidharamurthy et al. 2011). These therapies can activate the immune response against TAAs (Boon et al. 2003; Van Baren et al. 2005) and generate specific anticancer CTLs (Coulie et al. 2002; Godelaine et al. 2003).

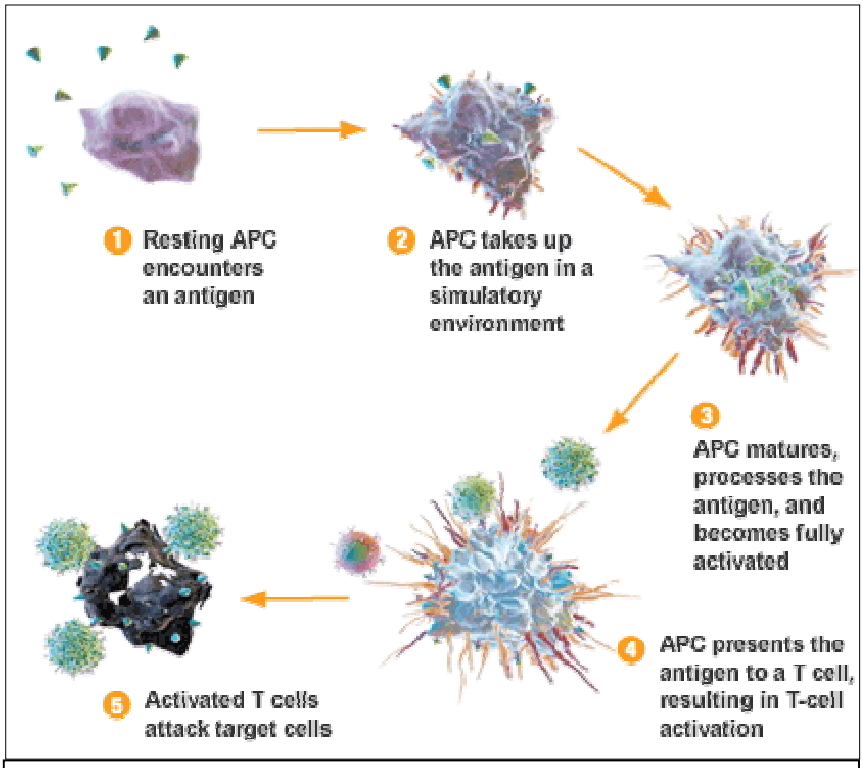

Figure 1: Activation of T cell for target lysis. From Pharma's Cutting Edge (http://www.pharmaweblog.com/?p=278)

2.3.3 Reduced success of Immunotherapy in clinical trials

In spite of the development of different immunotherapies, there has not been a significant improvement in the overall success rate of these therapies (Rosenberg and Dudley 2004; Rivoltini et al. 2005; Hersey et al. 2005). Passive immunotherapy with different antibodies showed an objective response of 5-15%, with just a 3% of complete response (Fong et al. 2006; Choi et al 2007; Peggs et al. 2009). Cytokine therapy have an overall objective response in 9-16% of patients, INF α2b shows a 16% of objective response, and 5% of complete response (King et al. 2008; Tarhini et al. 2009; Rosenberg and Dudley 2009). IL-2 treatment has a 9,7% of responding patient, this percentage growth to 22% when the therapy

is combined with peptide vaccination, however complete response is only observed in a 5% of patients (Lee et al 1999; Bhatia et al. 2009). Cancer vaccine therapies only shows an overall response rate of 3,3%. Even the adoptive therapy with pre-treated DCs shows no more than a 7% of success (Claesson 2009; Rosenberg et al. 2004). Adoptive T cell therapy has achieved the higher response rate in preliminary studies. The objective response rates vary from 13 to 70% depending on the different approach used. However a complete response is reduced to a 10-32% of patients (Rosenberg and Dudley 2009; Morgan et al. 2010).

It appears that the generation of antitumor T cells in cancer patients is necessary but not always sufficient to mediate the regression of established cancers (Lee et al. 1999). Immunoselection of immune escape mechanism by the tumor cells could impair the correct function of these T cell and could be the explanation of the poor outcome immunotherapy (Pawelec 2004; Ahmad et al. 2004; Rosenberg and Dudley 2004; Morgan et al. 2006; Rosenberg and Dudley 2009; Geldmacher et al. 2011). In addition immunotherapy protocols induce toxicity in a high percentage of patients, therefore this kind of therapy has to be administrated with caution and a strait monitoring of the patient is required.

2.4. Tumor Immune escape

Tumor cells have the ability to develop sophisticated immune escape mechanisms which enable proliferation in the presence of the immune system (Seymour et al. 1999; Villunger and Strasser 1999; Mapara and sykes 2004; Zou 2005). Tumor escape mechanisms can be subdivided into six different categories (Pawelec 2004; Drake et al. 2006).

2.4.1. Alteration of MHC class I and tumor antigen expression

The class I molecules encoded by the Major Histocompatibility complex (MHC) are cell surface glycoproteins that play a fundamental role in the regulation of immune responses. MHC class I molecules are necessary for the presentation of peptide antigens to cytotoxic T lymphocytes (CTLs) (Townsend et al. 1986) and for the immune regulatory activity exerted by NK cells (Ljunggren and Kärre 1990).

MHC class I molecules contain the classical (class Ia) human leukocyte antigens (HLA)-A, -B, and -C in humans and H-2K, D, and L in mice, and the non classical (class Ib) E, F, and G in humans (Bjorkman et al. 1987). They form a trimolecular complex consisting of a 45-kDa heavy chain (HC), peptide antigen, and the nonpolymorphic 12-kDa β2-microglobulin (β2-m) light chain. The HLA Class Ia is highly polymorphic (Bjorkman and Parham 1990). HCs are encoded by genes located on chromosome 6, whereas β2-m is encoded by a gene mapped on chromosome 15. The classical HLA class I molecules are predominantly expressed at the surface of most mammalian cells with only a few exceptions (Daar et al. 1984; Le Bouteiller 1994). Classical studies were mainly focused on studying the overall surface expression, without analyzing the behavior of the different loci or alleles in detail, or quantifying the expression levels. Only recently our group started to apply more advanced techniques to study HLA class I expression in normal tissue to elucidate locus-specific gene expression (Garcia-Ruano et al. 2010; Saenz-Lopez et al. 2010). It is estimated that up to 250,000 HLA class I molecules of each type are expressed on the surface of a somatic cell (Parham and Ohta 1996).

Antigenic peptides which are presented to CTLs on the surface of cancer cells can be recognized and bound by molecules of MHC class I. The formation of these peptides is called antigen processing (figure 2) and starts with the degradation of endogenous proteins by the antigen processing machinery (APM) (Maffei et al. 1997; Van Endert, 1999; Kessler et al.

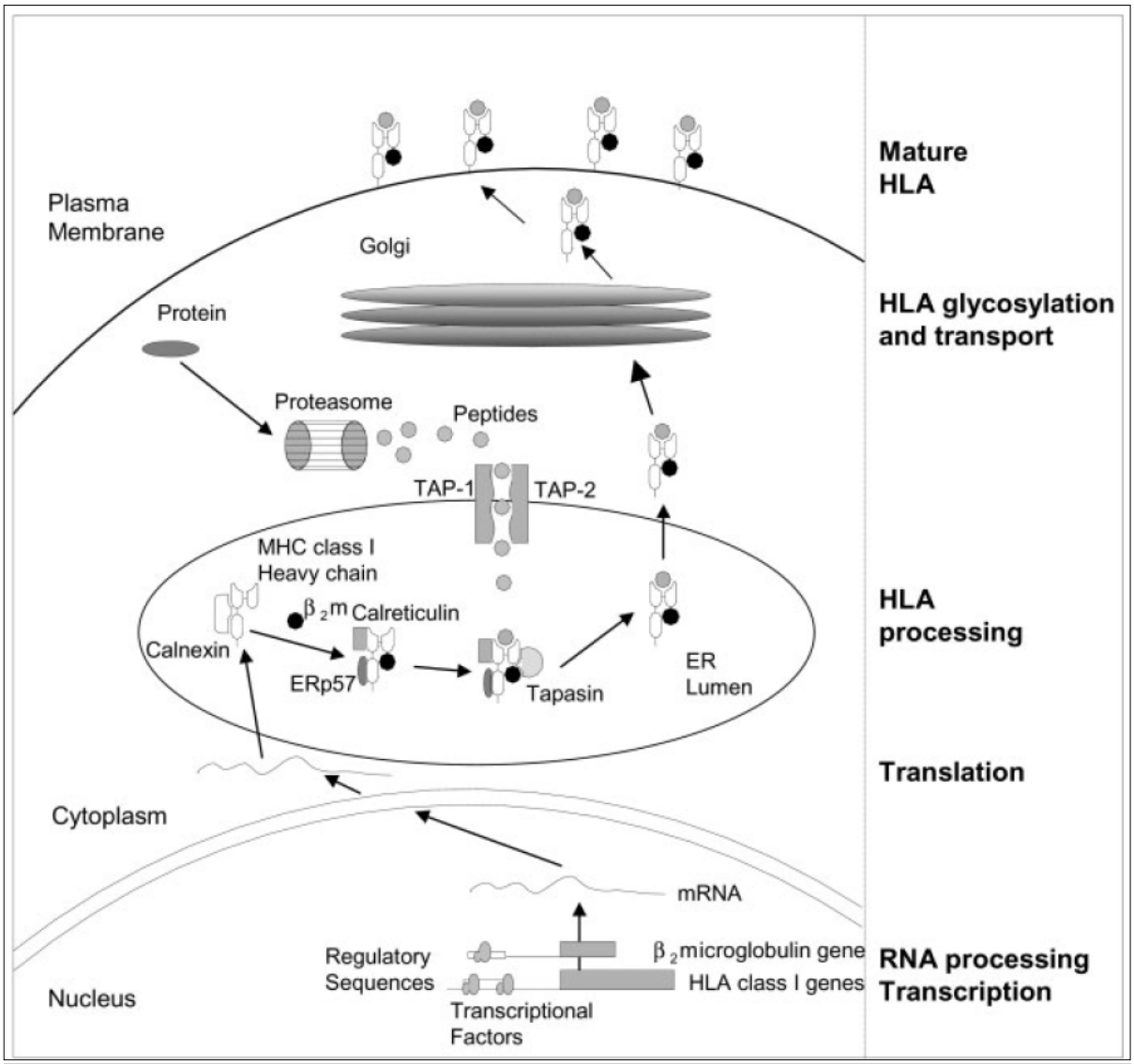

Figure 2: Antigen processing and presentation. From Garcia-Lora et al. 2003

2011). Protein fragments are transported into the endoplasmatic reticulum (ER) by the transporter associated with antigen processing-1 and -2 (TAP1 and TAP2) (Maffei et al. 1997; Van Endert, 1999). The produced peptides are transported into endoplasmatic reticulum (ER) by TAP1 and TAP2 (Abele and Tampé 1999). The folding and assembly of a complete MHC class I molecule depends on the association of the HC first with b2-m and then with the peptide in the ER. For this process various accessory proteins with a chaperone-like function are necessary, such as calnexin, calreticulin, endoplasmic reticulum protein 57 (ERp57) and tapasin (Grandea and Van Kaer 2001). Trimolecular complex leaves from the ER through the Golgi secretory pathway and displays on the cell surface. This event is referred to as antigen presentation. Any defect in these processes will prevent the expression of MHC class I molecules on the cell surface (Koopman et al. 1997; Parmer and Cresswell 1998). The

interaction between HLA and T cell receptor (TCR) triggers a cascade of T-signaling events that lead to cell proliferation, cytokine production, and lysis of target cells (Johnsen et al. 1999; Seliger et al. 2000).

MHC class I antigens also regulate the lytic activity of NK immune cells (Moretta et al. 1996; Lopez-Botet et al. 2000; Vilches and Parham 2002; Schwinn et al. 2009), which is related to their ability to kill target cells lacking MHC class I expression via immunoglobulin-like receptor (KIR). The detection of targets by NK cells is complex and it is regulated by many different membrane receptors and cytokines (Bashirova et al. 2006; Parham 2008). Alterations in the expression of any of the MHC class I subunits can affect both T and NK cell-mediated immunity. These alterations may affect the tumorigenic phenotype as well as metastatic capacity (Villunger and Strasser 1999; Garrido and Algarra 2001).

The loss of MHC class I molecules is a frequent mechanism found in experimental and spontaneous tumors to escape recognition by the immune system (Garrido et al. 1993 and 1997). This escape strategy is used widely by tumor cells, allowing them to not being recognized as targets by immune effectors. The loss of an MHC antigen associated with the H-2Kk class I molecule was first described for the first time in 1976 in a mouse lymphoma (Garrido et al. 1976). One year later, in 1977, HLA losses were also detected in human tumors (Pellegrino et al. 1977). In following years, the frequency of such losses was evaluated by studying series of tumor samples using monoclonal antibodies (mAbs) directed against HLA class I molecules.

Initial works showed only a small fraction of HLA class I loss (Ferron et al. 1989; López-Nevot et a. 1988). For these studies antibodies against a common HLA epitope were used and could therefore just detect total losses. The development of antibodies directed specifically against HLA-A or -B locus or against HLA allelic epitopes has increased the rate of HLA class I alterations in different human tumor samples (Garrido et al. 1993; Garrido et al. 1997; Koopman et al. 2000). The rates of HLA class I losses in some tumors were estimated to be 96% in cervical carcinomas (Koopman et al. 2000), 96% in breast carcinomas (Cabrera et al. 1996), 87% in colorectal carcinomas (Cabrera et al. 1998), and 70% in laryngeal carcinomas (Cabrera et al. 2000).

To define the MHC class I deficiencies of tumor cells carefully is of particular importance, since heterogeneous populations of tumor cells exist in tumor tissues. Our

laboratory has developed new strategies which allowed extensive analyses of the MHC altered phenotypes found in a variety of human tumors (Cabrera et al. 2003a). The description of MHC altered phenotypes is useful in order to select additional strategies to analyze the molecular mechanisms responsible for such alterations. Seven major altered HLA class I phenotypes have been defined in different tumor tissues (Figure 3) (Garrido et al. 1997; Garrido and Algarra 2001).

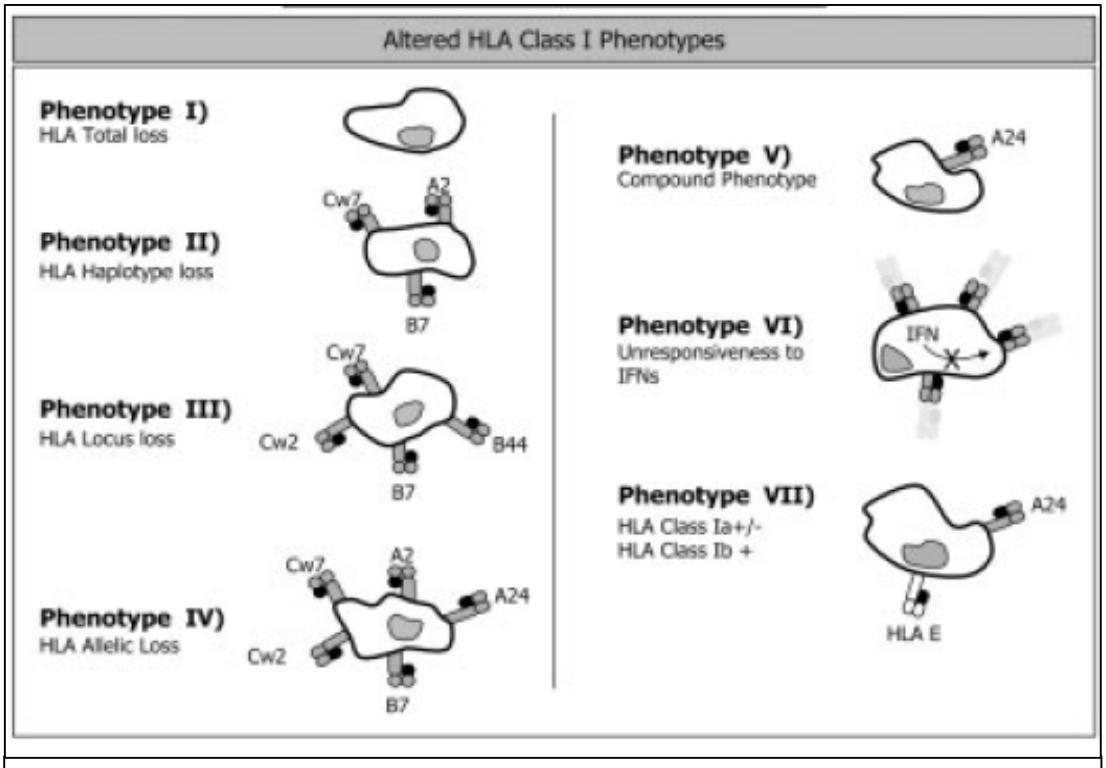

Figure 3: Altered HLA class I phenotypes. From Garcia-Lora et al. 2003

❖ **Phenotype I: HLA class I total loss.** This phenotype is characterized by the absence of any HLA class I antigen expression in tumor cells.

❖ **Phenotype II: HLA haplotype loss**. Tumors can partially or entirely lose one HLA haplotype.

❖ **Phenotype III: HLA locus loss**. This altered phenotype is found when both allele products of HLA A, B, or C loci are coordinately downregulated.

❖ **Phenotype IV: HLA allelic loss**. This alteration is defined as the loss of a single HLA class I allele.

❖ **Phenotype V: Compound phenotypes**. This phenotype appears by the combination of two different alterations.

- **Phenotype VI:** HLA class I downregulation with no response to interferon.
- **Phenotype VII:** HLA class I downregulation with expression of non-classical HLA class I molecules.

2.4.2. Deregulated expression of adhesion / accessory molecules by tumor and/or antigen-presenting cells

Tumor-antigen presentation by dendritic cells is critical for a correct CTLs activation. The expressions of accessory molecules by tumor cells also increase their susceptibility to these CTLs. Both DCs and tumor cell accessory/costimulatory molecule expression can be deregulated in cancer and contributes fundamentally to tumor escape. DCs can lose MHC class II expression (Ciavarra et al. 2003) or costimulatory molecules CD80 (B7.1) or CD86 (B7.2) (Chaux et al. 1996) and induce T cell anergy. Tumor cells frequently feature a decreased level of adhesion molecules, such as inter-cellular adhesion molecule 1 (ICAM-1) (Vora et al. 1997), which lead to a worse prognosis (Fujihara et al. 1999). The absence of TNF receptor superfamily member 5 (CD40) on epidermal tumors has been suggested to facilitate escape (von Leoprechting et al. 1999; Holub et al. 2003; Linderoth et at. 2003). Complete knowledge of all possible receptors and interactions might be beneficial in manipulating responses in the desired direction (Watanabe et al. 2003).

2.4.3 Secretion of immunosuppressive soluble factors either by tumor cells or infiltrating T cells or both

Cancer patients can exhibit a notable variety of immunosuppressive factors. Soluble forms of adhesion molecules such as lymphocyte function-associated antigen 3 (LFA-3) and others may correlate with disease progression (Grothey et al. 1998; Sanchez-Rovira et al. 1998). Further, an inhibitory effect of gangliosides at the level of antigen-presenting cell function has been discussed (Shen et Ladisch 2002; Peguet-Navarro et al. 2003). In addition to that, serum levels of soluble Fas (CD95) may also contribute to tumor escape (Tsutsumi et al. 2000; Bergmann-Leitner et Abrams 2001). The immunmodulatory enzyme 2, 3-indoleamone dioxygenase (IDO) appears to inhibit T-cell responses through the tryptophan catabolism (Munn et Mellor 2007). Adenosine can inhibit IL-12 and stimulate IL-10 production by monocytes, contributing to these suppressive effects (Link et al. 2000).

Many immunoregulatory cytokines can be secreted by either tumor, immune system or both (Pawelec 2004). The best known examples are tumor growth factor (TGF)-β and IL-10 (Carbone et al. 1999; de Vita et al. 2000; Steinbrink et al. 1999; Shariat et al. 2001), but also other ones like IL-6 are associated with a worse chance of survival and a greater extent of disease (Fayad et al. 2001; Salgado et al. 2003).

2.4.4. Induction of immune nonresponsiveness via anergy induction or clonal deletion of responding T cells

The clonal deletion of T lymphocytes was first described by Lauritzsen and coworkers (Lauritzsen et al. 1998). He discovered that typical thymus proteins are secreted from tumors. These proteins cause clonal deletion of newly generated T cells. The interaction of Fas ligand with the death receptor Fas can induce T cell apoptosis. Many types of tumors have been reported to express Fas ligand. (Reichmann 2002).

mors can also induce T cell anergy. It is possible that, T cell activation and effector function are ultimately controlled by a balance between costimulatory molecules (e.g. CD28) and coinhibitory molecules (Chen et al. 2004). The inhibitory pathway is mediated by the interaction between cytotoxic T lymphocyte antigen 4 (CTLA-4) and its ligands (B7-1 and B7-2) on antigen presenting cells. CTLA-4 blockade exerts a pronounced antitumor effect (Chambers et al. 2001). Clinical trials have finally developed anti-CTLA-4 monoclonal antibodies for clinical use (Callahan et al. 2010). Another inhibitory receptor is programmed death 1 (PD1), which serves as a marker for T cells that have been exhausted by persistent exposure to viral antigen. PD1/B7-H1 interaction prevents the lysis of target cells by CD8 T cells. In vivo blockade of either PD1 or of the PD1 ligand B7-H1 potentiates an antitumor immune response (Dong et al. 2002; Hirano et al. 2005)

Antigen-specific CD8 T cells do not appear to be specifically deleted, but rather enter in exhaustion. This process impairs CD8 T cell response to persistent tumor antigens (Drake et al. 2006). The phenomenon of CD8 T cell exhaustion is very interesting from the perspective of immunotherapy. Den Boer and coworkers (den Boer et al. 2004) showed that CD8 T cells isolated from tumor bearing hosts could still be functional in tumor free animals, mediating protection from tumor challenge. If the immunotherapy is able to revert CD8 exhaustion, these cells will attack the tumor.

2.4.5. Induction of suppressor cells

T regulatory (Treg) cells play an important role in many aspects of immunological tolerance. They regulate the immune response to avoid an excessive response and autoimmunity (Sakaguchi et al. 2001; von Boehmer 2005). Treg cells in tumors promote the tolerance against tumor cells (Mougiakakos et al. 2010). Different studies revealed that depletion of $CD4^{+}CD25^{+}$ can prevent tumor progression in mouse and human (Onizuka et al. 1999; Shimizu et al. 1999; Terabe and Berzofsky 2004). The presence of such regulatory T cells in ascitis fluid of women with advanced ovarian cancer was associated with decreased survival (Curiel et al. 2004). T helper cells type 2 (Th2) can also suppress the cellular immune response because of IL-4 and IL-10 secretion. Therefore, an infiltration of Th2 cells inside the tumor could impair the immune cellular response.

Suppressor macrophages may induce alterations in molecules involved in signal transduction. Macrophages with the capacity to suppress immune responses in tumor bearing hosts have been extensively described (Mantovani et al. 1992; Alleva et al. 1995). Tumor infiltrating macrophages can decrease CD3ζ expression on T cells (Aoe et al. 1995). Chronic inflammatory conditions in advanced cancer will alter the redox potential of macrophages, causing them to exert an immunosuppressive effect (Pawelec 2004).

2.4.6. Changes in T cell signal transduction molecules

The observation that T cell signal transduction is compromised in tumor-bearers (Mizoguchi et al. 1992) has subsequently been confirmed and extended to a variety of human tumors (Whiteside et al. 1992), Down-regulated TCR signal transduction may be related to down-regulation of CD28 (Hakansson et al. 1999). T cells were found to be CD3ζ deficient, which could be at least partially reversed by stimulation with CD3 mAb, IL-2 and IFN-α (Chen et al. 2000). This may reflect T cell replicative senescence caused by continuous antigen activation, which could contribute to tumor escape from immunosurveillance (Effros and Pawelec 1999; Pawelec 1999).

2.4.7. Immune stimulation

It has been suggested that the immune system can exert a dual effect on tumors, sometimes encouraging their growth (Ponzio et al. 2000; Guindi et al. 2000). An efficient

immune response lead to tumor progression. In contrast an incomplete, inefficient immune response will not eliminate the tumor and can even promote the tumoral cells expansion. The effect of certain cytokines produced by T cells on tumors may encourage tumor growth. One example for this in hematopoietic tumors is the response of B CLL to IL-4 produced by T cells. IL-4 is involved in protection of cancer cells against apoptosis and promotion of further growth (Dancescu et al. 1992). It has been reported that IL-10 may function as a growth-stimulating factor for melanoma as well as reducing cell surface expression of HLA and adhesion molecules (Yue et al. 1999). Tumor infiltrated lymphocytes (TILs) can also secrete angiogenic factors contributing to vascularisation of the tumors as basic fibroblast growth factor or vascular endothelial growth factor (Freeman et al. 1995) and factors directly stimulating tumor cells (Peoples et al. 1995). Tumors may also subvert the immune response by expressing receptors for T cell growth factors (Capelli et al. 1999).

2.5. Overlay and objectives of the thesis

During the last years researchers have developed several cancer immunotherapy protocols to improve disease-free survival of patients. However, the effectiveness of immunotherapy protocols has been below expectations. Only a small percentage of patients develop tumor regression after the treatment. There exists also a group of patients displaying a mixed response after the treatment, which consist in the regression of some metastasis and the progression of other ones. They represent an interesting subset of patients, allowing analyses of different tumor behaviors in the same patient at the same time. This allows us to consider exclusively the tumor's determining factors in immune responsiveness obviating variations in the genetic background of different patients or external variables affecting the potency of the treatment.

Generation of immune escape mechanism by tumor cells is believed to be an important explanation for the poor outcome of the T cell based immunotherapy. One of the best documented routes of cancer immune escape is the alteration of HLA class I antigen expression on tumor cells.

General objective:

- Analyze the role of the immune system during tumor regression and progression and the effects of HLA class I alterations on the response of transformed cells to the immunotherapy.

Specifics objectives:

- Characterize the HLA class I phenotype in bladder tumors and melanoma metastases from patients undergoing immunotherapy.
- Analyze the molecular mechanism responsible for alterations in HLA class I expression.
- Compare the HLA class I pattern between bladder tumors obtained before and after BCG immunotherapy.
- Evaluate the whole transcriptional pattern in progressing and regressing melanoma metastasis after immunotherapy.

Chapter 3.

Material Methods and Results

3.1. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy

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Abstract

Despite the potential efficacy of cancer immunotherapy in preclinical studies, it did not show yet significant positive clinical results in humans with only a small number of cancer patients demonstrating objective tumor regression. This poor clinical outcome can be explained by the generation of sophisticated tumor immune escape mechanism, in particular, abnormalities in the expression of HLA class I antigens. We have studied the expression of HLA class I antigens in ten metastatic lesions obtained from a melanoma patient undergoing immunotherapy. Five lesions were obtained after Interferon-alpha-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 PCR and immunohistological techniques. These results indicate a strong association between HLA class I expression and progression or regression of the metastatic lesions. Our data support the hypothesis that the level of HLA class I expression is an important parameter of tumor immune escape that needs to be monitored.

Keywords: Melanoma. HLA. Metastasis. Cancer.

Immunotherapy

Introduction

Metastatic melanoma has a poor prognosis, with a range for overall survival from 5 to 11 months (Sun and Schuchter 2001). In patient with progressing melanoma, high-dose interferon-alpha-2b is frequently used; however, a considerable proportion of patients fail this type of immunotherapy. Other types of cancer therapy, such as autologous vaccines, peptide vaccines, dendritic cells vaccination, vaccination with plasmid DNA and viral vector encoding tumor antigens, were developed as a more specific alternative treatment option

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(Rosenberg and Dudley 2004; Buteau et al. 2002; Terando et al. 2007). Different immunotherapy protocols can generate immune T cells capable of recognizing antigenic peptides presented on tumor cells (Marchand et al. 2001; Lonchay et al. 2004; Liu et al. 2004); however, these protocols did not generate the expected results (Rivoltini et al. 2005; Hersey et al. 2005; Rosenberg et al. 2004). Generation of immune escape mechanism by the tumor cells is believed to be an important explanation of the poor outcome of the T-cell based immunotherapy (Ahmad et al. 2004). One of the key mechanism of tumor immune escape is the alteration of HLA class I expression on tumor cells during cancer progression (Aptsiauri et al. 2007). Downregulation of HLA class I expression correlates with a poor response to immunotherapy (Benitez et al. 1998; Cabrera et al. 2007).

It is known that Interferon-alpha-2b inhibits tumor cells proliferation, induces inhibition of oncogenes, activates suppressor genes, and increases HLA class I expression (Gutterman 1994). The interferon-alpha-2b is also implicated in generation, activation, and survival of cytotoxic lymphocytes (Palmer et al. 2000). M-VAX is a vaccine composed of autologous melanoma cells treated with the hapten dinitrophenyl and Bacille Calmett-Guerin (BCG). This vaccine produces interferon-gamma-mediated T cell inflammatory response followed by delayed-type hypersensitivity either against modified melanoma cells (in the majority of the cases) or against non-modified melanoma cells (in about 50% of the cases). For vaccine details, see Berd D (Berd 2004).

There is little information on the different patterns of HLA class I alteration in metastases and its correlation with the progression of cancer (Cabrera et al. 2007; Mendez et al. 2001), since it is difficult to obtain several metastatic samples from a cancer patient during immunotherapy, especially regressing ones. Here we report the results of the analysis of HLA class I expression in ten metastases obtained from a melanoma patient as a result of treatment with two different types of immunotherapy. Five of them were obtained after treatment with Interferon-alpha-2b and five more after autologous vaccination plus BCG. We also analyzed the correlation between the HLA class I expression and tumor progression.

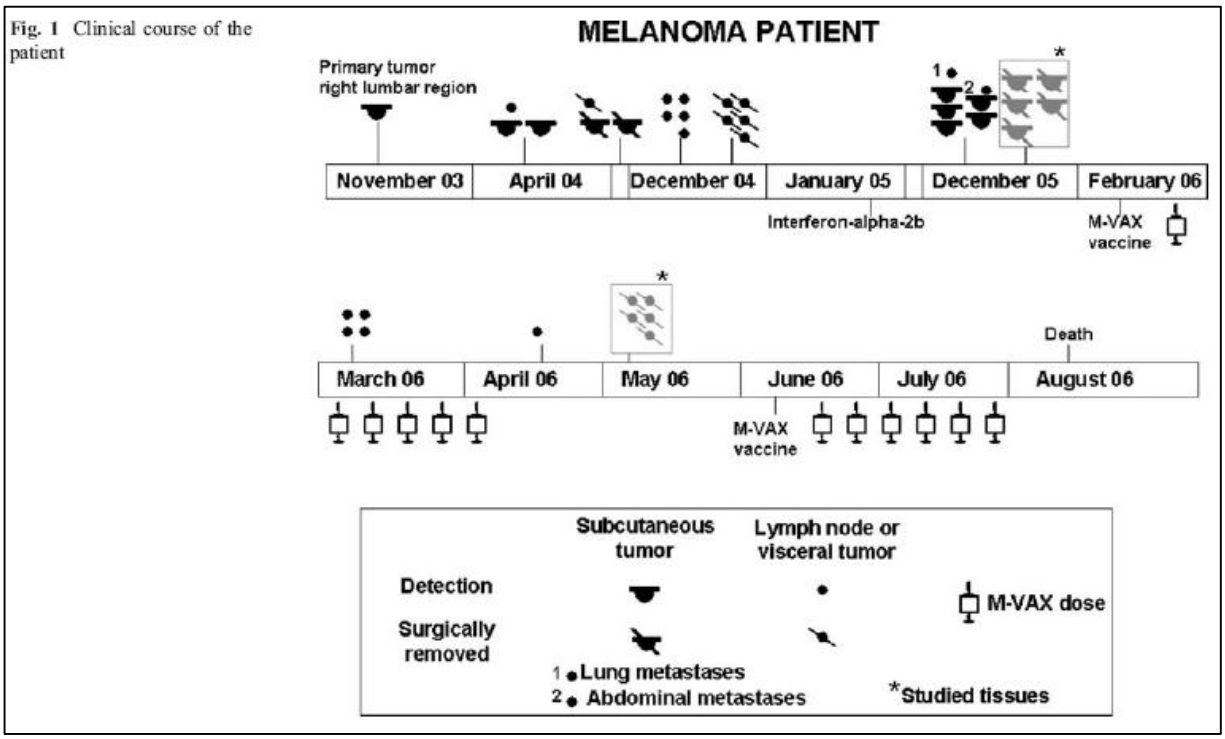

Fig. 1 Clinical course of the patient

Materials and methods

Patient, clinical protocols, and tumor specimens

We obtained ten metastases from a patient treated consequently with Interferon-alpha-2b and M-VAX vaccine. Five metastases were subcutaneous lesions removed from the patient after the Interferon-alpha-2b immunotherapy, and the other five were lymph node lesions obtained after autologous vaccination. Samples were collected at the Department of Dermatology, Hospital Virgen Macarena, Sevilla, Spain. Informed consents and approval of the research protocol by the institutional review board were obtained. Tumor samples were snap-frozen in liquid nitrogen-cooled isopentane.

In Fig. 1 we show a summary of the clinical condition of the patient. Primary tumor, on the right lumbar surface, was removed in November of 2003 (not available). From April of 2004 to December of 2004, several metastases were detected and removed. In January of 2005, the patient started a treatment with interferon-alpha-2b (Intron®) that lasted for 1 year, following the schedule of high doses of Kirkwood. In December of 2005, new metastases were detected, including lung and intestinal lesions. Five subcutaneous metastases were removed and sent to our laboratory. Four of these metastases (designated as MR-1, MR-2, MR-3, and MR-4) were regressing at the time of excision according to PET and CT scans, whereas one was progressing (designated as MP-5). From February to April of 2006, the patient was receiving the M-VAX vaccine according to the manufacturer's recommendations. During the administration of the vaccine, metastatic lesions were detected in five lymph nodes. In May of 2006, these lymph nodes were removed and sent to our laboratory. Four of these metastases (designated MR-7, MR-8, MR-9, and MR-10) were regressing at the time of excision according to PET and CT scans, whereas one (designated as MP-6), which is believed to be a relapse of MP-5, was progressing. Similarly, it was observed that some of the previously detected small lung nodules had regressed during the administration of the vaccine, while other nodules had progressed (Fig. 2). Between June and the end of August of 2006, the patient received another round of vaccination according to the manufacturer's recommendations. The patient died on August of 2006.

HLA typing of peripheral blood lymphocytes and immunohistological analysis

Autologous peripheral blood lymphocytes (PBLs) were isolated from the patient and

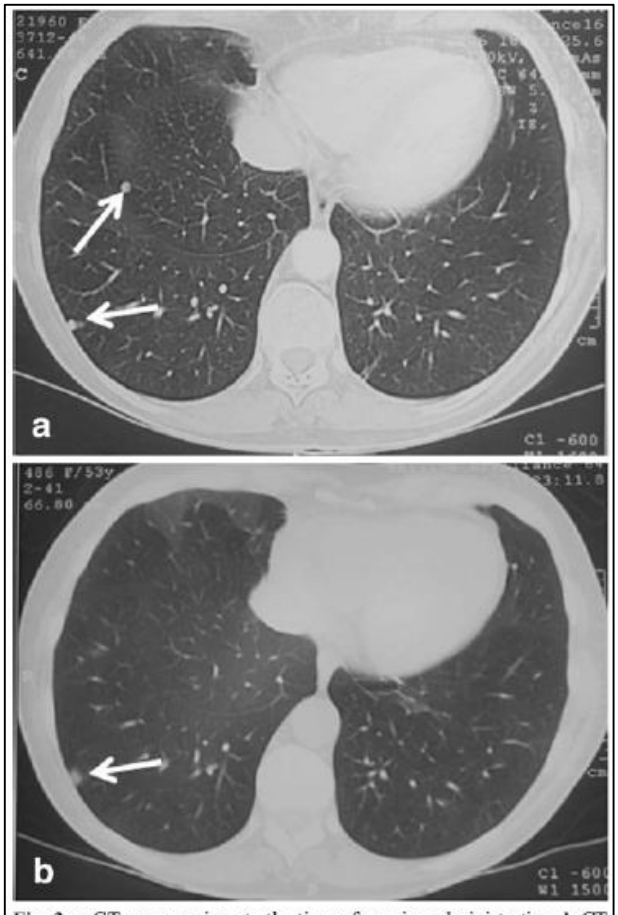

Fig. 2 a CT scan previous to the time of vaccine administration. b CT scan 3 months after starting vaccine administration. Note the progressing and regressing lung metastatic nodules indicated by the *arrows* before and 3 months after administration of the vaccine

HLA-typed in our laboratory using a low-resolution genomic sequence specific oligonucleotide analysis (SSO). Patient typing results: A 0201/2902; B 0702/4403; Cw 0702/1601; DR 1301/1501 DQ 0602/0603.

Immunohistological techniques were performed with the Biotin–Streptavidin System (supersensitive Multilink-HRP/ DAB kit, BioGenex). HLA class I expression was studied with the following mAbs: W6/32 (anti-HLA-ABC), GRH1 (anti-β2m), 1082C5 and A131 (anti-HLA-A locus; Lozano et al. 1989; Spear et al. 1985), 42-IB5 (anti-HLA-B locus; Lozano et al. 1990), HC-10 (anti-HLA-BC free heavy chain; Stam et al. 1986), H66 (anti-HLA-B12, One Lambda), 30-13-38 (De Pablo et al. 2003) and CR 11–357 (anti-HLA-A2), BB7-1 and KS-4 (anti-HLA-B7, kindly provided by Dr. Ferrone, University of Pittsburgh Cancer Institute, Pittsburgh, USA), MARB-7 (anti-HLA-Bw4), and 2BC4 (anti-HLA-Bw6, kindly provided by Dr. UchanskaZiegler, Humbolt University, Berlin, Germany). For HLA class II expression, we used GRB-1 mAb (anti-HLA-DR).

Tumor cells microdissection and DNA isolation

Cryopreserved tissue sections of 4–8 µm thick were stained with a 0.05% w/v solution of toluidine blue and were microdissected using a Laser micromanipulator (PALM Microlaser Systems, Olympus); microdissected fragments were collected in PALM Adhesive Caps. These fragments were used for RNA and DNA isolation. Normal tissue in the microdissected tumor section was not available.

DNA extraction was performed using the QIAamp DNA Mini Kit (QIAGEN, Westburg, Leusden, The Netherlands) following manufactured recommendation.

Microsatellite analysis

To determine the possible loss of heterozygocity (LOH) in chromosomes 6 and 15, DNA obtained from microdissected tumor and PBLs was studied with seven short tandem repeats (STRs), (D6S291, D6S273, C.1.2.C, C.1.2.5, D6S265, D6S105, and D6S276) mapping HLA region and D6S311 located in 6q. For β2-microglobulin studies, five STR markers that flanked the gene (DS15209, DS15126, DS15146, DS151028 and DS15153) were used. Methods of PCR reactions, electrophoresis, and data analysis were described previously (Maleno et al. 2006; Koene et al. 2004). LOH was assigned when a signal reduction of more than 25% in one allele was seen in tumor sample compared with control sample.

RNA isolation and reverse transcription

Total RNA was extracted from microdissected fragments using Absolutely RNA Nanoprep Kits (Stratagene, La Jolla, CA, USA) according to the manufacturer's recommendations. The extracted RNA was dissolved in 10 µl elution buffer supplied by the manufacturer. cDNA synthesis was performed with the RNA Reverse Transcription (RT) System (Promega Corporation, Madison, WI, USA) using 1 ηg RNA and following the manufacturer's instructions. Real-time quantitative PCR Various sections corresponding to each metastasis were used to obtain microdissected tissue areas for further RNA isolation and RT-PCR assay. RT products from the ten metastases were analyzed for the expression of various target genes (HLA class I heavy chain, β2m, HLA locus A, B, and C) by quantitative real-time PCR. The results of the analysis represent the mean of two

Table 1: Primers used for HLA class I locus -specific amplification

Primer	Sequence 5´ to 3´
A locus Fw 950	TC(C/T)TTGGAGCT(G/A)TG(A/T)TC(A/G)CT
A locus Bw 1146	AAGGGCAGGAACAACTCTTG
B locus Fw 950	TCCTAGCAGTTGTGGTCATC
B locus Bw 1089	TCAAGCTGTGAGAGACACAT
C locus Fw 957	TCCTGG(C/T)TGTCCTAGCTGTC
C locus Bw 1107	CAGGCTTTACAAGTGATGAG

independent microdissection experiments. To control for variations in the amounts of RNA, G6PDH and HPRT were tested as a housekeeping gene. All PCR reactions were performed in a Light Cycler using DNA Master Probes Kit (Roche Diagnostics, Manheim, Germany). For G6PDH, HPRT and β2m amplification we used commercial kits (Roche Diagnostics and Search LC, GmbH Heidelberg). Amplification reactions of HLA class I heavy chain was described previously (Cabrera et al. 2007). Amplification reactions of HLA class I specific locus were performed in a Light Cycler instrument using DNA Master Sylver Green kit (Roche Diagnostics, Manheim, Germany) in a final volume of 5 µl containing 1 µl cDNA, 4 mmol/lMgCl2, 0.5 µmol/l of each specific primer, and 1 µl LC-FastStart DNA master SYBR green. Primers used for HLA class I locus specific amplification are described in Table 1. After 10 min of initial denaturation at 95°C, the cycling conditions consisted of denaturation at 95°C for 6 s, annealing at 58°C for 10 s, elongation at 72°C 12 s and reading at 85°C. After amplification, the temperature was slowly raised above the melting point of the PCR product to measure the fluorescence for melting curve. mRNA levels for the target genes were calculated according to the calibration curves. All PCR products were checked by melting point analysis and by gel electrophoresis to verify the correct size of the products. PCR products of the HLA-B locus were sequenced for confirm specific amplification.

Results

We studied ten metastases from a melanoma patient who had undergone two types of immunotherapy. Five subcutaneous metastases were obtained after interferon-alpha-2b immunotherapy, and five affected lymph nodes were removed after autologous melanoma cell vaccination plus BCG (Fig. 1). During each immunotherapy protocol four metastases were regressing at the time of excision (MR-1, MR-2, MR-3, and MR-4 after interferon-alpha-2b and MR-7, MR-8, MR-9, and MR-10 after autologous vaccination), as evaluated by PETand CT, whereas two other ones (MP-5 after interferon-alpha-2b and MP-6 after autologous vaccination) were progressing.

Immunohistological techniques using specific mAbs directed against HLA-ABC, β2m, HLA-A locus, HLA-B and C free heavy chain, and HLA-A2 showed a strong positive staining in all the regressing metastases, whereas only weak positive staining was observed in the two progressing lesions (Table 2, Fig. 3). All metastases were HLA-B locus negative, as observed using immunohistological technique with anti-HLA-B locus, anti-B7, anti-Bw4, and anti-

Table 2: Immunohistochemical study of HLA Class I expression in the ten metastasis lesions (from MR-1 to MR10)

	HLA phenotypes after IFN-α-2b treatment					**HLA phenotypes after autologous vaccine**				
mAb	**MR-1**	**MR-2**	**MR-3**	**MR-4**	**MP-5**	**MP-6**	**MR-7**	**MR-8**	**MR-9**	**MR-10**
Anti-HC	+++	+++	++	++	+/-	+/-	+++	+++	++	++
Anti β2m	++	+++	+++	++	+/-	+/-	++	+++	+++	++
Anti-BC	++	++	++	+++	+/-	+/-	++	++	++	+++
Anti-A	+++	+++	+++	+++	+/-	+/-	+++	+++	+++	+++
Anti-B	-	-	-	-	-	-	-	-	-	-
Anti-Bw4	-	-	-	-	-	-	-	-	-	-
Anti-Bw6	-	-	-	-	-	-	-	-	-	-
Anti-B7	-	-	-	-	-	-	-	-	-	-
Anti-A2	+++	++	+++	++	+/-	+/-	++	+++	++	+++
Anti-DR	-	-	-	-	-	-	-	-	-	-

(-) Negative expression, (+/-)very weak expression, (+) positive expression

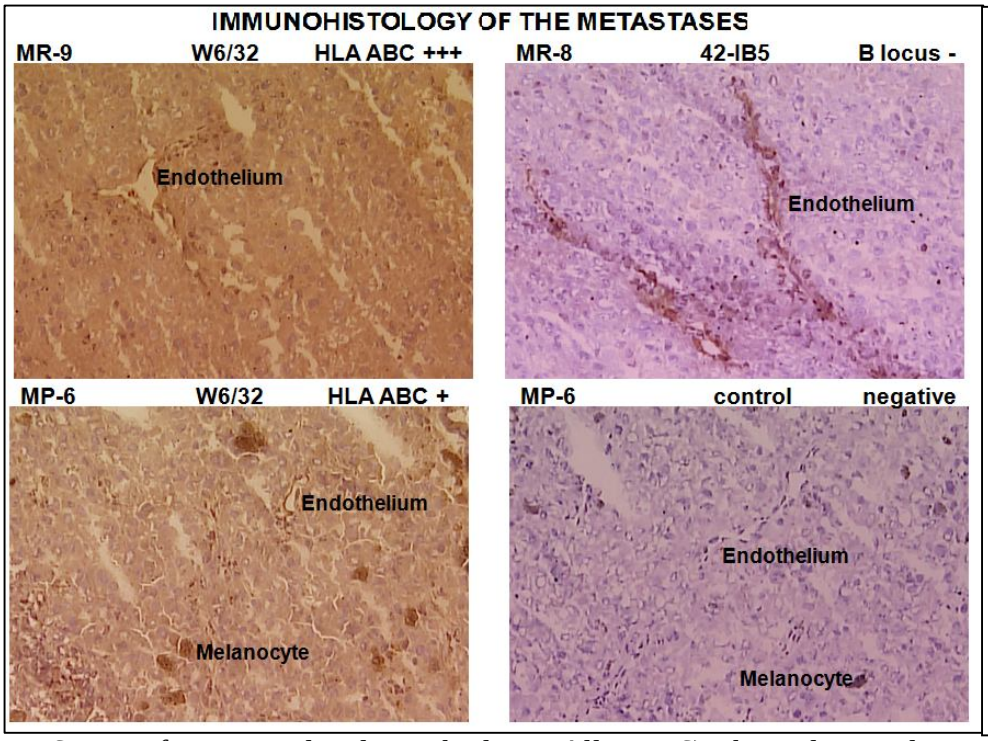

Fig. 3 Immunohistochemistry of frozen sections of the studied metastases: positive labelling of tumor cells with anti-HLA-ABC mAb in Regressing metastasis (MR-9), and weak staining in case of progressing metastasis (MP-6). All the metastases were negative for HLA-B locus.

Bw6 specific monoclonal antibodies. All metastases were negative for HLA class II expression.

Loss of heterozygocity was analyzed with eight microsatellite markers for chromosome 6 and five markers for chromosome 15. We found no LOH for chromosome 6 or 15 in none of the metastases.

cDNA obtained from microdissected tumor tissues was analyzed for heavy chain, β2m and HLA-A, HLA-B, and HLA-C loci. Figure 4 shows the results using a pairs of consensus primers for HLA-ABC and β2m. RQ-PCR assays of regressing metastasis (MR) showed higher levels of HLA-ABC mRNA in comparison with the progressing metastasis (MP) after both immunotherapy protocols (interferon-alpha-2b or autologous vaccination). Similar results were observed for β2m transcription. Results were obtained in two independent assays. We also studied HLA class I locus specific transcription (Fig. 5). Metastases obtained after M-VAX treatment had higher levels of HLA-A, B, and C mRNA as compared to the post-interferon-alpha-2b metastases, with the highest mRNA levels corresponding to locus C. Importantly, the two progressing metastases (MP-5 and MP-6) showed very low levels of mRNA corresponding to each studied locus. Thus, the downregulation in progressing metastasis affect the three HLA class I loci. These results correlate with the heavy chain data. All the transcription results were referred to the expression of G6PDH as a housekeeping gene.

mRNA levels corresponding to HLA-A locus correlated with the surface expression of the protein. In contrast, HLA-B locus, which showed similar mRNA levels than A locus, showed negative membrane expression by immunohistological techniques in all the metastases (Figs. 3 and 5).

Discussion

Growth of a particular tumor is not due, in most cases, to the existence of a deteriorated immune system, but rather to the acquisition by the tumor cells of new genetic and phenotypic features that allow them to escape antitumor immune responses (Garrido et al. 1997; Seymour et al. 1999). Evolution of genetically unstable cancer cells leads to the

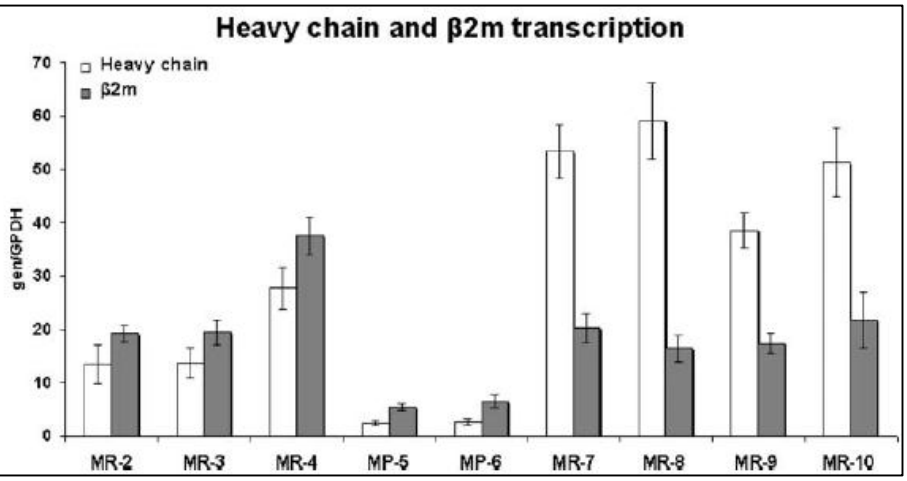

Fig. 4 HLA class I heavy chain (*white*) and β2m (*black*) transcription in the two groups of metastases: after interferon and after autologous vaccination. The figure shows the mean of two independent experiments

development of immune escape variants in tumor lesions that are selected out by the immune system. Consequently, different altered MHC class I tumor phenotypes may be produced (Garrido and Algarra 2001; Seliger et al. 2002). It has been proposed that the main contributor to the emergence of these tumor clones with MHC class I altered expression is T cell immunoselection (Garcia-Lora et al. 2002; Zitvogel et al. 2006). T cells can recognize tumor antigens presented by MHC class I positive tumor cells, thus performing effective immune surveillance. As a result, tumor cells that have better survival characteristic are selected.

Numerous attempts to develop an effective immunotherapy for the treatment of cancer patients based on stimulation of T cell reactivity against cancer antigens have been reported. However, the outcome of this type of treatment usually has little clinical success (Marchand et al. 2001; Lonchay et al. 2004; Liu et al. 2004). It is well known that a high proportion of tumors have alterations in the expression of HLA class I molecules and that these alterations represent an important tumor escape mechanism since they influence the interactions between tumor cells and specific T and NK cells in the course of malignant disease (Khong and Restifo 2002; Algarra et al. 2004; Aptsiauri et al. 2007). However, there is no sufficient information about patterns of HLA class I alterations at different metastatic sites within the same patient receiving immunotherapy. We have recently reported that the development of HLA class I alteration in melanoma metastases after autologous vaccination had a strong correlation with the progression of the disease (Cabrera et al. 2007). Here we report that HLA class I antigen expression on metastatic melanoma lesions could determine the course of the disease in a patient undergoing two types of immunotherapy protocols: interferon-alpha-2b and autologous vaccination plus

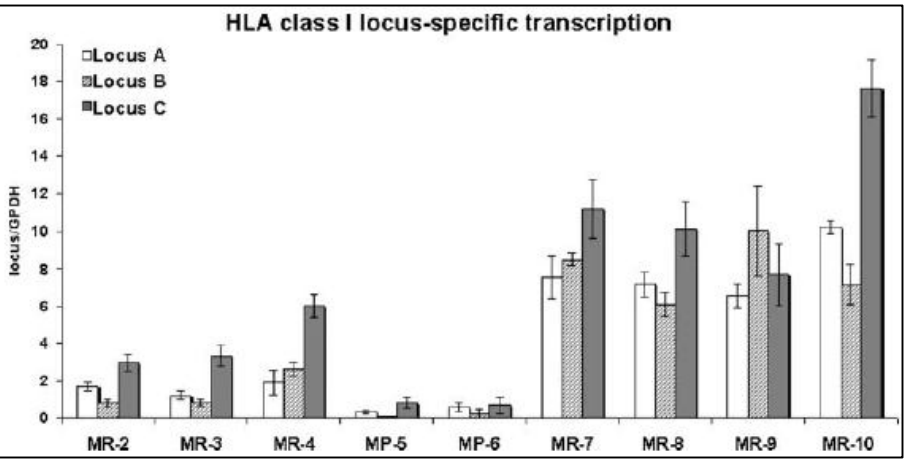

Fig. 5 HLA class I locus-specific transcription in the two groups of metastases: after interferon and after autologous vaccination. The figure shows the mean of two independent experiments

BCG (M-VAX). The progressing metastases (two out of ten) showed weak HLA class I expression, whereas the regressing ones (eight out of ten) showed a strong class I expression. Importantly, this correlation did not depend on the type of immunotherapy, since there was one progressing metastasis and four regressing ones in both types of treatment.

All the metastases showed an absence of the membrane expression of HLA-B locus. This alteration has been described in a variety of human tumors (Lopez-Nevot et al. 1989; Festenstein and Garrido 1986), in particular, in melanoma (Méndez et al. 2008; Versteeg et al. 1989). Therefore, we believe that downregulation of HLA-B locus detected in all the studied metastases have originated in the primary tumor (Fig. 6).

Staining with mAbs recognizing determinants of class I antigens has always been interpreted with caution, since the intensity of staining in immunohistochemical assays is a subjective parameter. Therefore, we also investigated mRNA transcription levels of HLA class I molecules. cDNA from microdissected tumor tissues was obtained and analyzed. We observed a significant difference in the mRNA levels of HLA heavy chain and β2m between progressing and regressing metastases. Progressing metastases had lower transcription levels of MHC class I and β2m than regressing ones.

We also have studied mRNA levels of each HLA class I specific locus and found that mRNA levels of HLA-A locus have a positive correlation with cell surface expression. In contrast, we did not observe such correlation for the HLA-B

locus; we detected high mRNA levels for HLA-B locus and no surface expression. Similar alteration has been found in a panel of melanoma cell lines where decreased HLA-B surface expression coincided with high mRNA levels (unpublished data). It has been described that B locus downregulation is the most frequent HLA alteration in melanoma (Marincola et al. 1994; Méndez et al. 2008). Other authors have showed that this alteration could be due to a lack of locus B transcription (Griffioen et al. 1999), but in our study, we detected B locus transcription, and we believe that a post-transcriptional mechanism that controls surface expression of the HLA-B locus plays a role in this case. The HLA processing machinery is important for MHC surface expression, and the loss of various chaperones, like tapasin, has been correlated with alterations in the correct expression of specific HLA alleles (Cabrera et al. 2005; Park et al. 2003).

We favor the idea that autologous vaccination and interferon-alpha-2b

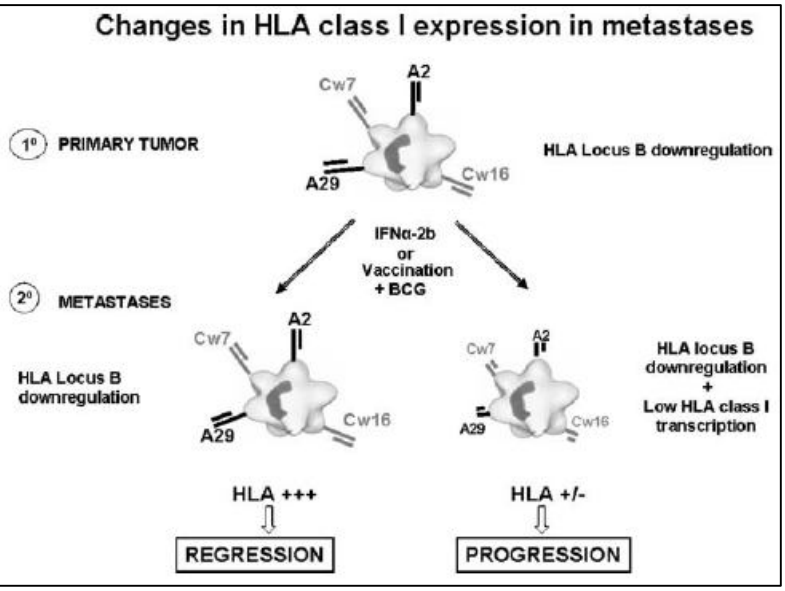

Fig. 6 Hypothetical chain of alterations in the HLA class I expression during cancer immunotherapy. The original tumor lost HLA-B molecules. This phenotype is also detected in regressing and progressing metastases. In addition, progressing metastases showed downregulation of HLA-A and C locus expression

upregulated tumor HLA class I expression and induced T cell mediated rejection of tumor cells sensitive to locally released cytokines. In contrast, HLA class I expression level remained very low in progressing metastases due to a failure in HLA genes transcription activation.

This hypothesis could explain the reduced levels of HLA class I expression in response to interferon-alpha-2b and MVAX (which produces an interferon-γ-mediated T-cell inflammatory response) in both progressing metastases. The underlying mechanism could be a defect in the HLA class I inductibility mediated by interferon. STAT-1 protein, could be absent or not phosphorylated, or have irreversible alterations. Previously we have reported the existence of such tumor phenotype (Rodríguez et al. 2007; Abril et al. 1998). Another mechanism could be a tumor antigen loss. In this case, the metastases fail to recruit tumor specific CD8 + cells to the site, and the IFN-gamma producing cells will not be present in the tissue. An extrinsic mechanism could be the induction of a strong local Treg response which inhibits local activation of HLA class I expression.

In this study, we show that there is a clear relationship between MHC class I expression and metastatic progression/ regression after immunotherapy. Similar results were obtained in a previous study of another patient, who had six post M-VAX vaccination metastases, three of them were regressing and the other three were progressing (Cabrera et al. 2007). Progressing metastases had much lower HLA class I expression than the regressing ones. Therefore, we have studied in total 16 metastases from two patients undergoing immunotherapy protocols, and in all of them we have found such correlation.

Both studies are consistent with the idea of HLA associated T and NK tumor escape mechanism. The regressing metastases with high HLA class I expression should be recognized and eliminated by cytotoxic T cells, while the progressing ones with low HLA class I expression could escape T and NK cells. In this context, some reports have indicated that low levels in HLA class I but not complete loss of class I expression correlates with a poor prognosis (Watson et al. 2006; Madjd et al. 2005) because this type of tumor cells could avoid both NK and T-cell-mediated immune surveillance.

Other reports have also analyzed HLA class I expression in metastases after immunotherapy (Belli et al. 2002), but including only progressing ones and using HC10 MoAb that recognize intracytoplasmic HLA class I free heavy chain. We believe this study cannot be compared with ours since we analyzed progressing and regressing metastases of the same patient using a broad panel of anti-HLA class I MoAbs defining monomorphic and allelic determinants as well as molecular techniques using DNA and RNA obtained after tumor tissue microdissection.

Poor clinical outcome of immunotherapy suggests that elimination of transformed cells in vivo is more difficult than it had been predicted in vitro (Rivoltini et al. 2002). It is possible that the selective pressure imposed by T cell-based immunotherapy will facilitate the appearance of resistant tumor metastatic variants with HLA class I alterations (Spear et al. 1985; Ruiz-Cabello et al. 2003). Monitoring HLA class I antigen expression and the knowledge of the molecular mechanism responsible for HLA class I alteration in malignant lesions is important. This information will help to define reversible versus nonreversible alteration of HLA class I in metastases that will determine which metastases progress or regress after immunotherapy.

Acknowledgement We would like to thank Ms. Ana Isabel Rodriguez for technical assistance and Ms. Natalia Aptsiauri for help in the preparation of the manuscript. This work was partially supported by the Fondo de Investigaciones Sanitarias (FIS), Consejeria de Salud, Junta de Andalucia, by Plan

Andaluz de Investigación (PAI), Proyecto de investigacion MEC I + D (SAF 2007-63262) in Spain, by the European Network for the identification and validation of antigens and biomarkers in cancer and their application in clinical tumor immunology (ENACT) project (European community contract number LSHC-CT-2004-503306), and by Cancer Immunotherapy project (European community contract number OJ 2004/c158,18234).

References

Abril E, Real LM, Serrano A, Jimenez P, Garcia A, Canton J, Trigo I, Garrido F, Ruiz-Cabello F (1998) Unresponsiveness to interferon associated with STAT1 protein deficiency in a gastric adenocarcinoma cell line. Cancer Immunol Immunother 47:113–120

Ahmad M, Rees RC, Ali SA (2004) Escape from immunotherapy: possible mechanisms that influence tumor regression/progression. Cancer Immunol Immunother 53:844–854

Algarra I, Garcia-Lora A, Cabrera T, Ruiz-Cabello F, Garrido F (2004) The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 10:904–910

Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz- Cabello F, Garrido F (2007) MHC class I antigens and immune surveillance in transformed cells. Int Rev Cytol 256:139–189

Aptsiauri N, Cabrera T, Mendez R, Garcia-Lora A, Ruiz-Cabello F, Garrido F (2007) Role of altered expression of HLA class I molecules in cancer progression. Adv Exp Med Biol 601:123– 131

Belli F, Testori A, Rivoltini L, Maio M, Andreola G, Sertoli MR, Gallino G, Piris A, Cattelan A, Lazzari I, Carrabba M, Scita G, Santantonio C, Pilla L, Tragni G, Lombardo C, Flavio A, Marciano A, Queirolo P, Bertolini F, Cova A, Lamaj E, Ascani L, Camerini R, Corsi M, Cascinelli1 N, Lewis JJ, Srivastava P, Parmiani G (2002) Vaccination of Metastatic Melanoma Patients With Autologous Tumor-Derived Heat Shock Protein gp96- Peptide Complexes: Clinical and Immunologic Findings. J Clin Oncol 20(20):4169–4180

Benitez R, Godelaine D, Lopez-Nevot MA, Brasseur F, Jiménez P, Marchand M, Oliva MR, van Baren N, Cabrera T, Andry G, Landry C, Ruiz-Cabello F, Boon T, Garrido F (1998) Mutations of the beta2-microglobulin gene result in a lack of HLA class I molecules on melanoma cells of two patients immunized with MAGE peptides. Tissue Antigens 52:520–529

Berd D (2004) M-VAX: an autologous, hapten-modified vaccine for human cancer. Expert Rev Vaccines 3:521–527

Buteau Ch, Suetomir N, Markovic, Celis E (2002) Challenges in the development of effective peptide vaccines for cancer M. Clin Proc 77:339–349

Cabrera CM, Lopez-Nevot MA, Jimenez P, Garrido F (2005) Involvement of the chaperone tapasen in HLA-B44 allelic losses in colorectal tumors. Int J Cancer 113:611–618

Cabrera T, Lara E, Romero JM, Maleni I, Real LM, Ruiz-Cabello F, Valero P, Camacho FM, Garrido F (2007) HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination. Cancer Immunol Immunother 56:709–717

De Pablo R, Compte M, Muro R, Solis R, Moreno ME, Kreisler M, Vilches C (2003) Cytofluorometric characterization of two monoclonal antibodies recognising polymorphic epitopes of HLA class I molecules: 30.13.38 (anti-A*02, A*68 and A*69) and 29.30.6 (anti-Bw6). Inmunología 22:321–328

Festenstein H, Garrido F (1986) MHC antigens and malignancy. Nature 322(6079):502–503

Garcia-Lora A, Añgarra I, Gaforio JJ, Ruiz-Cabello F, Garrido F (2002) Immunoselection by T lymphocytes generates repeated MHC Class I-deficient metastatic tumor variants. Int J Cáncer 91:109–19

Garrido F, Algarra I (2001) MHC antigens and tumor escape from immune surveillance. Adv Cancer Res 83:117–158 Garrido F, Ruiz-Cabello F, Cabrera T, Pérez-Villar JJ, López-Botet M, Duggan-Keen M, Stern PL (1997) Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today 18(2):89–95

Griffioen M, Ouwerkerk IJ, Harten V, Gobin SJ, van den Elsen PJ, Schrier PI (1999) Role of the interferon-stimulated response element in HLA-B downregulation in human melanoma cell lines. Immunogenetics 49(4):287–294

Gutterman JU (1994) Cytokine therapeutics: lessons from interferonalpha. Proc Natl Acad Sci USA 91:1198–205

Hersey P, Menzies SW, Coventry B, Nguyen T,

Farrelly M, Collins S, Hirst D, Johnson H (2005) Phase I/II study of immunotherapy with T cell peptide epitopes in patients with stage IV me1anoma. Cancer Immunol Immunother 54:208–218

Khong HT, Restifo NP (2002) Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 3:999–100

Koene GJ, Arts-Hilkes YH, van der Ven KJ, Rozemuller EH, Slootweg PJ, deWeger RA, TilanusMG (2004) High level of chromosome 15 aneuploidy in head and neck squamous cell carcinoma lesions identified by FISH analysis: limited value of beta2-microglobulin LOH analysis. Tissue Antigens 64(4):452–461

Liu M, Acres B, Balloul JM, Bizouarne N, Paul S, Slos P, Squiban P (2004) Gene-based vaccines and immunotherapeutics. Proc Natl Acad Sci USA 101(Supp12):14567–14571

Lonchay C, van der Bruggen P, Connerotte T, Hanagiri T, Coulie P, Colau D, Lucas S, Van Pel A, Thielemans K, van Baren N, Boon T (2004) Correlation between tumor regression and T cell responses in melanoma patients vaccinated with MAGE antigen. Proc Natl Acad Sci USA 101(Supp12):14631–14638

Lopez-Nevot MA, Esteban F, Ferron A, Gutierrez J, Oliva MR, Romero C, Huelin C, Ruiz-Cabello F, Garrido F (1989) HLA class I gene expresión on human primary tumours and autologous metastases: demostration of selective losses of HLA antigens on colorectal, gastric and laryngeal carcinomas. Br J cancer 59:221–226

Lozano F, Borche L, Places L, Alberola-Ila J, Gayá A, Vilella R, Vives J (1990) Biochemical and serological characterization of a public antigenic determinant present on HLA-B molecules. Tissue Antigens 35(4):193–5

Lozano F, Santos-Aguado J, Borche L, Places L, Doménech N, Gayá A, Vilella R, Vives J (1989) Identification of the amino acid residues defining an intralocus determinant in the alpha 1 domain of HLA-A molecules. Immunogenetics 30(1):50–53

Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG (2005) Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. Int J Cancer 117(2):248–255

Maleno I, Romero JM, Cabrera T, Paco L, Aptsiauri N, Cozar JM, Tallada M, López-Nevot MA, Garrido F (2006) LOH at 6p21.3 region and HLA class I altered phenotypes in bladder carcinomas. Immunogenetics 58(7):503–510

Marchand M, Brichard V, Van Baren N, Coulie PG (2001) Biological and clinical developments in melanoma vaccines. Expert Opin Biol Ther 1:497–510

Marincola FM, Shamamian P, Alexander RB, Gnarra JR, Turetskaya RL, Nedospasov SA, Simonis TB, Taubenberger JK, Yannelli J, Mixon A et al (1994) Loss of HLA haplotype and B locus downregulation in melanoma cell lines. J Immunol 153(3):1225–1237

Méndez R, Rodríguez T, Del Campo A,Monge E, Maleno I, Aptsiauri N, Jiménez P, Pedrinaci S, Pawelec G, Ruiz-Cabello F, Garrido F (2008) Characterization of HLAclass I altered phenotypes in a panel of human melanoma cell lines. Cancer Immunol Immunother 57:719–729

Mendez R, Serrano A, Jager E, Maleno I, Ruiz-Cabello F, Knuth A, Garrido F (2001) Analysis of HLA class I expresion in different metastases from two melanoma patients undergoing peptide immunotherapy. Tissue Antigens 57:508–519

Palmer KJ, Harries M, Gore ME, Collins MK (2000) Interferon-alpha (IFN-alpha) stimulates anti-melanoma cytotoxic T lymphocyte (CTL) generation in mixed lymphocyte tumour cultures (MLTC). Clin Exp Immunol 119(3):412–418

Park B, Lee S, Kim E, Ahn K (2003) A single polymorphic residue within the peptide-binding cleft of MHC class I molecules determines spectrum of tapasin dependence. J Immunol 170 (2):961–978 Erratum in: J Immunol 170(9):4869

Rivoltini L, Canese P, Huber V, Iero M, Pilla L, Valentini R, Fais S, Lozupone F, Casati C, Castelli C, Parmiani G (2005) Escape strategies and reasons for failure in the interaction between tumour cells and the immune system: how can we tilt the balance towards immune-mediated cancer control? Expert Opin Biol Ther 5:463–476

Rivoltini L, Carrabba M, Huber V, Castelli C, Novellino L, Dalerba P, Mortarini R, Arancia G, Anichini A, Fais S, Parmiani G (2002) Immunity to cancer: attack and escape in T lymphocyte–tumor cell interaction. Immunol Rev 188:97–113

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

Rosenberg SA, Dudley ME (2004) Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor 1ymphocytes. Proc Natl Acad Sci USA 101(Supp12):14639– 14645

Rosenberg SA, Yang JC, Restifo NP (2004) Cancer immunotherapy: moving beyond current vaccines. Nature Medicine 10(9): 909–915

Ruiz-Cabello F, Travesari C, Garrido F (2003) Multiple mechanisms are responsable for the alteration in the expresión of HLA class I antigens in melanoma. Int J cancer 105:432–433

Seliger B, Cabrera T, Garrido F, Ferrone S (2002) HLA class I antigen abnormalities and immune escape by malignant cells. Semin Cancer Biol 12(1):3–13

Seymour K, Pettit S, O'Flaherty E, Charnley RM,

Kirby JA (1999) Selection of metastatic tumour phenotypes by host immune systems. Lancet 354(9194):1989–1991

Spear BT, Kornbluth J, Strominger JL, Wilson DB (1985) Evidence for a shared HLA-A intralocus determinant defined by monoclonal antibody 131. J Exp Med 162(6):1802–1810

Stam NJ, Spits H, Ploegh HL (1986) Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. J Immunol 137:2299–2306

Sun W, Schuchter LM (2001) Metastatic melanoma. Curr Treat Options Oncol 2(3):193–202

Terando AM, Faries MB, Morton DL (2007) Vaccine therapy for melanoma: current status and future directions.Vaccine 25(Suppl 2): B4–B16

Versteeg R, Krüse-Wolters KM, Plomp AC, van Leeuwen A, Stam NJ, Ploegh HL, Ruiter DJ, Schrier PI (1989) Suppression of class I human histocompatibility leukocyte antigen by c-myc is locus specific. J Exp Med 170(3):621–635

Watson NF, Ramage JM,Madjd Z, Spendlove I, Ellis IO, ScholeWeld JH, Durrant LG (2006) Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. Int J Cancer 118:6–10

Zitvogel L, Tesniere A, Kroemer G (2006) Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 6(10):715–727

3.2. Regressing and progressing metastatic lesions: resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations

Natalia Aptsiauri, Rafael Carretero, Angel Garcia-Lora, Luis M. Real, Teresa Cabrera, Federico Garrido

Abstract

Despite the significant efforts to enhance immune reactivity against malignancies the clinical effect of anti-tumor vaccines and cancer immunotherapy is still below expectations. Understanding of the possible causes of such poor clinical outcome has become very important for improvement of the existing cancer treatment modalities. In particular, 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, especially in response to immunotherapy. In this report, we illustrate that two types of metastatic lesions are commonly generated in response to immunotherapy according to the pattern of HLA class I expression. We found that metastatic lesions, that progress after immunotherapy have low level of HLA class I antigens, while the regressing lesions demonstrate significant upregulation of these molecules. Presumably, immunotherapy changes tumor microenvironment and creates an additional immune selection pressure on tumor cells. As a result, two subtypes of metastatic lesions arise from pre- existing malignant cells: (a) regressors, with upregulated HLA class I expression after therapy, and (b) progressors with resistance to immunotherapy and with low level of HLA class I. Tumor cells with reversible defects (soft lesions) respond to therapy by upregulation of HLA class I expression and regress, while tumor cells with structural irreversible defects (hard lesions) demonstrate resistance to immunostimulation, fail to upregulate HLA class I antigens and eventually progress. These two types of metastases appear independently of type of the immunotherapy used, either non-specific immunomodulators (cytokines or BCG) or autologous tumor vaccination. Similarly, we also detected two types of metastatic colonies in a mouse fibrosarcoma model after in vitro treatment with IFN-_. One type of metastases characterized by upregulation of all MHC class I antigens and another type with partial IFN-_ resistance, namely with lack of expression of Ld-MHC class I molecule. Our observations may shed new light on the understanding of the mechanisms of tumor

This article is a symposium paper from the conference “Progress in vaccination against cancer 2007 (PIVAC 7)”, held in Stockholm, Sweden, on 10–11 September 2007.

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escape and might have implications for improvement of the efficacy of cancer immunotherapy.

Keywords HLA class I · Metastatic melanoma · Cancer immunotherapy

Introduction

Poor clinical outcome of current protocols of cancer immunotherapy

Cancer still remains to be a leading cause of death in humans with rare tumor regression in response to treatment. It is characterized by metastatic spread in different organs and tissues that actually causes lethal outcome in patients. Usually primary tumors without metastases are removed surgically, while patients with metastatic cancer spread are treated with immunomodulating therapy along with conventional chemotherapy. For many years clinicians used non-specific systemic immunomodulators of anti-tumor immunity, such as BCG [1], IL-2 [2], or IFNs [3] aimed to activate different branches of immune system to fight malignancy. Currently, new immunotherapy protocols are adopted based on the knowledge that T cell mediated response against tumor antigens complexed with HLA class I molecules is a key factor of anti-tumor immune reaction. Identification of various types of tumor associated antigens that are shared by different types of tumor and can be recognized by cytotoxic T cells (CTLs) prompted basic and clinical investigator to develop new peptide-based vaccines aimed to boost specific anti-tumor T cell responses [4]. Most of these vaccines use peptides restricted to by a specific HLA class I allele. Vaccines based on DCs pulsed with tumor-specific peptides or transduced with tumor antigens have also been tested with various degrees of success [5, 6].

Various reports during the past decade indicate that various types of immunomodulators, specific or non-specific, in the majority of clinical trials do not lead to expected clinical improvement in cancer patients. Only in some cases immunotherapy leads to a partial tumor regression or to incomplete elimination of secondary metastases [7, 8]. Paradoxically, immunization-induced CTLs frequently are able to recognize or even eliminate autologous or HLA matched tumor cells in vitro, but in most of the cases cannot lead to a complete metastatic regression in vivo [9–11]. Thus, the effect of peptide-based immunization is mostly limited to stimulation of anti-tumor cellular immune responses that scarcely lead to a clinical tumor regression. Survival of the patients only slightly improved during the past 20 years. According to Rosenberg et al. [12] cancer immunotherapy demonstrated only 3–4% of overall objective response rate.

It has become obvious that immune-mediated cancer rejection is a complex biological phenomenon that has to be studied more extensively from both sides—the tumor microenvironment and the host immune system. It is established that tumor sites are infiltrated by tumor-specific lymphocytes (TILs) [13]. Nevertheless, tumor growth progresses in these conditions suggesting the role of tumor microenvironment. Emerging evidence suggests that patients' responsiveness to immunotherapy is influenced by various factors that regulate preexisting immune status and the microenvironment of the primary tumor and of secondary metastatic lesions [14]. Given the low number of cancer patients achieving significant benefit from specific or non-specific immunotherapy, considerable recent effort has focused on identifying predictors of therapeutic response and key molecular mechanisms involved in tumor escape from treatment. Currently, an active intense investigation is ongoing to improve strategies of cancer vaccination and to enhance clinical

efficacy of immunization-induced T cell responses [15]. These efforts could be successful if tumor antigens and HLA class I molecules that are necessary for antigen-presentation to CTL would have been normally expressed on malignant cells. It has been described that these molecules are frequently lost or downregulated on tumor cells during cancer progression due to structural or regulatory defects [16, 17].

HLA class I altered expression on cancer cells represents one of the important mechanisms of tumor escape from the immune response that eventually leads to accumulation of new variants with low immunogenicity and high capability for metastatic progression [18–20]. Cancer cells escape from immunosurveillance through the outgrowth of poorly immunogenic tumor cell variants, which emerge due to a growth advantages created by a combination of certain features of tumor cells and the factors of tumor environment [21]. Total or partial loss of HLA class I expression has been described in almost all types of cancer and in some cases it has been associated with poor clinical prognosis [17, 21, 22]. Interestingly, it has been recently reported that patients with HLA class I positive bladder cancer had longer recurrence-free survival after BCG therapy than those with negative expression in 5-year-follow-up [23]. Cells that are highly immunogenic and express high levels of MHC class I are eliminated by CTLs. Malignant cells with total MHC class I loss are susceptible to NK cell lysis because of inactivation of KIRs. However, frequently a complex balance between activating and inhibiting signals in NK cells leads to failure of natural cytotoxic reactions against malignant cells. Another immunoselection route might be provided by the partial loss of HLA class I antigens that allows tumor cells to escape both CTL and NK attack. For instance, a recent study of colorectal cancer showed that a high level of HLA I expression or total loss of HLA class I was associated with similar disease-specific survival times, possibly due to T cell reactivity or NK cell mediated clearance of class I-positive and -negative tumor cells, respectively. However, tumors with intermediate HLA class I expression were reported to be associated with a poor prognosis, suggesting that these tumors may avoid both NK- and T cell-mediated immune surveillance [24].

It has been known for many years that changes in MHC class I profile affects the metastatic capacity. In particular, several reports from Dr. Feldmans group published in the 1980s indicate that high and low metastatic phenotypes in a mouse tumor mode are associated with defined MHC class I expression [25, 26]. It has been also demonstrated in various types of cancer that metastatic lesions are less sensitive to CTL killing than a primary tumor indicating that during tumor progression metastatic cancer cells may acquire various characteristics that

Patient #1

Autologous tumor vaccine plus BCG

Regressors/ progressors	HLA-ABC	β-2m
R1, R2, R3	+++	+++
P1, P2, P3	+/-	+/-

Patient #2 **1) IFN- α-2b treatment**

Regressors/ progressors	HLA-ABC	β-2m
R1, R2, R3, R4	+++	+++
P5	+/-	+/-

2) Autologous tumor vaccine plus BCG

	HLA-ABC	β-2m
R7, R8, R9, R10	+++	+++
P6	+/-	+/-

R - Regressors
P - Progressors

Fig. 1 HLA class I expression in progressing/regressing metastases after immunotherapy of two melanoma patients

help to evade CTL mediated destruction [7]. Metastatic progression has been demonstrated to correlate with HLA class I downregulation in breast carcinoma [27]. Decreased expression of HLA heavy chain and beta2-microglobulin mRNA was found in patients with metastatic renal cell carcinoma [28]. Therefore, it is much more complicated to treat metastatic disease that localized cancer. Thus, the complex nature of tumor metastasis necessitates a comprehensive approach to achieve successful immune intervention. There have been various reports demonstrating that different metastases in the same patient have various mechanisms that underlie the generation of tumor escape variants and the existing immunophenotypes of metastatic lesions [29, 30]. However, there is not sufficient data on a correlation between HLA expression and advancement of metastatic lesions, as well as changes in this expression in response to immunotherapy, since it is not always possible to obtain primary tumor and several metastatic samples from cancer patients during immunotherapy.

In this report based on analysis of metastases in cancer patients and from animal studies we classified metastatic lesions that develop under immunotherapy regimen in two groups: progressing with reduced HLA class I expression and regressing with normal expression of class I antigens.

Identification of two types of metastatic melanoma lesions based on different HLA class I expression: implications for cancer immunotherapy

Recently, we have observed an interesting association of HLA class I expression and metastatic progression in two melanoma patients, whose subcutaneous metastases responded differently to autologous tumor cell vaccination [31, 32]. In both patients, we were able to obtain and analyze both progressing and regressing metastatic lesions for HLA class I expression using immunohistochemistry, tissue microdissection, LOH analysis, and other molecular techniques. Figure 1 summarizes the immunohistochemical characteristics of the HLA class I expression in the studied metastatic lesions from both patients. In the first patient we analyzed three progressing (P1, P2, P3) and three regressing (R1, R2, R3) metastases obtained after treatment with autologous tumor vaccine combined with BCG. We observed that the progressing lesions have low transcription and protein expression levels of HLA class I heavy chain and beta2-microglobulin, and demonstrate higher frequency of LOH in chromosome 15 [31]. Malignant cells with such irreversible structural genetic defects in HLA class I are not likely to respond to immunotherapy aimed at enhancing anti-tumor T cell immune response and will generate a progressing type of metastases. In regressing lesions the expression level of class I molecules was significantly higher. LOH at chromosome 6 was detected in all six studied metastases, suggesting that this defect may also be found in the primary tumor, contributing to the mechanism of tumor escape and metastatic progression. Real-time quantitative PCR of the samples obtained from microdissected tumor showed lower mRNA levels of HLAABC heavy chain and 2 m in progressing metastases than in regressing ones, confirming the immunohistological findings.

In a second patient (Patient #2) we

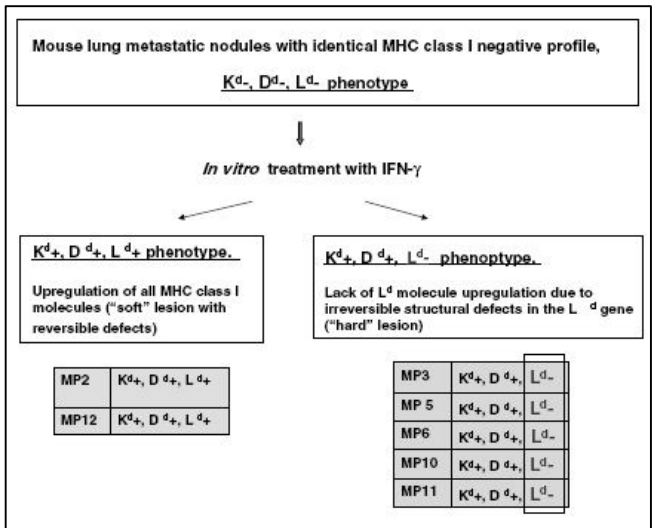

Fig. 2 Two types of mouse metastatic clones generated after in vitro IFN-γ treatment: one with up-regulated cell surface expression of K^d, D^d and L^d molecules (K^d+, D^d+, L^d+) **a**, and second one with the absence of Ld expression (L^d−) **b**. The lack of IFN-γ inducibility is due to the existence of an underlying structural irreversible defect in the L^d gene

analyzed ten metastases: five after treatment with IFN-2b (four regressing ones, R1, R2, R3, R4; and one progressing one, P5) and five after autologous vaccine (M-VAX) plus BCG (four regressing ones, R7,R8,R9,R10; and one progressor, P6). We observed once again a positive correlation between low HLA class I expression and metastatic progression independently of the type of therapy used. Accordingly, after immunotherapy metastatic lesions can be classified in two types: regressors with high expression of HLA class I molecules and progressors with low class I expression. We favor an idea that immunotherapy leads to modulation of HLA class I expression on different tumor cells subsets and leads to emergence of different responders to treatment based on preexisting "soft" or "hard" HLA alterations. It is expected that malignant cells with irreversible "hard" genetic defects in HLA class I genes will not respond to immune modulation by upregulation of HLA class I molecules.

Melanoma is characterized by diversity of malignant cells in many ways. It has been shown that metastases also differ and respond differently to treatment. Thus, the treatment leads to regression of some metastases. There are some factors that make cancer cells susceptible in a subpopulation with specific characteristics. Based on our observations we think that these characteristics include HLA class I expression and its modulation in response to cytokine stimuli.

We cannot totally exclude a possibility that progression and regression of certain metastatic lesion happened independently of the therapy. However, we believe that immunotherapy put an additional immunoselective pressure leading to emergence of highly proliferating metastatic cells with preexisting defect in HLA class I expression. From our animal studies we obtained evidence supporting our hypothesis.

Two types of metastases found in a mouse fibrosarcoma model

Earlier, we also identified two H-2 metastatic phenotypes in a murine cancer model [33]. Briefly, one H-2-class I-negative fibrosarcoma tumor clone generated H-2 class I-negative spontaneous lung metastases in immunocompetent BALB/c mice [34–36]. Cell lines from these metastatic nodes with apparently identical MHC class I expression pattern generated two distinct and reproducible H-2 phenotypes after in vitro IFN- treatment. The first (17%) was similar to that of the B9 tumor clone from which they derived and was characterized by the cell surface expression of Kd, Dd or Ld molecules. The second type of MHC expression profile was present in 83% of the colonies and was characterized by absence of expression of Ld class I molecule after IFN- treatment (Fig. 2). Therefore, vitro incubation of the metastatic clones with IFN- revealed a

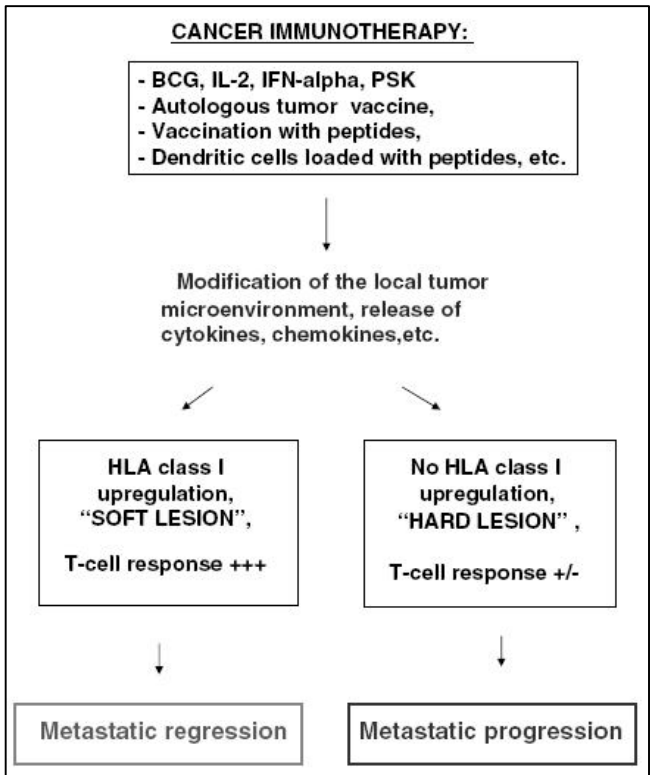

Fig. 3 Proposed mechanism of immunotherapy-mediated generation of progressing and regressing metastases

presence of non-responder cells to cytokine stimulation. We believe that the second type of metastatic clone with "hard" lesion in MHC Ld gene would eventually give rise to a progressor type of metastatic lesion. This altered phenotype was highly reproducible and repeated in different metastases from different animals and in different experiments, suggesting that MHC genetic alterations observed in a given metastasis are non-random and can be predicted [33]. The loss of expression of the Ld antigen was originated as a consequence of two independent events: at First a deletion of a large fragment of one chromosome 17 telomeric to Dd genes that had been found in the primary tumor and in all the metastatic clones, and secondly a new deletion involving only the Ld gene of the other chromosome 17 that had been detected in metastatic clones with no Ld expression. For details see reference [33].

Discussion and conclusions

Based on the data obtained from our animal experiments and clinical cases, we have identified two types of metastases according to the expression pattern of HLA class I antigens: progressing metastatic lesions with low HLA class I level and regressing ones with upregulated expression of these antigens. Our observations suggest that no matter what type of immunotherapy is administered, specific immunization or systemic immune stimulation, the outcome should be expected the same, namely, a modification of tumor microenvironment of each metastatic lesion leading to a release of immunostimulating cytokines. Depending on the preexisting HLA class I expression level and presence of reversible ("soft") or irreversible ("hard") alterations in the HLA class I system, its expression will be either upregulated by the cytokines leading to regression of metastatic nodule, or remained unchanged due to resistance to cytokine treatment which will stimulate progression of metastases with irreversible HLA class I alterations (Fig. 3). In some cases additional structural defects in molecules involved in MHC class I transcriptional activation could limit the response of metastases to immunotherapy. For example, STAT-1 protein, that plays an important role in IFN--mediated HLA class I upregulation could be absent or not phosphorilated, or have irreversible structural defects [37, 38]. Our data could at least partially explain why despite the constant improvement of existing protocols of cancer immunotherapy the clinical efficacy remains to be low. Among other possible explanations could be a loss of tumor antigens that would lead to the lack of recruitment of tumor specific CD8+ cells to the site of metastatic lesion [39] or induction of a T cell anergy in these cells. An extrinsic mechanism could be the induction of strong local Treg response that would inhibit IFN- production by tumor specific CD8+ cells thereby leading to failure in HLA class I upregulation and to inhibition of the downstream effector processes that cause tumor regression [40].

Metastatic melanoma consists of a heterogeneous population of cells with respect to gene and protein expression, including HLA class I antigens that may be of importance to disease progression [30, 41, 42]. Even if tumor specific antigen is present and CTL response is activated by vaccination, absence or low level of HLA class I allele necessary for presentation prevents tumor elimination.

Interestingly, in many cases peptide-based vaccination produces mixed responses in the same melanoma patients: regression of some malignant lesions and progression of others. Melanoma patients vaccinated with MAGE-3 peptides, or with autologous DC pulsed with MAGE-3 peptides, or with ALVAC virus encoding MAGE antigens demonstrated a mixed clinical response to treatment, with

some metastases regressing while others progressing [8, 43, 44]. Currently, there is no clear explanation for this diversity of responses. Interestingly, in some patients this treatment triggers a strong activation of T cells against nonvaccine tumor peptide, or epitope spreading. Both vaccine specific and new CTLs were detected at tumor sites even in progressing lesions suggesting that their activity was not sufficient for killing of the metastatic cells [45, and Dr. D. Godelaine, personal communication]. One possible explanation of the inability of CTLs to destroy tumor cells is failure of HLA class I upregulation on metastatic cells in response to immunomodulation. It is believed that in case of epitope spreading the recognition of an antigen on a target cell by T cells generates conditions such as cytokine release and target cell destruction, which in turn, facilitates the presentation of additional antigens of the target cells. Similar phenomenon of T cell response shifting has been previously described in other reports on melanoma patients [46]. We believe that our observations support the idea that cytokine release at the tumor site may lead to upregulation of HLA class I expression necessary for presentation of newly released tumor antigens or of peptides that previously lacked appropriate HLA class I allele for adequate presentation to T cells. However, such HLA upregulation would not be possible if malignant cells contain irreversible structural alterations in HLA class I genes or demonstrate resistance to cytokine-mediated upregulation due to defects in signal transduction pathways.

Therefore, taken together, these data supports our working hypothesis that the mixed response to immunotherapy in melanoma patients is predetermined by the presence of irreversible “hard” structural defects in HLA class I expression, which would give rise to progressing lesions.

Hence, to optimize cancer immunotherapy strategies evasion from newly developed immunoresistant tumor phenotypes needed to be considered. Monitoring HLA class I expression in metastatic lesions during course of cancer immunotherapy may help to identify immunoresistant lesions and predict, or even improve, the clinical outcome of treatment.

Acknowledgments This work was supported by grants from the Fondo de Investigaciones Sanitarias (FIS), Red Genomica del Cancer (RETIC RD 06/0020), Plan Andaluz de Investigacion (Group CTS-143), Consejeria Andaluz de Salud (SAS), Proyecto de Excelencia de Consejeria de Innovacion (CTS-695), Proyecto de investigacion I+D (SAF 2007-63262) in Spain; and from the Integrated European Cancer Immunotherapy project (OJ2004/C158, 518234).

References

1. Herr HW, Laudone VP, Badalament RA, Oettgen HF, Sogani PC, Freedman BD, Melamed MR, Whitemore WF Jr (1988) Bacillus Calmette–Guérin therapy alters the progression of superWcial bladder cancer. J Clin Oncol 6(9):1450–1455 2. Rosenberg SA, Yang JC, Topalian SL, Schwartzentruber DJ,

2. Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE (1994) Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin. JAMA 271(12):907–913

3. Atzpodien J, Korfer A, Palmer PA, Franks CR, Poliwoda H, Kirchner H (1990) Treatment of metastatic renal cell cancer patients with recombinant subcutaneous human interleukin-2 and interferon-alpha. Ann Oncol 1(5):377–378

4. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Palen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643–1647

5. Esche C, Shurin MR, Lotze MT (1999) The use of dendritic cells for cancer vaccination. Curr Opin Mol Ther 1:72–81

6. Godelaine D, Carrasco J, Lucas S, Karanikas V, Schuler-Thurner B, Coulie PG, Schuler G, Boon T,

Van Pel A (2003) Polyclonal CTL responses observed in melanoma patients vaccinated with dendritic cells pulsed with a MAGE-3.A1 peptide. J Immunol 171(9):4893–4897

7. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. Annu Rev Immunol 24:175–208

8. Kruit WH, van Ojik HH, Brichard VG, Escudier B, Dorval T, Patel P, van Baren N, Avril MF, Piperno S, Khammari A, Stas M, Ritter G, Lethe B, Godelaine D, Brasseur F, Zhang Y, van der Bruggen P, Boon T, Eggermont AM, Marchand M (2005) Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma. Int J Cancer 117(4):596–604

9. Rosenberg SA, Sherry RM, Morton KE, Scharfman WJ, Yang JC, Topalian SL, Royal RE, Kammula U, Restifo NP, Hughes MS, Schwartzentruber D, Berman DM, Schwarz SL, Ngo LT, Mavroukakis SA, White DE, Steinberg SM (2005) Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol 175(9):6169–6176

10. Anichini A, Vegetti C, Mortarini R (2004) The paradox of T-cellmediated antitumor immunity in spite of poor clinical outcome in human melanoma. Cancer Immunol Immunother 53(10):855–864

11. Wang E, Phan GQ, Marincola FM (2001) T-cell-directed cancer vaccines: the melanoma model. Expert Opin Biol Ther 1(2):277– 290

12. Rosenberg SA, Yang JC, Restifo NP (2004) Cancer immunotherapy: moving beyond current vaccines. Nat Med 10(9):909–915

13. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlechnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313(5795):1960–1964

14. Wang E, Miller LD, Ohnmacht GA, Mosellin S, Perez-Diez A, Petersen D, Zhao Y, Simon R, Powell JI, Asaki E, Alexander HR, Duray PH, Herlyn M, Restifo NP, Liu ET, Rosenberg SA, Marincola FM (2002) Prospective molecular profiling of melanoma metastases suggests classiWers of immune responsiveness. Cancer Res 62(13):3581–3586

15. Berzofsky JA, Terabe M, Oh S, Belyakov IM, Ahlers JD, Janik JE, Morris JC (2004) Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J Clin Invest 113(11):1515–1525

16. Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, Lopez-Botet M, Duggan-Keen M, Stern P (1997) Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today 18(2):89–95

17. Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz-Cabello F, Garrido F (2007) MHC class I antigens and immune surveillance in transformed cells. Int Rev Cytol 256:139–189

18. Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, Stern PL (1993) Natural history of HLA expression during tumour development. Immunol Today 14:491–499

19. Chang CC, Ferrone S (2007) Immune selective pressure and HLA class I antigen defects in malignant lesions. Cancer Immunol Immunother 56(2):227–236

20. Garrido F, Algarra I (2001) MHC antigens and tumor escape from immune surveillance. Adv Cancer Res 83:117–158

21. Seliger B, Cabrera T, Garrido F, Ferrone S (2002) HLA class I antigen abnormalities and immune escape by malignant cells. Semin Cancer Biol 12(1):3–13

22. Rolland P, Deen S, Scott I, Durrant L, Splendlove I (2007) Human leukocyte antigen class I antigen expression is an independent prognostic factor in ovarian cancer. Clin Cancer Res 13(12):3591–3596

23. Kitamura H, Torigoe T, Honma I, Sato E, Asanuma H, Hirohashi Y, Sato N, Tsukamoto T (2006) Effect of human leukocyte antigen class I expression of tumor cells on outcome of intravesical instillation of bacillus calmette–guerin immunotherapy for bladder cancer. Clin Cancer Res 12(15):4641–4644

24. Watson NF, Ramage JM, Madjd Z, Splendlove I, Ellis IO, ScholeWeld JH, Durrant LG (2006) Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. Int J Cancer 118(1):6–10

25. De Baetselier P, Katzav S, Gorelik E, Feldman M, Segal S (1980) DiVerential expression of H-2 gene products in tumour cells in associated with their metastatogenic properties. Nature 288(5787):179–181

26. Eisenbach L, Hollander N, Greenfield L, Yakor H, Segal S, Feldman M (1984) The differential expression of H-2 K versus H-2D antigens, distinguishing high-metastatic from low-metastatic clones, is correlated with the immunogenic properties of the tumor cells. Int J Cancer 34(4):567–573

27. Saio M, Teicher M, Campbell G, Feiner H, Delgado Y, Frey AB (2004) Immunocytochemical demonstration of down regulation of HLA class-I

molecule expression in human metastatic breast carcinoma. Clin Exp Metastasis 21(3):243–249

28. Romero JM, Aptsiauri N, Vazquez F, Cozar JM, Canton J, Cabrera T, Tallada M, Garrido F, Ruiz-Cabello F (2006) Analysis of the expression of HLA class I, proinflammatory cytokines and chemokines in primary tumors from patients with localized and metastatic renal cell carcinoma. Tissue Antigens 68(4):303–310

29. Mendez R, Ruiz-Cabello F, Rodriguez T, Del Campo A, Paschen A, Schedendorf D, Garrido F (2007) Identification of different tumor escape mechanisms in several metastases from a melanoma patient undergoing immunotherapy. Cancer Immunol Immunother 56(1):88–94

30. Chang CC, Campoli M, Restifo NP, Wang X, Ferrone S (2005) Immune selection of hot-spot beta 2-microglobulin gene mutations, HLA-A2 allospecificity loss, and antigen-processing machinery component down-regulation in melanoma cells derived from recurrent metastases following immunotherapy. J Immunol 174(3):1462–1471

31. Cabrera T, Lara E, Romero JM, Maleno I, Real LM, Ruiz-Cabello F, Valero P, Camacho FM, Garrido F (2007) HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination. Cancer Immunol Immunother 56(5):709–717

32. Carretero R, Romero JM, Ruiz-Cabello F, Maleno I, Rodríguez F, Camacho FM, Real LM, Garrido F, Cabrera T (2008) Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. Immunogenetics (in press)

33. Garcia-Lora A, Algarra I, Gaforio JJ, Ruiz-Cabello F, Garrido F (2001) Immunoselection by T lymphocytes generates repeated MHC class I-deficient metastatic tumor variants. Int J Cancer 91(1):109–119

34. Garrido A, Perez M, Delgado C, Garrido ML, Rojano J, Algarra I, Garrido F (1986) Influence of class I H-2 gene expression on local tumor growth. Description of a model obtained from clones derived from a solid BALB/c tumor. Exp Clin Immunogenet 3(2):98–110

35. Perez M, Algarra I, Ljunggren HG, Mialdea MJ, Gaforio JJ, Klein G, Karre K, Garrido F (1990) A weakly tumorigenic phenotype with high MHC class-I expression is associated with high metastatic potential after surgical removal of the primary murine Wbrosarcoma. Int J Cancer 46(2):258–261

36. Algarra I, Gaforio JJ, Garrido A, Mialdea MJ, Perez M, Garrido F (1991) Heterogeneity of MHC-class-I antigens in clones of methylcholanthrene-induced tumors. Implications for local growth and metastasis. Int J Cancer (suppl) 6:73–81

37. Rodriguez T, Mendez R, Del Campo A, Jiménez P, Aptsiauri N, Garrido F, Ruiz-Cabello F (2007) Distinct mechanisms of loss of IFN-gamma mediated HLA class I inducibility in two melanoma cell lines. BMC Cancer 7:34

38. Abril E, Mendez R, Garcia A, Serrano A, Cabrera T, Garrido F, Ruiz-Cabello F (1996) Characterization of a gastric tumor cell line defective in MHC class I inducibility by both alpha- and gammainterferon. Tissue Antigens 47(5):391–398

39. Khong HT, Wang QJ, Rosenberg SA (2004) Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother (1997). 27(3):184–190

40. Terabe M, Berzofsky JA (2004) Immunoregulatory T cells in tumor immunity. Curr Opin Immunol 16(2):157–162

41. De Vries TJ, Fourkour A, Wobbes T, Verkroost G, Ruiter DJ, van Muijen GN (1997) Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1, and tyrosinase in human melanoma cell lines and in human melanocytic lesions.Cancer Res 57(15):3223–3229

42. Bittner M, Metzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakini Z, Ben-dor A, Sampas N, Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D, Sondak V (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature 406(6795):536–540

43. Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, Bender A, Maczek C, Schreiner D, von den Driesch P, Brocker EB, Steinman RM, Enk A, Kampgen E, Schuler G (1999) Vaccination with mage-3A1 peptide-pulsed mature, monocytederived dendritic cells expands specfic cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190(11):1669–1678

44. Van Baren N, Bonnet MC, Dreno B, Khammari A, Dorval T, Piperno-Neumann S, Lienard D, Speiser D, Marchand M, Brichard VG, Escudier B, Negries S, Dietrich PY, Maraninchi D, Osanto S, Meyer RG, Ritter G, Maingeon P, Tarlaglia J, van der Bruggen P, Coulie PG, Boon T (2005) Tumoral and immunologic response after vaccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T cells. J Clin Oncol 23(35):9008–9021

45. Lurquin C, Lethe B, De Plaen E, Corbiere V,

Theate I, van Baren N, Coulie PG, Boon T (2005) Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 201(2):249–257

46. Yamshikov GV, Mullins DW, Chang CC, Ogino T, Thompson L, Presley J, Galavotti H, Aquila W, Deacon D, Ross W, Patterson JW, Engelhard VH, Ferrone S, SlingluV CL Jr (2005) Sequential immune escape and shifting of T cell responses in a long-term survivor of melanoma. J Immunol 174(11):6863–6871

3.3. BCG Immunotherapy of bladder cancer induces selection and escape of HLA class I-deficient tumor cells.

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Abstract

Bacillus Calmett-Guerin immunotherapy is a standard treatment for high-risk non-muscle-infiltrating bladder cancer patients. Although the outcomes are good, cancer relapse is observed in around 40% of patients. We present the comparative analysis of HLA class I expression in recurrent bladder tumours in patients treated with mitomycin or BCG. HLA class I expression was analyzed by RT-Q-PCR and immunohistochemical techniques. Loss of Heterozigosity (LOH) was determined by microsatellite amplification of markers in chromosome 6 and 15. We found more profound alterations in HLA class I expression in the post-BCG recurrent tumours than in the lesions before the therapy. In contrast, mitomycin treatment did not change the HLA class I expression pattern. Post-BCG recurrent tumours also showed a higher incidence of structural defects underlying altered HLA class I expression. We propose that the immunotherapy-activated immune system recognizes and eliminates tumour cells with reversible HLA class I changes, but transformed cells with additional, irreversible alterations do not respond to the immunostimulation, leading to tumour relapse. To our knowledge, this is the first clinical evidence of immunotherapy-induced immunoselection of HLA class I loss tumour variants in bladder cancer.

Introduction

Mycobacterium Bovis Bacillus Calmett-Guerin (BCG) is one of the most effective intravesicular therapies for preventing local recurrences and tumour progression following transurethral resection of non-muscle invasive bladder cancer. It is commonly used to treat T1 grade 3 tumours and is effective to reduce post-surgical tumour recurrence (1,2), which is observed within 5 years in around 40% of patients versus around 70% of patients treated with mitomycin (3,4). The mechanisms of the resistance to BCG have yet to be elucidated (5).

BCG enhances the Th1 cytokine response by increasing secretion of IL-2, IL-12, INF-γ, and TNF, promoting cellular immune response and upregulation of MHC class I expression on urothelial cells (6,7,8). The anti-tumour CTL response requires optimal cell surface expression of HLA class I antigen tumor antigen-peptide complexes (9). Loss of HLA class I expression is a frequent event during malignant transformation and is one of the key mechanisms of cancer immune escape during the natural history of tumour development (10,11,12,13). Altered expression of HLA class I antigens has been

previously reported in bladder carcinoma (14,15). A positive correlation between reduced HLA class I expression and poor prognosis in bladder cancer patients undergoing BCG therapy has been reported by Kitamura and co-workers (16). Nevertheless, only a few studies have analyzed how immunotherapy affects escape mechanisms and HLA class I expression during tumour progression. (17,18)

In this study, we analyzed HLA class I expression in primary and recurrent tumours from bladder patients undergoing chemotherapy and/or BCG immunotherapy. Detailed analysis of frozen tumour samples revealed that BCG immunotherapy induces the selection of highly malignant cells with new and more profound alterations in HLA class I expression.

Materials and methods

Patients, clinical protocols, and tumour specimens

Surgical specimens were obtained from 18 patients with bladder cancer. Tumour grade and stage was characterized according to the standard TNM classification, where T reflects the primary tumour invasiveness and G the tumour differentiation grade. Among patients treated with mitomycin, only patients who developed recurrent tumours were selected for the study (patients 1-5) (table 1). Among 13 patients treated with BCG (patients 6-18), five patients had recurrent tumours (patients 6-10), and eight patients remained relapse-free (patients11-18) (Table 1). Patients receiving BCG therapy had six intravesicular BCG instillations at 4 weeks right after transurethral resection of bladder tumours, followed by 3 instillations every 3 months up to 1 year (6+3 treatment protocol). The median follow-up was 65 (82 ±41.81) months. Around 35% of the bladder cancer patients in the Urology Department of our hospital develop post-BCG cancer relapse.

HLA typing of peripheral blood

Table 1: Clinical history of the patients

Treatment/ recurrence	Pat	Primary tumour stage	Treatment	Months to 1º relapse (stage)	Treatment	Months to 2º relapse (stage)	Treatment	Months to 3º relapse (stage)
MITOMYCIN WITH RELAPSE	1	pTaG2	MMC	9 (pTaG2)	MMC			
	2	pTaG2	MMC	10* (pT2G3)	MMC	4 (pT2G3)		
	3	pT1G2	MMC	6* (pT1G2)	MMC	74 (pT1G2)		
	4	pTxG1	MMC	6* (pT1aG1)	MMC	9* (pT1G1)	MMC	4 (pT2G3)
	5	pT1G1	MMC	24* (pT2G3)	MMC	10 (pT2G3)		
BCG WITH RELAPSE	6	pT1G2	MMC	8* (pT1G3)	BCG	71 (pT4G3)		
	7	pTaG1	MMC	20* (pTaG2)	BCG	31 (pT1G2)		
	8	pT1G3	BCG	8 (pT1G1)	MMC	11* (pT1G3)		
	9	pT1G3	BCG	36 (pT4G3)				
	10	*pT1G1	MMC	6 (pT1G3)	BCG	5 (pT2G3)	MMC	4* (pT2G3)
BCG WITHOUT RELAPSE	11	pT1G2	MMC	7* (pT1G2)	BCG			
	12	pT1G3	BCG					
	13	pT1G3	BCG					
	14	pT1G3	BCG					
	15	pT1G3	BCG					
	16	*pT1G1	MMC	12 (pT1G3)	BCG			
	17	pT1G3	BCG					
	18	pT1G3	BCG					

* tumours not available

lymphocytes and immunohistological analysis

Immunohistological technique with the Novolink Polymer Detection System (Leica Microsystems Inc. Bannockburn, USA) was employed to analyze HLA class I expression using the following mAbs: W6/32 (anti-HLA-ABC), L-368 and GRH1 (anti-β2m), 1082C5 (anti-HLA-A locus), 42-IB5 (anti-HLA-B locus), HC-10 (anti-HLA-BC free heavy chain), SRF-8 (anti-HLA-Bw6, hybridoma from ATCC), and 548-ha (anti-HLA-Bw4, One-lambda, Canoga Park, USA). Although with these antibodies we can determine the HLA loci status, there no exist antibodies against all the allospecificities, therefore we cannot exclude loss of single HLA class I alleles.

Stained tissues were analysed by two independent researcher and HLA class I expression was determined for each antibody following these parameters: (-) negative expression was established when tumor cells staining were totally negative. (+) low HLA expression was established when tumor cells staining were at least a 75% lower than positive control. (++) medium HLA expression was established when tumor cells staining were at least a 40% lower than positive control. (+++) high HLA expression was established when tumor cells staining was similar than positive control. (H) heterogeneous expression pattern was established when tumor cells have a different staining aereas.

Tumour microdissection, DNA/RNA isolation, and reverse transcription

Cryopreserved 6 µm thick tissue sections were stained with a 0.05% wt/vol solution of toluidine blue and microdissected using the Laser micromanipulator PALM Microlaser Systems (ZEISS, Dublin, USA). Microdissected fragments were collected in PALM Adhesive Caps. These fragments were used for RNA and DNA isolation. DNA and RNA extraction was performed using the QIAamp DNA Mini Kit (QIAGEN, Westburg, Leusden, Netherlands) and Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, USA) respectively. cDNA synthesis was performed with the RNA Reverse Transcription System (Promega Corporation, Madison, USA) following the manufacturer's instructions.

Microsatellite analysis

To determine possible loss of heterozygosity (LOH) in chromosomes 6 (MHC class I heavy chain) and 15 (β2microglobulin), DNA obtained from microdissected tumour and peripheral blood was studied using 12 short tandem repeats (STRs), mapping HLA region of chromosome 6 and β2-microglobulin region of chromosome 15. STR, methods of PCR reactions, electrophoresis and data analysis were previously described (15,19). LOH was assigned when a signal reduction of >25% in three alleles was seen in tumour versus PBL sample.

Quantitative PCR

RT-PCR products from the tumours were analyzed by quantitative PCR for the expression of two target genes: HLA class I heavy chain and β2m. To control for variations in the amounts of mRNA, G6PDH and HPRT were tested as housekeeping genes. PCR reactions were performed in a Light Cycler using DNA Master Probes Kit (Roche Diagnostics, Manheim, Germany). Commercial kits were used for G6PDH and HPRT amplification (Roche Diagnostics and Search LC, GmbH Heidelberg). Amplification reactions of HLA class I heavy chain was previously described (17). Amplification reactions of β-2microglobulin were performed in following manufacturer's recommendations for Universal probe library (Roche Diagnostics, Manheim, Germany). mRNA levels for the target genes were

Table 2: Immunohistochemical analysis of HLA class I expression in bladder tumours.

Treatment/ recurrence	Patient	Tumour	HLA-ABC	β2m	HLA-A	HLA-B	HLA-B,C free HC
MITOMICYN WITH RELAPSE	1	Before	+++	+++	+++	++	+++
		After	+++	+++	+++	++	+++
	2	Before	++	++	++	++	++
		After	++	++	++	++	++
	3	Before	+++	++	-	++	+++
		After	+++	++	-	++	+++
	4	Before	+++	+++	+++	-	-
		After	+++	+++	+++	-	-
	5	Before	++	++	-	-	++
		After	++	++	-	-	++
BCG WITH RELAPSE	6	Before	++	++	++	++	++
		After	-	-	-	-	-
	7	Before	++	++	++	-	+
		After	-	-	-	-	-
	8	Before	++	++	+	+	+
		After	-	-	-	-	-
	9	Before	++	++	++	++	++
		After	++	++	-	++	++
	10	Before*	+++	+++	++	++	+++
			++	++	+	-	++
		After	++	++	-	-	++
BCG WITHOUT RELAPSE	11	Before	+++	+++	+++	+++	+++
	12	Before	+++	+++	+++	+++	+++
	13	Before	+++	+++	+++	+++	+++
	14	Before	+++	+++	+++	++	+++
	15	Before	++	+++	++	-	+++
	16	Before	++	+++	+++	-	++
	17	Before	H++/+	H++/+	-	-	H++/-
	18	Before	H++/+	H++/+	-	-	H++/-

*This sample has tumour nest with distinct HLA class I expression patterns
(-) negative expression.
(+) low HLA expression.
(++) medium HLA expression.
(+++) high HLA expression.
(H) heterogeneous expression pattern

calculated according to the calibration curves. All PCR products were checked by melting point analysis and by gel electrophoresis to verify the PCR specificity.

Statistical analysis

We used the Mann–Whitney U-test with a significance threshold of $p < 0.05$ to evaluate possible differences between primary tumours obtained from relapse-free patients and patients with relapse after BCG treatment. Statistical Package for the Social Sciences (SPSS) was used for statistical comparison.

Results

BCG but not mitomycin induces more profound HLA class I alterations in recurrent lesions than in primary tumours

Immunohistochemistry studies demonstrated the same HLA expression phenotype in post-mit

omycin recurrent tumours (patients 1-5) as in the primary tumours (Table 2). In contrast, tumours recurring after BCG treatment (patients 6-10) showed new HLA class I alterations, including loss of locus A (patients 9, 10) or total loss of expression (patients 6,7 and 8) (Table 2, Fig. 1). In patient 10, the primary tumour had two distinct tumour nests, one strongly HLA-positive and the other with HLA-B locus loss, and reduced expression of HLA-A locus. After BCG treatment, the recurrent tumour in this patient showed a total loss of both HLA-A and B loci.

Higher expression of HLA class I molecules in primary tumours from post-BCG relapse-free patients

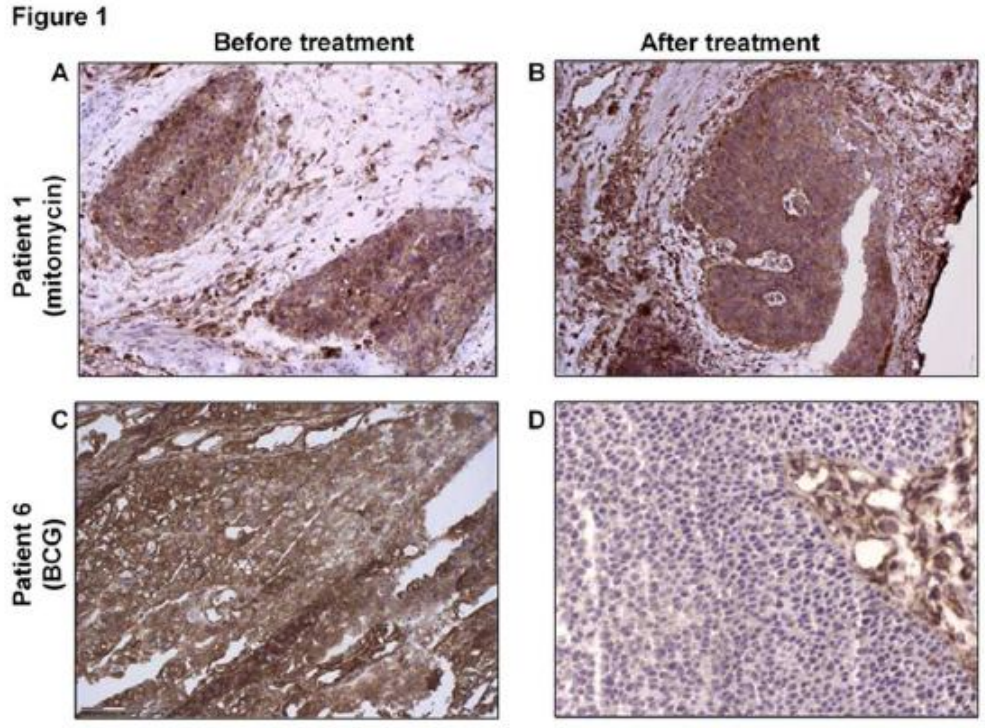

Figure 1. Immunohistochemical staining of tumours before and after treatment. Patient 1 showed positive tumour staining with antibody against HLA-class I cell surface complex (W6/32) both before (A) and after (B) mitomycin treatment. In Patient 6, we observed a positive tumour immunolabeling before BCG therapy (C) and loss of HLA class I expression in post-BCG relapsed tumour (D). 84x64mm (300 x 300 DPI)

The immunostaining of primary tumours from showed: strong expression of all HLA class I molecules in patients 11, 12, 13 and 14; positive expression of all HLA class I molecules except HLA-B locus in patients 15 and 16; and a heterogeneous pattern of HLA class I expression in different areas of the tumour nest in patients 17 and 18 this heterogeneous pattern consist in the co-localization of HLA class I positive tumour cells and CD-3 infiltrating cells (SFig 1). Therefore some BCG relapse free patients showed no alterations and other showed B locus alterations or heterogeneous expression.

The HLA class I status of these patients showed clearly a higher expression than the patients with relapse after BCG. When we compare mitomicyn treated patients we saw showing no alterations or lack of expression of some HLA molecules, as the mitomycin therapy is independent of the immune system, the immune status of patients showing relapse present a higher diversity.

Primary tumours relapsing after BCG therapy have higher incidence of LOH in chromosomes 6 and 15 and lower HLA class I mRNA levels.

Loss of heterozygosity was analyzed using eight microsatellite markers for chromosome 6 and five markers for chromosome 15. LOH in

chromosome 6 and chromosome 15 was detected

in two of the five mitomycin-treated patients (Table 3). Among the five BCG-treated patients with recurrence,

LOH in chromosome 6 was detected in the primary tumour of three patients (60%) and LOH in chromosome 15 in the primary tumour of four (80%) (Table 3); one of the patients without LOH in chromosome 6 in primary tumour (patient 8) showed this alteration in the post-BGC recurrent tumour. Importantly, only two (25%) of the eight relapse-free BCG-treated patients showed LOH in chromosome 6 in primary tumour and only two patients (25%) had LOH in chromosome 15 (Table 3), with one patient having LOH in both chromosomes.

Table 3: Frequency of LOH in chromosomes 6 (HLA class I heavy chain) and 15 (β2-microglobulin) in bladder cancer patients.

Treatment/recurrence	Primary tumour		Relapse	
	Chr 6	Chr 15	Chr 6	Chr 15
MITOMICYN WITH RELAPSE	40%	40%	40%	40%
BCG WITH RELAPSE	60%	80%	80%	80%
BCG WITHOUT RELAPSE	25%	25%		

As depicted in Fig. 2, mRNA expression levels of HLA class I heavy chain and β2m were significantly lower in primary tumours from BCG-treated patients with recurrence than from those without (p=0.005, Mann-Whitney's U test).

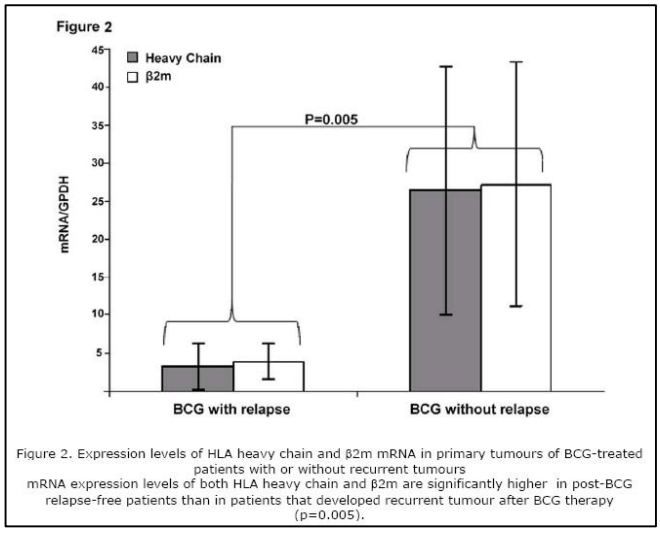

Figure 2. Expression levels of HLA heavy chain and β2m mRNA in primary tumours of BCG-treated patients with or without recurrent tumours
mRNA expression levels of both HLA heavy chain and β2m are significantly higher in post-BCG relapse-free patients than in patients that developed recurrent tumour after BCG therapy (p=0.005).

Discussion

The discovery of tumour-associated antigens recognized by T lymphocytes as

peptides presented by major histocompatibility complex (MHC) molecules (20,21) opened up new ways to understand the concept of tumour immune surveillance proposed by Burnet and Thomas in 1957. However, the effectiveness of immunotherapy protocols designed to increase anti-tumour T-cell responses (22,23,24,25,26) has been below expectations (27,28), which has been attributed to the development of highly sophisticated tumour immune escape mechanisms. These include the loss of tumour-associated antigen (TAA) expression, loss of costimulatory signals and/or adhesion molecules, alterations in the apoptosis program, an immunosuppressive tumour microenvironment, deficiency in the signal transduction pathway of CD8+ cytotoxic T cells (CTL), and induction of T-cell tolerance (29,30,31,27). One of the best documented routes of cancer immune escape is the alteration of HLA class I antigen expression on tumour cells (11,29,13,32), which may have a negative effect on tumour rejection by the immune system and pose a significant challenge to the success of cancer immunotherapy.

In this study of HLA class I expression in bladder tumours from patients treated with mitomycin or BCG, we observed that: 1) BCG treatment prevented the recurrence of bladder tumours expressing a high level of HLA class I molecules but not of tumours with a low expression of these molecules and with structural alterations (LOH) in chromosomes 6 and/or 15 (Table 2, Table 3, Fig. 1); and 2) HLA class I expression showed new alterations in BCG-treated recurrent tumours versus primary tumours,

Figure 3

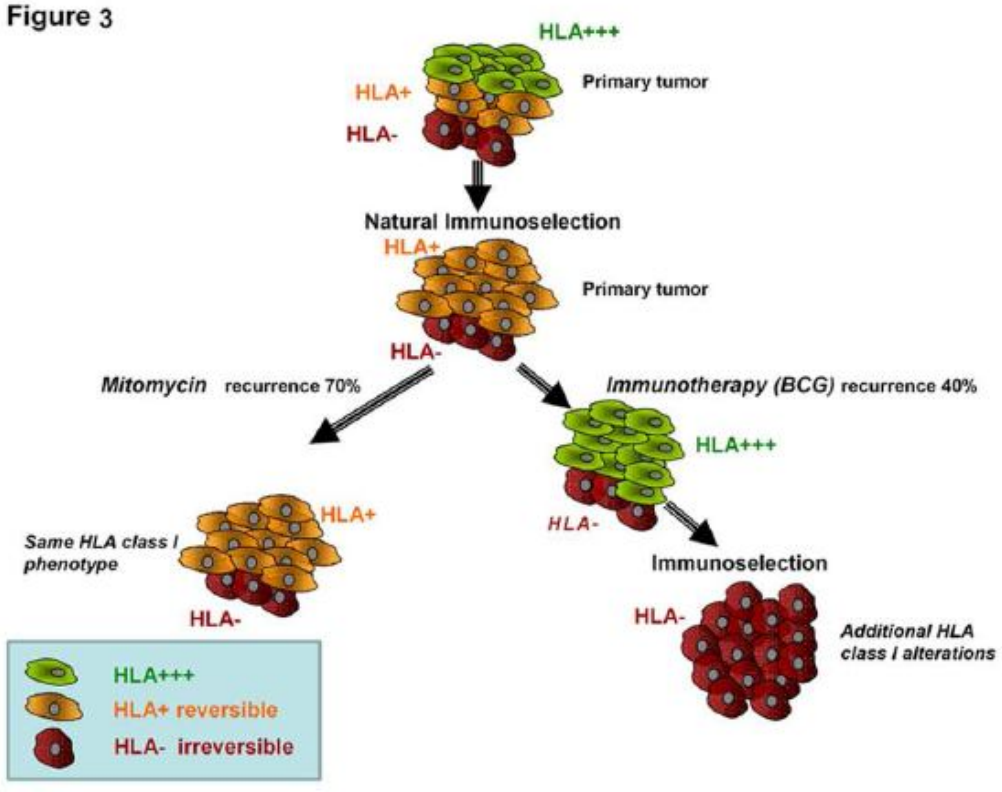

Figure 3. Changes in HLA class I expression during tumour development. During natural tumour development, transformed cells with normal HLA pattern (HLA+++) are eliminated, while malignant cells with altered HLA class I expression are immunoselected (HLA + and HLA -). Immunotherapy stimulates an additional strong cycle of immunoselection, eliminating cells with low and/or reversible HLA changes (HLA+). However, cancer cells with more profound HLA class I alterations, including irreversible defects (HLA-), escape the immune system and develop recurrent tumours. 84x64mm (300 x 300 DPI)

whereas there was no change in this expression profile after mitomycin treatment (Table 2).

During natural tumour development, the clonal progression of new tumour cell immune escape variants is favoured by a combination of a low immunogenicity of tumour cells, certain tumour environmental factors, and T-cell immunoselection (10,11,31). We favour the proposition that immunotherapy induces an additional cycle of immune selection of tumour cells with reduced or negative HLA class I expression, which are potentially able to escape from T-cell recognition. However, if tumour cells have a reversible HLA alteration ("soft" lesions), BCG therapy induces the local release of cytokines leading to the recovery of normal HLA class I expression, increased tumour cell immunogenicity, and immune rejection of tumour cells (33,34). In contrast, when tumour cells have irreversible HLA class I defects ("hard" lesions), including LOH and/or gene mutations, immunotherapy will fail to induce sufficient upregulation of the tumour HLA class I antigen expression, and the tumour cells will escape from immune recognition and relapse. Therefore, the activation of immune surveillance after BCG therapy leads to the immunoselection and elimination of tumour cells with upregulated HLA class I expression and to the outgrowth of cancer cells with structural HLA alterations (Fig. 3). This hypothesis is supported by our observation of: 1) a higher frequency of LOH in primary tumours in patients with recurrent cancer after BCG treatment than in relapse-free patients; and 2) an increased percentage of LOH in recurrent tumours (Table 3), suggesting that tumour cells with reversible alterations were eliminated.

In this context, we recently obtained clinical and laboratory data evidencing the immunoselection of HLA-class I-deficient tumour variants during immunotherapy. Thus, two patients with metastatic melanoma each developed two types of secondary lesions, progressing and regressing, in response to systemic immunotherapy. The progressing metastases showed a lower level of HLA class I expression and more profound alterations (including LOH) in comparison to the regressing lesions (17,18).

Although this study analysed a small sample size, The obtained results strongly support a major role for tumour HLA class I expression in the response to immunotherapy. The HLA class I status of tumour cells should therefore be taken into account before cancer immunotherapy and monitored during clinical trials. HLA class I losses have been described in a wide variety of tumours derived from HLA class I-positive epithelia. To our knowledge, this is the first clinical evidence of immunotherapy-induced immunoselection of HLA class I loss tumour variants in bladder cancer.

However, investigation of correlations between tumour recurrence/spread and HLA class I expression is hampered by the difficulty in obtaining progressing/regressing metastatic samples or relapsed tumours in patients undergoing immunotherapy. We propose in this paper the major importance of could obtain and analyse the tumor immune status of patients treated with immunotherapy protocols in order to enlarge the data available and could stablish tumors inner parameters of response to this kind of therapies

Acknowledgements The authors would like to thank Ana Isabel Rodriguez for technical assistance. This work was supported by grants from the Fondo de Investigaciones Sanitarias (FIS PI 07/0436 and PI 08/1265), Red Genomica del Cancer (RETIC RD 06/0020), Plan Andaluz de Investigacion (Group CTS 143), Consejeria Andaluz de Salud (SAS), Proyecto de Excelencia de Consejeria de Innovacion (CTS 695) in Spain, and from the Integrated European Cancer Immunotherapy project (OJ2004/C158, 518234).

References

1.- Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou-Redorta J EAU guidelines on non-muscle-carcinoma of the bladder. Actas Urol Esp 2009;33:361-71

2.- Demkow T, Alter A, Wiechno P. Intravesical bacillus Calmette-Guérin therapy for T1 superficial bladder cancer. Urol Int 2008;80:74-9

3.- Jarvinen R, Kaasinen E, sankila A. Rintala E. Long-term efficacy of maintenance Bacillus Calmette-Guerin versus maintenance mitomycin C instillation therapy in frequently recurrent TaT1 tumours without carcinoma in situ: a subgroup analysis of the prospective, randomised FinnBladder i study with a 20-year follow-up. Eur. Urol 2009;56:260-5

4.- Malmström PU, Sylvester RJ, Crawford DE, Friedrich M, Krege S, Rintala E, Solsona E, Di Stasi SM, Witjes JA. An individual patient data meta-analysis of the long-term outcome of randomised studies comparing intravesical mitomycin C versus Bacillus Calmette-Guerin for non-muscle-invasive bladder cancer. Eur. Urol 2009;56: 247-56

5.- Zlotta AR, Fleshner NE, Jewett MA. The management of BCG failure in non-muscle-invasive bladder cancer: an update. Can Urol Assoc J 2009;3:199-205

6.- Patard JJ, Saint F, Velotti F, Abbou CC, Chopin DK. Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. Urol Res 1998;26:155-9

7.- Böhle A, Brandau S. Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer J Urol 2003;170:964-69

8.- Videira PA, Calais FM, Correia M, Ligeiro D, Crespo HJ, Calais F, Trindade H. Efficacy of bacille Calmette-Guérin immunotherapy predicted by expression of antigen-presenting molecules and chemokines. Urology 2009;74:944-50

9.- Lonchay C, van der Bruggen P, Connerotte T, Hanagiri T, Coulie P, Colau D, Lucas S, Van Pel A, Thielemans K, van Baren N, Boon T. Correlation between tumour regression and T cell responses in melanoma patients vaccinated with MAGE antigen. Proc Natl Acad Sci USA 2004;101:14631-8

10.- Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, Stern PL. Natural history of HLA expression during tumour development. Immunol Today 1993;14:491-9

11.- Garrido F, Ruiz-Cabello F, Cabrera T, Pérez-Villar JJ, López-Botet M, Duggan-Keen M, Stern PL. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today 1997;18:89-95

12.- Garrido F, Algarra I. MHC antigens and tumour escape from immune surveillance. Adv Cancer Res 2001; 83:117-58

13.- Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz- Cabello F, Garrido F MHC class I antigens and immune surveillance in transformed cells. Int Rev Cytol 2007;256:139-89

14.- Cabrera T, Pedrajas G, Cozar JM, Garrido A, Vicente J, Tallada M, Garrido F. HLA class I expression in bladder carcinomas. Tissue Antigens 2003;62:324-7.

15.- Maleno I, Romero JM, Cabrera T, Paco L, Aptsiauri N, Cozar JM, Tallada M, López-Nevot MA, Garrido F. LOH at 6p21.3 region and HLA class I altered phenotypes in bladder carcinomas. Immunogenetics 2006;58:503-10

16.- Kitamura H, Torigoe T, Honma I, Sato E, Asanuma H, Hirohashi Y, Sato N, Tsukamoto T. Effect of human leukocyte antigen class I expression of tumour cells on outcome of intravesical instillation of bacillus calmette-guerin immunotherapy for bladder cancer. Clin Cancer Res 2006;12:4641-4

17.- Cabrera T, Lara E, Romero JM, Maleni I, Real LM, Ruiz-Cabello F, Valero P, Camacho FM, Garrido F. HLA class I expression in metastatic melanoma correlates with tumour development during autologous vaccination. Cancer Immunol Immunother 2007;56:709–17

18.- Carretero R, Romero JM, Ruiz-Cabello F, Maleno I, Rodriguez F, Camacho FM, Real LM, Garrido F, Cabrera T. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. Immunogenetics 2008;60:439-47

19.- Koene GJ, Arts-Hilkes YH, van der Ven KJ, Rozemuller EH, Slootweg PJ, de Weger RA, Tilanus MG. High level of chromosome 15 aneuploidy in head and neck squamous cell carcinoma lesions identified by FISH analysis: limited value of beta2-microglobulin LOH analysis. Tissue Antigens 2004;64:452-61

20.- Boon T, van der Bruggen P. Human tumour antigens recognized by T lymphocytes. J Exp Med 1996;183:725-9

21.- Renkvist N, Castelli C, Robbins PF, Parmiani G. A listing of human tumour antigens recognized by T cells. Cancer Immunol Immunother 2001;50:3-15

22.- Coulie PG, Hanagiri T, Takenoyama M. From tumour antigens to immunotherapy Int J Clin Oncol

2001;6:163-70

23.- Coulie PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, Van Baren N, Boon T. Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen. Immunol Rev 2002;188:33-42

24.- Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002;298:850-4

25.- Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature 2001;411:380-4

26.- Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 2009;21:233-40

27.- Rivoltini L, Canese P, Huber V, Iero M, Pilla L, Valenti R, Fais S, Lozupone F, Casati C, Castelli C, Parmiani G. Escape strategies and reasons for failure in the interaction between tumour cells and the immune system: how can we tilt the balance towards immune-mediated cancer control? Expert Opin Biol Ther 2005;5:463-6

28.- Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nature Medicine 2004;10:909-15

29.- Marincola FM, Jaffe EM, Hicklin DJ, Ferrone S. Escape of human solid tumours from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol 2000;74:181-273

30.- Zou W. Immunosupressive networks in the tumour environment and their therapeutic relevance. Nature Reviews Cancer2005; 5:263-74

31.- Drake CG, Jaffee E, Pardoll DM. Mechanism of immune evasion by tumours. Adv Immunol 2006;90:51-81

32.- Seliger B. Molecular mechanisms of MHC class I abnormalities and APM components in human tumours. Cancer Immunol Immunother 2008;57:1719-26

33.- Aptsiauri N, Carretero R, Garcia-Lora A, Real LM, Cabrera T, Garrido F. Regressing and progressing metastatic lesions: resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations. Cancer Immunol Immunother 2008;57:1727-33

34.- Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: Implications for immunotherapy. Int J Cancer 2010;127:249-56

3.4. Regression of melanoma metastases is associated with activation of antigen presentation and immune-mediated rejection genes.

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Key words: Melanoma, metastasis, Immunotherapy, rejection, HLA

Abstract.

Purpose: New and more advanced cancer immunotherapies have been generated in recent years, however clinical benefit has been observed in only a small fraction of patients. Distinct mechanisms of tumor escape from immune recognition were proposed as responsible for failure of immunotherapy. In order to elucidate the tumor response after immunotherapy, we have characterized the transcriptional pattern of progressing and regressing melanoma metastases from 2 patients showing mixed response after immunotherapy.

Experimental Design: We have studied 10 regressing and 6 progressing metastases obtained from 2 patients showing mixed response. Transcriptional pattern was analyzed by whole genome microarray hybridization. Obtained results were confirmed by Q-PCR and immunohistological analysis

Results: Whole genome transcriptional analysis showed that regressing metastases from both patients have upregulated genes involved in antigen presentation and immune rejection pathways. Up-regulation of these genes was associated with the expression of interferon-regulatory factor (IRF-1). Quantitative PCR and immunohistochemical analysis showed an increased expression of HLA class I in tumor cells from regressing lesions and a high degree of tumor infiltrating immune cells.

Conclusions: Our results clearly indicate that regression of melanoma metastases is due to an acute immune rejection associated with an increase in HLA Class I expression. We favor the idea that the nature of HLA Class I lesions in the tumor cells, and not the type of immunotherapy used, determine the success or failure of immunotherapy.

Introduction

Immunotherapy has been extensively used for the treatment of various cancers. Different treatments strategies have been tested spanning non-specific therapies, such as interferon alpha 2b, interleukin 2 or 12 to more specific ones, such as autologous vaccination with naked peptides, dendritic cells, or plasmid DNA. In addition, adoptive transfer of tumor-specific cytotoxic T cells has been used with promising results in the context of advanced melanoma (1-3).

Active-specific immunization with tumor antigen has been shown to generate immune T cells capable of recognizing antigenic peptides presented on tumor cells (3). However, successful enhancement of tumor-specific T cells does not linearly correlate with tumor regression and only a small percentage of patients demonstrate objective tumor regression. It is well documented that tumor cells develop a variety of sophisticated tumor escape mechanisms (4). One important tumor escape mechanism is represented by the loss or downregulation of Major Histocompatibility Complex (MHC) class I antigens in tumor cells that have been frequently observed in a variety of human malignancies derived from HLA class I positive epithelia (5).

Some patients display a mixed response to the same treatment i.e. regression and progression of different metastatic lesions following immunotherapy (6,7). Mixed responders represent an interesting subset of patients, in whom synchronous lesions respond differently to the same therapy. These rare cases are important to study because different behaviors are seen in the same patient at the same time. This allows us to consider solely the tumor's determining factors in immune responsiveness excluding variation in the genetic background of different patients or external variables affecting the potency of identical treatments provided at different times (8).

Our laboratory has previously described a correlation between HLA class I expression and the response of synchronous melanoma metastases to autologous vaccination plus BCG (M-VAX) and alfa-IFN-2b immunotherapy in two melanoma patients (9,10). Progressing metastases displayed defects leading to low HLA class I expression while regressing tumors bore high HLA-I levels. To date, no comparative analysis of synchronous lesions displaying discordant behavior has been performed at the global transcript level. Existing data on the transcriptional activation specific to regressing lesions is based on comparative studies among different patients whose lesions responded differently to therapy. These studies suggest that the mechanisms associated with tumor rejection parallel the acute inflammatory process occurring during allograft and pathogen rejection (11,12).

To obtain a comprehensive view of the molecular process leading to response and tumor rejection after immunotherapy, we analyzed the expression profile of progressing and regressing melanoma lesions and validated the salient findings by quantitative PCR and immunohistochemistry.

Material and methods.

Patients, clinical protocols and tumor specimens: We studied 15 metastases from 2 patient treated with M-VAX (24) and alpha-INF-2b. Both patients showed a mixed response to immunotherapy, with some lesions disappeared, others reduced their size while some new ones had appeared or growth. Five metastases were removed from patient 1 and sent to our laboratory. Two of these metastases (designated P1-VAX.R1, and P1-VAX.R2) were regressing at the time of excision, whereas the other three were progressing (designated P1-VAX.P1, P1-

VAX.P2, P1-VAX.P3). We obtained 10 lymph node metastases from patient 2, five of them were obtained after alpha-INF-2b and another 5 after M-VAX treatment. We obtained 1 progressing and 4 regressing lesions after each treatment. Samples obtained after INF treatment were designated as P2-INF.R1 P2-INF.R2 P2-INF.R3 P2-INF.R4 and P2-INF.P1 meanwhile samples obtained after M-VAX were named P2-VAX.R1 P2-VAX.R2 P2-VAX.R3 P2-VAX.R4 and P2-VAX.P1. The detailed clinical history of the patient was previously described (8,9). Samples were obtained from the Department of Dermatology, Hospital Virgen de la Macarena, Sevilla, Spain. Informed consents and approval of the research protocol by the institutional review board were obtained. All metastases were removed from the patient at the same time after immunotherapy and snap-frozen in liquid nitrogen-cooled isopentane. Tumor status were established according to PET and CT scans

Tumor cells micro-dissection: Cryopreserved 6 µm thick tissue sections were fixed in 70% ethanol and stained with a 0.05% wt/vol solution of toluidine blue and microdissected using a Laser micromanipulator (PALM Microlaser Systems, ZEISS). Microdissected fragments were collected in PALM Adhesive Caps.

Total RNA isolation and amplification: Total RNA (tRNA) from frozen sections and micro-dissected tumor cells were isolated using the miRNeasy Mini kit (QIAGEN, Westburg, Leusden, The Netherlands). The quality of tRNA was tested with Agilent Bioanalyzer 2000 (Agilent Technologies, Santa Clara, USA). For gene expression studies, tRNA obtained from the frozen section was amplified into antisense RNA (aRNA) as previously described (25). Reference for human arrays consisted of pooled PBMCs from four normal donors. Human reference total RNA was also amplified into antisense RNA.

Microarray performance and data processing: Array quality was documented as previously described (26). Both reference and test aRNA were directly labeled using ULS aRNA Fluorescent Labeling kit (Kreatech) with Cy3 for reference and Cy5 for test samples and co-hybridized at 45°C for 18 hours into 36k whole genome human array (27). After incubation, the arrays were washed and stained in the fluidics station using GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, USA). The data were uploaded to the mAdb databank http://nciarray.nci.nih.gov, further analyzed using BRBArray-Tools developed by the Biometric Research Branch, National Cancer Institute http://linus.nci.nih.gov/BRBArrayTools.html (28) and clustered by TreeView software (29). Unsupervised analysis was used for class discovery using the Stanford Cluster program applying standard gene filtering parameters (80% gene presence across all experiments and at least 1.5-fold ratio change) and Treeview program for visualization. Average gene ratios were normalized and displayed according to uncentered correlation algorithms. Class comparison was performed using parametric unpaired Student's t test to identify differentially expressed genes among progressing and regressing tumors. Validation by quantitative polymerase chain reaction (q-PCR) of the gene sets were not performed due to the fact that we have previously shown the present method for RNA amplification is robust and yields results comparable to those obtained by qPCR (26). Moreover, salient gene products were validated at the protein expression level by immunostaining. Gene function interpretation was based on GeneOntology software, while pathway analysis was based on Ingenuity Pathways Analysis (IPA) software. Primary microarray data are available in NCBI's Gene Expression Omnibus public database (microarray platform, GPL7088; microarray data,

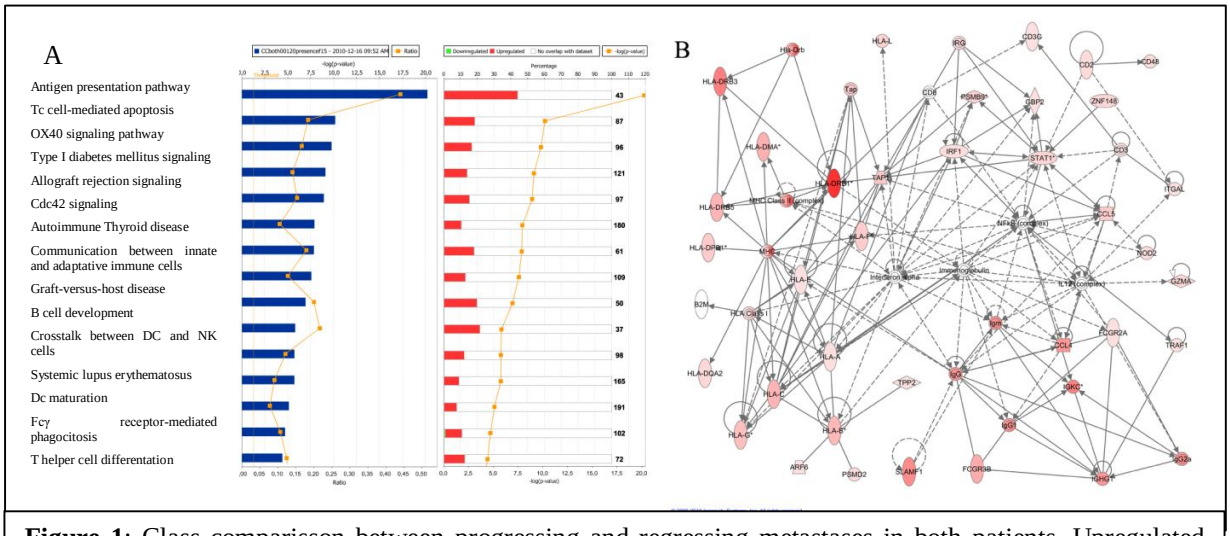


Figure 1: Class comparisson between progressing and regressing metastases in both patients. Upregulated genes in regressing metastases are represented in red whereas those downregulated are represented in green. A) Top 15 first canonical pathways ranking according to significance level (Fisher exact test –log p-value) based on the 579 genes using gene enrichment (p=0,001) analysis B) Antigen presentation plus IRF-1 self organizing network according to IPA analysis based on the 579 genes derived from the low stringency analysis.

GSE26383).

Quantitative RT-PCR of isolated tumor cells: RT products from the five metastases were analyzed for the expression of HLA locus A, B, and C by quantitative PCR. To control for variations in the amounts of RNA, G6PDH and HPRT were tested as a housekeeping gene. All PCR reactions were performed in a Light Cycler instrument using LC-FastStart DNA Master Probes Kit and LC-FastStart DNA SYBR Green I Kit (Roche Diagnostics, Manheim, Germany). For G6PDH and HPRT we used commercial kits (Roche Diagnostics, Manheim, Germany). Amplification reactions of HLA loci were described previously (9).

Immunohistological analysis: Immunohistological analysis was performed using Novolink® Polymer Detection System kit (Novocastra, Newcastle, United Kingdom). For HLA class II staining we used GRB-1 mAb (anti-HLA-DR). Infiltration pattern was studied with, OKT8 mAb (anti CD8) OKT3 mAb (anti CD3) (ATTC, Manassas, Virginia, USA) and RPA-T4 mAb (anti CD4) (BD Biosciences Pharmingen, Belgium).

Online supplemental material: Supp. table 1 shows genes significantly expressed between progressing and regressing metastases. Suppl. Fig. 1 showed chromosomal distribution of genes differentially expressed between progressing and regressing metastases. Suppl. Fig. 2 showed class comparisson between regressing metastases treated with interferon versus those treated with M-VAX.

Results

Regressing metastases display up-regulation of antigen presentation and immune rejection patterns.

High stringency class comparison between the progressing and regressing metastases from both patients was performed using as nominal cut-off significance level of ≤ 0.001. This analysis identified 167 genes differentially expressed between regressing and non regressing lesions – most of which were associated with antigen processing and presentation function (table S1) (Fig S1). To gain a more comprehensive portrait of the

transcriptional signatures associated with rejection, we performed gene enrichment analysis comparing regressing and non-regressing lesions at a nominal significance cut-off p-value $\leq$ 0.01. This analysis identified 540 transcripts differentially expressed between the two phenotypes (Table S1). The enriched data set was ranked according to significance of gene enrichment (Fisher exact test) by IPA; the top 15 canonical pathways among the approximately 400 included in IPA related to mechanisms associated with immune-mediated, tissue–specific destruction (Fig. 1A). The top ranking self-organizing network by IPA based on this data set clearly demonstrated antigen presentation as the primary determinant of immune responsiveness centered on the dominant transcription factor Interferon regulatory factor 1 (IRF-1). Figure 1B shows the upregulation of IRF and antigen presentation networks. Metastases

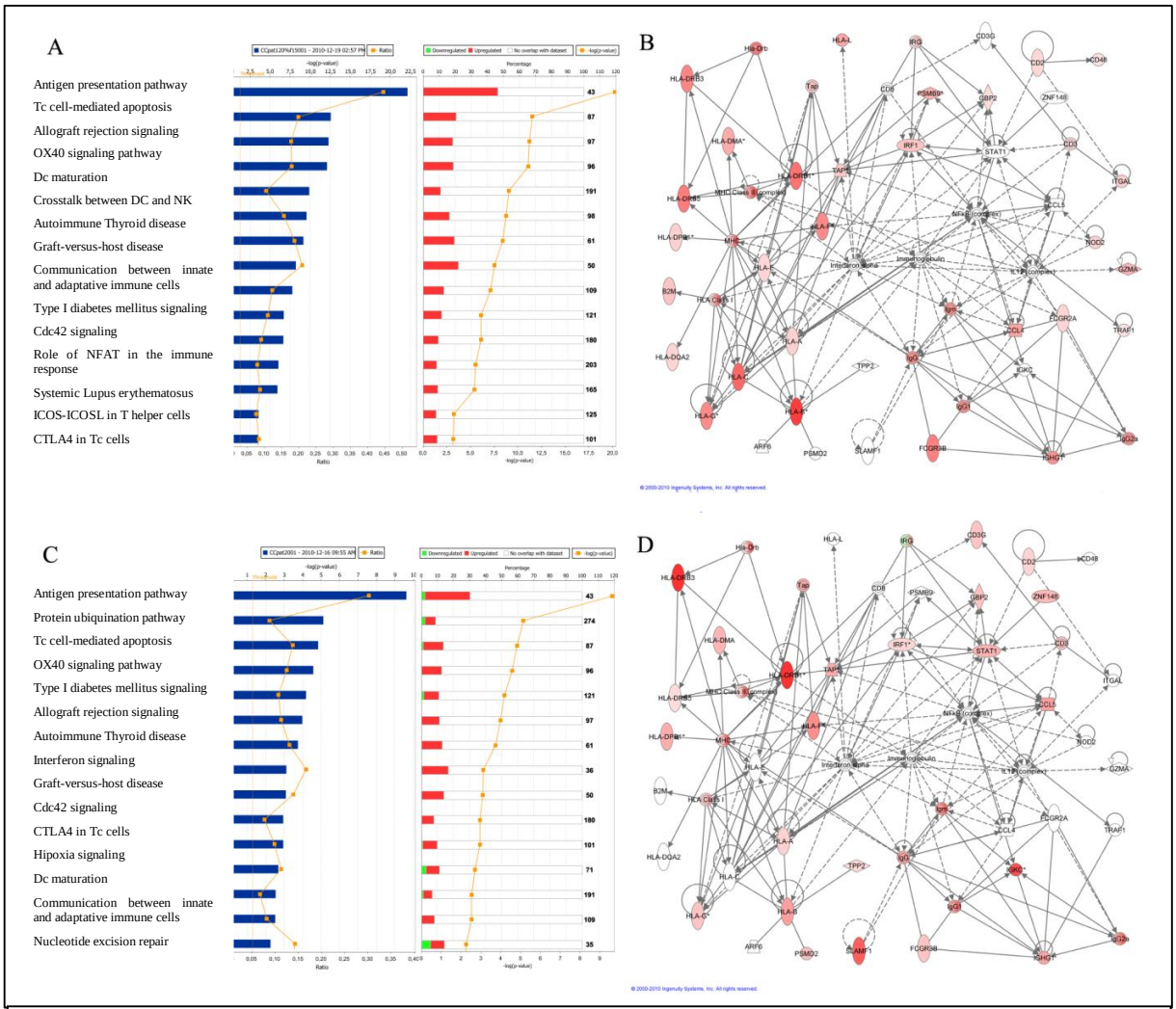


Figure 2: Class comparisson between progressing and regressing metastases in each individual patient. Upregulated genes in regressing metastases are represented in red whereas those downregulated are represented in green. A) Patient 1 top 15 first canonical pathways ranking according to significance level (Fisher exact test –log p-value) based on the 522 genes using gene enrichment (p=0,001) analysis B) Antigen presentation plus IRF-1 self organizing network according to IPA analysis with the 522 genes derived from the low stringency analysis in patient 1. C) Patient 2 top 15 first canonical pathways ranking according to significance level (Fisher exact test –log p-value) based on the 935 genes using gene enrichment (p=0,001) analysis D) Antigen presentation plus IRF-1 self organizing network according to IPA analysis with the 935 genes derived from the low stringency analysis in patient 2.

To determine the immune response against the regressing lesions in each patient, we compared progressing versus regressing lesions inside each patient separately. The results are showed in figure 2. Both patients showed similar differences between the two groups of metastases. Canonical pathways were similar between comparisons (Fig. 2 A and C) and antigen presentation and IRF activation networks were mostly involved in discriminating responding from non responding lesions in both patients in spite of the different genetic background and different treatments (Fig. 2 B and D). Chromosomal distribution of genes differentially expressed between progressing and regressing metastases demonstrated enrichment for transcripts located in chromosome 6 (Fig. S2), where antigen presentation and many inflammation genes are located.

The results showed an acute immune response in regressing metastasis, with most transcripts associated with immune rejection response being upregulated. These included activation of antigen presentation, interferon stimulated genes (ISGs), immune effector function related genes (IEFs) and several chemokines (Figures 1 and 2, network analyses).

To investigate the differences between regressing metastases after interferon and autologous vaccination we compared regressing metastases from patient 2 after interferon versus those after M-VAX. IPA canonical pathways analyses using the significant genes from the high stringency comparison showed a borderline significant upregulation of antigen presentation in M-VAX treated regressing lesions. We found no other significant different pathways between regressing lesions after interferon and M-VAX (Table S1) (fig. S2). Therefore there not exist major differences in the regressing pathway response after interferon and M-VAX treatment.

Immunohistological validation of immune infiltrates shows a large T-cell population inside regressing tumors.

To confirm the enhancement of immune activity in regressing lesions, we studied the immune infiltrate in cryo-preserved tissue sections. Staining with antibodies against HLA class II DR indicated that both

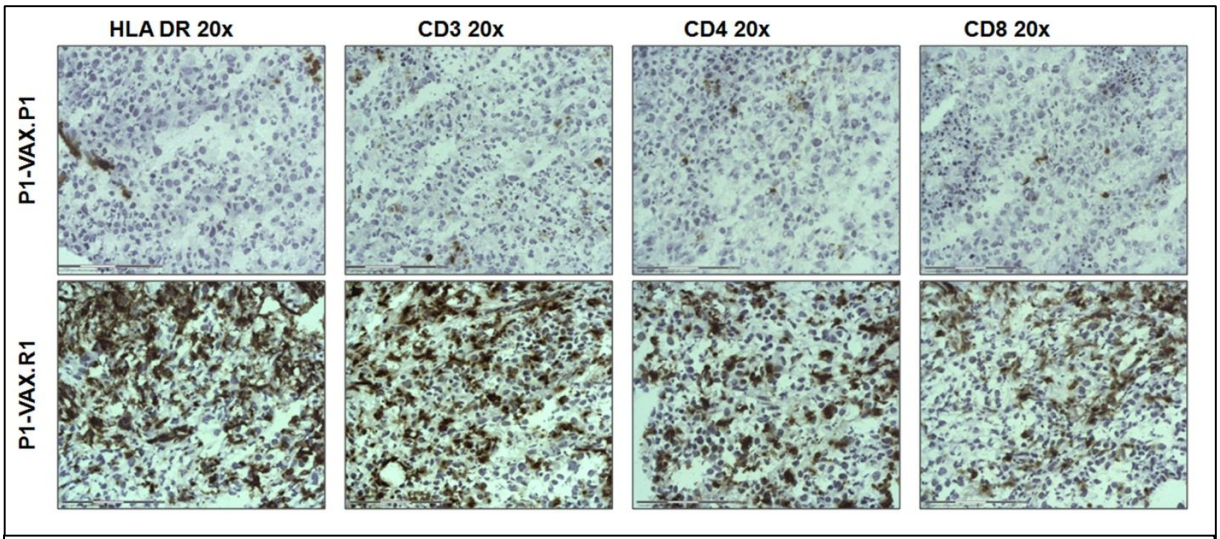


Figure 3: Immunoperoxidase staining of frozen sections of metastases: A) both progressing and regressing tumor cells have no expression of HLA-DR. The staining shows a high degree of HLA-DR positive infiltration in regressing metastasis, in contrast the progressing lesion have very low infiltration. B) CD3 staining demonstrates infiltrating cells consist mostly in T lymphocytes C) and D) The number of CD4 positive cells in the infiltrate is higher than the CD8 cells.

progressing and regressing tumor cells were negative for HLA class II expression, while the immune infiltrate showed strong positive staining (Figure 3). There was a dramatic difference in the number of infiltrating cells between phenotypes; progressing lesions showed low number of infiltrating cells, and vice versa for regressing lesions. Therefore, differences in HLA class II expression documented by transcriptional analysis of bulk mRNA were not associated with enhanced tumor cell expression, but rather with the enrichment of the tumor environment in immune cells. The composition of the infiltrate in regressing metastases consisted mostly in CD3+ T cells (80% of CD4+ and 20% of CD8 + cells) (Fig. 3).

Microdisected isolated tumor cells from progressing metastases showed a reduced expression of HLA class I A, B and C loci

Immune cell infiltration in tumor impair the analysis of HLA class I expression in tumor cells. For this reason we isolated tumoral cells by laser microdisection. Quantitative PCR analysis of isolated tumor cells demonstrated low messenger RNA levels for HLA-A locus transcripts with residual transcription of HLA-B and -C loci by progressing tumor cells in patient 1 (Fig. 4). In contrast, cancer cells from regressing lesions demonstrated enhancement in HLA-A, HLA-B and HLA- C transcription. Progressing tumor cells from patient 2 have a residual transcription of the three loci. In contrast regressing metastasis have high mRNA levels of the HLA loci. HLA levels are higher in post M-VAX regressing lesions. These data confirmed the array results and showed the differential HLA transcription in isolated tumoral cells. mRNA levels also confirmed immunohistochemical staining of HLA Class I molecules described previously (8,9). HLA levels in tumoral cells from patient 2 were previously published (9).

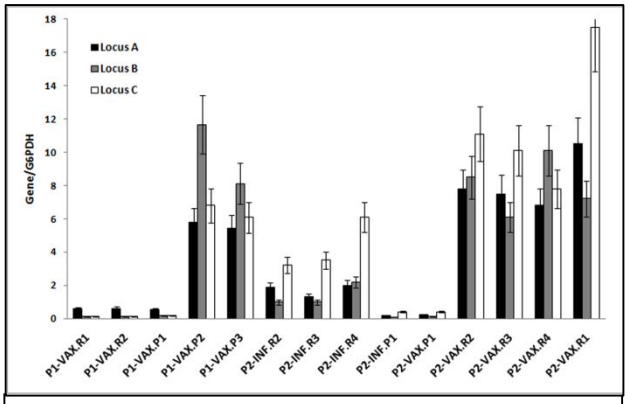


Figure 4: Normalized mRNA levels of tumor cells isolated by laser microdissection. The progressing metastasis showed low transcription levels of HLA-A locus and residual levels of HLA-B and -C loci. In contrast the regressing tumor cells displayed a high transcription of the tree HLA loci.

Discussion

Results obtained from the comparison of melanoma metastases with different behaviors in response to therapy in 2 patients who experienced a mixed response, indicate that the variation between regressing and progressing lesions are dramatic and predominantly due to the differential activation of genes involved in acute inflammatory processes (Fig. 1 and 2). Among 30.000 studied genes we mostly detected upregulation of those with immune rejection function in regressing metastases. We propose that these differences are due to efficient recognition and elimination of regressing metastases by the activated immune system triggered by HLA Class I upregulation. In contrast, progressing lesions do not upregulates HLA and are not recognized by the immune cells.

The transcriptional pattern expressed by regressing metastases was quite similar independently of the type of immunotherapy used. This suggests that the mechanisms leading to tumor rejection converge in a unique pathway (Fig 5). In this context, the expression pattern of regressing lesions was also similar to that observed after imiquimod administration in

basal cell carcinoma (12,13), allograft rejection, graft versus host disease, autoimmunity or acute infection resulting in clearance of pathogen (14). This have been postulated as the existence of an immunologic constant of rejection (15, 16). The molecular mechanisms used to eliminate all of them converge in a final pathway consisting of the expression of ISGs and IEFs.

Immunohistochemistry staining of tumors showed the correlation between tumor infiltration by immune cells and the expression of genomic transcripts implicated in tumor rejection (ISGs and IEFs). Several studies have described the tumor microenvironment having the presence of tumor-antigen specific cytotoxic T lymphocytes. Yet, these cells are unable to eliminate tumor cells (13). In this study, immune cells potentially induced or activated by the vaccine can recognize and eliminate lesions that previously were ignored. However, this process occurred only in a subset of metastases, while another group continued to grow in spite of systemic activation of immune surveillance mechanisms. Therefore it should be intra-tumoral factors impairing the correct function of immune response inside the progressing metastases. Potential mechanisms have been extensively described in tumor cells that may allow their evasion from antigen-specific recognition (17-20).

Our observations suggest that immunotherapy promotes a modification of tumor microenvironment, leading to a release of immune stimulating factors; if tumors have reversible alterations of HLA class I expression under treatment ("Soft" lesions), HLA expression can be up-regulated and tumor cells will be recognized and destroyed by the antigen specific T cells. These T cells, as they recognize tumor cells, produce more pro-inflammatory factors such as IFN-γ, IL-2, TNF-α and GM-CSF (21). This in turn triggers a positive and self perpetuating feedback between tumor and immune cells until tumor rejection occurs. In contrast, if cancer cells bear irreversible defects in HLA class I genes ("Hard" lesions), antigen presentation remains defective after immunotherapy impairing the amplification of the immune response in situ and promoting their escape from immune recognition (21).

Genome transcriptional analysis suggests that antigen presentation is the most critical pathway to determine tumor regression. Tumor tissue isolated by microdissection confirm that specific HLA-A,B and C high transcription is associated with immune infiltrate and tumor regression, meanwhile a low or negative transcription correlate with tumor progression.

Our prediction is that "soft" and "hard" HLA class I tumor lesions will coexist during the natural history of tumor development. However after immunotherapy tumor cells with "soft" lesion will upregulate antigen presentation and can be rejected. In contrast, those tumor cells bearing "hard" and irreversible genetic defects will prevail and

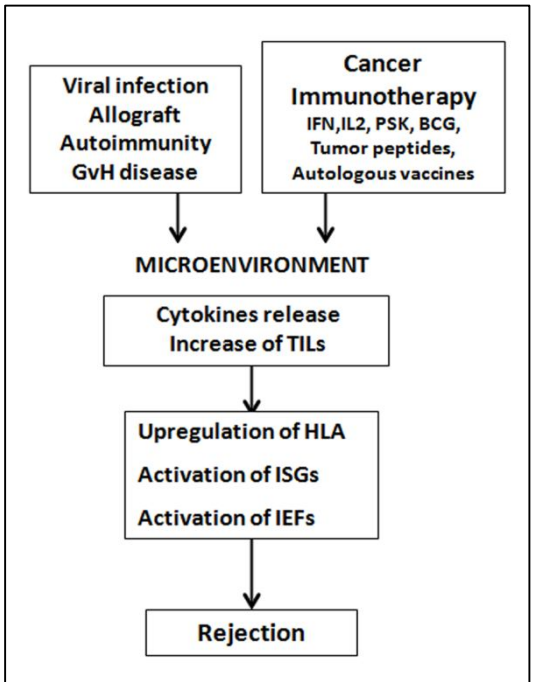


Figure 5: Different pathological situation show a common pathway of T cell mediated rejection.

progress to kill the host (22). This clearing would lead to the selection of a tumor variant with enhanced alteration of the expression of HLA class I or other transcripts associated with antigen processing and presentation (23). To our knowledge, this is the first comparison of the transcriptional profile of a disparate reaction to treatment in melanoma mixed responders. Our results, strongly suggest that the genetic makeup of individual tumor cells is a major factor determining immune responsiveness. This study, however, does not exclude that other factors related to the genetic background of the host may also affect differences in immune responsiveness observed among different patients.

Acknowledgements

We would like to thank Jingo Zhao for technical assistance and Dr. Harvey Alter for his helpful advice. This work was supported by grants from Proyecto de investigacion MEC I + D (SAF 2007-63262), Red Genomica del Cancer (RETIC RD 06/0020), Plan Andaluz de Investigacion (Group CTS 143), Proyecto de Excelencia de Consejeria de Innovacion (CTS 695), in Spain; and from the Integrated European Cancer Immunotherapy project (OJ2004/C158, 518234).

References

1.Terando AM, Faries MB, Morton DL. Vaccine therapy for melanoma: current status and future directions. Vaccine. 2007;25:4-16.

2.Kirkwood JM, Tarhini AA, Panelli MC, Moschos SJ, Zarour HM, Butterfield LH, et al. Next generation of immunotherapy for melanoma. J Clin Oncol. 2008; 26:3445-55.

3.Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol. 2009;21:233-40.

4.Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. Adv Immunol. 2006;90:51-81

5.Garrido F, Algarra I. MHC antigens and tumour escape from immune surveillance. Adv Cancer Res. 2001;83:117-58

6.Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nature Medicine . 2004; 10: 909–15

7.Kruit WH, van Ojik HH, Brichard VG, Escudier B, Dorval T, Dréno B et al. Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma. Int J Cancer. 2005;117:596-604.

8.Yuan J, Page DB, Ku GY, Li Y, Mu Z, Ariyan C et al. Correlation of clinical and immunological data in a metastatic melanoma patient with heterogeneous tumor responses to ipilimumab therapy. Cancer Immun. 2010;10:1.

9.Cabrera T, Lara E, Romero JM, Maleno I, Real LM, Ruiz-Cabello F, et al. HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination. Cancer Immunol Immunother. 2007;56:709–17

10.Carretero R, Romero JM, Ruiz-Cabello F, Maleno I, Rodriguez F, Camacho FM et al. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. Immunogenetics. 2008;60:439-47

11.Panelli MC, Stashower ME, Slade HB, Smith K, Norwood C, Abati A et al. Sequential gene profiling of basal cell carcinomas treated with imiquimod in a placebo-controlled study defines the requirements for tissue rejection. Genome Biol. 2007;8:R8.

12.Panelli MC, Wang E, Phan G, Puhlmann M, Miller L, Ohnmacht GA et al. Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration. Genome Biol. 2002;3: 1-17

13.Pilla L, Patuzzo R, Rivoltini L, Maio M, Pennacchioli E, Lamaj E et al. A phase II trial of vaccination with autologous, tumor-derived heat-shock protein peptide complexes Gp96, in combination with GM-CSF and interferon-alpha in metastatic melanoma patients. Cancer Immunol Immunother. 2006;55:958-68.

14.Marincola F.M. Wang E. Immunologic Signatures of Rejection: New York: Springer-Verlag: 2010.

15.Wang E, Worschech A, Marincola F.M. The immunologic constant of rejection. Trends Immuno. 2008;29:256-62.

16.Saint-Mezard P, Berthier CC, Zhang H, Hertig A, Kaiser S, Schumacher M et al. Analysis of

independent microarray datasets of renal biopsies identifies a robust transcript signature of acute allograft rejection. Transpl Int. 2009;22:293-302.

17.Garrido F, Ruiz-Cabello F, Cabrera T, Pérez-Villar JJ, López-Botet M, Duggan-Keen M et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today. 1997;18:89-95

18.Zou W. Immunosupressive networks in the tumor environment and their therapeutic relevance. Nature Reviews Cancer. 2005;5:263-74.

19.Rivoltini L, Canese P, Huber V, Iero M, Pilla L, Valenti R et al. Escape strategies and reasons for failure in the interaction between tumour cells and the immune system: how can we tilt the balance towards immune-mediated cancer control? Expert Opin Biol Ther. 2005;5:463-76

20.Aptsiauri N, Carretero R, Garcia-Lora A, Real LM, Cabrera T, Garrido F. Regressing and progressing metastatic lesions: resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations. Cancer Immunol Immunother. 2008;57:1227-33.

21.Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: Implications for immunotherapy. Int J Cancer. 2010;127:249-56.

22.Khong H.T, Restifo N.P Natural selection of tumor variants in the generation of tumor escape" phenotypes. Nat Immunol. 2002;3:999–1005

23.Dunn G.P, Old L.J, Schreiber R.D. The immunobiology of cancer immunosurveillance and immunoediting. Immunit. 2004;21:137-48.

24.Berd D. M-VAX: an autologous, hapten-modified vaccine for human cancer. Expert Rev Vaccines. 2004;3:521–7

25.Wang E, Miller L, Ohnmacht G.A, Liu E, Marincola F.M. High fidelity mRNA amplification for gene profiling using cDNA microarrays. Nature Biotech. 2000;17:457-9.

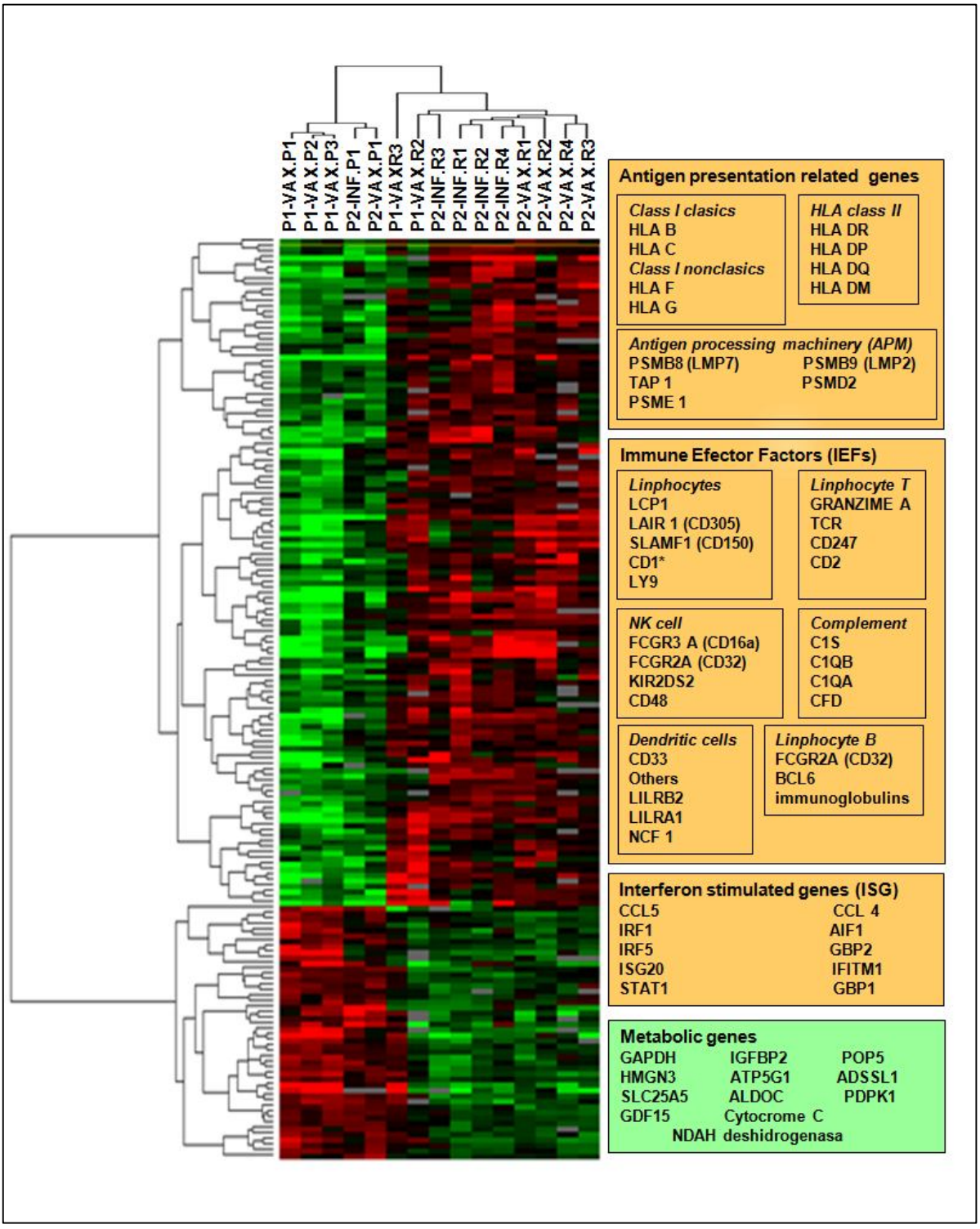
26.Jin P, Zhao Y, Ngalame Y, Panelli MC, Nagorsen D, Monsurró V et al. Selection and validation of endogenous reference genes using a high throughput approach. BMC Genomics. 2004;5:55.

27.Worschech A, Kmieciak M, Knutson KL, Bear HD, Szalay AA, Wang E et al. Signatures associated with rejection or recurrence in HER-2/neu-positive mammary tumors. Cancer Res. 2008;68:2436-46.

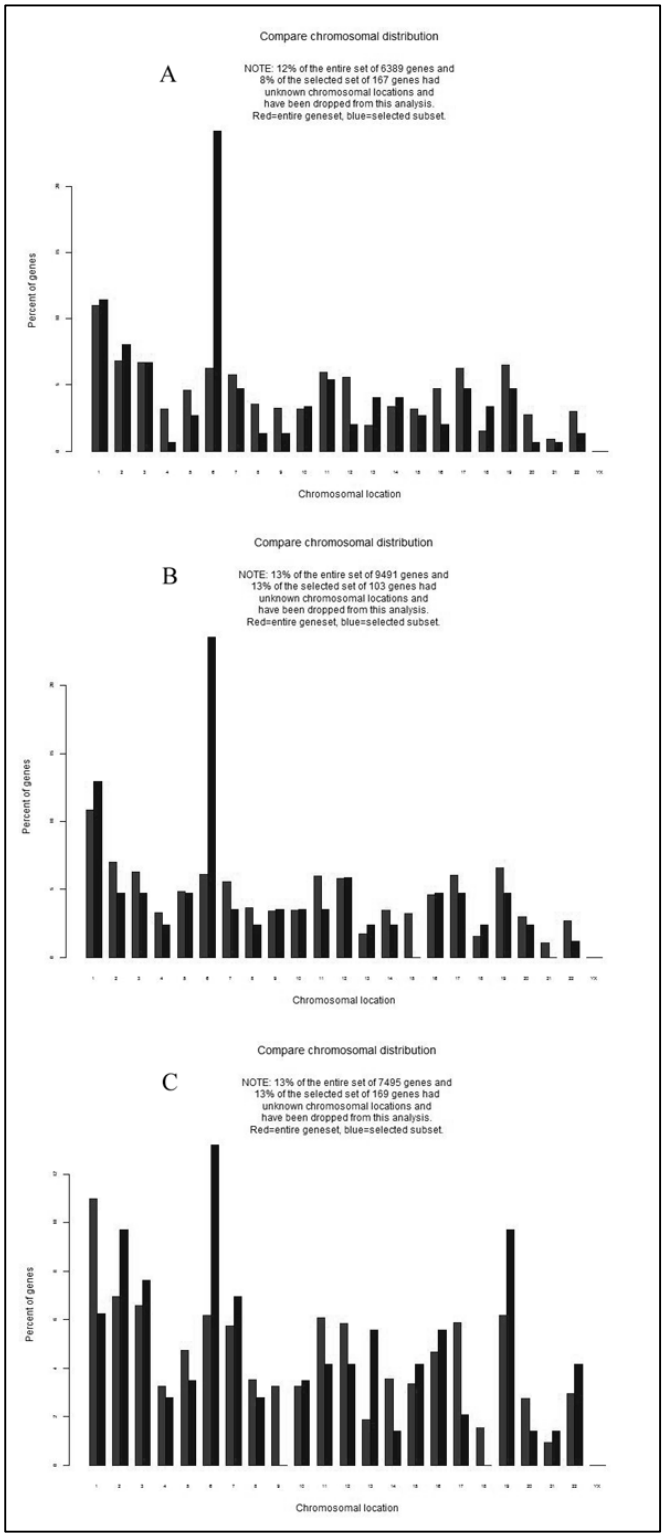
28.Simon R, Lam A, Li MC, Ngan M, Menenzes S, Zhao Y. Analysis of gene expression data using BRB-array tools. Cancer Inform. 2007;3:11-7.

29.Eisen M.B, Spellman P.T, Brown P.O, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA. 1998;95:14863-8.

Supplementary Figure 1: Supervised expression patter clustering of melanoma metastases obtained from both patients (p=0,001). Genes involved in immunological rejection are included in the figure.



Supplementary Figure 2: Chromosomal distribution of genes differentially expressed between regressing and progressing metastases at a high stringency nominal significance cut off level of ≤ 0.001. Red bars refer to the percent of gene distribution in individual chromosomes based on the entire gene set (expected frequency) and the blue bars refer to the observed distribution of genes differentially expressed between the two metastatic phenotypes in: A) Both patients. B) Patient 1. C) Patient 2.



Supplementary Figure 3: Class comparisson between regressing metastases treated with interferon versus those treated with M-VAX in patient 2. Top 15 first canonical pathways ranking according to significance level (Fisher exact test –log p-value) based on the 253 genes identified using high stringency (p=0,001) analysis. No mayor differences were detected between regressing metastases after two different immunotherapy protocols.

Chapter 4.

General Discussion

The identification of the cause of poor clinical response to immunotherapy protocols is essential for the improvement of this kind of treatments.

In the present study, we analyzed tissue samples from melanoma and bladder cancer patients receiving immunotherapy. We obtained 11 regressing and 5 progressing metastases from 2 melanoma patients after immunotherapy. We also studied 18 patients with bladder urothelial cancer. 13 patients were treated with BCG immunotherapy. Among them, 8 patients were relapse-free and 5 had recurrent tumors. 5 patients undergoing mitomycin, a non-based therapy, were selected as the control group.

A strong correlation between HLA Class I expression pattern and melanoma response after immunotherapy was shown. Progressing metastases had a more profound alteration in HLA Class I expression. This correlation was also found in bladder tumor treated with BCG. Tumors with recurrence after treatment have more HLA class I alterations and a higher frequency of LOH in chromosome 6 and 15 than tumors from relapse-free patients. The major escape mechanism in these tumors is the irreversible alteration in HLA Class I.

We have discovered that tumors relapsing after BCG have a more altered pattern of HLA Class I expression than the lesion obtained before the treatment. It suggests that tumoral cells with low HLA altered phenotypes were eliminated by the immune system, but tumoral cells with additional alterations are not rejected and promote the tumor recurrence.

In addition, we have analyzed the transcriptome in metastases from both melanoma patients. We discovered a predominant activation of antigen presentation and immune rejection pathways in regressing metastasis, which confirmed our previous results, demonstrating that melanoma regression is mediated by the immune system.

4.1. HLA class I expression correlates with tumor response after immunotherapy

Epidemiological data show that tumor growth is not restricted to immunedeficient patients. Several studies have proposed that the development of tumor is, in most cases, not due to a deteriorated immune system, but rather to the development of mechanisms that allow transformed cells to escape anti-tumor immunosurveillance (Garrido et al. 1997 Pawelec 2004; Drake et al. 2006). It is known that a high proportion of tumors have alterations in the expression of HLA class I molecules. These alterations represent an important tumor escape mechanism, for they impair the interaction between tumor and immune cells (Khong and Restifo 2002; Aptsiauri et al. 2007). However, the information about the relation between HLA and immunotherapy is still limited.

Here we report for the first time the patterns of HLA class I alterations for various melanoma tumors from the same patient receiving immunotherapy and from bladder tumors before and after BCG therapy. We show that HLA class I antigen expression on metastatic melanoma correlates with their response to therapy. All progressing metastases analyzed showed some residual HLA class I expression, whereas the regressing ones showed a strong class I expression. Most importantly, this correlation did not depend on the type of immunotherapy, since the same response was observed after interferon and autologous vaccination.

We previously described the frequency of HLA class I alteration in a panel of 91 melanoma cell lines (Mendez et al. 2009). 67% of the cell lines showed alteration in HLA class I. 11% showed total loss of HLA class I (phenotype I), 13% are homozygous for HLA (phenotype II), 35% showed loss of one HLA locus (phenotype III) and 6% showed a compound phenotype (phenotype V). LOH frequency was 35%. Although LOH cannot be determined in the study by comparing normal with tumor DNA, microsatellite amplification and FISH showed alteration in chromosome 6 in most of HLA homozygous cell lines (Rodriguez et al. 2005). In addition we and other lab showed that different mechanism can lead to an irreversible HLA class I loss (Rodriguez et al. 2007; Respa et al. 2011).

In the characterization of HLA class I expression of bladder tumors from patients treated with BCG, we observed that the immunotherapy prevents the recurrence of bladder tumors expressing a high level of HLA class I molecules but not of those with an altered

phenotype. Alterations in HLA Class I expression have been related to a more severe prognosis in head and neck squamous cell carcinoma (Bandoh et al. 2010), breast cancer (de Kruijf et al. 2010), ovarian cancer (Shehata et al. 2009), colorectal cancer (Kloor et al. 2010), medulloblastoma (Smith et al. 2010), renal cell carcinoma (Kitamura et al. 2007) and bladder cancer (Homma et al. 2009). However, there is little information regarding the relationship between HLA class I expression and tumor prognosis after immunotherapy. Our results are consistent with the description by Kitamura et al, where the HLA Class I downregulation in 30 bladder cancer patients was significantly correlated with a worsened prognosis after BCG treatment (Kitamura et al. 2006).

Most of prognosis studies are carried out in paraffin-preserved tissues due to the broad availability of these samples. However, paraffin-embedded tissues do not provide all the details about HLA expression, since there are no antibodies against the specific loci and alleles that could be used in paraffin. As a result, many HLA alterations would not be recognized in these tumors. In addition, paraffin does not preserve RNA and DNA well (Huang et al. 2010), making the analysis of these sequences very difficult. In contrast, cryopreserved tissues are ideal for both the qualitative investigation of different HLA alleles as well as RNA analysis. Typically, the mRNA transcription levels of HLA class I correlate with the expression of surface proteins. However, we were able to detect mRNA expression for locus B in all the melanoma metastases from Patient 2, without surface protein expression. We also observed high levels of mRNA expression for HLA-A, B and C in the relapsed bladder tumor sample from Patient 8 without detectable membrane staining. Since APM alterations can block the correct expression of HLA (Romero et al. 2003), specifically HLA-B alleles (Cabrera et al. 2005; Park et al. 2003), we believe that a post-transcriptional alteration is impairing the membrane expression of HLA-B.

Our lab previously described the frequency of HLA class I alteration in bladder tumors (Cabrera et al. 2003b; Maleno et al. 2006). 25% of tumors showed total loss of HLA class I (phenotype I), 17,5% had haplotype loss (phenotype II), 7% showed loss of one HLA locus (phenotype III), 14,5% had loss of one allele (phenotype IV) and 13% showed a compound phenotype (phenotype V). LOH frequency was 35%. In the present study we describe a 40% of LOH among tumors treated with mitomycin, a 25% among relapse free patients after BCG and an 80% among patients with relapse after BCG.

The LOH analyses of bladder tumor showed that this alteration is much more frequent in tumors that relapsed after BCG immunotherapy. LOHs were detected in chromosomes 6 in four out of five patients and in chromosome 15 in three out of five patients with relapse after BCG, whereas only two out of the eight samples from relapse-free BCG-treated patients showed this alteration. We also found that progressing melanoma metastases from Patient 1 showed an additional LOH in chromosome 15 not found in the regressing tumors. LOH in chromosome 6 and 15 are both frequent alterations found in different human tumors (Wang et al. 2006; Mendez et al. 2008; Maleno et al. 2011). If LOH in β2m is combined with other mutation event in the remaining allele, it will promote an irreversible total loss of HLA class I expression (Paschen et al. 2003 and 2006). There could also be additional structural defects in molecules involved in MHC class I activation not detected in this study. For example, STAT-1 protein, which plays a major role in IFN-mediated HLA class I upregulation, may either be absent, not phosphorylated, or it may have irreversible structural defects (Abril et al. 1996; Rodriguez et al. 2007). These structural defects can inhibit the upregulation of antigen presentation.

4.2. Immune selection of tumor variants with additional/hard irreversible HLA class I alterations after immunotherapy

The existence of cancer immunosurveillance and the description of tumor escape mechanism lead to the establishment of the Cancer Immunoediting Hypothesis (Dunn et al. 2002). This hypothesis proposes three phases between a functional immune system and tumor cells.

In the Elimination Phase, the immune system recognizes tumor cells due to the release of danger signals during the tissue remodeling (Vicari and Caux 2002). This recognition is followed by an innate response (Girardi et al. 2001; Smyth et al. 2005) and later by a tumor-specific acquired response (Shankaran et al. 2001). However, the immune system is not able to eliminate all the tumor cells. The survival-transformed cells enter in the Equilibrium Phase. During this stage, immune cells act as a pressure factor in selecting tumor cells resistant to the immune system. The Equilibrium Phase is supported by the "dormancy period" of the tumor (Uhr et al. 1997) and by murine models where mice free of disease for long periods experience a tumor relapse immediately after immune system depletion (Koebel et al. 2007). Immunosupression after transplantations in human also lead to the relapse of tumor (Cozar at al. 2008). In the final Escape Phase, the immune-resistant tumor cells escape from the immune system and form the tumor.

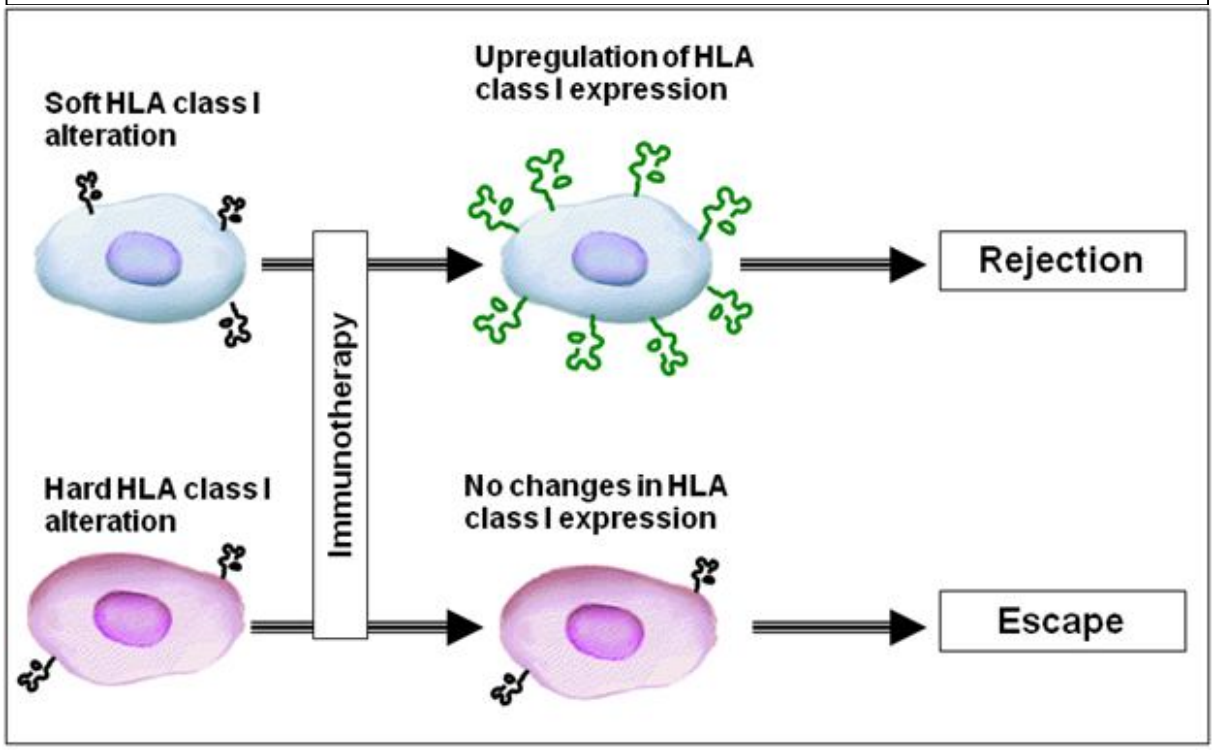
The major problem in verifying this hypothesis is that tumor cells have never been studied before the Escape Phase. We have analyzed the effect of immunotherapy in different melanoma metastases, showing that the immune system can specifically eliminate some of them, thereby demonstrating the existence of the immune selection. In addition, we studied bladder tumors before and after BCG immunotherapy. We described that relapses after BCG acquire additional alterations in HLA Class I expression than the tumor before treatment. In contrast, when a form of non-immune-based therapy, cytostatic mitomycin, was administrated, the HLA Class I phenotype remains the same.

We support the idea that regardless the type of immunotherapy used, it will promote modification of tumor microenvironment leading to an increment of immune-stimulating cytokines. If tumor cells bear reversible ("soft") alterations in the HLA class I system, HLA expression will be upregulated by the cytokines leading to regression of the lesion. In contrast, if transformed cells have structural, irreversible ("hard") alterations, HLA expression

will remain unchanged, and the tumor will progress in spite of the immunotherapy (Garrido et al. 2010). After immunotherapy, the immune system was able to recognize and eliminate previously growing tumors. However, some tumors continue progressing even after the immune stimulation, as shown in the melanoma metastases. This lead to new round of immunoselection in which tumor variants with irreversible altered phenotypes will be selected, such as those found in bladder cancer relapses after BCG.

Our data could explain why clinical efficacy is only restricted to a low percentage of patients, despite the improvements in cancer immunotherapy protocols. It also could explain the detection of specific CTLs against tumor antigen that were unable to eliminate tumor cells. (van der et al. 1991; Ohnmacht et al. 2001; Lurquin et al. 2005).

Figure 4: tumor cells with soft HLA class I alterations will upregulate antigen presentation and be rejected. In contras, tumor cells bearing hard alterations will not respond, impairing immune rejection



4.3. Tumor regression is associated with HLA class I activation and acute immune rejection genes.

Several studies have linked the immune system with tumor rejection. These studies were based on the differential tumor occurrence in immunodeficient and immunocompetent mice and humans (Kauffman 2006; Shankaran et al. 2001) and the description of several tumor-associated antigens (TAAs) (Bioley et al. 2009). However, the mechanisms leading to tumor rejection are not yet well known, mainly due to the little information regarding tumor-responding lesions, the extreme heterogeneity of tumor cells, and tumor-bearing individuals (Jin and Wang 2003; Perez-Diez et al. 2002).

There are only a few studies on the entire transcriptomic response in tumors after immunotherapy. Sullivan and coworkers (Sullivan et al. 2003) and Panelli and coworkers (Panelli et al. 2007) studied the effect of imiquimod, a nonspecific immune stimulator, administration in BCC. Biopsies from imiquimod-treated patients showed an activation of T and NK cells and the induction of IFN-γ-stimulated genes. It was also described that BCG enhances the Th1 cytokine response by increasing the secretion of IL-2, IL-12, INF-γ, and TNF, thereby promoting cellular immune response and upregulation of MHC class I expression on urothelial cells (Patard et al 1998, Videira et al. 2009).

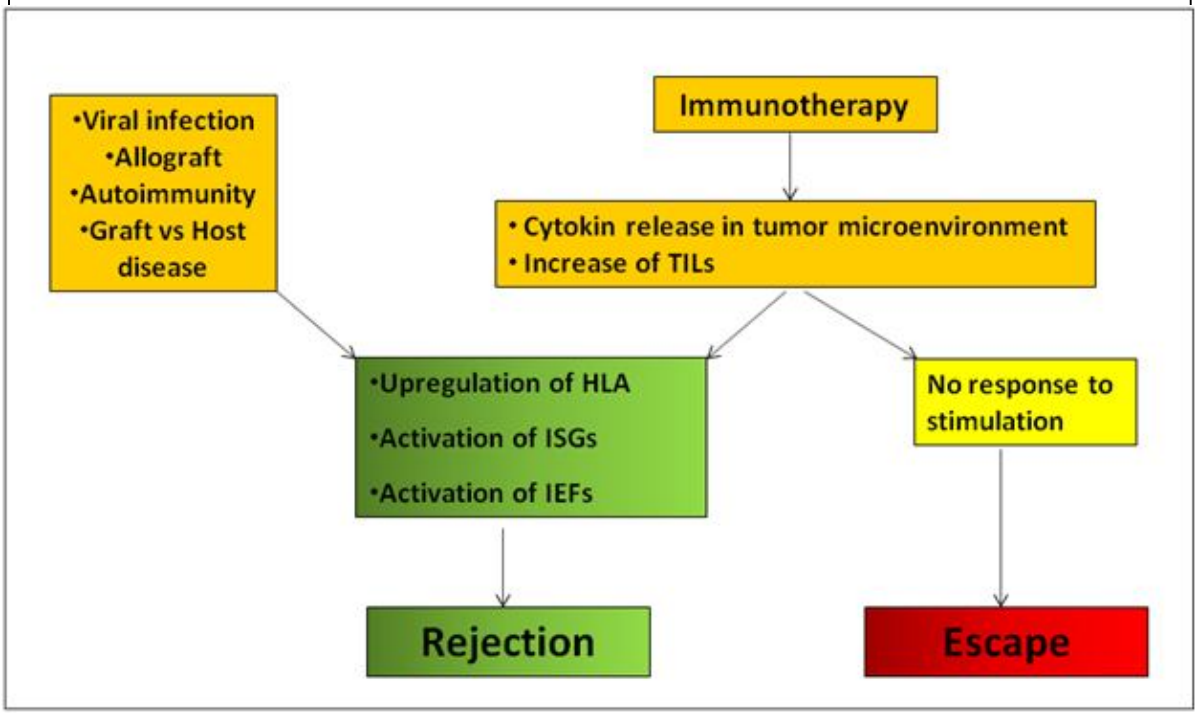
Here we documented the transcriptional patterns of 5 progressing and 10 regressing melanoma metastases from 2 patients following autologous vaccination plus BCG (M-VAX) and IFN-2b. Metastases response (progression versus regression) to immunotherapy was established according to PET and CT scans. In these mixed responders, we could analyze for the first time the tumor response after immunotherapy with exclusion of extrinsic variants. Whole genome transcriptional analysis comparing progressing and regressing lesions showed that regressing metastases were being rejecting by the immune system. Regressing metastases from both patients showed a predominant activation of antigen presentation and immune rejection pathways such as cytotoxic T lymphocytes-mediated apoptosis of target cells and allograft rejection signaling.

The expression pattern of regressing lesions was similar to that observed during allograft rejection (Sarwal et al. 2003; Saint-Mezard et al. 2009), suggesting that the immune system follows a similar process in the elimination of tumors as it does in the induction of allograft rejection. Results obtained in this study and other reports on post-immunotherapy

tumors (Pilla et al. 2006; Panelli et al. 2007) analyzed at the global transcriptional level showed that tumor regression is a facet of the broader phenomenon of rejection that can be observed during allograft rejection, graft versus host disease, autoimmunity and acute infection as a result of pathogen clearance. The molecular mechanisms used by the immune system to eliminate the "non-self" are related to the activation of interferon-stimulated genes (ISGs), particularly interferon regulatory factor (IRF)-1, HLA antigen presentation, and Immune effectors factors (IEFs) like granzymes and other transcripts specific for activated CTLs or NK cells. (Wang et al. 2008; Marincola and Wang 2010).

These results lead to the establishment of the Immunologic Constant or Rejection Hypothesis by Marincola and coworkers (Wang et al. 2008; Marincola and Wang 2010). This hypothesis states that: 1) Tissue-specific destruction could be directed against self or quasiself; 2) The requirements for the induction immune recognition differ from those necessary for the activation of the immune effector response; 3) Although the way of recognition may vary in different pathologic states, the effector immune response converges into a single pathway that includes the activation of adaptive and innate cytotoxic mechanisms; 4) Adaptive immunity is not always sufficient or necessary for tissue

Figure 5: Immunotherapy promotes tumor recognition. Once tumor is recognize by T cells, it will be eliminate by an acute immune response



destruction.

The relevance of IEFs in tumor response was also supported by others authors like Galon and coworkers (Galon et al. 2006), who described that colon cancer prognosis is associated with the density and location of CTLs, whose activations is associated with IFN-γ, IRF-1 and granzymes.

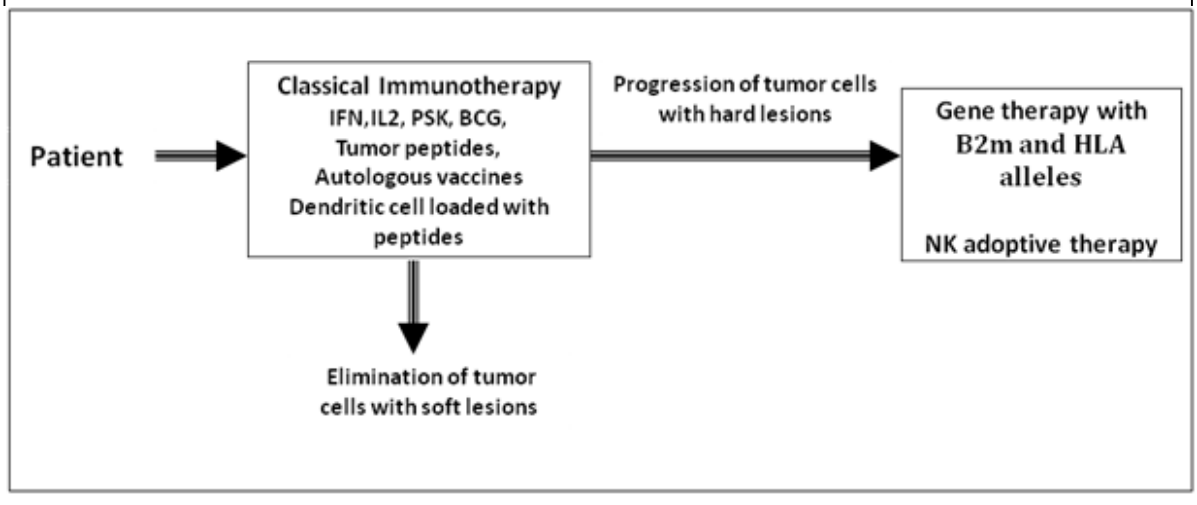
4.4. Monitoring tumor immune escape mechanism could lead to an improvement of immunotherapy efficacy.

It has been extensively demonstrated that immunotherapy leads to an activation of the immune system (Videira et al. 2009; Bhöle et al. 2003; Panelli et al. 2007). However, enhancement of tumor-specific T cells is not enough to eliminate all tumor lesions (Drake et al. 2006). We believe the therapy produce a cytokine release and the homing of immune cells to the tumor site. If tumor cells are recognized during this initial activation, the immune cells will release factors such as IFN-γ, IL, TNF-α, GM-CSF(Garrido et al. 2010). This will promote a positive and self-perpetuating feedback between the tumor and the immune cells until tumor rejection. In contrast, if tumoral cells did not respond to this initial activation, the immune response inside the tumor will be impaired.

We propose that the knowledge of tumor cell alterations and their molecular mechanisms will lead to an improvement of immunotherapy efficacy. Patients bearing tumors with reversible alterations should be treated with classic immune-based therapies. In contrast, those patients with tumor bearing irreversible HLA Class I alterations should seek alternative treatments, such as NK adoptive therapy in place of T cell adoptive therapy.

In our research group, we are developing a transduction gene therapy aimed to recover HLA Class I in tumors with irreversible HLA Class I total loss due to genetics alteration in *β2m*. The therapy will use a viral vector bearing the human *β2m*. When the virus infects HLA

Figure 6: Classical immunotherapy can eliminate tumor with soft lesions. Tumor bearing irreversible alterations should be treated with alternative therapies



negative tumor cells *in vitro*, it can recover the HLA Class I expression in those tumors(del Campo et al. 2010). We are currently testing the efficacy of the treatment *in vivo.*

Chapter 5.

Conclusions/Conclusiones

5.1. Conclusions

1. **Progressing melanoma metastases showed more profound alterations in HLA class I antigen presentation than regressing ones. Progressing lesions from both patients showed very weak surface expression of HLA molecules and residual mRNA levels. HLA class I expression correlated with the response of melanoma metastases after immunotherapy.**

2. **Bladder tumors with relapse after immunotherapy had more HLA class I alterations and a higher frequency of LOH in chromosome 6 and 15. The response to BCG therapy in bladder tumors may be impaired by HLA Class I alterations.**

3. **The correlation between HLA Class I expression and the response of the tumors to therapy occurred independently from the tumor tissue origin (melanoma or bladder) and the type of immunotherapy.**

4. **Immunotherapy up-regulated HLA Class I expression in tumor bearing reversible "soft" lesions, leading to their recognition and destruction by the immune system. In contrast, HLA Class I expression remained defective in cancer cells with irreversible "Hard" defects in HLA class I, promoting their escape from immune system recognition.**

5. **We have found a correlation between mRNA levels of HLA and protein surface expression in the majority of the tumors studied. However, tumors from melanoma patient 2 had high levels of mRNA expression for HLA-B without membrane expression, and the relapse tumor sample bladder cancer patient 8 also showed high mRNA expression levels for the HLA heavy chain with negative surface protein expression. Post transcriptional mechanism could be impairing the correct surface expression of HLA in these patients.**

6. **The activation of the immune response after immunotherapy triggered a new round of tumor immunoselection. The immune system rejected tumor cells that were growing previously, and only those tumors bearing additional, more profound HLA Class I losses could progress. Bladder cancer relapses after BCG treatment showed additional alterations in HLA Class I.**

7. **Genome transcriptional analysis showed that regressing metastasis caused a predominant activation of antigen presentation and immune rejection pathways. Expression patterns leading to tumor regression were shared by those involved in allograft rejection, Graft versus host disease, autoimmunity and pathogen clearance. These results demonstrated that tumor rejection is mediated by the immune system and support the "constant of immune rejection" hypothesis.**

5.2. Conclusiones

1. **Las metástasis de melanoma en crecimiento muestran mayores alteraciones en la presentación de antígenos que aquellas que están siendo eliminadas. Las metástasis en progresión tiene una expresión muy baja de HLA en superficie. La respuesta de las metástasis de melanoma a la inmunoterapia se correlaciona con su expresión de HLA de clase I.**

2. **Los tumores que recidivan a pesar del tratamiento muestran más alteraciones en HLA y una mayor frecuencia de pérdida de heterozigosidad en los cromosomas 6 y 15.Las alteraciones de la expresión de HLA de clase I pueden ser las responsables del fallo de la terapia con BCG en tumors uroteliales de vejiga.**

3. **La correlación entre la expresión de HLA de clase I se da de manera independiente al tipo de inmunoterapia usado y del origen del tumor. La respuesta inmune tras el tratamiento en melanoma es la misma a pesar del uso de distintas inmunoterapias**

4. **La activación de la respuesta inmunitaria por el tratamiento provoca un Nuevo proceso de inmunoselección. El sistema inmune elimina células transformadas que antes crecían, solamente las células tumorales con grandes alteraciones en HLA pueden continuar creciendo. Las recidivas estudiadas tras BCG mostraron unos patrones de expresión de HLA de clase I mucho mas alterados.**

5. **En la mayoría de los tumores estudiados hemos encontrado una correlación entre los niveles de ARNm de HLA y su expresión en membrana. Sin embargo, el paciente de melanoma 2 tiene altos niveles de ARNm del locus B sin expresión en membrana y el paciente con tumor vesical 8 tiene altos niveles de ARNm de cadena pesada siendo negativo para la proteína. Creemos que alteraciones**

postrascripcionales pueden estar impidiendo la correcta expresión en membrana de la proteína.

6. **La inmunoterapia puede activar la expresión de HLA de clase I en aquellos tumores con lesiones reversibles o "blandas", permitiendo su reconocimiento y eliminación. Sin embargo, la expresión de Clase I no puede ser activada en aquellas células tumorales que tengan alteraciones irreversibles o "duras", permitiendo su escape del sistema inmunitario.**

7. **Los análisis transcriptómicos muestras que las metástasis de melanoma en regresión tienen activadas las vías de presentación antigénica y rechazo inmunitario. EL patrón de expresión es el mismo en tumores, trasplantes, enfermedades autoinmunes, síndrome del injerto contra huésped y en la eliminación de patógenos. Los datos obtenidos demuestran que los tumores en regresión son eliminado por el sistema inmunitario y apoyan la hipótesis de la constante del rechazo inmunitario.**

Chapter 6.

References

Abele R, Tampe R. Function of the transport complex TAP in cellular immune recognition. Biochim Biophys Acta (1999) 1461:405–419.

Abril E, Mendez R, Garcia A, Serrano A, Cabrera T, Garrido F, Ruiz-Cabello F. Characterization of a gastric tumor cell line defective in MHC class I inducibility by both alpha- and gammainterferon. Tissue Antigens (1996) 47:391–398.

AJCC Staging manual (2010) Springer (7º Edithion)

Ahirwar DK, Agrahari A, Mandhani A, Mittal RD. Cytokine gene polymorphisms are associated with risk of urinary bladder cancer and recurrence after BCG immunotherapy. Biomarkers (2009) 14:213-8.

Ahirwar D, Kesarwani P, Manchanda PK, Mandhani A, Mittal RD. Anti- and proinflammatory cytokine gene polymorphism and genetic predisposition: association with smoking, tumor stage and grade, and bacillus Calmette-Guérin immunotherapy in bladder cancer. Cancer Genet Cytogenet (2008) 184:1-8.

Ahmad M, Rees RC, Ali SA. Escape from immunotherapy: possible mechanisms that influence tumor regression/progression. Cancer Immunol Immunother (2004) 53:844–854.

Alleva DG, Walker TM, Elgert KD. Induction of macrophage suppressor activity by fibrosarcoma-derived transforming growth factor-beta 1: contrasting effects on resting and activated macrophages. J Leukocyte Biol (1995) 57:919.

American Cancer Society. Smoking and Cancer Mortality Table. http://ww2.cancer.org/docroot/PED/content/PED_10_2X_Smoking_and_Cancer_Mortality_Table.asp

Antonia SJ, Mirza N, Fricke I, Chiappori A, Thompson P, Williams N, Bepler G, Simon G, Janssen W, Lee JH, Menander K, Chada S, Gabrilovich DI. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res (2006) 12: 878–887.

Aoe T, Okamoto Y, Saito T. Activated macrophages induce structural abnormalities of the T cell receptor-CD3 complex. J Exp Med (1995) 181:1881.

Aptsiauri N, Cabrera T, Mendez R, Garcia-Lora A, Ruiz-Cabello F, Garrido F. Role of altered expression of HLA class I molecules in cancer progression. Adv Exp Med Biol. (2007) 601:123-131.

Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou-Redorta J EAU guidelines on non-muscle-carcinoma of the bladder. Actas Urol Esp (2009) 33:361-371.

Bandoh N, Ogino T, Katayama A, Takahara M, Katada A, Hayashi T, Harabuchi Y. HLA class I antigen and transporter associated with antigen processing downregulation in metastatic lesions of head and neck squamous cell carcinoma as a marker of poor prognosis. Oncol Rep (2010) 23:933-939.

Bashirova AA, Martin MP, McVicar DW, Carrington M. The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. Annu Rev Genomics Hum Genet (2006) 7:277-300.

Berd D. M-Vax: an autologous, hapten-modified vaccine for human cancer. Expert Rev Vaccines (2004) 3:521-527.

Bergmann-Leitner ES, Abrams SI. Positive and negative consequences of soluble Fas ligand produced by an antigen-specific CD4+ T cell response in human carcinoma immune interactions. Cell Immunol (2001) 209:49-62.

Bhatia S, Tykodi SS, Thompson JA. Treatment of metastatic melanoma: an overview. Oncology (Williston Park) (2009) 23:488-496.

Bioley G, Dousset C, Yeh A, Dupont B, Bhardwaj N, Mears G, Old LJ, Ayyoub M, Valmori D. Vaccination with recombinant NY-ESO-1 protein elicits immunodominant HLA-DR52b-restricted CD4+ T cell responses with a conserved T cell receptor repertoire. Clin Cancer Res (2009) 15:4467–4474.

Birkeland SA., Storm HH, Lamm LU, Barlow L, Blohme I, Forsberg B, Eklund B, Fjeldborg O, Friedberg M, Frodin L. Cancer risk after renal transplantation nin the Nordic countries,1964–1986 Int J Cancer (1995) 60:183–189.

Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. Nature (1987) 329:506–512.

Bjorkman PJ, Parham P. Structure, function, and diversity of class I major histocompatibility complex molecules. Annu Rev Biochem (1990) 59:253–288.

Böhle A, Brandau S. Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer J Urol (2003) 170:964-969.

Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol (2006) 24:175–208

Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. J Exp Med (1996) 183:725-729.

Boshoff C, Weiss R. AIDs-related malignancies. Nature Rev Cancer (2002) 2:373-382.

Brennan P, Bogillot O, Cordier S, et al. Cigarette smoking and bladder cancer in men: a pooled analysis of 11 case-control studies. Int J Cancer (2000) 86:289-294.

Brenner MK, Heslop HE. Adoptive T cell therapy of cancer. Curr Opin Immunol (2010) 22:251-257.

Burnet, FM. Cancer- a biological approach. Brit Med J (1957) 1:841-847.

Burnet, FM. Immunological factors in the process of carcinogenesis. Br Med Bull (1964) 20:154-158.

Burnet, FM. The concept of immunological surveillance. Prog Exp Tumor Res (1970) 13:1-27.

Burstein NA, Law LW. Neonatal thymectomy and non-viral mammary tumours in mice. Nature (1971) 231:450-452.

Cabrera T, Sierra A, Fernandez MA, Herruzo A, Fabra A, Garrido F. High frequency of altered HLA class I phenotypes in invasive breast carcinomas. Human Immunol (1996) 50:127–134.

Cabrera T, Collado A, Fernandez MA, Ferron A, Sancho J, Ruiz-Cabello F, Garrido F. High frequency of altered HLA class I phenotypes in invasive colorectal carcinomas. Tissue Antigens (1998) 52:114–123.

Cabrera T, Salinero J, Fernandez MA, Garrido A, Esquivias J, Garrido F. High frequency of altered HLA class I phenotypes in laryngeal carcinomas. Human Immunol (2000) 61:499–506.

Cabrera T, Lopez-Nevot MA, Gaforio JJ, Ruiz-Cabello F, Garrido F. The analysis of HLA expression in human tumor tissues. Cancer Immunol Immunother (2003a) 52:1–9.

Cabrera T, Pedrajas G, Cozar JM, Garrido A, Vicente J, Tallada M, Garrido F. HLA class I expression in bladder carcinomas. Tissue Antigens (2003b) 62:324-327.

Cabrera CM, Lopez-Nevot MA, Jimenez P, Garrido F. Involvement of the chaperone tapasen in HLA-B44 allelic losses in colorectal tumors. Int J Cancer (2005) 113:611–618.

Callahan MK, Wolchok JD, Allison JP. Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. Semin Oncol (2010) 37:473-84.

Capelli E, Civallero M, Barni S, Ceroni M, Nano R. Interleukin-2 induces the growth of human glioblastoma cells in culture. Anticancer Res (1999) 19:3147.

Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature (2000) 407:249-57.

Carbone E, Terrazzano G, Ruggiero G, Zanzi D, Ottaiano A, Manzo C, Karre K, Zappacosta S. Recognition of autologous dendritic cells by human NK cells. Eur J Immunol (1999) 29:4022.

Chambers CA., Kuhns MS, Egen JG, and Allison JP. CTLA-4 mediated inhibition in regulation of T cell responses: Mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol (2001) 19: 565–594.

Chang YM, Newton-Bishop JA, Bishop DT, Armstrong BK, Bataille V, Bergman W, Berwick M, Bracci PM, Elwood JM, Ernstoff MS, Green AC, Gruis NA, Holly EA, Ingvar C, Kanetsky PA, Karagas MR, Le Marchand L, Mackie RM, Olsson H, Østerlind A, Rebbeck TR, Reich K, Sasieni P, Siskind V, Swerdlow AJ, Titus-Ernstoff L, Zens MS, Ziegler A, Barrett JH. A pooled analysis of melanocytic nevus phenotype and the risk of cutaneous melanoma at different latitudes. Int J Cancer (2009) 124:420–428.

Chaux P, Moutet M, Faivre J, Martin F, Martin M. Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7-1 and B7-2 costimulatory molecules of the T-cell activation. Lab Invest (1996) 74:975.

Choi BK, Kim YH, Kang WJ, Lee SK, Kim KH, Shin SM, Yokoyama WM, Kim TY, Kwon BS. Mechanisms involved in synergistic anticancer immunity of anti-4-1BB and anti-CD4 therapy. Cancer Res (2007) 67:8891–8899.

Choudhury A, Elliott F, Iles MM, et al. Analysis of variants in DNA damage signalling genes in bladder cancer. BMC Med Genet (2008) 9:69.

Chen X, Woiciechowsky A, Raffegerst S, Schendel D, Kolb HJ, Roskrow M. Impaired expression of the CD3-zeta chain in peripheral blood T cells of patients with chronic myeloid leukaemia results in an increased susceptibility to apoptosis. Br J Haematol (2000) 111:817.

Chen, L. Cohinhibitory molecules of the B7/CD28 family in the control of T-cell immunity. Nat Rev Immunol (2004) 4:336–347.

Ciavarra RP, Brown RR, Holterman DA, Garrett M, Glass WF 2nd, Wright GL Jr, Schellhammer PF, Somers KD. Impact of the tumour microenvironment on host

infiltrating cells and the efficacy of flt3-ligand combination immunotherapy evaluated in a treatment model of mouse prostate cancer. Cancer Immunol Immunother (2003) 52:535.

Claesson MH. Why current peptide-based cancer vaccines fail: lessons from the three Es. Immunotherapy (2009) 1:513-516.

Clark WH Jr, Elder DE, Guerry D 4th, Epstein MN, Greene MH, Van Horn M. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum Pathol (1984) 15:1147-1165.

Cloosen S, Arnold J, Thio M, Bos GM, Kyewski B, Germeraad WT. Expression of tumor-associated differentiation antigens, MUC1 glycoforms and CEA, in human thymic epithelial cells: implications for self-tolerance and tumor therapy. Cancer Res (2007) 67:3919–3926.

Coley W.B., The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases.1893,Clin Orthop Relat Res (1991) 3–11.

Coulie PG, Hanagiri T, Takenoyama M. From tumour antigens to immunotherapy Int J Clin Oncol (2001) 6:163-70

Coulie PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, Van Baren N, Boon T. Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen. Immunol Rev (2002) 188:33-42

Cozar JM, Aptsiauri N, Tallada M, Garrido F, Ruiz-Cabello F. Late pulmonary metastases of renal cell carcinoma immediately after post-transplantation immunosuppressive treatment: a case report. J Med Case Reports (2008) 2:111.

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med (2004) 10:942–949.

Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of HLA-A,B,C antigens in normal human organs. Transplantation (1984) 38:287–292.

Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, Sarfati M. Interleukin-4 protects chronic lymphocytic leukemic B-cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med (1992) 176:1319.

de Vita F, Orditura M, Galizia G, Romano C, Roscigno A, Lieto E, Catalano G. Serum interleukin-10 levels as a prognostic factor in advanced non-small cell lung cancer patients. Chest (2000) 117:365.

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. Scand J Immunol (2009) 70:125-135.

Delclos GL, Lerner SP. Occupational risk factors. Scand J Urol Nephrol Suppl (2008) 218:58-63.

den Boer AT, van Mierlo GJ, Fransen MF, Melief CJ, Offringa R., Toes RE. The tumoricidal activity of memory CD8þ T cells is hampered by persistent systemic antigen -free

environment. J Immunol (2004) 172:6074-6079.

Demkow T, Alter A, Wiechno P. Intravesical bacillus Calmette-Guérin therapy for T1 superficial bladder cancer. Urol Int (2008) 80:74-79.

di Carlo E, de Totero D, Piazza T, Fabbi M, Ferrini S. Role of IL-21 in immune-regulation and tumor immunotherapy. Cancer Immunol Immunother (2007) 56:1323-1334.

Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity (1994) 1:447-456.

Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. Tumour-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med (2002) 8:793.

Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. Adv Immunol (2006) 90:51-81

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol (2002) 3:991-998.

Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity (2004) 21:137-148.

Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol (2004) 22:329–360.

Ebensen T, Guzmán CA. Immune modulators with defined molecular targets: cornerstone to optimize rational vaccine design. Hum Vaccin (2008) 4:13-22.

Effros R, Pawelec G. Replicative senescence of T lymphocytes: does the Hayflick Limit lead to immune exhaustion? Immunol Today(1997) 18:450.

Ehrlich, P Ueberden jetzigen stand der karzinomforshung. Ned Tijdschr Geneeskd (1909) 5:273-290.

Engel AM, Svane IM, Rygaard J, Werdelin O. MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice. Scand J Immunol (1997) 45:463-470.

Fayad L, Keating MJ, Reuben JM, OBrien S, Lee BN, Lerner S, Kurzrock R. Interleukin-6 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with phenotypic characteristics and outcome. Blood (2001) 97:256.

Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov (2004) 3:391–400.

Ferris RL, Jaffee EM, Ferrone S. Tumor antigen-targeted, monoclonal antibody-based immunotherapy: clinical response, cellular immunity, and immunoescape. J Clin Oncol (2010) 28:4390-4390.

Ferron A, Perez-Ayala M, Concha A, Cabrera T, Redondo M, Oliva MR, Ruiz-Cabello F, Garrido F MHC class I and II antigens on gastric carcinomas and autologous mucosa. J Immunogenet (1989) 16:413-423.

Fong L, Kavanagh B, Rini BI, Shaw V, Weinberg V, Small EJ. A phase I trial of combination immunotherapy with CTLA-4 blockade and GM-CSF in hormone-refractory prostate

cancer. J Clin Oncol, (2006) ASCO Annual Meeting Proceedings (2006) 24:2508.

Fujihara T, Yashiro M, Inoue T, Sawada T, Kato Y, Ohira M, Nishiguchi Y, Ishikawa T, Sowa M, Chung KH. Decrease in ICAM-1 expression on gastric cancer cells is correlated with lymph node metastasis. Gastric Cancer (1999) 2:221–225.

Gallagher RP, Spinelli JJ, Lee TK. Tanning beds, sunlamps, and risk of cutaneous malignant melanoma. Cancer Epidemiol Biomarkers Prev (2005) 14:562–566.

Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pagès F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science (2006) 313:1960–1964.

Gandini S, Sera F, Cattaruzza MS, et al. Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. Eur J Cancer (2005) 41:2040–2059.

Garbe C, Terheyden P, Keilholz U, Kölbl O, Hauschild A. Treatment of melanoma. Dtsch Arztebl Int (2008) 105:845-851.

Garcia JA, Mekhail T, Elson P, Wood L, Bukowski RM, Dreicer R, Rini BI. Phase I/II trial of subcutaneous interleukin-2, granulocyte-macrophage colony-stimulating factor and interferon-α in patients with metastatic renal cell carcinoma. BJU Int (2011) Jan 18. [Epub ahead of print]

Garcıa-Closas M, Malats N, Silverman D, et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet (2005) 366:649–659.

García-Ruano AB, Méndez R, Romero JM, Cabrera T, Ruiz-Cabello F, Garrido F. Analysis of HLA-ABC locus-specific transcription in normal tissues. Immunogenetics (2010) 62:711-719.

Garrido F, Festenstein H, Schirrmacher V. Further evidence for derepression of H-2 and Ia like specificities of foreign haplotypes in mouse tumor cell lines. Nature (1976) 261:228–230.

Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, Stern PL. Natural history of HLA expression during tumor development. Immunol Today (1993) 14:491–493.

Garrido F, Ruiz-Cabello F, Cabrera T, Pe´rez-Villar J, Lo´pez-Botet M, Duggan-Keen M, Stern PL. Implications for immunosurveillance of altered class I phenotypes in humans tumors. Immunol Today (1997) 18:89–95.

Garrido F, Algarra I. MHC antigens and tumor escape from immune surveillance. Adv Cancer Res (2001) 83:117–158.

Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy. Int J Cancer (2010) 127:249-256.

Geldmacher A, Freier A, Losch FO, Walden P. Therapeutic vaccination for cancer immunotherapy: Antigen selection and clinical responses. Hum Vaccin (2011) Jan 1:7. [Epub ahead of print]

Geller MA, Cooley S, Judson PL, Ghebre R, Carson LF, Argenta PA, Jonson AL,

Panoskaltsis-Mortari A, Curtsinger J, McKenna D, Dusenbery K, Bliss R, Downs LS, Miller JS. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. Cytotherapy (2011) 13:98-107.

Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC. Regulation of cutaneous malignancy by gammadelta T cells. Science (2001) 294:605-609.

Godelaine D, Carrasco J, Lucas S, Karanikas V, Schuler-Thurner B, Coulie PG, Schuler G, Boon T, Van Pel A Polyclonal CTL responses observed in melanoma patients vaccinated with dendritic cells pulsed with a MAGE-3.A1 peptide. J Immunol (2003) 171:4893–4897

Goldstein AM, Chan M, Harland M, et al. High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. Cancer Res (2006) 66:9818–9828.

Goldstein AM, Chan M, Harland M, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet (2007) 44:99–106.

Golijanin DJ, Kakiashvili D, Madeb RR, Messing EM, Lerner SP. Chemoprevention of bladder cancer. World J Urol (2006) 24:445-472.

Gomes AQ, Martins DS, Silva-Santos B. Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. Cancer Res (2010) 70:10024-10027.

Grandea AG, Van Kaer L. Tapasin: An ER chaperone that controls MHC class I assembly with peptide. Trends Immunol (2001) 22:194–199.

Guindi M. Role of activated host T cells in the promotion of MALT lymphoma growth. Semin Cancer Biol (2000) 10:341.

Grant GA, Miller JF. Effect of neonatal thymectomy on the induction of sarcomata in C57 BL mice. Nature (1965) 205:1124-1125.

Green J, Banks E, Berrington A, et al. N-acetyltransferase 2 and bladder cancer: an overview and consideration of the evidence for gene-environment interaction. Br J Cancer (2000) 83:412–417.

Grothey A, Heistermann P, Philippou S, Voigtmann R. Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1, CD54) in patients with non-small-cell lung cancer: correlation with histological expression of ICAM-1 and tumour stage. Br J Cancer (1998) 77:801.

Hakansson A, Gustafsson B, Krysander L, Hjelmqvist B, Rettrup B, Hakansson L. On down-regulation of the immune response to metastatic malignant melanoma. Cancer Immunol Immunother (1999) 48:253.

Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol (2000) 18:975-1026.

Heaton KM, Grimm EA. Cytokine combinations in immunotherapy for solid tumors: a review. Cancer Immunol Immunother (1993) 37:213-219.

Hersey P, Menzies SW, Coventry B, Nguyen T, Farrelly M, Collins S, Hirst D, Johnson H. Phase I/II study of immunotherapy with T cell peptide epitopes in patients with stage IV

me1anoma. Cancer Immunol Immunother (2005) 54:208–218.

Hershkovitz L, Schachter J, Treves AJ, Besser MJ. Focus on adoptive T cell transfer trials in melanoma. Clin Dev Immunol (2010) 2010:260267.

Hirano, F., Kaneko, K., Tamura, H., Dong, H., Wang, S., Ichikawa, M., Rietz, C., Flies, D. B., Lau, J. S., Zhu, G., Tamada, K., and Chen, L. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res (2005) 65:1089–1096.

Hocker T, Tsao H. Ultraviolet radiation and melanoma: a systematic review and analysis of reported sequence variants. Hum Mutat (2007) 28:578–588.

Holub M, Zakeri SM, Lichtenberger C, Pammer J, Paolini P, Leifeld L, Rockenschaub S, Wolschek MF, Steger G, Willheim M, Gangl A, Reinisch W. Heterogeneous expression and regulation of CD40 in human hepatocellular carcinoma. Eur J Gastroenterol Hepatol (2003) 15:119.

Homma I, Kitamura H, Torigoe T, Tanaka T, Sato E, Hirohashi Y, Masumori N, Sato N, Tsukamoto T. Human leukocyte antigen class I down-regulation in muscle-invasive bladder cancer: its association with clinical characteristics and survival after cystectomy. Cancer Sci (2009) 100:2331-2334.

Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science (1994) 264:961-5.

Huang WY, Sheehy TM, Moore LE, Hsing AW, Purdue MP. Simultaneous recovery of DNA and RNA from formalin-fixed paraffin-embedded tissue and application in epidemiologic studies. Cancer Epidemiol Biomarkers Prev (2010) 19:973-977.

Hudis CA. Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med (2007) 357:39–51.

IARC Working Group on artificial ultraviolet (UV) light and skin cancer. The association of use of sunbeds with cutaneous malignant melanoma and other skin cancers: a systematic review. Int J Cancer (2007) 120:1116–11122.

Jacobs BL, Lee CT, Montie JE. Bladder cancer in 2010: how far have we come? CA Cancer J Clin (2010) 60:244-272.

Jemal A, Devesa SS, Hartge P, Tucker MA. Recent trends in cutaneous melanoma incidence among whites in the United States. J Natl Cancer Inst (2001) 93:678-683.

Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA Cancer J Clin (2002) 52:23-47.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin (2009) 59:225-249.

Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin (2010) 60:277-300.

Jin, P. Wang, E. Polymorphism in clinical immunology. From HLA typing to immunogenetic profiling. J Transl Med (2003) 1:8.

Johnsen A, Templeton DJ, Sy MS, Harding CV. Deficiency of transporter for antigen

presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis. J Immunol (1999) 163:4224–4231.

Jinushi M, Tahara H. Cytokine gene-mediated immunotherapy: current status and future perspectives. Cancer Sci (2009) 100:1389-1396.

Kaplan HS. Role of immunologic disturbance in human oncogenesis: some facts and fancies. Br J Cancer (1971) 25:620-634.

Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A (1998) 95:7556-7561.

Karve SJ, Feldman SR, Yentzer BA, Pearce DJ, Balkrishnan R. Imiquimod: a review of basal cell carcinoma treatments. J Drugs Dermatol (2008) 7:1044-1051.

Kessler JH, Khan S, Seifert U, Le Gall S, Chow KM, Paschen A, Bres-Vloemans SA, de Ru A, van Montfoort N, Franken KL, Benckhuijsen WE, Brooks JM, van Hall T, Ray K, Mulder A, Doxiadis II, van Swieten PF, Overkleeft HS, Prat A, Tomkinson B, Neefjes J, Kloetzel PM, Rodgers DW, Hersh LB, Drijfhout JW, van Veelen PA, Ossendorp F, Melief CJ. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. Nat Immunol (2011) 12:45-53.

Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol (2002) 3:999-1005.

King J. Waxman J. Stauss H. Advances in tumour immunotherapy Q J Med (2008) 101:675–683.

Kirkwood JM, Strawderman MH, Ernstoff MS, Smith TJ, Borden EC, Blum RH. Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. Natl Acad Sci U S A (1998) 95:7556-7561.

Kirkwood JM, Manola J, Ibrahim J, Sondak V, Ernstoff MS, Rao U; Eastern Cooperative Oncology Group. A pooled analysis of eastern cooperative oncology group and intergroup trials of adjuvant high-dose interferon for melanoma. Clin Cancer Res (2004) 10:1670-7677.

Kirkwood JM, Tarhini AA, Panelli MC, Moschos SJ, Zarour HM, Butterfield LH, et al. Next generation of immunotherapy for melanoma. J Clin Oncol (2008) 26:3445-3455.

Kitamura H, Torigoe T, Honma I, Sato E, Asanuma H, Hirohashi Y, Sato N, Tsukamoto T. Effect of human leukocyte antigen class I expression of tumor cells on outcome of intravesical instillation of bacillus calmette-guerin immunotherapy for bladder cancer. Clin Cancer Res (2006) 12:4641-4644.

Kitamura H, Honma I, Torigoe T, Asanuma H, Sato N, Tsukamoto T. Down-regulation of HLA class I antigen is an independent prognostic factor for clear cell renal cell carcinoma. J Urol. (2007) 177:1269-1272.

Kloor M, Michel S, von Knebel Doeberitz M. Immune evasion of microsatellite unstable colorectal cancers. Int J Cancer (2010) 127:1001-1010.

Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. Adaptive immunity maintains occult cancer in an equilibrium state. Nature (2007) 450:903-907.

Koenig S, Woods RM, Brewah YA, Newell AJ, Jones GM, Boone E, Adelsberger JW, Baseler MW, Robinson SM, Jacobson S. Characterization of MHC class I restricted cytotoxic T cell responses to tax in HTLV-1 infected patients with neurologic disease. J Immunol (1993) 151:3874-3883.

Koopman JO, Hammerling GJ, Moburg F. Generation, intracellular transport, and loading of peptides associated with MHC class I molecules. Curr Opin Immunol (1997) 9:80–88.

Koopman LA, Corver WE, van der Slik AR, Giphart MJ, Fleuren GJ. Multiple genetic alterations cause frequent and heterogeneous human histocompatibility leukocyte antigen class I loss in cervical cancer. J Exp Med (2000) 20:961–976.

Kurai J, Chikumi H, Hashimoto K, Yamaguchi K, Yamasaki A, Sako T, Touge H, Makino H, Takata M, Miyata M, Nakamoto M, Burioka N, Shimizu E. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. Clin Cancer Res (2007) 13:1552–1561.

Kauffman H.M. Malignancies in organ transplant recipients . J Surg Oncol (2006) 94:431–433.

de Kruijf EM, van Nes JG, Sajet A, Tummers QR, Putter H, Osanto S, Speetjens FM, Smit VT, Liefers GJ, van de Velde CJ, Kuppen PJ. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res (2010) 16:1272-1280.

Lauritzsen GF, Hofgaard PO, Schenck K, Bogen B. Clonal deletion of thymocytes as a tumour escape mechanism. Int J Cancer (1998) 78:216.

Le Bouteiller P. HLA class I chromosomal region, genes, and products: Facts and questions. Crit Rev Immunol (1994) 14:89–129.

Lee KH, Wang E, Nielsen MB, Wunderlich J, Migueles S, Connors M, et al. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. J Immunol (1999) 163:6292–6300.

Leppert JT, Shvarts O, Kawaoka K, Lieberman R, Belldegrun AS, Pantuck AJ. Prevention of bladder cancer: a review. Eur Urol (2006) 49:226-234.

Li J, Mookerjee B, Wagner J, Flomenberg N. In vitro methods for generating highly purified EBV associated tumor antigen-specific T cells by using solid phase T cell selection system for immunotherapy. J Immunol Methods (2007) 328:169-181.

Liao RY, Mao C, Qiu LX, Ding H, Chen Q, Pan HF. TGFBR1*6A/9A polymorphism and cancer risk: a meta-analysis of 13,662 cases and 14,147 controls. Mol Biol Rep (2010) 37:3227-3232.

Linderoth J, Jerkeman M, Cavallin-Stahl E, Kvaloy S, Torlakovic E. Immunohistochemical expression of CD23 and CD40 may identify prognostically favorable subgroups of diffuse large B-cell lymphoma: a Nordic Lymphoma Group study. Clin Cancer Res (2003) 9:722.

Link AA, Kino T, Worth JA, McGuire JL, Crane ML, Chrousos GP, Wilder RL, Elenkov IJ. Ligand-activation of the adenosine A2a receptors inhibits IL-12 production by human monocytes. J Immunol (2000) 164:436.

Ljunggren HG, Kärre K. In search of the ‘‘missing self’’: MHC molecules and NK cell

recognition. Immunol Today (1990) 11:237–244.

Lopez-Botet M, Llano M, Navarro F, Bellon T. Nk cell recognition of non-classical HLA class I molecules. Semin Immunol (2000) 12:109–119.

López-Nevot MA, Garcia E, Romero C, Oliva MR, Serrano S, Garrido F. Phenotypic and genetic analysis of HLA class I and HLA-DR antigen expression on human melanomas. Exp Clin Immunogenet (1988) 5:203-212.

Love WE, Bernhard JD, Bordeaux JS. Topical imiquimod or fluorouracil therapy for basal and squamous cell carcinoma: a systematic review. Arch Dermatol. (2009) 145:1431-1438.

Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG, Boon T. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med (2005) 201:249–257.

Maffei A, Papadopoulos K, Harris PE. MHC class I antigen processing pathways. Hum Immunol (1997) 54:91–103.

Maleckar JR, Sherman LA. The composition of the T cell receptor repertoire in nude mice. J Immunol (1987) 138:3873-3876.

Maleno I, Romero JM, Cabrera T, Paco L, Aptsiauri N, Cozar JM, Tallada M, López-Nevot MA, Garrido F. LOH at 6p21.3 region and HLA class I altered phenotypes in bladder carcinomas. Immunogenetics (2006) 58:503-510.

Maleno I, Aptsiauri N, Cabrera T, Gallego A, Paschen A, López-Nevot MA, Garrido F. Frequent loss of heterozygosity in the β2-microglobulin region of chromosome 15 in primary human tumors. Immunogenetics. (2011) 63:65-71.

Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumour-associated macrophages. Immunol Today (1992) 13:265.

Mapara MY, Sykes M. Tolerance and cancer: mechanisms of tumor evasion and strategies for breaking tolerance. J Clin Oncol (2004) 22:1136–1151.

Marchand M, Brichard V, Van Baren N, Coulie PG. Biological and clinical developments in melanoma vaccines. Expert Opin Biol Ther (2001) 1:497–510

Marcus PM, Hayes RB, Vineis P, et al. Cigarette smoking, N-acetyltransferase2 acetylation status, and bladder cancer risk: a case-series meta-analysis of a gene-environment interaction. Cancer Epidemiol Biomarkers Prev (2000) 9:461–467.

Mariani L, Venuti A. HPV vaccine: an overview of immune response, clinical protection, and new approaches for the future. J Transl Med (2010) 8:105.

Marincola F.M. Wang E. Immunologic Signatures of Rejection (2010) New York: Springer-Verlag

Matzinger P.Tolerance, danger, and the extended family. Annu Rev Immunol (1994) 12:991-1045.

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) 57:719-729.

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) 58:1507-1515.

Meyskens FL Jr, Berwick M. UV or not UV: metals are the answer. Cancer Epidemiol Biomarkers Prev (2008) 17:268–270.

Michaud DS, Spiegelman D, Clinton SK, Rimm EB, Curhan GC, Willett WC, Giovannucci EL. Fluid intake and the risk of bladder cancer in men. N Engl J Med (1999) 340:1390-1397.

Mizoguchi H, OShea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. Alterations in signal transduction molecules in lymphocytes-T from tumour-bearing mice. Science (1992) 258:1795.

Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, Moretta L. Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol (1996) 14:619–648.

Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, Rosenberg SA, Cancer regression in patient safter transfer of genetically engineered lymphocytes. Science (2006) 314126–314129.

Morgan RA, Dudley ME, Rosenberg SA. Adoptive cell therapy: genetic modification to redirect effector cell specificity. Cancer J (2010) 16:336-341.

Mostafa MH, Sheweita SA, O'Connor PJ. Relationship between schistosomiasis and bladder cancer. Clin Microbiol Rev (1999) 12:97-111.

Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res (2010) 107:57-117.

Morgan RA, Dudley ME, Rosenberg SA. Adoptive cell therapy: genetic modification to redirect effector cell specificity. Cancer J (2010) 16:336-341.

Mumberg D, Wick M, Schreiber H. Unique tumor antigens redefined as mutant tumor-specific antigens. Semin Immunol (1996) 8:289-293.

Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest (2007) 117:1147-54.

Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res (1998) 58:3491-3494.

Nilsson S, Ullen A. Chemotherapy-induced bladder cancer. Scand J Urol Nephrol Suppl (2008) 218:89-92.

Nishizuma, Y Nakakuki, K Usui, M enhacing effect of thymectomy on hepatotumorigenesis in Swiss mice following neonatal injection of 20-methylcholantrene. Nature (1965) 20:1236-1238

O'Neill DW. Dendritic cells and T cells in immunotherapy. J Drugs Dermatol (2010) 9:1383-1392.

Old LJ. Cancer vaccines 2003: opening address. Cancer Immun (2003) 3 Suppl 2:1.

Olsen CM, Zens MS, Stukel TA, Sacerdote C, Chang YM, Armstrong BK, Bataille V, Berwick M, Elwood JM, Holly EA, Kirkpatrick C, Mack T, Bishop JN, Østerlind A, Swerdlow AJ, Zanetti R, Green AC, Karagas MR, Whiteman DC. Nevus density and melanoma risk in women: a pooled analysis to test the divergent pathway hypothesis. Int J Cancer (2009) 124:937–944.

Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumour rejection by in vivo administration of anti-CD25 (Interleukin-2 receptor alpha) monoclonal antibody. Cancer Res (1999) 59:3128.

Outzen HC, Custer RP, Eaton GJ, Prehn RT. Spontaneous and induced tumor incidence in germfree "nude" mice. J Reticuloendothel Soc (1975) 17:1-9.

Osen W, Soltek S, Song M, Leuchs B, Steitz J, Tüting T, Eichmüller SB, Nguyen XD, Schadendorf D, Paschen A. Screening of human tumor antigens for CD4 T cell epitopes by combination of HLA-transgenic mice, recombinant adenovirus and antigen peptide libraries. PLoS One (2010) 5:e14137

Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med (2005) 353:2654-2666.

Panelli MC, Stashower ME, Slade HB, Smith K, Norwood C, Abati A, Fetsch P, Filie A, Walters SA, Astry C, Aricó E, Zhao Y, Selleri S, Wang E, Marincola FM. Sequential gene profiling of basal cell carcinomas treated with imiquimod in a placebo-controlled study defines the requirements for tissue rejection. Genome Biol (2007) 8:R8.

Pardoll D. Does the immune system see tumors as foreign or self? Annu Rev Immunol (2003) 21:807-839.

Parham P, Ohta T. Population biology of antigen presentation by MHC class I molecules. Science (1996) 272:67–74.

Parham P. The genetic and evolutionary balances in human NK cell receptor diversity. Semin Immunol (2008) 20:311-316.

Park B, Lee S, Kim E, Ahn K. A single polymorphic residue within the peptide-binding cleft of MHC class I molecules determines spectrum of tapasin dependence. J Immunol (2003) 170:961–978.

Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin (2005) 55:74-108.

Parmer E, Cresswell P. Mechanisms of MHC class I restricted antigen processing. Annu Rev Immunol (1998) 16:323–358.

Paschen A, Méndez RM, Jimenez P, Sucker A, Ruiz-Cabello F, Song M, Garrido F, Schadendorf D. Complete loss of HLA class I antigen expression on melanoma cells: a result of successive mutational events. Int J Cancer. (2003) 103:759-67.

Paschen A, Eichmuller S, Schadendorf D. Identification of tumor antigens and T-cell epitopes, and its clinical application. Cancer Immunol Immunother (2004) 53:196-203.

Paschen A, Arens N, Sucker A, Greulich-Bode KM, Fonsatti E, Gloghini A, Striegel S, Schwinn N, Carbone A, Hildenbrand R, Cerwenka A, Maio M, Schadendorf D. The

coincidence of chromosome 15 aberrations and beta2-microglobulin gene mutations is causative for the total loss of human leukocyte antigen class I expression in melanoma. Clin Cancer Res (2006) 12:3297-32305.

Patard JJ, Saint F, Velotti F, Abbou CC, Chopin DK. Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. Urol Res (1998) 26:155-159.

Pawelec G. Tumour escape from the immune response: the last hurdle for successful immunotherapy of cancer? Cancer Immunol Immunother (1999) 48:343.

Pawelec G. Tumour escape: antitumour effectors too much of a good thing? Cancer Immunol Immunother (2004) 53: 262–274.

Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. Clin Exp Immunol (2009) 157:9-19.

Peguet-Navarro J, Sportouch M, Popa I, Berthier O, Schmitt D, Portoukalian J. Gangliosides from human melanoma tumours impair dendritic cell differentiation from monocytes and induce their apoptosis. J Immunol (2003) 170:3488

Pellegrino MA, Ferrone S, Reisfeld MA, Irie RF, Golub SH. Expression of histocompatibility (HLA) antigens on tumor cells and normal cells from patients with melanoma. Cancer (1977) 40:36–41.

Penn I. Sarcomas in organ allograft recipients. Transplantation (1995) 60:1485-1491.

Penn I. Solid tumors in cardiac allograft recipients. Ann Thorac Surg (1995) 60:1559-1560.

Penn I. Malignant melanoma in organ allograft recipients. Transplantation (1996) 61:274-278.

Penn I. Posttransplant malignancies. Transplant Proc (1999) 31:1260-1262.

Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. T lymphocytes that infiltrate tumours and atherosclerotic plaques produce heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor: a potential pathologic role. Proc Natl Acad Sci U S A (1995) 92:6547.

Perez-Diez, A., Spiess, P. J., Restifo, N. P., Matzinger, P., Marincola, F. M. Intensity of the vaccine-elicited immune response determines tumor clearance. J Immunol (2002) 168:338–347.

Pescovitz MD. Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. Am J Transplant (2006) 6:859–866.

Pettit KS, O'Flaherty E, Charnley RM, Kirby JA. Selection of metastatic tumor phenotypes by host immune system. Lancet (1999) 353:1989–1991.

Pilla L, Patuzzo R, Rivoltini L, Maio M, Pennacchioli E, Lamaj E et al. A phase II trial of vaccination with autologous, tumor-derived heat-shock protein peptide complexes Gp96, in combination with GM-CSF and interferon-alpha in metastatic melanoma patients. Cancer Immunol Immunother. (2006) 55:958-968.

Ponzio NM, Thorbecke GJ. Requirement for reverse immune surveillance for the growth of germinal center-derived murine lymphomas. Semin Cancer Biol (2000) 10:331.

Quezada SA, Peggs KS, Curran MA, Allison JP. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. J Clin

Invest (2006) 116:1935–4195.

Raimondi S, Sera F, Gandini S, Iodice S, Caini S, Maisonneuve P, Fargnoli MC. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer (2008) 122:2753-2760.

Reichmann E. The biological role of the Fas/FasL system during tumour formation and progression. Semin Cancer Biol (2002) 12:309.

Repp R, Valerius T, Wieland G, Becker W, Steininger H, Deo Y, Helm G, Gramatzki M, Van de Winkel JG, Lang N, et al. G-CSF-stimulated PMN in immunotherapy of breast cancer with a bispecific antibody to Fc gamma RI and to HER-2/neu (MDX-210) J Hematother (1995) 4:415-421.

Respa A, Bukur J, Ferrone S, Pawelec G, Zhao Y, Wang E, Marincola FM, Seliger B. Association of IFN-gamma signal transduction defects with impaired HLA class I antigen processing in melanoma cell lines. Clin Cancer Res (2011). [Epub ahead of print]

Rivoltini L, Canese P, Huber V, Iero M, Pilla L, Valenti R, Fais S, Lozupone F, Casati C, Castelli C, Parmiani G. Escape strategies and reasons for failure in the interaction between tumour cells and the immune system: how can we tilt the balance towards immune-mediated cancer control? Expert Opin Biol Ther (2005) 5:463–476.

Rodriguez T, Méndez R, Roberts CH, Ruiz-Cabello F, Dodi IA, López Nevot MA, Paco L, Maleno I, Marsh SG, Pawelec G, Garrido F. High frequency of homozygosity of the HLA region in melanoma cell lines reveals a pattern compatible with extensive loss of heterozygosity. Cancer Immunol Immunother (2005) 54:141-148.

Rodriguez T, Mendez 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) 7:34.

Romero JM, Jiménez P, Cabrera T, Cózar JM, Pedrinaci S, Tallada M, Garrido F, Ruiz-Cabello F. Coordinated downregulation of the antigen presentation machinery and HLA class I/beta2-microglobulin complex is responsible for HLA-ABC loss in bladder cancer. Int J Cancer (2005) 113:605-610.

Rosenberg SA.A new era of cancer immunotherapy: converting theory to performance. CA Cancer J Clin. (1999) 49:70-73.

Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med (2004) 10:909–915.

Rosenberg SA, Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci USA (2004) 101:14639–14645.

Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol (2009) 21:233-240.

Rutter JL, Bromley CM, Goldstein AM, et al. Heterogeneity of risk for melanoma and pancreatic and digestive malignancies: a melanoma case-control study. Cancer (2004) 101:2809–2816.

Rygaard J, Povlsen CO. The mouse mutant nude does not develop spontaneous tumours. An argument against immunological surveillance. Acta Pathol Microbiol Scand B Microbiol

Immunol (1974) 82:99-106.

Sáenz-López P, Gouttefangeas C, Hennenlotter J, Concha A, Maleno I, Ruiz-Cabello F, Cózar JM, Tallada M, Stenzl A, Rammensee HG, Garrido F, Cabrera T. Higher HLA class I expression in renal cell carcinoma than in autologous normal tissue. Tissue Antigens (2010) 75:110-118.

Saint-Mezard P, Berthier CC, Zhang H, Hertig A, Kaiser S, Schumacher M et al. Analysis of independent microarray datasets of renal biopsies identifies a robust transcript signature of acute allograft rejection. Transpl Int (2009) 22:293-302.

Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumour immunity, and transplantation tolerance. Immunol Rev (2001) 182:18.

Salgado R, Junius S, Benoy I, Van Dam P, Vermeulen P, Van Marck E, Huget P, Dirix LY. Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. Int J Cancer (2003) 103:642.

Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. Annu Rev Immunol (2000) 18:593-620.

Sanchez-Rovira P, Jimenez E, Carracedo J, Barneto IC, Ramirez R, Aranda E. Serum levels of intercellular adhesion molecule 1 (ICAM-1) in patients with colorectal cancer: Inhibitory effect on cytotoxicity. Eur J Cancer (1998) 34:394.

Sanford BH, Kohn HI, Daly JJ. Soo SF. long-term spontaneous tumor incidence in neonatally thymectomizad mice. J. immunol (1973) 110:1437-1439.

Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, Salvatierra O Jr. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. (2003) 349:125–138

Schietinger A, Philip M, Schreiber H. Specificity in cancer immunotherapy. Semin Immunol (2008) 20:276–285.

SEER Cancer Statistics Review, 1975-2006. Bethesda, MD: National Cancer Institute. http://seer.cancer.gov/csr/1975_2006/.

Schwinn N, Vokhminova D, Sucker A, Textor S, Striegel S, Moll I, Nausch N, Tuettenberg J, Steinle A, Cerwenka A, Schadendorf D, Paschen A. Interferon-gamma down-regulates NKG2D ligand expression and impairs the NKG2D-mediated cytolysis of MHC class I-deficient melanoma by natural killer cells. Int J Cancer (2009) 124:1594-1604.

Seliger B, Maurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today (2000) 21:455–464.

Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature (2001) 410:1107-1111.

Shariat, S. F., Kim, J. H., Andrews, B., Kattan, M. W., Wheeler, T. M., Kim, I. Y., Lerner, S. P., and Slawin, K. M. Preoperative plasma levels of transforming growth factor beta strongly predict clinical outcome in patients with bladder carcinoma. Cancer (2001) 92:2985–2992.

Shashidharamurthy R, Bozeman EN, Patel J, Kaur R, Meganathan J, Selvaraj P. Immunotherapeutic strategies for cancer treatment: A novel protein transfer approach for cancer vaccine development. Med Res Rev. 2011. [Epub ahead of print]

Shehata M, Mukherjee A, Deen S, Al-Attar A, Durrant LG, Chan S. Human leukocyte antigen class I expression is an independent prognostic factor in advanced ovarian cancer resistant to first-line platinum chemotherapy. Br J Cancer (2009) 101:1321-1328.

Sheil AG. Cancer after transplantation . World J Surg (1986) 10:389-396.

Shen W, Ladisch S. Ganglioside GD1a impedes lipopolysaccharide- induced maturation of human dendritic cells. Cell Immunol (2002) 220:125.

Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumour immunity by removing CD25+ CD4+ T cells: a common basis between tumour immunity and autoimmunity. J Immunol (1999) 163:5211.

Shokeir AA. Squamous cell carcinoma of the bladder: pathology, diagnosis and treatment. BJU Int (2004) 93:216-220.

Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer (2005) 5:615-625.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med (2001) 344:783–792.

Smalley KS, Nathanson KL, Flaherty KT. Genetic subgrouping of melanoma reveals new opportunities for targeted therapy. Cancer Res. (2009) 69:3241-3244.

Smith C, Santi M, Rushing EJ, Cornelison R, Macdonald TJ, Vukmanovic S. Characterization of signaling function and expression of HLA class I molecules in medulloblastoma. J Neurooncol (2010) [Epub ahead of print]

Smyth MJ, Wallace ME, Nutt SL, Yagita H, Godfrey DI, Hayakawa Y.Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. J Exp Med (2005) 201:1973-1985.

Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol (2002) 2:185-194.

Stanley M. Potential mechanisms for HPV vaccine-induced long-term protection. Gynecol Oncol (2010) 118:S2-7.

Steinbrink K, Jonuleit H, Muller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8+ T cells resulting in a failure to lyse tumour cells. Blood (1999) 93:1634.

Storkus WJ, Howell DN, Salter RD, Dawson JR, Cresswell P. NK susceptibility varies inversely with target cell class I HLA antigen expression. J Immunol (1987) 138:1657–1659.

Street SE, Cretney E, Smyth MJ. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. Blood (2001) 97:192-197.

Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science (1974) 183:534-536.

Stutman O. Immunodepression and malignancy. Adv Cancer Res (1975) 22:261-422.

Sullivan TP, Dearaujo T, Vincek V, Berman B: Evaluation of superficial basal cell carcinomas after treatment with imiquimod 5% cream or vehicle for apoptosis and lymphocyte phenotyping. Dermatol Surg (2003) 29:1181-1186.

Tarhini AA, Kirkwood JM. Clinical and immunologic basis of interferon therapy in melanoma. Ann N Y Acad Sci (2009) 1182:47-57.

Terabe M, Berzofsky JA. Immunoregulatory T cells in tumor immunity. Curr Opin Immunol (2004) 16157–162.

Terando AM, Faries MB, Morton DL. Vaccine therapy for melanoma: current status and future directions. Vaccine (2007) 25:4-16.

Thomas, L. in Celular and Humoral Aspects of the Hypersensitive States (ed. Lawrence, H. S.) 529-532 (1959) (Hoeber-Harper, New York,

Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell (1986) 28:959–968.

Trainin N, Linker-Israeli M, Small M, Boiato-Chen L. Enhancement of lung adenoma formation by neonatal thymectomy in mice treated with 7,12-dimethylbenz(a)anthracene or urethan. Int J Cancer (1967) 2:326-336.

Trofe J, Beebe TM, Buell JF, Hanaway MJ, First MR, Alloway RR, Gross TG, Woodle ES. Post transplant malignancy. Prog Transplant (2004) 14:193–200.

Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. N Engl J Med (2004) 351:998-1012.

Tsutsumi S, Kuwano H, Shimura T, Morinaga N, Mochiki E, Asao T. Circulating soluble Fas ligand in patients with gastric carcinoma. Cancer (2000) 89:2560.

Tucker MA, Goldstein AM. Melanoma etiology: where are we? Oncogene (2003) 22:3042-3052.

Turtle CJ, Riddell SR. Artificial antigen-presenting cells for use in adoptive immunotherapy. Cancer J (2010) 16:374-381.

Uhr, J. W., Scheuermann, R. H., Street, N. E., and Vitetta, E. S. Cancer dormancy: Opportunities for new therapeutic approaches. Nat Med (1997) 3:505–509.

Van Baren N, Bonnet MC, Dreno B, Khammari A, Dorval T, Piperno-Neumann S, Lienard D, Speiser D, Marchand M, Brichard VG, Escudier B, Negries S, Dietrich PY, Maraninchi D, Osanto S, Meyer RG, Ritter G, Maingeon P, Tarlaglia J, van der Bruggen P, Coulie PG, Boon T Tumoral and immunologic response after vaccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T cells. J Clin Oncol (2005) 23:9008–9021

Van den Eynde B, Brichard VG. New tumor antigens recognized by T cells. Curr Opin Immunol (1995) 7:674-681.

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (1991) 254:1643–1647.

van der Bruggen P. T cell defined tumour antigens (2009) (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm)

Van Endert P. Genes regulating MHC class I processing of antigen. Curr Opin Immunol (1999) 11:82–88.

Vergati M, Intrivici C, Huen NY, Schlom J, Tsang KY. Strategies for cancer vaccine development. J Biomed Biotechnol (2010) 2010pii: 596432.

Vicari AP, Caux C. Chemokines in cancer. Cytokine Growth Factor Rev (2002) 13:143-154.

Videira PA, Calais FM, Correia M, Ligeiro D, Crespo HJ, Calais F, Trindade H. Efficacy of bacille Calmette-Guérin immunotherapy predicted by expression of antigen-presenting molecules and chemokines. Urology (2009) 74:944-950.

Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol (2002) 20:217-251.

Villunger A, Strasser A. The great escape: Is evasion required for tumour progression? Nat Med (1999) 5:874–875.

Von Boehmer, H. Mechanisms of suppression by suppressor T cells. Nat. Immunol (2005) 6:338–344.

von Leoprechting A, van der Bruggen P, Pahl HL, Aruffo A, Simon JC. Stimulation of CD40 on immunogenic human malignant melanomas augments their cytotoxic T lymphocyte-mediated lysis and induces apoptosis. Cancer Res (1999) 59:1287.

Vora AR, Rodgers S, Parker AJ, Start R, Rees RC, Murray AK. An immunohistochemical study of altered immunomodulatory molecule expression in head and neck squamous cell carcinoma. Br J Cancer (1997) 76:836.

Wang E, Worschech A, Marincola F.M. The immunologic constant of rejection. Trends Immuno (2008) 29:256-262.

Wang E, Selleri S, Sabatino M, Monaco A, Pos Z, Worschech A, Stroncek DF, Marincola FM. Spontaneous and treatment-induced cancer rejection in humans. Expert Opin Biol Ther (2008) 8:337-349.

Wang XC, Zhang JQ, Shen YQ, Miao FQ, Xie W. Loss of heterozygosity at 6p21.3 underlying HLA class I downregulation in gastric cancer. J Exp Clin Cancer Res (2006) 25:115-119.

Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, Hurchla MA, Zimmerman N, Sim J, Zang X, Murphy TL, Russell JH, Allison JP, Murphy KM. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol (2003) 4:670.

Weiss JM, Subleski JJ, Wigginton JM, Wiltrout RH. Immunotherapy of cancer by IL-12-based cytokine combinations. Expert Opin Biol Ther (2007) 7:1705-1721.

Whiteside TL. Signaling defects in T lymphocytes of patients with malignancy. Cancer Immunol Immunother (1999) 48:346.

Wu XR. Urothelial tumorigenesis: a tale of divergent pathways. Nat Rev Cancer (2005) 5:713-725.

Yin R, Zheng W, Hao F, Yang XC, Zhong BY, Li QJ. HPV16E7 tumor antigen modified by

KDEL sequence induce specific cytotoxic T lymphocytes-dependent antitumor immunity. J Dermatol Sci (2009) 55:116-122.

Yoshimoto T, Morishima N, Okumura M, Chiba Y, Xu M, Mizuguchi J. Interleukins and cancer immunotherapy. Immunotherapy (2009) 1:825-844.

Yue FY, Dummer R, Geertsen R, Hofbauer G, Laine E, Manolio S, Burg G. Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-1, HLA class-II and ICAM-1 molecules. Int J Cancer (1997) 71:630.

Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer (2005) 5:263-274.

Chapter 7.

Apendix

7.1. List of publications

Articles related with the Thesis published in international journal

- Aptsiauri N, Carretero R, Garcia-Lora A, Real LM, Cabrera T, Garrido F. Regressing and progressing metastatic lesions: resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations. Cancer Immunol Immunother. 2008 Nov;57(11):1727-33.
- Carretero R, Romero JM, Ruiz-Cabello F, Maleno I, Rodriguez F, Camacho FM, Real LM, Garrido F, Cabrera T. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. Immunogenetics. 2008 Aug;60(8):439-47.
- Carretero R, Cabrera T, Gil H, Saenz-Lopez P, Maleno I, Aptsiauri N, Cozar JM, Garrido F. BCG immunotherapy of bladder cancer induces selection of HLA class I-deficient tumor cells. Int J Cancer. 2010 Oct 18. [Epub ahead of print].
- Carretero R, Wang E, Rodriguez AI, Reinboth J, Ascierto ML, Engle AM, Liu H, Camacho FM, Marincola FM, Garrido F, Cabrera T. Regression of melanoma metastases is associated with activation of antigen presentation and immune-mediated rejection genes. Sended to journal

Others articles published in journals during the research.

- Romero JM, Sáenz-López P, Cózar JM, Carretero R, Canton J, Vazquez F, Concha A, Tallada M, Garrido F, Ruiz-Cabello F. A polymorphism in the interleukin-10 promoter affects the course of disease in patients with clear-cell renal carcinoma. Hum Immunol. 2009 Jan;70(1):60-4.
- Sáenz-López P, Carretero R, Cózar JM, Romero JM, Canton J, Vilchez JR, Tallada M, Garrido F, Ruiz-Cabello F. Genetic polymorphisms of RANTES, IL1-A, MCP-1 and TNF-A genes in patients with prostate cancer. BMC Cancer. 2008 Dec 19;8:382.
- Bernal M, Garrido P, Jiménez P, Carretero R, Almagro M, López P, Navarro P, Garrido F, Ruiz-Cabello F. Changes in activatory and inhibitory natural killer (NK) receptors may induce progression to multiple myeloma: implications for tumor evasion of T and NK cells. Hum Immunol. 2009 Oct;70(10):854-7.
- Sáenz López P, Vázquez Alonso F, Romero JM, Carretero R, Tallada Buñuel M, Ruiz Cabello F, Cózar Olmo JM. [Polymorphisms in inflammatory response genes in metastatic renal cancer]. Actas Urol Esp. 2009 May;33(5):474-81.
- Sáenz-López P, Carretero R, Vazquez F, Martin J, Sánchez E, Tallada M, Garrido F, Cózar JM, Ruiz-Cabello F. Impact of interleukin-18 polymorphisms-607 and -137 on clinical characteristics of renal cell carcinoma patients. Hum Immunol. 2010 Mar;71(3):309-13.

7.2 Agradecimientos/Acknowledgment

A pesar del gran esfuerzo personal que requiere la realización de una Tesis doctoral, sería totalmente imposible realizarla sin la colaboración un grupo de grandes profesionales y el apoyo de unas magnificas personas.

Al Dr. Federico Garrido por darme la oportunidad de trabajar en su grupo, por sus consejos y su asesoramiento y por estar al frente del grupo de investigación.

A la Dr. Teresa Cabrera, por la confianza depositada en mí y por su apoyo y formación durante la realización de esta tesis. Muchas gracias por ser una persona cercana, y por las clases de esqui☺.

Al Dr. Jose M. Cozar, por llevar tantos años colaborando en la investigación y por su esfuerzo en desarrollar la relación entre los servicios.

Me gustaría agradecer especialmente la ayuda del Dr. Francisco Ruiz-Cabello por su inestimable ayuda y colaboración en la investigación, a la Dra. Natalia Aptsiauri por no utilizar jamás la palabra no. Muchisimas gracias por ayudarme con la redacción del ingles sin importar lo ocupada que estuvieras. Tambíen al Dr. García-Lora por estar siempre pendiente de nosotros e informándonos de todas las convocatorias. Agradezco al Dr. Francisco Camacho su implicación en el proyecto y en la investigación en general.

A todos los miembros del servicio y del grupo de investigacion por haberme acompañado durante este largo viaje. Gracias por vuestra ayuda y compañerismo. A mis "niñas terribles" de la habitación de genética por todos los buenos ratos durante las PCRs y por alimentarme (a pesar de que terminarais quitando mi foto… que fuerte…). A la Dr. Pilar por ser tan cercana y sacar una sonrisa cada vez que usaba el microscopio.

A mis compañeros por hacer de la investigación un esfuerzo conjunto. Me gustaría agradecer especialmente a mis coleguillas Pablo y Anabel por alegrar la habitación. Anabel, no se si eres cosciente de que sin tu implicación en el proyecto (especialmente meses antes de las estancias), habría sido imposible realizarlo, y sin tu amistad hubiera sido mucho mas duro. Pablo, gruñon, aunque fuera siempre de los primeros en irme, no sabes cuanto aprecio que siempre andaras organizando planes y relacionando a todos con todos. Por muy "radical" que seas, el grupo es mas grupo gracias a ti.

A "mein Freund" Isa, Ana, Anita, Víctor, Mónica, Ana Belén y Cristina, gracias por

demostrarme que sois mis amigos en los buenos y, sobre todo, en los malos momentos. No me puedo imaginar estar trabajando en algún sintió sin vosotros.

I also want to thanks Dr. Franco Marincola and Dra. Ena wang for allow me to work in their lab and for, no matter how busy you could be, you always keep the door open for us. This thesis also belongs to both of you.

Jenny, Lore, BBB, Heidi, Alyson, Jaime, Sara, Ping, Davide, Mary Free, Francesca, Dany, Paolo and all the NIH friends, thank you so much for your friendship and for making me feel I have a home there. I will never forget you. Lore, Barbara (the grunchies), Sara and little latinoide ☺, thank you for taking care of the poor little Spanish guy and MF for hosting the dangerous latieuropoide. Jenny, thank you so much for your help in the thesis and thank you so much for all the good times and for be my best friend and my little little sister. Heidi, thank you so much for correcting the english in this work and for being the taller little sister ☺ (we are a really mixed family). Jaime and Aly, thanks for all the funny moments in the lab.

Quiero agradecer especialemte el apoyo de mi familia. A mis padres muchas gracias por guiarme durante este largo camino por apoyarme siempre y por quererme tanto. A mis hermanos Javi y Migue, por todo su cariño y todas las horas que hemos pasado juntos con nuestras frikadas. Espero que sigamos asi siempre. Tambien a Bea y Laura, por vuestra simpatía y vuestra santa paciencia aguantando a ese par ;-).

A Alexandra. Gracias por hacer este viaje posible, ya que sin todas esas horas de estudio que pasaba solamente por poder estar contigo jamás hubiera llegado hasta aquí. Gracias por enseñarme que el esfuerzo es la clave del exito. Gracias por todo tu entusiasmo y ánimos. Y sobre todo, gracias por alegrar mi vida y estar a mi lado. Sin ti todo esto no tendría sentido.

7.3. List of abreviations

AIF-1: allograft inflammatory factor 1.
AJCC: american joint committee on cancer.
APM: antigne proccesing machinery.
B-CLL: B-cell chronic lymphocytic leukemia.
BCC: basa cell carcinoma.
BCG: bacillus Calmette-Guerin.
β2m: beta 2 microglobulin.
CTL: cytotoxic T lymphocyte.
CTLA-4: cytotoxic T lymphocyte antigen 4.
DC: dendritic cells.
DNP: dinitrophenyl.
EBV: Epstein Barr virus.
ER: endoplasmic reticulum.
FISH: fluorescent in situ hibridation
G-CSF : granulocyte colony-stimulating factor.
GM-CSF: granulocyte macrophage colony-stimulating factor.
HC: heavy chain.
HLA: human leukocyte antigen.
HPV: Human papillomavirus.
HTLV-1: Human T-cell Lymphotropic Virus Type 1.
ERB-2: Human Epidermal growth factor Receptor 2.
IL: interleukin.
INF: interferon.
ICAM-1: inter-cellular adhesion molecule 1.
IDO: 2, 3-indoleamone dioxygenase.
IRF-1: Interferon regulatory factor 1.
KIR: immunoglobuline-like receptor.
LMP2: latent membrane proteins 2.
LOH: loss of heterozigosity.
mAbs: monoclonal antibodies
MAGE: melanoma antigen gene.
MART-1: melanoma antigen recognized by T cells.
MHC: major histocompatibility complex.
NK: natural killer.
PD1: programmed death 1.

STAT-1: signal transducer and activator of transcription 1.
SCC: squamous cell cancers.
SCID: combined immune deficiency mutation.
TAA: tumor associated antigen.
TAP: transporter associated with antigen processing
TSA: Tumor-Specific Antigens.
TCR: T cell receptor.
TIL: Tumor infiltrated lymphocytes.
TNF: tumor necrosis factor.
TGF: tumor growth factor.
WHO: worl health organization.