DOCTORAL THESIS

STUDY OF THE IMMUNOLOGICAL PHENOTYPE OF METASTASES OBTAINED FROM MICE RECEIVING DIFFERENT IMMUNOTHERAPY PROTOCOLS

"ESTUDIO DEL FENOTIPO INMUNOLÓGICO DE METÁSTASIS OBTENIDAS DE RATONES SOMETIDOS A DIFERENTES PROTOCOLOS DE INMUNOTERAPIA"

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UNIVERSIDAD DE GRANADA

Universidad de Granada

PROGRAMA OFICIAL DE DOCTORADO EN INMUNOLOGÍA

ABRIL 2013

Editor: Editorial de la Universidad de Granada
Autor: Cristina Pilar Garrido López
D.L.: GR 2218-2013
ISBN: 978-84-9028-652-4

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A MI FAMILIA

COMPAÑEROS Y AMIGOS

ACKNOWLEDGEMENTS

I do thank to Dr. Federico Garrido for accepting and homing me in his research group and helping me to get the pre-doctoral grant to realize my PhD training.

I do also thank him and Dr. Ángel M. García-Lora as my thesis directors for forming me as a scientific research and for the guidance during the development of the PhD.

I do thank Isabel Linares and Ana Rodriguez for technical assistance in experimentation during the PhD.

I do thank Dr. Irene Romero for the continuous collaboration and advisement during the PhD.

I do thank to Dr. Ignacio Algarra for his collaboration with Dr. Ángel M. García-Lora with the planning of experimentation and the writing of paper manuscripts.

I do thank to Dr. Antonia Collado and Encarnación Arias for their help with the animal experimentation.

I do thank others members of Dr. Federico's research group that have occasionally help in the experimental work, scientific formation or results discussion: Dr. Natalia Aptsauri, Dr. Francisco Ruíz-Cabello, Dr. Teresa Cabrera, Dr. Antonio Garrido, Dr. Pilar Jiménez, Dr. Rafael Carretero, Dr. Isabel Maleno, Dr. Rosa Méndez, Dr. Teresa Rodríguez, Dr. López Nevot, Pablo Sáenz-López, Mónica Bernal, Enrique Berruguilla, Victor Cabrera, María Martínez, Javier Carretero, Ana Belén Del Campo, Ana Belén Rodríguez and Svetlana Zinchenko.

I do thank to Dr. Michael Shurin from the University of Pittsburgh, for accepting me in his research group for four month during my PhD training, for the research work I could do there and the welcoming he and his group showed to me.

I do thanks to Dr. Greta Garrido from the Center of Molecular Immunology in Havana for the collaboration research projects we made during her stays in Dr. Federico Garrido laboratory.

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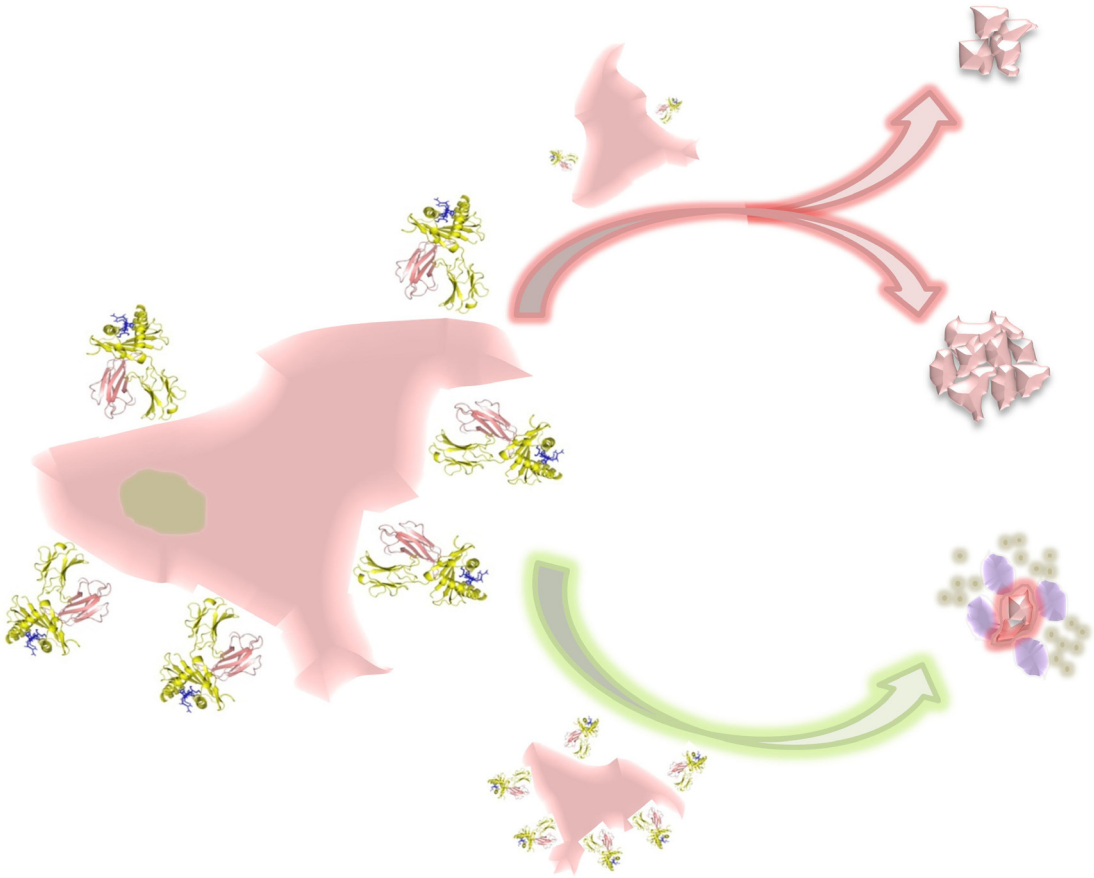
ABBREVIATIONS

AAM: alternatively activated macrophage
ABL: Abelson leukaemia-virus protein
ACT: adoptive cell transfer
Ad5-IFNγ: Ad vector expressing interferon-gamma
APM: antigen processing machinery
APC: antigen-presenting cell
ATM: ataxia telangiectasia mutated
ATP: adenosine 5'-triphosphate
ATR: ATM-and Rad3-related
53BP1: p53 binding protein 1
β_2-m: β_2-microglubulin
BCG: Bacillus Calmette-Guérin
BRC: kinases B-cell receptor
BRCA: breast cancer 1
c-FLIP: cellular FLICE-inhibitory protein
CCL: Chemokine (C-C motif) ligand
CDK: cyclin-dependent kinase
CHK: checkpoint kinase
CKI: CDK inhibitor
CML: chronic myelogenous leukemia
CpG: cytosine guanosine dinucleotide
CRM1: chromosome region maintenance/exportin 1
CTL: cytotoxic T lymphocyte
CTLp: cytotoxic T lymphocytes precursor
CTLA-4: cytotoxic T-lymphocyte antigen-4
CXCL: chemokine (C-X-C motif) ligand
DAC: 2'-deoxy-5-azacytidine
DC: dendritic cell
DISC: death-inducing signaling complex
DNA: deoxyribonucleic acid
DSB: double-strand break
E2f1: E2f transcriptional factor 1
EBV: Epstein-Barr virus
ECM: extracellular matrix
EDTA: ethylenediaminetetraacetic acid
EGFR: epidermal growth factor receptor
ER: endoplasmic reticulum
ERAP-1: endoplasmic reticulum resident aminopeptidase-1
FADD: Fas-associated death domain
FasL: Fas ligand
FDA: Food and Drug Administration
FITC: fluorescein isothiocyanate
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GDP: guanosine-diphosphate
GJIC: gap junctional intercellular communication
GM-CSF: granulocyte-macrophage colony-stimulating factor
H-2: histocompatibility antigen-2
HC: heavy chain

HDACi: histone deacetylase inhibitor
HLA: human leucocyte antigen
HIF-1: hypoxia inducible factor-1
HPV: human papiloma virus
HSP70 or HSPA: 70kDa heat-shock protein
ICAM1: intercellular adhesion molecule
ID1: inhibitor of differentiation 1
IDO: indoleamine 2,3-dioxygenase
IFN: interferon
IFNR: interferon receptor
IGF: immunoglobuline growth factor
Ii: invariant chain (also known as CD74)
IL: interleukin
ILT: immunoglobuline like transcript
iMC: immunosuppressive monocyte
ING4: inhibitor of growth 4
IRF: interferon-regulated factor
ITAM: immunoreceptor tyrosine-based activating motif
ITIM: immunoreceptor tyrosine-based inhibitory motif
JAK: janus kinase
Kpna1: karyopherin importin-α 1
LMP: low molecular mass protein
LNM: lymph node metastasis
LOH: loss of heterozygosity
M-CSF: macrophage colony-stimulating factor
M1: macrophages type 1
M2: macrophages type 2
mAb: monoclonal antibody
MCA: methylcholantrene
MDC1: mediator of DNA damage checkpoint 1
MDSC: Myeloid-derived suppressor cell
MECL1: multicatalytic endopeptidase complex subunit 1 (also known as LMP10)
MetAP2: methionyl aminopeptidase 2
MFI: mean fluorescence intensity
MHC: major histocompatibility complex
MIC: MHC class I chain-related molecule
MRD: minimal residual disease
mRNA: messenger ribonucleic acid
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cell
NKAR: NK activation receptor
NKIR: NK inhibition receptor
ODN: oligodeoxynucleotide
PBS: phosphate buffered saline
PD-1: programmed death-1
PD-L1: programmed death-1 ligand
PDGFR: platelet derived growth factor receptor
PI3K: phosphatidylinositol-3-OH kinase
PM: pulmonary metastasis
PSK: Protein-bound polysaccharide K
PSMA: prostate specific membrane antigen

PTP: protein tyrosine phosphatase
Rb: retinoblastoma protein
RNA: ribonucleic acid
RNS: reactive nitrogen specie
ROS: reactive oxygen specie
RT: radiotherapy
SD: standard deviation
sHLA-G1: soluble HLA-G1
STAT: Signal transducers and activators of transcription
SOCS: suppressors of cytokine signaling
TA: tumor antigen
TAA: tumor-associated antigen
TAM: tumor-associated macrophage
TAP: transporter associated with antigen processing
TBP: TATA-binding protein
TC: tumor cell
TCR: T cell receptor
TGF: transforming growth factor
Th: T-helper
TIL: tumor-infiltrating lymphocyte
TLR: toll-like receptor
TNF: tumor necrosis factor
TPT: Topotecan
TRAILR: TNF-related apoptosis-inducing ligand receptor
Treg: regulatory T cell
VCAM-1: vascular cell adhesion protein 1
VEGF: vascular endothelial growth factor
VEGFR: VEGF receptor



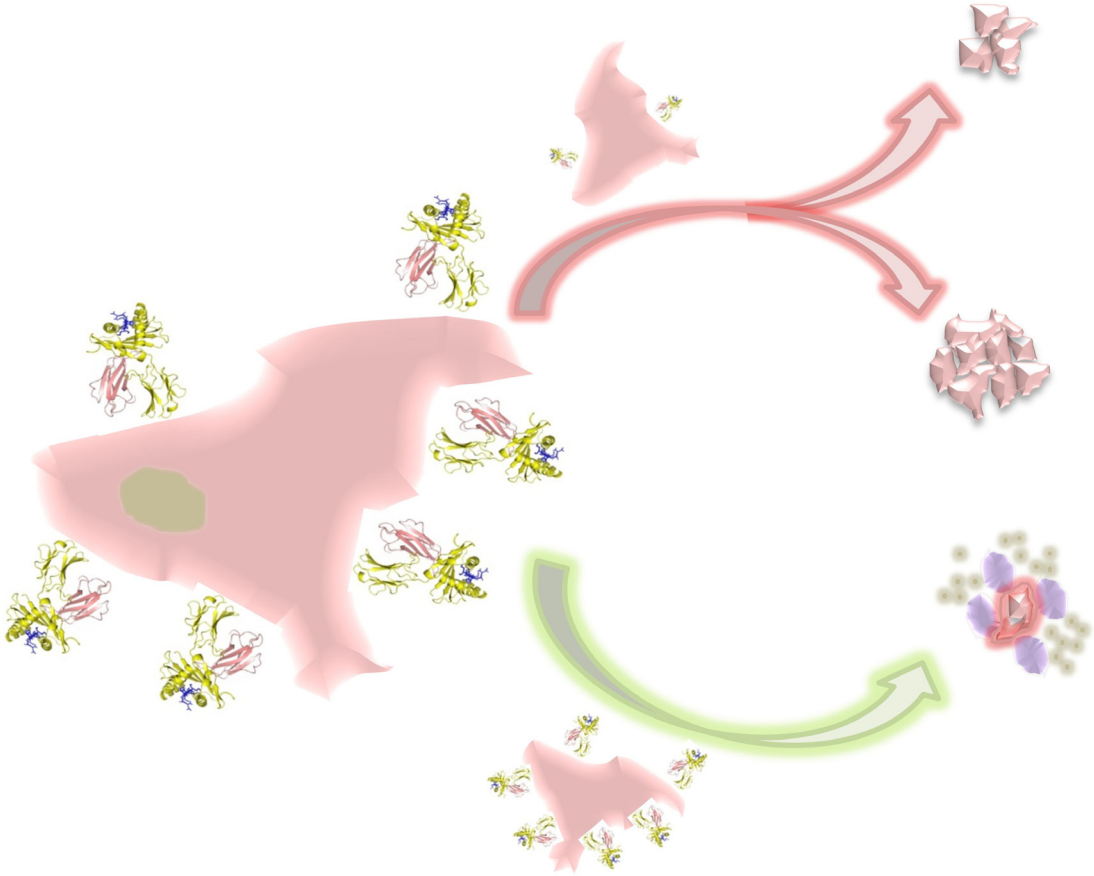
Abstract

Major Histocompability Complex class I (MHC-I) molecules are essential for the development of an effective antitumor immunoresponse. Loss of MHC-I expression is one of the most important immunoescape mechanisms developed by cancer cells to avoid the immune response. Previous observations by our group showed changes in the surface expression of human leukocyte antigen class I (HLA-I) molecules in a human melanoma cell line after growth in immunodeficient mice. The present results show losses in HLA-I surface expression in different human melanoma cell lines after growth in immunodeficient mice. The resulting HLA-I-altered tumor cells showed greater malignancy in comparison to the parental melanoma cell lines. These results are important in translational research for testing new anticancer therapeutic procedures in which oncogenic and immunological components are evaluated. Moreover, this phenomenon showed an association between loss of HLA-I expression and the oncogenicity of tumor cells. Transfection of the HLA-A2 allele in a melanoma cell line promoted a decrease in the proliferation, migratory, and invasive capacities and the tumorigenicity of these cells. HLA-A2-transfected cells revealed the induced expression of cyclin A1, AP-2α, and p21WAF1/CIP1 tumor suppressor genes. HLA-I surface expression of three cell lines derived from a common human melanoma cell line showed an inverse correlation with their oncogenicity. In brief, we suggest that MHC-I molecules may act as tumor suppressor genes, directly suppressing tumor progression.

Our observations demonstrate that the effect of ionizing radiation on tumor cells depends on their MHC-I phenotype. Tumor cells with high expression of MHC-I molecules evidenced a greater induction of MHC-I surface expression and a higher sensitivity to the cytotoxic effects of radiation treatment. MHC radio-induction was caused by an elevated transcription of β2-microglobulin, H-2 class I heavy chains, and some components of the antigen processing machinery. Our findings demonstrate that the surface expression of MHC-I molecules may act as a radio-sensitizing factor, influencing the capacity of ionizing radiation to kill tumor cells by proliferation arrest and inducing the MHC-I surface expression of irradiated tumor cells. These results, together with the important immunological component of radiotherapeutic effects, indicate that the surface expression of MHC-I molecules by tumor cells may exert a major influence on the efficacy of radiotherapy.

Finally, we studied the role of the MHC-I phenotype of tumor cells in the success of antimetastatic immunotherapeutic treatments. We performed preclinical assays to test immunotherapeutic treatments of spontaneous metastases from two GR9 fibrosarcoma subclones with different MHC-I surface expressions. A7, an intensely MHC-I positive subclone with high metastatic capacity, was completely inhibited by the different immunotherapy treatments. A7 tumor progression produced losses in the MHC-I expression of tumor cells and an immunosuppression of the host that was completely restored and further induced by the immunotherapies. These same treatments only achieved a partial inhibition of the metastatic spread of B7 tumor cells, a moderately MHC-I positive subclone with lesser metastatic capacity. B7 tumor growth and metastasis progression produced stronger host immunosuppression, and the treatments were not able to completely restore the immune response. These results suggest that the success of immunotherapy as antimetastatic treatment depends on the MHC class I surface expression of the primary tumor cells.

All these findings show that MHC-I molecules play a dual antitumor role: on one hand, they induce the antitumor immunoresponse; on the other hand, they act as tumor suppressor genes directly inhibiting the intrinsic oncogenic characteristics of tumor cells. Hence, alterations in the surface expression of MHC-I molecules provide advantage for tumor progression: facilitating tumor immunoescape and promoting oncogenicity; and consequently, favoring the appearance of resistance to antitumor therapies.



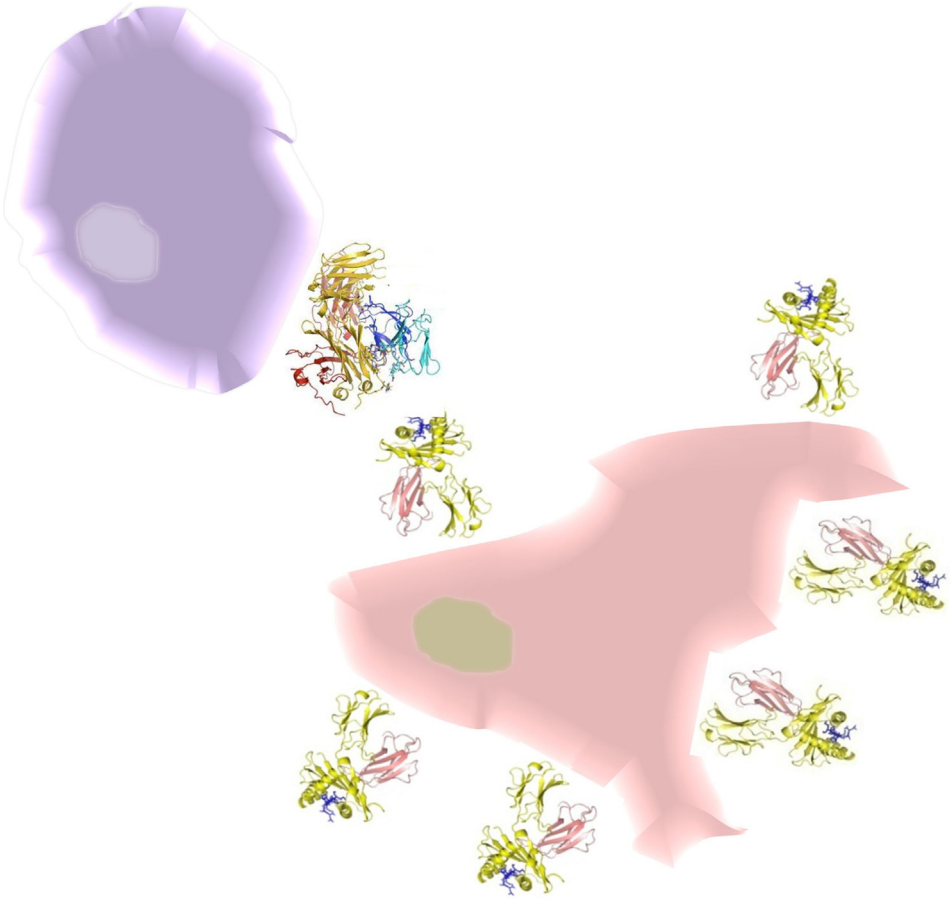
Resumen

El Complejo Mayor de Histocompatibilidad de clase I (MHC-I) juega un importante papel en la respuesta inmunológica antitumoral presentando antígenos tumorales a los linfocitos T citotóxicos. La pérdida de expresión de moléculas MHC-I es el mecanismo más frecuente desarrollado por las células tumorales para evadir la respuesta inmunológica antitumoral. Resultados previos de nuestro grupo mostraron que se originaron pérdidas de la expresión de antígenos leucocitarios humanos de clase I (HLA-I) en una línea celular de melanoma humano después de su crecimiento en ratones inmunodeficientes. Estas pérdidas se originaron en ausencia de una respuesta inmunológica autóloga y de una respuesta por parte del huésped. Nuestros resultados muestran cómo diferentes líneas de melanoma humano pueden sufrir pérdidas en la expresión de moléculas HLA-I durante su crecimiento en ratones inmunodeficientes. Este fenómeno es frecuente y ocurre de manera reproducible. Se obtuvieron nuevas líneas de melanoma, después del crecimiento en ratones inmunodeficientes, con pérdida de expresión de moléculas HLA-I, que presentaban mayor capacidad oncogénica que las líneas parentales de las que fueron obtenidas. Estos resultados son importantes en investigación translacional, para el estudio de nuevos tratamientos anticancerígenos, donde la oncogenicidad e inmunogenicidad de las células tumorales es evaluada. Además estos fenómenos muestran una asociación, pero no evidencian una implicación, entre la pérdida de expresión HLA-I con la oncogenicidad de la célula tumoral. La transfección génica del alelo HLA-A2 en una línea de melanoma que había perdido su expresión conllevó una disminución de la proliferación celular, del crecimiento tumoral in vivo y de las capacidades de migrar e invadir de las células tumorales transfectadas. La transfección de HLA-A2 indujo la expresión de los genes supresores de tumores ciclina A1, AP-2α y p21WAF1/CIP1. La expresión en superficie de moléculas MHC de clase I, en tres líneas de melanoma humano derivadas desde la misma línea de melanoma, se correlacionaba de forma inversa con su capacidad oncogénica. Así podemos deducir que las moléculas MHC-I pueden actuar *per se* como genes supresores de tumores, mostrando una acción directa de supresión de la progresión tumoral. A su importante papel en la inmunogenicidad de las células tumorales, ahora podemos añadir que las moléculas MHC-I desempeñan también directamente un importante papel como genes supresores de tumores.

Además, nuestros resultados muestran que los efectos de la radiación ionizante sobre células tumorales dependen de su expresión en superficie de moléculas MHC-I. Células tumorales con mayor expresión en superficie de moléculas MHC-I al ser radiadas in vitro presentan una mayor inducción de la expresión de estas moléculas y una mayor sensibilidad a los efectos citotóxicos de la radiación. El aumento de la expresión de moléculas MHC-I es debido a una inducción transcripcional coordinada de las cadenas pesadas MHC-I, de la β_2-microglobulina y de varios componentes de la maquinaria de presentación y procesamiento antigénico. Teniendo en cuenta estos resultados y que los efectos antitumorales de la radiación tiene una importante componente inmunológica, podemos sugerir que la expresión en superficie de las moléculas MHC-I de las células tumorales puede tener gran importancia en los efectos antitumorales de la radioterapia.

También se ha querido investigar cómo puede influir la expresión en superficie de moléculas MHC-I del tumor primario y de las metástasis en la eficacia de inmunoterapia como tratamiento antimetastásicos. Se han realizado estudios en el modelo de fibrosarcoma murino GR9, compuesto por diferentes clones tumorales que presentan diferente expresión de MHC-I. Los estudios con el clon A7, con una alta expresión de moléculas MHC-I y alta capacidad metastásica, mostraron que diferentes inmunoterapias lograron erradicar completamente las metástasis, curando a los animales. El crecimiento y desarrollo metastásico de las células A7 provocó una inmunosupresión en los huéspedes, que fue totalmente revertida por los tratamientos de inmunoterapia. En cambio, cuando los mismos ensayos fueron realizados con el clon B7, con una expresión intermedia de moléculas MHC-I y una menor capacidad metastásica, se consiguió una inhibición parcial del proceso metastásico, disminuyendo el número de ratones con metástasis. B7 también produjo la inmunosupresión de los huéspedes, pero esta fue solamente parcialmente revertida por los tratamientos de inmunoterapia. Estos resultados indican que una alta expresión superficial de moléculas MHC-I en el tumor primario puede ser muy importante para el éxito de inmunoterapia como tratamiento antimetastásico.

Los resultados obtenidos muestran que las moléculas MHC-I desempeñan un doble papel en la respuesta antitumoral: por un lado como activadoras de la respuesta inmunológica antitumoral; por otro lado, como genes supresores de tumores inhibiendo directamente la progresión tumoral. Estos hechos hacen que los tumores que pierden expresión de moléculas MHC-I presenten una doble ventaja para el crecimiento del propio tumor: escape de la respuesta inmunológica antitumoral, y escape de los mecanismos de supresión tumoral; en consecuencia presentan una mayor resistencia frente a las terapias antitumorales usadas en clínica.



Introduction

CARCINOGENESIS

Carcinogenesis, the hallmarks of cancer disease

Cancer is a disease in which abnormal cells divide without control and can invade nearby tissues. Cancer cells can also spread to other parts of the body through the blood and lymph systems (NCI dictionary). It is widely assumed that in carcinogenesis process DNA damage and down-regulated DNA repair pathways and cell cycle checkpoints destabilize cell genome allowing DNA disruption, point mutations and chromosome changes making possible that oncogenes gain functions while tumor suppressor genes lose theirs. Although the genetic and molecular mechanisms that involved the acquisition of malignant properties are not clear enough; molecular, biochemical and cellular traits are acquired on the way from normality through pre-malignant states to invasive cancers. Six essential alterations were resolute to dictate malignant growth, the hallmarks of cancer (Fig. 1) (Hanahan and Weinberg 2000):

1) Self-sufficiency in growth signals
2) Insensitivity to growth-inhibitory signals
3) Evasion of programmed cell death
4) Limitless replicative potential
5) Sustained angiogenesis
6) Tissue invasion and metastasis

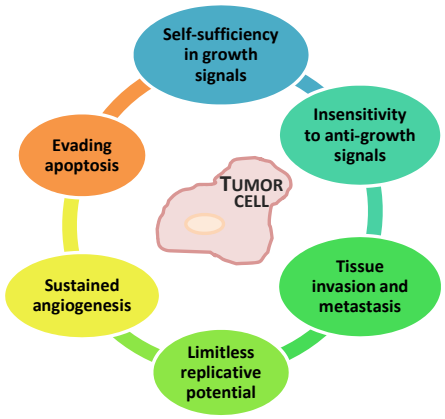


Figure 1. Most cancer cells acquire the same functional capabilities during their development, and characteristic phenotype that identifies malignancy through the six hallmarks of cancer. Adapted from *Hanahan and Weinberg*

Furthermore some disagreements have come up pointing that first five hallmarks are common characteristics with benign tumors, being only the capacity of invade and metastasize what differences malignant tumors (Lazebnik 2010).

Chronic inflammation

Is generally accepted the paradigm that cancer and inflammation maintain a close relation. Indeed, not only inflammation is considered as a risk factor for certain cancers, but as well an inflammatory component is present in the microenvironment of tumors. Thus defining the seventh hallmark of cancer (Colotta et al. 2009):

7) Cancer-related inflammation.

The key orchestrators of this before mentioned inflammatory process are transcriptional factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Signal transducers and activators of transcription (STAT)3), cytokines (interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α), chemokines (Chemokine (C-C motif) ligand (CCL)2, chemokine (C-X-C motif) ligand (CXCL)8) and infiltrating leucocytes (Myeloid-derived suppressor cells (MDSC), regulatory T lymphocytes (Treg), tumor-associated macrophages (TAM)). There exit two different pathways linking inflammation and cancer:

a) Intrinsic pathway: the overexpressed oncogenes guide the progress of inflammatory circuits in the tumor microenvironment.
b) Extrinsic pathway: Chronic inflammation due to viral or bacterial infections, autoimmune diseases or with uncertain origin can trigger genetic destabilization (microsatellite or chromosomal instability) inducing tumor development.

Inflammatory process contributes to the accumulation of random genetics alterations, accelerating the expansion of genomically heterogeneous population of cancer cells.

Stem-cell carcinogenesis theory

Despite the diversity of the cells within a tumor, carcinogenesis responds to the clonal theory that stated that cancer cells are clonally derived from a single initiated cell. Accordingly to dedifferentiated cell hypotheses, carcinogenic process requires the immortalization of a normal cell before neoplasticaly transformation. In normal tissue conditions immortal stem-cells stay in protect niches and maintain a low proliferation by negative growth factors secreted by themselves, terminally differentiated daughters or stroma cells. By asymmetrically division a stem-cell produce a new stem-cell and a progenitor or transit cell. Again, growth is going to be controlled, in this case by gap junctional intercellular communication (GJIC). By reestablishing the telomerase activity, these progenitor cells finally give rise to specialized terminally differentiated cells. Tumor promoters could inhibit growth control factors leading to initiated stem or progenitor cells. This stage could be reversed by connexin genes or restoring GJIC, if resistance to growth controls remain permanent cell will entered in the progression phase (Fig. 2).

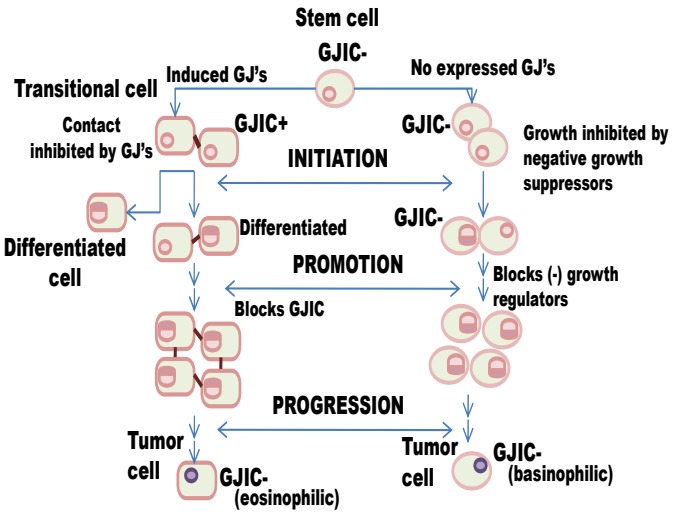


Figure 2. Diagram indicating how both stem cells and very early transit cells can be reversely initiated through lacking the expression of connexins or functional GJICs as well as inhibiting negative growth signals. If these initiated cells are continuously expose to proliferative signals, they promote, accumulating mutations that activate oncogenes or inactivate tumor suppressor genes. Finally cells arise in uncontrolled progression leading to cancer disease. Adapted from *Trosko et al. 2004*.

The stem-cell carcinogenesis theory discovers two more hallmarks of malignant phenotype (Trosko et al. 2004):

8) Prevention of the mortalization of stem-cells
9) Abrogation of GJIC

Carcinogenesis is a multimechanism and multistage process structured in initiation, promotion and progression phases (Fig. 3) (Trosko et al. 2004).

a) In the initiation stage a single cell is irreversible blocked from terminal differentiation.

b) During the promotion stage, the combination of growth stimulation and apoptosis inhibition lead to a potentially reversible or interruptible clonal expansion of the initiated cells.

c) Finally the progression phase will be achieved with the accruing of several mutations and epigenetic alterations that provide cells the phenotypes of invasiveness and metastasis.

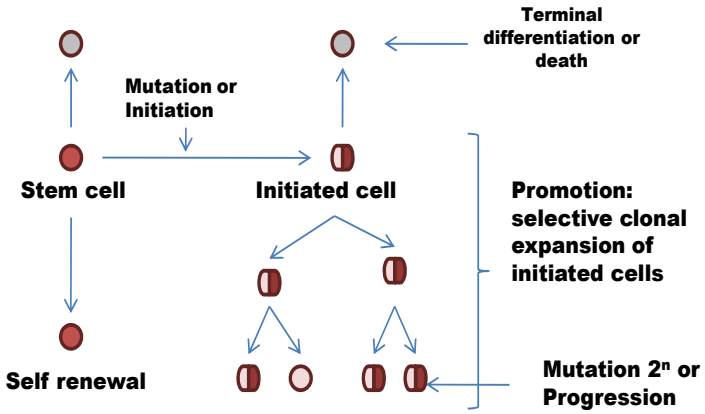


Figure 3. Stem cells could initiate by blocking terminal differentiation or cell death. The combination of growth stimulation and apoptosis inhibition lead the promotion of clonal expansion. Progression will be driven by several mutations that provide cells invasiveness and metastasis phenotypes. Adapted from *Trosko et al. 2004*

Immunological aspects of carcinogenesis

It has become increasingly clear that the immune system plays an important role in the control and destruction of cancer what forces to cancers cells to develop skills to evade immune mechanisms. Thus three immune hallmarks have been defined (Fig. 4) (Cavallo et al. 2011). The before mentioned chronically inflamed microenvironment (7) and:

10) Ability to evade immune recognition. Immunological system achieves a function of detection and destruction of cancer cells. Immune cells are activated by recognizing and interacting with specific markers in malignant cells. So cancer cells elaborate mechanisms of defense against the immunological response.

11) Ability to suppress immune reactivity. With the same aim before mentioned, to avoid immune response, tumors not only growth hidden from immune recognition, but cancer cells acquire the ability to release factors that actively corrupt the immunological anti-tumor reaction.

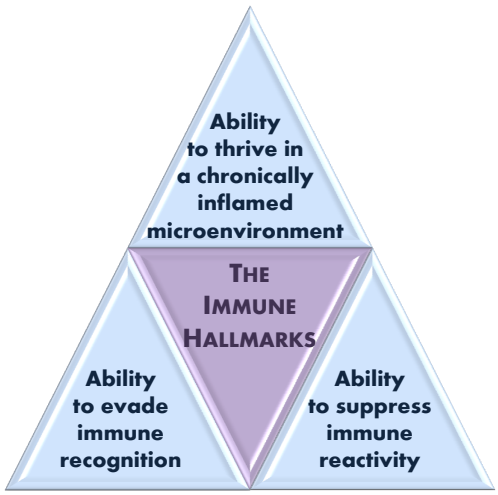


Figure 4. Immune hallmarks: three capabilities and acquisition of the genetic changes required are constant and essential features of natural and experimental cancers.

METASTATIC PROCESS

Metastasis is a multiphase and complex process, which occurs through a series of sequential steps that include the invasion of adjacent tissues, intravasation, transport through the circulatory system, and arrest at a secondary site, extravasation and growth in a secondary organ. Metastases are the cause of 90% of human cancer deaths (Mehlen and Puisieux 2006). Despite its clinical importance, the genetic and biochemical determinants of metastasis are not completely understood, although important advances had been made. Understanding the many molecular players and processes involved in metastasis could lead to effective, targeted approaches to prevent and treat cancer metastasis (Chiang and Massagué 2008).

An overlook

In 1991 Sean E. Egan and colleges reviewed the role of oncogenic transformation with metastatic conversion. They proposed that the instability of malignant cells facilitated the tumor progression evolution with activating mutations in oncogenes and inactivating mutations or loss of tumor-suppressor genes (i.e. mutations of K-ras, Rb, p53 or HER/neu growth factor receptor or myc genes). These alterations contribute in the progression of tumors at different stages of tumor evolution. One specific genetic lesion may be able to promote metastatic phenotype; however, events preceding this ultimate change are important to the maintenance and regulation of metastatic dissemination (Egan et al. 1991).

In the same year, Garth L. Nicolson highlighted the fact that colonization properties depend on a complexity amount of specific characteristics of multiple tumor cell, host cell and stromal molecules that are differentially expressed by particular tumor and organ cells and by the organ extracellular matrix (Nicolson 1991). According to Paget's "seed" versus "soil" hypothesis, noted compatibilities between disseminated cancer cells (the seed) and specific distant organs (the soil) have long influenced in the metastatic process (Fig. 5) (Paget 1889).

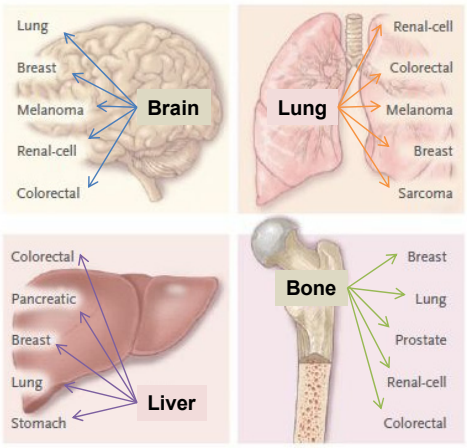


Figure 5. There are characteristic target organs for metastasizing in dependence of the type of cancer. Adapted from *Chiang and Massagué 2008.*

Several cancer cells are released into the circulation, but a tiny minority colonizes a distant organ. Metastasis cascade required discernible biological discrete steps: loss of cellular adhesion, increased invasiveness and motility, entry and survival in the circulation, exit in a new tissue and established and colonized this distant site (Fig. 6) (Chambers et al. 2002, Fidler 2003). The complexity of this process goes further than an accumulation of mutations in cancer cells. Multiple layers mechanisms challenge metastatic progression; these barriers can

be cell intrinsic, but also cells extrinsic barriers mean important limits of cancer cell progression. Although many of metastasis steps remain unknown, deeper understanding of molecular and cellular happens involving metastasis has been achieved (Gupta and Massagué 2006, Chiang and Massagué 2008).

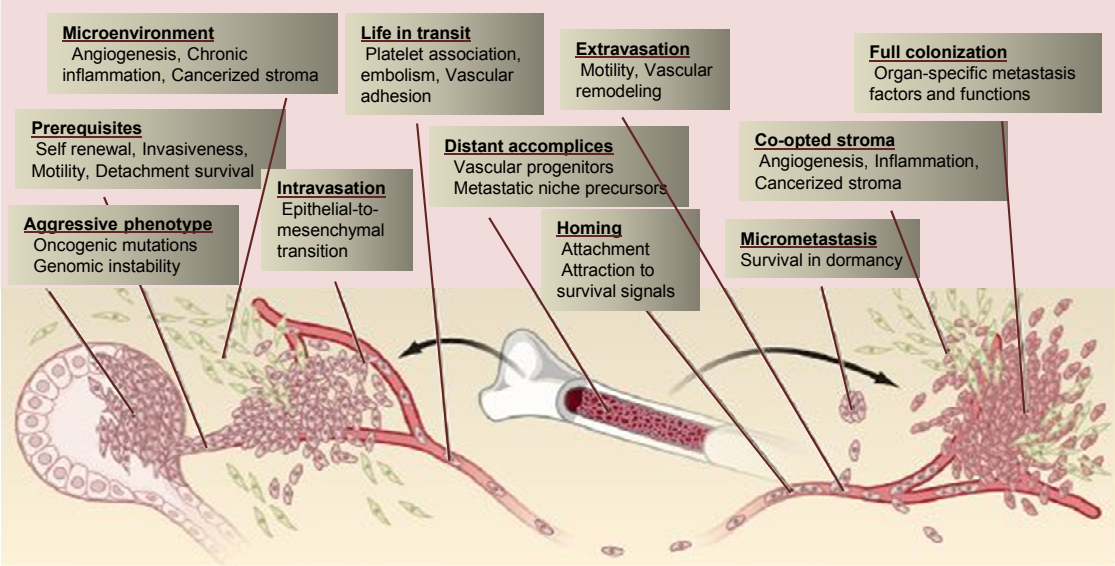


Figure 6. Cells from the primary tumor need to acquire several traits that drive the proceeds of metastasizing. The acquisition of these factors should follow an order to implement the single steps as part of a continuous biological sequence. The specific steps that are rate limiting for the metastatic cascade could vary between different types of tumors. Adapted from *Gupta and Massagué 2006.*

Cellular heterogeneity and selective pressures

Primary tumors consist in very heterogeneous populations of cells, amply supplied by the intrinsic instability of cancer genomes. DNA integrity can be compromised by aberrant cell-cycle progression, telomeric crisis, inactivation of DNA repair genes, and altered epigenetic control mechanisms. These alterations allow the apparition of genetic mutations necessary to acquire metastatic capacity. Cell intrinsic and extrinsic pressure barriers will then select those cells with an aggressive phenotype. Cancer cells need to evolution to overcome genotoxic stress induced by oncogenes, the expression of growth inhibitory, apoptotic and senescent pathways and telomere attrition. Moreover there are microenvironmental factors that limit tumor progression, including extracellular matrix components, basement membranes, reactive oxygen species, limited availability of nutrients and oxygen and the attack of the immune system. Tumors also suffer physical pressures from the well-organized tissues as tensional forces (Fig. 7). For example, hypoxia is a strong selective pressure that benefits the progression of cancer cells developing resistance by stabilization of the hypoxia inducible factor-1 (HIF-1) transcriptional complex, promoting angiogenesis, anaerobic metabolism, cell survival and invasion (Harris 2002). The lysyl oxidase has been implicated as an HIF-1 target in the promotion of breast metastasis under hypoxia conditions trough changes in collagen fibrils maturation and focal adhesion kinases activity (Erler et al. 2006). Inhibition of cell death, apart from its function in primary tumour development, is a crucial characteristic of metastatic

cancer cells, thus survival capabilities of cancer cells could determine the organ-specific lodging of metastases, and moreover, metastatic cancer cells could be expected to display increased chemoresistance (Mehlen and Puisieux 2006).

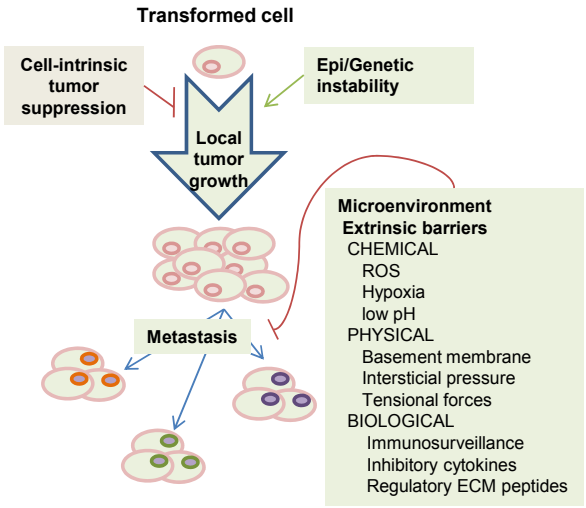


Figure 7. Promotion of cancer cells arise by corrupting intrinsic mechanisms that limit aberrant proliferation. But also the microenvironment provides extrinsic barriers to preserve normal tissue structure. These barriers can be broadly classified in chemical, biological and physical. They act as selective factors for cancer cells capable to overcome them. The more aggressive cells will resist these pressures dominating the cancer population and priming metastatic evolution. Adapted from *Gupta and Massagué 2006.*

Invasion and motility

Invasive properties are acquired by cancer cells by diminishing intercellular adhesiveness. Loss of E-cadherin disrupts intercellular adhesion and leads to early invasion and metastasis (Perl et al. 1998) and can be repressed as part of epithelial-to-mesenchymal transition, a process whereby epithelial cells acquire a mesenchymal progenitor-cell phenotype, enabling detachment and reorganization of epithelial-cells sheets during embryonic development, as well as tumor invasion (Thiery 2002).

Migration is a fundamental capacity to metastasis that involves the motility of cancer cells from one site to another. Integrins lead to cell movement by contacts with extracellular-matrix components such as fibronectin, collagen and laminin. They also attach with the actin cytoskeleton by interacting with focal adhesion kinases and SRC family kinases. Through calcium-dependent guanosine triphosphatases extracellular-matrix signals changes cytoskeleton to form filopodia and lamellipodia, structures that are important to migratory movement. Proteolytically disruption of basement membrane also facilitates tumor invasion and metastasis. In addition proteases, such as the matrix metalloproteinases generate cleaved peptides which could modulate migration, cancer-cell proliferation, cancer-cell survival and tumor angiogenesis (Egeblad and Werb 2002).

Angiogenic switch, intravasation and surviving in the transit

Angiogenic switch concept means the acquisition of an angiogenic phenotype that induces outgrowth of preexisting vasculature as the generation of neo-vasculature (Hanahan and Folkman 1996). Once cancer cells become motile, they would be attracted to blood vessels due to chemoattractive gradients, leading to intravasation. Then tumor cells have to survive several stresses as hemodynamic shear forces or immuno-mediated killing. Co-opting platelets and using them as shields, forming tumor emboli give greater metastatic potential to tumor cells (Nash et al. 2002).

Extravasation, tissue niche and dormancy

Once malignant cells have invaded and endured the circulation, they must escape from endothelial vasculature into a target tissue in a process called extravasation. The cytoskeletal anchoring proteins facilitate this escape. There are also signals from metastatic cells that induce changes in endothelial vasculature as the expression of vascular endothelial growth factor (VEGF) that activates Src family kinases in endothelial cells disrupting cell junctions (Criscuoli et al. 2005). Disseminated cells must productively interact with the new environment in order to extract growth and survival advantages. Because different organs may impose distinct requirements and the developmental history of a cell also predispose it to activate the expression of specific metastasis-promoting mechanisms, different primary tumor cells could be predisposed to colonized specific target tissues (Fig. 8). Metastatic initiation involves the mobilization of hematopoietic progenitors from bone marrow into targets sites for metastatic colonization in response to hormonal factors produced by the primary tumor. These hematopoietic cells home and precondition sites prior to dissemination generating a viable niche for cancer cells and contributing to organ-specific metastatic behavior (Fig. 8).

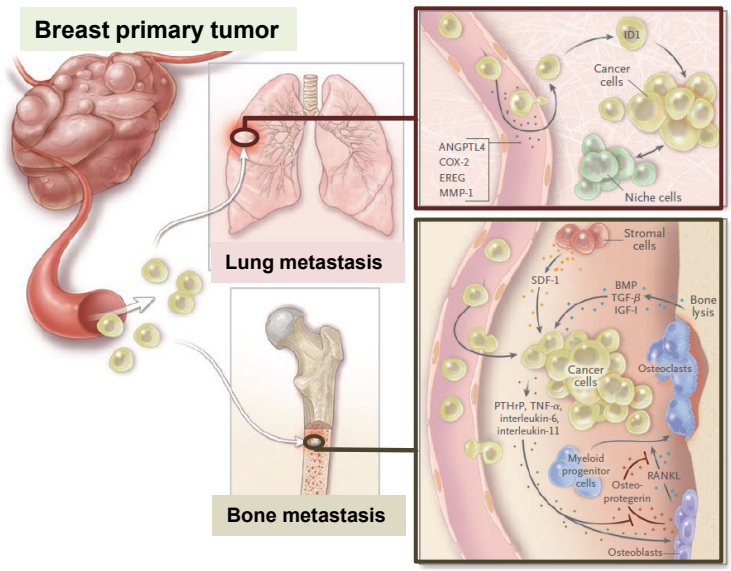


Figure 8. Breast cancer cells share specific traits that equipped them to preferentially invade lungs and bones. Whereas breast cancer cells express factors that help altering the integrity of lung microcapillary epithelia, they easily infiltrate bone marrow, due to its fenestrate vasculature. Extravasated cells are promoted by activator factors specific of each organ that activate the expression of pro-metastatic genes leading the colonization. Adapted from *Chiang and Massagué 2008.*

Extravasation *per se* is not enough to guarantee the colonization of the new site. In several carcinomas, cancer cells can be detected in the bone marrow before develop metastases (Braun et al. 2005). This minimal residual disease (MRD) predicts disease recurrence and less survival, and it is an indicator of systemic disease (Pantel and Brakenhoff 2004). This cancer cells could be dormant for years, due to the inability to induce angiogenesis, immune-surveillance tightly control or because MRD cell population consist primarily in quiescent cells – staying in a G0 cell-cycle arrest state –. Cancer cells could disseminate from a

tumor very early in its life, thus exposing to selective pressures after the departure. Such cancer cells could be detected in a dormant state in the host bone marrow. In patients with metastatic disease, it was found that metastatic cells were more genetically disparate of the matched in primary tumors than bone marrow-derived cancer cells present in those patients (Klein et al. 2002, Schimdt-Kittler et al. 2003). In fact the fusion between cancer cells with bone marrow-derived cells is under intensive study. It was proposed as the theory that explains the acquisition of metastatic phenotype within primary tumor cells (Goldenberg 1968, Mekler 1971).

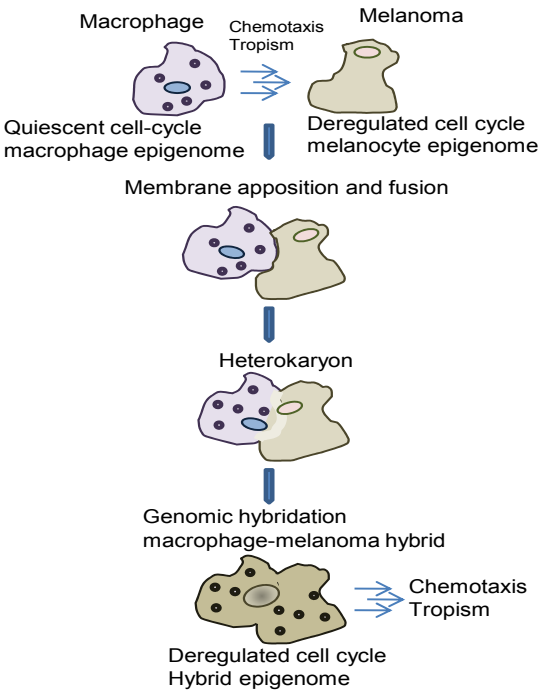


Figure 9. Melanoma cells are capable to fuse with melanoma-associated macrophages forming a heterokaryon – with two nucleic separate in the cytoplasm – that after genetic hybridization lead to macrophage-melanoma hybrids that share genomes of both parental lines, exhibiting myeloid chemotaxis and tropism, characteristic of metastatic cells. Adapted from *Pawelek and Chakraborty 2008.*

Fusion between cancer cells with bone marrow-derived cells represents a non-mutational mechanism that could explain aberrant gene expression patterns associated to metastatic cells. Moreover the resulting cells will co-express both fusion partner genomes, carrying healthy myeloid capacities that are associated with tumor progression as angiogenesis, motility, chemotaxis and tropism, immune signaling, matrix degradation and remodeling, responses to hypoxia, and multi-drug resistance to chemotherapy (Fig. 9) (Pawelek 2005, Pawelek and Chakraborty 2008). Even so, some of the genes that promote metastasis are coexpressed within subsets of primary tumors. One possible explanation is that the genes that induce metastasis were selected in the primary tumor for selectable growth advantage. In the other hand intrinsic colonizing functions of metastatic cells may allow them to constantly reseed the primary tumor.

Integrated model

Metastatic evolution of tumors is orchestrated by a complex of random genetic and epigenetic alterations in cancer cells in combination with a plastic and responsive microenvironment. The genes needed at individual steps along metastatic process are being identified. These genes are classified in three main categories: initiation, progression and virulence (Fig. 10). The genes necessary for certain functions such as vascular remodeling are present in both the primary tumor as well as the metastasis; these genes are metastasis-progression genes and could be enriched in primary tumors. These genes could give cancer cells particular advantages and influenced in metastatic destination. For example, breast tumor cells could express ID1 (inhibitor of differentiation 1), the sole transcriptional regulator in lung-metastatic signature. Suppression of ID1 expression inhibits the initiation of mammary tumors and metastases in the lungs (Gupta et al. 2007). Mapping altered expression genes through metastatic process help to built gene-expression signatures of cancer cells, related with specific cancer-cell capabilities and the prediction of clinical outcome. This gene framework is useful for the design of treatments that target metastasis-specific genes.

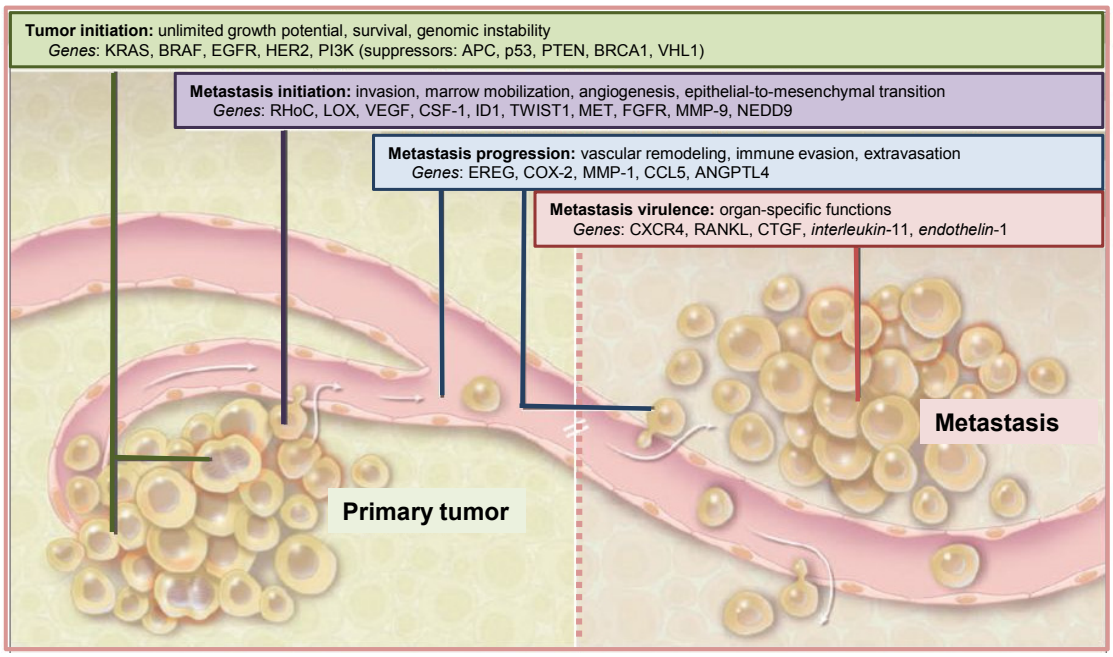


Figure 10. Along the tumor progression cells acquire specific functions altering the expression of different genes. Firstly, the tumor initiation is provided by oncogenic mutations and inactivation of tumor-supressor genes. Associated with the metastatic initiation genes that promote invasion, hypoxia resistance and tumor growth are overexpressed. Genes that provide cancer cells to local advantage in the target organs are essential during the promotion. Finally cells develop the functions associated with metastatic virulence that ensure adaptation and takeover a specific organ microenvironment. Adapted from *Chiang and Massagué 2008.*

CELL CYCLE

In an adult human body, the majority of cells are not dividing; they reside in an "out-of-cycle" state. Only a minority are actively proliferating. These cycling cells are located in the stem-transit amplifying compartments of cell-renewing tissues such as epithelia and bone marrow (Potten and Loeffler 1990). By contrast, most functional cells have withdrawn into a reversible quiescent state (G0), as the glial cells, thyroid follicular cells or hepatocytes; or have irreversibly withdrawn into a terminal differentiated state, as the neurons myocytes or surface colonic epithelial cells of the skin or the mucosa (Hall and Watt 1989).

Cell cycle phases

Cell cycle has four sequential phases (Fig. 11). Most important phases are S phase, when the DNA is replicated; and M phase, when the cell divides into two daughter cells. Separating S and M phases are two gap phases, which allow for the repair of possible DNA damage and replication errors. G1 phase incorporates after mitosis (M phase). During G1 period, many signals intervene to influence cell division and the deployment of cells developmental programs, and it is when is decided whether to enter S phase or pause, leading to self-renewing, differentiation or die (Massagué 2004). G2 is the gap after S phase, when cell prepares for entry in mitosis (Fig. 11). When there is a high cell density or mitogen deprivation, cells reversibly entry in G0; or they irreversibly withdraw from the cell cycle into terminally differentiated or senescent out-of-cycle states (Williams and Stoeber 2007).

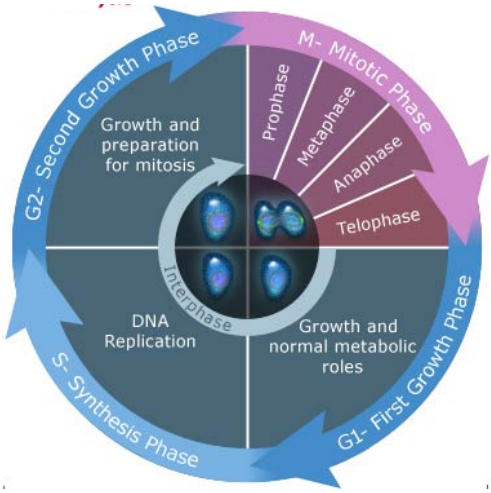


Figure 11. The cell cycle is a sequence of four phases that ensure the correct replication of the DNA material and organelles of a cell before their separation in two identical cells.

Drivers of the cells cycle

Progression through the cell cycle is driven by the cyclin-dependent kinase (CDK) family of serine/threonine kinases, which activity requires binding to their regulatory partners the cyclins. In humans CDK and cyclin genes are encoded in multiple loci (13 and 25 loci, respectively). But only some subsets of CDK-cyclin complexes are directly involved in cell cycle. CDK family is formed by the interphase CDKs (CDK2, CDK4 and CDK6) and a mitotic CDK (CDK1). Cyclins are synthesized and destroyed at specific times during the cell cycle, thus regulating the CDKs activity in a timely manner. Cyclin family is formed by 10 components that belong to four main different classes (A-, B-, D- and E-types cyclins) (Malumbres and Barbacid 2009).

Specific CDK-cyclin complexes drive the interphase in a sequential and orderly fashion. First mitogenic signals are sensed by expression of the D-type cyclins (D1, D2 and D3) that bind and activate CDK4 and CDK6 during G1 phase to prepare the cell for the DNA synthesis

(Fig.12). These complexes partially inhibit the pocket proteins (RB, RBL1 and RBL2), to allow the expression of E-type cyclins (E1 and E2) which bind to CDK2. Cyclin E-CDK2 complexes further phosphorylate, completely inhibiting the pocket proteins. Cyclin E availability is tightly controlled and limited during early stages of DNA synthesis and CDK2-cyclin E complexes are essential to drive the G1/S transition. Subsequently CDK2 is activated by cyclin A2, during the period G2, to drive the transition from S phase to mitosis, in the late stages of DNA replication. Finally, CDK1 through A-type cyclin activation, facilitate the onset of mitosis at the end of interphase. When the nuclear envelope is breakdown, A-type cyclins are degraded, allowing B-type cyclins to complex with CDK1. CDK1-cyclin B complexes are the responsible to drive cells through mitosis (Fig. 12) (Malumbres and Barbacid 2005).

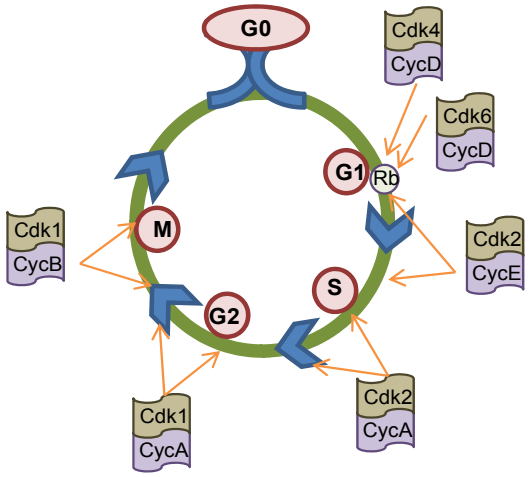


Figure 12. CDK-Cyclin complexes have been proposed as the drivers of the cell cycle along the different phases. Cdk4–CyclinD, Cdk6–CyclinD and Cdk3–CyclinC complexes regulate the G0–G1 transition (in quiescent cells) and the early phases of G1 (in proliferating cells) by phosphorylating the retinoblastoma protein (Rb). Cdk2–CyclinE complex completes the phosphorylation of Rb leading the G1–S transition. Cdk2 later associates with Cyclin A during progression through S phase. Cdk1 participates in the S–G2 and G2–M transitions by sequential binding to Cyclin A and Cyclin B. Adapted from *Malumbres and Barbacid 2005.*

CDK activity is regulated by two families of inhibitors: INK4 proteins, including INK4A (p16), INK4B (p15), INK4C (p18) and INK4D (p19); and the Cip and Kip family composed by p21 (Cip1), p27 (Kip1) and p57. These cell-cycle inhibitors have been shown to block proliferation of adult stem cells in different tissues. CDK inhibitors (CKIs) may be the responsible in maintenance of the quiescent state of stem cell population (Kippin et al. 2005, Walkley et al. 2005). This is important to the homeostasis within adult tissues in which a quiescent stem cell pool intermittently generates daughter progenitor cells with high proliferative capacity. While CDK down-regulation may result in defective homeostasis of specific tissues, hyperactivation of CDKs leads to unscheduled stem or progenitor cells division favoring tumor development.

Cell-cycle checkpoints

The term “cell-cycle checkpoint” refers to mechanisms by which cells actively halt progression of cell cycle until they can ensure that earlier processes, such as DNA replication or mitosis are complete (Kastan and Bartek 2004). Different stresses produce DNA-damage that acts as the signal initiation of specialized pathways that ensure DNA-repair. DNA can be damaged in a variety of ways. First, some chemical or physical agents can break the phosphodiester bonds in the backbone of the DNA helix. If two breaks are close to each other, and in opposite DNA strands, a double-strand break (DSB) is formed in the DNA molecule. Second, bifunctional alkylating chemicals can cause intra-strand or inter-strand crosslinks. And third, the inhibition of DNA topoisomerase activity could lead to single or DSBs.

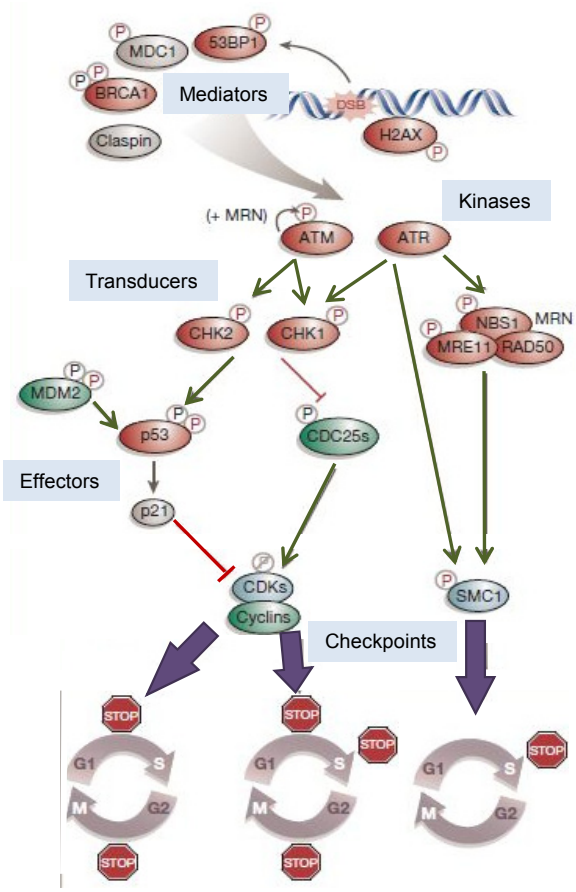


Figure 13. Cell-cycle checkpoints are orchestrators of DNA damage responses. In the case of DSBs, the proximal ATM and ATR kinases phosphorylate through CHK2 and CHK1 transducing kinases their final substrates, p53, CDC25s or SMC1 proteins to induce cell-cycle arrests. Mediators as MRCA1, MDC1, 53BP1 or Claspin modulate the signaling network in a spatio-temporal arrangement. Cell-cycle checkpoint network affects DNA repair (BRCA1 and p53), transcription, chromatin assembly and cell death (p53). Adapted from *Kastan and Barket 2004.*

The first steps in the activation of signal transduction pathways after DNA-damage are PI3K (phosphatidyl-inositol-3-OH kinase)-like kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM-and Rad3-related). ATM kinase is primarily activated by DNA damage, while ATR kinase has a critical function in cellular response to the arrest of DNA replication forks (DNA structures form during replication) (Abraham 2001).

To efficiently spread and orchestrate the global response to DNA damage, checkpoint kinases cooperate with the checkpoint mediators and the transducers kinases CHK1 and CHK2, which are targeted by ATM and ATR, respectively (Bartek and Lukas 2003). Checkpoint mediators modulate ATM/ATR activity, facilitate the interaction between them and their substrates (in some cases the same mediators), and mediate spatio-temporal assembly of multiprotein complexes in the chromatin regions surrounding the sites of DNA damage. ATM mediators include MDC1 (mediator of DNA damage checkpoint 1), 53BP1 (p53 binding protein 1) and BRCA (breast cancer 1). ATR has one of this checkpoint mediator factors, the claspin protein. Finally, regulatory phosphorylation of the downstream checkpoint targets, the effector proteins that can trigger cell-cycle arrest, DNA-repair and cell-death machineries. Effector proteins involved the CDK inhibitors, as p16 or p21, the complex p53/Mdm2 or the transcriptional complex Rb/E2F, which can reversibly halt the cell cycle progression (Fig. 13).

Along the cell cycle phases, there are specific checkpoint networks that ensure the correct function during the phase, otherwise promoting an arrest in response to DNA damage signals (Fig. 13) (Kastan and Bartek 2004).

Into the G1 phase, dominant checkpoint response to DNA damage is the ATM(ATR)/CHK2(CHK1)-p53/MDM2-p21 pathway. Phosphorylation of p53 and Mdm2 (MDM2 binds p53 and ensures a rapid p53 turnover) contribute to the accumulation of p53, inducing transcription of the p21 CKI, that blockade cyclin E/CDK2, preserving the Rb/E2F active, in its growth suppressing mode, thereby causing G1 sustained blockade.

S phase checkpoint can be mediated by two pathways. Through ATR/CHK1 inhibiting phosphorylation of the CDC25A phosphatases, an activator of cyclin E(A)/CDK2 complex, arresting the G1/S transition. On the other hand, once in the *intra-S-phase*, ATM phosphorylates NBS1 and the cohesion protein SMC1 leading a non-well understood pathway, which blockades cell cycle in the S phase.

Finally G2 checkpoint prevent from initiating mitosis when DNA have not been appropriately replicated. In this cases ATM(ATR)/CHK2(CHK1) or p28-kinase mediated subcellular sequestration, degradation or inhibition of the CDC25 phosphatases that normally activate CDK1 at G2/M boundary. p53 and BRCA pathways are also implicated through CHK-mediated phosphorylation in the checkpoints of S and G2 phases.

Targets of cell cycle checkpoints, as Rb1, BCRA and p53, are genes which mutations are strongly relate with human cancer (Kastan and Bartek 2004).

Cell cycle and cancer

Tumor cells accumulate mutations that result in constitutive mitogenic signaling and defective to anti-mitogenic signals producing an unscheduled proliferation. Cells have developed elegant but not perfect mechanisms to repair DNA damage. The attack of exogenous and endogenous genotoxic agents induce diverse alterations in the DNA molecule activating cell-cycle checkpoints signaling pathways that leads to cell cycle arrest. If repair is unsuccessful, cells may enter senescence or undergo apoptosis. Contrary, accumulation of DNA alterations may result in genomic instability leading to cell transformation and oncogenesis. Furthermore if mutations occur once the genetic material is duplicated, affecting chromosome segregation may provoke unequal inheritance and chromosomal instability, finally facilitating tumor progression by accumulating numerical chromosomal aberrations (Fig. 14) (Kastan and Bartek 2004).

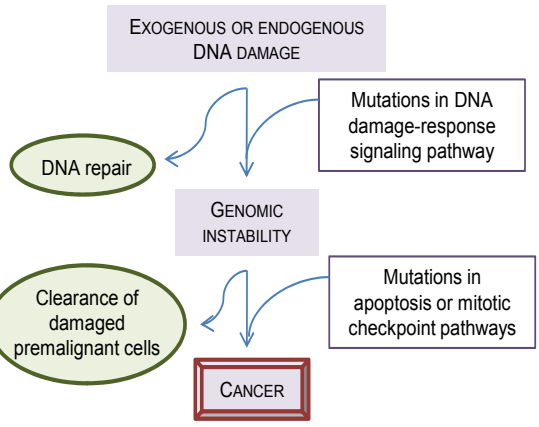


Figure 14. DNA damage occurs in genes involved in cell-cycle checkpoint or DNA-repair pathways induce genomic instability and mutations that generate resistance to cell-cycle arrest and to programmed cell death, promoting oncogenesis. Adapted from *Kastan and Barket 2004.*

Mutations that induces cell-cycle progression

Mutations in CDK and their regulators may contribute in tumor progression. Alterations in CDK4 and CDK6 have been implicated in a wide variety of tumors (Ortega et al. 2002). Although no mutations in CDK2 have been found, E-type cyclins are often overexpressed in human tumors; and the expression of p21 and p27 is normally silenced during tumor development, in some cases accompanied by a deregulation of CDK1 activity (Malumbres and Barbacid 2001, Martin et al. 2005).

Mutations that avoid cell cycle arrest

Mutations in apoptosis, DNA-damage responses or in mitotic-checkpoint pathways, allow the survival or the growth of cells with genomic abnormalities, thereby enhancing the chance of malignant transformation. Loss of ATM predisposes to the development of lymphoma in human and mice and to other malignancies (Shiloh 2003). Mice that lack the expression of 53BP1 show cell-cycle defects and cancer predisposition (Ward et al. 2003). In women the inheritance of a single mutated BCRA1 or BCRA2 increase the incidence of breast and ovarian cancers (King et al. 2003).

Mutations that induce apoptosis resistance

Cell proliferation and cell death are opposed cellular fates with overlaps between the mechanistic pathways. Both processes are coupled at various levels through individual molecular players that are often targets for oncogenic mutations. Both, apoptosis and senescence are intrinsic tumor suppressor mechanisms that can be spin off by oncogenic mutations (Lowe et al. 2004). Apoptosis could be triggered by two main distinct programs, the intrinsic program responds to signals of survival factors, cell stress and injury (Cory et al. 2003, Danial and Korsmeyer 2004). The mitochondrion is the central conduit of this pathway. The balance between the pro-apoptotic Bax/Bak proteins and the anti-apoptotic Bcl2/BclXL change mitochondrial permeability, and regulate the release of pro-apoptotic effectors as cytochrome-*c* that acts together with the cell-death adaptor Apaf-1 to trigger the activation of the cascade caspase-9, caspase-3 and caspase-7. Activated caspases cleave proteins important for cell and genome integrity, orchestrating the orderly death and engulfment of the cell (Green and Kroemer 2004).

p53 is a transcription factor that establishes programs for apoptosis, senescence, and repair in response to DNA damage, hypoxia, and nutrient deprivation (Vogelstein, Lane and Levine 2000). Transcriptional targets for p53 in promoting apoptosis include various pro-apoptotic Bcl2 members, including puma, noxa, bid and bax (Evan et al. 1992), as well as components of death-receptor signalling (for example, DR5, Fas/CD95), the apoptotic-effector machinery (for example, caspase-6, Apaf-1, PIDD). p53 is also induced by many oncogenes, including E1A, Myc and E2F. Moreover, p53 inactivation severely compromises oncogene-induced apoptosis in many instances. Inactivation of p53 potently cooperates with diverse oncogenes to promote transformation *in vitro* and tumorigenesis *in vivo*.

An especially important mediator of oncogene-dependent activation of p53 is the tumour suppressor ARF (Sherr 2001). ARF is an autonomous sensor for aberrant proliferative signalling that discerns when there is an elevated mitogenic signalling. It is transcriptionally up-regulated in response to many oncogenes (Lowe and Sherr 2003). ARF is not expressed in

normal proliferating tissues, but is rapidly induced in response to aberrant signals such as activated Myc (Zindy et al. 2003).

E2F can induce several downstream effectors of the apoptotic machinery, including various caspases (Nahle et al. 2002). It has pleiotropic effects on the expression of pro- and anti-apoptotic members of the Bcl2 family (Eischen et al. 2001, Croxton et al. 2002). Common downstream E2F effectors are also part of the apoptotic pathways activated by deregulated Myc expression or inactivated tumour suppressing retinoblastoma protein.

Many survival factors prevent apoptosis by triggering receptor tyrosine kinases that ultimately signal through Ras and the PI(3)K signalling cascade (Vivanco and Sawyers 2002). A key mediator of PI(3)K signalling is the Akt/PKB kinase, which phosphorylates multiple effectors leading to pleiotropic changes in proliferation, metabolism, cell growth and survival. Akt promotes survival by coordinating programs that directly inhibit apoptotic effectors, suppress transcription of pro-apoptotic genes, and modulate the translation of cell-death regulatory messenger RNAs78. Additionally, Akt survival signalling is potentiated by its effects on cellular bioenergetics, and its modulation of the mTOR pathway, which controls the cell response to nutrients (Plas et al. 2002). Some cytokines also trigger PI(3)K independent activation of STATs and NF-κB, transcription factors that promote cell survival by modulating the transcription of the Bcl2-related proteins and other anti-apoptotic genes (Grad et al. 2000).

Mutations that induce senescence resistance

Both Rb and p53 tumour suppressors are key regulators of the senescence program. Also oncogenic Ras promotes cellular senescence in non-immortal human and rodent cells in a manner that depends on one or both products of the INK4a/ARF locus, which encodes the tumour suppressor proteins p16 and ARF. The mitogen-activated protein kinase (MAPK) signalling cascade appears to be the principal Ras-effector pathway responsible for cellular senescence by inducing p16 and/or ARF, and ultimately by activating Rb and p53, respectively (Shay and Roninson 2004). p53 and Rb then promote senescence by controlling a number of effectors, including p21CIP1/WAF1, PML, and various chromatin-modifying factors that produce a repressive state that buffers proliferative genes from mitogenic signalling (Ferbeyre et al. 2000, Pearson et al. 2000, Paramio et al. 2001, Itahana et al. 2003, Narita et al. 2003). Escape from oncogene-induced senescence is a prerequisite for the transformation of cells that probably explains the oncogenic cooperation between Ras and so-called ‘immortalizing’ oncogenes in vitro. High Ras levels are frequently observed in tumour cells and are probably required for malignant conversion (Elenbaas et al. 2001).

These data support the concept that alterations in DNA damage-response pathways increase the risk of cancer developing. The cell cycle engine has become in a diagnostic and therapeutic target in cancer. It is a convergence point of the oncogenic signaling networks, and the alteration of components belong these pathways lead up to aberrant cell proliferation and cell death resistance characteristic of cancer cells (Williams and Stoeber 2012).

MHC MOLECULES: DESCRIPTION, TYPES AND FUNCTIONS

Major histocompatibility complex (MHC) is a genetic region that encodes molecules involved in the antigen presentation to T lymphocytes. First research works that revealed their existence rose from the interest in transplant rejection. So in the decade of 1940, George Snell developed experiments of xenotransplantation in mice, defining the histocompatibility genes as responsible of the recognition of a foreign tissue, that were hereditable and different in each individual (Snell 1944, Snell et al. 1948). It was in 1952 when for the first time Jean Dausset found a reaction of agglutination of leucocytes when exposed to the serum of a patient preimmunized by several transfusions (Dausset 1952). In 1958 he discovered in the surface of leucocytes the antigen MAC, subsequently named human leucocyte antigen (HLA) molecules involved in the recognition of the own and the rejection of the foreign. The discovery of the molecules of the polymorphic HLA family, and their implications in the rejection of transplants and in the immunological reaction against pathogens made him worthy of the Nobel Prize in Physiology and Medicine in 1980 shared with George Snell and Baruj Benacerraf (Benacerraf 1977, Benacerraf and Germain 1978, Snell 1980).

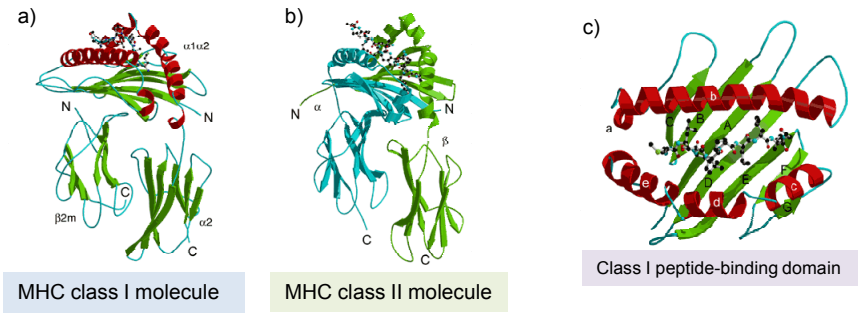


Figure 15. Protein structure of MHC class I HLA-A2 molecule (a) and MHC class II HLA-DR molecule (b), both with an antigenic peptide. View of the peptide-binding domain of HLA-A2 molecule (c). Adapted from *Sun et al. 2004.*

The molecules of the major histocompatibility complex play a critical role in immune recognition. They present antigenic peptides coming from fragments of proteins to T cells. Indeed, during development in the thymus, T cells that recognize complexes of MHC molecules with peptides derived from self proteins are eliminated, resulting in a set of mature T cells in the periphery that respond to combinations of MHC molecules with foreign peptides. In general, upon T-cell receptor binding of a foreign peptide bound to a class I MHC molecule, cytotoxic T cells are activated to lyse the cell, while T-cell recognition of a peptide-MHC class II molecule complex results in the secretion of lymphokines by T-helper cells (Bjorkman and Burmeister 1994).

MHC class I

Advances in X-ray crystallography permitted the determination of the structures of human and murine class I MHC molecules (Bjorkman et al. 1987, Garrett et al. 1989, Fremont et al. 1992, Madden et al. 1992, Zhang et al. 1992, Young et al. 1994). Class I molecules consist of an extracellular heavy chain of 44kDa, organized into three globular domains (α_1, α_2 and α_3) linked noncovalently to the β_2-microglubulin (β_2-m), a 12kDa polypeptide. The hydrophobic sections in α3 and β_2-m anchor in to the cell membrane and a short hydrophilic sequence carries the C-terminus into the cytoplasm. α1 and α2 form a groove of two extended α-helices above a floor created by a β-pleated sheet that will house a linear peptide (Fig. 15 and 16).

Top and side view of an MHC class I molecule

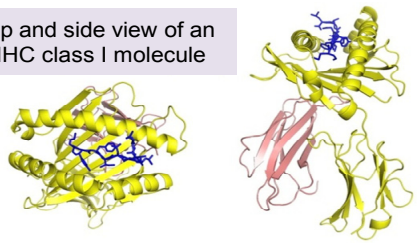


Figure 16. MHC class I molecules consist in an extracellular heavy chain, with three α-domains, associated with the β_2-microglobulin.α1 and α2 form a groove that will house a linear peptide. Adapted from *Rudolph et al. 2006*

There are three different α-chain genes HLA-A, HLA-B and HLA-C in human and histocompatibility antigen-2 (H-2)K, H-2D and H-2L in mice. Heavy chains genes are in chromosome 6 in humans while in the 17th in mice. β_2-m gene is out of the MHC region, being code in chromosome 15 in humans and in chromosome 2 in mice. The MHC-I genes are co-dominant, what means that the product of each allele, on each chromosome, is expressed on a cell at the same time. A human individual would express three different HLA-I alleles, in the case of homocigosity, until six different HLA-I alleles if heterocigosity in all loci. Most individuals inherit all the alleles encoded in only a MHC locus as two sets, one from each parent. Each set of alleles is referred to as a haplotype (Zhang 2007)**.** Although the overall fold of these proteins is very similar there exist sequence differences in the peptide cleft between different alleles (Bjorkman and Burmeister 1994). Class I molecules bind short peptides, usually 8-10 amino acids, within this cleft. Within the sequences of peptides there are critical or anchor residues that define binding positions into the cleft pockets of a particular class I allele (Fig. 16) (Rammensee et al. 1993).

MHC class I molecules report intracellular self/non-self antigens to CD8+ cytotoxic T lymphocytes (CTL) interacting with the T cell receptor (TCR) (Groothuis et al. 2005, Jensen 2007, Nitta et al. 2010, Neefjes et al. 2011). This is mediated by different molecular processes and involves a number of distinct molecules, the antigen processing machinery (APM) components. Most of class I antigenic peptides are generated upon ubiquitination of mainly intracellular proteins followed by their degradation via the multicatalytic proteasome complex yielding precursor peptides with a HLA class I compatible correct C-terminus, but an extended N-terminus. These peptides could be further trimmed by different cytoplasmic peptidases. Then, they are translocated by the heterodimeric transporter associated with antigen processing (TAP)1/TAP2 subunits from the cytosol into the lumen of the endoplasmic reticulum (ER). In the ER the peptides could be further processed and loaded onto the newly synthesized β_2-m-associated MHC class I heavy chain (HC) with the assistance of the chaperones ERp57, calnexin, calreticulin and tapasin. Upon peptide loading HLA class I/β_2-m/peptide complex travels via the trans-Golgi to the cell surface for presentation to CD8+ CTL

(Wearsch and Cresswell 2007, Scholz and Tampe 2009, Saunders and van Endert 2011, Yewdell 2011, Bukur et al. 2012). Under physiological conditions β_2-m, MHC class I heavy chains and APM components genes are constitutively expressed in all adult nucleated cells and tissues with the exception of immune privileged tissues/organs.

MHC class II

The determination of the MHC class II molecules structure (Brown et al. 1993, Stern et al. 1994) allowed an examination of the similarities and differences between protein structure and peptide binding by class I and class II MHC molecules (Stern et al. 1994). Class II molecules are a dimer of two transmembrane polypeptides α and β chains, with a weigh of 34kDa and 29kDa, respectively. α_1 and β_1 form a groove mimicking class I molecules; and α_2 and β_2 are the nearest to the membrane with anchorage domains (Fig. 15 and 17).

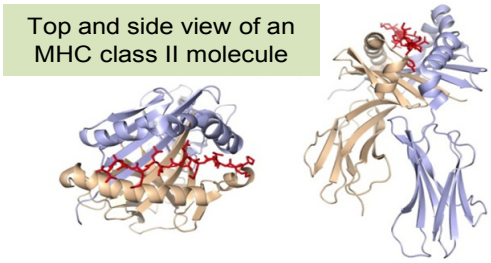


Figure 17. MHC class II molecules are a dimer of two transmembrane polypeptides α and β chains. α_1 and β_1 form a groove mimicking class I molecules. Adapted from *Rudolph et al. 2006*

In human there are three chain genes HLA-DQ, HLA-DP and HLA-DR, and a pair in mice H-2A (I-A) and H-2E (I-E). Before the structural determination, a model for the class II peptide binding site based upon the class I structure was proposed in 1988 (Brown et al. 1988). Whereas class I molecules bind short peptides, class II molecules bind longer peptides of variable length. MHC class II molecules present exogenously derived 10-15 residue antigenic peptides (Fig. 18). Besides class II MHC molecules interact with molecules called superantigens which bind as intact proteins to a region outside the class II peptide binding cleft not interfering with peptide binding, they are virulent polypeptides that are produced by a variety of infectious organisms. The result of superantigen binding is a nonspecific stimulation of a large number of T cells, due to superantigens interact with a surface of the T-cell receptor predicted to be outside the hypervariable combining site (Jardetzky et al. 1994).

MHC class II molecules sample the extracellular milieu and present antigens to CD4+ T cells (Jensen 2007). The molecular expression of MHC class II molecules is mostly restricted to professional antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs). Microorganisms can transiently exist in the extracellular space and be taken up by DCs into the endocytic pathway, where relevant antigens are loaded onto MHC class II molecules in endolysosomes. In addition, DCs possess the capacity to take up these antigens and transfer them to the MHC class I pathway through a process referred to as cross-presentation stimulate naive T cells (Vyas et al. 2008).

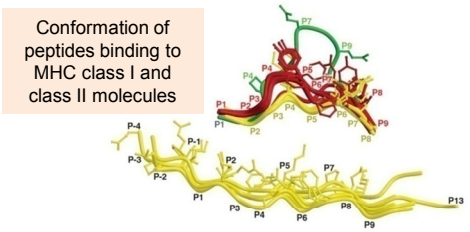


Figure 18. Comparison of peptide conformations as observed in class I (top) and class II (bottom) TCR/pMHC complexes. Class I bound peptides of 8, 9, and 13 residues. Peptides from class II complexes are 10–15 residues length. Adapted from *Rudolph et al. 2006.*

Similar to MHC class I molecules, the α- and β-chain of MHC class II molecules are synthesized in the ER where they associate with the invariant chain (Ii; also known as CD74) for proper folding, trafficking and protection of the antigen-binding groove (Schmid et al. 2007). Newly assembled MHC class II molecules are then delivered by vesicular transport to endolysosomal compartments that supply peptide antigens. Following peptide loading, peptide/MHC class II complexes are delivered to the cell surface. Despite the involvement of different molecules and cellular compartments, the generation of peptide/MHC class II complexes can be stratified as those for peptide/MHC class I complexes. Others pathways of antigen processing and presentation have recently been extensively reviewed (Jensen 2007, Savina and Amigorena 2007); as, for example, the contribution of the autophagy. Autophagy can target pathogens that reside in the cytosol or within phagosomes for lysosomal degradation and can therefore participate in the effective elimination of viruses, bacteria and parasites (Levine and Deretic 2007, Schmid and Munz 2007, Schmid et al. 2007).

Polymorphism of MHC class I and class II genes

MHC molecules compound a highly polymorphic system based on multiples alleles offering variability between individuals. Both MHC class I and II exhibit the same basic structure and participate on peptide presentation to T cells. Differences on the groove architecture defined the range of peptide they will present. MHC genes are the most polymorphic on human genome, new alleles with differences in the α-helices or β-pleated sheet of the central cavity offered an increased fitness for protection against the plethora of infectious organisms. HLA class I has multiple allelic variants, B molecule presents over 600 variants, while -A is over 300, and -C has near 200 allelic variants. So are highly polymorphic class II β chains and much lesser α chains -DP and -DQ. HLA-DRα and β_2m have an invariant structure (Peter et al. 2006).

Nonclassical MHC molecules

Nonclassical MHC molecules are encoded by a set of genes in MHC region and others on a different chromosome to the MHC. Nonclassical molecules encoded in MHC region are H-2T, -Q and -M loci in mice; and HLA-E, -F, -G and -H, MICA and MICB molecules in human. They exert a limited polymorphism and less peptide diversity, than MHC class I molecules. In mice H-2T22 and H-2T10, for example, are inducible and are recognize by γδ TCR. HLA-E is oligomorphic with two well-defined alleles HLA-E107R and HLA-E107G. With the exception of erythrocytes, HLA-E is ubiquitiously expressed in tissues, like kidney, skin, liver, thyroid,

bladder, stomach, endometrium, spleen, lymph nodes as well as in endothelial, B and T cells, monocytes, macrophages and megakaryocytes. HLA-E binds a nine-amino acid peptide derived from the peptide processing pathway of HLA-I molecules, and is recognized by the receptor of the NK and T cells CD94/NKG2, and by the αβ TCR on some cytotoxic T cells (Kaiser et al. 2008). The murine homolog, Qa-1, has similar functions. HLA-F is physiologically expressed only on the surface of B cells, monocytes and in vivo on extravillous trophoblasts, while constitutive HLA-F expression was mainly found in the cytoplasma of B and T lymphocytes, NK cells and monocytes. Upon activation of these immune cells HLA-F expression is up-regulated at the cell surface. HLA-F has been shown to bind to the inhibitory ILT-2 and ILT-4 receptors suggesting a potential role of HLA-F in regulating immune cell function (Lepin et al. 2000, Ishitani et al. 2006). HLA-G exerts a limited polymorphism and limited peptide diversity. HLA-G consists of 7 isoforms, which were generated by alternate splicing, 4 membrane-bound (HLA-G1, -G2, -G3 and -G4) and 3 soluble isoforms (HLA-G5, -G6 and -G7) (Menier et al. 2010). HLA-G expression is highly tissue restricted and found mainly in immune-privileged organs, in organs during development and in cells of the hematopoietic lineage. HLA-G exerts immune suppressive properties due to its capability to bind to inhibitory receptors as immunoglobuline-like receptors, the immunoglobuline like transcripts (ILT)2, ILT4 and KIR2DL4, expressed in B- and T lymphocytes as well as in peripheral NK cells and monocytes. By binding to these receptors HLA-G inhibits the cytotoxicity of CD8+ T cells as well as NK cells and the allogenic proliferative response of CD4+ T cells. Furthermore, HLA-G can induce apoptosis of activated CD8+ T cells and NK cells. In addition, HLA-G affects the function of DCs, in particular their maturation, migration, trafficking, antigen presentation as well as their cross-talk between T and NK cells (Carosella et al. 2011). Another soluble HLA-G is generated by the proteolytical cleavage of the HLA-G1 surface antigen (sHLA-G1). Recently, sHLA-G has also been shown to down-regulate the chemokine receptor expression in T cells, which lead to an impaired chemotaxis (Bukur et al. 2012). HLA-H possesses an extremely narrow groove which is unable to bind peptides, however, it binds the transferring receptor, and it appears to be involved in iron uptake.

The stress-inducible MICA and MICB (MHC class I chain-related molecules) have the same domain structure as classical class I molecules and display a relatively high level of polymorphism. They are present on epithelial cells, mainly in the gastrointestinal tract and in the thymic cortex, and are recognized by NKG2D activating receptor, promoting NK- and T-cell mediated immuno response.

Within those molecules we can found the CD1 family proteins which acquire a similar structure of MHC-I molecules combining with the β_2-m protein. They are encoded in chromosome 1 in humans and 3 in mice. They also take part in T cells presentation, but they have a groove riches in hydrophobic amino acids that binds lipids or glycolipids, and that there is accessible only through a narrow entrance. Human appear to express four different CD1 molecules, CD1a, -b and -c are present on cortical thymocytes, DCs and a subset of B cells, while CD1d is expressed on the intestinal epithelium. Mice only express two different CD1 molecules CD1d1 and CD1d2.

Gen map of the MHC

MAIN GENETIC REGIONS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX												
HUMAN	MHC CLASS	II			III				I			CHROMOSOME 6
	HLA	DP	DQ	DR	C'	HSP	TNF	etc	B	C	A	
MOUSE	MHC CLASS	I	II		III				I			CHROMOSOME 17
	H-2	K	A	E	C'	HSP	TNF	etc	D	L		

MHC genetic region is in the short arm of chromosome 6 in humans, and 17 in mice. Complete sequence of MHC comprises 224 gene loci, about a 40% of which are predicted to be expressed. Class I genes are located in the most telomeric position, while class II is in the most centromeric region. Class III genes are between them (Peter et al. 2006).

HUMAN	HLA GENE	MICB	MICA	B	C	E	A	G	F
	GENE PRODUCT	MICB	MICA	HLA-B	HLA-C	HLA-E	HLA-A	HLA-G	HLA-F

MOUSE	H-2 GENE	TAPASIN	K	D	L	Q	T	M
	GENE PRODUCT	TAPASIN	H-2K	H-2D	H-2L	Q	T	H-2M

MHC class I gene map. Comprised by the classical or class Ia genes HLA-A, -B, -C in humans and H-2K, -D, -L in mice, and the nonclassical or so called class Ib genes HLA-E, -F and -G, HFE, MICA and MICB in humans and H-2T, -Q or -M in mice. Murine tapasin molecule, involved in MHC-peptide joining, is also encoded in this region.

HUMAN	HLA GENE	TAPASIN	DPB	DPA	DOA	DMA	DMB	LMP2	TAP1	LMP7	TAP2	DOB	DQB	DQA	DRB	DRA
	GENE PRODUCT	TAPASIN	DPβ	DPα	Doα	DMα	DMβ	PROTEOSOME COMPLEX AND PEPTIDE TRANSPORTER				Doβ	DQβ	DQα	DRβ	DRα
			HLA-DP		HLA-DO	HLA-DM						HLA-DO	HLA-DQ		HLA-DR	

MOUSE	H-2 GENE	Oa	Ma	Mb2	Mb1	LMP2	TAP1	LMP7	TAP2	Ob	Ab	Aa	Eb	Ea
	GENE PRODUCT	Oα	DMα	DMβ2	DMβ1	PROTEOSOME COMPLEX AND PEPTIDE TRANSPORTER				B	Aβ	Aα	Eβ	Eα
		H-2O	H-2DM							H-2O	H-2A		H-2E	

MHC class II gene map. Include in humans the genes HLA-DP, -DQ and -DR; and in mice H-2A (I-A) and H-2E (I-E). Both α and β chains are transcribed from closely locates gene. DRα and I-Eα chains pair with two different β chains. In this region there are also other genes related with peptide process: TAP1, TAP2, low molecular mass protein (LMP)7, LMP2 (sharing bidirectional promoter with TAP1), HLA-DM and HLA-DO in humans or their respective in mice H-2M and H-2O.

HUMAN	CYP21B	C4B	CYP21A	C4A	BF	C2	HSPA1B	HSPA1A	HSPA1L	LTB	TNF	LTA
MOUSE	CYP21B	C4B	CYP21A	C4A	BF	C2	HSPA1B	HSPA1A	HSPA1L	LTB	TNF	LTA

MHC class III gene map. Region III of MHC grouped genes related with the immune system: four genes coding for complement components C4A, C4B, C2 and factor B, tumor necrosis factor has complement factors, tumor necrosis factor, and lymphotoxin-α and -β and members of the 70kDa heat shock proteins (HSP70 or HSPA); and many genes that don't have any defensive function as valil tRNA synthetase, NOTCH4 regulatory protein or the extra cellular matrix protein tenascin.

Antigen processing and presentation in MHC-I molecules

Within the proteolytic structures, the proteasome is involved in the routine turnover and cellular degradation of proteins. First step in degrading proteins is the covalently link to several ubiquitin molecules in an ATP-dependent process. The ubiquitylation targets the polypeptides to the proteasome. Generally proteins destined to be degraded are cytosolic, including viral proteins, but they can also be membrane-bound or secretory proteins that are transported back from the endoplasmic reticulum to the cytosol, as can be proteins from the mitochondria. Proteasome degrade proteins into peptides that will precise further trimmed by cytosolic proteases including leucine- and aspartyl-aminopeptidases and by ER proteases as endoplasmic reticulum resident aminopeptidase-1 (ERAP-1), to give the peptides the optimal length to fit into the MHC-I groove (octamers or nonamers). Interferon (IFN)-γ induce the production of three specialized catalytic proteasome inducible subunits LMP2β1i, LMP7β5i and MECL1β2i, that will replace the constitutive homologous LMP2β1c, LMP7β5c and MECL1β2c, producing the immuno-proteasome which modifies the cleavage process to tailor the peptide production for MHC-I binding (Fig. 19) (Huber et al. 2012).

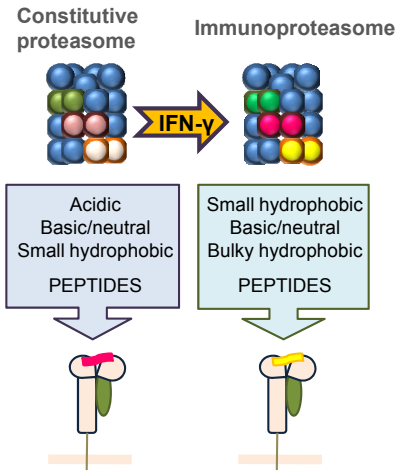


Figure 19. In the presence of IFN-γ constitutive proteasome subunits are replace by inducible subunits that result in a modification in the peptide production. Adapted from *Huber et al. 2012.*

Processed peptides are translocated into the ER by the transporters associated with antigen processing TAP1 and TAP2, involving also some heat-shock family members in this process. Into the proteasome the newly synthesized heavy chains are retained by the calnexin and calreticulin chaperons that assist in the formation of the disulfide bond and the assembly with the B_2m. Then ERp57 thiol reductase becomes associated to the complex. Tapasin intervenes linking TAP1/TAP2 dimer with the complex class I and chaperons to load the peptide, stabilizing the MHC-I molecules later transported across the Golgi to the cell surface. Thus six steps could summarize the biosynthesis of MHC-I molecules: 1) acquisition of antigenic peptides from proteins with errors; 2) the antigenic peptide are tagged for destruction by ubiquitylation; 3) proteolysis of ubiquitylated peptides into the proteasome and further process by aminopeptidases outside and into the ER; 4) delivery of peptides to the ER by the complex form by TAP1 and TAP2; 5) binding of peptides to MHC class I molecules with the mediation of the chaperons; and 6) display of peptide-MHC class I complexes across the Golgi on the cell surface (Fig. 20) (Vyas et al. 2008).

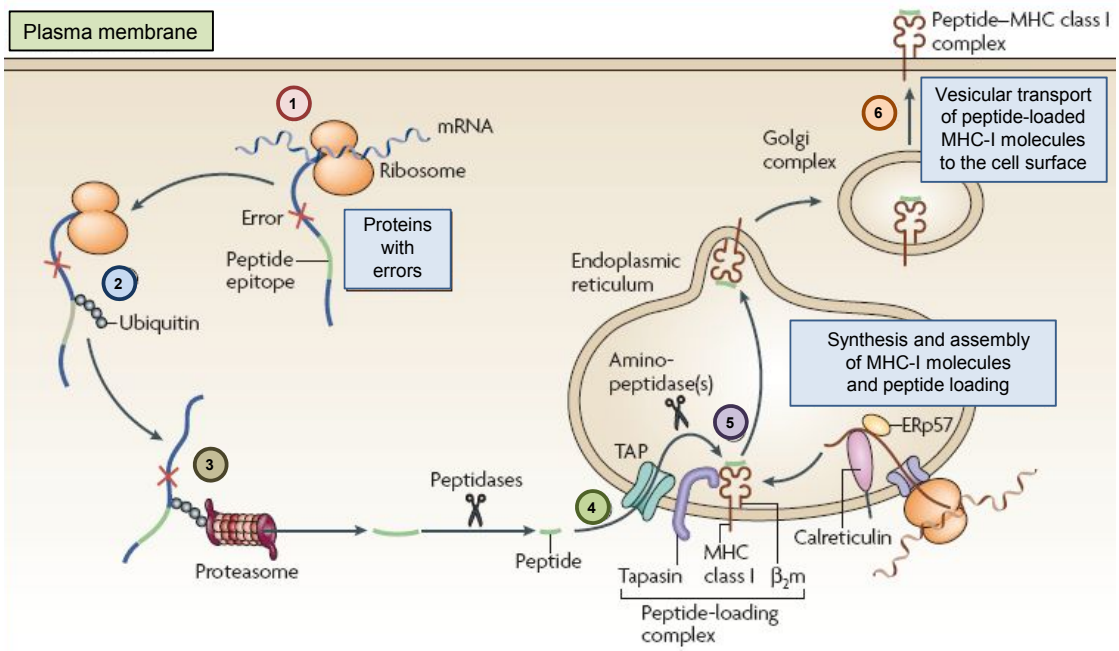


Figure 20. Final peptide-loaded MHC-I molecules onto the cell surface is the result of a six-step process. Step 1: Acquisition of antigens from proteins with errors. Step 2: Misfolded proteins are tagged with ubiquitin. Step 3: proteasome degrades ubiquitylated proteins in peptides. Step 4: Peptides are delivered into the ER by TAP1/TAP2 complex and further processed by peptidases. Step 5: Into the ER, nascent MHC-I molecules are assembled by calnexin, calreticulin and ERp57 and loaded with peptides by tapasin activity. Step 6: Peptide loaded-MHC-I molecules are transported via the Golgi to the cell surface. Adapted from *Vyas et al. 2008.*

It's been also proposed the fact that peptide could be acquire by alternative ways, both by intercellular protein channels and autophagy processes. GAPs are intercellular channels that connect the cytoplasm of neighboring cells. Some data support the role of these connexions as mediators of peptide translocation (Neijssen et al. 2007). Autophagy is implicated in the clearance of mitochondria and the prokaryotes organisms *Listeria monocytogenes* and *M. tuberculosis*, whose N-formylated peptides are presented by H-2M3a, a member of MHC-Ib family, suggesting that H-2M3a molecule might acquire its peptides by an autophagy-dependent process (Rodgers and Cook 2005, Gutierrez et al. 2004).

ANTI-TUMOR IMMUNORESPONSE

The concepts of immunosurveillance and immunoediting

Cancer disease is considered the result of multiple and accumulative genetic alterations in oncogenes, tumor-suppressor genes and genome-stability genes. These mutations help tumor cells to avoid cell-intrinsic control point that drive damaged cells to the death. Nowadays is widely known that cancer cells are also exposed to extrinsic pressures that inhibit malignant progression but also have an important effect in the selection of tumor cells that present growth advantages. One of the first times the idea that the immunity could play a role in the control and repression of tumor progression was proposed was in the 1909 by Paul Erlich (Erlich 1909). It was suggested that cellular immunity was the manager of maintaining tissue homeostasis and the protector from neoplastic disease (Thomas 1959). The fact that mice rejected syngeneic transplanted tumors mediated by cellular components of the immunity, participated in the establishment of the existence of tumor associated antigens (Old and Boyse 1964, Klein 1966). The emerging discoveries provide the basis of the hypothesis of "cancer immunosurveillance". In 1970, Macfarlane Burnet finally defined the concept of immunosurveillance as immunological mechanisms that eliminate and inactivate the potentially dangerous premalignant mutant cells (Burnet 1970).

Numerous researches were done to test the immunosurveillance hypothesis, but the advances came slowly and the results were not fully well understand due to the lack of learning of the Immunology. The development of athymic nude mouse enables the study of tumor generation in genetic immunodeficient mice (Flanagan 1966). Immunodeficient mouse showed major susceptibility to virally induced tumors and to develop lymphomas, which was associated to the more susceptibility to viral and bacterial infections. On one hand, transforming virus could induce the malignancy; in the other hand, during the infection the lymphocytes quickly proliferate and could acquire somatic mutations that finally lead to the formation of lymphomas (Stutman 1975). Working with CBA/H strain nude mice, no significant differences were found on the incidence of methylcholantrene (MCA)-induced tumor formation between nude and wild-type mice (Stutman 1979), convincing the scientific community to give up the immunosurveillance hypothesis. The limited understanding of this mouse strain makes them not to bear in mind that the CBA/H strain is very sensible to MCA carcinogenic action that overcame the immunity of the wild-type mice and that the periods they established for spontaneous tumor formation could be too short.

The renaissance of cancer immunosurveillance came with the sequence of some key findings. Experiments with nude BALB/c mice showed a major predisposition of immunodeficient mice to MCA-induce sarcomas (Engel et al. 1996). It was found that endogenously produce IFN-γ exerted host protection against transplanted tumor and chemical-induced tumors. Blocking IFN-γ molecules by specific antibodies induced a faster growth of transplanted fibrosarcomas in mice (Dighe et al. 1994). Moreover the lack of perforin in C57BL/6 mice made them to be prone to MCA-induced tumor formation compare with their wild-type counterparts (van der Broek et al. 1996). Spontaneous metastasis assays in immunodeficient nu/nu and immunocompetent BALB/c mice with an H-2 class I negative fibrosarcoma tumor clone showed that the incidence of metastatic disease was higher in immunodeficient mice, and these colonies were more immunogenic than the metastasis

generated in immunocompetent mice (García-Lora et al. 2001). Moreover, the metastases from immunocompetent BALB/c mice remained MHC-I negative phenotype, while in athymic nu/nu mice, metastatic nodes were MHC-I positive (García-Lora et al. 2001). Thus, the existence of T cell response would appear as the responsible of the control of the metastasis disease in immunocompetent mice, and of the selection of cancer cell colonies with phenotypic alterations that provide these cancer cells with a low immunogenic profile.

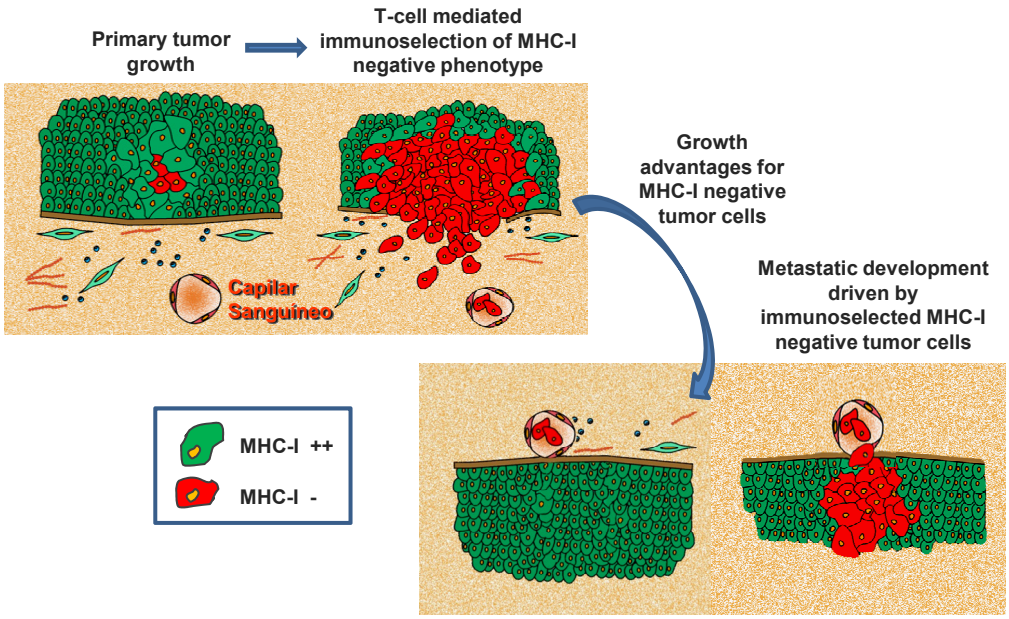


Figure 21. In the presence of immune response, T cells kill MHC-I positive tumor cells selecting those that harbor alterations in the MHC-I phenotype, which will develop the metastatic disease. Adapted from *Algarra Master Classes archives 2012.*

Mice lacking RAG-2 cannot correctly rearrange lymphocyte antigen receptors and lack the NKT, T and B lymphocytes populations. RAG-$2^{-/-}$ mice developed more rapidly and more immunogenic MCA-induced sarcomas or spontaneous neoplasia than immunocompetent mice (Shankaran et al. 2001). Similar results were found using genetically modified mice lacking IFN responsiveness (IFNGR$1^{-/-}$ or STAT1 $^{-/-}$) or lymphocytes (RAG-$2^{-/-}$) or both RAG-2 and STAT1. Each of the four lines of gene-modified mice generated three times more chemically induced tumors than syngeneic wild-type mice, with no significant differences detected between the four immunodeficient groups (Kaplan et al. 1998). In humans, it was found that individuals with severe immunodeficiency (as transplanted or AIDS patients) had higher probability of developing viral-induced cancers (Penn 1999, Boshoff and Weiss 2002). Taken together, these results show: first, T lymphocytes intervened in tumor immunosurveillance preventing the formation of spontaneous or chemically-induced tumors and the development of metastases; and second, immunity eliminate highly immunogenic cancer cells selecting those cells with less immunogenicity, like for example, those that carried alterations in the expression of MHC-I molecules or in the IFN-γ receptor signaling pathway.

In this context, the concept of "cancer immunoediting" was proposed to describe the dual host-protecting and tumor-sculpting actions of the immune response, preventing but, as well as molding cancer disease (Dunn et al. 2002). Cancer immunoediting was proposed as a three-step process with a very complex network of cellular and molecular dynamics.

Immunoediting process was envisioned as the sequence of elimination, equilibrium and escape phases (Fig. 22). Elimination phase encloses the concept of immunosurveillance, that was thought to function protecting the host and only at the earliest stages of malignant transformation. If tumor is eliminated by immune system, this would complete the process. During equilibrium phase immunity held in check the expansion of transformed cells (Koebel et al. 2007). This phase could long for years, and maintain tumor occult in organs, that could be the responsible of post-transplanted tumor formation. Cancer cells in equilibrium are unedited and highly immunogenic. They interfere with adaptative immunity, causing a chronic inflammation that exert tumor-promoting actions, so cancer cells can spontaneously exit equilibrium acquiring an edited and less immunogenic phenotype (Koebel et al. 2007). In the escape process, surviving cancer cells have acquired an evasive phenotype developing different immunoescape mechanisms that result in an undetectable and uncontrolled expansion of cancer disease.

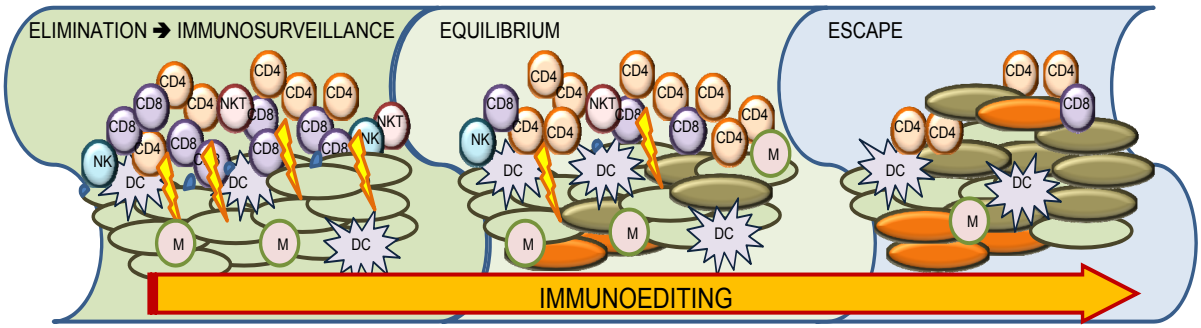


Figure 22. Cancer immunoediting is a continuous process that encompasses three steps: 1) Elimination, which corresponds to the traditional concept of immunosurveillance, with an active immunity killing malignant cells. 2) Equilibrium represents when the immune response selects or promotes the generation of tumor variants (orange or brown cells) that maintain a "pulso" with immune cells. 3) Escape is when the immuno-sculpted tumor cells expand in an uncontrolled manner. Adapted from *Dunn et al. 2002.*

Anti-tumor immunoresponse

In the tumor microenvironment different immune effectors and regulators control the development and progression of the cancer pathogenesis. Into the tumor microenvironment cells from both innate (DCs, NK cells, macrophages, neutrophils) and adaptative immunity (B and T lymphocytes) have been found (Disis 2010). Immune cells are present during the cancer progression process, from the first alterations as the genomic instability, to the supply of growth signals, the maintenance of the intratumoral angiogenesis, and the promotion of metastatic process (Fig. 24). Tumor cells, presenting tumor antigens by MHC-I molecules interact with the DCs antigen presenting cells. Under appropriated conditions DCs undergo further maturation and migrate to secondary lymphoid tissues where they present antigens to T cells inducing their clonal proliferation and the immune response (Steinman 1991). Naïve CD4+ and CD8+ T cells proliferate to antigen-specificity. Macrophages are also presented in the tumor infiltrated as the primary source of secreted pro-inflammatory cytokines. Tumor associated macrophages may acquire type 1 (M1) phenotype. M1s activated T-helper 1 (Th1) adaptive immunity by secreting cytokines such as interleukin 12 (IL-12) that promote CTL cytotoxic effect to tumor cells (Sica et al. 2008). An effective adaptative T cell response is

composed by both cytotoxic CD8+ and CD4+ T lymphocytes. CD4+ T cell are also called T-helper cells because they secrete cytokines involved in the regulation and propagation of the immune response. Th cells could manifest several phenotypes Th1 and Th2, further described bellow; Th17 cells, that secrete IL-17, participate in autoimmune diseases; and Treg cells damp the anti-tumor immunoresponse by the secretion of inhibitory cytokines as IL-10 and tumor growth factor (TGF)-β (Sallusto and Lanzavecchia 2009). Type 1 T helper (Th1) cells produce IFN-γ, IL-2, and TNF-β, which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. By contrast, type 2 Th (Th2) cells produce IL-4, IL-5, IL-10, and IL-13, which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses. Th1 cells mainly develop following infections by intracellular bacteria and some viruses, whereas Th2 cells predominate in response to infestations by gastrointestinal nematodes. Polarized Th1 and Th2 cells not only exhibit different functional properties, but also show the preferential expression of some activation markers and distinct transcription factors. Several mechanisms may influence the Th cell differentiation, which include the cytokine profile of "natural immunity" evoked by different offending agents, the nature of the peptide ligand, as well as the activity of some costimulatory molecules and microenvironmentally secreted hormones, in the context of the individual genetic background (Romagnani 1999). The prime movers of cancer immunosurveillance are CD8+ T adaptative immunity cells and NK innate immunity cells. At the initiation of malignancies tumors express tumor-associated antigens (TAAs) in the context of MHC class I molecules that could be recognized by the DCs and adaptive immune system cells. CD8+ T lymphocytes become activated in the lymph nodes by tumor antigen cross-presentation by mature and activated DCs, promoting the clonal expansion of tumor antigen specific CTLs. So then, the migration of CTLs into tumor lesions leads to the recognition of TAA presented by the tumor cells, mediated by TCR/MHC-I interaction. Later, costimulatory signals will finally induce an effective cancer cell killing. The role of NK cells in immunosurveillance process is slowly becoming clear, due the complexity of this cell population. NK cells have both stimulatory and inhibitory receptors (Fig. 23).

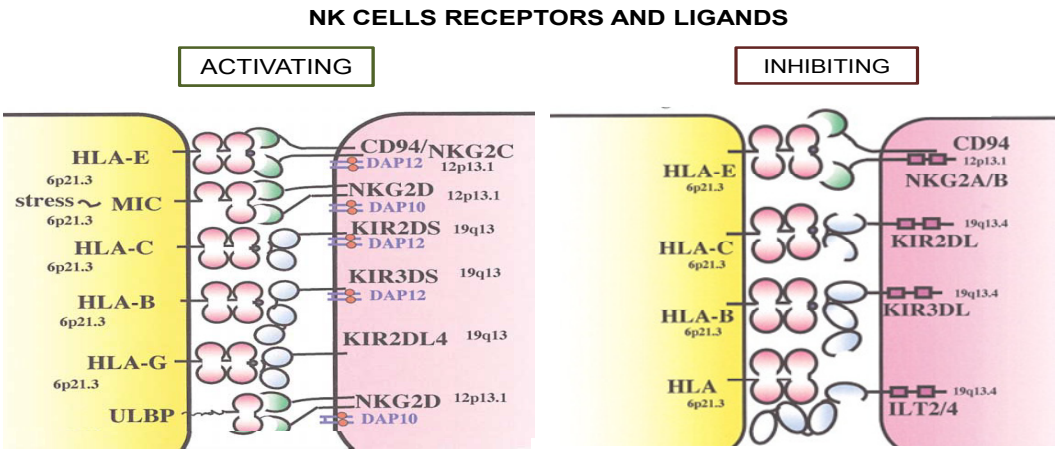


Figure 23. Interactions between MHC Class I molecules and: Activating Receptors (left side), a range of activating receptors are shown along with their chromosomal positions and class I ligands (KIR2DL4 has not yet been confirmed as activating receptor); or Inhibitory Receptors (right side), both 2- and 3-domain Ig superfamily and lectin receptors are shown along with chromosomal assignments. Adapted from *Trowsdale 2001.*

NK receptors bind both classical and nonclassical MHC class I molecules blocking the killing of target cells (Lanier 1998). Inhibitory receptors (CD94/NKG2A/B, KIR2DL, KIR3DL, ILT2/4) are associated with ITIM domains (immunoreceptor tyrosine-based inhibitory motifs) in the cytoplasmic tail. However, certain members of the NK receptor family (CD16, KIR2DS, Ly49D/H, and CD94/NKG2C) are associated with the adapter molecule DAP12, which has an ITAM domain (immunoreceptor tyrosine-based activating motif) capable of activating NK cells (Bakker et al. 2000). NKG2D receptor gene complex is expressed by NK cells as well as by CD8+ αβ and γδ T cells and induced the ability to kill tumor cells. The two best characterized NKG2D ligands are MICA and MICB, nonclassical MHC human molecules, which are induced by classic stress stimuli such as heat shock (Pardoll 2001). In mouse NKG2D does bind to many products of the retinoic acid inducible gene family *Rae-1*α−ε as well as to the product of the *H60* gene (Girardi et al. 2001). Changes in activating or inhibiting NK receptors may have implications for tumor evasion (Bernal et al. 2009).

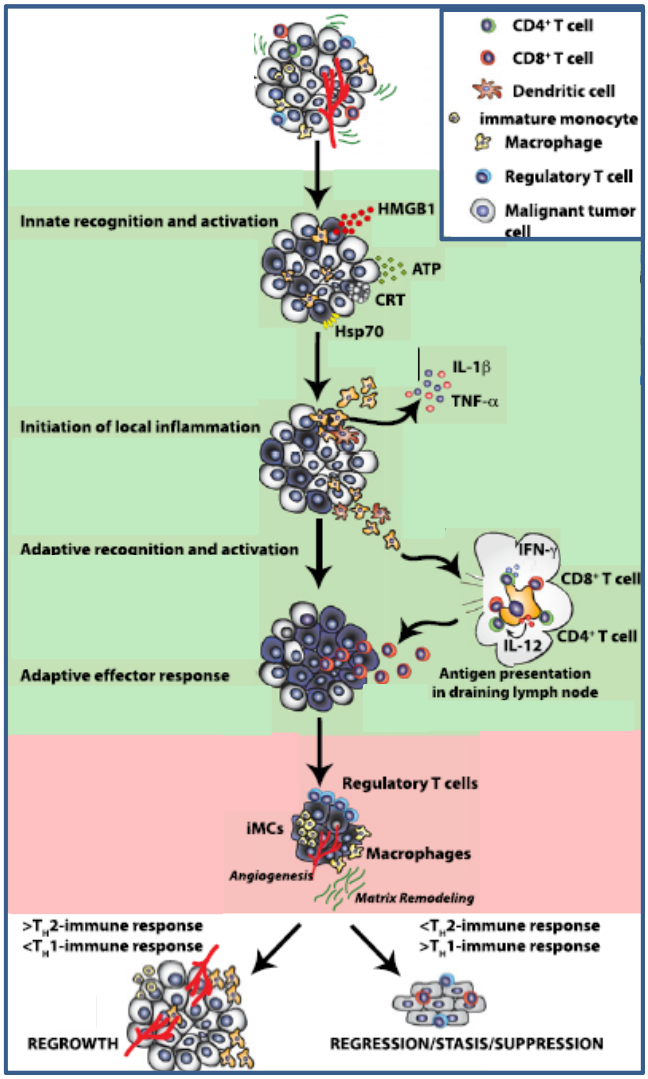


Figure 24. Tumor cells release inflammatory mediators (HMGB1, calreticulin, ATP, and Hsp70) that activate immune cells such as DCs and tissue macrophages, and the release of TNF-α and IL-1, which further recruits peripheral blood leukocytes (PBLs) from the circulation. Activated DCs and macrophages migrate to the lymphoid tissue bearing tumor antigens that present to CD4+ and CD8+ T cells, leading to their activation and infiltration of the tumor to eliminate tumor cells. Throughout this process, immunosuppressive cells are recruited into the tumor (e.g., Treg cells and various subtypes of myeloid cells), and these become increasingly dominant as the tumor is cleared by a cytotoxic response (primarily CD8+ T cells and NK cells) and function to reduce the anti-tumor cytotoxic response. Incomplete eradication of malignant cells leads to tumor regrowth at the primary site or metastases at distal sites. Adapted from *Shiao et al. 2011.*

Immune cells could have either a potent anti-tumor function activating the immune response, but also a pro-tumor function blocking the anti-tumor immunity (Fig. 24). Regulatory population is formed by regulatory/tolerogenic dendritic cells, regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), and they induced a T cell unresponsiveness, anergy or tolerance by different networks. DCs can exist in a multitude of functional states,

additionally to immature or mature phenotypes; they may be conditioned to tolerant or suppressed states (Shurin et al. 2012). Tumor associated macrophages could acquire a type 2 (M2) phenotype. M2s are frequently present in the tumor microenvironment and secrete immunosuppressive cytokines and proteases or angiogenic factors that promote tumor and metastasis progression (Allavena et al. 2008). MDSCs are also innate immune cells that are elicited during chronic inflammatory states due to the blocking of the differentiation of immature myeloid precursors into mature myeloid. MDSCs inhibit the adaptive immune response via multiple mechanisms: direct secretion of substances that affect T cell function as well as the induction of adaptive T regulatory cells (Ostrand-Rosenberg and Sinha 2009).

Immunoescape mechanisms

Cancer cells develop along their transformation process, several immunoescape mechanisms that abrogate the anti-tumor immunoresponse. Thus immune response localized to the tumor may not be able to inhibit cancer growth; indeed, some types of inflammation induced in a tumor may also lead to cancer proliferation, invasion, and dissemination. Two major groups of mechanisms could be depicted, on one hand those mechanisms that directly or indirectly inhibit the anti-tumor immunity and, on the other hand the intrinsic cancer cells mechanisms to avoid or to hide from immune cells.

Cell-mediated programs that blunt anti-tumor immunity

Cancer cells have the ability of thrive in a chronically inflammation microenvironment sustained by the infiltration of cells with immunosuppressive activity and that ensue neoplastic progression (Coussens and Werb 2002). Cells that significantly participate in suppressing anti-tumor immunoresponse are regulatory T cells, alternatively activated macrophages, immunosuppressive monocytes and regulatory dendritic cells (Fig. 25).

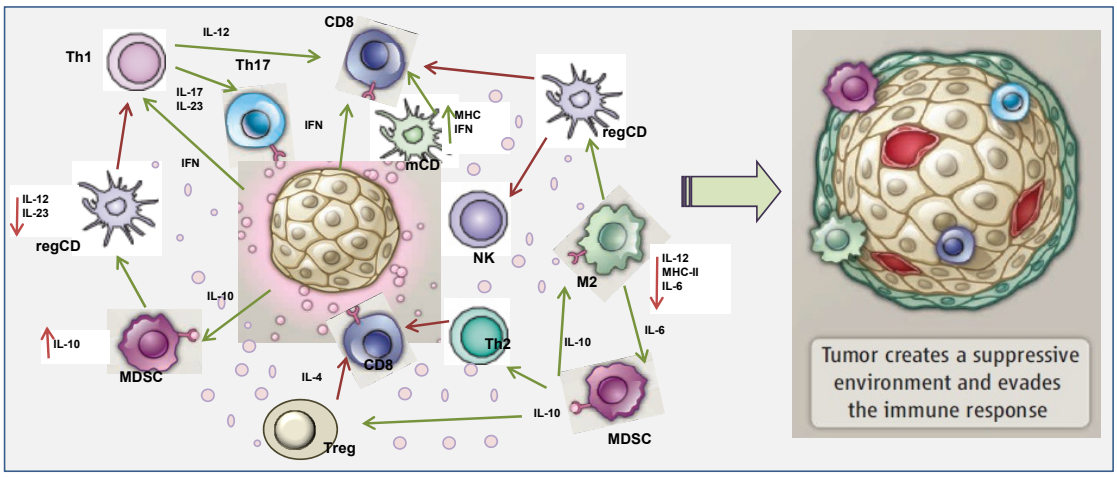


Figure 25. Tumor cells and immunosuppressive cells as MDSC or Treg, through a variety of soluble factors and cell contact-dependent mechanisms polarize immunity towards a type 2 response and the impairing function of DCs, macrophages, and cytotoxic CD8+ T cells or NK cells, leading to a chronic inflammatory microenvironment that promotes the tumor progression. Integration and adaptation from *Zindl and Chaplin, 2010* and *Ostrand-Rosenberg et al. 2012.*

Regulatory-T cells. Treg cells are a subset of CD4+ T-cell population that constitutively express the high-affinity IL-2 receptor (CD25), cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor, and the lineage-specific transcription factor Foxp3. Treg cells prevent autoimmunity by the inhibition of responses to self-antigens (Hori et al. 2003). Tumour specificity of the immune response resides in the recognition of tumour antigens. Viral proteins in tumors caused by viruses and mutated proteins from oncogenes or other genes, as well as nonmutated but abnormally expressed self proteins are found on all tumors (Finn 2012). Malignant cells may express self-antigens, inducing Treg to hamper anti-tumor immunity (Shiao et al. 2011). Evidence of tolerance induction to nonmutated p53 peptides were found using $p53^{-/-}$ and $p53^{+/+}$ HLA-A2.1/Kb transgenic mice (Theobald et al. 1997). Furthermore, it was reported that an HLA-A2-restricted CTL clone specific for the nonmutated $p53_{264-272}$ peptide LLGRNSFEV, established from a healthy donor, could recognize p53-overexpressing squamous cell carcinoma cell lines derived from head and neck cancers (Ropke et al. 1996). It remained to be evaluated how often these p53-specific CTL capable of recognizing tumor cells can be induced from patients with cancer (Kawakami and Rosenberg 1997). Tumors can also support the development of Treg cells by the secretion of chemokines as CCL2 (Curiel et al. 2004a) and cytokines such as TGF-β enhancing Treg infiltration (Ghiringhelli et al. 2005).

Alternatively activated macrophages. When the tumor milieu showed Th2 cytokine profile (IL-4, IL-13, etc.), macrophages become alternatively activated (AAMs) (Qian and Pollard 2010) and block CD8+T cell proliferation or infiltration, and express proliferative, proangiogenic and tissue remodeling mediators that provide survival advantages to tumor cells (DeNardo et al. 2009, Ruffel et al. 2010).

Immunosuppressive monocytes. Immunosuppressive monocyte (iMC) population is characterized by the expression of CD11b and Gr1 surface markers (Gabrilovich and Nagaraj 2009), and include MDSCs, inflammatory monocytes and neutrophils (Ostrand-Rosenberg 2008). iMC suppress T- and NK cell proliferation via arginase I, inducible nitric oxide synthase expression and peroxynitrite as well as promote Treg generation (Mazzoni et al. 2002, Doedens et al. 2010, Lu et al. 2010). In the tumor microenvironment frequently the tumor and host components interact to generate a highly immune suppressive environment that frustrates T cell cytotoxicity and promotes tumor progression. MDSCs are a major host component contributing to the immune suppressive environment that amplifies the immune suppressive activity of macrophages and dendritic cells via cross-talk (Ostrand-Rosenberg et al. 2012, Gabrilovich et al. 2012). MDSC suppress CD4+ or CD8+ T cells in an antigen dependent or non-dependent manner (Nagaraj and Gabrilovich 2012). One key characteristic of MDSCs is the generation of reactive oxygen (ROS) and reactive nitrogen species (RNS) in the tumor microenvironment. ROS and RNS are involved in induction of antigen-specific T-cell tolerance, inhibition of T-cell migration to the tumor site, and tumor cell evasion of recognition by cytotoxic T cells (Lu and Gabrilovich 2012).

Regulatory Dendritic cells. Regulatory dendritic cells in cancer may maintain T cell unresponsiveness by controlling T cell polarization, MDSC and Treg differentiation and activity. They affect specific microenvironmental conditions in premalignant niches where they may mediate genomic damage, support neovascularization, block anti-tumor immunity and stimulate cancerous cell growth and spreading (Shurin et al. 2012).

Expression or secretion of immunosuppressive factors or cytokines

Cancer cells may acquire the ability to express or release series of factors and cytokines to subvert normal immuno-reaction mechanisms. The secretion of colony-stimulating factors or vascular endothelial growth factors induces the expansion of the myeloid immature cells population that may not only help tumors to suppress immune reaction but also aid in the construction of new blood vessels for tumor growth (Curiel et al. 2004b, McLean and Buckanovich 2008). Tumor cells direct secretion or through activating such secretion in MDSCs, DCs or TAMs release TGF-β, IL-10, and indoleamine 2,3-dioxygenase (IDO), promoting Treg proliferation, MDSCs accumulation and block DCs maturation as well as lymphocytes functions (Serafini et al. 2006).

Fas and Fas ligand (FasL) are complementary receptor-ligand proteins (Suda et al. 1993). Fas is expressed in many types of tissues, whereas FasL is highly expresses in activated T lymphocytes, but also in some organs in the body as the lung, cornea or testes (French et al. 1996). Fas-induced apoptosis involves the binding of Fas receptor with its ligand, which induces the recruitment of Fas-associated death domain (FADD) and procaspase-8, leading to the activation of caspase-8 by autocatalytic cleavage. Activated caspase-8 can either directly cleave effector caspases or Bid. Cleaved Bid can then translocate to the mitochondria and induce the activation of capase-9, leading to the activation of effector caspases (Wajant 2002). A direct inhibitor of Fas-mediated apoptosis is cellular FLICE-inhibitory protein (c-FLIP), which functions by competitively binding to FADD and inhibiting the cleavage of procaspase-8 (Chang et al. 2002). Several cancer cell lines and tissues from patients have been found to over-express c-FLIP (Irmler et al. 1997, Scaffidi et al. 1999, Lavrik and Krammer 2011) which is associated with increased resistance to death receptor pathways. The Fas/FasL system plays a significant role in tumorigenesis. Effective anti-tumor response involves the induction of apoptosis of tumor cells by activated CTL by the engagement of Fas/FasL (Weigelin et al. 2011). Moreover, Fas expression is inversely correlated with metastatic potential of some cancer cells as the osteosarcoma cells (Worth et al. 2002). Research has shown that its impairment in cancer cells may lead to apoptosis resistance and contribute to tumor progression (Villa-Morales and Fernández-Piqueras 2012). Cholesterol- and sphingolipid-rich membrane domains, termed lipid rafts, have been recently involved in the triggering of death receptor-mediated apoptosis. There is a co-clustering of lipid rafts and Fas/CD95 death receptor. The adaptor molecule FADD protein and procaspase-8 were also recruited into lipid rafts and form the death-inducing signaling complex (DISC). An increasing number of antitumor drugs are been found to induce apoptosis through recruitment of Fas/CD95 into membrane rafts, and some of these compounds accumulated in raft membrane domains. Edelfosine was the first antitumor drug reported to induce apoptosis in cancer cells through co-clustering of lipid rafts and Fas/CD95 death receptor (Gajate et al. 2012). Combination of antitumor gene therapy, transfecting the inhibitor of growth 4 (ING4) tumor suppressor gene, with radiation therapy produced a substantial up-regulation of the pro-apoptotic genes Bax, Fas, FasL and cleaved caspase-3, as well as a down-regulation of the apoptosis inhibitor Bcl-2 gene, and better antitumor results than radiotherapy alone (Ling et al. 2012). The success of the Fas/FasL system targeting for therapeutics will require the understanding of the alterations conferring resistance in tumor cells. The most appropriate would be using sensitizing chemotherapeutic

or radiotherapeutic agents in combination with effective targeted therapies (Villa-Morales and Fernández-Piqueras 2012).

The called “immunecheckpoints” are points of control of the adaptive immunity in an attempt to limit the damage induced by inflammation and laterally these control mechanisms play a role in limiting tumor-specific immunity. As an example, programmed death-1 (PD-1) is a receptor that is found on the surface of T cells after encounter with an antigen (Fig. 25) (Saibil et al. 2007). When PD-1 is bound by ligands PD-L1 (expressed on cells of multiple lineages) or PD-L2 (expressed on macrophages and DCs), the function of T cells is inhibited. Over expression of PD-L1 has been reported in several tumor types and is associated with poor prognosis, presumably because of the inhibitory effect ligation would have on the antitumor immune response (Hamanishi et al. 2007, Gao et al. 2009). CTLA-4 is a negative costimulatory molecule expressed on both activated T cells and Treg cells that inhibits chronically activated and exhausted T cells (Fig. 26) (Engelhardt et al. 2006, Wherry et al. 2007) and promotes Treg function (Teft et al. 2006). Both markers PD-1 as CTLA-4 are being used to develop immunotherapeutic strategies such as the CTLA-4 blocking monoclonal antibody (mAb) Ipilimumab (Hodi et al. 2010).

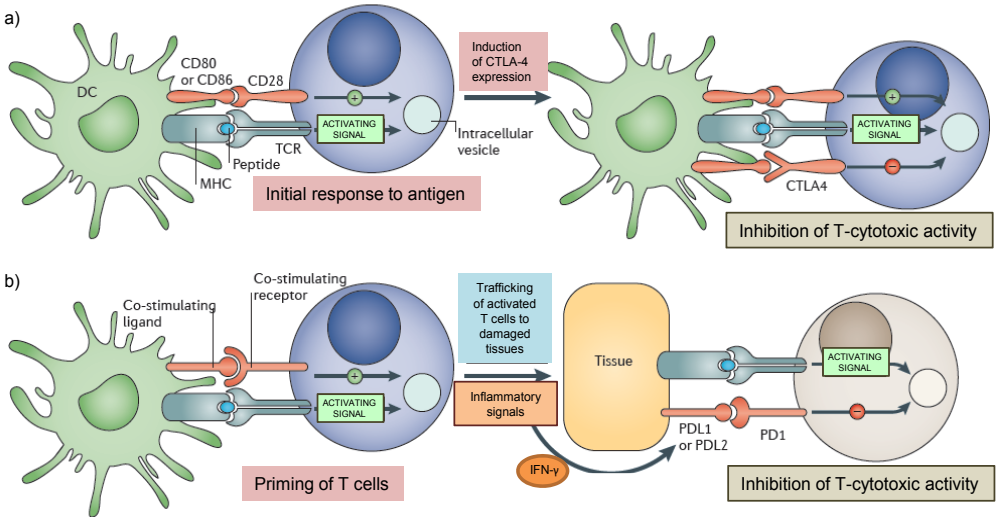


Figure 26. Immune checkpoints regulate different components in the evolution of an immune response. a) The cytotoxic T-lymphocyte-associated CTLA4-mediated immune checkpoint is induced in T cells at the time of their initial response to antigen. The level of CTLA4 induction depends on the amplitude of the initial TCR-mediated signalling, so high-affinity ligands induce higher levels of CTLA4. After the TCR is triggered by antigen encounter, CTLA4 is transported to the cell surface. b) By contrast, the major role of the programmed cell death protein PD1 pathway regulates inflammatory responses in tissues by effector T cells recognizing antigen in peripheral tissues. Activated T cells up-regulate PD1 and the inflammatory signals in the tissues induce the expression of PD1 ligands, which down-regulate the activity of T cells that, in the case of a microorganism infection limit collateral tissue damage. The best characterized signal for PDL1 induction is IFN-γ, which is predominantly produced by Th 1 cells. Excessive induction of PD1 on T cells in the setting of chronic antigen exposure can induce an exhausted or anergic state in T cells. Adapted from *Pardoll 2012*.

Evading immune recognition

Cancer cells develop non immunogenic phenotypes to hide from the immunosurveillance. An important mechanism of immunoescape process is the loss or alteration of the expression of the glycoproteins of the major histocompatibility complex or alterations of antigenic peptide-processing machinery (Garrido and Algarra 2001). Indeed, cancer cells may present losses in the expression of a specific tumor-specific antigen to avoid anti-tumor immunoresponse. This happen is associated to the phenomenon of the "Immunodominance" (Schreiber et al. 2002). An immunodominant response is preferentially directed to a determinate or immunodominant antigen that is associated with a specific MHC class I allele. Immunodominance may allow cancer cells to escape even after the loss of a single MHC class I allele associated with a specific antigen, because the surrounding antigen presenting cells retain the cross-presentation of the immunodominant antigen by this allele and sustains the immunodominant response. Immunodominance may also prevent the development of responses to new tumor-specific antigens that may arise during tumor progression (van Waes et al. 1996). Multiple alterations in the expression of MHC-I molecules have been detected in cancer cells that allow them remain invisible for immunity.

MHC-I and immunoescape

Malignant transformation involves a process of somatic evolution due to the genetic instability that promotes within the tumor cells population the development of variants that allow the immunoescape and that are selected by T-lymphocytes (Marincola et al. 2000, Seliger et al. 2002). MHC class I molecules presented the tumor-associated antigens to T-lymphocytes activating cell proliferation, cytokine production, and target cell lysis. Any alteration affecting the expression of MHC-I molecules, as the expression and function of APM components, or the expression of class I heavy chains or β_2-m, in tumor cells will have a profound effect in the recognition and killing of those tumor cells mediated by T-lymphocytes (Johnsen et al. 1999, Seliger 2008). But MHC-I molecules also regulate the activity of NK cells that generally target tumor cells lacking MHC-I expression, although NK-activity is not so clear due to the complexity of inhibiting and activating receptors they present (Lopez-Botet et al. 1996, Moretta et al. 1996). Based on function, NK cell receptors can be divided into activation receptors (NKAR) and inhibition receptors (NKIR) which ligands, among others, are the MHC-I molecules (Pan et al. 2002).The perpetual balance between signals derived from inhibitory and activating receptors controls NK cell responsiveness (MacFarlane and Campbell 2006). MHC class I alterations is a widespread immunoescape mechanism largely studied.

First detections of MHC-I lack in mouse tumors was described in 1976 (Garrido et al. 1976) followed by the detections of those alterations in human tumors in 1977 (Pellegrino et al. 1977). Since then several HLA class I deficiencies have been described in human tumors (Garrido et al. 1993, 1997), the frequency of the losses has been evaluated by studying series of tumor samples with different histological origin (Cabrera et al. 1996, 1998 and 2000, Koopman et al. 2000, Maleno et al. 2006). Patient tissue samples showed alterations in HLA-I from the 40% to the 90% of the human cancers (Algarra et al. 2000, Marincola et al. 2000). For example, the 97% of laryngeal tumors showed alterations in the HLA-I phenotype (Cabrera et

al. 2000, Maleno et al. 2002), the 96% of cervix carcinomas presented HLA-I alterations (Koopman et al. 2000), and same percentage was found for breast cancer (Cabrera et al. 1996), a 87% of colorectal carcinomas (Maleno et al. 2004) or a 72% of bladder carcinomas (Cabrera et al. 2003).

Different types of HLA-I deficiencies have been found in tumors, seven major HLA-I altered phenotypes were defined and so the different molecular mechanisms that led to those total or partial HLA-I losses (Fig. 27) (Garcia-Lora et al. 2003):

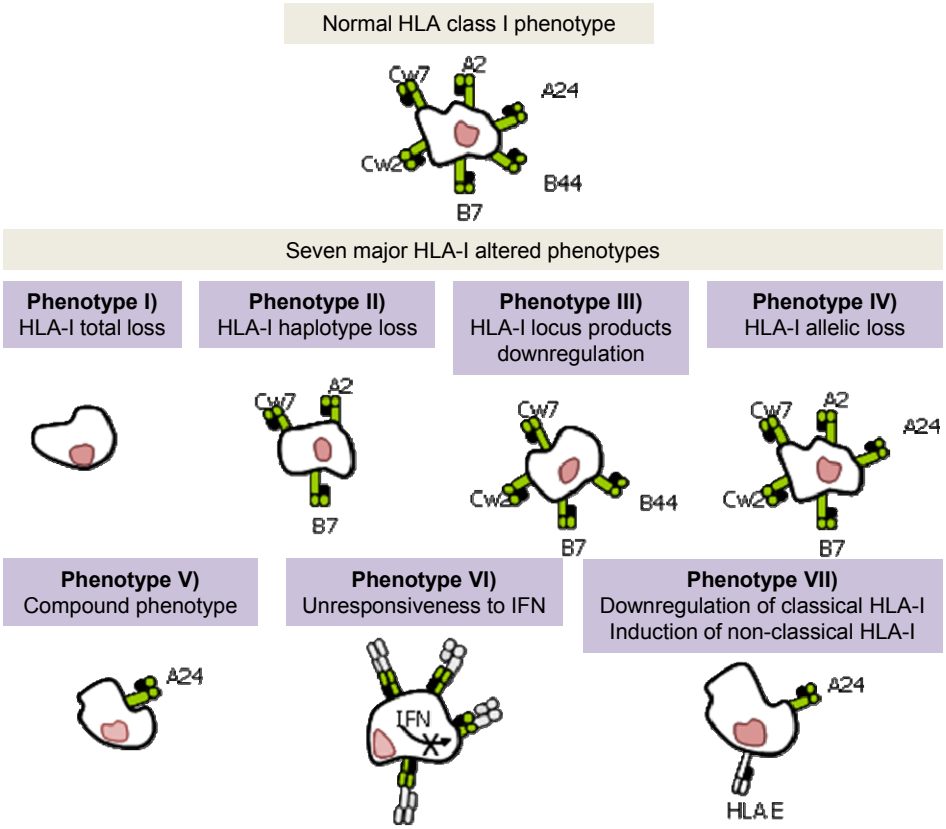


Figure 27. Normal cells express six HLA-I alleles. During malignancy disease, tumor cells develop mechanisms to avoid T cells recognition. They may partially or totally lose the expression of HLA-I molecules, develop resistance to IFN signals or express non-classical HLA-I molecules. Adapted from *Garcia-Lora et al. 2003.*

Phenotype I: HLA-I total loss. This phenotype is characterized by the absence of any HLA-I expression.

It can be associated with the lack or a truncated synthesis of β_2-m (Browning et al. 1996, Chen et al. 1996, Benitez et al. 1998, Wang et al. 1999). Alterations of the APM components could also induce a total loss of HLA-I expression (Seliger 2008). For example, the failure of peptide transport by TAPs or peptide processing by LMPs lead to HLA-I abnormalities (Restifo et al. 1993, Ritz et al. 2001, Vitale et al.

2002) correlated with reduced survival or increased disease stage, suggesting that APM deficiencies are of clinical relevance (Mehta et al. 2008, Meissner et al. 2008). Moreover, epigenetic events such as DNA hypermethylation or histone deacetylation may play a critical role in modifying HLA class I antigen (Magner et al. 2000, Chang et al. 2005, Chou et al. 2005, Khan, Gregorie and Tomasi 2008) and APM components (Tomasi et al. 2006, Setiadi et al. 2007) expression in malignant cells (Fig. 28) (Campoli and Ferrone 2008).

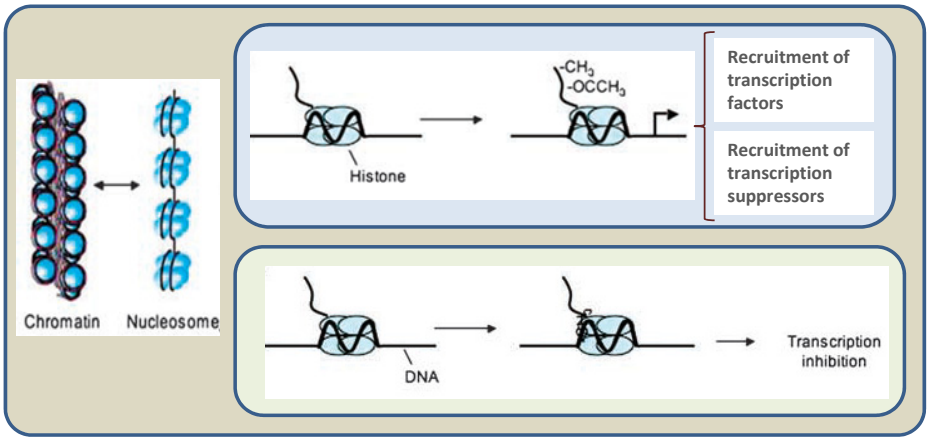


Figure 28. Epigenetic modulations underlie some abnormalities in MHC-I expression identified in malignant cells. Modification of histones through methylation or acetlylation results either in an open chromatin structure ready for expression or in a closed chromatin configuration impermeable to transcription factors. DNA methylation is the enzymatic addition of a methyl group to position 5 of cytosine incorporated into DNA. In mammals, methylation is largely limited to cytosines that are part of the symmetrical dinucleotide CpG (cytosine and guanine separated by a phosphate) resulting in permanent loss of gene expression. Adapted from *Campoli and Ferrone 2008.*

Phenotype II: HLA-I haplotype loss. The loss of one HLA-I haplotype characterizes tumors cells carrying phenotype II.

Phenotype II represent the most common HLA-I alteration in human tumors (Ramal et al. 2000). The majority of the analysis performed in tumors with this altered phenotype revealed loss of heterozygosity (LOH) by deletion of a full chromosome 6 or a large genomic region. It's been found in different humans tumors (Torres et al. 1996, Feenstra et al. 1999, Jimenez et al. 1999, Koopman et al. 2000, Mendez et al. 2001, Maleno et al. 2002).

Phenotype III: HLA-A, -B or -C locus products down-regulation. The two alleles of a specific HLA-I loci can be coordinately down-regulated in tumor cells.

This alteration is associated with HLA-I gene transcription since suppressor regulators bind to locus-specific DNA motifs decreasing the expression of an HLA-I locus (Soong and Hui 1992). For example, in melanomas the increased c-myc

transcription interferes as the promoter level with HLA-B transcription, selectively down-regulating HLA-B locus (Peltenburg and Schrier 1994).

Phenotype IV: HLA-I allelic loss. This alteration is characterized by the absence of a single HLA class I allele.

Point mutations, partial deletions of HLA-I genes, chromosomal breakage or somatic recombination can be some of the causes of HLA-I allelic loss (Brady et al. 2000, Serrano et al. 2000).

Phenotype V: Compound phenotype is defined by the combination of two phenotypes.

An example could be the combination of phenotypes II and III resulting in the down-regulation of HLA locus combined with an HLA haplotype loss. It has been observed in some tumors (Ikeda et al. 1997, Real et al. 1998).

Phenotype VI: Unresponsiveness to interferons. Describes tumor cells that have loss the capacity to up-regulate HLA-I molecules in response to IFNs.

Unresponsiveness to IFN treatment was analyzed in different tumors and can be frequently found in human cancers (Kaplan et al. 1998, Rodríguez et al. 2007). The involvement of IFN signal transduction pathways in the transcriptional regulation of APM promoters have been established, but there exist limited information about the underlying molecular mechanisms of defective IFN-inducible APM components expression (Seliger et al. 2008). Based on the current knowledge STAT1 and IRF1 are involved in the transcriptional regulation of the dual TAP1 and LMP2 promoter, deficiencies in these regulatory factors could lead the loss of expression of TAP1 and LMP2 (Dovhey et al. 2000). But tumors can display other multiple molecular mechanisms that cause the resistance to IFNs (Wong et al. 1997, Huang et al. 2002, Wellbrock et al. 2005, Lesinski et al. 2007).

Phenotype VII: Down-regulation of classical HLA-A, -B or/and -C expression and appearance of non-classical HLA-I molecules.

Some tumors develop the capacity of reducing class I classical molecules expression while inducing the expression of non-classical. This combination could lead to avoid T-lymphocyte cytotoxicity and as the same time the inhibition of NK cells mediated by the interaction of non-classical molecules (HLA-E)/inhibitory NK receptors (CD94/NKG2a) (Marin et al. 2003).

ANTI-TUMOR TREATMENTS

Traditional anti-tumor treatments

Anti-tumor conventional therapies currently involve the combination of surgery, local radiotherapy and chemotherapy.

Surgery can be used to diagnose, treat, or even help in the prevention of cancer in some cases. Most people with cancer will have some type of surgery. It often offers the greatest chance for cure, especially if the cancer has not spread to other parts of the body. Surgical treatment could present post-operative problems as morbidity or functional deterioration, or in some cases mortality. The most common surgical techniques are laser surgery, cryosurgery, electrosurgery, laparoscopic surgery, and thorascopic surgery (American Cancer Society, Understanding cancer surgery). Minimal invasive surgical procedures are gaining importance in the last years and aim at minimizing the operative trauma and associated inflammatory reactions to achieve faster convalescence after surgery (Schneiter et al. 2012). Within the scope of modern multimodal treatment concepts radical surgical resection is essential and the main pillar of curative treatment of several malignancies as lung cancer (Lardinois 2012), prostate carcinoma (Namiki and Yoshioka 2012) or papillary thyroid carcinoma (Abboud and Tannoury 2011).

Radiation therapy is a physical agent used as a local treatment to kill the cancer cells (Bernier et al. 2004). The ionizing radiation forms ions (electrically charged particles) and deposits energy in the cells of the tissues it passes through, producing damages in the genetic material of cells and thus blocking their ability to divide and proliferate (Jackson 2009). Although radiation damages both normal cells as well as cancer cells, normal cells usually can repair themselves at a faster rate and retain its normal function status than the cancer cells. One of the goals of radiation therapy is to maximize the radiation dose to abnormal cancer cells while minimizing exposure to normal cells (Begg et al. 2011). In this direction, a possible approach is the use of radioimmunotherapy, which involves the administration of radiolabeled monoclonal antibodies that are directed specifically against tumor-associated antigens or against the tumor microenvironment (Pouget et al. 2011). Radiation can be given with the intent of cure as well as an effective modality of palliative treatment to relieve the symptoms caused by the cancer. There are two ways to deliver radiation treatments. External beam radiation is delivered from outside the body by aiming high-energy rays (photons, protons or particle radiation) to the location of the tumor. This is the most common approach in the clinical setting. Internal radiation or brachytherapy is delivered from inside the body by administrating radioactive sources, sealed in catheters or seeds directly into the tumor site. This is used particularly in the routine treatment of gynecological and prostate malignancies as well as in situations where retreatment is indicated, based on its short range effects.

Chemotherapy is a kind of treatment that uses drugs to attack cancer cells. Chemotherapy may be used to: keep the cancer from spreading, slow tumors growth, kill cancer cells that may have spread to other parts of the body, relieve symptoms such as pain or blockages caused by cancer. From the first time, in 1942, when nitrogen mustard chemotherapy was applied to x-ray resistant patients in terminal stages of lymphosarcoma reducing the size of the local tumors and distant nodes, and increasing the survival of the

patients, chemotherapy has become in the main anti-tumor therapy. Cytotoxic compounds could be classified into several groups depending on their chemical nature and on their pharmacological action mechanism (Zitvogel et al. 2008a):

Antimetabolites: involve a group of agents that are analogous to endogenous substrates but that instead of taking part of cellular processes act as inhibitors of those. Examples of this kind of drugs are 5-fluorouracil, a fluoropyrimidine analogue of endogenous pyrimidine nucleoside that perturbs ribonucleic acid (RNA) and DNA synthesis; gemcitabine (Gemzar, Eli Lilly) that is another synthetic pyrimidine analogue or methotrexate, which inhibits dihydrofolate reductase depleting cells of reduced folates, which are required for DNA synthesis.

Alkylating agents: these include agents chemically related to nitrogen mustard as melphalan (Alkeran, Celgene) and cyclophosphamide. Hepatic metabolism converts these drugs in aldophosphamide and phosphoramide mustards, active metabolites that bind to DNA inhibiting DNA replication and initiating cell death.

Anthracyclines: Synthetic agent based in samine and tetrahydronaphthacenedione, which intercalate between the base pairs of the DNA or RNA strand inhibiting DNA or RNA synthesis and, thereby the cell replication. They also produce DNA and membrane damage by creating iron-mediated free oxygen radicals.

DNA methyltransferase inhibitors: group of drugs which pharmacological action is the inhibition of the DNA transferasa, so causing demethylation which in turn leads to the reactivation of silenced genes, including some tumor-suppressor genes. An example of this drug family is the 2'-deoxy-5-azacytidine (DAC), an analogue of cytidine nucleoside which incorporates in RNA or DNA strands exerting its cytotoxic activity.

Platinum compounds: group of cytotoxic compounds as the cisplatin or the oxaliplatin (Eloxatin, Sanofi-Aventis), which active derivatives form both inter- and intra-strand platinum-DNA crosslinks inhibiting DNA replication and transcription.

Spindle poisons: this group corresponds to the family of the taxanes as paclitaxel (Taxol, Bristol-Myers Squibb) and docetaxel (Taxotere, Sanofi-Aventis). Their action mechanism is the stabilization of guanosine-diphosphate (GDP)-bound tubulin microtubules. Microtubules are essential structures for cell division, so taxanes stop the cell mitosis

Vinca alkaloids: this group of drugs inhibits cell division by destroying the mitotic spindles. These compounds include vinblastine, vincristine, vindesine, and vinorelbine.

Glucocorticoids: Used in the treatment of several lymphoproliferative diseases. Moreover glucocorticoids are prescribed to cancer patients to attenuate chemotherapy-associatednausea and vomiting. Examples of glucocorticoids are dexamethasone, betamethasone, prednisolone or cortisone.

Immunological aspects of chemotherapy and radiotherapy

Many chemotherapeutic agents have important immunosuppressive side effects, inhibiting or killing immune effector cells, or provoking immune-anergy or -blockade. Actually some cancer chemotherapeutics are also used for severe autoimmune diseases as immuno-suppressant medicaments. This is the case of cyclophosphamide and methotrexate which impair proliferation and functions of peripheral T lymphocytes. Glucocorticoids are part of the chemotherapeutic cocktails to attenuate the nausea and vomit side effects associate with chemotherapy. Glucocorticoids suppress the production of proinflammatory cytokines (IFN-α,

IFN-β, IL-1) and chemokines (CXCL8, CCL7, CCL8, CCL11 or CCL20) (Galon et al. 2002). They severely impaired differentiation and antigen presenting function of dendritic cells despite the induction of the toll-like receptors (TLR) (Rozkova et al. 2006). Moreover glucocorticoids block immunity repressing the expression of MHC-II, TCR, STAT-1, CD40 and eliciting production of TGF-β that suppress T and NK cells (Zitvogel et al. 2008a). Chemotherapy may also indirectly immunosuppress by inducing non-immunogenic cell death, for example, protein tyrosine kinase inhibitors, proteasome inhibitors or DNA-damaging drugs produce mitochondrial, lysosomal or endoplasmic reticulum stress inducing apoptosis, which is accompanied by the exposure of phosphatidylserine which triggers phagocytosis by macrophages and the production of anti-inflammatory cytokines. Furthermore, chemotherapy might lead to the sudden and systemic release of numerous dying tumor cells and high amount of tumor antigens resulting immune-tolerance (Morgan et al. 1999, Zitvogel et al. 2008a).

Besides the immunosuppressive effects of traditional therapies, accumulating evidences indicate that some cytotoxic compounds as well as radiotherapy promote specific anticancer immune responses that contribute to the therapeutic effects. Even the mere surgical removal of the primary tumor can reverse the tumor-induced immuno tolerance, restoring the antibody- and cell-mediated immune response (Danna et al. 2004).

In the case of radiotherapy, accumulating data support that adaptive immunity and more specifically CD8+ T cells are required to the efficacy of radiation treatments (North 1984, Sechler et al. 1999, Lugade et al. 2005, Pandey et al. 2005). Furthermore, tumor radiation provokes several immunological effects on immunologic and malignant cells while on the tumor microenvironment (Shiao and Coussens 2010, Finkelstein et al. 2011) (Fig. 29). Actually, it has been shown that radiation induces the expression of IFN-γ on tumor micro-environment and consequently modulates genes with immunomodulatory properties such as MHC-I, adhesion molecules on tumor vasculature as vascular cell adhesion protein 1 (VCAM-1) or chemo-attractants as MIG and IP-10 (Lugade et al. 2008). Several studies have shown *in vivo* and *in vitro* up-regulation of MHC-I surface expression on irradiated tumor cells and its beneficial effects on anti-tumor immune-response. Histological studies showed an up-regulation of gene transcription and surface expression of cancer-testing antigens and HLA-I of paired biopsies from sarcoma patients before and after radiation, correlated with a major infiltration by CD4+ and CD8+ T cells and granzyme production (Sharma et al. 2011). Radiation enhances the degradation of pre-existing proteins and induces the production of new proteins increasing peptide pool what results in the induction of antigen presentation by MHC-I molecules (Reits et al. 2006). Radiation of established invasive mouse glioma induced the expression of Beta2-microglobulin, down-regulated during *in vivo* growth, increased the levels of CD4+ and CD8+ T cells and NK cells in tumor infiltration, as well as reduced the tumor size and increased the survival of mice. Better results were obtained when radiotherapy treatment was combined with peripheral vaccination based on irradiated granulocyte-macrophage colony-stimulating factor (GM-CSF)-transduced tumor cells (Newcomb et al. 2006). Thus, through inducing immunogenicity of tumor cells and modifying immunological tumor microenvironment, radiotherapy could result useful combined with immunotherapy (Chakraborty et al. 2004, Meng et al. 2005, Sharkey et al. 2011).

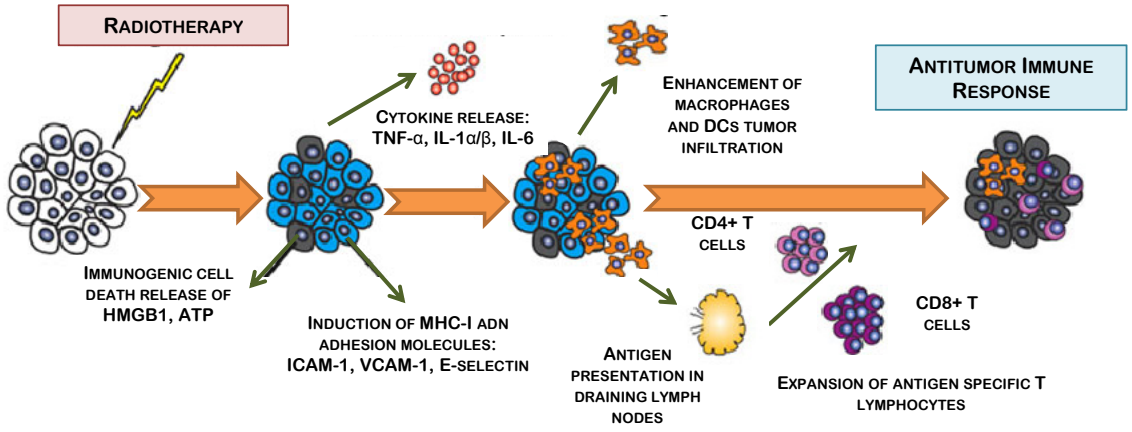


Figure 29. Tumor cells respond to radiotherapy by inducing cytokines, adhesion molecules and MHC-I surface expression. The death of tumor cells also generates the secretion of HMGB1 as well as ATP. Macrophages and DCs are recruited and activated resulting in their migration to draining lymph nodes were CTLs, which should infiltrate the tumor, are activated. Adapted from *Shiao and Coussen 2010.*

Chemotherapy and radiotherapy may contribute with anticancer immunity in different ways. For example DNA damage as result of radiation or topoisomerase inhibitors stimulates DNA repair signaling factors as ATM, CHK1, or p53 that have tumor suppressor characteristics. This DNA-damage response induces the expression of NKG2D ligands on tumor cells, which would interact with NKG2D receptors on NK, NKT, γδ T, and CD8+ T cells (Gasser et al. 2005) (Fig. 30). The activation of p53 promotes tumor cell senescence and tumor regression by activating NK cells, neutrophils, and macrophages populations and the production of proinflammatory cytokines (IL-15, macrophage colony-stimulating factor (M-CSF)), adhesion molecules (intercellular adhesion molecule 1 (ICAM1), VCAM1) and chemokines (CCL2, CXCL1) (Xue et al. 2007) (Fig. 30). Low doses of cyclophosphamide decrease the amount and the actions of Treg, by down-regulating the expression of FoxP3 and of the glucocorticoid-induced TNF-receptor-related protein (Lutsiak et al. 2005). Cyclophosphamide induces IFN-α production, augmenting antibody responses and persistence of memory T cells (Schiavoni et al. 2000) which have a synergy effect with specific immunotherapies in the eradication of tumors (Sutmuller et al. 2001). The anthracycline doxorubicin, for example, enhances the antitumor potency of GM-CSF-transfected tumor-cell vaccine (Nigam et al. 1998). And various cytotoxic agents as cisplatin, cyclophosphamide, paclitaxel or doxorubicin improve the antileukaemic effect of IL-12 (Zagozdzon et al. 1998). It has been found that those cytotoxic agents elicit an immunogenic cell death (Casares et al. 2005, Obeid et al. 2007), which could explain the synergy effect with the immunotherapies. In this situation, the stressed or dying tumor or stromal cells, under the influence of cytotoxic agents, induce the expression of eat-me signals (calreticulin, HSPs, HMGB1) and antigen transfer, as well as the antigen processing and presentation which promote DC maturation, co-stimulation, polarization and trafficking (Apetoh et al. 2007, Obeid et al. 2007) (Fig. 30). In addition, stressed tumor cells may stimulate the expression of NKG2D, CD95 and TRAILR, increasing the stimulation of NK cells and their susceptibility to lysis by immune effectors. Paclitaxel binds to mouse TLR4, mimicking LPS signal and activating macrophages and DCs (Byrd-Leifer et al. 2001). Paclitaxel also sensitizes to tumor vaccines that express GM-CSF (Machiels et al. 2001) in breast HER2/neu tolerant

cancer and improves the efficiency of intratumor DC inoculations, which benefited in the treatment of breast (Yu et al. 2003) and lung cancer (Zhong et al. 2007). The synthetic pyrimidine gemcitabine inhibits B cell proliferation and antibody production in response to tumor antigens (Nowak et al. 2002), reduces the frequency of MDSCs (Suzuki et al. 2005) and enhance the DC cross-presentation of tumor antigens (Nowak et al. 2003). These immunostimulatory effects have been confirmed in patients with pancreatic (Plate et al. 2005), non-small-cell lung (Levitt et al. 2004) or colon (Correale et al. 2005) carcinoma receiving gemcitabine in combination with recombinant cytokines or vaccines. The antineoplastic effect of DAC involves reversing DNA hypermethylation, reactivating the expression of tumor suppressor genes, but also inducing the expression of MHC-I molecules and cancer testing antigens (Serrano et al. 2001) (Fig. 30) and restoring IFN-γ-mediated apoptotic-cell-death pathway (Lubbert 2000). Treatment of leukaemia and melanoma in mice with DAC, was potentiated by combining with IL-12, obtaining a synergistic stimulation of T cell-mediated antitumor activity (Kozar et al. 2003).

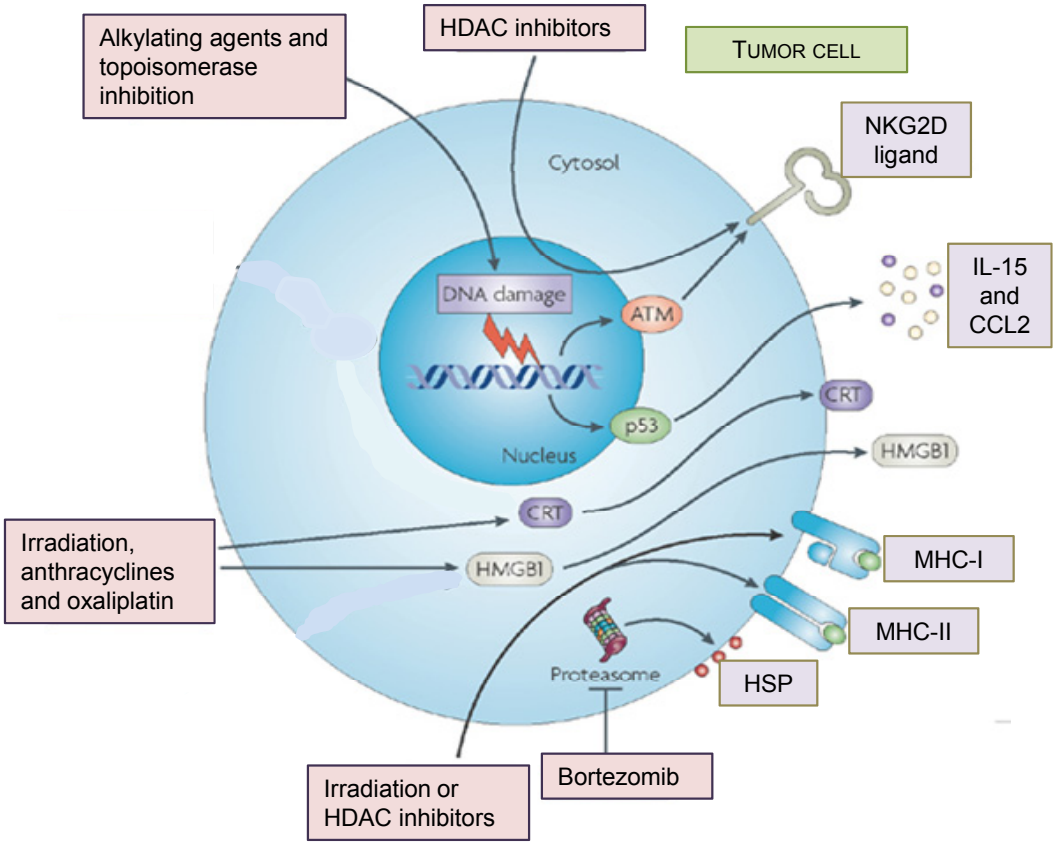


Figure 30. Cytotoxic agents may produce different effects over tumor cells that finally have an immunological consequence. DNA damages would trigger DNA repair signaling up-regulating p53 and ATM which induce NKG2D ligand expression and the secretion of immunostimulatory cytokines (IL-15, CCL2), enhancing NK-cell and T-cell mediated tumor cell killing. Cellular death may constitute the expression of "eat-me" signals as HMGB1 or calreticulin activating DC-mediated cross-presentation of apoptotic tumors to T cells. Some drugs or radiation could also induce the expression of MHC-I, MHC-II and HSP molecules facilitating antigen uptake and maturation of DCs. Adapted from *Zitvogel et al. 2008a.*

Looking for new strategies and targets

Although traditional therapies have extensive curative potential, there are limiting factors as the side effects, the adverse response of the tumor microenvironment to the treatments and tumor-acquired mechanisms of evasive resistance. So, it has become essential the development of novel therapeutic strategies for tumor sensitizing to traditional therapies, incorporating the involvement of the immune response and the role of cancer stem cells, as well as direct targeting of signal transduction pathways (Linkous and Yazlovitskaya 2012). Understanding genomic alterations in cancer cells that allow them to blunt intrinsic and extrinsic barriers, as well as to avoid or interfere with the anti-tumor immunoresponse, means the main clue for the identification of new cancer cell targets to develop new therapeutic strategies.

Targeted therapy

The identification of appropriate targets is based on a detailed understanding of the molecular and genetic changes underlying cancer: how oncogene and tumour suppressor gene networks influence the cancer cells to proliferate or die, the impact of the tumour microenvironment, the stress signals, such as DNA damage, the subpopulation of cancer cells with stem-cell-like properties that may be critical for triggering tumour development and the anti-tumor immune response developed by the patient (immune cells, cytokines, chemokines, regulatory or tolerogenic signals). All this information provides a conceptual framework within which experimental cancer therapeutics can consider the design of targeted agents (Sawyers 2004).

Targeting mutant oncogenes. In breast cancer, over-expression of HER2 (ErbB2) occurs in approximately 25% of patients and is associated with shorter survival (Nahta et al. 2006) The epidermal growth factor receptor (EGFR) is widely up-regulated in solid tumors and mediates many characteristics of malignant phenotype, including proliferation, protection from apoptosis, and tumor cell motility. These findings lead to the development of antibodies that target HER2 and EGFR. The validity of both growth factor receptors as therapeutic targets is illustrated by the successes of trastuzumab and cetuximab (Laird and Cherrington 2003).

The majority of growth factor receptors are composed of extracellular, transmembrane, and cytoplasmic tyrosine kinase domains. Receptor tyrosine kinase activation regulates many key processes including cell growth and survival and their deregulation has been found in a wide range of cancers. Low-molecular-weight Kinase inhibitors, such as imatinib, targeting tumors with mutant c-Kit, and gefitinib, targeting non-small cell lung cancer with mutant EGFR, have received marketing approval. Hepatocyte growth factor, fibroblast growth factor receptor, and insulin-like growth factor-I receptor are frequently genetically altered or otherwise deregulated in advanced cancers, suggesting them to be attractive therapeutic targets (Christensen et al. 2005). Tyrosine kinase inhibitors of these receptors have not yet been approved for marketing; however, many anticancer drug candidates are currently undergoing clinical trials (Takeuchi et al. 2012). Chronic myelogenous leukemia (CML) is caused by a chromosomal translocation that promotes an abnormal fusion protein between the kinases B-cell receptor (BRC) and cellular Abelson leukaemia-virus protein (ABL), which displays constitutive activation of the Abl kinase leading to uncontrolled proliferation of the leukemia cells. Gastrointestinal stromal tumors are driven by activating point mutations in the c-Kit or

platelet derived growth factor receptor (PDGFR)-α kinases. Imatinib mesylate (Gleevec, Novartis) effectively blocks the activity of all three kinases and produces positive clinical responses in these situations, the effects of the treatment correlate precisely with the presence of the BCR-ABL, c-Kit or PDGFR-α kinases mutations in the tumour (Sawyers 2003). Extending this treatment to larger numbers of cancer patients would require establishing the frequency of kinase mutations in human cancers. It has been found B-Raf kinase mutations in melanoma patients (Davies et al. 2002), or fusion of mutant FIPL1/PDGFR-α kinases in hypereosinophilic syndrome (Cools et al. 2003). Moreover, clinical responses are also observed when tumors contain mutations in genes that activate the kinase indirectly, as for example, the chromosome translocation that causes overproduction of the kinase ligand PDGF in dermatofibrosarcoma protuberans (Rubin et al. 2002). Sadly, Gleevec resistance has been found in CML patients with disease relapse caused by the expansion of tumor subclones that contain single amino-acid mutations in the BCR-ABL kinase domain that prevent enzyme inhibition by Gleevec (Gorre et al. 2001, Branford et al. 2002, Shah et al. 2002). It has been developed a second generation of dual Src/Abl kinase inhibitors that retain activity against the Gleevec-resistant mutants (Shah et al. 2004).

EGFR activation via ligand binding results in signaling through various pathways ultimately resulting in cellular proliferation, survival, angiogenesis, invasion, and metastasis. Aberrant expression or activity of EGFR has been strongly linked to the etiology of several human epithelial cancers including but not limited to head and neck squamous cell carcinoma, non-small cell lung cancer, colorectal cancer, breast cancer, pancreatic cancer, and brain cancer (Brand et al. 2011). Gefitinib (Iresa, AstraZeneca) and Erlotinib (Tarceva, Roche) are EGFR inhibitors developed for the therapy of advanced non-small cell lung cancer harboring epidermal growth factor receptor mutation or overexpression (Ma et al. 2012). Clinical responses with these treatments are associated with point mutations in the EGFR kinase domain and present a modest 10% response rate in all patients (Paez et al. 2004, Sordella et al. 2004). Monoclonal antibodies anti-EGFR (i.e. Nimotuzumab or Cetuximab) are other alternative for the inhibition of this receptor inducing apoptosis of tumor cell lines, and which mechanisms of action depends on the action of T lymphocytes (Talavera et al. 2009). Designing mouse anti-EGFR antibodies have allowed a profound study of molecular mechanisms underlying anti-tumor activity and mechanisms of resistance to anti-EGFR (Garrido et al. 2004).

Inhibiting pathway components. The pathway-mutation paradigm could be applied for example to tumors with molecular lesions in the primary cell-cycle machinery which should be susceptible to CDK inhibition. Melanomas have mutations in CDKs (Wolfel et al. 1995), mantle cell lymphomas have translocations leading to increased cyclin D1 expression (Williams et al. 1993), and other tumors with loss-of-function mutations in p16 or Rb. Signalling pathways more commonly mutated in human cancers are Myc, Ras, TGF-β through receptor mutations or Smad 4 loss, that impinge directly on cell-cycle regulation. A third example is to use inhibitors of kinases in the PI(3)K/Akt/mTOR pathway to treat tumors with mutations in the tumor suppressor gene PTEN, the negative principal regulator of this pathway. Proof of concept for this approach has been demonstrated in numerous murine models using rapamycin analogues that block mTOR activity (Sawyers 2003). Because mTOR receives signalling inputs from several signalling pathways, tumors with a number of distinct molecular lesions could be sensitive to treatment.

Epigenetics drugs for cancer treatment. Epigenetic mechanisms involve DNA methylation and covalent histone modifications, and vary the transcription of genes leading to phenotypic variations and changes in the interactions with the environment (Berger et al. 2009). The disruption of such changes underlies a wide variety of pathologies, including cancer (Jones and Baylin 2007, Bird 2002). Epimutations are reversible, in contrast with genetic mutations. Epigenetics drugs would restore the normal epigenetic landscape in cancer cell by inhibiting the enzymes that drive epigenetic machineries. Four drugs have been approved by the FDA for cancer treatment: Two DNA methyltransferasas inhibitors, vidaza and decitabine (Garcia et al. 2010) (5-aza- and 5-aza-2'-deoxycytidine, respectively) for the treatment of myelodisplastic syndrome; and two histone deacetylase inhibitors (HDACi) vorinostat (suberoylanilide hydroxamic acid) (O'Connor et al. 2006) and romidepsin (FK-228) (Byrd et al. 2005, Piekarz et al. 2009) for the treatment of hematological malignancies. The effects on gene transcription of vidaza and decitabine involve differential effects on genes relevant to leukemogenesis (Flotho et al. 2009). In the other hand, the main anticancer effects of HDACi are cell cycle arrest in G1 or G2-M, induction of differentiation and apoptosis, but they can also inhibit angiogenesis and metastasis and enhance the sensitivity to chemotherapy (Nebbioso et al. 2005). The use of epigenetic drugs will required further knowledge about optimal doses for single or combined therapy, as well as sequence of delivery in combined therapies, the design of new less-toxic agents against specific enzymes of the epigenetic machinery or even against different isoforms or mutated variants of particular enzymes involved in very specific types of cancers (Rodríguez-Paredes and Esteller 2011).

Targeting the microenvironment. The stroma can exert profound effects on the initiation and progression of epithelial malignancies. Elucidation of the molecular circuitry of this crosstalk could profoundly influence in targeted cancer therapy, and may provide new prevention strategies. As an example, TGF-β exerts direct anti-proliferative effects on epithelial cells but also has immunosuppressive properties that may hamper host immunosurveillance. Remarkably, the tumour-suppressive effect of TGF-β is critical in stromal cells and adjacent epithelium, as epithelial malignancy can develop in certain organs when the TGF-β receptor is deleted only in stromal fibroblasts (Bhowmick et al. 2004). Furthermore, earlier studies that demonstrate pro-oncogenic characteristics of cancer-associated fibroblasts isolated from human prostate tumors (Olumi et al. 1999). Antisense oligodeoxynucleotide specific for TGF-β2 (Trabedersen, Antisense Pharma) has a profound response affecting both stromal cells and cancers overexpressing TGF-β2 as pancreatic carcinoma, melanoma and glioma. Upon the influence of hypoxia, tumor cells secrete cytokines that activate stromal cells to produce proteases and angiogenic factors. The proteases degrade the stromal extracellular matrix (ECM) and participate in the release of various ECM fragments, named matrikines, capable to control tumor invasion and metastasis dissemination. Peptide fragments derived from the ECM protein collagen have an anti-cancer activity that comprises anti-proliferative effect on tumor cells and on endothelial cells by induction of cell apoptosis or cell cycle blockade and the induction of a loss of their migratory phenotype. As examples, endostatin (Endostar, Simcere) derived from collagen XVII is currently used in China, and canstatin, tumstatin and tetrastatin, respectively derived from the NC1 domains of α2, α3 and α4 chains of collagen IV are in clinical trials (Monboisse et al. 2012). Angiogenesis is critical to the growth of human tumors and the development of metastasis. Amongst the many proangiogenic mechanisms identified, the VEGF signaling pathway has been implicated as the key regulator of tumor

neovascularisation. Various therapeutic agents targeting the VEGF pathway have been successfully developed, with many now approved and in routine clinical use. In general, VEGF-mediated angiogenesis can be inhibited by 2 approaches: antibodies directed against VEGF ligands or VEGF receptors (VEGFRs) and tyrosine kinase inhibitors targeting the VEGFRs (Tie and Desai 2012). In 2004, the monoclonal antibody directed VEGF-A ligand (which is essential for endothelial cell proliferation) bevacizumab (Avastin, Genentech) became the first Food and Drug Administration (FDA)-approved angiogenesis inhibitor. Bevacizumab has been used for the treatment of colon cancer, in conjunction with chemotherapy (Hurwitz et al. 2004). In 2005, sorafenib (Nexavar, Bayer) was approved by the FDA as a small-molecule kinase inhibitor of many intracellular and extracellular kinases. Most affected are Raf kinases, VEGFR and PDGFR, so reducing tumor growth as angiogenesis. Notably, the VEGF antibody and small molecule inhibitors targeting the VEGF tyrosine kinase receptor have both shown impressive single-agent activity in renal cancer (Yang et al. 2003). These tumors are highly vascular owing to the deletion of the von Hippel-Lindau tumour suppressor gene, the primary molecular lesion in these cancers. This leads to up-regulation of the HIF transcription factors and constitutive expression of VEGF in tumour cells (Kaelin 2002). So the anti-tumour properties of VEGF pathway drugs may not occur solely through effects on the stroma because these tumors often express VEGFRs.

Targeting cancer stem cells. Recent studies have demonstrated the existence of a minority of tumor cells possessing the stem cell properties of self-renewal and differentiation in leukemia and several solid tumors. Following transplantation tumor stem cells are capable of initiating tumorigenesis. Several signaling pathways which are involved in carcinogenesis, including Wnt/β-catenin, Notch and Oct-4 signaling pathways are crucial in normal stem cell self-renewal decisions, suggesting that breakdown in the regulation of self-renewal may be a key event in the development of tumors (Meng et al. 2012). If these cells have unique patterns of cell-surface antigen expression, monoclonal antibodies might be designed to target them specifically. Perhaps, more promising, is the potential to target specific signalling pathways required for stem-cell function, as Hh and Wnt pathways, which have oncogenic potential based on known mutations in pathway components found in several human tumors. Furthermore, pharmacological blockade of these pathways (like that demonstrated with cyclopamine) seems feasible. The effects of a stem-cell drug may take long to become clinically evident, possibly months or years. An additional consideration is safety for delayed toxicity due to loss of normal stem-cell function in the relevant organ (Sawyers 2004).

Immunotherapy

Cancer progression is naturally hinder by immune cells and their soluble mediators, and cancer cells express tumor-specific and tumor-selective antigens as a consequence of genetic alterations and epigenetic deregulation that induce a specific adaptative immune response. So, the immunology community has been and is hardly working in the design of therapies that induce targeted antitumor immune response (Pardoll and Drake 2012).

Current cancer immunotherapies use a variety of strategies, including therapeutic monoclonal antibodies (mAbs), adoptive cell transfer (ACT) of *ex vivo* expanded autologous or allogeneic tumor-reactive lymphocytes, as well as cancer vaccines. A critical evaluation of these approaches revealed limited overall objective response rates (3,6%) across several early-

phase trials (Klebanoff et al. 2011). Although some positives results have been obtained with immunotherapy, the majority of phase III immunotherapy trials in patients with solid tumors have failed to demonstrate improved overall survival. The analysis of immunotherapy failure reveals that the limited success is due to the immune-inhibitory pathways in the tumor microenvironment that block CTL- and NK-cell-mediated killing, and the immunogenic loss that cancer cells develop during tumor progression, as the loss of surface expression of MHC-I molecules or the changes in the antigen processing (Aptsiauri et al. 2007, Shiao et al. 2011). Given the immunogenic potential that cytotoxic treatments exert, strategies augmenting the antitumor immunoresponse would synergize with the immunity generated by the cytotoxic therapy. Hence studies should focus their efforts in combining therapies and determining the optimal treatment schedule of chemo- or radio-immunotherapy, considering that the kinetics of the patient and the tumor response are critical determinants of the therapeutic response (Zitvogel et al. 2008b).

Augmenting the antitumor immune response. Immunotherapeutic strategies focus to the induction of antitumor immune response include, between others, the administration of cytokines, cell-based therapies, different kinds of vaccination, synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN). Recombinant cytokines or cytokine genes are one of the strategies for cancer therapy. Since cytokines are relatively unstable in vivo, cancer patients have to receive a large amount of the recombinant protein to maintain the required blood concentration for biological activity. Administration of the protein is thereby often toxic to the patients. In contrast, secretion of the cytokine from tumor or vehicle cells by gene transfer is another therapeutic strategy. However, several technical problems to express sufficient amounts of cytokines in appropriate target cells remain unresolved but the potential of cytokine gene therapy is being explored (Tagawa 2000). Among the heterogeneous group of interleukines in particular interleukin 2 has reached clinical practice as an immunostimulating agent in the therapy of metastatic renal cell carcinoma and skin melanomas (Lehners et al. 2011). Among the IFNs, IFN-α2 has been the most broadly evaluated clinically. At the molecular level, IFN-α has multiple effects in a variety of malignancies that range from antiangiogenic to potent immunoregulatory, differentiation-inducing, antiproliferative, and proapoptotic effects. A multitude of IFN-α2 regimens that may be classified as low dose, intermediate dose, and high dose have been evaluated as adjuvant therapy in melanoma (Tarhini et al. 2012). Over the last decade dozens of human clinical trials have been conducted with different CpG ODN in thousands of humans for applications ranging from vaccine adjuvant to immunotherapies for allergy, cancer, and infectious diseases. Along with many positive results have come some failures showing the limitations of several therapeutic approaches (Krieg 2012). Adoptive cell transfer therapy achieves T-cell stimulation *ex vivo* by activating and expanding autologous tumor-reactive T-cell population to a large amount of cells that are transferred back to the patient (Yee et al. 2002, Dudley and Rosenberg 2003, Bollard et al. 2004). It has been described a clear, reproducible response in a substantial percentage of patients, when therapies used tumor-infiltrating lymphocytes (TILs), or the treatment was preceded by non-myeloablative lymphodepletion with systemic chemotherapy (Dudley et al. 2002). The lymphopaenic environment benefits ACT by inducing the elimination of immunosuppressive cells as Treg or iMCs (Dudley et al. 2002, Gattinoni et al. 2005), the depletion of endogenous cells that competes for activating cytokines, and the increased function and availability of APCs (Gattinoni et al. 2006). The combination of several

chemotherapeutic agents with ACT in murine models reported that chemotherapy rendered tumor cells more susceptible to the cytolytic effects of CTLs by inducing mannose-6-phosphate receptors and perforin-independent permeability to granzyme B in malignant cells (Ramakrishnan et al. 2010). Cancer vaccines attempt to immunize patients against their own cancers. They can include killed tumor cells, proteins, peptides or DNA vaccines (Giaccone et al. 2005, Testori et al. 2008, Amato et al. 2010), as well as cellular vaccines based on APCs pulsed with tumor antigens. The inherent immunogenicity of melanoma and renal cell carcinoma has made these tumors a focus of considerable research in vaccine development. Melanoma has been a useful model for the identification of tumor-associated antigens and a number of putative renal cell antigens have been described more recently. These antigens have been targeted using a variety of vaccine strategies, including protein- and peptide-based vaccines, recombinant antigen-expressing vectors, and whole cell vaccine approaches. While evidence for clinical benefit has been disappointing to date, several current phase III clinical trials are in progress based on promising results from phase II studies (Kaufman 2012). Clinical trials testing vaccination with *ex vivo* generated DCs pulsed with tumor antigens provided evidences of increased T-cell infiltration and tumor specific immune response in sarcoma patients (Finkelstein et al. 2011). However, clinical benefit measured by regression of established tumors have shown a limited success of cancer vaccines. This string of negative results was broken in 2010 with the positive clinical results of the phase III trial of sipuleucel-T (Provenge, Dendreon), which was originally touted as a DC vaccine for prostate cancer (Kantoff et al. 2010). The FDA approved sipuleucel-T for marketing, which represented the first therapeutic cancer vaccine reaching this critical milestone.

Targeting immunosuppressive pathways and cells. The negative contribution that immunosuppressive cells and soluble factors have over tumor progression highlights the importance of redirecting immunity to an efficient anti-tumor immunoresponse. Strategies targeting Treg cells via anti-CD25 mAb led to syngeneic tumor rejection in several murine models (Casares et al. 2003) and in conjunction with cytotoxic therapy enhanced anti-tumor immune response in both murine models and humans (Kudo-Saito et al. 2005, Mackensen et al. 2006, Morse et al. 2008). Agonistic antibody against OX40, specifically induced Treg apoptosis and presented synergy effects in combination with cyclophosphamide (Hirschhorn-Cymerman et al. 2009). Blockade of CTLA-4 was developed as an antitumor strategy due to their functions limiting the amplitude of activation of naïve and memory T cells and promoting Treg population and function (Teft et al. 2006). Subsequently, a landmark study, demonstrated improved survival in patients with untreated advanced melanoma receiving a combination of dacarbazine with the mAb anti-CTLA-4 ipilimumab (Robert et al. 2011). Starting the clinical trials in 2000, the final results from the phase III trial evaluation led to the approval from the FDA of the mAb anti-CTLA-4 ipilimumab (Yervoy, Medarex/Bristol-Myers Squibb) for the treatment of advanced malignant melanoma (Hodi 2010), becoming in the first therapy in the history to demonstrate a statically significant survival benefit for patients with metastatic melanoma in randomized clinical trial (Pardoll and Drake 2012). The blockade of the inhibitory receptor PD-1 is other therapeutic strategy under trial evaluation. The major role of PD-1 is to limit the activity of T and NK cells in the peripheral tissues during inflammation in the presence of its ligands PD-L1 (also called B7-H1) and PD-L2, which are not constitutively expressed but rather are up-regulated after encounter with inflammatory stimuli (Okazaki and Honjo 2007). In tumors PD-1 is expressed on the majority of the TILs (Ahmadzadeh et al. 2009, Sfanos et al.

2009). In some types of tumors PD-L1 is driven by active oncogenic signalling pathways, as PI3k-AKT pathway in glioblastoma (Parsa et al. 2007) or ALK-STAT3 signaling in lymphomas (Marzec et al. 2008). PD-L2 is more commonly up-regulated in lymphoid malignancies in response to cytokine signals (Xerri et al. 2008). Both, anti-PD-1 and anti-PD-L1 antibodies are currently under clinical evaluation for multiple cancers (Brahmer et al. 2012). Other strategies less developed are for example agonistic antibodies anti-CD40 which activated MHC-II^{high}CD86+ macrophages and CD8+T cells in combination with gemcitabine in patients with pancreatic ductal adenocarcima (Beatty et al. 2011). Neutralizing CD11b mAbs inhibit the recruitment of CD11b+ myeloid suppressor cells into radiotherapy-treated tumors, slowing regrowth and improving the response to the therapy (Ahn et al. 2010).

Combined therapy

Anticancer treatments are facing enormous challenges. Traditional therapies are very dramatic solutions that entail damage of other healthy tissues apart from the tumor. For example surgery of the tumor mass entail extirpation of the organ tissue homing the tumor as well as, normally the removal of the draining lymph nodes, damaging the specific organ functions as well as the immunoresponse against the remaining disease or just to repair the damage from the surgery. In the case of radiotherapy, although is improving the tissue specificity by labeling radioactive molecules to antibodies directed to tumor hallmarks, the traditional administration schedule damages the tissues toward the tumor and produces immunosupression. Chemotherapeutic treatment also induces immunosupression, but moreover it has severe second effects in patients as hair lost, gastrointestinal alterations, or blood alterations. The targeted therapies as well as the immunotherapy represent an alternative to traditional treatments. Furthermore they could mean the curative treatment for most patients in combination with traditional therapies. Starting with an antiangiogenic agent and/or drugs that modify the microenvironment extracellular matrix could facilitate the administration and function of cytotoxic drugs. Antiangiogenic agents produce blood vessel reorganization enhancing the delivery of drugs to the tumor intersticium as well as the infiltration of immune cells. After this a cytotoxic agent would kill tumor cells. The combination would allow decrease the dose of radiotherapy or chemotherapy, which is beneficial because imply immunological consequences as immunogenic tumor cell death, or enhancing antitumor immunity cells and soluble factors. In this point targeted therapies would represent a higher specificity for killing only tumor cells or to kill the residual disease. To finish immunotherapy would activate an effective antitumor immunoresponse, boosted by dying tumor cells, and by the synergic effect of radiotherapy or chemotherapy. This immunotherapy should activate the immune effector by dendritic cell vaccines, adoptive cell transfer, immune cells growth factors or Th1 cytokines administration or transfected autologous tumor-cells based vaccines. But immunotherapy might also avoid the immunosuppressive tumor environment by blocking immune check-points as CTLA-4 or PD-1 and inhibiting immunosuppressive cells as Treg, MDSC, AAM or regulatory DC.

Despite the advances with numerous monotherapies, a multimodality approach that targets different aspects of tumor biology may yield the greatest clinical benefit for patients with late-stage disease. The addition of immunotherapy to standard-of-care radiation therapy has shown evidence of efficacy in some preclinical models, and the addition of angiogenesis

inhibitors therapeutic modality could further enhance the antitumor effects of these treatments. Exploiting new modalities safely and effectively remains an ongoing challenge (Fig. 31) (Kamrava et al. 2009). The combination of antiangiogenic agents with adoptive cell transfer treatment was beneficial in the treatment of established mouse melanoma tumor. Lymphodepletion by radiation preceded the transference of T lymphocytes modified by cell engineering to express TCR molecules that specifically recognize tumor specific antigens in conjunction with a monoclonal antibody anti-VEGFR, after which intratumoral injections of recombinant vaccine virus expressing a specific tumor antigen was applied. Combination scheduled presented advantage survival and inhibition of tumor growth (Shrimali et al. 2010). Serendipitous observations made in patients of clinical trials reported high rates of objective clinical responses when cancer vaccines were combined with chemotherapy (Wheeler et al. 2004, Arlen et al. 2006). Indeed, several commonly used chemotherapeutic agents as paclitaxel, doxorubicin and cisplatin; sensitize tumor cells to CTLs in mouse, by making them more permeable to granzyme B. So the CTLs could kill tumor cells by interacting with specific tumor antigens, but also by inducing cell lysis of neighboring tumor cells by secreting granzyme B. The final consequence is the synergy between immunotherapeutic strategies that enhance CTL activity with chemotherapy (Ramakrishnan and Gabrilovich 2010).

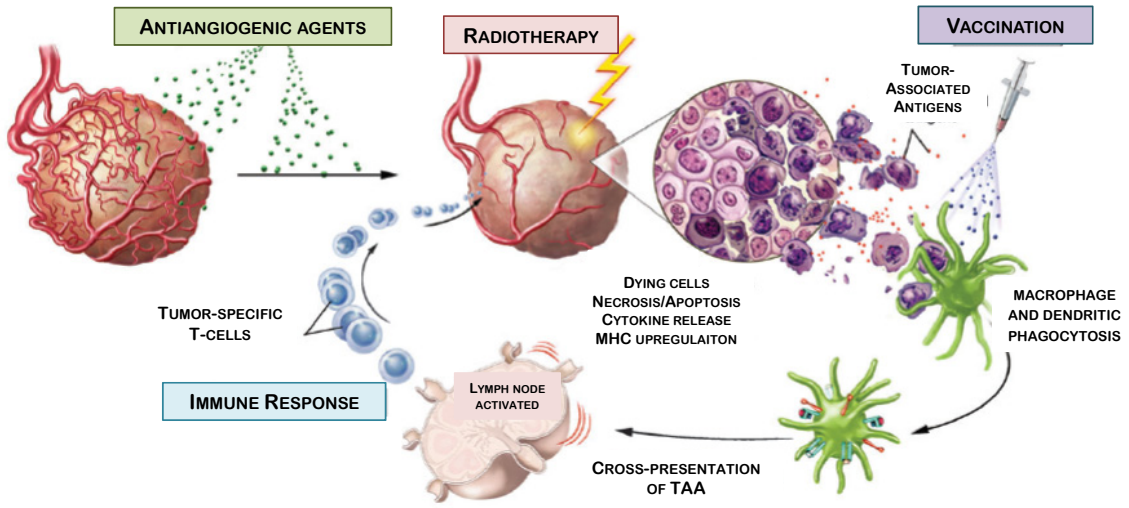


Figure 31. Angiogenesis inhibitors normalize blood flow and low intratumoral pressure, enabling T cells to gain efficient access to the tumor. Furthermore, by reducing hypoxia, antiangiogenic agents allow for optimization of radiotherapy, which killing tumor cells supply of tumor antigens. But radiotherapy also induce cytokine, adhesive molecules and MHC-I expression, synergizing with the administration of antitumor vaccines. Adapted from *Kamrava et al. 2009.*

ROLE OF MHC-I IN IMMUNOTHERAPY SUCCESS

Cytotoxic T lymphocytes can kill tumor cells by recognizing an assortment of MHC class I bound tumor-associated peptides and being activated under effective immunostimulatory conditions. So that, great efforts have been made to develop CTL-inducing immunization protocols, but so far, cancer patients have yielded only limited clinical benefits, underscoring the urge to improve current approaches for the effective induction of tumor-reactive CTLs (Speiser and Romero 2010). Defects in MHC-I antigen presentation represent a common feature of cancer and allow evasion from T cell recognition (Fig. 32) (García-Lora et al. 2003, Algarra et al. 2004, Poschke et al. 2011). Although loss of antigen presentation is known for many years, strategies to counteract these defects are scarce and largely unexamined. Now that the first T-cell-based immunotherapy show clinical efficacy and reach FDA approval, this issue deserves urgent awareness (Lampen and van Hall 2011).

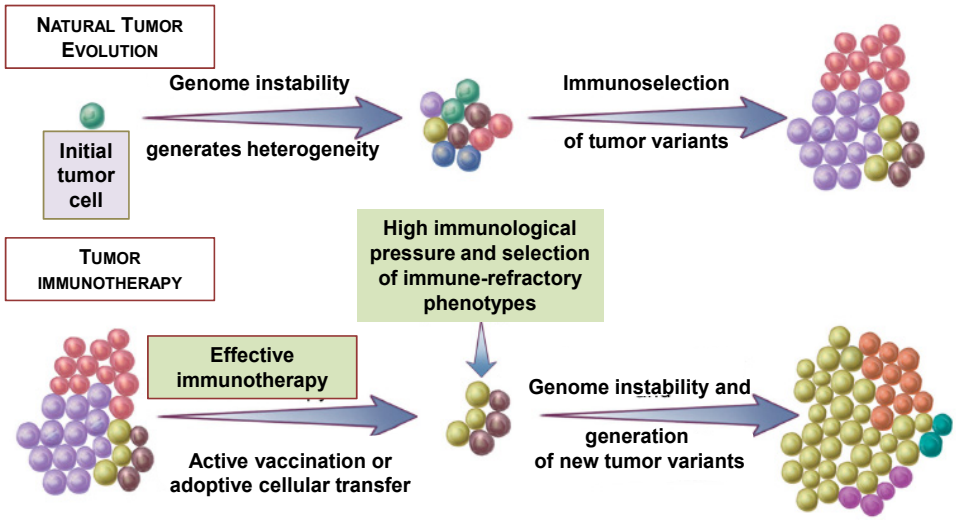


Figure 32. In the natural evolution of tumor growth, immunoresponse produces the selection of tumor cells more sensitive to immunity. Immunotherapy enhances antitumor immunity leading to a major immuno-pressure which will exert higher selection of tumor cell populations with immuno-resistant phenotypes and finally generating a different profile of tumor cells than when no intervention was made. Adapted from *Khong and Restifo 2002.*

Indeed, the FDA has approved a few tumor antigen (TA)-specific monoclonal antibodies for the treatment of several malignant diseases (Groner et al. 2004, Adams and Weiner 2005, Campoli et al. 2009). But the average of clinical success of this TA-specific mAbs manifests significant disease free interval and survival prolongation as well as reduction of tumor mass in a 30% of patients with ranges from 0 to 60% (Reichert et al. 2005). TA-specific mAbs therapy induce the generation of TA-specific CTLs (Dhodapkar et al. 2002, Gelderman et al. 2004), and although HLA-I abnormalities increased during T cell-based immunotherapy of cancer patients (Neller et al. 2008) the expression of HLA-I in tumor cells of patients receiving this type of therapy is not normally taken in consideration. Although only little is known about the mechanisms underlying the limited clinical response, the alterations HLA-I expression have been proposed as a critical mechanism that produce the TA-specific mAbs therapy failure (Fig. 33) (Campoli et al. 2010). Several examples of therapies directed to induce a specific immunity as DNA-based vaccine that induce the expression of single or several tumor antigens (Lichtor

and Glick 2012), as well as monoclonal antibodies specifically targeting human papiloma virus peptides (Lei and Zhang 2012), generally overlook the implications that the surface expression of HLA-I molecules could play on the response to the therapy (Cabrera et al. 2007a) (Fig. 32). For example, a clinical trial involving vaccination of melanoma patients with autologous DC expressing melan A and gp100 showed a reduction of the tumor volume in 3 of the 25, in which they found a T lymphocyte response and reactivity restricted to both antigens. Although they considered the importance of the expression of both HLA class I and II molecules for the results of the therapy, they did not monitorized this variable during the trial (Steele et al. 2011).

Experimental evidences from murine tumor models indicate that cancer progression is accompanied of the acquisition of additional MHC-I alterations with reversible-soft and irreversible-hard lesions in metastatic colonies (García-Lora et al. 2001). The nature of the alterations in MCH-I expression, irreversible or "soft" *versus* irreversible or "hard" may determine the response to immunotherapy (Garrido et al. 2010a, Garrido et al. 2010b). A melanoma patient following a treatment with autologous tumor vaccine (M-VAX) plus Bacillus Calmette-Guérin (BCG) for several subcutaneous metastases presented different responses within the metastatic lesions, while three of them progressed, other three presented considerable regression. Although all metastases harbored a loss of an HLA-I haplotype, regressing nodes presented a high level of surface expression of HLA-I molecules, while progressing nodes presented additional alterations as an LOH in chromosome 15 and HLA B locus down-regulation (Cabrera et al. 2007a). Moreover, in another melanoma patient treated for metastatic lesions with IFN-α-2b followed by autologous vaccine plus BCG, five metastases autopsies were obtain after IFN-α-2b and other five after autologous vaccine plus BCG. Eight of these metastases were regressing after the immunotherapy, while two were progressing. Newly regressing metastases presented a high level of HLA-I surface expression, while progressing metastases had low HLA-I levels (Fig. 33) (Carretero et al. 2008, 2011a and 2012). Other studies showed how the ACT of transgenic T cells specific for P1A, used in the treatment of tumors with distinct histological origin, caused the appearance of tumor escape variants with antigen drift or antigen loss (Bai et al. 2006).

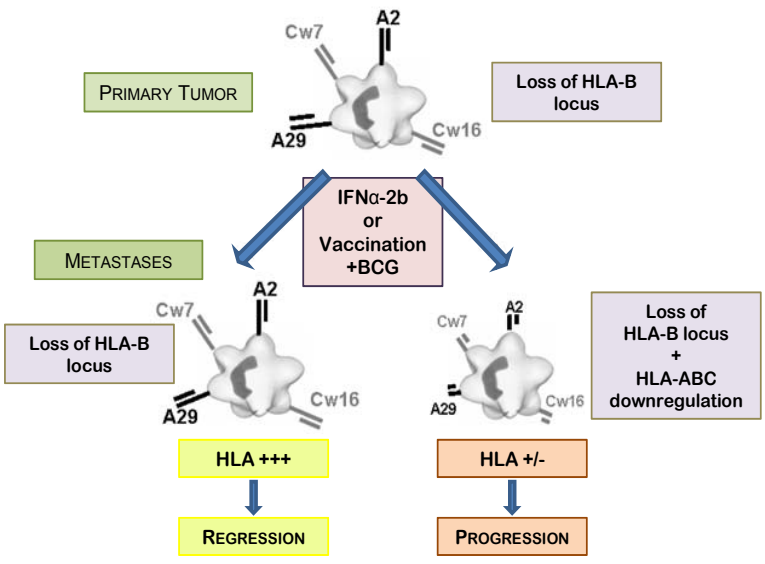


Figure 33. Hypothetical chain of alterations in the HLA class I expression during cancer immunotherapy. The original tumor lost HLA-B molecules, phenotype that is detected in regressing and progressing metastases. In addition, progressing metastases showed down-regulation of HLA-A and C locus expression. Adapted from *Carretero et al. 2008.*

The vision of the role of MHC-I alterations is gradually gain attention between the immunologists and the clinicians, and the analysis of the HLA-I surface expression is turning as an important factor to take into consideration in the evaluation of anticancer therapies. Indeed, it has been found that, Topotecan (TPT), a topoisomerase I-targeting drug, in low doses, stimulates MHC-I expression in breast cancer cells through elevated expression/secretion of IFN-β and activation of type I IFN signaling. TPT-treated cells also exhibit elevated expression of multiple cytokines such as IFN-β, TNF-α, IL-6 and IL-8. Studies have also demonstrated that other chemotherapeutic agents (e.g. etoposide, cisplatin, paclitaxel and vinblastine) as well as γ-radiation therapy similarly induce increased IFN-β secretion and elevated MHC-I expression (Wan et al. 2012). These results suggest that much cancer therapeutics induce elevated tumor antigen presentation through MHC-I, which could represent a common mechanism for enhanced antitumor immune response through T cell cytotoxicity during metronomic chemotherapy, as well as increased efficacy of combined chemotherapy or radiotherapy with immunotherapy (Wang et al. 2012).

Furthermore, recent studies showed that in patients with chronic myelogenous leukemia drug-resistant mutations in BCR-ABL could lead to disease relapse after imatinib treatment. Peptides derived from the BCR-ABL mutations would bind to autologous HLA alleles, as for example the E255K mutation-derived peptide would bind HLA-A3 with high affinity, and showed that this peptide is endogenously processed and presented. E255K-specific CD8+ T cells were detected in two imatinib-resistant HLA-A3+ CML patients concurrent with an effective anti-CML response to further therapy, which suggest the possibility of immunizing relapsed patients with CML against newly acquired tumor neoantigens (Cai et al. 2012).

HER2 is overexpressed in 20-30% of breast cancers and is considered a promising target for immunotherapeutic interventions with T cell-based approaches. However, several previous studies including ours showed that HER2-overexpressing tumors may escape cytotoxic T lymphocyte-mediated lysis by down-regulating MHC class I and components of the antigen-processing machinery. It has been found that small-interfering RNAs targeting HER2 as well as an inhibitor of HER2 signaling resulted in MHC class I up-regulation on breast cancer cell lines. Thus, agents that target the MAPK signaling pathway may increase MHC class I expression in breast cancer cells (Inoue et al. 2012). Moreover, overexpression of the receptor tyrosine kinases HER2 and HER3 is associated with a poor prognosis in several types of cancer. So thus, HER2- as well as HER3-targeted therapies are in clinical practice or evaluated within clinical trials, including treatment with mAbs mediating growth inhibition and/or activation of Ab-induced innate or adaptive cellular immunity. It has been seen that genetic silencing of HER3 but not HER2 down-regulated the expression of MHC class I-related chain A and B (MICA/B) in breast cancer cell lines. The MICA and MICB molecules act as key ligands for the activating receptor NK group 2, member D (NKG2D) and promote NK cell-mediated recognition and cytolysis. So that signaling via the HER2/HER3 pathway in breast carcinoma cell lines may lead to enhanced NKG2D-MICA/B recognition by NK cells and T cells (Okita et al. 2012).

Protein-bound polysaccharide K (PSK), a glycoprotein purified from the mushroom *Coriolus versicolor* that has antitumor immunoenhancing effects, is clinically used in Japan, in combination with anticancer agents following gastric cancer surgery. A clinical trial, comprising 349 patients with stage II/III gastric cancer, who had received oral chemotherapy or chemotherapy plus PSK adjuvant therapy following curative resection between 1995 and 2008,

evaluates the MHC class I expression in the primary lesion and the clinical outcomes were compared between these two groups. Expression-negative cases demonstrated 3-year recurrence-free survival rates of 65% in the PSK group and 47% in the chemotherapy-only group. Differences were higher when compare between the expression-negative cases with advanced lymph node metastasis. Thus, PSK adjuvant immunochemotherapy resulted effective in MHC class I-negative patients, who were in a state of antitumor immunological tolerance, and more even in patients with advanced lymph node metastasis (Ito et al. 2012).

In a clinical trial, treating with anti-cytotoxic T-lymphocyte antigen-4 antibody Ipilimumab metastatic melanoma patients pre-vaccinated with gp100 DNA (IMF-24), gp100(209-217) and tyrosinase peptides plus GM-CSF DNA (IMF-32), or NY-ESO-1 protein plus imiquimod (IMF-11), it was found that vaccination induced a measurable antigen-specific T-cell response that increased following CTLA-4 blockade and produced polyfunctional intracellular cytokines. Furthermore, they found that primary and metastatic tumors expressed tyrosinase but not gp100 or class I/II MHC molecules, and considered that tumor escape could be related to antigen loss or lack of MHC expression necessary for immune activity (Yuan et al. 2011).

Current information shows how therapy schedules that involve the monitoring of the HLA-I surface expression and the analysis of molecular lesions causing HLA-I altered phenotypes are taken importance in the evaluation of anticancer therapeutic outcome. Moreover, new therapies that increase the expression of HLA-I molecules are being developed. As an example, there is a cancer immunotherapeutic, Velimogene aliplasmid lipid complex (Allovectin, Vical), currently completing a pivotal phase III study for metastatic melanoma. It consists of a bicistronic plasmid encoding both MHC class I heavy and light chains (HLA-B7 and β2-microglobulin, respectively) formulated with a cationic lipid-based system and that it is designed for direct intratumoral administration. Following injection into a single lesion, the product is intended to induce anti-tumor immune responses against both treated and distal lesions. Both the plasmid and lipid components of Allovectin contribute to the biological activity of the drug and its therapeutic activity is hypothesized to derive from the induction of both cytotoxic T-cell and innate immune responses directed against allogeneic as well as tumor-derived targets, consequences of both an increased MHC class I expression on tumor cells and the induction of a localized immune/inflammatory response (Doukas and Rolland 2012).

Other case is a new proposed approach for human prostate tumor vaccine and gene therapy trials using ex vivo methods to prime dendritic cells with prostate specific membrane antigen (PSMA) was cancer vaccination with tumor antigens by delivering PSMA via a CD40-targeted adenovirus vector directly to DCs in combination with an Ad vector expressing interferon-gamma (Ad5-IFNγ). Thus maximizing antigen presentation in target cells, inducing both MHC class I and TAP protein expression and efficiently activating high levels of tumor-specific CTL responses against prostate cancer cells pretreated with Ad5-IFNγ (Williams et al. 2012).

These approaches are very important to the results of immunotherapy treatments, but also, to the traditional treatments as chemotherapy and radiotherapy which had a proved immunological component within their therapeutic effects.

TRANSLATIONAL RESEARCH AND ANIMAL MODELS

Experimental mouse tumor models have provided key mechanistic insights into tumor initiation and progression process (as somatic evolution, metastasis process, dormancy, tumor microenvironment and angiogenesis or antitumor immunoresponse), that have guide the development of novel treatment strategies (Dranoff 2012). Nowadays, mouse tumor models remain almost obligatory stepping stone for anticancer therapies before undertaking studies in cancer patients. But those animal model systems have so strengths as well as limitations that would be considered to predict the effects of therapies in patients. Animal experimentation involve three main model systems: transplantable tumors, genetically engineered tumor models and humanized mouse models of cancer.

Transplantable tumor models are widely used. They consist in the injection of cells with tumorigenic capacities that normally belong to a cell line selected for efficient propagation (as for example, B16 melanoma, CT26 colon carcinoma, 4T1 breast carcinoma, Lewis lung carcinoma or methylcholanthrene-induced fibrosarcoma cell lines) (Fig. 34). An easy issue is the subcutaneous injection of the tumor cells that facilitates growth monitoring, possible surgeries and intratumoral therapies. Another option is orthotopic injection of the tumor cells, as for example the intracranial injection of glioma cells, which present the advantageous of mimicking the growth of tumors within a relevant microenvironment (Wilmanns et al. 1992). Bioluminescent techniques allow, by cell engineering to express a luminescent protein and specialize imaging systems, the following of visceral disease (Craft et al. 2005). Implanted tumor cells tend to grow rapidly and thus do not mimic the much slower doubling times for most human tumors, consequently these animal tumors may result more sensitive to most cancer-therapy drugs that target dividing cells and the local inflammation affects to immunotherapeutic responses (Ostrand-Rosenberg 2004).

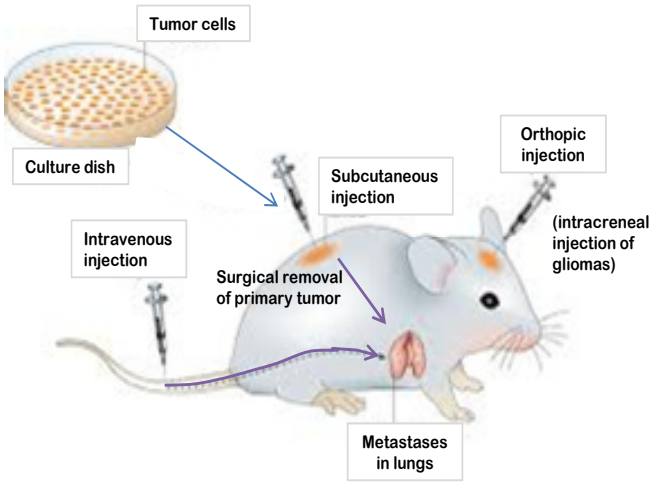


Figure 34. Tumor cell lines are easy to grow in culture and form tumors after inoculation into syngeneic mice. Cells may be injected subcutaneously, intravenously or orthopically to induce tumors and/or metastases in different microenviroments. These models allow the evaluation of therapeutic strategies to prevent or treated established tumors or metastatic development. Adapted from *Dranoff 2012.*

Other challenge the experimental models present is that fail to reflect clinical trials of patients with advanced metastatic disease while the treated animals normally just present the primary local tumor (Sleeman and Steeg 2010). Possible alternatives are spontaneous or experimental metastases assays. Spontaneous metastases assay involve surgical resection of subcutaneous primary tumor, while experimental metastasis assay is performed by injecting tumor cells directly in the blood torrent by intravenous injection, thus avoiding important steps of the metastatic process as the acquisition of motility, migration, blood vessels intravasation and extravasation, and surviving in these inhospitable milieus (Welch 1997, Steeg and Theodorescu 2008). Several studies have used advanced disease models to test new therapeutic regimens (Man et al. 2007). Preclinical models of spontaneous metastases assays presented important advantage on the study of drugs. Spontaneous metastasis models allow researchers to test therapies in laboratory conditions that mimic disease present in most clinical trials. Metastatic spread follows natural route and mechanisms; it is possible to examine all steps of metastatic cascade and the process is slow which gives more time to the administration of therapies and the development of a complete and effective immune response (Francia et al. 2011).

Genetically engineered tumor models have been developed over the knowledge of gene signature of tumor cells, tumor microenvironment and antitumor immunoresponse (Fig. 35). A large number of mouse strains that harbor mutations in specific genes have been used to investigate mechanisms associated with the expression of these genes. In one approach gene-knockout mice, lacking for example, a gene decisive for the immunoresponse, are exposed to carcinogens, and then the frequency of tumor formation is measured. In a complementary approach, advance in cancer genetic have allow a wealth of new mouse tumor models that compile genetic lesions characteristic of human cancers. These models, by cell engineering enforce the expression of oncogenes and/or the loss of expression of tumor suppressor genes.

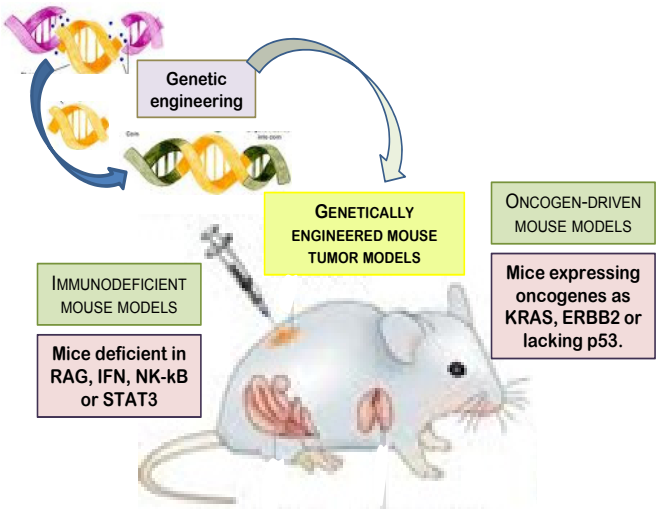


Figure 35. Genetic modifications of mice allow working with mice lacking the expression of some genes, as the example of immunodeficient models as well as mice overexpressing oncogenes that drive the tumor development. These models help in the analysis of the role of those immunological components, oncogenes or tumor supressor genes in the progression of cancer disease. Adapted from *Dranoff 2012.*

Humanized mouse models of cancer. The work with human cancer cell lines entails human tumor xenografts, implanted in immunodeficient mice (Fig. 36). Immunodeficient mice include between others athymic nude mice, NOD mice, SCID mice, NOD/SCID mice, Beige mice, recombination-activating gene-deficient (Rag $^{-/-}$) mice and NOD-Scid Il2rg $^{-/-}$ mice. These experimental models are widely used in studies of cancer biology and targeted or cytotoxic therapy. In this regard, several efforts are directed towards reconstituting these mice with a human immune system and evidence the antitumor immunological response as well as the interactions and functions of dendritic cells, B and T lymphocytes with human cancer cells (Legrand et al. 2009). Humanized mouse models represent high potential to clinical investigations and translation of therapeutic strategies to patients, but require additional studies as the impact of mouse cytokines and cell-surface molecules or microenvironmental factors on human cancer cells.

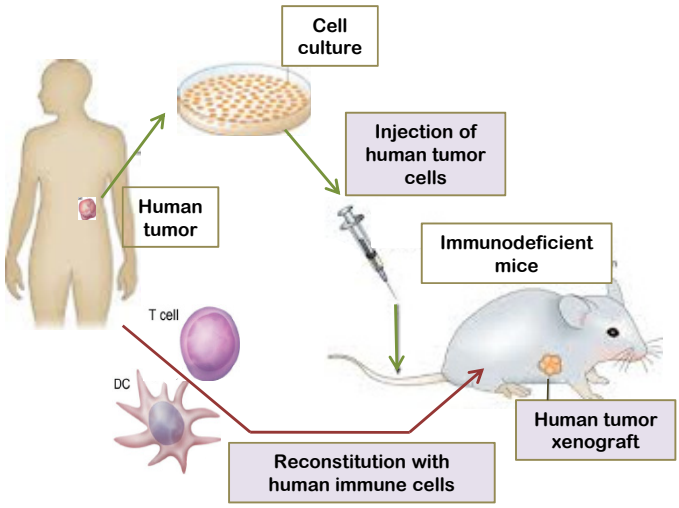


Figure 36. Immunodeficient mice are used to study in vivo therapeutic strategies with human tumor cell lines, by human tumor xenograft models. These mice could be reconstituted with human immune cells to mimic human tumor microenvironment. Adapted from *Dranoff 2012.*

Better understanding of strength and limitations of mouse tumor models would accelerate the translation of clinical benefit findings to cancer patients.

HUMAN MELANOMA MODEL ANDO-2

Ando-2 human melanoma xenograft model was developed and described by Paco L et al. (Paco et al. 2007). Ando-2 is a human melanoma cell line obtained from a left supraclavicular lymph node metastasis of a patient with malignant melanoma, and Ando-EBV cell line is the result of Epstein-Barr virus (EBV)-transformed autologous B lymphocytes. Ando-2 and Ando-EBV were kindly provided by Dr P. Coulie [Universite´ Catholique de Louvain, Brussels, Belgium] to our laboratory. Ando-EBV cells were genomically typed as HLA-A*0201,3201; HLA-B*1302,4001; HLA-Cw*0304,0602; HLA-DRB1*0101,1501 and HLA-DQB1*0501,0602. Melanoma cell line Ando-2 presented a hemizygous typing: HLA-A*3201, HLA-B*4001, HLA-Cw*0304, HLA-DRB1*1501 and HLA-DQB1*0602. Phenotype analysis showed that Ando-2 presented surface expression of HLA-A32, HLA-B40 molecules, but did not present surface expression of HLA-A2, HLA-B13 or HLA class II molecules (Fig. 37) (Paco et al. 2007).

Ando-2

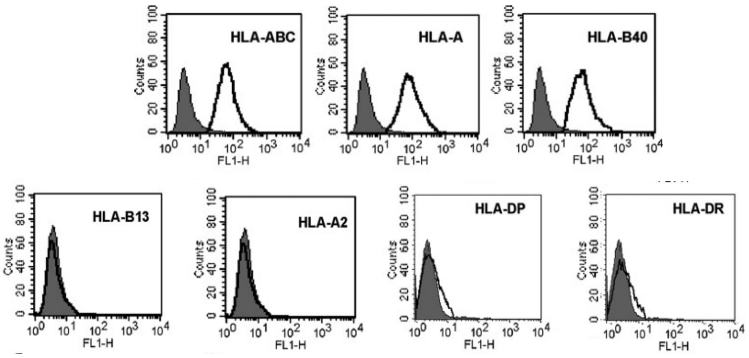


Figure 37. Ando-2 tumor cell line presented surface expression of HLA class I (-A, and -B40) but it did not express HLA-A2 or -B13 and neither HLA class II molecules. Adapted from *Paco et al. 2007.*

There was an LOH pattern in Ando-2 melanoma cell line in comparison with autologous EBV cell line. Ando-2 presented a deletion in 6p21.3 and 6q24 regions (Fig. 38). β_2-m gene exhibited a normal pattern in Ando-2 cells (Paco et al. 2007).

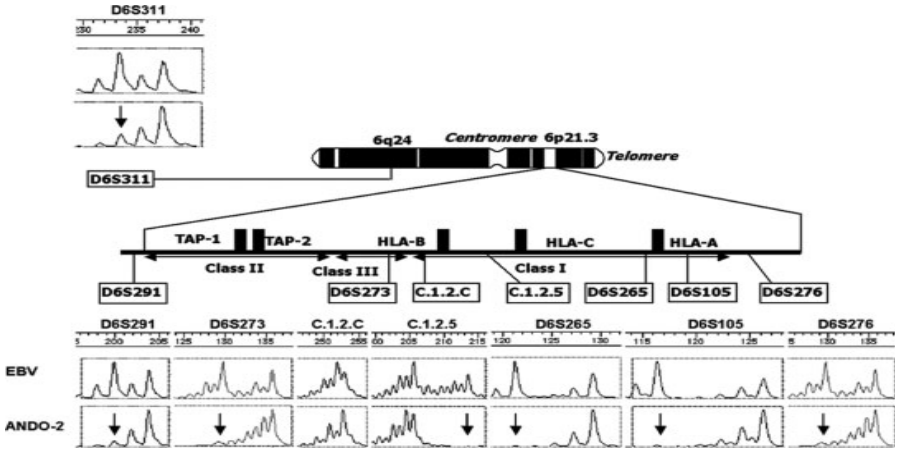


Figure 38. Microsatellite analysis showed LOH in 6p21.3 and 6q24 regions in Ando-2 melanoma cell line versus autologous EBV cell line, suggesting complete deletion of one chromosome 6. Adapted from *Paco et al. 2007.*

Ando-2 cells were subcutaneously injected into immunodeficient nude mice, excising the primary tumor that was adapted to tissue culture and cloned. The melanoma cell lines obtained were designated Ando-nude1, 2, 3, 4 and 5. Surprisingly, Ando-nude cell lines presented no surface expression of either HLA class I allele and recovered expression of HLA class II molecules, HLA-DP and HLA-DR (Fig. 39). HLA phenotype was reproducible after multiple in vitro passages. Surface expression of HLA class I molecules was recovered by IFN-γ treatment (Paco et al. 2007).

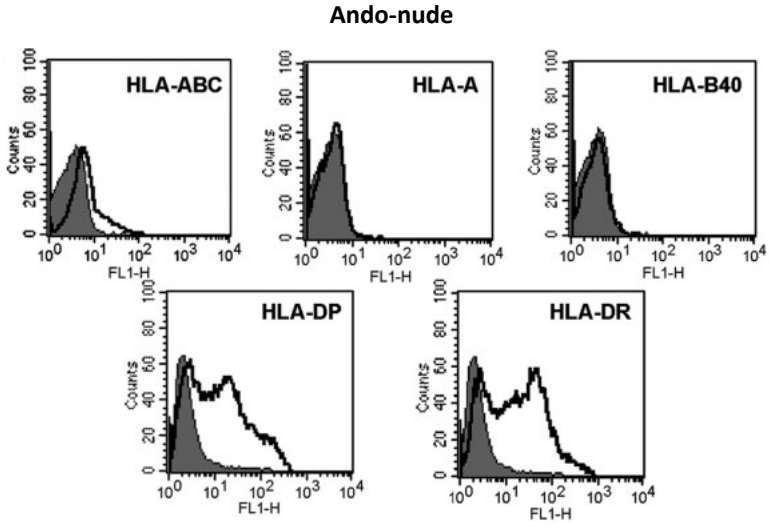


Figure 39. Ando-Nude tumor cell line derived from nude mice lost the surface expression of HLA class I, while recuperated HLA class II molecules. Adapted from *Paco et al. 2007.*

When Ando-2 melanoma cells were grown in SCID-Beige mice, resulted tumor cell lines showed similar phenotype that those obtain from nude mice, with the exception that the expression of HLA-A32 allele was not completely negative although it was hardly down-regulated. Analysis of transcriptional level of HLA heavy chains, β_2-m and APM components showed a coordinated down-regulation of these genes in HLA-I negative Ando-nude cell lines.

GR9 FIBROSARCOMA MOUSE MODEL

GR9 fibrosarcoma mouse model was developed by our group at the beginnings of the eighties. GR9 is a methilcholantrene-induced fibrosarcoma in BALB/c mice which was directly adapted to tissue culture from the primary tumor without any *in vivo* passage in syngeneic or allogeneic mice. GR9 cell line was cloned using a phase contrast microscope and taking isolated cells that were grown in culture plaques (Pérez et al. 1985). GR9-derived clone cell lines A2, A3, A6, A7, B3, B4, B5, B6, B7, B9, B10, B11, C5, C11, D6, D8, F9, G2, G10 were adapted to tissue culture and criopreserved in liquid nitrogen. The GR9 fibrosarcoma tumor model (GR9 cell line and some of GR9-derived clones) has been extensively studied and characterized by our group (Bonal et al. 1986, Delgado et al. 1986, Garrido et al. 1986, Pareja et al. 1987, Caballero et al. 1989, Perez et al. 1989, Mialdea et al.1992). The H-2 class I phenotype of the different cell lines was analyzed founding a profound heterogenic composition of GR9 tumor (Garrido A. et al. 1986). Those analyses were confirmed by posterior flow cytometry analysis of H-2K^d, H-2D^d and H-2L^d molecules (Romero 2012). GR9 cell line presented surface expression of the three H-2 class I molecules and is composed by clones with heterogenic H-2 class I phenotype that can be classified in four groups: highly positive clones (D8, A7, G2), middle positive clones (B10, B3, B7), low positive clones (G10, B6, C5, C11, B11) and negative clones (B9) (Fig. 40)

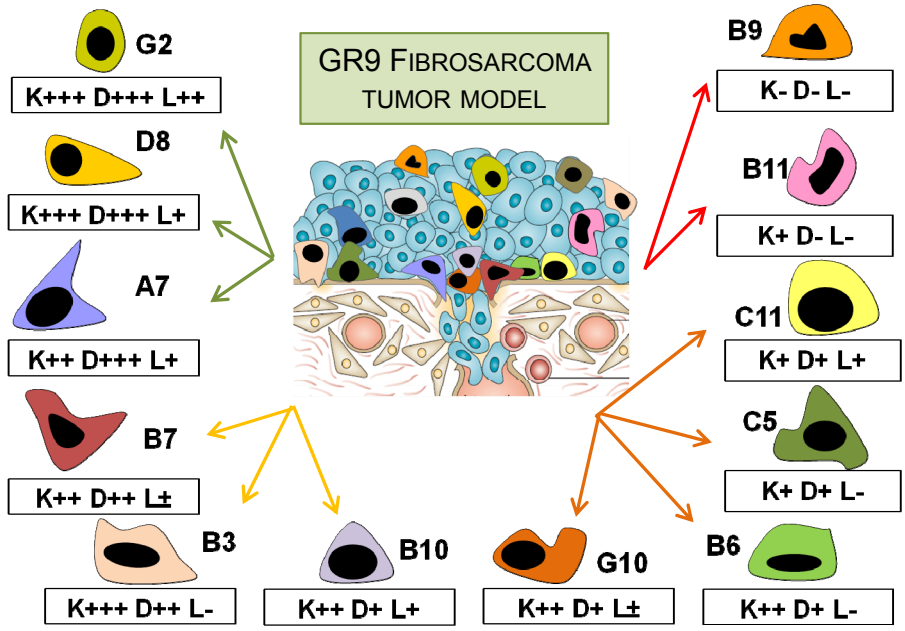


Figure 40. GR9 is a methilcholantrene-induced fibrosarcoma in BALB/c mice which was directly adapted to tissue culture from the primary tumor. GR9 cell line was cloned under a phase contrast microscope. GR9-derived clone cell lines presented heterogeneity in their H-2 class I phenotype. Adapted from *Romero Doctoral thesis 2012.*

Transcriptional analysis of the H-2 class I heavy chains, β_2-m and APM components genes showed a correlation between the expression of these genes and the surface expression of MHC-I molecules (Romero 2012). The coordinated down-regulation of the transcription of H-2L^d heavy chain, calreticulin, LMP-2 and TAP-1 was found in B7, C5 and B11 clones in comparison with A7 clone (Romero 2012).

Local tumor growth in syngeneic immunocompetent BALB/c mice showed a correlation between the MHC-I phenotype of GR9-clones and their tumorigenic capacity (Garrido A. et al. 1986, Romero 2012). Comparing the progression of local tumor growth after the subcutaneous injection of 6,25 x 10^5 cells of A7, B7, C5 and B11, A7 and B7 showed similar growth rate, but different than C5 and B11 that grew slower (Romero 2012).

Spontaneous metastasis assays showed different metastatic capacities within the GR9-derived clones A7, B7, C5 and B11. A7 (H-2 high phenotype) produced a high number of metastatic colonies in different organs (lungs, spleen, lymph nodes) B7, C5 (H-2 middle or low phenotype) produced low metastatic development and only in the lungs, finally B11 (negative for H-2D^d and H-2L^d molecules) did not produced any metastasis (Romero 2012). It was found that there was a correlation between the metastatic capacity *in vivo* with the migratory and invasive capacities of these tumor cell lines *in vitro* (Romero 2012). The metastatic progression of these tumor clones lead to the generation of a second variety of H-2 class I phenotypes (Pérez et al. 1990, Romero 2012). They found four different types of H-2 class I phenotypes within the generated metastases: phenotype I, metastases with similar phenotype than the original clone (A7- and B7-metastases); phenotype II, metastases with a down-regulation in the MHC-I surface expression (A7- and B7-metastases); phenotype III, metastases with a down-regulation of H-2K^d and H-2D^d and with a reversible loss of H-2L^d allele (soft lesion) (A7- and B7-metastases); and finally, phenotype IV metastases with down-regulation of H-2K^d and H-2D^d and with a irreversible loss of H-2L^d allele (hard lesion) (only B7-metastases) (Romero 2012).

The expression of different oncogenes was studied in GR9 model. Within the GR9-derived clones it was found that the cell-surface expression of MHC class-I antigens correlated inversely with levels of c-myc mRNA transcripts. On the other hand, mRNA levels of c-fos were correlated directly with the amount of mRNA of MHC class I. Treatment of the B9 clone with gamma interferon increased mRNA transcription and surface expression of H-2 class-I antigens, while c-myc transcription was simultaneously down-regulated. In contrast, c-fos mRNA levels remained unaltered (Gaforio et al. 1991). In spontaneous metastasis assays with B9 and G2 clones it was found that the amplification of the c-myc proto-oncogene was similar in original tumor clones than in all metastatic nodes and that metastatic cells showed an overexpression of K-ras proto-oncogene that was associated to a significantly reduced *in vitro* sensitivity to NK cells as compared with the tumor clones (Algarra et al. 1991).

Profound studies were performed to analyze the behavior of GR9 clones in experimental metastasis assays, as well as the function of NK cells in the metastatic process. Fibrosarcoma clones with no expression of MHC-I molecules (B9) presented sensitivity to NK-mediated lysis while clones with high levels of MHC-I expression (G2) or IFN-γ treated B9 cells, which recuperated H-2 class I expression, were relatively resistant. In experimental metastasis assays demonstrated less colonization by the H-2 high positive clones than H-2 low or negative clones which correspond with the sensitivity to NK-mediated lysis (Algarra et al. 1989, Pérez et al. 1990). It was found that an oral dose of a chemical compound, a tilorone analogue, produced a boosting of splenic NK-cells activity and given one day before i.v. injection, completely eliminated lung colonization of the H-2-negative B9 clone. The effects were abrogated by the administration of the NK inhibitor anti-asialo GM1 (Algarra et al. 1993).

Those experiments were extended to other GR9 clones and tilorone analogues obtaining similar results (Algarra et al. 1994 and 1996). The effect of protein-bound polysaccharide K (PSK), other immuno-stimulator treatment that activates NK-cell activity used in China traditional medicine, was studied. Pretreatment of mice with PSK inhibited metastatic colonization derived from B9 tumor cells. The effect of PSK was attenuated when high numbers of cells were injected and with anti-asialo GM1 serum (Algarra et al. 1997). These studies were extended to other fibrosarcoma induced cell lines founding that PSK pretreatment prolonged survival and inhibited metastasis formation in mice injected with sarcoma cells and that this activity presented an inverse correlation with H-2 antigen expression and direct correlation with in vitro NK sensitivity (Algarra et al. 1999).

In the early 2000s, the alteration of MHC class I molecules expression was known as a widespread mechanism used by tumor cells to evade T cell responses. And it has long been proposed that the origin of these MHC class I-negative or -deficient tumor variants was T cell immune selection. However, there were no experimental or clinical data to substantiate that hypothesis. The exhaustive study of the behavior of B9 clone in spontaneous metastasis assays allow to the understanding of some elements of those phenomena.

It was reported that B9 H-2 class I-negative fibrosarcoma tumor clone generated MHC class I-negative spontaneous lung metastases (0-1 metastases per mouse) in immunocompetent syngeneic BALB/c mice, while, interestingly, it generated MHC class I-positive metastatic nodes (5-7 metastases per mouse) in athymic nu/nu BALB/c mice (García-Lora et al. 2001) (Fig. 41).

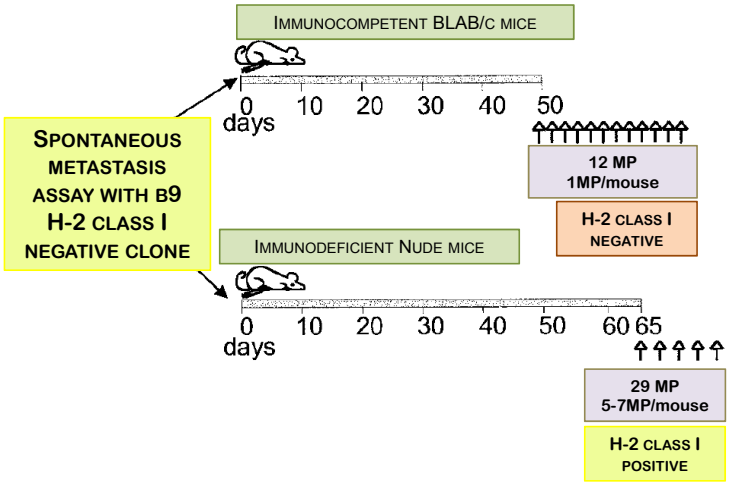


Figure 41. B9 H-2 class I negative fibrosarcoma tumor clone produced 0-1 metastasis per mouse in BALB/c mice, while between 5-7 metastases in athymic nu/nu mice. Metastases from immunocompetent host kept MHC-I negative phenotype. But metastases from immunodeficient mice were MHC-I positive. Adapted from *Garcia-Lora et*

In addition, two different phenotypes were found in the metastatic nodules obtained from immunocompetent mice. One phenotype, present in 83% of the colonies, was characterized by resistance of the H-2L^d molecule to IFN-γ induction, due to a deletion of the gene. The second phenotype (17% of the colonies) was similar to the original B9 clone and was characterized by the response of K, D and L class I genes to IFN-γ (Garcia-Lora et al. 2001). These findings supported the hypothesis that the H-2 phenotype of metastatic nodes was influenced by the T cell repertoire of the host, since in the absence of this T cell pressure (i.e., in nude mice) the metastatic nodes 'recovered' H-2 class I expression (García-Lora et al. 2001).

Then, molecular alterations responsible for the changes of MHC class I molecules was analyzed. Metastatic nodes were analyzed for the messenger ribonucleic acid (mRNA) level of H-2 class I heavy chains, β_2-m, and several components of the antigen processing machinery. Analyses showed that TAP-1, TAP2, LMP-2, LMP7, LMP10, tapasin and calnexin genes mRNA was absent in metastases produced in immunocompetent mice. In contrast, it was highly positive in metastatic nodes from immunodeficient mice. Interestingly, the MHC class I-positive or negative phenotypes of the metastatic colonies correlated with *in vivo* immunogenicity. H-2 positive metastases locally grew more slowly in syngeneic immunocompetent animals than the H-2 negative ones, and were finally rejected (Fig. 42) (García-Lora et al. 2003).

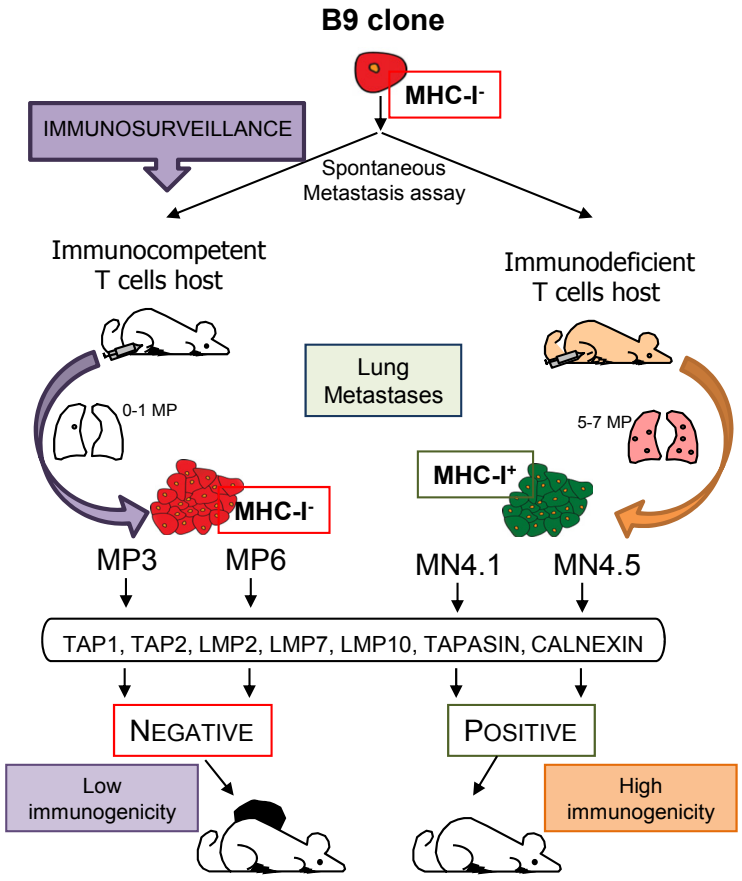
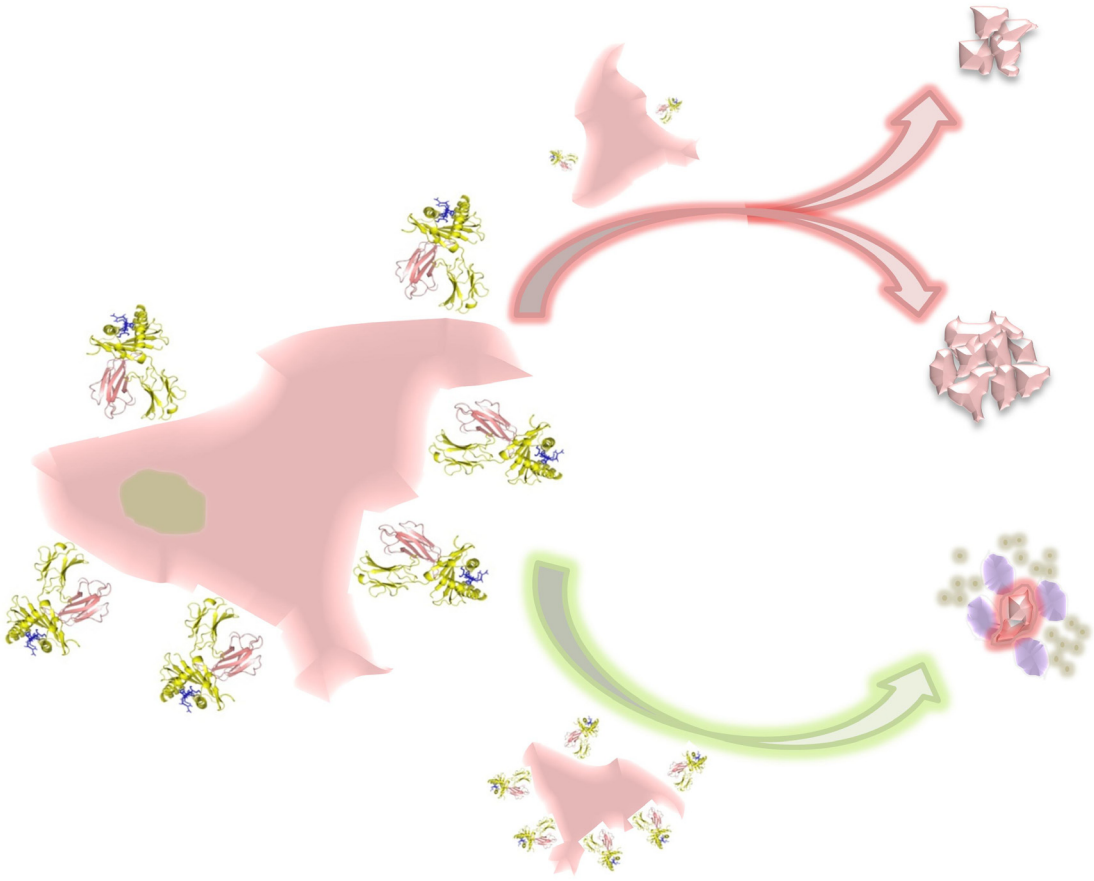


Figure 42. The spontaneous metastases of an H-2 class I negative fibrosarcoma tumor clone in BALB/c mice kept MHC-I negative phenotype, while in athymic nu/nu mice, metastatic nodes were MHC-I positive. Metastatic colonies from immunodeficient mice resulted high immunogenic and didn't grew when transplanted to an immunocompetent host. Contrary, metastases generated in immunocompetent mice were low immunogenic and presented local growth when transplanted. Adapted from *Garcia-Lora et al. 2003.*

These results supported the idea that the immunogenicity of a particular tumor depends on the immune status of the host, producing tumor escape variants of low immunogenicity when growing in immunocompetent individuals. In addition, the data highlighted the potential clinical usefulness of the analysis of MHC phenotype in tumors and of elucidating the molecular mechanisms used to escape immune surveillance, especially when heterogeneous populations exist in individual cancer patients. GR9 tumor model could provide a powerful tool for understanding the role of antigen processing defects in tumor immunity and for developing new immunotherapeutic strategies (García-Lora et al. 2003).



HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

Immunodeficient mice are commonly used for study of therapeutic strategies against human tumors. Our group injected Ando-2 human melanoma cells in immunodeficient mice to study in the future their biological and immunological behavior in humanized mice. Previous results showed that Ando-2 human melanoma cells completely lost HLA-I surface expression after growth in immunodeficient mice (Paco et al. 2007). The following questions arose: is this phenomenon exclusive for Ando-2 melanoma cells, or is it extensive to other melanoma cell lines? Is it a reproducible phenomenon? Analysis of Ando-Nude cells revealed that these cells presented a higher *in vivo* oncogenic capacity than Ando-2 cells. But, is this higher oncogenicity due to the loss of HLA-I surface expression, or both phenomenon are simply associated? Can play a direct role the MHC-I molecules suppressing the growth of cancer cells? We will try to answer these questions.

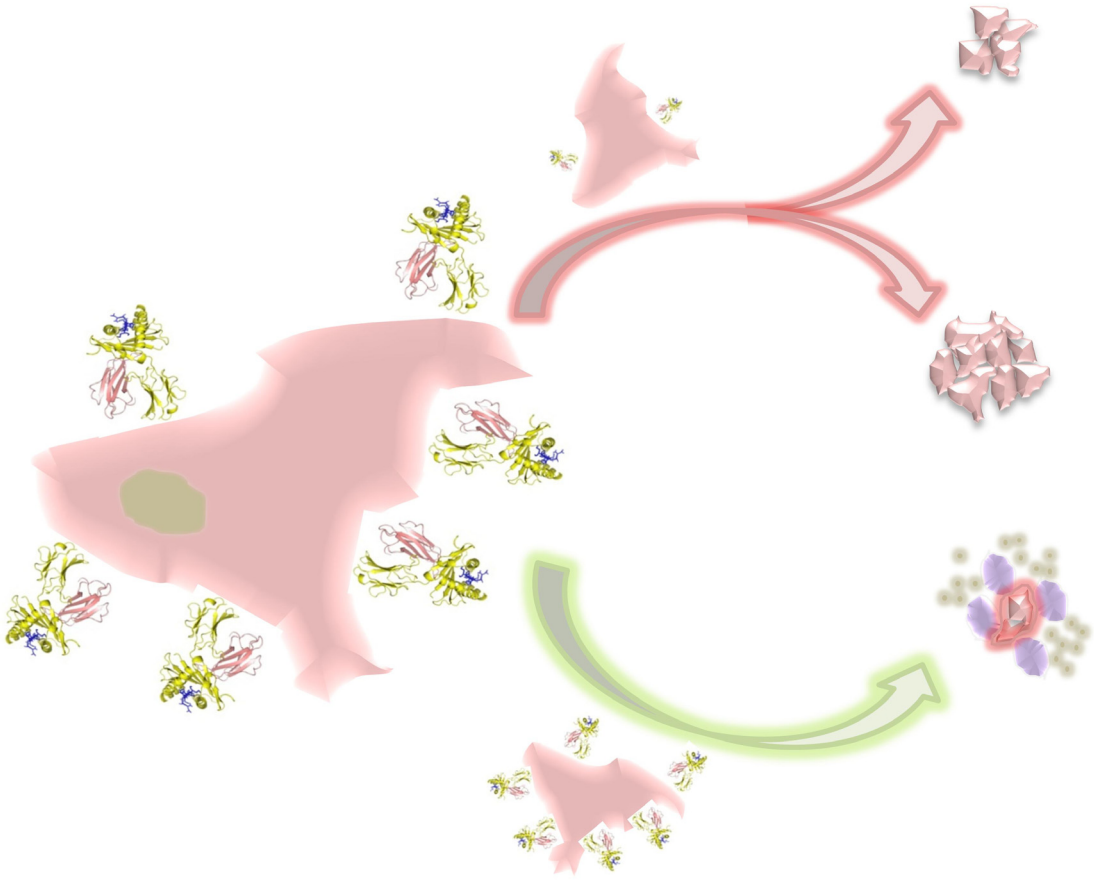
Studies in two melanoma patients showed that alterations in the MHC-I phenotype of cancer cells may determine the success of immunotherapies as anticancer treatments. Metastases or tumor cells harboring highly positive HLA-I phenotype and/or reversible-soft alterations regressed with the treatments, whereas those cells that presented low or negative HLA-I phenotype and/or irreversible-hard alterations progressed in the patients (Cabrera et al. 2007, Carretero et al. 2008). We consider defining the role of MHC-I surface expression on primary tumor in the success of immunotherapy as anti-metastatic treatment. GR9 tumor model, composed by several tumor clones with different MHC-I surface expression, might be very useful to answer this question.

SPECIFIC OBJECTIVES

- To determinate the frequency and the reproducibility of the loss of HLA-I surface expression on tumor cells during their growth in immunodeficient mice. We would perform *in vivo* local tumor growth assays with several human melanoma cell lines in nude and SCID-Beige mice. We will analyze the HLA-I phenotype, the transcriptional expression of class I heavy chains, β_2-microglobulin and APM components genes, and the *in vivo* tumorigenicity of the different human melanoma cell lines obtained.

- To analyze if MHC-I surface expression on tumor cells may determine their intrinsic oncogenicity. We would use the Ando-2 melanoma model to compare oncogenic characteristics of different melanoma cell lines, derived from a same melanoma cell line, with a distinct MHC class I phenotype. We will perform: *in vivo* local tumor capacity, *in vitro* proliferation rate, migratory and invasive capacities the cell cycle distribution, and the transcriptional level of cell cycle genes

- To evaluate the implication of cancer cell MHC-I phenotype in the success of immunotherapy as antimetastatic treatment. Using the GR9 tumor model, different immunotherapies will be administered during spontaneous metastasis assays, analyzing immune response and efficacy promoted by the treatment.



Methodology and Results

COMPARATIVE ANALYSIS OF HLA-I PHENOTYPE OF DIFFERENT MELANOMA CELL LINES BEFORE AND AFTER TUMOR GROWTH IN IMMUNODEFICIENT MICE

Previous results from our group showed how alterations in HLA-I expression may also occur in absence of autologous immune response. Using Ando-2 human melanoma cells we showed that these cells developed a total loss of HLA class I cell surface expression after growth in immunodeficient nude or SCID-Beige mice. We wondered if this phenomena was specific of Ando-2 melanoma cell line or if it could be extensible to others human tumor models. In order to determine the frequency and reproducibility of this phenomenon, we performed local tumor growth assays in immunodeficient mice with different human melanoma cell lines (Ando-2, E-033, E-179 and E-195), analyzing the HLA-I phenotype before and after the *in vivo* growth.

We found that three of the four tested cell lines (Ando-2, E-179, and E-195 melanoma cells) presented the loss of HLA-I surface expression after growth in immunodeficient mice due to a coordinate down-regulation of the transcriptional level of HLA class I heavy chains, β_2-microglobulin and different components of the APM. HLA-I alterations were reversible with IFN-γ treatment and they were produced by epigenetic modifications that could be reversed by treatment with histone deacetylase inhibitors. Finally, we found that the HLA-I negative tumor cells resulted from the growth in immunodeficient mice presented higher *in vivo* local growth capacities.

Cancer Immunol Immunother (2010) 59:13–26
DOI 10.1007/s00262-009-0716-5

Alterations of HLA class I expression in human melanoma xenografts in immunodeficient mice occur frequently and are associated with higher tumorigenicity

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Received: 12 December 2008 / Accepted: 16 April 2009 / Published online: 9 May 2009

Abstract Animal models are widely used to study the biological behavior of human tumors in vivo. Murine immunodeficient models are used to test novel human anti-tumor therapies, and humanized mice are employed to study immunotherapeutic protocols. We find that human melanoma cell lines lose HLA class I surface expression after growth in immunodeficient mice and that this phenomenon occurs frequently and is reproducible. This HLA loss is due to a coordinated down-regulation of APM and HLA heavy chain expression at the transcriptional level. It is produced by epigenetic modifications and can be reversed by treatment with histone deacetylase inhibitors or IFN-gamma. These HLA alterations only appear during in vivo growth and not during successive in vitro passages. Interestingly, these new tumor variants with HLA class I loss show higher tumorigenicity per se and may represent a more advanced state of the original tumor. Lack of MHC class I expression on tumor cells represents a frequent escape mechanism from the immune response. Our results indicate that tumor variants with alterations in MHC can also appear in vivo after the immunoescape phase in the absence of anti-tumor immune response. Our findings suggest that any studied parameter, i.e., HLA expression, of malignant cells in xenograft models, has to be evaluated before and after growth in immunodeficient mice, in order to design more appropriate immunotherapy and chemotherapy treatments against tumor cells growing in vivo.

Keywords HLA alterations · Human tumor · Immunodeficient mice · Oncogenicity

Abbreviations

HLA	Human leukocyte antigens
APM	Antigen processing machinery
TSA	Trichostatin A
HDCAi	Histone deacetylase inhibitors

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Introduction

One current view of the clonal expansion of tumor cells takes into consideration intrinsic characteristics of these cells (growth signals, ignoring of growth inhibitory signals, avoidance of cell death, and unlimited replication producing angiogenesis and invasion of tissue) and immune selection of tumor variants [4, 5, 12]. Cancer progression occurs despite an active and normal immune response, with the growth of tumor cells that invade and metastasize in a healthy host [17]. The acquisition by cancer cells of genetic and phenotypic alterations allows tumors to escape the anti-tumor immune response [30, 34]. This approach may be

useful to explain why acquisition of an immune-escape tumor phenotype is a critical step in the natural progression of human and experimental cancer.

During cancer initiation, tumor cells express tumor-associated antigens that are recognized by the adaptive immune system [22]. Tumor cells have been observed to use a variety of mechanisms to evade a specific T cell immune response [7, 19]. Alterations in MHC expression represent an important escape mechanism that is frequently observed during cancer progression [2, 10, 11]. MHC-altered tumor variants emerge after an immunoselection process in which tumor cells with standard MHC-expression levels are eliminated by the immune system. Several altered HLA class I phenotypes have been detected in human tumors, resulting from the active immune surveillance system in these patients [8]. These findings strongly support the hypothesis that the selection of tumor variants with different MHC class I patterns is dependent on the type and intensity of the immune response of the tumor-bearing host [1]. The immune selection process is followed by an immune-escape step where tumor cells are not recognized by immune system [4, 5].

However, alterations in HLA expression may also occur in absence of autologous immune response. Our earlier studies using Ando-2 human melanoma cells showed that these cells develop a total loss of HLA class I cell surface expression after growth in immunodeficient nude or SCID-Beige mice [23]. In order to learn whether this is just an isolated observation or represents a more general phenomenon and may occur in other cancer cell lines, we compared the HLA phenotype in three different melanoma cell lines (E-033, E-179 and E-195) before and after in vivo growth in immunodeficient mice. Our results indicate that alterations in HLA expression occur frequently in human melanoma cells after growth in nude mice and that these melanoma cells are more tumorigenic in vivo.

Materials and methods

Mice

Athymic 6- to 8-week-old Balb/c nu/nu mice (average weight 20 g) were purchased from Charles River (CRIVER, Barcelona, Spain). Mice were housed under specific pathogen-free conditions, and all work with the animals conformed to guidelines approved by our institution.

Cell lines and reagents

Melanoma cell lines Ando-2, E-033 (FM-93/2), E-179 (URKV-Mel-13) and E-195 (Ma-Mel-48a) were obtained from patients with malignant melanoma. Ando-2 melanoma cell line was kindly provided by Dr. P. Coulie (Universite Louvain (UCL), Brussels, Belgium). The other three melanoma cell lines are publically available at The European Searchable Tumor cell Line Database (ESTDAB) and Cell Bank (http://www.ebi.ac.uk/ipd/estdab) [24]. Cell lines were maintained in ISCOVE tissue culture (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy) and antibiotics. In some experiments, cell lines were treated for 48 h with 800 U/ml IFN-γ (Roche Applied Science, Mannheim, Germany) or Trichostatin A (TSA) (Sigma, St Louis, MO) at concentrations of 50, 250 and 500 nM for 48 h.

HLA typing and microsatellite analysis

Conventional complement-mediated microtoxicity assay was used for serological HLA typing. Sequence specific oligonucleotide analysis was performed using DYNAL RELI® HLA-A, B, C, DR and DQ with DNA obtained from Ando-2 and autologous EBV cell lines. Genomic typing was done using the PMP 5.1 program.

DNA from Ando-2 and autologous EBV cell lines was diluted to 0.50 µg/µl and studied with eight STR markers mapping chromosome 6 (D6s311 located at 6q24; D6s291 at 6p21.2; D6s273, C.1.2.c, C.1.2.5, D6s265, D6s105 at 6p21.3 and D6s276 at 6p22); and two markers located on chromosome 15 (D15S209, D15S126). PCR data were analyzed on an ABI PRISM 377 using ABI PRISM 377 GENESCAN and GENOTYPER programs. LOH was assigned when there was a >25% signal reduction of one allele in tumor versus control sample. Allelic reduction in three or more STR markers in chromosome 6 was defined as haplotype loss.

In vivo tumor growth

5×10^6 cells of each tumor cell line (Ando-2, E-033, E-179 and E-195) were subcutaneously (s.c.) injected into the footpad in groups of five nude mice. When local tumors reached 10 mm in large diameter, they were extirpated and disaggregated and the cells were cultured. These tumor cell lines were designated by adding N to the name of original melanoma cell line, e.g., E-179-N1. All tumor cells were maintained in ISCOVE tissue culture supplemented with 10% fetal bovine serum and antibiotics.

In in vivo oncogenicity assays, different doses of cells, 1- 2.5- and 5×10^6 were s.c. injected into footpad in groups of five nude mice. The large tumor diameter was measured every 2 days.

Surface HLA expression

Surface HLA class I and II expression on cultured cells was determined by indirect immunofluorescence using the

appropriate anti-class-I monoclonal antibody (mAb) and fluorescein isothiocyanate-labeled rabbit antimouse Ig (Fab2) fragments (Sigma). Fluorescence was analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) using standard methods. Thus, 5×10^5 cells were washed twice in PBS supplemented with 2% fetal calf serum (FCS) and 0.1% sodium azide. Cells were incubated with the primary mAb at saturating concentration for 30 min at 4°C, using mAbs against: HLA class I (W6/32), HLA-A (Tu-155), HLA-B (42-IB5), HLA-A2 (30.13.38 [Kre-501] and CR11-357), HLA-B40 (HB 115) and HLA-B8 (MRE4). The secondary antibody was used at a 1:80 dilution and incubated with cells for 30 min at 4°C in the dark. In control experiments, a primary antibody was replaced by the isotype-matched non-immune mouse IgG. In addition, cells labeled with only the fluorescein-conjugated antibody were always used as a control. Instrument alignments were checked with caliBrite beads, and the calibration was set by FACSComp software. CellQuest software was used to generate plots. Flow cytometry histograms were generated with the logarithmic amplification of fluorescence emitted by single viable cells. Each sample analyzed consisted of a minimum of 10^4 cells.

NK cytotoxicity assay

Cytotoxicity assays were performed according to a standard ^{51}Cr-release method as mentioned above. Ando-2 and Ando-nude cell lines were used as target cells. Splenocytes (effector cells) were isolated from all mice by mechanical dissociation and lysing of erythrocytes. Splenocytes were fractionated by density centrifugation at $500 \times g$ for 20 min with Ficoll Hypaque and interface lymphocytes were obtained. YAC-1 cells (ATCC), a mouse lymphoma line sensitive to the cytotoxic activity of NK cells, were used as positive control. YAC-1 cells were maintained in RPMI 1640 supplemented with 10% FCS. After 4 h of culture, the supernatant was removed from each well and counted in a gamma counter for the determination of ^{51}Cr release. Lysis was considered significant when >10%.

CTL cytotoxicity assay

Cytotoxicity of CTL was assessed using the standard ^{51}Chromium-release assay. Briefly, one million target cells were labeled at 37°C for 1 h with 100 µCi Na_2 $^{51}CrO^4$ (New England Nuclear, Boston, MA, USA). Target cells were washed and resuspended in CM at 5×10^4 cells/ml. Five thousand target cells per well (100 µl) were added to a 96 well plate (Costar, Cambridge, MA, USA) following the appropriate number of effector cells (100 µl/well). Cells at the defined effector:target (E:T) ratios were plated in triplicate. Cytotoxicity assays were performed at 37°C for 4 or 12 h. After incubation, cell-free supernatants were collected using a Skatron harvester and analyzed in a gamma counter (LKB Wallac CliniGamma 1272, Wallac, Finland). The percentage of the specific lysis was calculated using the following equation: $(ER-SR)/(MR-SR) \times 100$, where ER = experimental release, SR = spontaneous release and MR = maximum release. For maximal release, target cells were treated with 0.3% Triton X-100 (Sigma). Spontaneous release of radioactivity by target cells was determined in the absence of effector cells. Results are shown as an average percentage of the specific lysis ±SE of triplicate determinations.

For the first screening assay, the labeled-target cells were mixed with cold (unlabeled) K562 cells at a 1:20 labeled-target:cold-target ratio to decrease the impact of the nonspecific killing by the natural killer cells.

Ando-2 and Ando-nude cell lines were used as target cells. CTL clones used as effector cells were obtained from co-cultures of $CD8^+$ lymphocytes (purified by negative selection with $CD8^+$ T Cell Isolation Kit, human, Myltenyi-Biotech, Germany) of melanoma patient Ando-2 with irradiated (10,000 rads) autologous melanoma cells Ando-2. Cultures were weekly stimulated with irradiated autologous tumor cells in RPMI 1640 medium-containing IL-2 (50 U/ml) and IL-7 (10 ng/ml). After 4 weeks of stimulation, 4 h cytotoxic assays were performed against Ando-2 and Ando-2 EBV cells and those cultures that exhibited specific cytotoxic activity were selected and expanded in cell culture. The CTL clones were restimulated weekly by addition of feeder cells and irradiated autologous melanoma cells Ando-2.

RT and quantitative real-time PCR

The mRNA isolation kit (Myltenyi-Biotech) was used to extract mRNA from tumor cell lines under basal conditions and after 48-h IFN-γ treatment. First-strand cDNA was synthesized with 100 ng of mRNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 µl. These cDNAs were diluted to a final volume of 100 µl. Real-time quantitative PCR analyses for β_2-microglobulin, HLA-A, HLA-B, HLA-C, TAP1, TAP2, LMP2, LMP7 and Tapasin genes were performed by means of the 7500 Fast System (Applied Biosystems), using GAPDH and TBP genes (Kit from Applied Biosystems) as housekeeping genes. PCR reactions were performed in quadruplicate, and values obtained were expressed as mean ± SD (standard deviation). Quantitative PCR was performed with the Power SYBR Green Master mix (Applied Biosystems). Primers, amplicon size and annealing temperature for each gene are shown in Table 1. PCR conditions were 40 cycles of 15 s of denaturation at 95°C and 60 s at 60°C.

Table 1 Oligonucleotides used in Q-PCR

Gene	Oligo	Sequence	Position	Fragment size
Beta-2m	Forward	TGACTTTGTCACAGCCCAAGAT	374–459	86
	Reverse	CAATCCAAATGCGGCATCTTCA		
TAP1	Forward	GCCTCACTGACTGGATTCTAC	964–1123	160
	Reverse	TCTCCCTGCAAGTGGCTGTG		
TAP2	Forward	GGTCGTGTGATTGACATCCTG	642–870	229
	Reverse	TCAGCTCCCCTGTCTTAGTCT		
Tapasin	Forward	TCCAGCCTCTTGCGACCACA	577–713	137
	Reverse	CTCAAGTCCAGCAGAGCATCT		
LMP2	Forward	ATGGGTTCTGATTCCCGAGTG	139–260	122
	Reverse	GCTTGGGCATCAGCAGCTGA		
LMP7	Forward	CCTTCAAGTTCCAGCATGGAG	740–875	136
	Reverse	GCTGCACAGCCAGACATGGT		
HLA-A	Forward A2	CTCTTTGGAGCTGTGATCACT	949–1147	198
	Forward A32	TCTCTTTGGAGCTATGTTCGCT	948–1147	199
	Reverse	GAAGGGCAGGAACAAMTCTTG		
HLA-B	Forward	GTCCTAGCAGTTGTGGTCATC	949–1089	140
	Reverse	TCAAGCTGTGAGAGACACATCA		
HLA-C	Forward	TCCTGGCTGTCCTAGCTGTC	950–1100	150
	Reverse	CAGGCTTTACAAGTGATGAGAG		

Table 2 HLA class I genomic typing of melanoma cell lines

Melanoma cell line	HLA-A	HLA-B	HLA-C
E-033	0201, 2601	4001, 4402	0304, 0501
Ando-2	3201	4001	0602
E-179	0201	4001	0304
E-195	0101	0801	0701

Results

HLA genotype and phenotype of human melanoma cell lines

HLA genomic typing of human melanoma cell lines Ando-2, E-179, E-195 and E-033, are presented in Table 2. Analysis of microsatellites of chromosome 6 showed loss of one chromosome 6 in Ando-2, E-179 and E-195 cell lines (data not shown).These melanoma cell lines present the loss of one HLA haplotype. The E-033 melanoma cell line does not show losses and maintains the two HLA haplotypes.

HLA class I surface expression on these melanoma cell lines was measured by indirect immunofluorescence and flow cytometry. Since the isotype-matched antibody control and FITC-conjugated secondary Ab showed identical results, we showed only one control in the histograms and plots. The Ando-2 melanoma cell line showed surface expression of one HLA class I haplotype, expressing HLA-A32 and HLA-B40 alleles (Fig. 1). E-179 melanoma cell line showed surface expression of one HLA class I haplotype, corresponding to HLA-A2 and HLA-B40 alleles (Fig. 1). E-195 melanoma cell line showed surface expression of one HLA-A allele, HLA-A1, but no surface expression of locus B (Fig. 1). In all melanoma cell lines, treatment with IFN-γ induced surface expression of HLA class I alleles expressed in baseline conditions (data not shown). E-033 melanoma cell line showed surface expression of both HLA-A alleles but no surface expression of locus B (Fig. 1).The HLA class I phenotype of these melanoma cell lines was assessed at different time points during in vitro cell culture. HLA phenotypes were identical and did not change during in vitro passages.

Changes in HLA class I phenotype after growth in nude mice

5×10^6 cells of each melanoma cell line were injected in groups of five nude mice and after local growth of the tumors, they were extirpated and the cells adapted to tissue culture, thus each tumor generated a new melanoma cell line. Each human melanoma cell line obtained after growth in nude mice was labeled with suffix -N. The HLA phenotype of tumor cells was analyzed immediately after extirpation and again after adaptation to tissue culture. The same HLA phenotype was found in all of the melanoma cell lines. Ando-2 melanoma cells showed total loss of HLA class I expression after growth in nude mice (Ando-2-N1 human melanoma cells) (Fig. 2). Both HLA-A and HLA-B

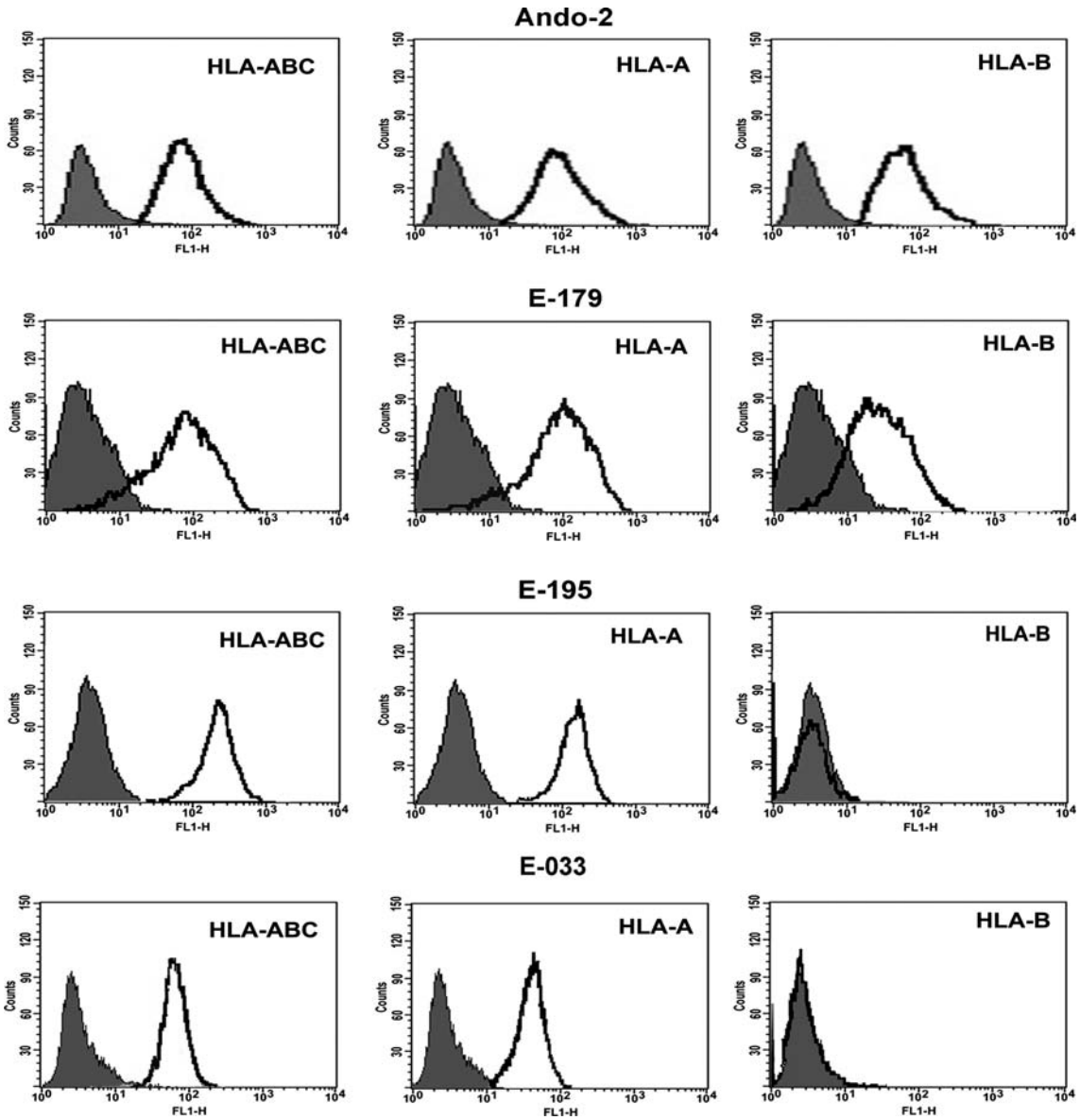

Fig. 1 Surface expression of HLA class I antigens on Ando-2, E-033, E-179 and E-195 human melanoma cell lines. Melanoma cells were stained with specific HLA antibodies and isotype controls and analyzed by flow cytometry. Isotype controls are shown as *shaded peaks*, and *heavy lines* represent expression determined by specific antibody staining. Data represent more than three independent experiments

alleles were induced after IFN-γ treatment (Fig. 2). The same HLA class I phenotype was found in other studied human melanoma cells, from Ando-2-N2 to -N5 (Table 3).

Tumor cells grown in nude mice after injection of E-179 melanoma cells showed total loss of surface expression of locus B and strong down-regulation of locus A (Fig. 2) and only weak surface expression of one HLA-A allele, HLA-A2. HLA-A and HLA-B alleles were induced after IFN-γ treatment (Fig. 2). The same HLA phenotype was found in the five melanoma cell lines E-179-N (Table 3).

E-195 melanoma cells showed surface expression of one HLA-A allele, HLA-A1 and treatment with IFN-γ induced HLA-A and HLA-B surface expression (Fig. 3). After growth in nude mice, the cells had two different HLA class I phenotypes: (1) total loss of HLA class I expression and no induction of HLA expression after IFN-γ treatment (E-195-N1 cells; Fig. 3); and (2) down-regulation of surface expression of HLA-A1 allele, no expression of HLA-B8 allele and induction of HLA-A and HLA-B alleles with IFN-γ treatment (E-195-N2 cells; Fig. 3). This is the only melanoma cell line showing two different HLA phenotypes after growth in nude mice. In five melanoma cell lines E-195-N, three showed total loss of HLA class I expression and two had HLA class I down-regulation (Table 3). All assays were repeated at least three times with similar results.

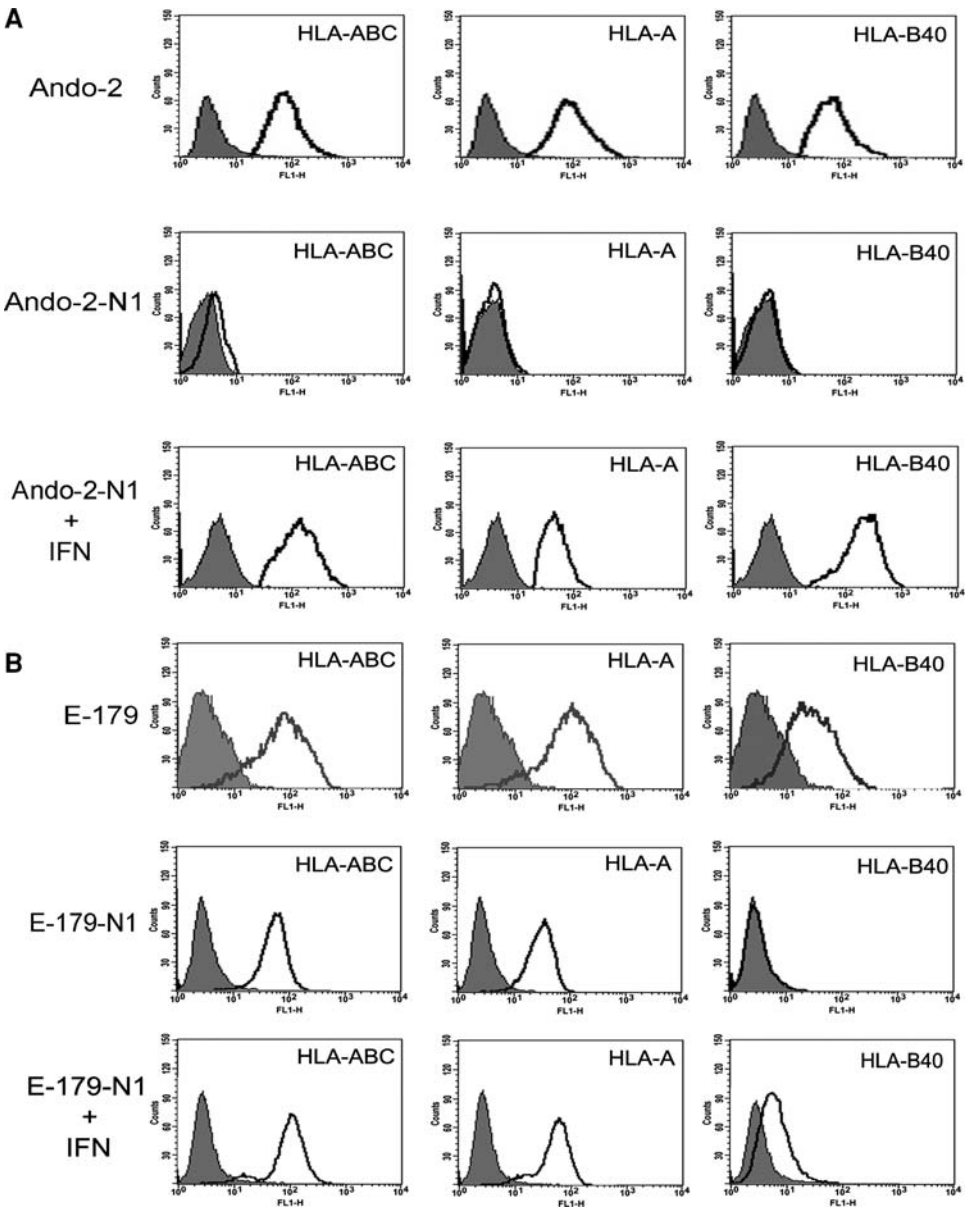

Fig. 2 Growth in nude mice decreases surface expression of MHC class I antigens. Ando-2-N1 and E-179-N1 melanoma cells derived from nude mice (corresponding to mouse 1 of each group) were stained with specific antibodies and isotype controls and analyzed by flow cytometry. Isotype controls are shown as *shaded peaks*, and *heavy lines* represent expression determined by specific antibody staining. Treatment with IFN-γ produces an increase in HLA-A and HLA-B expression. Data for other melanoma cell lines, -N2 to -N5 (obtained from mouse 2–5) presented very similar results (Table 3). Data represent more than three independent experiments

Table 3 depicts the mean fluorescence of the melanoma cell lines before and after growth in nude mice. Three melanoma cell lines (Ando-2, E-179 and E-195) lost HLA class I surface expression after growth in nude mice. E-033 melanoma cells showed no changes in HLA class I phenotype after growth in nude mice, expressing only locus A (Fig. 1, Table 3).

In vitro 4 h cytotoxic assays were performed to determine whether Ando-2 or Ando-2-N cells might be recognized in vitro by mononuclear cells from spleen of nude mice. The results showed that none of the studied melanoma cell lines were recognized by the spleen cells (lysis <10%; data not shown). These cytotoxic assays were also performed using effector cells derived from nude mice previously injected with Ando-2 or Ando-2-N melanoma cells for different periods of time (1–4 weeks), and the results were negative once again. According to these findings, neither Ando-2 nor Ando-2N melanoma cells are recognized in vitro by mononuclear cells derived from spleen of nude mice. Similar results were found in case of E-179, E-195 and E-033 melanoma cells.

Molecular mechanisms implicated in the loss of HLA class I expression

The mRNA expression of HLA class I heavy chains (HLA-A, -B and -C), was determined by quantitative RT-PCR to explore whether transcriptional mechanisms underlie the loss of HLA class I surface expression after growth in nude mice. A strong transcriptional down-regulation of all three

Table 3 Mean fluorescence of HLA class I surface expression

Fluorescence intensity (mean)					
Cell lines[a]	HLA-ABC	HLA-A	HLA-A2	HLA-B40	HLA-B8
Ando-2	87	60	–	66	–
Ando-2-N1	6	0	–	0	–
Ando-2-N2	8	0	–	1	–
Ando-2-N3	5	1	–	0	–
Ando-2-N4	5	1	–	1	–
Ando-2-N5	3	0	–	0	–
E-179	84	84	151	102	–
E-179-N1	46	28	41	1	–
E-179-N2	46	56	29	0	–
E-179-N3	69	56	61	2	–
E-179-N4	48	53	35	1	–
E-179-N5	41	35	25	0	–
E-195	185	169	–	–	1
E-195-N1	3	1	–	–	2
E-195-N2	79	68	–	–	1
E-195-N3	4	0	–	–	0
E-195-N4	3	3	–	–	1
E-195-N5	46	28	–	–	2
E-033	78	56	47	2	–
E-033-N1	80	60	50	3	–
E-033-N2	72	55	40	1	–
E-033-N3	70	50	41	1	–
E-033-N4	65	48	39	0	–
E-033-N5	70	55	45	2	–

[a] N1–N5 melanoma cell lines obtained after in vivo growth in nude mice

HLA loci was observed in Ando-2-N, E-179-N, E-195-N1 and -N2 cells as compared to Ando-2, E-179 and E-195 melanoma cells, respectively. Figure 4 presents the mean values ±SD measured in these three melanoma systems. The down-regulation was around 10-fold for locus B, 6-fold for locus C, and 2-fold for locus A. In E-033 system no changes were detected (Fig. 4).

The mRNA expression of APM components (TAPs, LMPs and Tapasin) was also determined by RT-PCR, finding a strong down-regulation of TAP1, LMP2, LMP7, and Tapasin after growth in nude mice for Ando-2-N, E-179-N and E-195-N cells (Fig. 4). The down-regulation was greatest for LMP2, at around 20-fold, followed by TAP1, LMP7 and Tapasin. No change in Tap2 mRNA expression was observed (Fig. 4). The transcriptional expression of β_2-microglobulin was also modified but at a lesser degree, with the tumor cells showing half of their original mRNA expression after growth in nude mice (Fig. 4). In E-033 tumor system, we did not find any changes in the transcriptional level of neither APM components nor β_2-microglobulin gene (Fig. 4). According to these findings, the loss of HLA surface expression is mainly due to a strong coordinated transcriptional down-regulation of HLA class I heavy chains and APM components.

Previous studies showed that surface MHC class I expression on tumor cell lines can be enhanced by treatment with histone deacetylase inhibitors (HDCAi) [14] and that HDCAi induces coordinated mRNA expression of APM and MHC class I heavy chains [13, 16]. The role of histone deacetylation in the loss of MHC class I expression after growth in nude mice was examined by treating the Ando-2-N1 tumor cells with different doses of TSA (50, 250 and 500 nM) for 48 h. All treatments induced HLA class I surface expression in the Ando-2-N1 cells at various levels. Figure 5 depicts, as an example, the results of 48-h treatment with 500 nM TSA, after which positive expression of HLA class I molecules was detected in 42% of tumor cells. HLA-A and HLA-B allele expression was enhanced after TSA treatment. Similar results were obtained with other Ando-2-N human melanoma cells. E-179-N1 melanoma cells do not have cell surface expression of locus B, but after treatment with TSA practically all cells recovered surface expression of allele HLA-B40 (Fig. 5). Similar results were found for other E-179-N melanoma cells. A 40% of E-195-N1 melanoma cells showed positive surface expression of HLA class I molecules after treatment with TSA (Fig. 5). These findings suggest that histone deacetylase-mediated epigenetic mechanisms may be involved in the down-regulation of the MHC class I expression in these tumor cells. When E-033-N1 to -N5 melanoma cells were treated with TSA, no changes in HLA surface expression were found (Fig. 5).

Absence of recognition by CTLs and increased in vivo oncogenicity of tumor cell lines after growth in immunodeficient mice

The Ando-2-N cells are not expected to be recognized by the autologous CTLs generated against Ando-2 melanoma cells due to the total cell surface loss of HLA class I molecules. We tested this possibility in cytotoxic assays using different CTL clones as effector cells against Ando-2 and different Ando-2-N melanoma cells as target cells. These CTLs specifically recognized Ando-2 cells but did not recognize Ando-2-N1 cells (Fig. 6). As controls, NK-susceptible target (K562) and LAK-sensitive target (Daudi) were used (Fig. 6). None of the other melanoma cell lines (Ando-2-N2 to-N5) were recognized by these CTLs. The Ando-2 human melanoma cells are not recognized by autologous CTLs after growth in nude mice.

We evaluated the in vivo oncogenicity of the human melanoma cells obtained before and after growth in nude

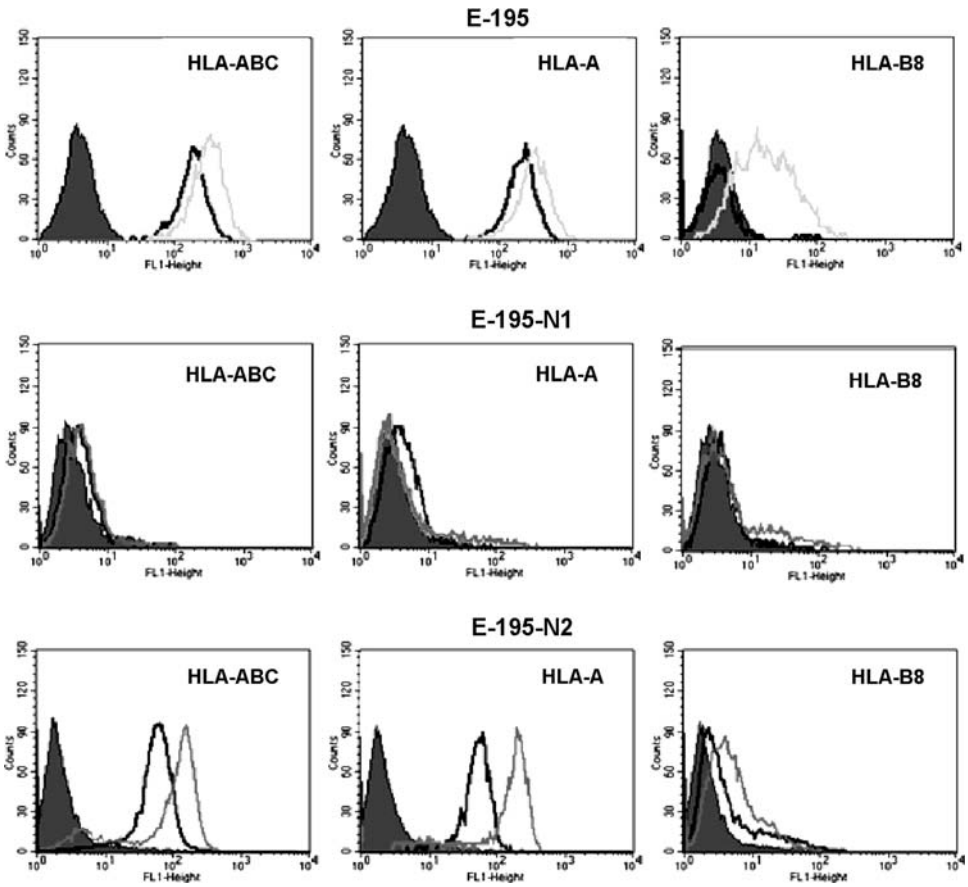

Fig. 3 E-195 human melanoma cells show two different altered HLA class I phenotypes after growth in nude mice. Isotype controls are shown as *shaded peaks*, expression levels without treatment (baseline conditions) are shown as heavy lines, and *dotted lines* represent expression after IFN-γ treatment. E-195-N1 (derived from mouse 1) do not present HLA class I surface expression under baseline conditions or after IFN-γ treatment. E-195-N2 (derived from mouse 2) show down-regulation of HLA-A class I allele and IFN-γ induced enhancement of HLA-A and HLA-B alleles. Data for other melanoma cell lines, -N3 to -N5 (obtained from mouse 3–5) presented very similar results (Table 3). Data represent more than three independent experiments

mice. 10^6, 2.5×10^6 and 5×10^6 cells were injected into different groups of immunodeficient mice, five mice per group and the local tumor growth rate was measured every 2 days. Figure 7 shows the results of local tumor growth as a mean value ±SD from each group of five mice. Ando-2 melanoma cells generated a solid tumor only at a dose of 5×10^6 cells, with no tumor growth when fewer cells were injected (Fig. 7). The Ando-2 cells initially grew very slowly, with an increased growth rate after 20 days, reaching a diameter of 10 mm at day 60 after the injection (Fig. 7). In contrast, Ando-2-N1 cells grew into a solid tumor at all cell doses, even when only 10^6 cells were injected (Fig. 7). Ando-2-N1 melanoma cells grew rapidly, reaching a diameter of 10 mm by day 30 (Fig. 7). In E-179 and E-195 tumor system, we observed similar results, E-179-N1 and E-195-N1 cells presented a higher in vivo oncogenicity and local growth than E-179 and E-195 cells, respectively (Fig. 7). In addition, we obtained very similar results when we used SCID-Beige mice (data not shown). Therefore, the studied human melanoma cells obtained after growth in immunodeficient mice showed a more rapid local growth and higher oncogenic potential in vivo. On the contrary, E-033 and E-033-N1 human melanoma cells showed the same in vivo oncogenicity and local growth (Fig. 7).

Discussion

Mouse models of cancer have consistently been used to qualify new antitumor therapies for study in human clinical trials. The most frequently used models include transplantable murine tumors grown in syngeneic hosts and xenografts of human tumors grown in immunodeficient mice or humanized mice [29, 32]. However, the phenotype of human tumor cells growing in immunodeficient mice is not usually analyzed or compared with their previous phenotype. Our results describe differences in the HLA phenotype and tumorigenicity of human melanoma cells before and after their transplantation into immunodeficient mice. Three melanoma cell lines showed various types of HLA class I loss after growth in nude mice. One of them, Ando-2, showed a total loss of HLA class I surface expression recoverable after IFN-γ treatment [23]. Another cell line, E-179, presented a total loss of HLA locus B expression and a strong down-regulation of HLA-A locus. The third line, E-195, showed two distinct patterns of changes in the HLA class I expression: down-regulation of locus A or total loss of HLA class I surface expression. Only one melanoma cell line, E-033, showed no changes in HLA class I surface expression after growth in nude mice, indicating that these HLA alterations in human melanoma cell lines

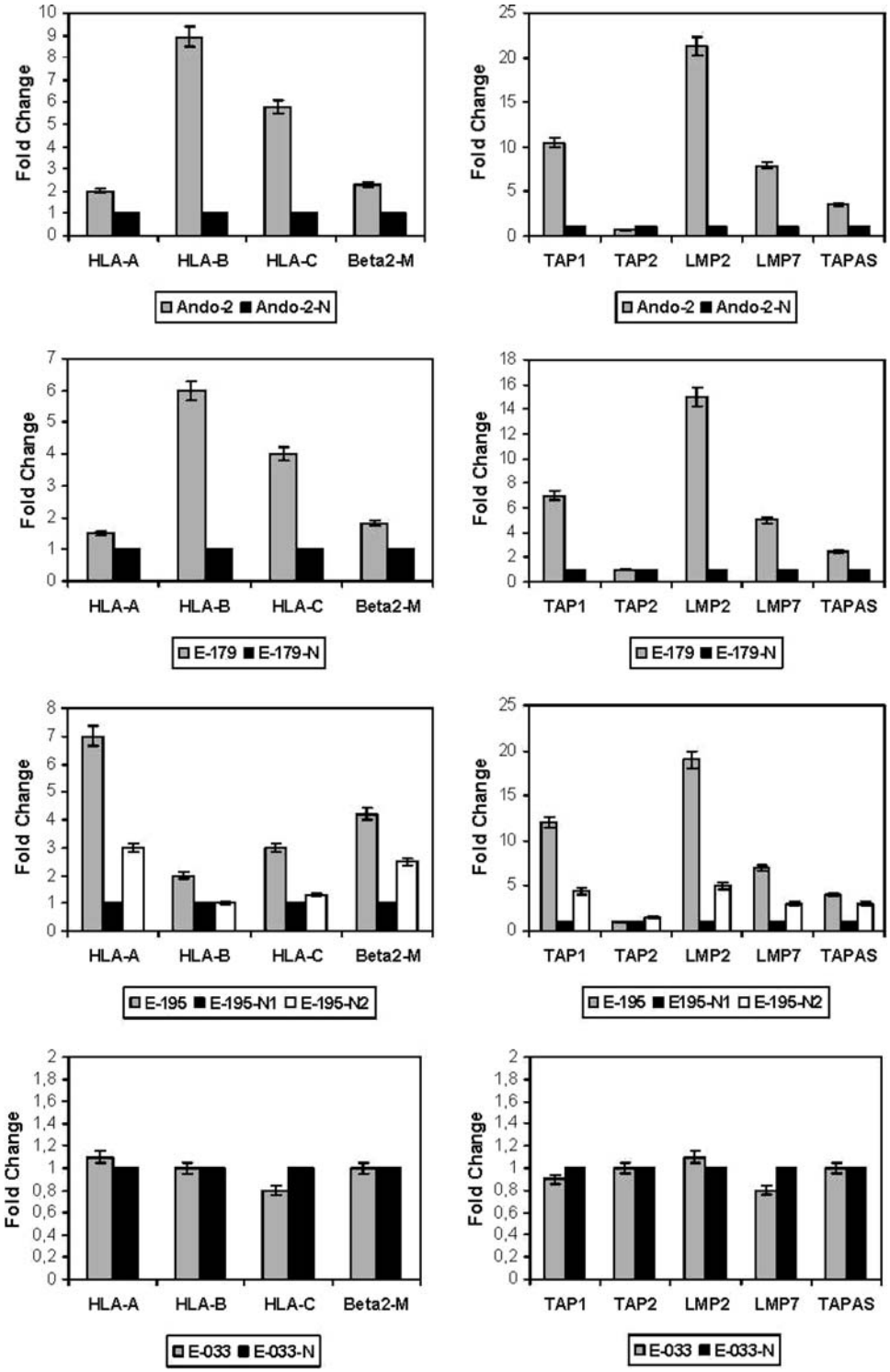

Fig. 4 Detection of HLA class I, β2-m and APM component mRNAs by quantitative RT-PCR. This figure depicts the results for melanoma cell lines -N1 to -N5 as mean ± SD in each case (values corresponding to data with -N), except for E-195 where two different means are determinate according to different HLA phenotypes found (N1 and N2). After growth in nude mice of Ando-2, E- 179 and E-195, the human melanoma cells show approximately two- to ninefold decrease in mRNA of HLA-class I heavy chains and β2-microglobulin. The decrease is approximately 5- to 20-fold in APM genes. Only TAP2 gene showed no change in expression. In E-033 system, we did not detect any change at mRNA levels. These RT-PCR experiments were repeated at least three times with similar results

are frequent and reproducible but do not always occur. Interestingly, unlike the other studied cell lines, E-033 did not have HLA haplotype loss before being injected into nude mice, while the other investigated melanoma cell lines had developed this escape mechanism to avoid anti-tumor immune attack. These results might indicate that E-033 melanoma cells might have developed a different escape mechanism. In all melanoma cell lines, except for E-195-N1, -N3 and N-4 the HLA class I expression was inducible after IFN-γ treatment. According to our results, the loss of HLA class I surface expression in the studied cell lines was due to a transcriptional mechanism, i.e., the coordinated downregulation of LMP, TAP, Tapasin and HLA class I heavy chain.

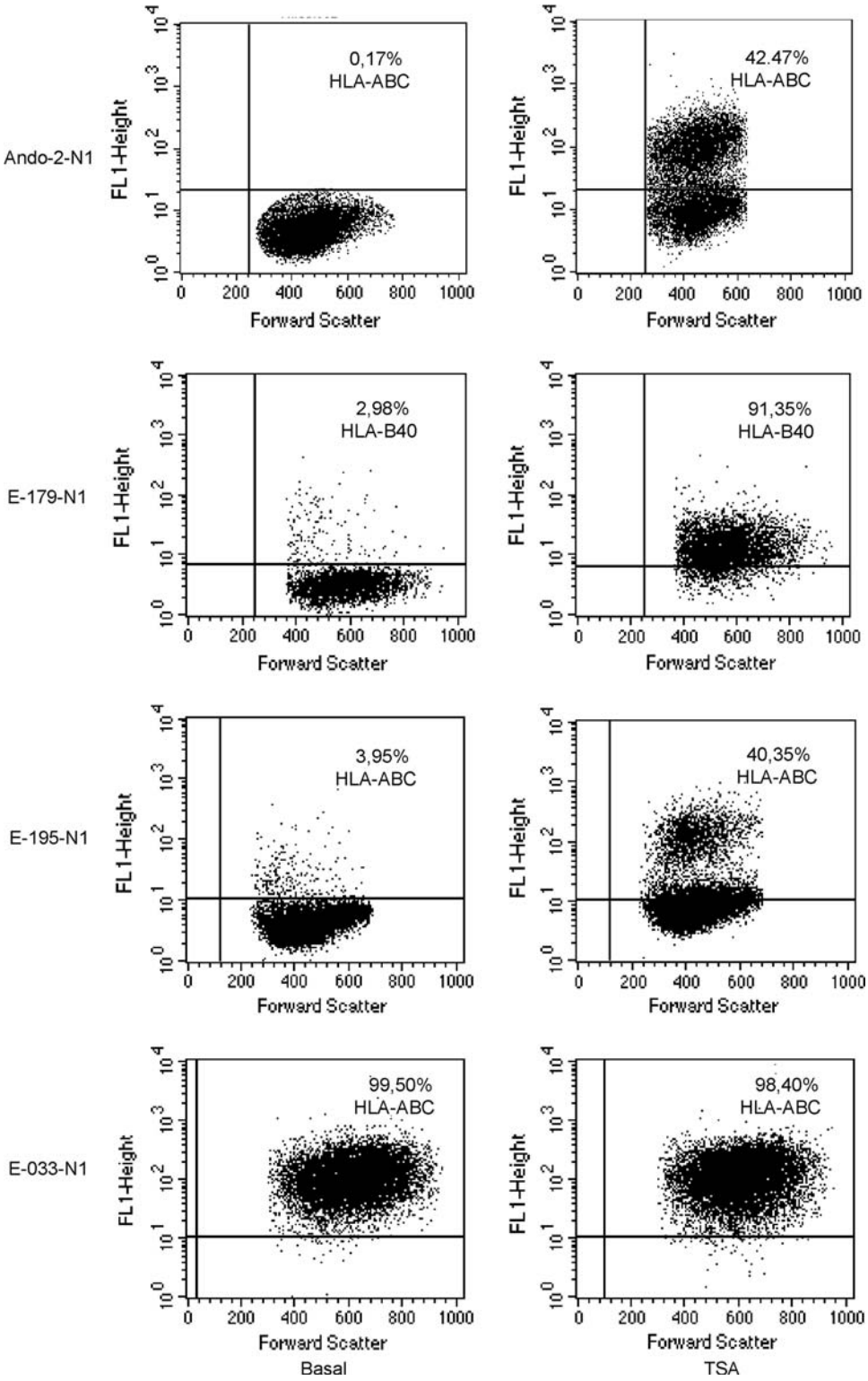

Fig. 5 TSA treatment enhances expression of down-regulated MHC class I molecules. Ando-2-N1, E179-N1, E195-N1 and E-033-N1 melanoma cells were stained with specific antibodies (W6/32 or HB 115) and isotype controls before and after treatment with TSA (500 nM for 48 h) and analyzed by flow cytometry. Values in the dot-plot are the percentage of cells positive for the respective antibody relative to untreated cells. E-033-N1 cells did not show changes after TSA treatment. The results found for the other melanoma cell lines, named -N2 to -N5, were identical. Data represent more than three independent experiments

Multiple epigenetic mechanisms have been described to underlie changes in HLA antigens on tumor cells and they have been shown to impair the recognition of tumor cells by the components of the adaptive immunity [3]. It was recently reported that TSA treatment of murine cells induces MHC class I surface expression by enhancing the coordinated expression of TAP and LMP molecules [13, 16]. In our experiments, TSA treatment recovered HLA class I expression on human melanoma cell lines derived from nude mice, suggesting that HDAC-mediated chromatin

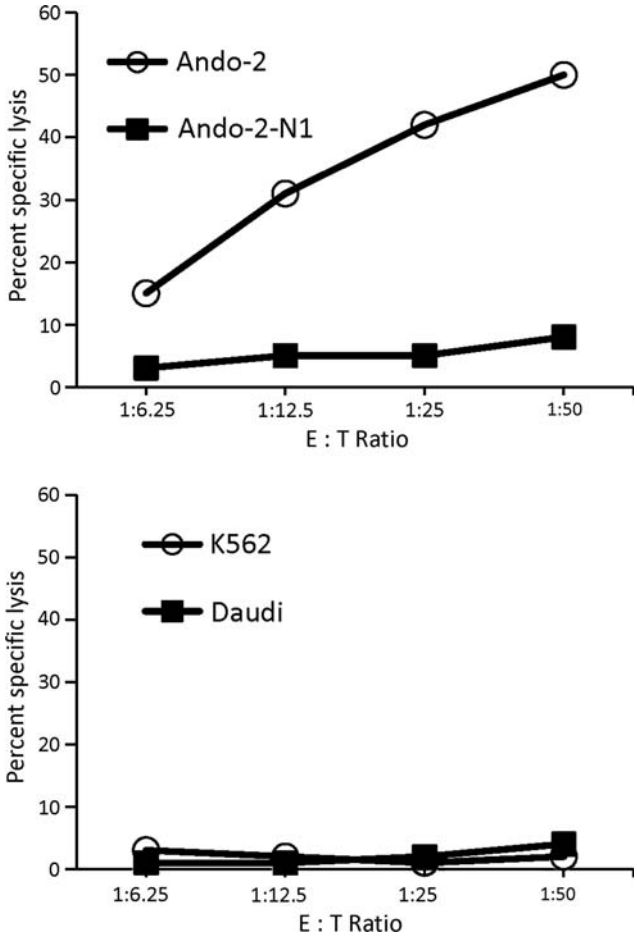

Fig. 6 Cytotoxicity of autologous CTL in the ^{51}Cr-release assay. CTL cell line obtained against Ando-2 was utilized as effector cells. Various numbers of CTL cells were tested against 5×10^3 Ando-2 or Ando-2-N1 cells (target cells) for 4 h at 37°C. Results are expressed as the mean percentage specific lysis of triplicate samples, in which the SEs of the means were consistently below 10% of the value of the means. Data is representative of three experiments. Similar results were obtained for other Ando-2-N melanoma cell lines and other different autologous CTLs

regulation is involved in the suppression of class I antigen processing genes in these cells.

These alterations in HLA class I expression did not occur during in vitro culture, they are produced solely when human tumor cells were grown in vivo in the absence of an autologous immune response. B lymphocytes and NK cells of the mice do not appear to be implicated, since the HLA expression changes were also observed in SCID-Beige mice [23]. Recently, it has been reported that stromal cells may have a major influence on the growth and progression of tumors [33], and that their genetic characteristics determine the prognosis and malignancy of tumors [6, 25]. The HLA alterations observed in the present study might have been caused by interaction with stromal cells. In vitro cell culture maintains the HLA phenotype of tumor cells, whereas in vivo growth in immunodeficient mice can produce phenotypic changes in tumor cells, possibly due to interactions with fibroblasts, epithelial cells and macrophages in the stroma.

Importantly, in all cases where we found alterations in MHC after growth in immunodeficient mice, the melanoma cells acquired a phenotype more tumorigenic per se with higher oncogenic capacity. Based on the obtained results, we can propose a hypothetical path of the step-by-step changes that might take place in the studied melanoma cells during the course of cancer progression in the patients. First, the tumor cells with HLA haplotype loss were selected during the autologous immunoselection stage in the patient. Indeed, the haplotype loss of HLA class I antigen has been described widely as escape mechanism in different tumors [15, 21, 26, 31]. During the next stage, called immunoescape, these cells evade the immune reactivity due to the growth advantage that gives them altered HLA phenotype. After the extirpation from patient and an in vitro culture, these tumor cells maintained their HLA phenotype. However, when these tumor cells were then injected into immunodeficient mice, new HLA alterations had appeared during their in vivo growth indicating that loss of HLA expression can also occur after the immunoescape step. This hypothesis is supported by previous reports describing that two successive mechanisms are implicated in total loss of MHC class I expression in tumor cells: loss of one MHC haplotype and downregulation of APM and MHC heavy chains [9, 15, 18, 27]. This new phase that comes after the immunoescape phase, we have called "Immunoblindness" phase, since such tumor cells are invisible to immune system. We believe that this is an important stage of the tumor progression because the human melanoma cells obtained after growth in nude mice were more oncogenic in vivo than their respective original melanoma cells, indicating a higher grade of malignancy and a more evolved state of the tumor.

The observed HLA alterations have great importance in immunotherapeutic procedures used in humanized-immunodeficient mice against human tumor cells, where immune response component must be evaluated. Moreover, it also must be considered in chemotherapy protocols given the importance of the immune system in this type of therapy [35, 36]. Recently, it has been reported that chemotherapy enhances vaccine-induced antitumor immunity in melanoma patients [20]. The dacarbazine administration before peptides vaccination was able to induce a long-lasting enhancement of memory $CD8^+$ T cell responses to cancer vaccines. We propose that it is crucial to apply a combined anti-tumor treatment including chemo- and radiotherapy along with immunotherapy treatment at that particular stage. This approach might prevent an outgrowth of the most aggressive tumor cell variants and allow prediction of the progression of a specific tumor.

*Fig. 7: Note at the end of the article

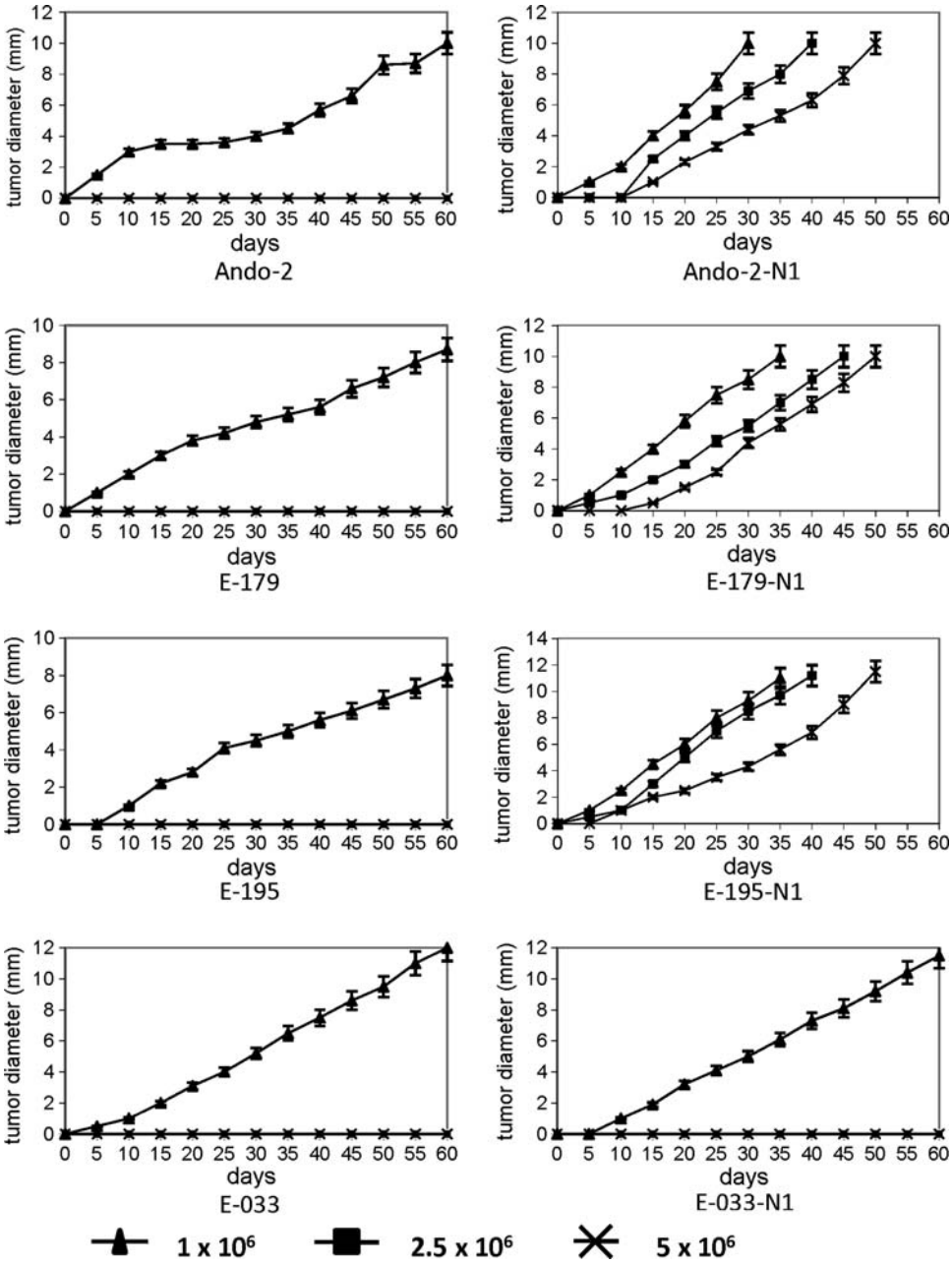

Fig. 7 In vivo growth of human melanoma cell lines derived from nude mice. Three different doses of human melanoma cells (1×10^6, 2.5×10^6 and 5×10^6) were implanted in nude mice and the tumor growth rate was assessed. Data depict mean ± SD from five mice from each group. **a** Ando-2 tumor cells grew only after injection of 5×10^6 cells, reaching a tumor diameter of 10 mm in 60 days. **b** Ando-2-N1 tumor cells grew at all cell doses, reaching a tumor diameter of 10 mm at 30–50 days. Very similar results were obtained for E-179, E-179-N1, E-195 and E-195-N1 human melanoma cells. E-033 and E-033-N1 melanoma cells did not present differences in in vivo growth in nude mice. The assays were performed in groups of five mice and were repeated at least three times

In addition, recently it has been reported a direct relation between defective MHC class I expression and cellular survival promoting resistance to apoptosis [28]. Our results show that human melanoma cells with lower MHC class I expression are more tumorigenic in vivo in immunodeficient mice, suggesting in this xenogenic model an inverse relation between HLA class I expression and tumor oncogenicity per se.

Our findings suggest that any studied parameter, i.e., HLA expression, of malignant cells in xenograft models, has to be evaluated before and after transplantation into immunodeficient mice. These changes in tumor cell phenotype must be taken into account in order to design more appropriate immunotherapy and chemotherapy treatments against tumor cells growing in vivo. Moreover, our results could indicate new implications of HLA losses in oncogenicity and survival of tumor cells. These experimental tumor models will also be useful to study additional functions of HLA class I molecules in tumor progression and the underlying molecular mechanisms.

Acknowledgments The authors thank I. Linares and E. Arias for technical advice. They also thank Dr. Natalia Aptsiauri for helpful discussion. This work was partially supported by grants from the Fondo de Investigaciones Sanitarias (FIS, CP03/0111), Red Genómica del Cancer RTICC (RETIC RD 06/020), Consejeria de Salud and by Plan Andaluz de Investigación (PAI, Group CTS-143 and project CTS-695)

from the Junta de Andalucia, in Spain; by the ENACT project (LSHC-CT-2004-503306), and by the Cancer Immunotherapy project (OJ 2004/c158,18234) from the European Community. A. G. L. was supported by FIS-Research Contract CP03/0111 and C. G. was supported by FPU grant from MICINN (AP2007-03102).

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*The authors would like to indicate that in the Figure 7 there is a mistake in the legend, corresponding the triangle to the highest dose of injected tumor cells (five million of cells) and the asterisk to the lowest dose of injected tumor cells (a million of cells).

ANALYSIS OF THE IMPLICATION OF MHC-I MOLECULES IN THE INTRINSIC TUMORIGENIC CAPACITIES OF TUMOR CELLS

Previous results showed two surprising phenomena: firstly the loss of HLA-I surface expression of different melanoma cell lines during growth in immunodeficient mice; and secondly, that the HLA-I negative derived cell lines showed more quickly *in vivo* growth than their respective HLA-I positive ones. If these melanoma cells were growing in absence of an immune response, why they lost HLA-I surface expression? Moreover, were the alterations in HLA-I phenotype which induced the higher oncogenic capabilities of these melanoma cell lines?

To answer these questions, we analyzed different oncogenic characteristics of the HLA-A2 stably transfected Ando-2, Ando-TA2 cell line, and compared it with Ando-2 and Ando-Nude cell lines. Ando-2, Ando-TA2 and Ando-Nude cell lines compound a human melanoma tumor model formed by three melanoma cell lines with a common origin but with different HLA-I expression, and the only difference between Ando-2 and Ando-TA2 cell lines is the gene transfection of HLA-A2 allele.

We found that the transfection of HLA-A2 allele promoted cell cycle arrest and induced the up-regulation of AP-2α, p21WAF1/CIP1 and cyclin A1 tumor supressor genes. In the contrast, these genes were down-regulated in Ando-Nude cells and they showed increased cell cycle progression. Furthermore, we found an inverse correlation between the HLA class I phenotype and the intrinsic tumorigenic capacities of these three melanoma cell lines: 1) *in vivo* growth in immunodeficient mice, 2) *in vitro* proliferation rate and, 3) *in vitro* migratory and invasiveness capacities.

For the first time, we showed that alterations of HLA-I expression in tumor cells play a direct role in cancer progression, increasing the intrinsic oncogenicity, migratory and invasive capacities of cancer cells.

Carcinogenesis vol.33 no.3 pp.687–693, 2012
doi:10.1093/carcin/bgr318

MHC class I molecules act as tumor suppressor genes regulating the cell cycle gene expression, invasion and intrinsic tumorigenicity of melanoma cells

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The alteration of MHC class I (MHC-I) expression is a frequent event during cancer progression, allowing tumor cells to evade the immune system. We report that the loss of one major histocompatibility complex haplotype in human melanoma cells not only allowed them to evade immunosurveillance but also increased their intrinsic oncogenic potential. A second successive defect in MHC-I expression, MHC-I total downregulation, gave rise to melanoma cells that were more oncogenic *per se in vivo* and showed a higher proliferation rate and greater migratory and invasive potential *in vitro*. All these processes were reversed by restoring MHC-I expression via human leukocite antigen-A2 gene transfection. MHC-I cell surface expression was inversely correlated with intrinsic oncogenic potential. Modifications in the expression of various cell cycle genes were correlated with changes in MHC-I expression; the most important differences among the melanoma cell lines were in the transcriptional level of AP2-alpha, cyclin A1 and p21WAF1/CIP1. According to these results, altered MHC-I expression in malignant cells can directly increase their intrinsic oncogenic and invasive potential and modulate the expression of cell cycle genes. These findings suggest that human leukocite antigen class I molecules may act directly as tumor suppressor genes in melanoma.

Introduction

MHC class I (MHC-I) molecules present cell surface self/foreign peptides to cytotoxic T lymphocytes and are crucial in the immune response (1). During cancer initiation and progression, MHC-I molecules present tumor-associated antigens that are recognized as foreign antigens, promoting lysis of tumor cells by cytotoxic T lymphocytes (2–4). The involvement of MHC-I expression in the immunogenicity of tumor cells is well documented (5–9). Seven altered human leukocite antigen (HLA) class I phenotypes have been described in human tumor cells, and multiple molecular mechanisms have been implicated in these HLA defects (10,11).

The loss of one MHC-I haplotype can often be followed by total loss of MHC-I expression due to other molecular mechanisms, e.g. antigen-processing machinery (APM) component downregulation (12–14). Accordingly, the initial loss of MHC-I molecules may allow cells to evade the initial immune response, leading to the emergence of tumor escape variants (15,16) that the immune system might not subsequently recognize during the so-called 'immunoblindness phase' of tumor progression (17). During this phase, the immune system loses control over tumor cells, which progress and invade and colonize tissues, producing metastases (18). There may also be HLA class I expression losses unrelated to immune escape. Our group reported that loss of HLA class I surface expression on human melanoma cells grown in immunodeficient mice with no autologous immune response gave rise to more oncogenic tumor cells *in vivo* (17,19), although a direct causative relationship was not established. The biological significance of these new HLA losses has yet to be elucidated.

The present investigation describes two successive stages of HLA loss in melanoma cells: (i) loss of one HLA class I haplotype during tumor progression in a patient and (ii) transcriptional downregulation of APM components and HLA class I heavy chain genes during tumor growth in immunodeficient mice, leading to total loss of HLA class I cell surface expression. HLA gene transfection experiments demonstrated that the first HLA class I alteration (loss of one haplotype) permitted tumor cell escape from the patient's immune system. Importantly, both stages of HLA class I alterations increased the *in vivo* intrinsic oncogenic capacity and *in vitro* proliferation rate of the cells and augmented their migratory and invasive potential.

Abbreviations: APM, antigen-processing machinery; CTLp, cytotoxic T lymphocyte precursor; EBV, Epstein-Barr virus; HLA, human leukocyte antigen; MHC, major histocompatibility complex; MHC-I, MHC class I; NK, Natural Killer.

[†]These authors contributed equally to this work.

Materials and methods

Cell lines and reagents

The Ando-2 melanoma cell line, from a patient with malignant melanoma, was kindly provided by Dr P. Coulie [Université Catholique de Louvain (UCL), Brussels, Belgium]. Ando-Nude melanoma cell lines were established *in vitro* after growth in nude mice (19). Cell lines were maintained in ISCOVE culture medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Life Technologies, Milan, Italy) and antibiotics. Epstein-Barr virus (EBV)-transformed autologous B lymphocytes (Ando-EBV) and K-562 cell lines were cultured in RPMI medium supplemented as described above. Mixed lymphocyte-tumor cell culture experiments were performed in ISCOVE medium as described above but with human serum replacing fetal calf serum. All cell lines were characterized by PCR assay using short tandem repeat markers, and they were also regularly tested for MHC-I genotype and surface expression.

*Cloning of HLA-A*0201 complementary DNA and transfection into autologous Ando-2 melanoma cells*

HLA-A*0201 complementary DNA was cloned from Ando-EBV cells. The PCR product was purified and cloned using the pcDNA3.1/D Expression kit (Invitrogen, Paisley, UK), and the plasmid obtained was designated pcDNA-A2. The empty vector was used as control vector (pcDNA-control). Next, Ando-2 melanoma cells (1×10^6/ml) were cultured in six-well plates at 37°C in 5% CO_2 atmosphere and then transfected with 1 μg of digested pcDNA-A2 plasmid or pcDNA-control, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfected melanoma cells were designated Ando-TA2 and Ando-Tmock, respectively. See Supplementary Information, available at *Carcinogenesis* Online, for additional details.

Analysis of surface HLA expression

Surface HLA class I expression on cultured cells was determined by indirect immunofluorescence using the appropriate anti-class-I monoclonal antibody and fluorescein isothiocyanate-labeled rabbit antimouse Ig (Fab2) fragments (Sigma, St Louis, MO). A more detailed description is given in the Supplementary Information, available at *Carcinogenesis* Online.

Analysis of antitumor cytotoxic T lymphocytes with mixed lymphocyte-tumor cell cultures

Mixed lymphocyte-tumor cell cultures were set up to estimate the blood frequency of cytotoxic T lymphocyte precursors (CTLps), specifically directed against the tumor. Blood $CD8^+$ lymphocytes were purified from thawed peripheral blood mononuclear cells by magnetic beads (Miltenyi Biotec, GmbH, Germany). A previously reported assay protocol (20) was followed, priming $CD8^+$ lymphocytes with Ando-2 or Ando-2-TA2 melanoma cells. Results for percentages of specific lysis are the mean values

(±SD) of triplicate determinations. ^{51}Chromium-labeled target cells included Ando-2, Ando-Nude, Ando-TA2 and Ando-Tmock melanoma cells, Ando-EBV autologous lymphocyte cells and K-562 cells. See Supplementary Information, available at *Carcinogenesis* Online, for additional details.

Natural Killer cytotoxicity assay

Cytotoxicity assays were performed according to a standard ^{51}Chromium-release method. Ando-2, Ando-Nude, Ando-TA2 and Ando-Tmock melanoma cell lines were used as target cells. See further description in the Supplementary Information, available at *Carcinogenesis* Online.

Mice

Athymic 6- to 8-week-old Swiss nu/nu mice and SCID-Beige mice (average weight, 20 g) were purchased from Charles River (CRIVER, Barcelona, Spain). The breeding and care of animals complied with European Community Directive 86/609/CEE and Spanish laws (Ley 32/2007 and Real Decreto 1201/2005) for the use of laboratory animals. Housing and all experimental procedures involving animals were performed according to protocols approved by the hospital's animal care committee and in compliance with the guidelines on animal welfare of the National Committee for Animal Experiments.

In vivo *local tumor growth assay*

For *in vivo* oncogenicity assays, different doses of cells (1×10^6, 2.5×10^6 and 5×10^6 cells) were subcutaneously injected into the footpad in groups of 10 nude mice each. Growth of local tumors was monitored in all animals and recorded three times per week. When the maximum diameter of local tumors, measured with electronic calipers, reached 10 mm, animals were anesthetized [0.04 ml of diazepam (Valium; Roche, Madrid, Spain) and 0.1 ml of ketamine (Ketolar; Pfizer, Spain)] and killed by cervical dislocation. These assays were repeated at least three times.

In vitro *proliferation test*

The proliferation rate was determined by using the non-toxic colorimetric-based assay Alamar Blue (Biosource, Camarillo, CA) as described previously (21). See Supplementary Information, available at *Carcinogenesis* Online, for additional details.

Cell cycle analysis

Cells were incubated with 1 mM bromodeoxyuridine for 1 h at 37°C and processed using the fluorescein isothiocyanate bromodeoxyuridine flow kit (BD Biosciences, San Diego, CA) following the manufacturer's instructions. See Supplementary Information, available at *Carcinogenesis* Online, for additional details.

Gene expression profiling on Agilent microarrays and reverse transcription and quantitative real-time reverse transcription–PCR

The Agilent Whole Human Genome Oligo Microarray (G4112A) (Agilent Technologies, Santa Clara, CA) was used. Five micrograms of total RNA of each melanoma cell line were amplified, carrying out probe labeling and hybridization in accordance with the manufacturer's instructions. Fluorescence array images for Cy3 and Cy5 were scanned with an Agilent fluorescent scanner, and Feature Extraction software was used to grid, extract image intensities and normalize data.

The messenger RNA isolation kit (Miltenyi Biotec) was used to extract messenger RNA from tumor cell lines under baseline conditions. Real-time quantitative PCR analyses (for genes listed in Table I) were performed in the 7500 Fast System (Applied Biosystems), using Gliceraldehyde-3-phospate dehidrogenase and TATA-binding protein genes as housekeeping genes (TaqMan gene expression assays from Applied Biosystems). See further description in the Supplementary Information, available at *Carcinogenesis* Online.

Migration and invasion assays

Migration and invasion assays were performed using Boyden Chambers containing polycarbonate filters with 8 μm pore size (Becton–Dickinson). Each assay was performed in duplicate and repeated at least three times. See Supplementary Information, available at *Carcinogenesis* Online, for additional details.

Statistical analysis

Data were expressed as mean ± SD. The paired Student's *t*-test was used to compare mean values. $P \leq 0.05$ was considered significant. SPSS 16.0.2 (SPSS, Chicago, IL) was used for data analyses.

Results

MHC-I surface expression in Ando-2 tumor system

The Ando-2 melanoma cell line had a loss of heterozygosity in chromosome 6 and presented surface expression of only one HLA class I haplotype (Figure 1). The lost HLA haplotype was HLA-A2, HLA-B13 and HLA-Cw6 (19). Transfection of Ando-2 cells with plasmid pcDNA-A2 recovered expression of HLA-A2, one of the lost HLA alleles. Various clones with high surface expression of HLA-A2 allele from different transfection assays were selected and designated Ando-TA2 cells (Figure 1). Ando-2 melanoma cells transfected with the vector pcDNA-control, designated Ando-Tmock cells, showed practically identical surface HLA class I expression to that of Ando-2 cells (Figure 1). Ando-2 melanoma cells were injected in nude mice, obtaining Ando-Nude melanoma cell lines after local growth (19). These cells had total loss of HLA class I expression (Figure 1) due to the coordinated downregulation of several APM components (19). Transfection with HLA-A2 gene did not recover the expression of these APM components, and Ando-Nude-transfected cells therefore had the same expression as Ando-Nude cells, i.e. total loss of HLA class I surface expression (data not shown). Ando-Nude-TA2 cells and their corresponding Ando-Nude-Tmock cells had total loss of HLA class I surface expression and showed the same results as for Ando-Nude cells in all assays performed in this study, as detailed below.

The Ando-2 tumor system comprises three melanoma cell lines (with the exception of the controls), Ando-2, Ando-TA2 and Ando-Nude, which derive from the same cell line but have different HLA class I surface expressions. At least three additional clones or batches (i.e. cells from different transfection assays for Ando-TA2 or Ando-Tmock cells lines, from different *in vivo* growth assays for Ando-Nude cells and from different culture passages for Ando-2 cells) were tested for surface HLA class I expression and practically identical results were obtained.

Table I. Sequences of the cell cycle primers

Primer name		Sequence 5′–3′	Product length (bp)
Cyclin A1	Forward	GCCTTGCCTGAGTGAGCTTC	128
	Reverse	AACTGCAGGTGGCTCCATGAG	
Cyclin B2	Forward	GTAGCAGCAGCTGCTTCCTG	121
	Reverse	GCCATGTGCTGCATGACTTCC	
Cyclin D1	Forward	CTCTGTGCCACAGATGTGAAG	148
	Reverse	GTGAGGCGGTAGTAGGACAG	
Cyclin D2	Forward	GAGGAAGTGAGCTCGCTCAC	101
	Reverse	CTCAATCTGCTCCTGGCAAGC	
Cyclin D3	Forward	GAAGCCTCTCAGACCAGCTC	87
	Reverse	CTGTAGGAGTGCTGGTCTGG	
Cyclin E1	Forward	GTGCTACTGCCGCAGTATCC	122
	Reverse	ATACAAGGCCGAAGCAGCAAG	
CDK2	Forward	AGATGAGGTGGTGTGGCCAG	119
	Reverse	GCTCCGTCCATCTTCATCCAG	
CDK3	Forward	CAGCGGATCACAGCCAAGAC	89
	Reverse	TCGCTGCAGCACATACTGGC	
CDK4	Forward	ACCTGAGATGGAGGAGTCGG	93
	Reverse	GTGCTGCAGAGCTCGAAAGG	
CDK6	Forward	CACACCGAGTAGTGCATCGC	146
	Reverse	CTCTGTACCACAGCGTGACG	
CDC25A	Forward	CCTACTGATGGCAAGCGTGTC	84
	Reverse	CTCTCACATACCGGCACATGC	
CDC25B	Forward	CCTGTAGCCTGGACAAGAGAG	105
	Reverse	TTGACAGCACGGTCTCGTTCC	
p21	Forward	AAGACCATGTGGACCTGTCAC	123
	Reverse	TCTGTCATGCTGGTCTGCCG	
p16	Forward	CTTCCTGGACACGCTGGTG	166
	Reverse	GGCATGGTTACTGCCTCTGG	
E2F1	Forward	GCCACCATAGTGTCACCACC	81
	Reverse	GCTCCAGGCTGAGTAGAGAC	
Mdm2	Forward	GTGAGGAGCAGGCAAATGTGC	79
	Reverse	CCGAAGCTGGAATCTGTGAGG	
p53	Forward	GGCTCTGACTGTACCACCATC	109
	Reverse	CCACTGGAGTCTTCCAGTGTG	
Rb1	Forward	TTCAGAAGGTCTGCCAACACC	132
	Reverse	GAGCACACGGTCGCTGTTAC	

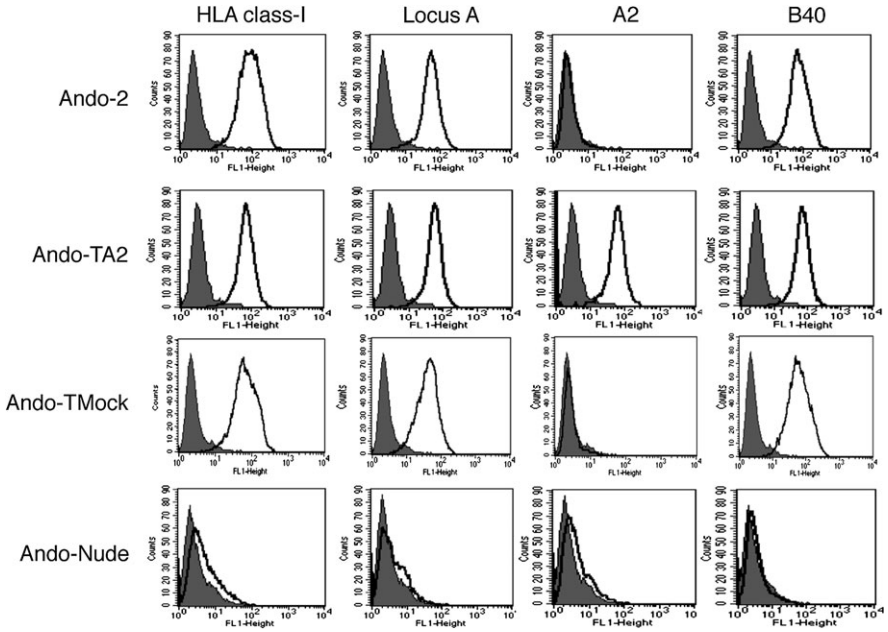

Fig. 1. MHC-I surface expression on melanoma cell lines of Ando-2 system. Surface expression of one HLA class I haplotype is observed in Ando-2 and Ando-Tmock cell lines and a high expression of HLA-A2 molecule is observed in Ando-TA2 cell line. No surface expression of any HLA class I molecule was detected in the Ando-Nude cell line. Several clones or batches of each melanoma cell line were tested and highly similar results were found. A representative example of three independent experiments is depicted.

Presence of specific CTLps against Ando-TA2 melanoma cells in the patient's blood

The presence of CTLps specifically recognizing Ando-TA2 cells was determined to test the possibility that Ando-2 melanoma cells were immunoselected in the patient after the loss of one HLA haplotype as escape mechanism. We performed mixed lymphocyte-tumor cell cultures to estimate the frequency in blood of CTLps directed specifically against the Ando-TA2 tumor cells, as described previously (20). Purified CD8 blood T cells were stimulated with irradiated Ando-TA2 tumor cells in limiting dilution conditions with 375–3000 lymphocytes per microculture. After three weekly stimulations, the lytic activity of the microcultures was tested against the following cells: Ando-TA2, Ando-Tmock, Ando-2, autologous EBV-transformed B cells (Ando-EBV) and Natural Killer (NK) target K-562. Precise criteria and representative results are shown in Figure 2. Out of 48 microcultures with 1500 lymphocytes, 5 were positive. Out of 48 microcultures with 375 lymphocytes, 1 was positive. Antitumor CTLps, which specifically recognize Ando-TA2 tumor cells, were present in the blood at a frequency of 6×10^{-5}, indicating the previous generation in this patient of an immune response against melanoma cells expressing the HLA-A2 molecule. These assays were repeated with at least three additional clones or batches of each cell line and the results obtained were always the same.

day	Cell line	unlabeled K562, 50-fold excess*	Lytic activity (% specific lysis) of 5 microcultures positive of 48				
20	Ando-TA2	+	52	20	34	29	45
	Ando-Tmock	+	8	2	0	5	0
	Ando-2	+	7	0	5	3	7
	Ando-2-EBV	+	3	0	4	0	3
	K562	+	0	0	0	0	0
27	Ando-TA2	—	45	24	30	32	51
	Ando-Tmock	—	6	0	6	3	5
	Ando-2	—	6	5	3	6	5
	Ando-2-EBV	—	0	0	3	0	5
	K562	—	5	3	0	7	4

* +: with unlabeled K562; —: without unlabeled K562

Fig. 2. Limiting dilution analysis of specific antitumor CTLps against Ando-TA2 cells. Lytic patterns obtained at days 20 and 27 with limiting dilution microcultures set up with 1500 blood CD8 lymphocytes and stimulated with irradiated autologous tumor cells. Microcultures were considered to contain tumor-specific CTLs against Ando-TA2 melanoma cells if they satisfied the following criteria on both day 21 and 27: lysis of Ando-TA2 >10% and lysis of Ando-2, Ando-EBV and K-562 less than one-third of lysis of Ando-TA2. Three independent experiments yielded similar results.

MHC-I expression determines in vivo local growth of melanoma cells in immunodeficient mice

We studied *in vivo* local growth in nude and SCID-Beige mice in order to analyze the *in vivo* tumorigenic potential of these melanoma cell lines in the absence of an autologous immune response. These immunodeficient mice were selected because Ando-Nude cells were obtained from them, and SCID-Beige mice are the usual choice for reconstitution assays with immune human cells. Three cell doses (1, 2.5 and 5 million) were injected in groups of 10 mice each and local growth was monitored. Ando-2 melanoma cells did not grow in the nude mice at doses of 1 or 2.5 million cells, showed a very low growth rate at a dose of 5 million cells and only reaching a maximum tumor diameter of 10 mm at day 60 (Figure 3a). The results for Ando-Tmock and Ando-2 melanoma cells were very similar (Figure 3c). Ando-Nude cells grew at all cell doses used at a higher growth rate versus Ando-2 cells, with local tumors reaching a maximum diameter of 10 mm in only 35 days (Figure 3b). Ando-Nude-TA2 and Ando-Nude-Tmock melanoma cells showed identical results to those found for Ando-Nude melanoma cells (data not shown). Ando-TA2 melanoma cells only grew at a dose of 5 million cells, with local tumors reaching 4 mm of diameter in 20 days and then regressing until their final disappearance (Figure 3d). These animals remained tumor-free throughout the study (>12 months). Ando-TA2-transfected melanoma cells showed no *in vivo* tumorigenic capacity in nude mice, whereas Ando-Nude melanoma cells evidenced a very high oncogenic potential, with Ando-2 melanoma cells occupying an intermediate position. *In vivo* local tumor growth assays were also performed in SCID-Beige mice to rule out the possible implication of NK or

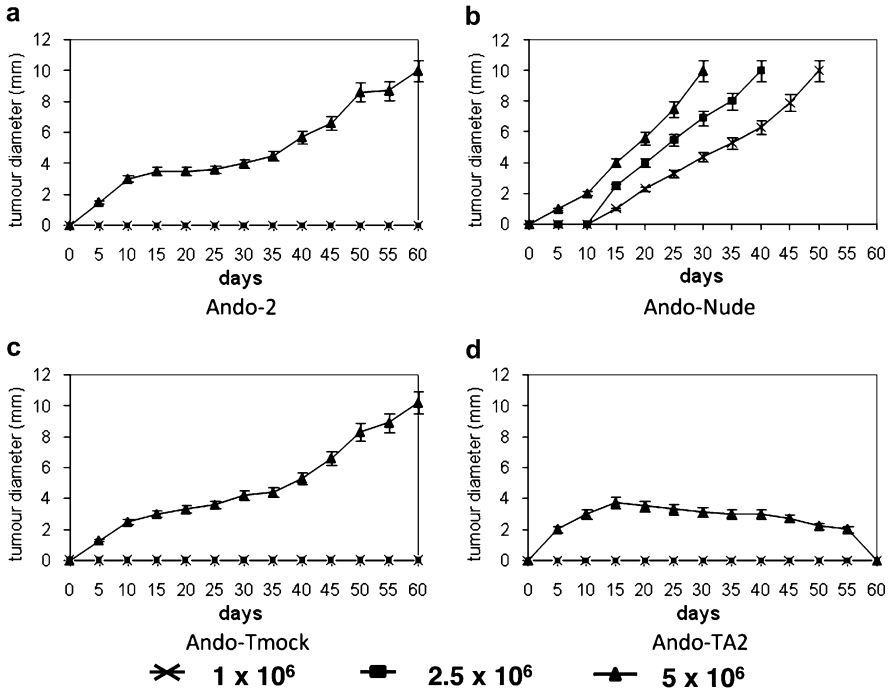

Fig. 3. *In vivo* tumor growth and tumorigenic potential of each melanoma cell line. Three doses of each human melanoma cell line (1×10^6, 2.5×10^6 and 5×10^6) were subcutaneously inoculated in nude mice, and the tumor growth rate was assessed. Data depict mean ± SD for 10 mice from each group. (**a**) Ando-2 tumor cells grew only after injection of 5×10^6 cells; (**b**) Ando-Nude tumor cells formed local tumor at all cell doses; (**c**) Ando-Tmock showed the same results as Ando-2 tumor cells. (**d**) Ando-TA2 melanoma cells grew and formed a tumor of 4 mm diameter that subsequently regressed and disappeared. The data shown are representative of three independent experiments.

B cells. No significant differences were found between the results for nude and SCID-Beige mice (data not shown). All the above *in vivo* local growth assays were repeated using other different clones or batches of each melanoma cell line, and the same results were always observed.

Splenocytes were isolated from nude mice previously injected with Ando-2, Ando-Nude, Ando-TA2 or Ando-Tmock human melanoma cells to rule out the possibility that NK cells or residual T cells from nude mice recognize some of these human melanoma cell lines and alter their *in vivo* local growth. We performed assays on CTLps frequency, using the methods described above for the patient-derived T-CD8$^+$ cells but in this case, using cells from the spleen of nude mice previously injected with each of these human melanoma cell lines. These assays detected no presence of CTLps against these human melanoma cell lines in the nude mice. Other cytotoxic activities (e.g. NK cytotoxic activity) were studied, using splenocytes or lymphocytes isolated from mice previously injected with each human melanoma cell line as effector cells and their corresponding human melanoma cells as target cells. Cytotoxic assays were performed at 4 and 16 h; in some assays, effector cells were previously restimulated *in vitro* with their corresponding target cells. All results were again negative, i.e. human melanoma cells were not recognized by the murine effector cells.

Differences in in vitro proliferation rate, cell cycle distribution and cell cycle gene expression among melanoma cell lines

In vitro proliferation rates from 24 to 144 h were compared among the cell lines, using the Alamar Blue assay. At 24 h, the growth rate was slightly higher in Ando-TA2 cells, followed by Ando-2 melanoma cells and Ando-Nude cells (Figure 4). At 48 h, the growth rate was highly similar between Ando-2 and Ando-Nude cells and slightly higher in Ando-TA2 cells (Figure 4). At 72 h, however, the proliferation rate was higher in Ando-Nude melanoma cells than in the other two cell lines, which did not differ between them. At 96 and 144 h, Ando-Nude cells showed an even higher value, followed by Ando-2 and Ando-TA2 melanoma cells (Figure 4). Results for Ando-Tmock and Ando-2 cells

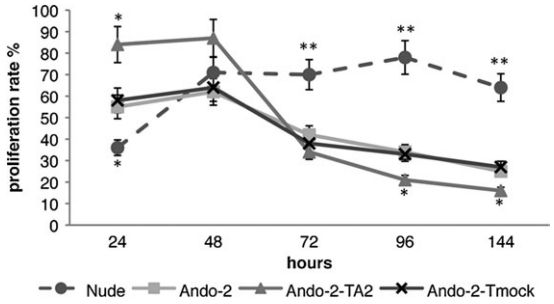

Fig. 4. Differences in *in vitro* proliferation rate among the melanoma cell lines. Ten thousand cells per well were seeded in 96-well plate and cell proliferation was evaluated with Alamar blue staining, quantifying % reduction after incubation for 5 h. Each graph represents three independent experiments, and data are expressed as mean ± SD. Statistically significant differences in % proliferation between Ando-2 tumor cells and Ando-TA2 or Ando-Nude cells (Student's *t*-test; $^*P < 0.01$, $^{**}P < 0.001$).

were the same in all assays (Figure 4). The data obtained for Ando-Nude, Ando-Nude-TA2 and Ando-Nude-Tmock cells were also practically identical (data not shown). Comparison of results obtained using Alamar Blue technique and 3-(4,5-dimethylthiazole-2-yl)-2,5-bdiphenyl tetrazolium bromide assay confirmed the validity of our findings (data not shown). These assays were repeated with at least three additional clones or batches of each cell line and the results obtained were always the same.

Exponentially growing tumor cells were plated and the cell cycle distribution was measured at 72–96 h post-incubation. Transfection of HLA-A2 gene in Ando-2 melanoma cells promoted cell arrest in G_0–G_1 phase. In comparison to Ando-2, Ando-TA2 melanoma cells showed a significant increase (50–60%) in the number of cells in the G_1 phase with a concomitant decrease (27–17%) in the number of cells in the S phase (Table II). In contrast, Ando-Nude cells showing a significant increase (27–40%) in the number of cells in S phase

accompanied by a decrease (50–39%) in the number of cells in G_1 phase (Table II). Values in Ando-2 and Ando-Tmock cells were practically identical (data not shown). There were no significant differences in the percentage of apoptotic cells (sub-G_0 phase) among the different Ando variants.

Complementary DNA microarray studies showed that the overall gene expression profile was highly similar among these melanoma cell lines, with few differentially expressed genes. Table I lists the differentially expressed genes selected for further verification and quantification by real-time reverse transcription–PCR, along with the primers and conditions used. Data were normalized by using TATA-binding protein and Gliceraldehyde-3-phospate dehidrogenase as housekeeping genes. Figure 5 depicts the real-time reverse transcription–PCR results, using the values for Ando-2 cells as reference (relative value of 1). Cyclin D2 was not expressed by these melanoma cell lines. Three genes were differentially expressed among all three melanoma cell lines: expression of AP-2α in Ando-TA2 cells was 4.5-fold higher than in Ando-2 cells and 7.5-fold higher than in Ando-Nude cells (Figure 5); expression of cyclin A1 in Ando-TA2 cells was 2-fold higher than in Ando-2 cells and 4-fold higher than in Ando-Nude cells and expression of p21WAF1/CIP1 in Ando-TA2 cells was 1.8-fold higher than in Ando-2 cells and 3-fold higher than in Ando-Nude cells (Figure 5). The expression of some other genes was higher in one cell line than in the other two: expression of Mdm2 gene was 2-fold higher in Ando-TA2 cells than in the other two melanoma cell lines and expressions of Cdk2, Cdc25A and E2F1 were around 3-, 2- and 2-fold higher, respectively, in Ando-Nude cells than in the other two melanoma cell lines (Figure 5). The results for Ando-2 and Ando-Tmock cells were again practically identical (Figure 5). In these assays, Ando-Nude melanoma cells and their corresponding transfected cells presented practically identical results. All these assays were repeated with at least three additional clones or batches of each cell line, always finding the same results.

Table II. Cell cycle distribution in Ando cell lines

Cell line	G_0/G_1	S	G_2/M
Ando-Nude	39.5 ± 2.1*	40.0 ± 1.9*	20.1 ± 1.1
Ando-2	50.2 ± 2.4	27.3 ± 1.1	22.0 ± 0.9
Ando-TA2	60.6 ± 3.0*	17.8 ± 0.7*	21.2 ± 0.7

Data are mean ± SD of at least three experiments performed.
*$P < 0.01$ compared with Ando-2 data.

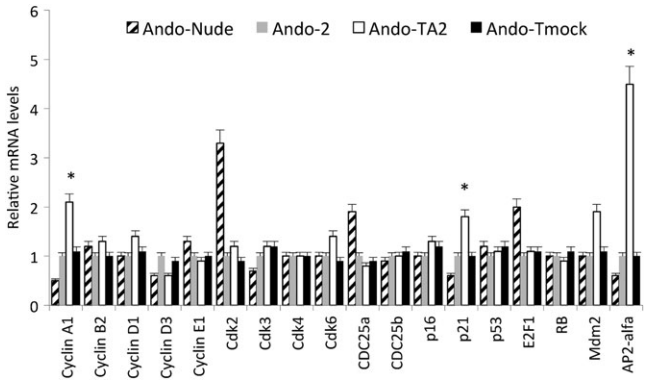

Fig. 5. Transcription levels of several cell cycle genes detected by real-time reverse transcription–PCR. Messenger RNA was extracted from melanoma cell lines and quantitative reverse transcription–PCR analyses were performed using specific primers (Table I). Expression level of the genes of interest was determined relative to levels of TATA-binding protein and Gliceraldehyde-3-phospate dehidrogenase housekeeping genes. Ando-2 cells are set to 1. Values are depicted as mean ± SD of three independent experiments performed in quadruplicate. Statistically significant differences in % proliferation observed between Ando-2 tumor cells and Ando-TA2 or Ando-Nude cells (Student's *t*-test; $^*P < 0.01$).

In vitro *migratory and invasive capacities increase with lower MHC-I expression on the melanoma cell lines*

The melanoma cell lines were allowed to migrate or invade through a Matrigel matrix, using fetal bovine serum-supplemented medium as the chemoattractant. In these migration assays, MHC-I expression was inversely correlated with an increase in the migratory properties of the cell lines (Figure 6a and b). In the first assay, we compared the migratory capacity by incubating 25 000 cells for 16 h and found significant differences among cell lines. The migratory capacity of Ando-Nude melanoma cells was 5.7-fold higher versus Ando-2 cells and 12.8-fold versus Ando-TA2 cells (Figure 6a). In the second assay, Ando-2 and Ando-TA2 cells were incubated for 40 h, and a 4-fold higher migratory capacity was found for Ando-2 than for Ando-TA2 cells (Figure 6b). In both assays, results were practically identical between mock-transfected and Ando-2 cells (Figure 6a and b).

Ando-Nude cells showed a higher invasive capacity versus the other cell lines, and the difference was even greater than for the migratory capacity (Figure 6c and d). In the 16 h invasion assay, the invasion capacity of Ando-Nude cells was 23-fold higher versus Ando-2 cells and 50-fold higher versus Ando-TA2 cells (Figure 6c). In the 40 h assay, the invasion capacity of Ando-2 cells was 7.5-fold higher versus Ando-TA2 cells (Figure 6d). In both assays, results were practically identical between mock-transfected and Ando-2 cells (Figure 6c and d). In brief, Ando-Nude cells presented the highest *in vitro* migratory and invasive capacities and Ando-TA2 cells the lowest (Figure 6). To rule out the possibility that the differences observed were due to variations in proliferation among the melanoma cell lines, the assays were repeated and cells counted after the incubation period. In the 16 h assay, the cell number was very similar among all melanoma cell lines, although slightly higher for Ando-TA2 cells, whereas in the 40 h assay, the cell number was practically identical among all three melanoma cell lines (results similar to those in Figure 4). In the migration and invasion assays, Ando-Nude melanoma cells and their corresponding transfected cells presented practically identical results. Again, all assays were repeated using at least three more different clones or batches of each cell line, finding the same results.

Discussion

The present data indicate that defects in MHC-I expression on human melanoma cells endowed them with a double advantage for tumor progression. First, Ando-2 melanoma cells presented loss of one HLA haplotype during tumor progression in the patient, evading the immune system, finding specific CTLps against Ando-TA2 melanoma

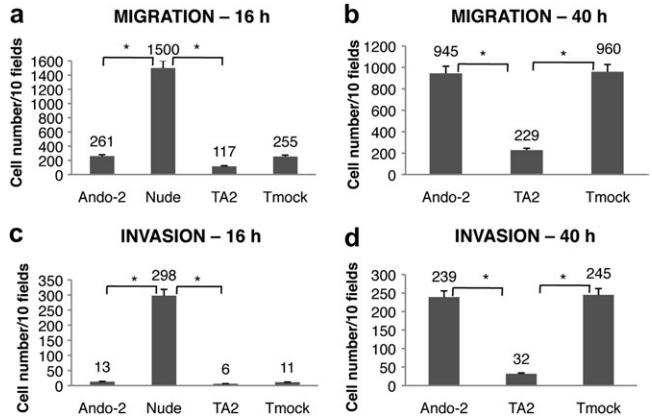

Fig. 6. Increased *in vitro* migratory and invasive potential of melanoma cells with lower MHC-I expression. The ability of the cells to migrate was evaluated in Boyden chamber for 16 h (**a**) or 40 h (**b**). The ability of cells to invade Matrigel was analyzed by chemoinvasion in blind well Boyden chamber for 16 h (**c**) or 40 h (**d**). Error bars represent mean ± SD; each cell was analyzed at least three times and in duplicate wells. Statistical analysis was performed by Student's *t*-test; $^*P < 0.001$.

cells in the patient's peripheral blood, i.e. Ando-2 cells transfected with HLA-A*0201 allele (one of the lost alleles). It was previously reported in similar *in vitro* assays that HLA haplotype loss in lung cancer cells allows them to escape from immune attack (22,23). Moreover, Ando-2 melanoma cells may enter a new stage of interaction with the immune system, the immunoblindness phase, where they may not be recognized by the immune system (17). No specific CTLps against Ando-2 melanoma cells were detected in the blood of our patient. An additional HLA defect (total loss of HLA class I surface expression) was found after growth in immunodeficient mice in the absence of an autologous immune response. Our group previously reported that alterations in HLA class I expression are frequent and reproducible in human melanoma cells grown in immunodeficient mice (17). Here, we demonstrate that melanoma cells with total HLA class I downregulation (Ando-Nude cells) are more oncogenic *per se* and have higher *in vivo* tumorigenicity, proliferation rate and migratory and invasive potentials. Ando-TA2 cells (Ando-2 melanoma cells transfected with HLA-A2 allele, one of the lost HLA alleles) showed the lowest *in vivo* tumorigenicity and the lowest *in vitro* proliferation rate and migratory and invasive capacities. According to these *in vitro* and *in vivo* findings, the order of direct oncogenic potential of these melanoma cells, which derive from the same cell line, is: Ando-Nude > Ando-2 > Ando-TA2. The present findings demonstrate an inverse correlation between HLA class I expression and these intrinsic oncogenic characteristics in this tumor system.

Hence, HLA class I losses may play an additional role in malignant progression besides immune system escape. MHC-I surface expression defects in murine cells were recently reported to be responsible for resistance to multiple apoptotic stimuli through elevated endoplasmic reticulum stress and defective p53 activation, conferring cell survival advantage (24). Metastatic cells derived from a single murine fibrosarcoma tumor clone were previously found to present total loss of MHC-I expression in immunocompetent mice and high MHC-I surface expression in immunodeficient mice. Metastatic cell lines without MHC-I expression showed higher *in vivo* local growth and metastatic potential in immunodeficient mice, whereas metastatic cell lines with high MHC-I surface expression were less oncogenic (13,25). Furthermore, MHC-I alterations in B16 murine melanoma cells impaired insulin receptor-regulated signal transduction and augmented resistance to apoptosis (26). In human studies, these relationships have been reported in neoplastic lymphoid cells but not in normal cells, with HLA molecules mediating programmed tumor cell death in the former when engaged by specific monoclonal antibodies (27–30). All these findings strongly suggest that major histocompatibility complex (MHC) molecules may play a role in the regulation of tumor survival and progression, acting directly as tumor suppressor genes.

The expression pattern of three tumor suppressor genes, AP-2α, p21WAP1/CIP1 and cyclin A1 was similar in the three melanoma cell lines, with a higher expression in Ando-TA2 cells and lower in Ando-Nude cells. Interestingly, the two first genes are located in chromosome 6 (regions 6p24 and 6p21, respectively) very close to the HLA region in 6p21, where HLA heavy chains and the majority of APM genes are located. In many other species, P21WAF1/CIP1 gene is located in the middle of MHC region. Hence, all these genes may share a common regulatory mechanism, and this possibility is supported by the transcriptional downregulation of all these genes in Ando-Nude cells and their transcriptional induction in Ando-TA2 melanoma cells. Furthermore, the loss of HLA class I haplotype in tumor cells, e.g. in Ando-2 melanoma cells, always coincides with the loss of one allele of p21WAP1/CIP1 and one allele of AP-2α. AP-2α gene showed the greatest differences in transcriptional expression among the cell lines under study. Loss of AP-2α expression has been widely implicated in melanoma progression toward a malignant, invasive and metastatic phenotype (31,32). The high induction of AP-2α transcriptional expression in Ando-TA2-transfected cells might indicate that the expression of this gene is indirectly regulated by HLA class I molecules. In a metastatic murine model developed in our laboratory, AP-2α gene was upregulated in the MHC-positive metastases from a fibrosarcoma tumor clone but downregulated in the MHC-negative metastases from the same clone (13,25) (I.Romero and A.M.Garcia-Lora, unpublished results). The expression pattern of p21WAF1/CIP1 was broadly similar to that of AP-2α. Regulation of p21WAF1/CIP1 is mainly at transcriptional level and may be independent of p53 (33,34), although it may be coregulated with AP-2α gene (35,36), which is highly upregulated in Ando-TA2 and downregulated in Ando-Nude melanoma cells. It has been demonstrated that p21WAF1/CIP1 upregulation stops proliferation and promotes senescence, whereas its downregulation is frequently associated with tumor progression and higher invasiveness (37–39).

Another gene that may be implicated is cyclin A1, which was 2- and 4-fold lower, respectively, in Ando-2 and Ando-Nude melanoma cells versus Ando-TA2 cells. Cyclin A1 may act as a tumor suppressor gene, given that downregulation of its expression has been reported in various cancers and associated with an invasive phenotype (40–42). The upregulation of AP-2α, p21WAF1/CIP1 and cyclin A1 in Ando-TA2 cells may be responsible for their lesser oncogenicity, migration and invasion and for the regression of these tumors in immunodeficient mice, in which hypoxia and nutrient deprivation prevail. Conversely, the downregulation of these genes in Ando-Nude melanoma cells may enhance their oncogenicity. The expression pattern of other cell cycle genes differed among the three melanoma cell lines. For instance, the expression of Mdm2 gene, associated with a reduced invasion capacity (43–45), was upregulated in Ando-TA2 cells, whereas the expression of E2F1, Cdk2 and Cdc25A genes, implicated in the transition from G_1 to S phase, suppression of cellular senescence (46,47) and melanoma progression (48,49), was upregulated in Ando-Nude cells. All these results, together with data published by Sabapathy and Nam (24), suggest a direct connection between MHC-I molecules and cell cycle gene expression.

Loss of MHC-I expression, frequent in cancer cells, is known to play a role in tumor escape from immune system. This study provides evidence that MHC-I defects may also play a direct role in cancer progression, increasing the growth, intrinsic oncogenicity and migratory and invasive potential of cancer cells. These new data on the role of MHC-I molecules in tumor survival and progression suggest their direct action as tumor suppressor genes. Future research efforts should focus on the restoration of MHC-I expression in tumor cells of different histological origin, examining its influence on immune recognition and on the intrinsic oncogenic characteristics of tumor cells, which are of major clinical relevance. Analysis of MHC-I surface expression of a tumor may prove of value to predict its clinical progression and to establish the most appropriate therapeutic approach in each case.

Supplementary material

Supplementary Materials and methods can be found at http://carcin.oxfordjournals.org/.

Funding

Fondo de Investigaciones Sanitarias ISCIII (CP03/0111, PI 08/1265, RETIC RD 06/020); Junta de Andalucía (Group CTS-143, CTS-695, CTS-3952, CVI-4740 grants);European Community (LSHC-CT-2004-503306, OJ 2004/c158,18234). A.M.G.-L. was supported by Miguel Servet Contract CP03/0111 and Contract I3 from Fundacion Progreso y Salud and C.G. by FPU–MEC 1631.

Acknowledgements

The authors thank I.Linares, A.B.Rodriguez and E.Arias for technical advice; Dr N.Aptsiauri for helpful discussion and R.Davies for editorial assistance.

Conflict of Interest Statement: None declared.

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Received July 12, 2011; revised December 14, 2011; accepted December 27, 2011

PRECLINICAL ASSAYS OF ANTIMETASTATIC IMMUNOTHERAPEUTIC TREATMENTS WITH GR9 FIBROSARCOMA CELL LINE, A7 AND B7 CLONES

Our group previously reported a different response to metastatic immunotherapeutic treatments in cancer patients depending on the HLA-I phenotype of the tumor cells. While tumors with high HLA-I expression and/or reversible-soft alterations regressed, tumors with low HLA-I expression and/or irreversible-hard alterations progressed (Teresa et al. 2007, Carretero et al. 2008). These results suggest an association between the MHC-I phenotype of the tumor cells with the effects of the immunotherapy.

To study the role of MHC-I surface expression of tumor cells in the success of antimetastatic immunotherapeutic strategies we performed preclinical assays with different antimetastatic immunotherapy treatments analyzing their ability to inhibit spontaneous metastases from GR9 fibrosarcoma, A7 and B7 clones. These tumor cells present different MHC-I phenotype and evolution during tumor progression. A7 clone, MHC-I highly positive, lead to the development of metastases that present high, middle and low MHC-I expression, that have reversible MHC-I alterations. B7 clone, MHC-I middle positive, produces metastases with middle and low MHC-I expression that, in some cases, present irreversible MHC-I alterations. Previous studies from Romero showed that we had to subcutaneously inject $1.25x10^6$ GR9 and A7 tumor cells, whereas $2.5x10^6$ B7 cells in order to have comparable metastatic progression and time periods (Romero 2012). In these conditions, these three cell lines produce several metastatic nodules in spontaneous metastasis assay, giving a wide range of therapeutic effect, and the period for metastatic development (around 45 days after the excision of the primary tumor) allows the administration of several treatment doses.

GR9, A7 and B7 tumor cells: MHC-I surface expression and tumor progression in spontaneous metastasis assays

Differences in MHC-I phenotype and expression of MHC-I related genes between GR9 fibrosarcoma and its clones A7 and B7

GR9 fibrosarcoma has surface expression of the three H-2K^d, H-2D^d and H-2L^d molecules, inducible after IFN-γ treatment (Fig. 43a). GR9 tumor has an heterogeneity composition and it is formed by several clones which were classified according to their different H-2 class I phenotype in highly positive, middle positive, low positive or negative H-2 class I phenotype. A7 clone belongs to the group of highly positive H-2 class I phenotype clones, and it has intensive surface expression of H-2 class I molecules (Fig. 43a). However, B7 has a middle positive H-2 class I phenotype, with intermediate intensities of expression of H-2 class I molecules (Fig. 43a). Both, A7 and B7 clones, keep the capability of induction of H-2 class I molecules after IFN-γ treatment (Fig. 43a).

To study the mechanisms implicated in the different MHC-I expression of these three cell lines, we analyzed the transcriptional gene expression of APM components, H-2 class I heavy chain, and β_2-microglobulin genes. The genes analyzed and primers used in real-time quantitative RT-PCR are shown in table 1a. Data were normalized to GAPDH and β-actin

housekeeping genes. Figure 43b shows the results using the values for GR9 cells as reference (assigned a relative value of 1). A7 clone presents higher expression of tapasin, LMP2, LMP7, TAP1 and TAP2 genes than GR9 cells; whereas a partial down-regulation of H-2D^d and H-2L^d heavy chains and calreticulin (Fig. 43b). B7 clone presents strong down-regulation of H-2D^d and H-2L^d heavy chains and LMP7 genes and partial down-regulation of LMP2 gene compared with GR9 fibrosarcoma (Fig. 43b).

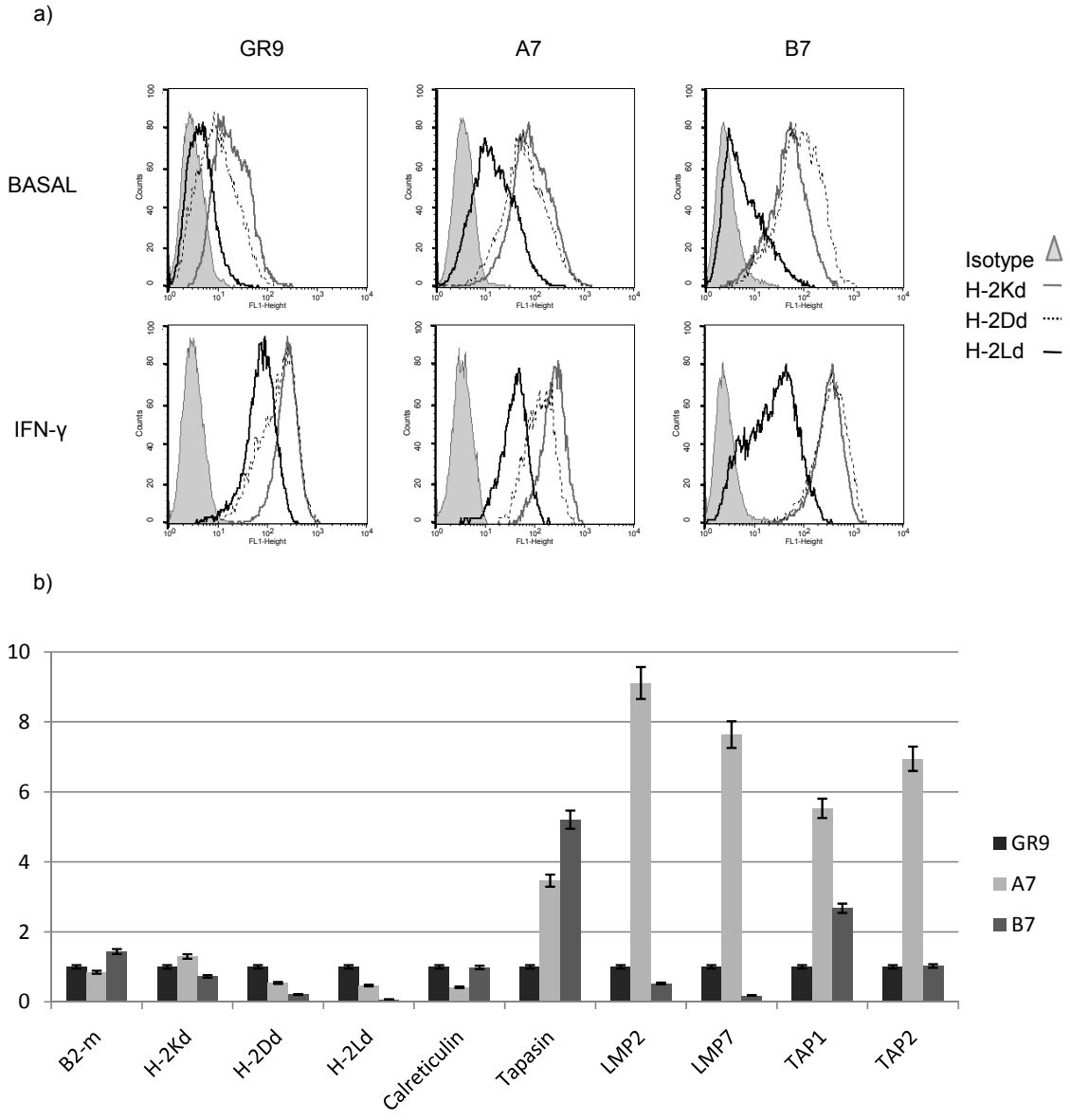


Figure 43. a) GR9 fibrosarcoma has positive expression of the three MHC-I molecules. A7 clone presents highly positive H-2 class I phenotype, whereas B7 clone has a middle positive H-2 class I phenotype. b) Transcriptional levels of MHC-I related genes were detected by qRT-PCR. Expression level of the genes was determined relative to levels of GAPDH and β-actin housekeeping genes, and the values for GR9 cells were set to 1. A7 clone has higher expression of tapasin, LMP2, LMP7, TAP1 and TAP2 genes and less expression of H-2Dd and H-2Ld heavy chains and calreticulin than GR9 cells. B7 clone presents less expression of H-2Dd and H-2Ld heavy chains, LMP2 and LMP7 genes than GR9 fibrosarcoma.

Table 1. Primers used in real time RT–PCR: a) β_2-m, MHC-I heavy chains and APM components genes; b) cell cycle genes, c) IFN-γ signalling pathway genes and d) Housekeeping genes.

a)

B_2-m, MHC-I heavy chains and APM

primer name	sequence 5' to 3'	product length [bp]
m-B2m-F	CTGGTGCTTGTCTCACTGACC	214
m-B2m-R	GACCAGTCCTTGCTGAAGGAC	
m-H2Kd-F	CCATCCACTGTCTCCAACACG	112
m-H2Kd-R	CCACCTGTGTTCCTTCTCATC	
m-H2Dd-F	GCCTCCTTCATCCACCAAGAC	81
m-H2Dd-R	CACAGCTCCAAGGATGACCAC	
m-H2Ld-F	CGTCCACTGACTCTTACATGG	74
m-H2Ld-R	CCACAGCTCCAATGATGGCC	
m-LMP2-F	CCTCTGCACCAGCACATCTTC	93
m-LMP2-R	CGTGTAGCTCCAGCTGGTAG	
m-LMP7-F	GGACCTCAGTCCTGAAGAGG	116
m-LMP7-R	CAACCGTCTTCCTTCATGTGG	
m-TAP1-F	GCTGTTCAGGTCCTGCTCTC	105
m-TAP1-R	CACTGAGTGGAGAGCAAGGAG	
m-TAP2-F	AGGAGCCTGTGCTGTTCTCG	116
m-TAP2-R	CTATGAAGTCGTCTGCACAGG	
m-Calnexin-F	GCAGCTGAAGAGCGTCCATGG	154
m-Calnexin-R	TCATCCTTCACATCTGGCTGG	
m-ERp57-F	TCATGCAGGAGGAGTTCTCG	141
m-ERp57-R	TGCTACCACAACCTTGACAGG	
m-Calreticulin-F	AGCAGATGAAGGACAAGCAGG	139
m-Calreticulin-R	CCTCTCATCTTCTTCGTCCTC	
m-Tapasin-F	CAGCTACCTCCAGTCACTGC	193
m-Tapasin-R	CCTAGCACCTTGAGGAGTCC	

b)

Cell Cycle

primer name	Sequence 5' to 3'	product length [bp]
m-Cdc25a-F	CCTACTGATGGCAAGCGTGTC	146
m-Cdc25a-R	CTTCAGGACATACAGCTCAGG	
m-Cdc25b-F	CCATCATGCCTTGTAGCCTGG	119
m-Cdc25b-R	AGTCGTTAGCTGCACGGTCC	
m-Cdk2-F	GGCCAGGAGTTACTTCTATGC	116
m-Cdk2-R	GCTCCGTCCATCTTCATCCAG	
m-Cdk3-F	CCAGATGCCTGACTATCAGAG	142
m-Cdk3-R	TCTTGGCTGAGATCCGCTGG	
m-Cdk4-F	CCAGAGATGGAGGAGTCTGG	111
m-Cdk4-R	CCTCCTTGTGCAGGTAGGAG	
m-Cdk6-F	TCTGGTGACCAGCAGTGGAC	101
m-Cdk6-R	TACCACAGCGTGACGACCAC	
m-Ccna1-F	TGCCTTGCCTGAGTGAGCTG	125
m-Ccna1-R	GAACTACAGGCGGCTCCATG	
m-Ccnb2-F	CTGCCTCTTGCCTGTCTCAG	91
m-Ccnb2-R	GCCATGTGCTGCATGACTTCC	
m-Ccnd1-F	CTCTGTGCCACAGATGTGAAG	126
m-Ccnd1-R	TTGTGCGGTAGCAGGAGAGG	
m-Ccnd2-F	AGCTGCTGGCCAAGATCACC	90
m-Ccnd2-R	TGCTGCAGGCTGTTCAGCAG	
m-Ccnd3-F	CCATCCATGATCGCCACAGG	143
m-Ccnd3-R	GATCTGTTCCTGGCAGGCTC	
m-Ccne1-F	TCTCCTCACTGGAGTTGATGC	129
m-Ccne1-R	CCGGAAGTGCTTGAGCTTGG	
m-E2f1-F	AAGCAGTATTGCCACGGATGG	87
m-E2f1-R	CTCCAGGAGTGAGTCAGCAG	
m-Metap2-F	GCGTGGTTCATGACGACATGG	140
m-Metap2-R	CACCTTCGGCAGAAGGCAAG	
m-Mdm2-F	GACGAGAGTGTGGAATCTAGC	106
m-Mdm2-R	AGGTGTCCAGTCTTGCCGTG	
m-p16-F	CTTCCTGGACACGCTGGTG	117
m-p16-R	TCGCACGATGTCTTGATGTCC	
m-p21-F	GTGGCCTTGTCGCTGTCTTG	107
m-p21-R	ATCTGTCAGGCTGGTCTGCC	
m-p53-F	CCGGCTCTGAGTATACCACC	111
m-p53-R	CCACTGGAGTCTTCCAGTGTG	
m-Rb1-F	TTCAGAAGGTCTGCCAACACC	215
m-Rb1-R	CCATCTGCTTCATCGGCTCC	

c)

IFN-γ Signaling Pathway

primer name	sequence 5' to 3'	product length [bp]
m-Ifngr1-F	CTGTCCTAGAGAGTGAGACG	87
m-Ifngr1-R	TCTTCCTGTTCTGCTGCTTCG	
m-Ifngr2-F	AGAGCAACTCCATTGTGCTGG	174
m-Ifngr2-R	ATCAGGATGACTTGCTGCAGC	
m-Jak1-F	CAGTCTCTGTGCTGACCAGG	88
m-Jak1-R	CACACTCAGGTTCTTGGAGTC	
m-Jak2-F	CAGCAAGCATGATGAGTCAGC	70
m-Jak2-R	CTCTCCACAGACACAGACACC	
m-Stat1-F	ACAACATGCTGGTGACAGAGC	87
m-Stat1-R	CTCAACACCTCTGAGAGCTGG	
m-Stat2-F	TGCAGCAGCAGAAGTCCTGC	126
m-Stat2-R	TCCTTCAGCTGCTTCAGTAGC	
m-Stat3-F	GATCGTGACTGAGGAGCTGC	108
m-Stat3-R	GTTGGAGATCACCACAACTGG	
m-Irf1-F	CAGACATCGAGGAAGTGAAGG	157
m-Irf1-R	TCCACACAGCTTCCTCTTGG	
m-Irf7-F	ATGGCAGGTGGAAGCTGTCC	85
m-Irf7-R	TGATGGTCACATCCAGGAACC	
m-Irf9-F	GTTCCTGGAGCATCAACTTCC	138
m-Irf9-R	ACTCCACCTGCTCCATGCTG	
m-Kpna1-F	GTACTAGCTGATGCTTGCTGG	82
m-Kpna1-R	ACTCCTGCATCGATGACTGC	
m-Socs1-F	TCGAGCTGCTGGAGCACTAC	170
m-Socs1-R	TCAGGTAGTCACGGAGTACC	
m-Socs3-F	GACCAAGAACCTACGCATCC	104
m-Socs3-R	ACCAGCTTGAGTACACAGTCG	
m-Ptpsh1-F	ACTACGTGAAGAACCAGCTGC	129
m-Ptpsh1-R	ACGATGACACGAGTGTTCTCC	
m-Ptpsh2-F	GACTGTGACATCGACGTTCC	79
m-Ptpsh2-R	TACTGTGCTTCTGTCTGGACC	
m-Irf2-F	TCAGCATGAGTGAGCTCTACC	153
m-Irf2-R	TGTTGCTGAGGTACTGCTTGC	
m-Crm1-F	TGAGCTCTACAGAGAGAGTCC	165
m-Crm1-R	TACAACCAGTACCTCCTCTGG	

d)

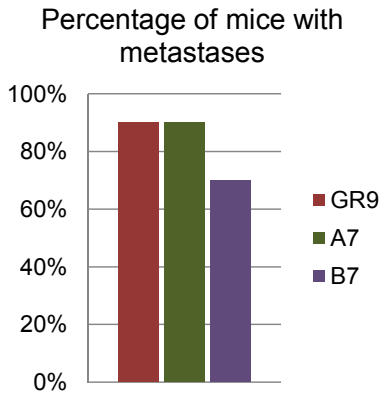
Housekeeping genes

primer name	Sequence 5' to 3'	product length [bp]
m-GAPDH-F	TCAAGAAGGTGGTGAAGCAGG	117
m-GAPDH-R	CGATCGAAGGTGGAAGAGTGG	
m-β-actin-F	CAACACAGTGCTGTCTGGTGG	62
m-β-actin-R	CTCCTTCTGCATCCTGTCAGC	
m-TATA-F	TCAAGATCCAGAACATGGTGG	90
m-TATA-R	TAGCTACTGAACTGCTGGTGG	

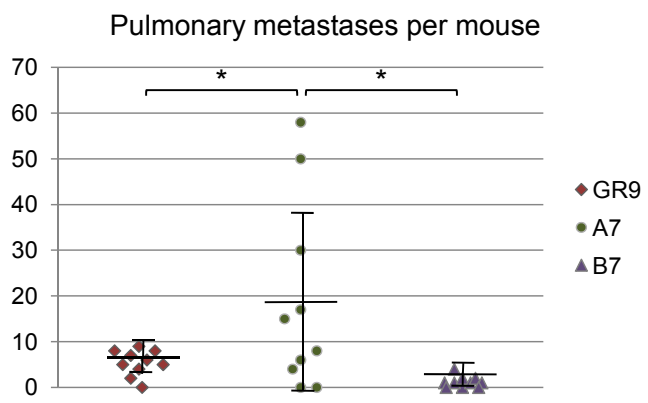
Differences in metastatic progression between GR9 fibrosarcoma tumor, A7 and B7 clones

In spontaneous metastases assays, GR9 fibrosarcoma subcutaneously injected into the foot pad of BALB/c mice, in a dose of $1.25x10^6$ cells, produced a primary tumor that was excised when reached a major diameter of 10 mm, between 19-23 days post-injection. Mice were euthanized on days 42-50. Metastatic progression was detected in the 90% of the animals that developed 2-9 pulmonary metastases (PMs) and 0-1 lymph node metastases (LNMs) (Fig. 44a-c). A7 was injected in a dose of $1.25x10^6$ cells; primary tumor reached a major diameter of 10 mm and was excised on days 20-25. Mice were euthanized on day 46-50 post-injection and 90% of mice developed 6-58 PMs and 0-4 LNMs (Fig. 44a-c). B7 was injected in a dose of $2.5x10^6$ cells; primary tumor was excised when reached a major diameter of 10 mm, in 19-25 days. B7-injected mice were euthanized on day 54-69, B7 clone produced metastases in 70% of mice in a range of 1-4 PMs (Fig. 44a-c).

a)



b)



c)

GROUPS	number of mice	mice with metastases	number of metastases *PM	number of metastases *LNM
GR9	10	9	2-9	0-1
A7	10	9	6-58	0-4
B7	10	7	1-4	0

*PM: pulmonary metastases; *LNM: lymph node metastases.

Figure 44. Progression of the cancer disease in spontaneous metastasis assays with GR9 fibrosarcoma, A7 and B7 clones. a) Percentage of experimental animals that developed metastatic disease. b) Number of metastatic colonies in mice injected with each tumor cell line. Significant differences (*) were found between the metastatic capacity of A7 higher than GR9 and B7. c) The table shows the number of mice per group, number of mice with metastases, and number of pulmonary (PMs) and lymph node (LNMs) metastases per mouse. These results were reproducible in other 2 independent experiments.

Metastatic progression leads to the apparition of a new H-2 class I phenotype variability

When mice were euthanized, at the end of the spontaneous metastasis assay, several metastatic nodules were adapted to cell culture and their MHC-I phenotype in basal conditions and after 48h-IFN-γ treatment was analyzed by flow cytometry. Metastases were classified in four different groups according to their MHC-I phenotype respecting to the tumor cell line injected: Phenotype I, with similar MHC-I phenotype than injected cells; Phenotype II, with partial and reversible MHC-I down-regulation; Phenotype III, with partial and reversible down-regulation of H-2K^d and H-2D^d molecules, and total and reversible loss of H-2L^d allele; Phenotype IV, with partial and reversible down-regulation of H-2K^d and H-2D^d molecules, and total and irreversible loss of H-2L^d allele.

4% of GR9-derived spontaneous metastases had similar MHC-I phenotype than GR9 fibrosarcoma (Phenotype I). The 83% of GR9-derived spontaneous metastases presented phenotype II, and the 13% presented phenotype III, with reversible total loss of H-2L^{d} molecule. All alterations were reversible after IFN-γ treatment (Fig. 45).

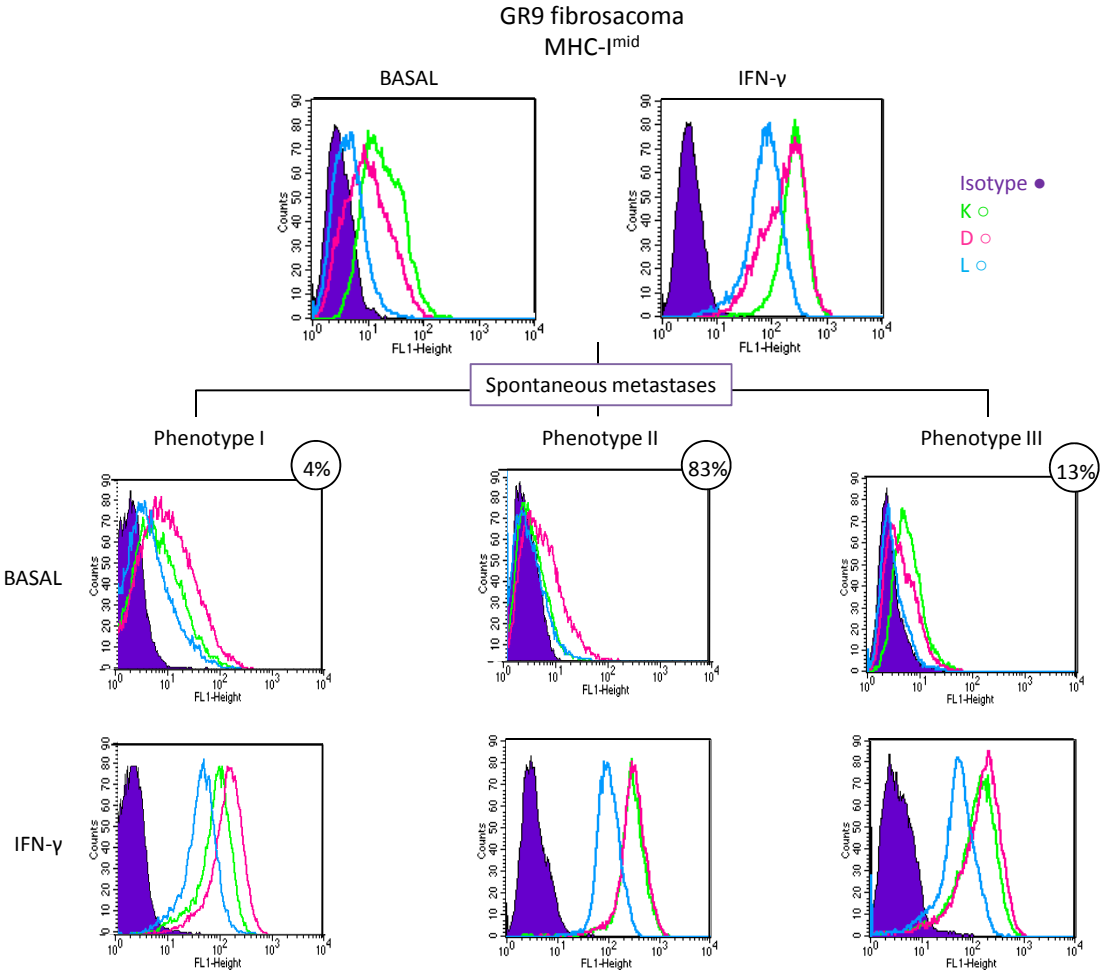


Figure 45. GR9-spontaneous metastases were classified according to their MHC-I phenotype. 4% of GR9-metastases presented phenotype I, with no alterations; 83% had phenotype II, with a down-regulation of MHC-I expression; and a 13% presented phenotype III, with a reversible total loss of H-2Ld allele. Alterations in MHC-I phenotype were inducible after IFN-γ treatment.

Between the A7-derived spontaneous metastases, the 29% of metastases had no alterations in their MHC-I phenotype. A 71% of metastases presented alterations in the MHC-I phenotype, the 36% presented phenotype II and the 35% presented phenotype III. All the alterations were reversible after IFN-γ treatment (Fig. 46).

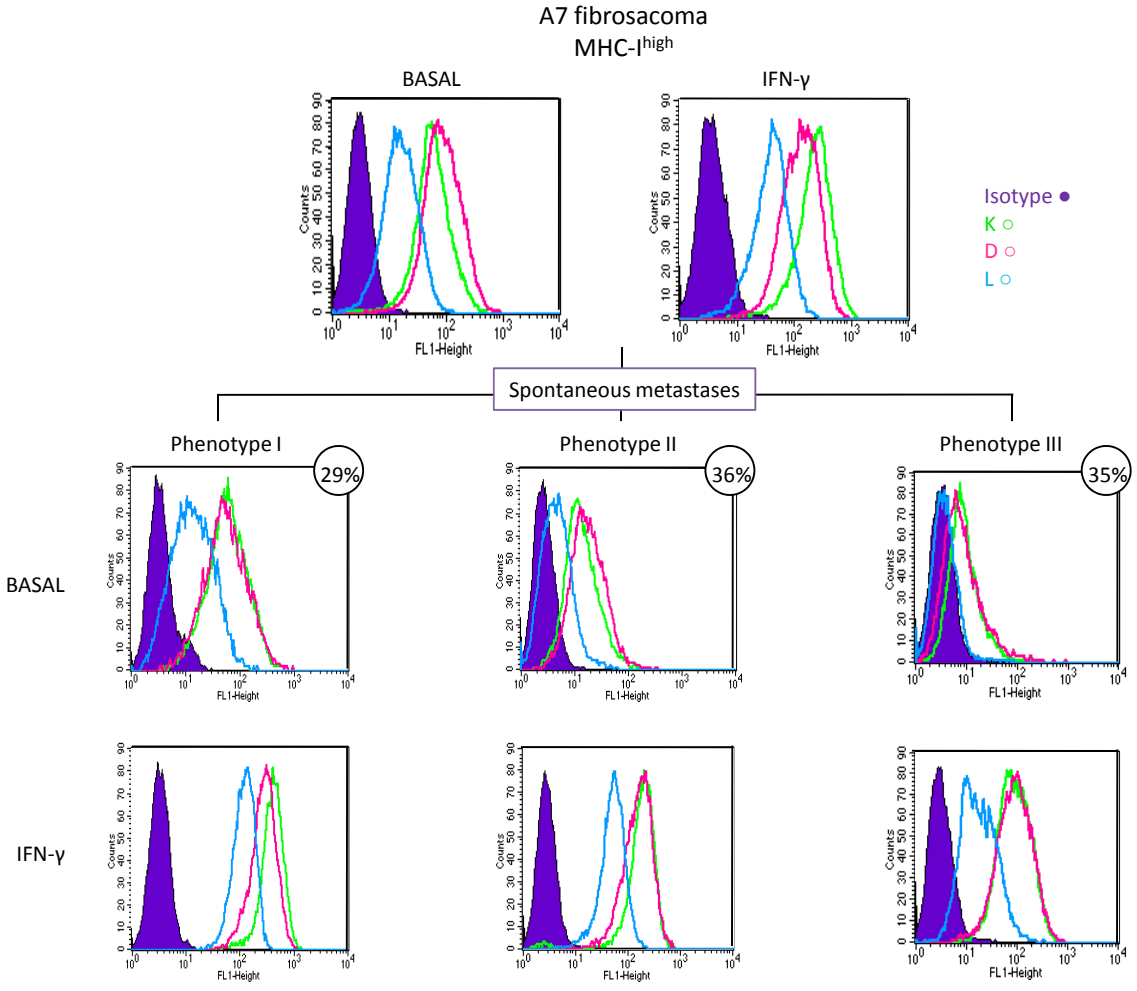


Figure 46. In spontaneous metastasis assay, A7 fibrosarcoma clone led to the development of metastases. 29% of A7-metastases had no alterations in their MHC-I expression (phenotype I). The 36% presented phenotype II, with a down-regulation of MHC-I expression; and a 35% presented phenotype III, with a reversible total loss of H-2Ld allele. Alterations in MHC-I phenotype were inducible after IFN-γ treatment.

The 80% of B7-derived spontaneous metastases presented alterations of the MHC-I phenotype compared to the injected B7 cells. The 20% of the metastases presented phenotype III, with total loss of the expression of H-2L^d molecule in basal conditions, reversible after IFN-γ treatment. The 10% of B7-derived metastases presented a total and irreversible loss of the H-2L^d molecule (Phenotype IV) (Fig. 47).

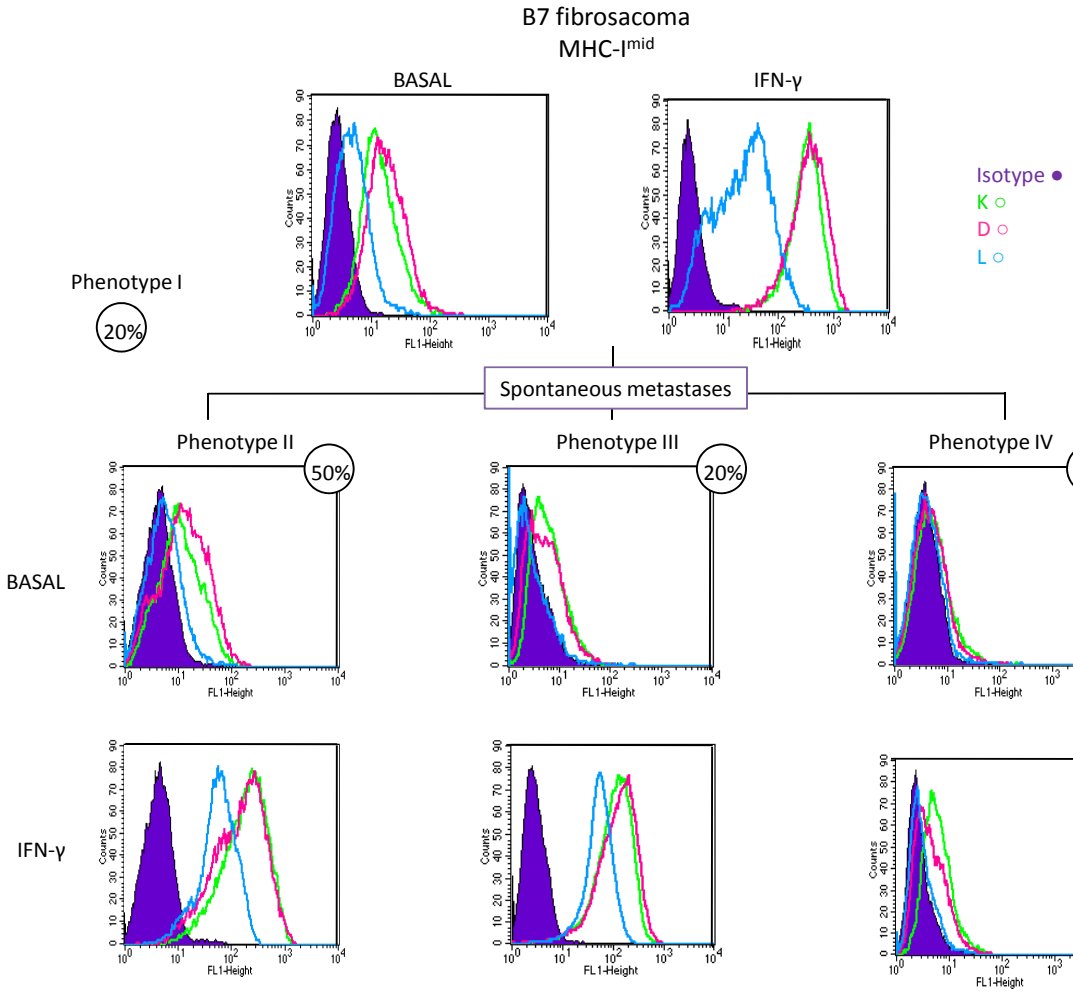
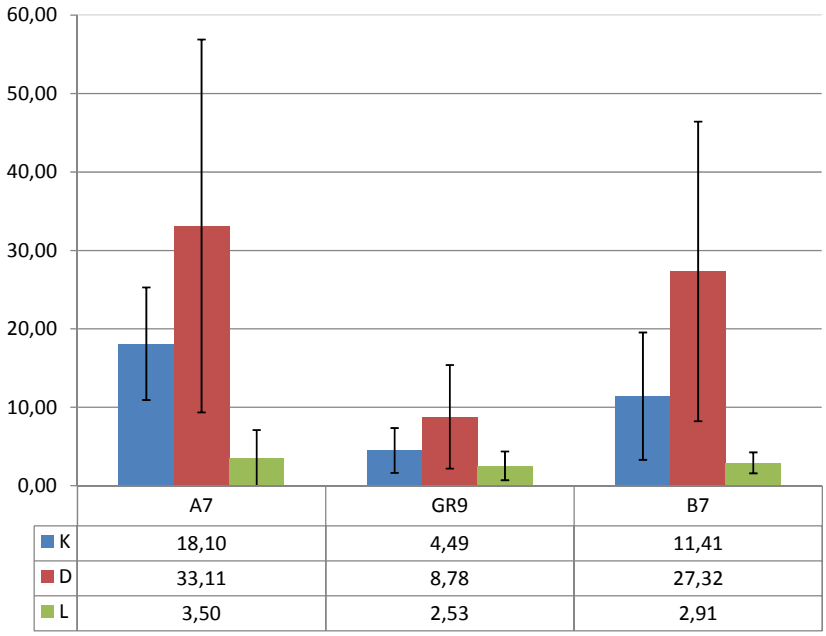


Figure 47. In spontaneous metastasis assay, B7 fibrosarcoma clone led to the development of metastases. The 50% of B7-metastases presented phenotype I. The 20% had phenotype III, with a reversible total loss of H-2Ld allele; and the 10% presented phenotype IV, with a total and irreversible loss of H-2Ld allele.

The averages of the mean fluorescence intensities (MFI) of H-2K^d, H-2D^d and H-2L^d molecules of the metastases derived from each cell line (GR9, A7 and B7-metastases) were compared (Fig. 48). We found that for H-2K^d molecule A7-metastases were the most positives (MFI: 18.10 ± 7.18), follow by B7-metastases (MFI: 11.41 ± 8.12) and finally by GR9-metastases (MFI: 4.49 ± 2.86). H-2D^d molecule, was more expressed in A7-metastases (MFI: 33.11 ± 23.77), followed by those derived from B7 (MFI: 27.32 ± 19.10) and finally by GR9 ones (MFI: 8.78 ± 6.60). The expression of H-2L^d molecule was higher in A7-metastases that presented an

average of expression of (MFI: 3.50 ± 3.59), followed by B7-metastases (MFI: 2.91 ± 1.34) and finally by GR9-metastases (MFI: 2.53 ± 1.83) (Fig. 48).



	A7	GR9	B7
K	18,10	4,49	11,41
D	33,11	8,78	27,32
L	3,50	2,53	2,91

Figure 48. The averages of the mean fluorescence intensities (MFI) of H-2Kd, H-2Dd and H-2Ld molecules of the metastases derived from each cell line (GR9, A7 and B7-metastases) were compared. The expression MHC-I molecules was higher in A7-metastases, followed by B7-metastases and finally by GR9-metastases (A7>B7>GR9).

Preclinical assays with different immunotherapeutic strategies for the treatment of A7-spontaneous metastases

To reveal the role of MHC-I expression on the efficacy of different immunotherapeutic approaches *in vivo,* we primary performed preclinical assays with different immunotherapeutic treatments with A7 clone, highly H-2 positive, that produces several metastases with only reversible MHC alterations. A7-injected mice were treated with CpG ODN 1826 plus A7-radiated cells, PSK, the combination of PSK plus docetaxel or with docetaxel alone. We found that the immunotherapy and the chemoimmunotherapy treatments administered to A7-injected mice completely inhibited metastatic development, whereas docetaxel treatment partially reduced A7-metastatic development. Analysis of lymphocyte populations showed that A7 tumor growth produced immunosupression in host mice that could be restored by the immunotherapeutic treatments.

These results suggest that immunotherapy induces an effective immune response inhibiting spontaneous metastatic colonization of tumor cells with high surface expression of MHC-I molecules or reversible MHC-I alterations.

Cancer Immunol Immunother (2011) 60:1257–1268
DOI 10.1007/s00262-011-1027-1

Immunotherapy eradicates metastases with reversible defects in MHC class I expression

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Received: 20 November 2010 / Accepted: 21 April 2011 / Published online: 8 May 2011

Abstract Tumor or metastatic cells lose MHC class I (MHC-I) expression during cancer progression as an escape mechanism from immune surveillance. These defects in MHC-I may be reversible by cytokines or different agents (soft lesions) or irreversible due to structural defects (hard lesions). The nature of these MHC-I alterations might determine the success or failure of immunotherapy treatments. In this study, we have used an MHC-I-positive murine fibrosarcoma tumor clone, GR9-A7, which generates multiple lung and lymph node metastases with reversible MHC-I alterations after treatment with IFN-γ. Four different antitumor treatments were carried out after primary tumor excision to determine their capacity to inhibit spontaneous metastatic colonization of the GR9-A7 tumor clone. We found that 2 different immunotherapy protocols (CpG plus autologous irradiated-GR9-A7 cells and protein-bound polysaccharide K (PSK) and 1 chemoimmunotherapy (docetaxel plus PSK) induced eradication of metastases. In contrast, chemotherapy with docetaxel alone produced only partial reduction in the number of metastases. Flow cytometric analysis of lymphocyte populations showed an immunosuppression in GR9-A7 tumor-bearing host, which could be reverted by immunotherapy treatments. Our results suggest that irreversible or reversible MHC-I alterations in tumor target cells may determine its progression or regression independently of the type of immunotherapy used.

Keywords MHC class I · Reversible defects · Metastases · Immunotherapy

Abbreviations

MHC-I	Major histocompatibility complex class I
H-2	Mouse leukocyte antigen
HLA	Human leukocyte antigen
PSK	Protein-bound polysaccharide K
BCG	Bacillus of Calmette-Guérin
MCA	Methylcholanthrene
PMs	Pulmonary metastases
LNMs	Lymph node metastases

Introduction

A variety of cancer immunotherapy protocols are widely used for activation of different branches of the immune system, especially in patients with metastatic disease, since primary tumors are usually surgically removed [1, 2]. New

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therapies have been developed based on the fact that tumor antigens recognized by antitumor lymphocytes are small peptides coupled with HLA class I molecules. Peptide-based vaccines have proven able to boost a specific antitumor T cell response, inducing specific CTLs able to recognize and eliminate autologous tumor cells in vitro [3, 4]. Nevertheless, the clinical effects of antitumor vaccines and cancer immunotherapies remain below expectations [5, 6]. To date, cancer immunotherapy has achieved a full response in < 5% of cases [7, 8].

Evidence has been accumulating that tumors generate sophisticated escape mechanisms to avoid attacks by different components of the immune system [9, 10]. Among those mechanisms, alterations in the expression of MHC-I molecules are frequently detected in primary tumors and metastatic lesions [11–13]. These molecules are known to play a crucial role in the presentation of tumor antigens to T lymphocytes and modulate NK cell function. Our group previously reported that the progression or regression of human melanoma metastases after immunotherapy correlates with the level of HLA class I antigen expression [14, 15]. Metastatic melanoma tissue samples were obtained from 2 patients undergoing autologous vaccination plus BCG and/or IFN-α treatment. Results demonstrated that regressing metastases expressed high levels of HLA-ABC molecules, while progressing lesions had low/intermediate levels of HLA class I and harbored structural defects (hard lesions) in MHC-I or β_2-microglobulin genes. Based on these data, we hypothesized that metastatic tumor cells with reversible defects in MHC-I expression (soft lesions) might respond to immunotherapy by upregulating MHC-I expression and promoting T cell-mediated rejection [16, 17].

In the present study, we have tested this hypothesis in a preclinical murine metastatic model. GR9-A7 fibrosarcoma clone presents surface expression of H-2 K^d, D^d, and L^d MHC molecules and generates multiple spontaneous lung and lymph node metastases. These metastases present defects in MHC-I expression that are always reversible by IFN-γ treatment. Three different immunotherapy and a chemotherapy protocols were applied after excision of the primary tumor: (a) immunotherapy with CpG plus irradiated autologous tumor cells GR9-A7; (b) immunotherapy with protein-bound polysaccharide K (PSK) [18]; (c) chemotherapy with docetaxel; and (d) chemoimmunotherapy with docetaxel plus PSK. PSK obtained from *Basidiomycetes*, also known as *Krestin*, has been used as an agent in the treatment for cancer in Asia for over 30 years [19–21]. PSK is derived from the fungus *Coriolus versicolor* and has documented anticancer activity in vitro in experimental models [22] and in human clinical trials [23, 24]. These studies have suggested the efficacy of PSK as principally an immunomodulator of biological response.

We report here that a mouse tumor clone (GR9-A7) with high MHC class I expression generates multiple metastases with reversible MHC lesions. These metastases can be eradicated with different immunotherapy protocols.

Materials and methods

Cell lines

GR9 cell line is derived from a mouse fibrosarcoma induced by methylcholanthrene in BALB/c mice, which has been developed and extensively characterized in our laboratory [25]. It is composed of cell clones with distinct H-2 class I expression patterns and metastatic capacities [26]. Spontaneous metastasis assays were performed with different GR9 cell clones and one of them, the GR9-A7 clone, was selected for this study. GR9-A7 is a clone obtained by a limited dilution method from GR9 cell line. Cell lines were maintained in Dulbecco's medium (Sigma–Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy), 2 mM glutamine (Sigma–Aldrich), and antibiotics. In some experiments, cell lines were treated with 100 U/ml IFN-γ for 48 h (Sigma–Aldrich) or irradiated with a dose of 100 Gy.

Mice

Eight-week-old male BALB/c mice (Charles River Laboratories, Barcelona, Spain) were used in experiments. Breeding and care of animals were undertaken in compliance with European Community Directive 86/609/CEE and Spanish law (Real Decreto 1201/2005) for the use of laboratory animals. Housing and all experimental procedures involving animals were performed according to protocols approved by the hospital's animal care committee and in compliance with the guidelines on animal welfare of the National Committee for Animal Experiments.

Spontaneous metastasis assay

1.25×10^6 GR9-A7 cells were injected into the footpad of syngeneic BALB/c mice. The growth of local tumors was monitored in all animals and recorded 3 times/week. For each tumor, the largest diameter was measured with electronic calipers. Tumors were excised when the largest diameter of each tumor reached 10 mm, at around 20–25 days postinjection. The model resembles metastatic development in humans where primary tumor is surgically removed. Before tumor excision, mice were anesthetized with 0.04 ml of diazepam (Valium, Roche, Madrid, Spain) and 0.1 ml of ketamine (Ketolar, Pfizer, Spain). Primary tumors were removed with sterilized instruments using

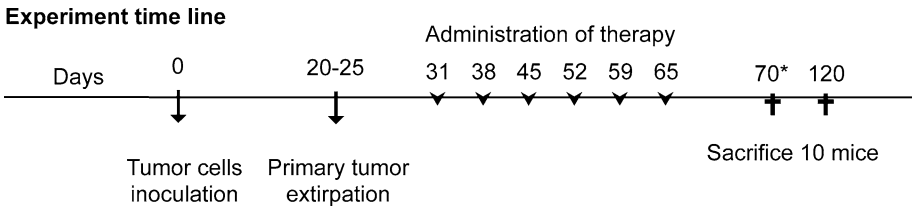


Fig. 1 Time schedule of the treatments. Therapies were administered weekly for 6 consecutive weeks by intraperitoneal injection, beginning 1 week after removal of the primary tumor. Ten mice were killed on day 70 to analyze treatment efficacy. Remaining 10 mice were killed on day 120 to evaluate the long-term effect. *In control and docetaxel group, all mice were killed on day 70

electrocautery to minimize bleeding, and the wounds were closed using surgical clips and adhesive. After surgery, each animal was housed alone until recovery from anesthesia. On day 70 postinjection, when signs of illness started to evidence, animals were anesthetized and euthanized by cervical dislocation. Complete necropsy was done, and the number of metastases was counted. Local tumors and macroscopically visible metastatic nodules were excised, disaggregated, and adapted to tissue culture. Following, mice lungs were fixed in Bouin's solution and micrometastases were counted.

Immunotherapy, chemotherapy, and chemoimmunotherapy protocols

Each of 4 different treatment protocols was applied in a group of 20 mice. The protocols used were as follows:

1. *PSK*: kindly provided by Kureha Chemical Ind. Co. (Tokyo, Japan). It is prepared by extracting cultured mycelia of *Coriolus versicolor* with hot water. The precipitate is separated from the clear supernatant with saturated ammonium sulfate, then desalted and dried. Protein-bound polysaccharide K was dissolved in saline solution and heated at 50°C for 30 min until a clear solution appeared. PSK solution was filtered by 0.22-μm filters (Millipore, Spain). PSK was administered intraperitoneally (i.p.) at 2.5 mg/mouse/week in 500 μl of saline solution.
2. *CpG ODN 1826 plus irradiated GR9-A7 cells*: 1 million GR9-A7 cells were collected in Dulbecco's medium and irradiated with a dose of 100 Gy. After irradiation, cells were maintained in culture for 48 h, washed with PBS, and resuspended in 200 μl saline solution. Twenty micrograms of CpG ODN 1826 (InvivoGen, San Diego, CA, USA) were resuspended in 200 μl saline solution. Both solutions were i.p. injected weekly in each mouse.
3. *Docetaxel*: (Taxotere, 80 mg/2 ml, Sanofi Aventis, Barcelona, Spain) was diluted to 10 mg/ml according to the manufacturer's instructions and further diluted to 625 μg/ml in sterile saline solution; 125 μg/mouse/week was i.p. injected.
4. *PSK plus docetaxel*: mice were simultaneously i.p. injected with PSK combined with docetaxel at the same doses as in the above monotherapies.

These agents were administered once a week during a 6-week period from day 7 after local tumor excision (i.e., days 31, 38, 45, 52, 59, and 65 after cell injection) (Fig. 1). Ten mice from each group were euthanized at 1 week after the end of the treatment (day 70 post-cell injection). The remaining 10 mice in each group were euthanized on day 120 post-cell injection (Fig. 1). In docetaxel group, all mice were euthanized on day 70 due to signs of illness. Twenty mice in a control group (injected with GR9-A7 cells) received i.p., two hundred microliter of saline solution. In this group, all mice were euthanized on day 70 post-cell injection. The same protocols were applied in tumor-free animals to evaluate the toxicity of the treatments and their effect on the survival rate.

Analysis of MHC-I surface expression

MHC-I surface expression was analyzed by indirect immunofluorescence using FACS (FACSCanto; Becton–Dickinson) according to a standard protocol. Primary antibodies used were anti-H-2 K^d (K9–18), anti-H-2 D^d (34-5-8), and anti-H-2 L^d (28.14.8 and 30.5.7), all obtained from the ATCC (Rockville, MD). Fluorescein isothiocyanate (FITC)-conjugate antibody (anti-mouse FITC IgG/Fab, Sigma–Aldrich) at a 1:120 dilution was used as secondary antibody. Isotype-matched nonimmune mouse IgG and cells labeled with only the fluorescein-conjugated antibody were used as controls. A minimum of 1×10^4 cells were analyzed with Cell-Quest Pro software. Each cell line was firstly tested after only one culture passage and retested for 3 times more during different passages. All cell lines were analyzed in basal conditions and after treatment with IFN-γ for 48 h.

Real time RT–PCR

An *mRNA isolation kit* (Myltenyi-Biotech) was used to extract mRNA from GR9-A7 fibrosarcoma clone, from metastases with both high- and low- MHC-I surface expression.

First-strand cDNA was synthesized with 100 ng of mRNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a total volume of 20 µl. These cDNAs were diluted to a final volume of 100 µl. Real-time quantitative RT–PCR analyses for β_2-microglobulin, H2-K^d, H2-D^d, H2-L^d, TAP1, TAP2, LMP2, LMP7, calnexin, calreticulin, and tapasin genes were performed in the 7500 Fast System (Applied Biosystems), using GADPH and β-actin genes as housekeeping genes. PCR reactions were performed in quadruplicate, and values obtained were expressed as means ± SD (standard deviation). Real-time quantitative RT–PCR was performed with the Power SYBR Green Master mix (Applied Biosystems). Primers and amplicon size for each gene are shown in Table 1. PCR conditions were 40 cycles of 15 s of denaturation at 95°C and 60 s at 60°C.

Analysis of lymphocyte populations

Splenocyte and peripheral blood lymphocyte populations were studied by direct immunofluorescence. Mice were anesthetized with 0.04 ml of diazepam and 0.1 ml of ketamine. Spleens were excised, cells were dissociated, and splenocytes were isolated. Blood was collected from the vena cava with a 25-G needle using 0.02 ml of heparin (Rovi, Madrid, Spain). Blood was centrifuged at 300×g for 15 min. Mononuclear cells from spleen and blood were isolated by gradient centrifugation at 500×g for 20 min on Ficoll Histopaque 1083 (Sigma–Aldrich), and they were washed 3 times with PBS. For direct immunofluorescence, the following labeled antibodies (Miltenyi Biotec, Madrid, Spain) were used: CD3ε-APC, CD4-FITC, CD8-PE, CD25-PE, FoxP3-APC, CD19-FITC, and CD49b-FITC. Isotype-matched nonimmune mouse IgGs conjugated with FITC, PE, or APC were used as controls. FcR Blocking Reagent was used to block unwanted binding of antibodies to mouse cells expressing Fc receptors. Immunofluorescence was done according to the manufacturer's instructions (Miltenyi Biotec), using FoxP3 staining buffer to obtain optimal results in FoxP3 immunofluorescent staining. Cells were analyzed on a FACSCanto cytometer (Becton–Dickinson). Each sample consisted of a minimum of 5 × 10^4 cells and was analyzed with CellQuest-Pro software.

Table 1 Primers used in real time RT–PCR

Primer	Name	Sequence 5′ → 3′	Product length (bp)
Gapdh	Forward	TCAAGAAGGTGGTGAAGCAGG	117
	Reverse	CGATCGAAGGTGGAAGAGTGG	
Actb	Forward	CAACACAGTGCTGTCTGGTGG	62
	Reverse	CTCCTTCTGCATCCTGTCAGC	
B2m	Forward	CTGGTGCTTGTCTCACTGACC	214
	Reverse	GACCAGTCCTTGCTGAAGGAC	
H-2 K^d	Forward	CCATCCACTGTCTCCAACACG	112
	Reverse	CCACCTGTGTTCCTTCTCATC	
H-2 D^d	Forward	GCCTCCTTCATCCACCAAGAC	81
	Reverse	CACAGCTCCAAGGATGACCAC	
H-2 L^d	Forward	CGTCCACTGACTCTTACATGG	74
	Reverse	CCACAGCTCCAATGATGGCC	
TAP1	Forward	GCTGTTCAGGTCCTGCTCTC	105
	Reverse	CACTGAGTGGAGAGCAAGGAG	
TAP2	Forward	AGGAGCCTGTGCTGTTCTCG	116
	Reverse	CTATGAAGTCGTCTGCACAGG	
LMP2	Forward	CCTCTGCACCAGCACATCTTC	93
	Reverse	CGTGTAGCTCCAGCTGGTAG	
LMP7	Forward	GGACCTCAGTCCTGAAGAGG	116
	Reverse	CAACCGTCTTCCTTCATGTGG	
Calnexin	Forward	GCAGCTGAAGAGCGTCCATGG	154
	Reverse	TCATCCTTCACATCTGGCTGG	
Calreticulin	Forward	AGCAGATGAAGGACAAGCAGG	139
	Reverse	CCTCTCATCTTCTTCGTCCTC	
Tapasin	Forward	CAGCTACCTCCAGTCACTGC	193
	Reverse	CCTAGCACCTTGAGGAGTCC	

Statistical analysis

Data were expressed as mean ± SD. The paired Student's *t* test was used to compare mean values. A significance level of $P \leq 0.05$ was assumed for all statistical evaluation. SPSS 16.0.2 (SPSS, Chicago IL) was used for the data analyses.

Results

GR9-A7 fibrosarcoma cells originate multiple spontaneous metastases with reversible defects in MHC-I expression

We performed spontaneous metastasis assays (as described in "Materials and methods") in groups of 10 BALB/c mice using GR9-A7 fibrosarcoma clone cell line. Mice were killed on day 70 postinjection and complete necropsy was done. Ninety percent of animals presented metastases. Mice developed pulmonary metastases (PMs, range 9–62, mean of 28) and lymph node metastases (LNMs, range 0–6, mean of 2) (Fig. 2). Lymph node metastases were localized in axillaries nodes, para-aortic nodes, and superior mesenteric nodes. The experiment was repeated twice with similar results.

Seventy-seven PMs and 27 LNMs were adapted to tissue culture, and their H-2 class I surface expression was analyzed and compared with GR9-A7 clone. GR9-A7 is characterized by positive H-2 K^d, D^d, and L^d surface cell expression (Fig. 3a), and these 3 molecules were upregulated after treatment with 100 U/ml IFN-γ for 48 h (Fig. 3a). There were no differences between pulmonary and lymph node metastases. Similar or higher H-2 class I surface expression to that of the GR9-A7 fibrosarcoma clone was found in 29% of the metastases (MHC^{high}) (Fig. 3b), whereas lower MHC-I expression versus GR9-A7 tumor cells was found in 71% of metastases (MHC^{low}), with half of them negative for L^d allele (MHC^{low}-L^{d-}) (Fig. 3b). Each metastatic cell line was tested after the first culture passage and retested for 3 times more during different passages, and the results were always practically identical. The MHC-I defects found in the metastases were always reversible after in vitro treatment with 100 U/ml IFN-γ for 48 h (Fig. 3b).

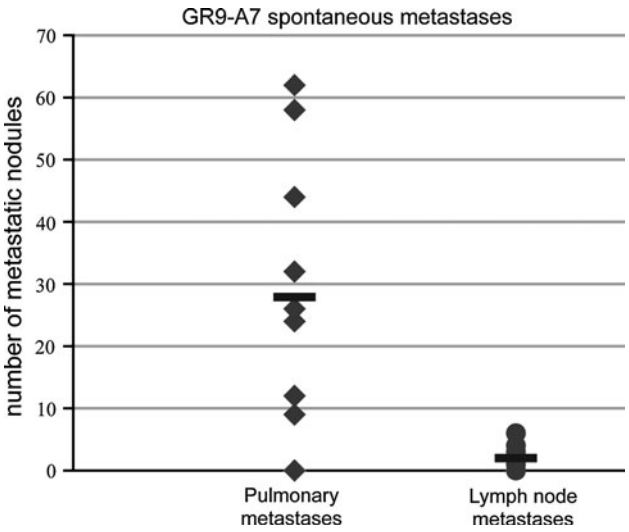


Fig. 2 Spontaneous metastases in mice bearing GR9-A7 fibrosarcoma clone. Seventy days postinjection of GR9-A7 cells, 10 mice were euthanized and metastatic nodules were counted. Nine of ten mice developed metastases (range, 9–62 pulmonary metastases and 0–6 lymph node metastases). This experiment was repeated twice more with similar results

To study the possible mechanisms implicated in the different MHC-I expression of these metastases, we analyzed the transcriptional gene expression of antigen-processing machinery (APM), H-2 class I heavy chain, and β_2-microglobulin genes. GR9-A7 fibrosarcoma clone was compared with 3 MHC^{high} metastases and 3 MHC^{low} metastases. Data were expressed as mean ± SD of 3 metastases by group and the 3 independent experiments. The genes analyzed and primers used in real-time quantitative RT–PCR are given in Table 1. Data were normalized to GADPH and β-actin housekeeping genes. Figure 4 shows the results using the values for GR9-A7 cells as reference (assigned a relative value of 1). MHC^{high} metastases had higher expression of β_2-microglobulin, LMP7, TAP1, and calreticulin genes (Fig. 4). There were no differences in the other genes versus GR9-A7. MHC^{low} metastases presented a downregulation of all MHC-I-related genes except for LMP7 and calreticulin (Fig. 4). These results evidence a direct relationship between H-2 class I surface expression and MHC-I-related transcriptional gene expression in these metastatic cell lines.

Eradication of metastases by immunotherapy and chemoimmunotherapy but not by chemotherapy treatments

The GR9-A7 fibrosarcoma clone produced multiple PMs and LNMs in spontaneous metastasis assays, with a downregulation of MHC-I surface expression in 71% of the metastases, including the absence of the L^d allele expression in 36% of the metastases. All of these alterations in MHC-I expression were reversible with IFN-γ treatment. We established a preclinical assay in mice with four different anticancer therapies: 2 immunotherapy protocols, CpG ODN 1826 (20 µg/mouse/week) + 1 million irradiated GR9-A7 cells, and PSK (2.5 mg/mouse/week); a chemotherapy protocol based on docetaxel (125 µg/mouse/week); and a chemoimmunotherapy protocol combining PSK plus docetaxel. The control group was also injected with GR9-A7 cells but received i.p., two hundred microliter of saline

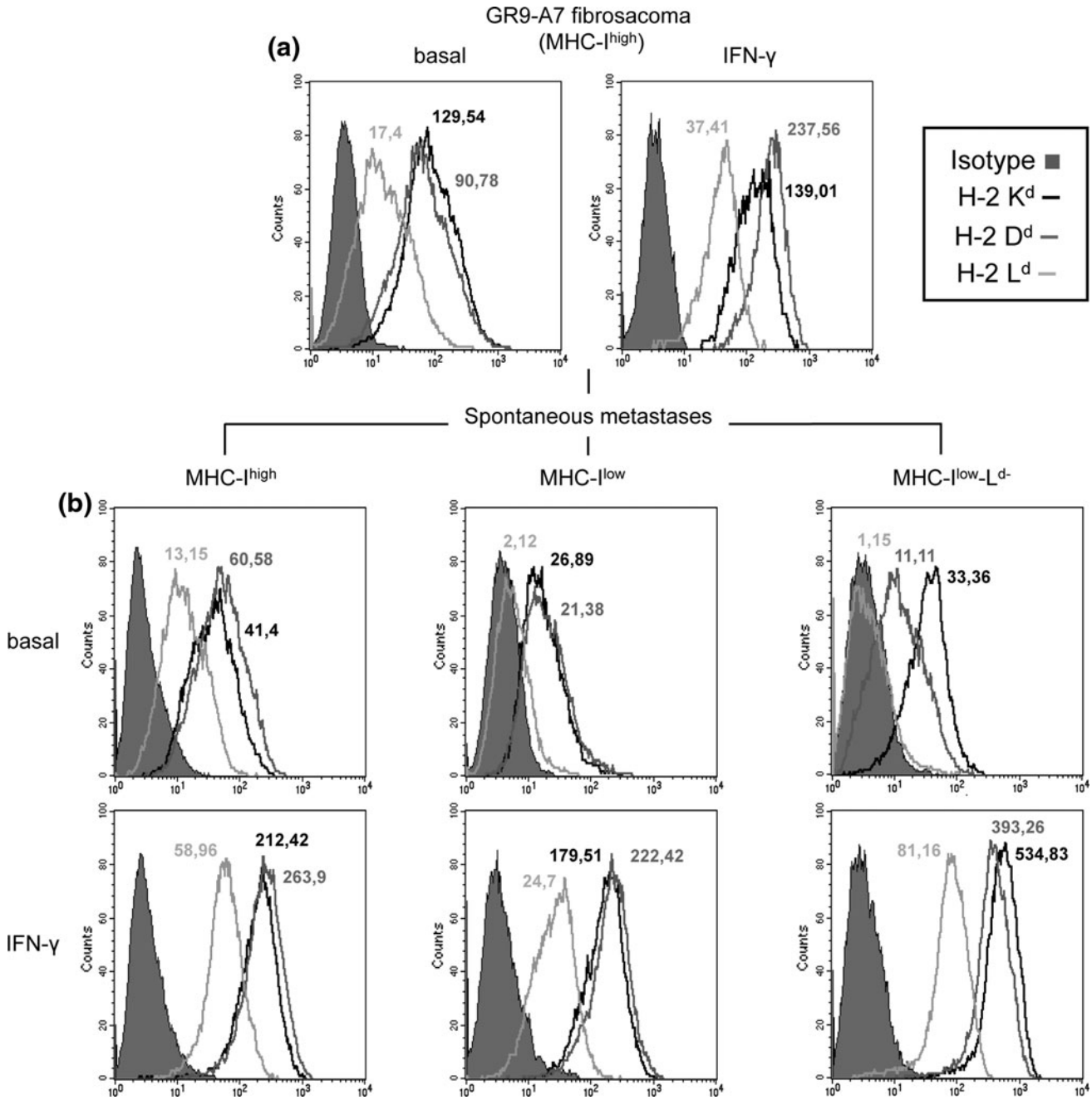


Fig. 3 MHC-I surface expression of GR9-A7 clone and GR9-A7 spontaneous metastases. **a** MHC-I phenotype of the GR9-A7 fibrosarcoma cell line in basal conditions and after treatment with IFN-γ (100 U/ml) for 48 h: H-2 K^d (*black line*), H-2 D^d (*gray line*), and H-2 L^d (*light gray line*); means of fluorescence are indicated. **b** MHC-I phenotypes of GR9-A7 spontaneous metastases: 3 different MHC-I phenotypes were found (MHC^{high}, MHC^{low}, and MHC^{low}-L^{d-}). A representative example of 3 independent experiments is depicted

solution. All therapies were administered after primary tumor excision, only during metastatic development and not during local tumor growth. These therapies comprised weekly intraperitoneal injections administered during a 6-week period starting at 1 week after local tumor excision (Fig. 1). None of the therapies applied in this study had any toxic effect in tumor-free mice or affected their survival rate.

Firstly, we performed in vitro assays analyzing MHC-I expression in irradiated GR9-A7 tumor cells after 48 h in tissue culture, since irradiation of cells may induce an increase in MHC-I surface expression [27]. We found an increased expression of K^d, D^d, and L^d molecules in comparison with baseline MHC-I expression (*data not shown*). This increase in MHC-I expression might induce an increase in specific immune response against these tumor

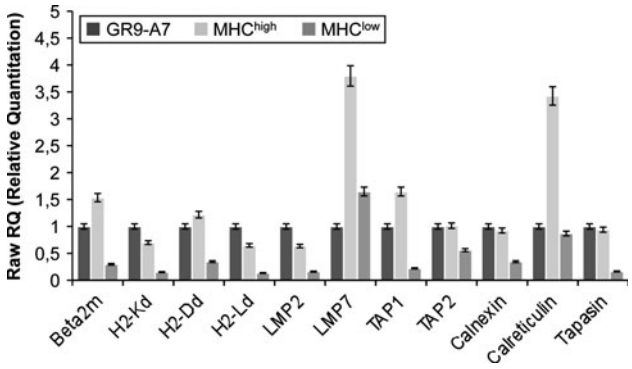


Fig. 4 Transcriptional expression of MHC-I and APM genes in GR9-A7 tumor cells and in its spontaneous metastases. APM, β_2-m, and H-2 class I heavy chain genes expression was analyzed by real-time quantitative RT–PCR. GR9-A7 fibrosarcoma clone (*black*) was compared with 3 MHC^{high} metastases (*light gray*) and 3 MHC^{low} metastases (*gray*). Data are expressed as mean ± SD of 3 metastases of each group. Data were normalized using GADPH and β-actin as housekeeping genes. Results are the average of 3 independent experiments

cells. PSK can produce a cytotoxic effect on tumor cells [28]; therefore, we tested in vitro cytotoxic activity of PSK on GR9-A7 cells at 2 different concentrations, 50 and 100 μg/ml, finding that PSK did not modify the proliferation rate of GR9-A7 cells (*data not shown*). Our group previously reported that PSK only slightly modified the proliferation rate of GR9-B9, another GR9 fibrosarcoma tumor clone [28].

Twenty mice from control group and docetaxel group were killed on day 70 postinjection. In control group, 18 of 20 mice developed pulmonary metastases (range 6–58) and lymph node metastases (range 0–4) (Fig. 5a, b). Seventeen of twenty mice in the docetaxel group had metastases (2–9 PMs; 0–6 LNMs) (Fig. 5a, c). Ten mice from each immunotherapy and chemoimmunotherapy treatment group were euthanized at the end of the treatment (day 70 post-cell injection). No metastases were found in the mice from the CpG + A7–100 Gy, PSK, or PSK + docetaxel groups (Fig. 5a, d). The other 10 mice of these groups were euthanized at day 120 post-cell injection, and newly, all mice were metastasis-free. The experiment was repeated twice with the same results. In a subsequent assay, we found that the mice in these treatment groups remained alive and metastasis-free at 12 months. In brief, immunotherapy and chemoimmunotherapy showed complete inhibition of metastasis development. In contrast, chemotherapy treatment with docetaxel only partially reduced the number of metastases.

Metastases obtained from docetaxel group were adapted to tissue culture, and MHC-I surface expression was analyzed. These metastases present the same 3 MHC-I phenotypes as found in control group (Fig. 3b) and at practically identical percentages: 31% MHC^{high}, 39% MHC^{low}, and 30% MHC^{low}-L^{d-}. According to these results, MHC-I expression of the metastases was highly similar between control and docetaxel groups, indicating that the MHC-I profile was not altered by docetaxel treatment for the mice.

Changes in lymphocyte populations in mice from different groups

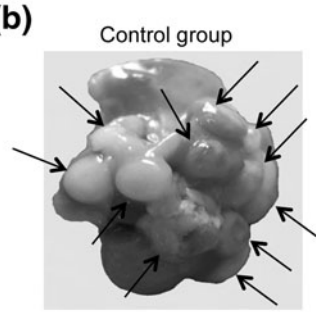
Ten mice from each group were killed at the end of treatment administration, on day 70 postinjection. Spleen and peripheral blood lymphocyte populations from these mice

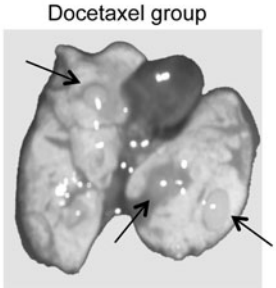
Fig. 5 Spontaneous metastases in mice treated with the different therapies. **a** The table shows the number of mice/group, number of mice with metastases, and number of pulmonary (PMs) and lymph node (LNMs) metastases found/mouse. These results were reproducible in other 2 independent experiments. **b**–**d** Representative picture of lungs from mice of control group (**b**), docetaxel group (**c**), and CpG plus A7 irradiated cells, PSK alone or in combination with docetaxel groups (**d**)

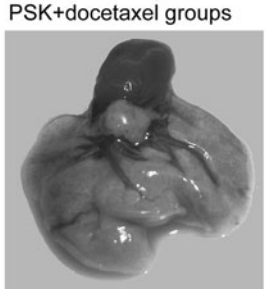
(a)

GROUPS	number of mice	mice with metastases	number of metastases *PMs	*LNMs
Control	20	18	6-58	0-4
Docetaxel	20	17	2-9	0-6
CpG+A7-100Gy	20	0	0	0
PSK	20	0	0	0
PSK+docetaxel	20	0	0	0

*PM: pulmonary metastases; *LNM: lymph node metastases.







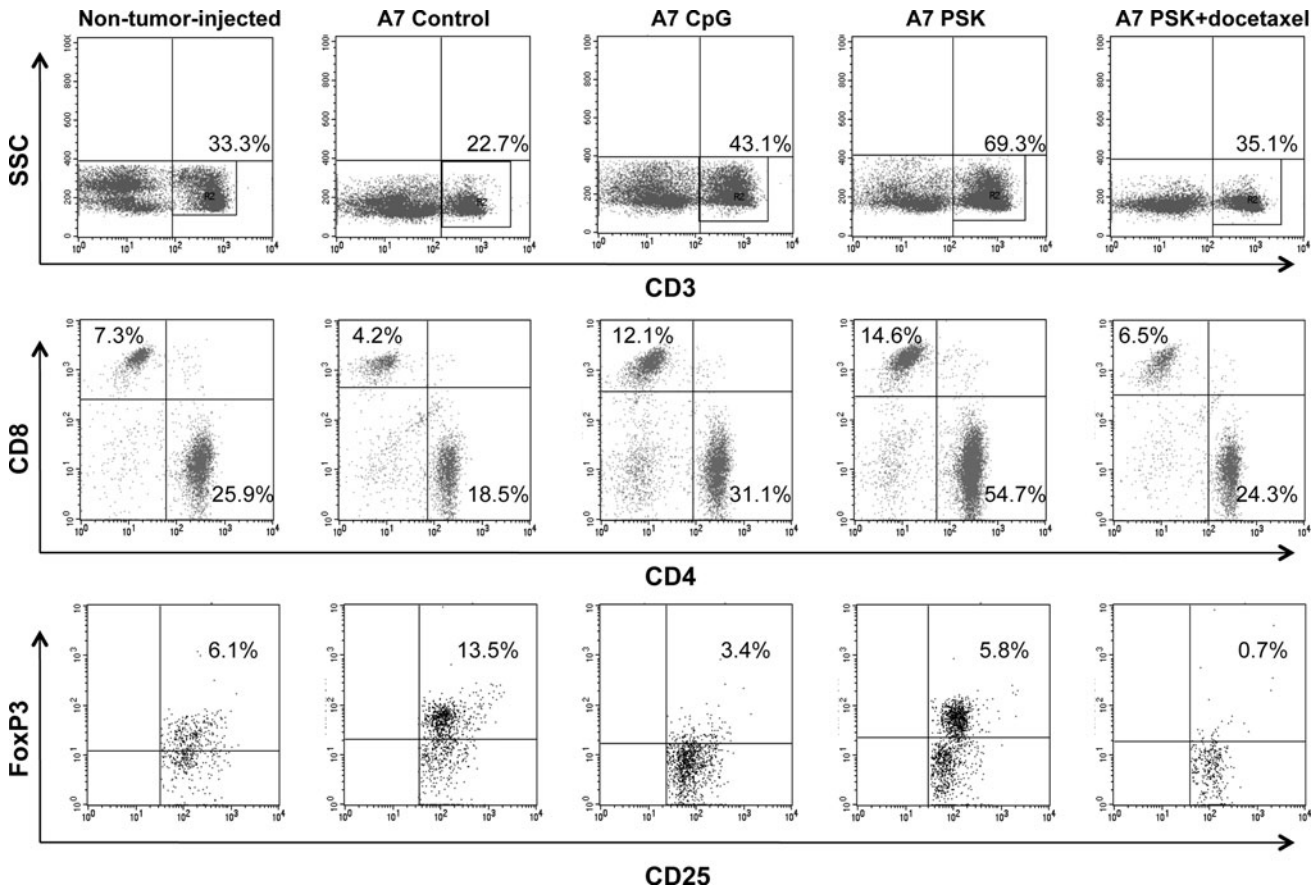


Fig. 6 Changes in spleen lymphocyte populations in the different groups of mice. A representative experiment showing T lymphocyte populations ($CD3^+CD4^+$ and $CD3^+CD8^+$) and T reg cells ($CD4^+CD25^+FoxP3^+$). All percentages are referred to total lymphocytes except for T reg cells that are among $CD4^+$ cells

Table 2 Spleen lymphocyte populations in the different mice groups (%)

Mice	$CD3^+$	$CD4^+$	$CD8^+$	$CD3^+CD4^+$ $CD25^+FoxP3^{+a}$	$CD3^-CD19^+$	$CD3^-CD49^+$	$CD3^+CD49^+$
Non-tumor injected	31.5 ± 4.5	9.1 ± 2.4	22.4 ± 3.1	6.1 ± 1.8	50.7 ± 4.5	8.3 ± 1.0	2.5 ± 1.1
A7 Control	25.3 ± 6.9*	4.9 ± 1.7*	20.4 ± 5.3	10.8 ± 4.2*	54.6 ± 5.9	3.4 ± 2.0*	0.9 ± 0.5*
A7 CpG	40.9 ± 5.4**	12.4 ± 2.2**	28.5 ± 3.9**	4.7 ± 3.8**	43.6 ± 12.3	3.4 ± 1.3	2.0 ± 0.8**
A7 PSK	51.6 ± 17.7**	12.2 ± 2.4**	39.4 ± 15.3**	9.5 ± 3.8	27.4 ± 15.7**	9.9 ± 0.6**	1.6 ± 0.1**
A7 PSK + docetaxel	32.2 ± 2.9**	5.7 ± 0.8	26.5 ± 2.2**	1.1 ± 0.3**	52.9 ± 5.3	2.7 ± 0.1	0.9 ± 0.1

Data are expressed as mean ± SD of 10 mice of each group

* $P < 0.05$ when A7 control group was compared to non-tumor-injected group

** $P < 0.05$ when each treatment group was compared to A7 control group

[a] Percentage among $CD4^+$ cells

were studied by flow cytometry. Mice from control group showed statistically significant changes on the lymphocyte populations as compared to animals not injected with tumor cells (non-tumor-injected mice) ($P < 0.05$): decrease in CD3+ lymphocytes (25.3 vs. 31.5%), principally CD8+ T lymphocytes (4.9 vs. 9.1%); also decrease in NK cells (3.4 vs. 8.3%); and an increase of $CD4^+CD25^+FoxP3^+$ regulatory cells (10.8 vs. 6.1% among $CD4^+$) (Table 2). The immunotherapy and chemoimmunotherapy treatments eradicated metastases in the mice, showing statistically significant differences in lymphocyte populations between treated and control mice ($P < 0.05$) (Table 2). In the CpG + A7-100 Gy group, the treatment produced an increase in $CD3^+$ (40.9 vs. 25.3%), $CD3^+CD8^+$ (12.4 vs. 4.9%), and $CD3^+CD4^+$ (28.5 vs. 20.4%) lymphocytes and a decrease in $CD4^+CD25^+FoxP3^+$ lymphocytes (4.7 vs.

10.8% among $CD4^+$) (Table 2). In the PSK group, the treatment increased $CD3^+$ lymphocytes to 51.6%, $CD3^+CD8^+$ to 12.2%, and $CD3^+CD4^+$ to 39.4% and markedly augmented NK cells, $CD3^-CD49^+$ (9.9 vs. 3.4%) (Table 2). In the PSK plus docetaxel group, a strong decrease in $CD4^+CD25^+FoxP3^+$ lymphocytes was detected (1.1 vs. 10.8% among $CD4^+$) and an increase in $CD3^+$ and $CD4^+$ lymphocytes (32.2 and 26.5%, respectively) (Table 2). A representative experiment depicting these changes is shown in Fig. 6. It should be borne in mind that these data correspond to the systemic level and not to the tumor site. Lymphocyte populations were also analyzed in peripheral blood, finding similar differences to those found in spleen (*data not shown*).

Discussion

The data presented in this paper indicate that the spontaneous metastatic colonization produced by a fibrosarcoma tumor clone (GR9-A7) was completely eradicated by different immunotherapy treatments but not by chemotherapy alone. To resemble the natural cancer progression in humans, we always initiated therapies after excision of large primary tumor when metastases are already established [29]. Importantly, all the metastases generated from GR9-A7 tumor cells had reversible defects in MHC-I expression, which could be restored after IFN-γ treatment. The MHC-I defects were due a coordinated transcriptional downregulation of several APM and H-2 class I heavy chains genes. We classified the metastases obtained in 3 groups according to the total MHC expression as high, low, or low-L^{d-} (when the L^d expression levels were undetectable). In all cases, the mean fluorescence obtained for L^d was lower that the level in the control GR9-A7 clone. All the studied metastases retained the capacity to upregulate the L^d gene after IFN-γ treatment (Fig. 3b). In the GR9-A7 metastatic system, the level of H-2 L^d expression could act as a restriction element for the presentation of the specific tumor antigen that influences the T cell activation.

We favor the idea that the level of MHC-I expression in a particular cancer cell target is a crucial factor that determines the capacity to activate specific antitumor T lymphocytes. In immunotherapy protocols with peptides derived from tumor antigens, the loss of expression of MHC-I molecules or the loss of the antigen itself predicts treatment failure [30–32]. Furthermore, previous results from our group indicate that irreversible "hard" defects in MHC-I expression play a major role in the appearance of progressing metastases after immunotherapy in melanoma patients [14, 15]. Similarly, we have recently reported that bladder tumor relapses after BCG immunotherapy present more profound irreversible "hard" MHC-I alterations [33]. In this context, the success or failure of immunotherapy to induce tumor rejection (specific immunization with tumor-derived peptides or polyclonal immune stimulation with immunomodulatory agents) might depend on the reversible "soft" or irreversible "hard" nature of preexisting MHC-I defects of the target tumor cell rather than on the type of therapy used. In the case of reversible lesions, MHC-I cell surface expression can be restored, leading to a CTL antitumor response and the regression of lesions [16, 17]. This could be the case for the metastases originated from GR9-A7 clone since all of them have the capacity to upregulate MHC-I after IFN-γ treatment. We also studied other clones of GR9 tumor model, with low MHC-I expression and irreversible MHC-I downregulation in metastases, in which immunotherapies did not eradicate metastatic colonization (unpublished observations).

We found an increase in some lymphocyte populations in immunotherapy and chemoimmunotherapy groups at systemic level. These data do not necessarily reflect events at the tumor site, but the metastases were totally eradicated in all of these mice, indicating that a strong immune response may also occur at metastatic sites. The immunotherapy used included PSK and CpG combined with autologous irradiated GR9-A7 cells. PSK is widely used in Asia and has dual anticancer effects, not only activating T cells, NKs, and dendritic cells [34–36], but also exerting a direct cytotoxic activity on tumor cells [28]. According to our assays, PSK had no cytotoxic activity on GR9-A7 tumor cells and therefore acted only as an immunomodulator, producing an increase in T and NK cells. GR9-A7 fibrosarcoma cells evidence in vitro sensitivity to antitumor T cells and resistance to NK cytotoxicity [37]. The action of PSK on T cells might induce the selection of metastatic colonies with low MHC-I phenotypes. In this context, we analyzed some metastases during the course of PSK treatment, in the halfway through the treatment, and all these metastases showed reduced MHC-I expression (*unpublished observations*). The activation of T and NK cells could locally release cytokines in the tumor microenvironment upregulating MHC-I molecules and, therefore leading to the elimination of these metastatic cells. Similarly, treatment with CpG ODN 1826 plus autologous irradiated GR9-A7 cells may produce in vivo a cytokine storm, mainly IFN type I [38, 39], which could markedly increase MHC expression in metastatic cells, favoring their elimination by CTLs. Other possibility is that activation of T cells and decrease in T reg cells might promote recognition of metastatic cells with low MHC-I expression.

In mice from PSK + docetaxel therapy group was detected a marked decrease in T reg CD4+ lymphocytes, which would further potentiate the immune response. Various clinical trials have shown that PSK enhances the antitumor effect of different chemotherapeutic agents [21, 40, 41].

It is also possible that docetaxel increases the PSK-induced immune response. Thus, several clinical and preclinical studies with chemoimmunotherapy have reported enhanced immune response and antitumor activity [42–46]. All of these data support the benefits of combined chemoimmunotherapy protocols. In our preclinical tumor model, the application of docetaxel alone did not block metastatic progression but partially reduced the number of metastases. Furthermore, the MHC-I phenotype of the metastases was similar to that of the control group, suggesting that the mechanism of action of this chemotherapeutic treatment is independent of the MHC-I phenotype.

Previous results from our laboratory showed that another tumor clone of this fibrosarcoma, GR9-B9 (H-2 class I negative but with capacity to upregulate K^d, D^d and L^d molecules after IFN-γ treatment), generated MHC-I-positive metastatic variants on immunodeficient nude/nude mice and MHC-I-negative metastases on immunocompetent syngeneic BALB/c mice [47]. These results using a deprived immune system showed that MHC-positive metastases progressed in the absence of T cells but were eliminated in immunocompetent mice, in which only MHC-negative metastases could progress. Interestingly, the metastases in nude mice were highly immunogenic when transplanted into immunocompetent mice [48]. Our previous and present results, alongside observations in human studies, suggest that the progression or regression of metastases after immunotherapy may depend not on the type of immunotherapy used but rather on the capacity to upregulate the MHC-I expression of tumor cells and on the immune status of the host [49].

The results presented in this paper may contribute to explain the apparently low effectiveness of current immunotherapeutic protocols. Analysis of MHC expression in primary tumors and metastases, especially the identification of irreversible versus reversible defects in MHC-I expression, might help to explain why some metastatic lesions are eradicated after immunotherapy and others are not. It will also open up the possibility of identifying metastases that would respond to immunotherapy and those that require gene therapy to replace the defective MHC-I gene or other mutated genes involved in antigen presentation.

Acknowledgments The authors thank I. Linares and E. Arias for technical assistance and staff of the Departments of Hospital Pharmacy and Radiotherapeutic Oncology of the Virgen de las Nieves University Hospital for their collaboration. They also thank Dr. Natalia Aptsiauri for helpful discussion and Richard Davies for editorial assistance. C. Garrido was supported by the MEC (FPU, 1631) and A.M. García-Lora by FIS-Research Contract CP03/0111 and Stabilization Contract of Fundación Progreso y Salud. This study was partially funded by grants from the FIS (CP03/0111; PI 08/1265; RTICC, RETIC RD 06/020), Consejería de Salud and PAI (Group CTS-143 and projects CTS-695 and CTS-3952) from the Junta de Andalucía in Spain; by the ENACT project (LSHC-CT-2004-503306) and by the Cancer Immunotherapy project (OJ 2004/c158, 18234) of the European Community; and by Kureha Chemical Industry, Tokyo, Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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Immunosuppression of metastasis-free mice from A7 groups after antimetastatic treatments

In the preclinical assay with different antimetastatic treatments in A7-injected mice, treatment with irradiated autologous tumor cell vaccine combined with CpG ODN 1826, monotherapy with PSK or the combination of PSK plus docetaxel completely inhibited metastatic development (Garrido et al. 2011). Ten mice of each group were maintained two months after the treatments finished. Eight mice of each group were euthanized and the mice were free of metastases nodules. Two mice from each group were maintained two months more, and animals did not present signs of illness. At that point, it was possible that metastatic tumor cells were being maintained in dormant state by the immune system. As a preliminary assay, these mice were depleted of CD4+ T lymphocytes by the administration of a treatment with the anti-CD4 monoclonal antibody GK1.5 during a month. At the end of the immunosupressor treatment, mice were euthanized, complete necropsy was done and the number of metastasis was counted. Macroscopically visible metastatic nodules were excised, disaggregated, and adapted to tissue culture. Following, mice lungs were fixed in Bouin's solution and micrometastases were counted.

Immunodepletion of CD4+ T cells induced the reappearance of pulmonary metastases in a PSK+docetaxel treated mouse

Two mice from preclinical assays with antimetastatic immunotherapeutic treatments from CpG+A7-100Gy, PSK and PSK+docetaxel groups were maintained alive for four months without signs of illness. Following, mice were depleted of CD4+ T lymphocytes by receiving weekly intraperitoneal doses of 100µl of GK1.5 ascitic fluid. Mice were euthanized after a month of immunosupressor treatment. Spleens were harvested and lymphocyte populations were analyzed showing a complete immunodepletion of CD4+ T lymphocyte population. Mice from CpG+A7-100Gy and PSK groups did not present any metastasis. However, one of the two mice from PSK+docetaxel group (A732) had developed four small lung metastatic nodules. The four pulmonary metastases (PM) were excised, adapted to cell culture and they were named PMA732a-d. Lungs from each mouse were fixed with Bouin's solution to check under microscope the existence of micro-metastases, but metastases nodules were not found.

Characterization of H-2 class I phenotype in metastasis obtained after immunodepletion

The surface expression of H-2 class I molecules of MPA732a, MPA732b, MPA732c and MPA732d cell lines was analyzed by flow cytometry. Compare with A7 clone, MPA732a, MPA732c and MPA732d showed a partial down-regulation in the surface expression of three H-2 class I molecules (Fig. 49). However, MPA732b presented very similar expression level of H-2 class I molecules than A7 (Fig. 49). Alterations in MHC-I phenotype were inducible after IFN-γ treatment (Fig. 49).

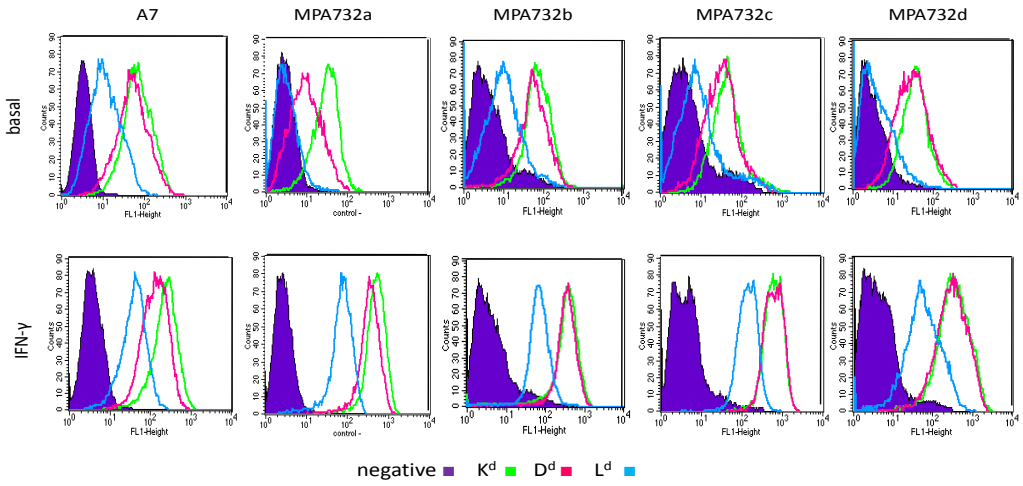


Figure 49. Flow cytometry analysis of MHC-I cell surface expression of A7 fibrosarcoma clone and MPA732 metastases in basal conditions and after IFN-γ treatment.

Molecular mechanisms underlying MHC-I alterations in MPA732 metastases

Transcriptional level of MHC-I related genes (H-2K^d, H-2D^d, and H-2L^d heavy chains, β_2-m, and the APM components TAP1, TAP2, LMP2, LMP7, calnexin, calreticulin, and tapasin) was analyzed by qRT-PCR for MPA732 metastases and compared with A7 clone. Primers and amplicon size for each gene are shown in table 1a. Results were obtained using the values for A7 cells as reference (assigned a relative value of 1). Compared with A7 cell line, MPA732a and MPA732d showed a down-regulation of class I heavy chains, LMP2, TAP1, TAP2, and tapasin genes (Fig. 50). MPA732a also presented LMP7 down-regulation and MPA732d had less expression of β_2-m and calnexin than A7 cells. MPA732c had down-regulated the transcriptional level of H-2L^d and LMP2 genes, but it had increased the levels of LMP7, calreticulin and tapasin. MPA732b presented down-regulation of LMP2 gene and up-regulation of LMP7, TAP1 and calreticulin (Fig. 50).

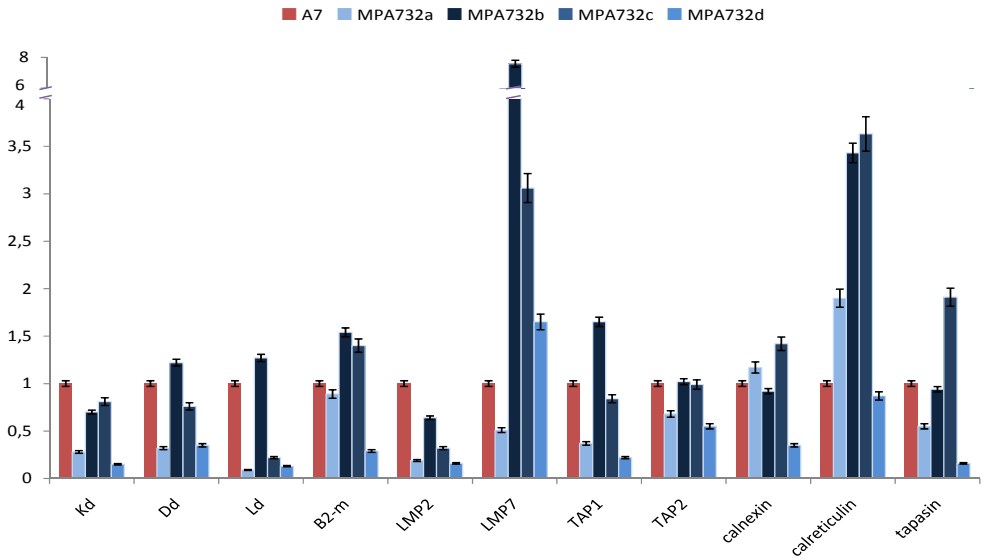


Figure 50. Transcriptional expression of H-2 heavy chains, β_2-m and APM genes in MPA732 metastases was analyzed by qRT-PCR and compared to A7 tumor cells. MPA732a and MPA732d presented down-regulation of heavy chains, LMP2, TAP1, TAP2, and tapasin genes. MPA732c cells present down-regulation of H-2Ld allele, LMP2. MPA732b cells present down-regulation of LMP2 gene.

Analysis of cell cycle genes in MPA732 metastases

MPA732a-d cell lines showed lesser proliferative rate than A7 cells and than other A7-metastases obtain from A7-control group. We studied the transcriptional level of cell cycle genes of MPA732a-d cell lines by qRT-PCR taking the values for A7 clone as reference (primers and amplicon size for each gene are shown in table 1b). Transcription of cell cycle genes were also studied for two A7-metastases obtain from A7-control group: MPA738b and MPA738c, which presented a normal proliferation rate in cell culture. MPA738b presented an MHC-I phenotype very similar to A7 cells, MPA738c presented a down-regulation of the three MHC-I molecules.

Compared with A7 cell line and with control A7-derived metastases (MPA738b and MPA738c), MPA732 metastases presented coordinated down-regulation of Cdc25a, Cdc25b, cdk2, cdk3, cdk4 and p53 genes (Fig. 51). In contrast, they presented an increase of p21, cyclin A1, cyclin B2, and Mdm2 genes (Fig.51).

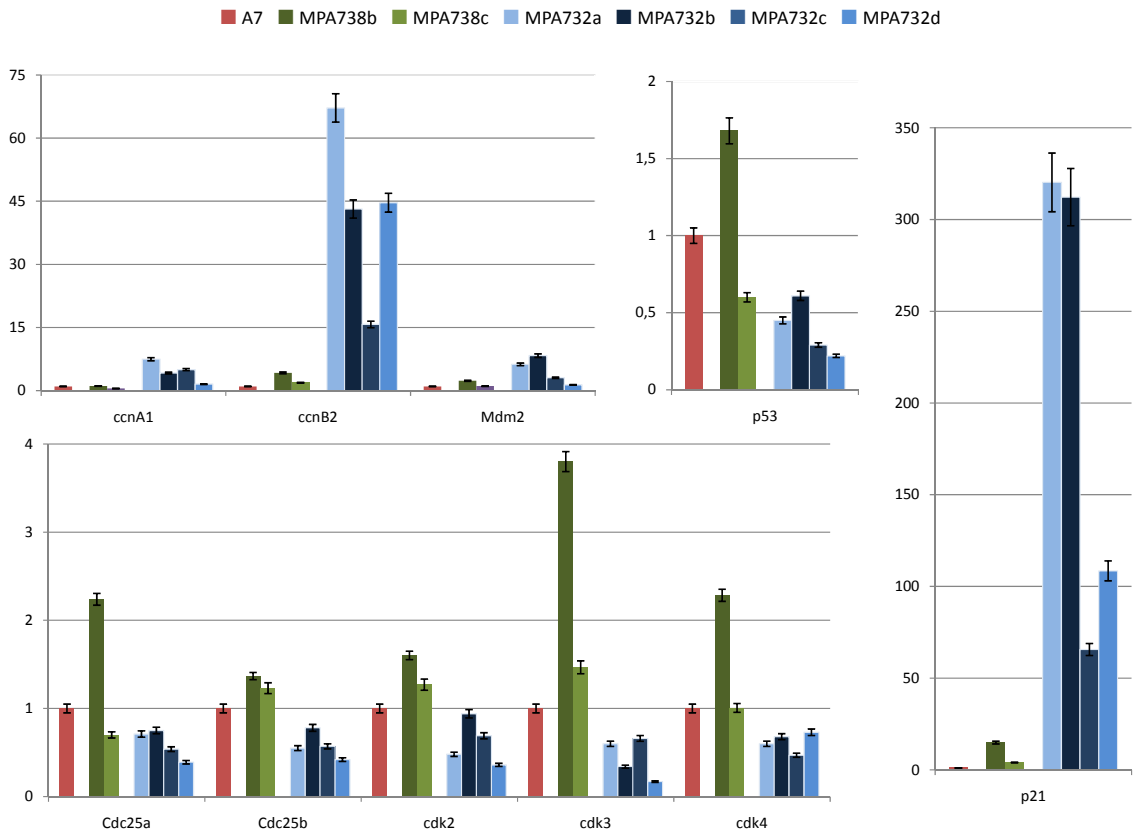


Figure 51. Transcription levels of cell cycle genes of MPA732a-d cells were analyzed by qRT-PCR and compared with A7 cells as well as with control A7-derived metastases (MPA738b and MPA738c). MPA732a, MPA732b, MPA732c and MPA732d presented an increase of the genes: p21, cyclin A1, cyclin B2, and Mdm2; as well as a down-regulation of p53, Cdc25a, Cdc25b, cdk 2, cdk3 and cdk4.

Immunotherapy and chemoimmunotherapy antimetastatic treatments of B7 and GR9 spontaneous metastases

Preclinical assays performed with the highly MHC-I positive clone A7, showed how different immunotherapies completely inhibited A7-metastatic spread. A7-derived metastases presented high/intermediate surface expression of MHC-I molecules, inducible with IFN-γ treatment. The different immunotherapy and chemoimmunotherapy treatments produced an increase of effector lymphocyte populations leading to the elimination of A7-metastatic cells. Following, we applied the same therapy protocols in spontaneous metastasis assays with B7 clone which had an intermediate MHC-I expression. Experiments were also performed with GR9 fibrosarcoma.

Therapies did not totally inhibit the metastatic development of GR9 fibrosarcoma or B7 clone

Preclinical assays of antimetastatic treatments were performed with groups of 10 mice undergoing spontaneous metastasis assays with GR9 or B7 fibrosarcoma tumor cells, and the results were compared with the obtained results for A7 cells. Treatment protocols: CpG plus irradiated autologous tumor cells (TC-100Gy), docetaxel, PSK or combination of PSK plus docetaxel) started after excising the primary tumor (19-25 days post-injection) and were weekly administered during six weeks (i.e., days 31, 38, 45, 52, 59, and 66 after cell injection). Mice were euthanized on day 70 post-injection, complete necropsy was done and metastatic nodules were counted. Table 2 shows the results of incidence of metastatic disease and the range of the number of pulmonary (PMs) and lymph node (LNMs) metastases found in the mice from each experimental group.

Table 2. Incidence of metastatic disease and number of metastases per mice from each experimental group.

Injected tumor cell line	Groups	mice with metastases [§]	number of metastases	
			*PM	*LNM
GR9	Control	9	2-9	0-1
	Docetaxel	7	4-23	0
	CpG+GR9-100Gy	7	3-9	0-2
	PSK	7	2-23	0-2
	PSK+Docetaxel	8	1-15	0-1
A7	Control	9	6-58	0-4
	Docetaxel	5	2-9	0-6
	CpG+A7-100Gy	0	0	0
	PSK	0	0	0
	PSK+Docetaxel	0	0	0
B7	Control	7	1-4	0
	Docetaxel	3	1-8	0
	CpG+B7-100Gy	8	1-4	0-1
	PSK	2	2-3	0-1
	PSK+Docetaxel	3	2	0

[§] Results referred to groups of ten mice.
*PM: pulmonary metastases; *LNM: lymph node metastases.

B7 clone produced metastases in the 70% of mice from B7-control group; 1-4 PMs per mouse were developed. Mice treated with PSK, docetaxel or the combination of PSK plus docetaxel presented a reduction in the incidence and in the number of metastases. Treatment with PSK reduced the development of metastases to a 20% of mice, in a range of 2-3 PMs and 0-1 LNMs. In B7-docetaxel group 3/10 mice developed 1-8 PMs. In B7-PSK+docetaxel group a

3/10 mice developed 2 MPs each animal. In CpG+B7-100Gy group results were worst than in B7-control group, the 80% of mice developed 1-4 PMs and 0-1 LNMs (Fig. 52c). B7-metastases obtained from treated mice were adapted to tissue culture, and MHC-I surface expression was analyzed. These metastases did not show significant differences with the MHC-I phenotype of B7-metastases from the control group.

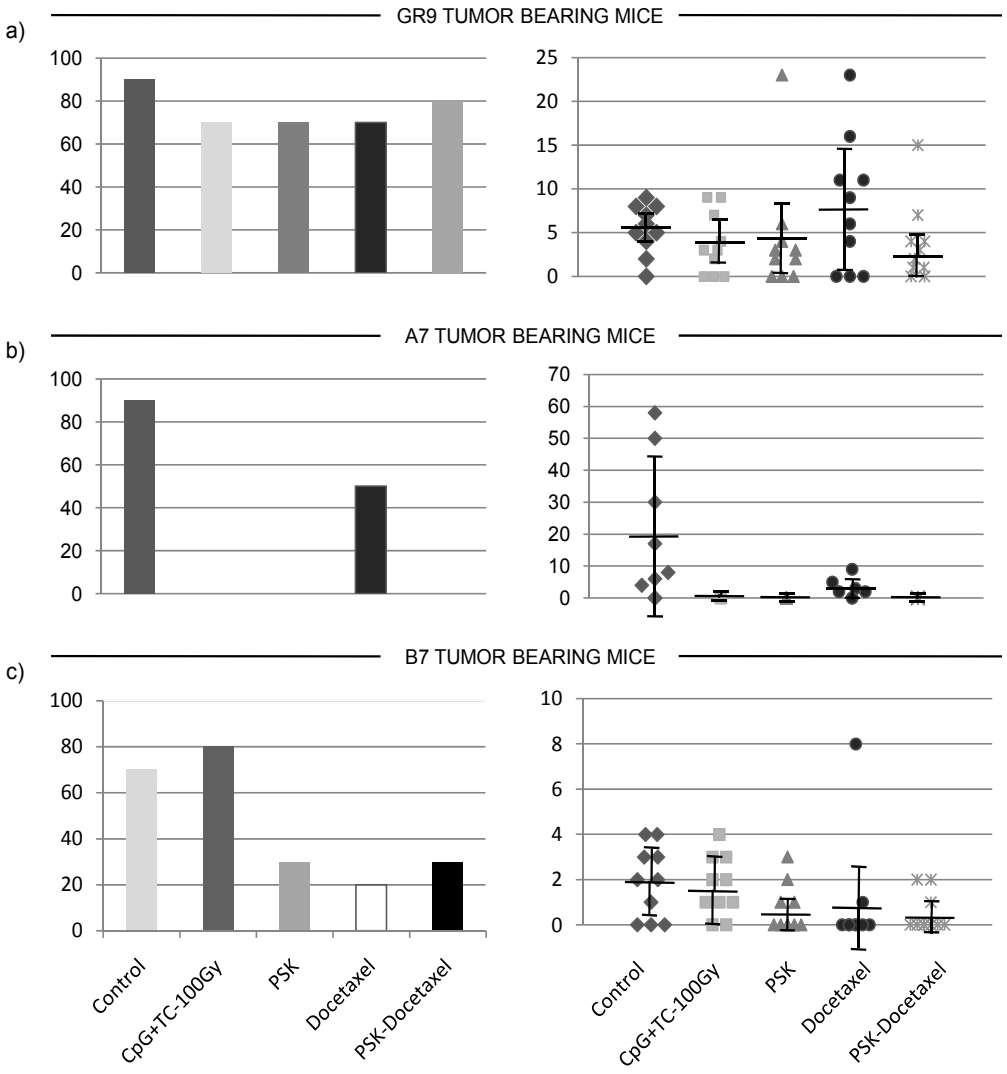


Figure 52. a) In GR9-control group 90% of mice presented 2-9 PMs and 0-1 LNMs. Treatments slightly reduced metastatic spread. b) In A7-control group 9/10 mice developed metastases. Treatments with CpG+A7-100Gy, PSK or the combination of PSK+docetaxel totally inhibited metastases, while docetaxel reduced the incidence of metastases to the 50% of mice. c) B7 clone produced metastases in the 70% of mice, generating a range of 1-4 PMs. Mice treated with PSK, docetaxel or the combination of PSK plus docetaxel presented a reduction in the incidence and in the number of metastases. In CpG+B7-100Gy group 8/10 mice developed 1-4 PMs and 0-1 LNMs. These results were reproducible in two independent experiments.

GR9 spontaneously metastasized in the 90% of mice generating 2-9 PMs and 0-1 LNMs. Treatments slightly reduced GR9 metastatic spread to a 70% of incidence in the case of CpG+GR9-100Gy (3-9 PMs and 0-2 LNMs), docetaxel (4-23 PMs and 0 LNMs) or PSK (2-23 PMs and 0-2 LNMs). PSK+docetaxel reduced the metastatic incidence to an 80% of mice that presented 1-15 PMs and 0-1 LNMs (Fig. 52a). The results were compared with the experiments performed with A7 clone, in which treatments with CpG+A7-100Gy, PSK or the combination of PSK+docetaxel completely inhibited metastatic development. The

chemotherapeutic treatment reduced metastatic incidence from a 90% of A7-control group with 6-58 PMs and 0-4 LNMs to a 50% of mice with 2-9 PMs and 0-6 LNMs in A7-docetaxel group (Fig. 52b). GR9-metastases obtained from treated mice were adapted to tissue culture, and MHC-I surface expression was analyzed. Again, these metastases did not show significant differences with the MHC-I phenotype of GR9-metastases from the control group.

According to these results, MHC-I expression of the B7- and GR9-metastases was highly similar between control and treatment groups, indicating that the MHC-I profile was not altered by these treatments for the mice.

Spleen lymphocyte populations in treated mice

When mice were euthanized, spleens were harvested, and spleen lymphocytes populations were analyzed. Flow cytometry was done to study CD3+CD8+ T cells, CD3+CD4+ T cells, CD4+CD25+FoxP3+ regulatory T cells, CD3-CD19+ B cells, CD3-CD49+ NK cells, and CD3+CD49+ NKT cells. Spleen lymphocyte populations were analyzed and compared between 1) BALB/c mice that were not injected with any tumor cell line, 2) the control groups, injected with tumor cells (GR9 or B7) that received saline as treatment, and 3) the therapy groups, animals injected with tumor cells (GR9 or B7), that were treated with CpG+TC-100Gy, docetaxel, PSK or PSK+docetaxel therapies. Table 3 and figure 53 show the results obtained.

Table 3. Spleen lymphocyte populations in the different mice groups (%)

GROUPS	SPLEEN LYMPHOCYTE POPULATIONS						
	$CD3^+$	$CD8^+$	$CD4^+$	[a] $CD3^+CD4^+$ $CD25^+FoxP3^+$	$CD3^-CD19^+$	$CD3^-CD49^+$	$CD3^+CD49^+$
Wild-type	31,46±10,49	9,10±3,28	22,36±7,99	6,11±2,54	50,67±9,89	8,31±2,04	2,47±1,03
GR9 Control	9,01±1,58*	1,65±0,49*	7,42±1,20*	10,63±2,48*	36,15±5,80*	4,79±1,58*	0,32±0,24*
CpG+GR9-100Gy	10,07±3,08	1,68±0,29	8,44±2,84	11,47±2,81	38,19±13,02	2,39±0,72**	0,19±0,07**
GR9 PSK	26,98±5,61**	5,19±0,70**	22,04±6,42**	9,05±1,06	51,73±0,58**	5,02±1,60	0,35±0,07
GR9 Docetaxel	26,50±9,04**	6,59±2,81**	20,11±6,87**	7,50±3,23**	49,76±4,43**	5,29±2,48	0,35±0,09
GR9 PSK+Doc	22,04±5,37**	6,12±2,81**	14,08±2,43**	10,07±5,17	42,77±5,13**	6,35±6,34**	0,76±1,12**
A7 Control	25,29±6,90*	4,91±1,65*	20,37±5,28*	10,84±4,19*	54,64±5,99*	3,41±2,02*	0,92±0,46*
CpG+A7-100Gy	40,96±5,35**	12,43±2,23**	28,53±3,91**	4,72±3,76**	43,55±12,29	3,44±1,28	2,01±0,84
A7 PSK	51,57±17,70**	12,21±2,36**	39,35±15,34**	9,54±3,76	27,40±15,73**	9,91±0,59**	1,61±0,11
A7 Docetaxel	64,93±10,16**	15,74±4,87**	49,18±5,28**	5,05±1,45	28,53±9,16**	2,69±0,28	1,12±0,06**
A7 PSK+Doc	32,15±2,97**	5,70±0,82	26,45±2,15**	1,06±0,33**	52,92±5,29	2,65±0,13	0,86±0,05**
B7 Control	20,13±7,42*	4,56±1,47*	15,72±6,92*	6,88±2,12*	53,24±9,50	6,11±1,91	1,01±0,44*
CpG+B7-100Gy	17,84±5,46	4,59±0,81	13,37±5,44	5,55±1,78	60,61±7,39**	3,76±1,66**	0,64±0,34**
B7 PSK	26,56±8,54	6,34±2,88	19,00±6,03	8,99±2,54**	51,57±8,26	4,44±1,59	0,73±0,30
B7 Docetaxel	31,42±7,87**	8,03±3,20**	24,98±6,32**	7,20±1,70	51,20±10,26	5,16±2,48	0,56±0,0,45**
B7 PSK+Doc	23,29±7,14	4,97±3,23	18,45±4,20	9,57±3,33**	62,71±5,67**	3,33±0,96**	0,44±0,27**

Averages data are expressed as mean ± SD of 10 mice of each group

* $P < 0.05$ when control groups were compared to non-tumor-injected group

** $P < 0.05$ when each treatment group was compared to their respective control group

[a] Percentage among $CD4_+$ cells

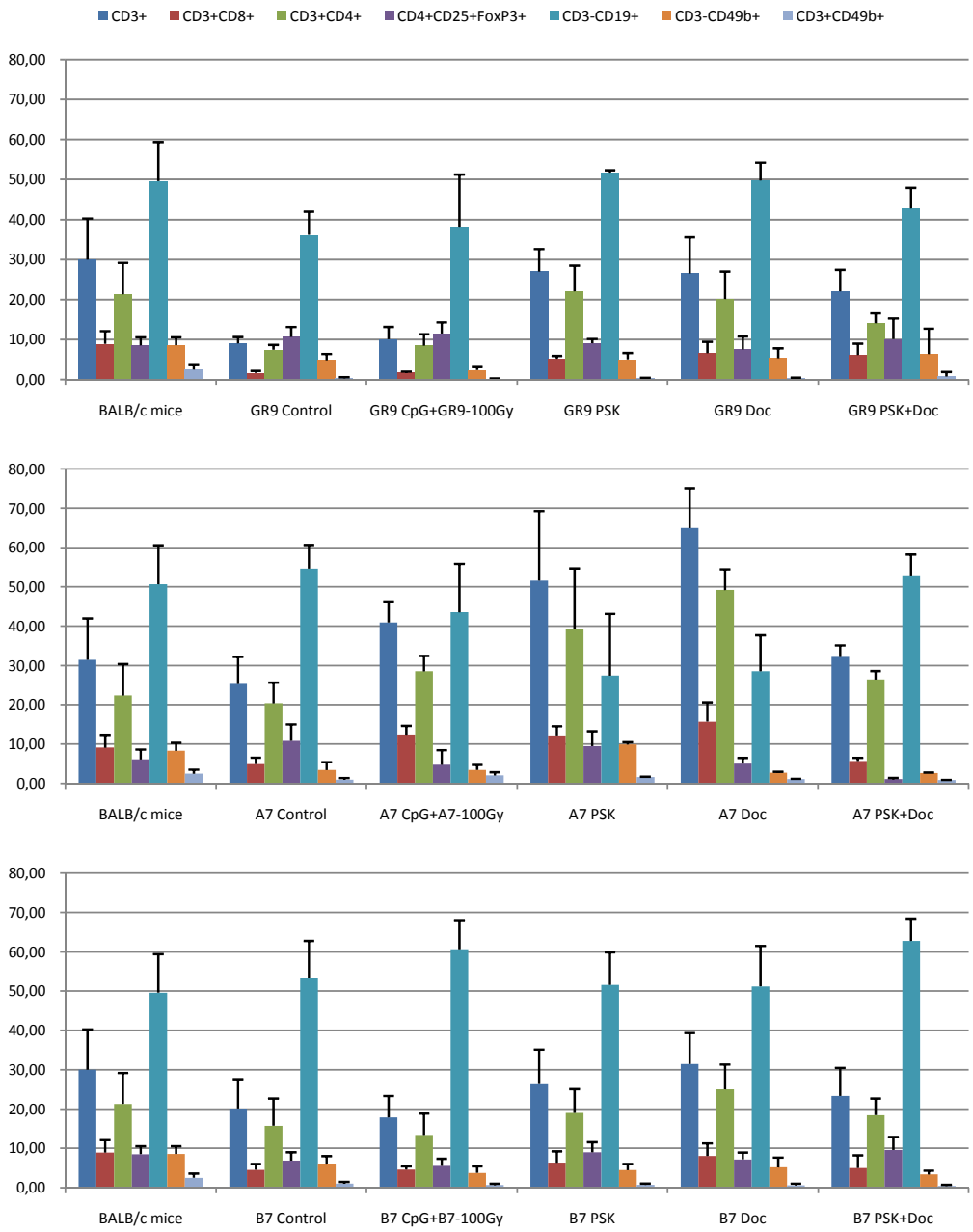


Figure 53. Spleen lymphocyte populations (%) (mean ± SD), compared between 1) BALB/c mice that were not injected with any tumor cell line, 2) the control groups, injected with tumor cells (GR9, A7 or B7) that received saline as treatment, and 3) the therapy groups, animals injected with tumor cells (GR9, A7 or B7), that were treated with CpG+TC-100Gy, docetaxel, PSK or PSK+docetaxel therapies.

Compared with non-injected BALB/c mice, GR9 and B7 tumor and metastatic progression induced a decrease of CD3+ T cells, CD3+CD8+ T cells, CD3+CD4+ T cells, CD3-CD49+ NK cells and CD3+CD49+ NKT cells (Table 3 and Fig. 53). Mice injected with A7 cells also showed a decrease in these population but not as strong as B7 or GR9-injected mice. The population of regulatory T cells increased in GR9- and A7-control mice *versus* non-injected BALB/c mice (Table 3 and Fig. 53).

In A7-groups the different therapies, compared to A7-control group, increased T CD8+, T CD4+, NK and NKT lymphocyte populations, while reduced Treg population, mainly with

PSK+docetaxel treatment (Table 3 and Fig. 53). The same treatments of mice injected with B7 clone and GR9 fibrosarcoma produced different effects over the lymphocytes populations. Comparing to GR9-control group, CpG+GR9-100Gy group presented a very slightly increase of T CD4+ and Treg population, whereas a decrease in NK and NKT cells. PSK alone and the combination of PSK with docetaxel produced an immunostimulation of T CD8+, T CD4+, and the combined treatment also increased NK and NKT populations (Table 3 and Fig. 53). In B7-injected mice, compared to B7-control group, treatment with CpG+B7-100Gy produced a decrease of CD3+ T, CD3+CD4+ T, NK and NKT cell populations. PSK alone or the combined treatment of PSK+docetaxel slightly induced CD3+ population, T CD8+ and T CD4+ lymphocytes by increasing Treg cells. NKT cell population was induced by these treatments, whereas NK cells population was decreased (Table 3 and Fig. 53).

CpG ODN 1826 plus irradiated autologous tumor cell vaccine have not positive antimetastatic effect against B7-metastases

A result that attracted our attention was the fact that CpG ODN 1826 plus irradiated autologous tumor cells-based vaccine induced protective immunity against A7-metastases while not against B7-metastases. Indeed mice treated with CpG+B7-100Gy presented higher metastatic development and decreased T cells and NK cells populations compared with B7-control group. In contrast, CpG+A7-100Gy completely inhibited A7-metastases and hardly induced T CD3+ population, especially T CD8+ cells. We performed some additional preclinical assays to discern if the bad results with this treatment in B7-injected mice were consequence of the CpG ODN 1826 or of the vaccine with B7-irradiated cells. Following the same schedule than before, groups of ten mice were subcutaneously injected with B7 cells. After the excision of the primary tumor, mice were treated with PSK alone or as adjuvant of autologous tumor cells based vaccines (PSK+A7-100Gy, PSK+B7-100Gy). Other animals groups received BCG (analogue to CpG that stimulates mainly Th1 immune response) alone or as adjuvant of anti-tumor vaccines with irradiated autologous tumor cells (BCG+A7-100Gy, BCG+B7-100Gy). Compared to B7-control group in which a 70% of animals developed metastases, PSK therapy alone reduced the incidence of metastasis disease to a 30%, while PSK+A7-100Gy and PSK+B7-100Gy reduced the incidence of metastasis disease to a 40 and 50%, respectively (Fig. 54a). In contrast, the 100% of the B7-injected mice treated with BCG, BCG+A7-100Gy or BCG+B7-100Gy developed metastases (Fig. 54b).

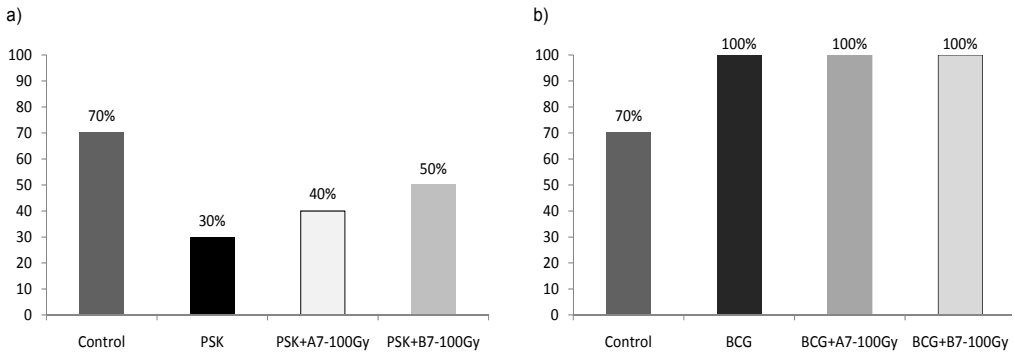


Figure 54. In B7-control group the 70% of mice developed metastases. a) PSK therapy reduced the incidence of metastasis disease to a 30%, while PSK+A7-100Gy and PSK+B7-100Gy reduced the incidence of metastasis disease to a 40 and 50%, respectively. b) The 100% of the B7-injected mice treated with BCG, BCG+A7-100Gy or BCG+B7-100Gy developed metastases. These results were reproducible in two independent experiments.

ANALYSIS OF THE EFFECTS OF IONIZING RADIATION IN GR9 FIBROSARCOMA SUBCLONES WITH DIFFERENT MHC-I PHENOTYPE

During preclinical experimentation with GR9, A7 and B7 fibrosarcoma cells, the radiation of these tumor cells was performed in order to develop an autologous tumor cells based vaccine. For the treatment GR9, A7 or B7 cells were irradiated with a dose of 100 Gy, and then the cells were maintained in culture for 48 h before the administration of the vaccine in combination with the immunostimulatory adjuvant CpG ODN 1826. Before starting the treatments we analyzed the MHC-I phenotype as well as the *in vitro* proliferation of irradiated tumor cells. We found that all the three cell lines (GR9, A7 and B7) experienced an up-regulation of the surface expression of H-2 class I molecules and the arrest of *in vitro* cell proliferation after the radiation treatment.

Several studies have shown *in vivo* and *in vitro* up-regulation of MHC-I surface expression on irradiated tumor cells and its beneficial effects on anti-tumor immune-response. But the molecular mechanisms that involved up-regulation of MHC-I surface expression by radiation, are not completely elucidated and constitute an open field of researching. We aimed to make a profoundly study of the molecular mechanisms underlying the effects of radiation associated to MHC-I surface expression. Experiments were done with representative clones of the heterogeneity in MHC-I phenotype within the variety of GR9 fibrosarcoma clones: $G2^{MHChigh}$, $B7^{MHCmid}$, $C5^{MHClow}$, $B11^{MHClow/neg}$ and $B9^{MHCneg}$.

MHC-I phenotype of GR9 fibrosarcoma and GR9-clones

The MHC-I surface expression of GR9 fibrosarcoma and G2, B7, C5, B11 and B9 GR9-clones was studied by flow cytometry in basal conditions and 48h after IFN-γ treatment. GR9 tumor cells present positive MHC-I phenotype (Fig. 55). These five clones are representative of the variety in MHC-I phenotypes of the tumor cells that compound the GR9 tumor. They present high, middle, low or negative MHC-I surface expression ($G2^{MHChigh}$, $B7^{MHCmid}$, $C5^{MHClow}$, $B11^{MHClow/neg}$, and $B9^{MHCneg}$) (Fig. 55). GR9 and GR9-clones respond to IFN-γ treatment inducing their MHC-I surface expression (Fig. 55).

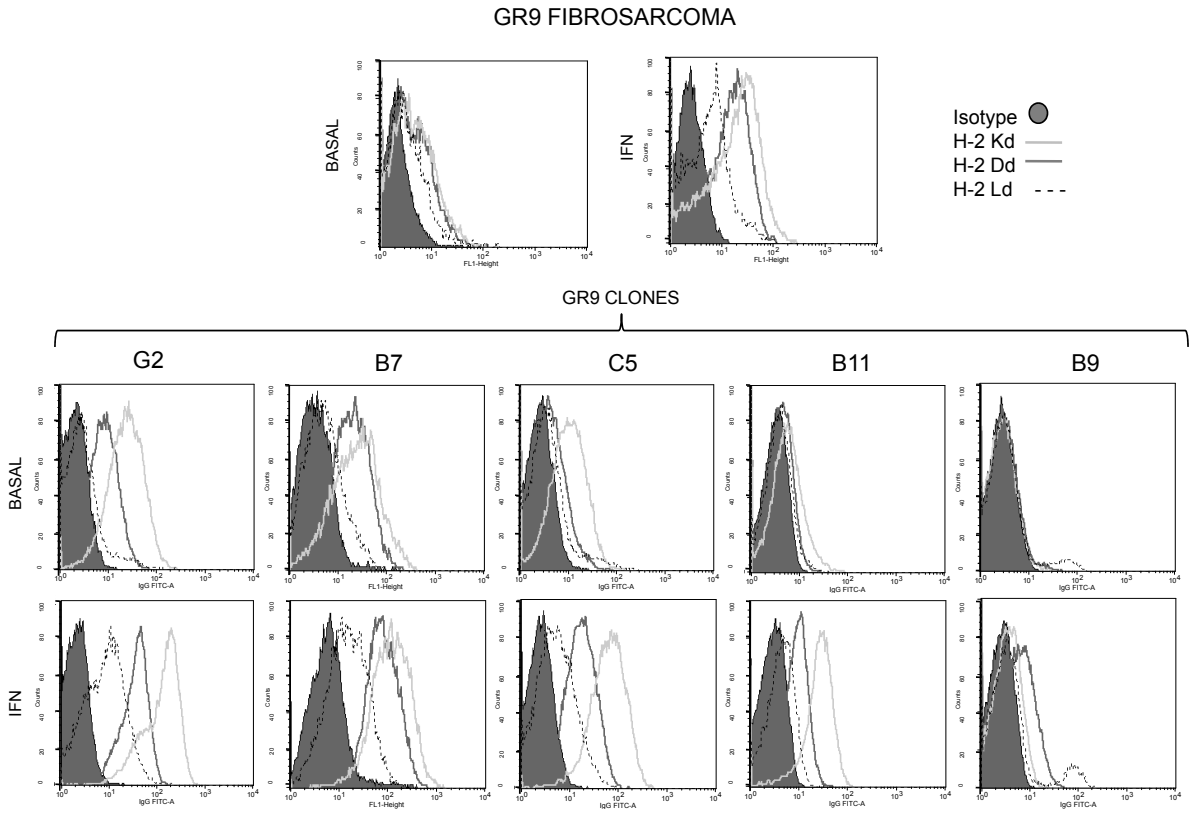


Figure 55. GR9 tumor cells present positive MHC-I phenotype. G2, B7, C5, B11 and B9 are representative clones for the variety in MHC-I phenotypes of the tumor cells that compound the GR9 tumor. They present high, middle, low or negative MHC-I surface expression. GR9 and GR9-clones respond to IFN-γ treatment inducing their MHC-I surface expression.

Radiation elevates MHC-I surface expression in GR9-clones according to their MHC-I phenotype and in a dose-dependent manner

Both *in vitro* and *in vivo* experiments have previously reported an up-regulation of MHC-I surface expression after radiation of human and murine tumor cells (Shiao and Coussens 2010). Studies comparing the radio-response of tumor cells carrying different MHC-I phenotypes have never been done before. For this study we analyzed the effects of radiation in GR9 and in GR9-clones: G2, B7, C5, B11 and B9. All tumor cells were irradiated at the dose of 22Gy and maintained in culture, analyzing the MHC-I surface expression by flow cytometry after 48 hours. GR9-irradiated tumor cells showed a slightly up-regulation in the surface expression of H-2K^{d}, the induction was stronger for H-2D^{d} and H-2L^{d} molecules (Fig. 56). G2^{MHChigh}, B7^{MHCmid}, B11$^{MHClow/neg}$ and B9^{MHCneg} clones presented an induction of the surface expression of the three MHC-I molecules, whereas C5^{MHClow} presented an increase only for H-2K^{d} expression when irradiated (Fig. 56).

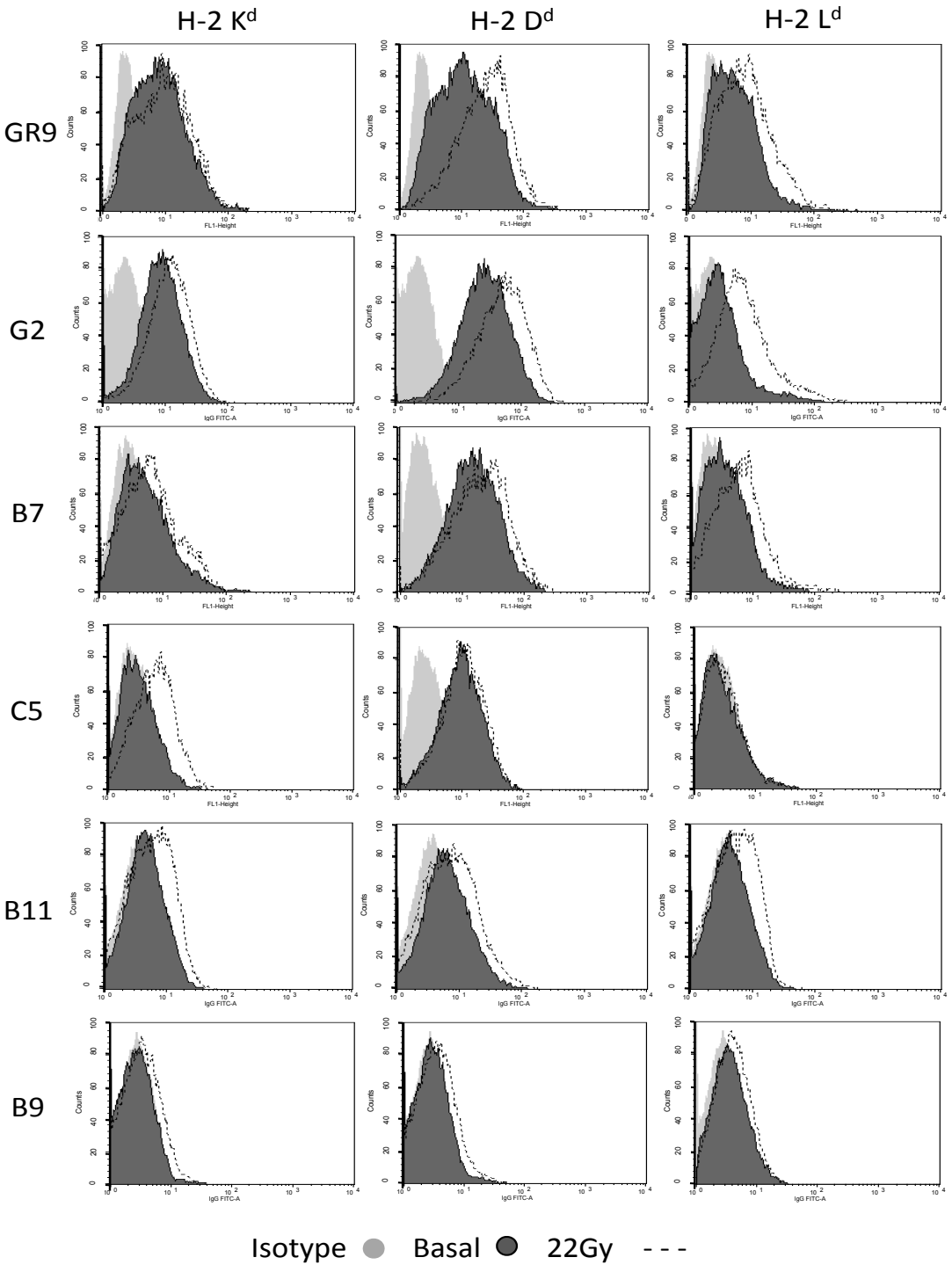


Figure 56. Flow cytometry of GR9 and GR9-clones analyzing surface expression of H-2Kd, H-2Dd and H-2Ld molecules in basal conditions and 48h after 22Gy-radiation treatment.

Complete radiotherapy protocols should involve a total radiation dose of 50-60 Gy. In order to obtain results more reliable to what happens in clinics, radiation treatment was extended to the doses of 44 and 66 Gy. Cells were irradiated at the crescent doses of 22, 44 and 66 Gy, and their MHC-I phenotype was analyzed 48h later. Generally MHC-I surface expression was induced in a dose-dependent way, although in some cases lower dose of radiation produced stronger effect than higher doses of radiation (i.e. GR9 cells presented higher response to 22Gy and 66Gy than to 44Gy radiation dose) (Fig. 57a-c).

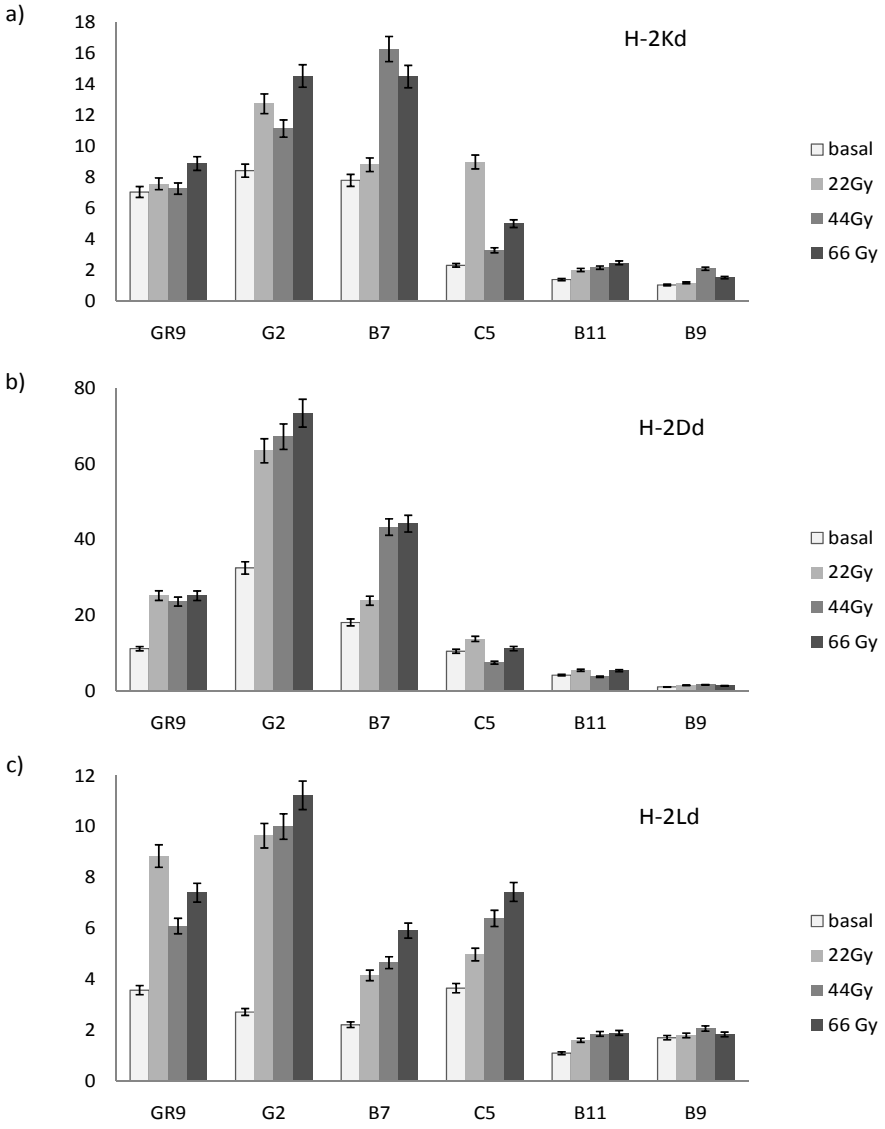


Figure 57. Mean of Intensity Fluorescence of a) H-2Kd, b) H-2Dd and c) H-2Ld molecules, of flow cytometry analyses of GR9 and GR9-clones in basal conditions and 48h after 22Gy-, 44Gy-, and 66Gy-radiation treatment.

The capacity of MHC-I induction within the GR9-clones after radiation treatments was analyzed and compared. The radio-induction of H-2K^d molecule was very similar between the different clones. Major differences were found in H-2D^d and H-2L^d molecules. For example, the radio-induction of H-2D^d molecule: in G2-irradiated cells was of 1.95 times fold at 22Gy, 2.06 times fold at 44Gy and 2.25 times fold at 66Gy, and it was gradually decreasing with the loss of MHC-I expression of the tumor cells until B9-irradiated cells that presented a radio-induction of H-2D^d molecule of 1.38 times fold at 22Gy, 1.49 times fold at 44Gy and 1.27 times fold at 66Gy. The radio-induction of H-2L^d molecule: was higher for G2-irradiated cells that presented a radio-induction of 3.56 times fold at 22Gy, 3.69 times fold at 44Gy and 4.14 times fold at 66Gy, and it was gradually decreasing along the clones accordingly with their MHC-I phenotype until B9-irradiated cells that presented a radio-induction of H-2L^d molecule of 1.05 times fold at 22Gy, 1.21 times fold at 44Gy and 1.08 times fold at 66Gy. These differences can be appreciated in figure 57. These results show that radio-response was stronger in those clones with a highly positive MHC-I phenotype, and more moderate in those clones that presented lower MHC-I surface expression.

Increased transcription of Beta2-microglobulin, H-2 heavy chains and APM components genes after radiation

Subsequently, we studied the effect of radiation in the complete battery of genes implicated in the surface expression of MHC-I molecules. Cells were 66Gy-irradiated or non-irradiated and 48h later total RNA was isolated and reverse transcript to cDNA. qRT-PCR was performed to analyze transcriptional level of Beta2-microglobulin (B_2-m), H-2K^d, H-2D^d and H-2L^d heavy chains and the APM components PA28α, LMP2, LMP7, TAP1, TAP2, calnexin, tapasin, calreticulin and ERp57 genes. The genes analyzed and primers used in real-time quantitative RT-PCR are shown in table 1a. Data were normalized to GAPDH and TATA binding protein housekeeping genes, and the values for non-irradiated GR9 cells were used as reference, receiving a relative value of 1. Irradiated GR9 tumor cells presented a coordinated up-regulation of B_2-m and H-2 heavy chains (Fig. 58a-d). G2 and C5-irradiated cells presented up-regulation of H-2D^d and H-2L^d heavy chains. B7-irradiated cells presented radio-induction of B_2-m and all H-2 heavy chains genes. B11-irradiated cells presented induction of B_2-m and H-2K^d genes, while B9 only presented radio-induction of H-2L^d heavy chain gene (Fig. 58b-d).

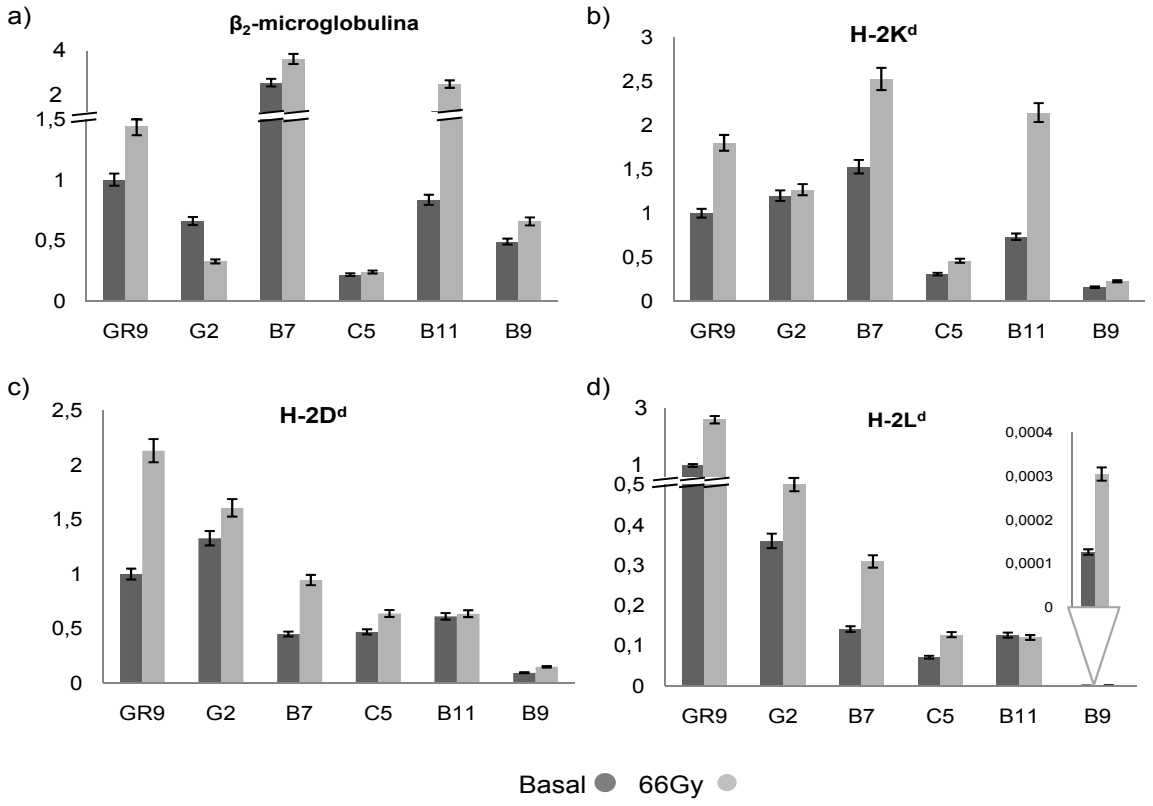


Figure 58. Transcription levels of β_2-microglobulin and MHC-I heavy chains genes were detected by qRT-PCR. Analyses were done to GR9 and GR9-clone cells in basal conditions and 48h after 66Gy-radiation treatment. Expression level of the genes was determined relative to levels of GAPDH and TATA housekeeping genes, and the values for GR9 cells were set to 1.

About the APM components genes, GR9-irradiated cells presented induction of LMP2, LMP7 and TAP1 genes (Fig. 59). G2-irradiated cells up-regulated LMP2 and LMP7 genes. B7-irradiated cells had increased the expression of LMP2, LMP7, TAP1 and tapasin genes. Irradiated C5 cells presented higher expression of LMP2, LMP7, TAP2 and calreticulin. B11-

irradiated cells increased the transcription of PA28α, LMP2, TAP1, calnexin, tapasin and ERp57 genes. And finally B9-irradiated cells presented radio-induction of LMP2, LMP7 and calreticulin genes (Fig. 59).

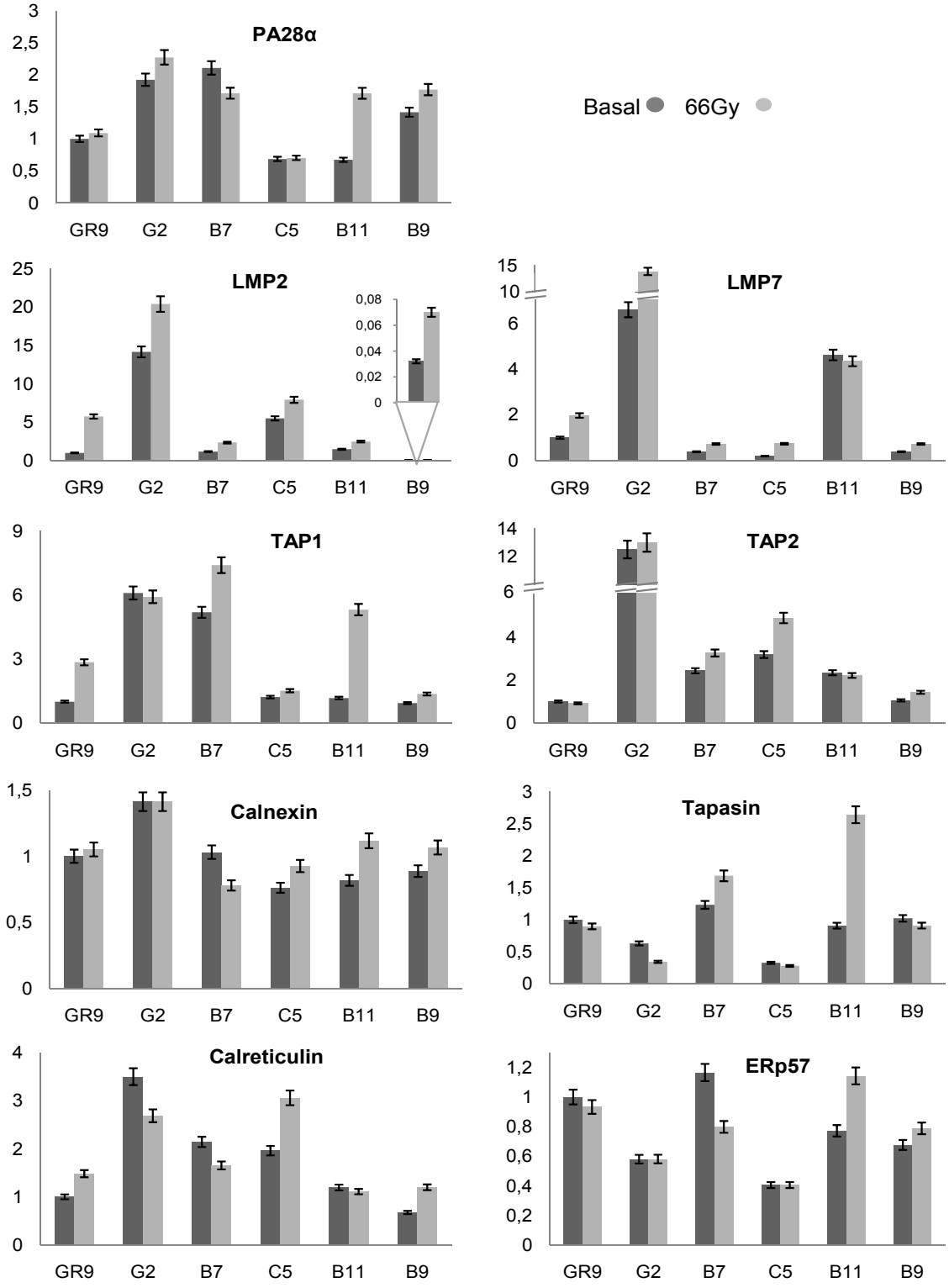


Figure 59. Transcription levels of the APM components: PA28α, LMP2, LMP7, TAP1, TAP2, calnexin, tapasin, calreticulin and ERp57 genes were detected by qRT-PCR. Analyses were done to GR9 and GR9-clones cells in basal conditions and 48h after 66Gy-radiation treatment. Expression level of the genes was determined relative to levels of GAPDH and TATA housekeeping genes, and the values for GR9 cells were set to 1.

Radiation effects on cell proliferation rate and cell cycle genes

It's largely known that ionizing radiation produces DNA-damage, what is translated in the induction of the cell cycle checkpoint signal transducing kinases ATM/ATR and the consequent cell cycle arrest (Kastan and Bartek 2004). We previously reported the new fashion of MHC-I molecules acting as tumor suppressor gene by regulating cell cycle gene expression in melanoma human cells (Garrido et al. 2012). The collection of GR9-clones, with similar genetic background but different MHC-I expression might help to elucidate the role of MHC-I phenotype in radiation-mediated cell cycle arrest.

5×10^5 cells were non-irradiated, irradiated at 22, 44 or 66 Gy or treated with IFN-γ and maintained in culture for 48h. Alamar blue assays were made to measure cell proliferation. Radiation had cytotoxic effects over GR9 tumor cells and over the clones $G2^{MHChigh}$, $B7^{MHCmid}$ and $C5^{MHClow}$ at all doses (Fig. 60). $B11^{MHClow/neg}$ presented less proliferation rate when irradiated at 22Gy, and a cytotoxic effect at 44 or 66Gy (Fig. 60). $B9^{MHCneg}$ tumor clone only presented cytotoxic effects at the dose of 66Gy and lesser *in vitro* proliferation at the doses of 22 and 44Gy (Fig. 60). IFN-γ treated cells were used as control, and they showed decreased proliferation rate or cytotoxic effects in all cell lines comparing with non-treated cells.

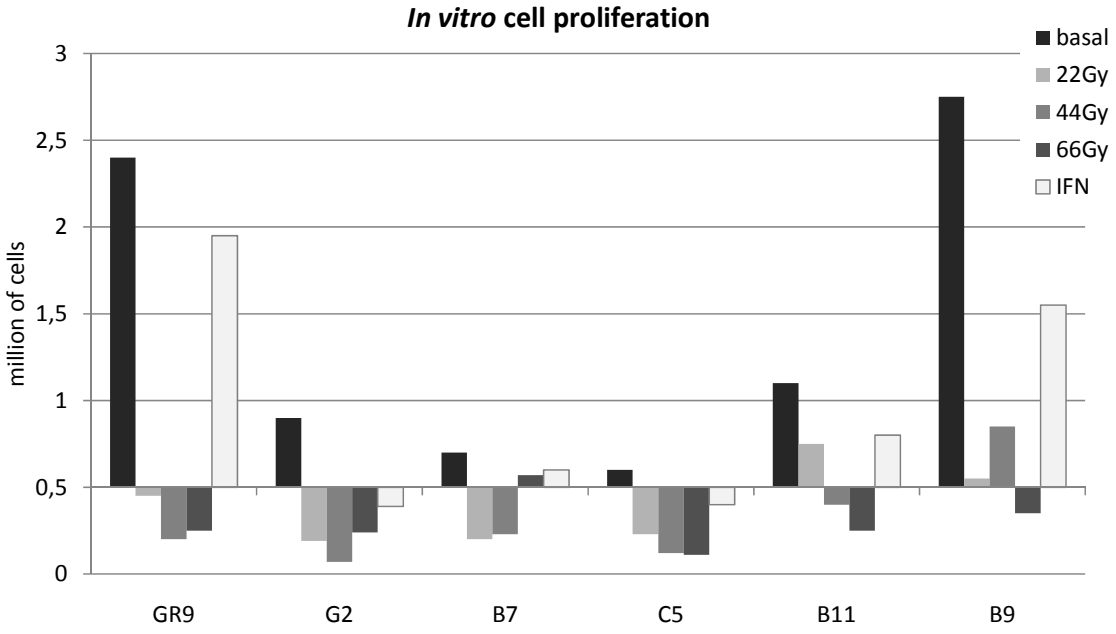


Figure 60. 5×10^5 cells were non-irradiated, irradiated at 22, 44 or 66 Gy or treated with IFN-γ and maintained in culture for 48h. Alamar blue assays were made to measure cell proliferation. These results were reproducible in three independent experiments.

Differences in the capacity of radiation to produce cell proliferation arrest in these tumor cells encouraged us to measure the transcriptional level of genes implicated in cell cycle by quantitative real time-PCR. The genes analyzed and primers used in real-time quantitative RT-PCR are shown in table 1b. cDNA was obtained from 48h-culture maintained tumor cells, non-irradiated or irradiated at the dose of 66Gy. Data were normalized to GAPDH and TATA binding protein housekeeping genes, and the non-irradiated cells values were used as reference, receiving a relative value of 1. All irradiated cells presented the up-regulation of the

transcriptional levels of p21 gene as well as the down-regulation of the transcriptional level of cdk4 and MetAP2 (Fig. 61). Irradiated tumor cells also presented differences in the transcription of other cell cycle genes specifics of each cell line (Fig. 61).

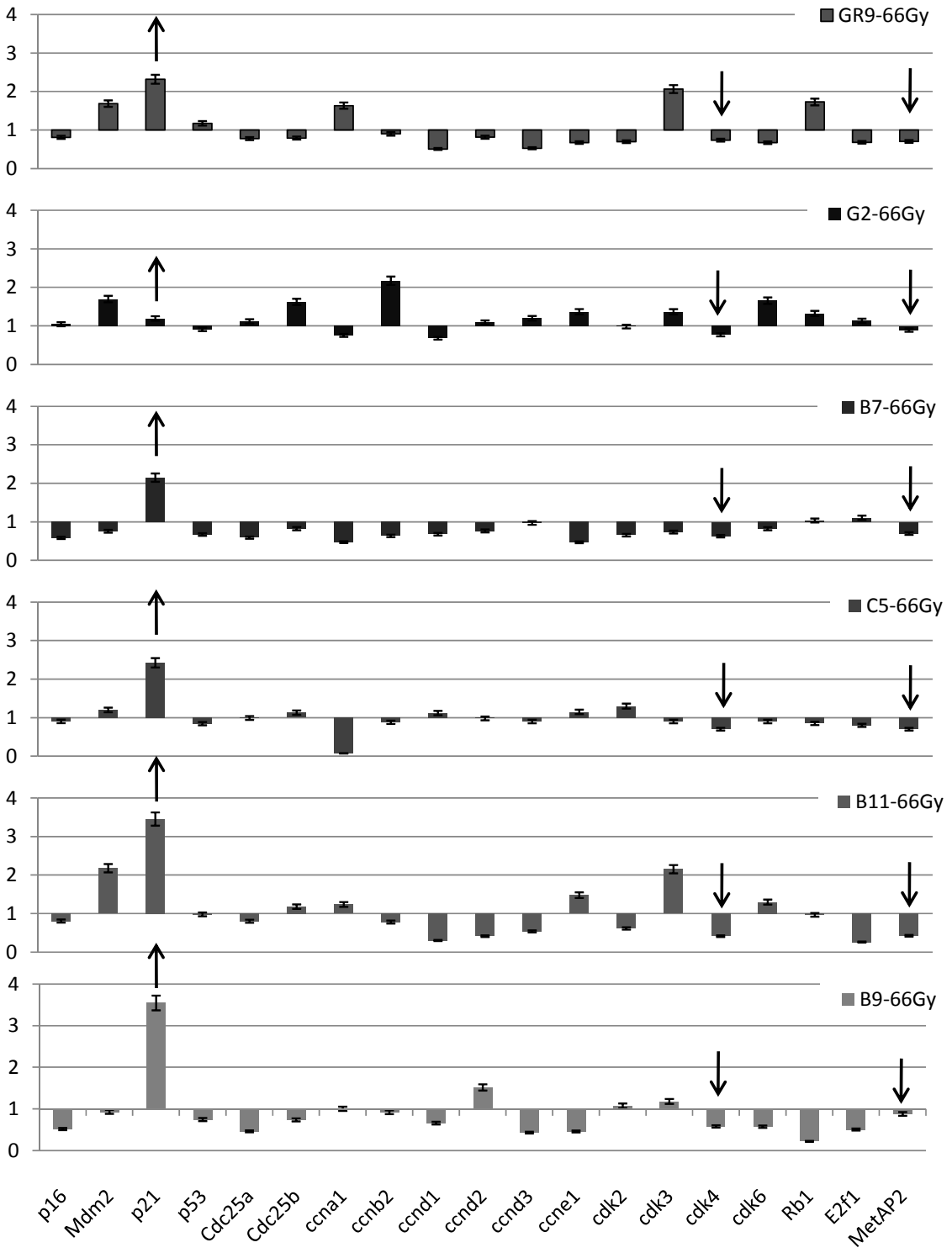


Figure 61. Transcription levels of the cell cycle genes were detected by qRT-PCR. Analyses were done to GR9 and GR-clone cells in basal conditions and 48h after 66Gy-radiation treatment. Expression level of the genes was determined relative to levels of GAPDH and TATA housekeeping genes, and the values for non-irradiated cells were set to 1.

Radiation effects on IFN-gamma signaling pathway genes

Previously, it has been described that radiation of B16/OVA tumors in IFN-γ$^{-/-}$ mice slightly increased the levels of pSTAT1 (Lugade et al. 2008). STAT1 is an important factor of the IFN-γ pathway, and it is especially implicated on the IFN-mediated induction of MHC class I expression. So, could the up-regulation of MHC-I be mediated by the radiation-induction of the IFN-γ cascade components? To answer this question, transcriptional level of the main genes of the IFN-γ pathway was analyzed by qRT-PCR for 66Gy-irradiated or non-irradiated cells. Since B7-irradiated cells showed strong changes in the transcription of MHC-I related genes, they were chose for these analyses. The genes analyzed and primers used in qRT-PCR are shown in table 1c. Data were normalized to GAPDH and TATA binding protein housekeeping genes, and B7 non-irradiated cells values were used as reference, receiving a relative value of 1. B7-irradiated cells showed a radio-induction of the transcriptional level of signal transducer and activator of transcription STAT1 and STAT2, interferon-regulated factors transcriptional activators Irf1and Irf7, Kpna1 importin and suppressor of cytokine signaling Socs1 (Fig. 62). Radiation treatment decreased the transcription of IFN-γ receptor Ifngr2, Jak1, Irf9, Ptpshp2 and Irf2 (Fig. 62). So, although the function of much of these proteins depends on their phosphorilated state, the variations of the transcriptional level of some IFN-γ cascade components could be related with the induction of the transcription of MHC-I related genes and of the MHC-I surface expression of the irradiated tumor cells.

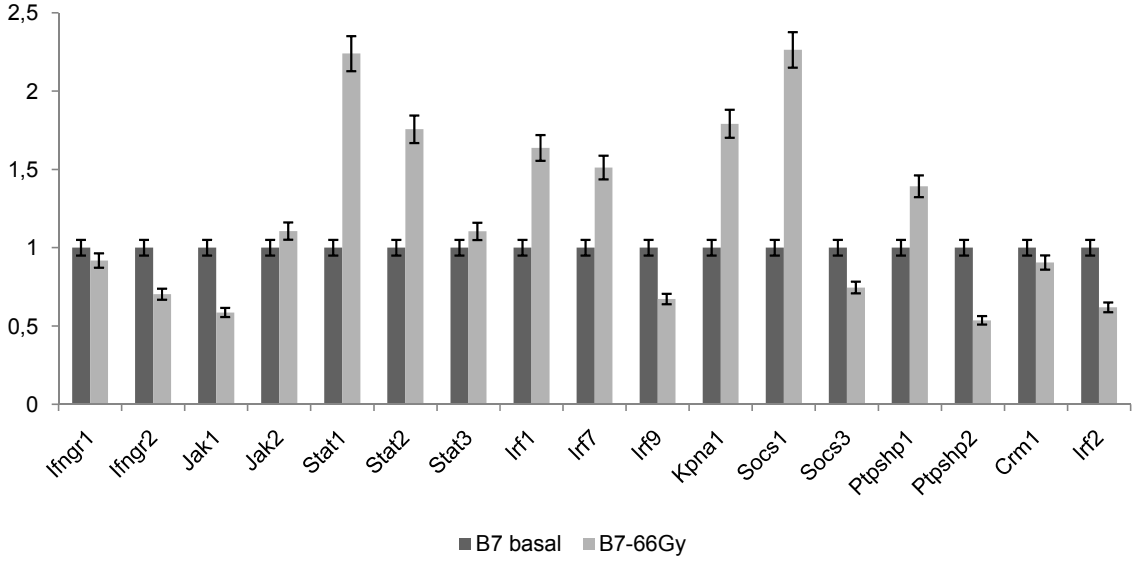
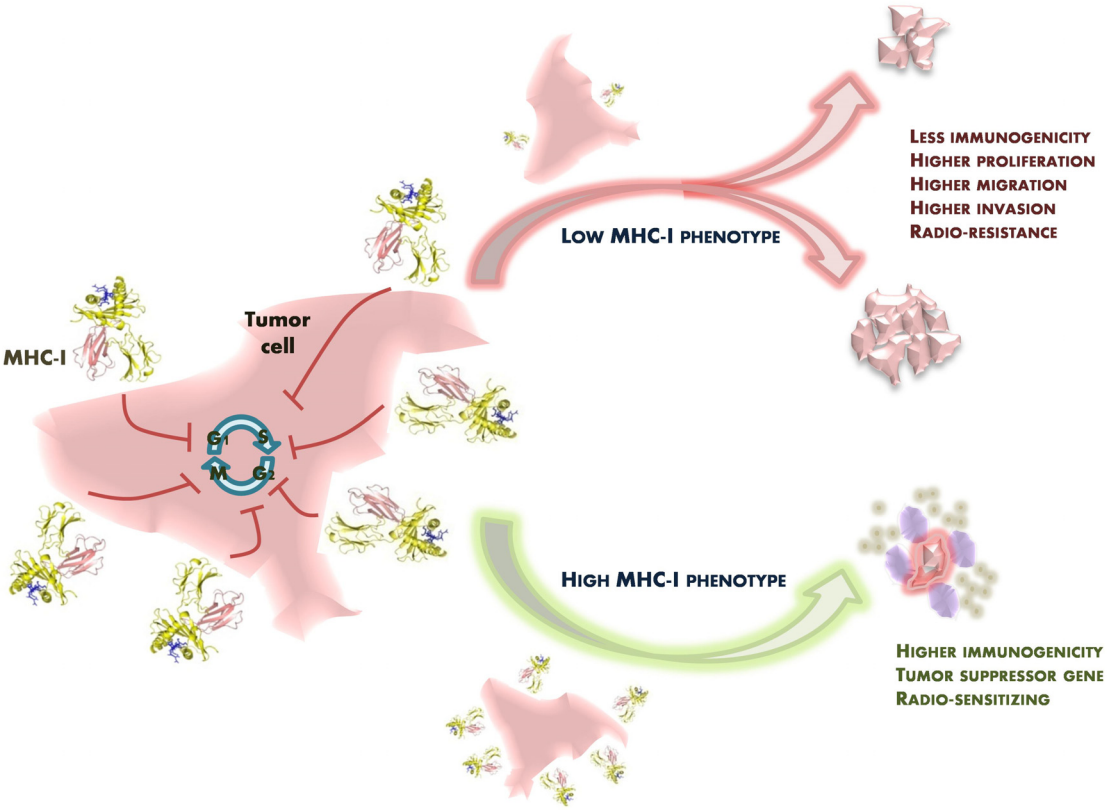


Figure 62. Transcription levels of the IFN-γ pathway genes were detected by qRT-PCR. Analyses were done to B7 cells in basal conditions and 48h after 66Gy-radiation treatment. Expression level of the genes was determined relative to levels of GAPDH and TATA housekeeping genes, and values for B7 cells in basal conditions were set to 1.


MHC-I
Tumor cell
G1
S
M
G2
LOW MHC-I PHENOTYPE
LESS IMMUNOGENICITY
HIGHER PROLIFERATION
HIGHER MIGRATION
HIGHER INVASION
RADIO-RESISTANCE
HIGH MHC-I PHENOTYPE
HIGHER IMMUNOGENICITY
TUMOR SUPPRESSOR GENE
RADIO-SENSITIZING

DISCUSSION

Cancer cells are able to develop immunoescape mechanisms that allow them to avoid the immunosurveillance and the efficacy of immunotherapeutic treatments. Furthermore cancer cells develop resistance mechanisms against different cytotoxic therapeutic agents as chemotherapeutic drugs, targeted-therapeutic drugs (i.e. imatinib, trastuzumab…) or radiation therapy. So thus, understanding the molecular mechanisms underlying these events represents a challenge for scientists and clinicians. Cancer researchers usually work with animal models, which allow modifying and controlling different conditions (tumor microenvironment, immune response, oncogene expression …) during the cancer progression. Immunodeficient or humanized mice have usually been used to study the biology of human cancers, including the expression of oncogenes, changes in the cell cycle, the expression of immunological factors; as well as for the evaluation of new therapeutic antitumor strategies (Colmont et al. 2012, Izzotti 2012, Khong et al. 2012, Rothenberg and Ellisen 2012). Mouse models are an attractive platform for validating candidate cancer genes, determining therapy efficacy, and defining mechanisms of drug resistance (Roper and Hung 2012, Van Miltenburg and Jonkers 2012) and great advances have been achieved in the generation of humanized mice and genetically engineered mouse models which accurately recapitulate the human cognate condition (Cook et al. 2012, Drake et al. 2012). However, our results suggest that these models should be used carefully because they could induce changes over transplanted human tumor cells. We found that three different melanoma cell lines (Ando-2, E-179, E-195) underwent changes in their HLA-I surface expression after growth in nude mice. These changes involved from locus-specific loss or down-regulation (E-179-N1, E-195-N2), to complete loss of HLA-I expression (Ando-N1, E-195-N1). B- and NK- immunological populations of the nude mice did not appear to be implicated, since the HLA expression changes were also observed in SCID-Beige mice. Only one of the four studied melanoma cell lines, E-033, showed no changes in HLA class I surface expression after growth in immunodeficient mice (Garrido C. et al. 2010). Contrary to the others cell lines that carried a HLA haplotype loss, E-033 cancer cells did not suffered any change in its HLA-I phenotype during the growth in the patient. In consequence, this melanoma cell line did not develop alterations in the surface expression of HLA-I molecules as immunoescape mechanism. Recently, Turrini *et al* have reported in epithelial tumor cell lines, as well as in cancers of B- and T-cell lineages, losses on MHC-I and MHC-II cell surface expression after passage in immunodeficient mice. They found from tumor cell lines with MHC complete loss or down-regulation, to cell lines without MHC alterations (Turrini et al. 2011). As we previously described they also showed that these phenotype alterations were underlying by epigenetic mechanisms and partially recoverable with epigenetic drugs. All these results indicate that immunodeficient mice may exert an influence over human tumor-grafts, modifying their immunogenicity and promoting direct consequences in the immunity of humanized models and in the study of immunotherapeutic strategies. Moreover, these MHC alterations also must be considered in the study of cytotoxic protocols, as chemotherapy or radiotherapy, given the importance of the immune system in this type of therapies. Recent reviews of humanized murine models highlight how they are extremely useful in basic and applied human disease research, but also remain the limitations present in these models and the challenges that has yet to be conquered, especially those referred to the immunity (Ito et al. 2012, Shultz et al. 2012).

Interestingly, the human melanoma cells with an altered MHC-I phenotype, obtained after growth in immunodeficient mice, were more oncogenic *in vivo* than their respective original melanoma cells, indicating a higher grade of malignancy (Garrido C. et al. 2010). Previous reports of our group described that two successive mechanisms may be implicated in complete loss of HLA class I expression in tumor cells: loss of one HLA haplotype and subsequent down-regulation of APM and HLA-I heavy chains (Maleno 2002, Romero et al. 2005, Méndez et al. 2008). We hypothesize that HLA-I altered tumor cells are selected during the autologous immunoselection stage. Then, during the immunoescape stage, these cells evade the immune reactivity due to the growth advantage that gives them the altered HLA-I phenotype. Later, we propose a last phase that we have called "Immunoblindness Phase", during which tumor cells are invisible to immune system but new alterations in the HLA-I phenotype could occur and seem to be associated with a higher grade of malignancy of these tumor cells. Our results suggest that HLA-I expression would play a direct role on the oncogenic characteristics of tumor cells. In fact, the transfection of Ando-2 cells with HLA-A2 allele (one of the lost HLA alleles during tumor progression in the patient) led to the new tumor cell line, Ando-TA2, which presented lower *in vivo* tumorigenicity, *in vitro* proliferation rate and migratory and invasive capacities than Ando-2 cells (Garrido C. et al. 2012). In contrast, Ando-Nude cells, with a complete HLA class I down-regulation, were more oncogenic and presented higher proliferation rate, migratory and invasive potentials, and higher *in vivo* tumorigenicity than Ando-2 cell line (Garrido C. et al. 2012). According to these findings, the order of direct oncogenic potential of these melanoma cells, which derive from the same cell line, is: Ando-Nude > Ando-2 > Ando-TA2.

These results demonstrate an inverse correlation between HLA class I expression and the intrinsic oncogenic characteristics in this tumor model. Actually, it has been previously reported that MHC-I surface expression defects could be responsible of apoptotic resistance or impaired insulin receptor-regulated signal transduction (Sabapathy et al. 2008, Assa-Kunik et al. 2003). It has been shown that MHC-I molecules are physically associated with some hormone or growth factor receptors, such as glucose transporters and insulin-like growth factor receptors (Centrella et al. 1989, Stagsted et al. 1993), epidermal growth factor receptor (Schreiber et al. 1984), IL-2 receptor (Sharon et al. 1988), suggesting that MHC-I-induced signaling may be transmitted through these receptors. Indeed the expression of HER2, member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, presented an inverse correlation with the expression of MHC-I molecules on breast cancer cells (Inoue et al. 2012, Okita et al. 2012). Both EGFR tyrosine kinase inhibitors and ligand-blocking antibodies (cetuximab) augmented the induction of MHC-I and MHCII molecules by IFN-γ in primary and malignant human keratinocytes (Pollack et al. 2011). Treatment with specific monoclonal antibodies against HLA molecules promoted that neoplastic lymphoid cells enter in programmed tumor cell death (Yang and Yi 2010). In the past decades, antibodies targeting surface MHC-I molecules on various malignant hematologic cell types have been generated and investigated showing tumoricidal activity *in vitro* and *in vivo* (animal models) (Woodle et al. 1997, Nagy et al. 2002, Matsuoka et al. 2003, Sekimoto et al. 2007, Spanoudakis et al. 2009, Stein et al. 2010). All these findings strongly suggest that MHC-I molecules or their components may play a role in the regulation of tumor survival and progression, further than in the regulation of the immune response, via MHC-I-mediated signaling.

Analyses of the transcriptional level of cell cycle genes showed a higher expression in Ando-TA2 cells (HLA-A2-transfected) and lower in Ando-Nude cells (HLA-I negative) of the three tumor suppressor genes AP-2α, p21WAP1/CIP1 and cyclin A1 (Garrido C. et al. 2012). Cyclin A1 expression is down-regulated in various cancers and associated with an invasive phenotype (Sriuranpong et al. 2004, Tokumaru et al. 2004, Kitkumthorn et al. 2006). AP-2α and p21WAP1/CIP1 genes are located in chromosome 6 (regions 6p24 and 6p21, respectively) very close to the HLA region in 6p21, where HLA heavy chains and the majority of APM genes are located. So thus, the loss of one HLA haplotype in tumor cells, e.g. in Ando-2 melanoma cells, always coincides with the loss of one allele of p21WAP1/CIP1 and one allele of AP-2α. Loss of AP-2α expression has been implicated in melanoma progression toward a malignant, invasive and metastatic phenotype (Berger et al. 2005, Melnikova et al. 2010). p21WAF1/CIP1 up-regulation stops proliferation and promotes senescence, whereas its down-regulation is frequently associated with tumor progression and higher invasiveness (Masuda et al. 2003, Saleem et al. 2008, Schaefer et al. 2010). The high induction of AP-2α and p21WAF1/CIP1 transcriptional expression in Ando-TA2-transfected cells might indicate that the expression of these genes is indirectly regulated by HLA class I molecules. Recent results of our group support this hypothesis, showing in GR9 fibrosarcoma tumour model a correlation between the MHC-I surface expression on tumor cell lines and the transcriptional expression of AP-2α gene. Moreover, silencing of AP-2α by siRNA did not modify the transcription of MHC-I or APM components, or the surface expression of MHC-I molecules. These results suggest that MHC-I expression may be implicated in AP-2α expression but not *vice versa* (Romero et al. 2012). Furthermore, using this same murine tumour model, our group demonstrated, for the first time, the direct implication of the Fhit tumour suppressor gene in the transcriptional regulation of APM components and MHC-I heavy chains and in the surface expression of MHC-I molecules (Romero et al. 2012). Since, Fhit expression was significantly lower in tumour cell lines with negative MHC-I expression than in those with positive MHC-I expression. Further, when Fhit expression was silenced by siRNA in a MHC-I positive tumor cell line, the transcription of APM components and MHC-I heavy chains and MHC-I surface expression was decreased and accompanied of a transcriptional down-regulation of p21WAF1/CIP1. All these results together suggest that a decrease in the Fhit expression may reduce MHC-I molecule expression, and consequently may produce a decrease in the expression of AP-2α and p21WAF1/CIP1 tumour suppressor genes. Analyses of transcriptional levels of Fhit, AP-2α and p21WAF1/CIP1 genes in Ando-2 model showed also a direct correlation with the HLA-I surface expression of the melanoma tumor cells (Romero et al. 2012).

These new data reveal the new role of MHC-I molecules in tumor survival and progression, suggesting their direct action as tumor suppressor genes. Moreover, they would imply that the MHC-I cell surface expression on a specific tumor might predict its clinical behavior, determining the immune recognition and the intrinsic oncogenic characteristics of these tumor cells. Actually, it has been shown a population of hormone-refractory prostate cancer cells that presents *in vitro* and *in vivo* resistance to docetaxel treatment. These tumor cells lacked HLA-I antigens while over expressed the Notch and Hedgehog signalling pathways, and their presence in tumor tissues was related with tumor aggressiveness and poor patient prognosis (Domingo-Domenech et al. 2012). Moreover, this HLA-negative cell population

presented a higher tumorigenicity *in vivo* in immunodeficient mice than the HLA-positive cell population.

So thus, MHC-I molecules on tumor cells, as tumor suppressor genes, could be beneficial for the effects of cytotoxic treatments as radiotherapy or chemotherapy. Radiotherapy represents a main resource in anti-cancer therapies, but several challenges are being encountered: to minimize toxicity to normal cells and tissues, to avoid radio-resistance phenomena or to drive cancer cells to an immunogenic death pathway (Baskar et al. 2012). Radio-resistance is one of the most desirable-to-overcome difficulties that radiotherapy treatments present. Radio-resistant tumor cells avoid radiation effects and escape to the treatment leading to the tumor outgrowth and to the metastatic spread. Since the role of CD8+ T lymphocytes has been determined essential for an effective radiation treatment (Lee et al. 2009), MHC-I surface expression of tumor cells passes to play an important role in the therapeutic efficacy of radiotherapy, because of its role in immune recognition. Furthermore, actually we must consider that MHC-I molecules may act directly as tumor suppressor genes, and in consequence play a role in radio-resistance of tumor cells. Current results show how 22Gy-radiation increased the surface expression of MHC class I molecules on the tumor cells of GR9 fibrosarcoma. However, into the heterogeneity of subclones forming the total tumor, we found that whereas MHC-I highly positive clones presented a higher MHC-I radio-induction, this effect was decreasing with the lost of basal MHC-I surface expression of the different GR9-clones. When the cells were radiated at crescent doses of 22, 44 and 66Gy, generally they presented a dose-dependent response, but there were some irradiated tumor cells or specific H-2 alleles that were more affected at the lowest or the intermediate radiation dose. Indeed, it has been previously shown that the efficacy as well as the immunological consequences of radiotherapy can be different depending on the radiation dose administrated and the schedule of the treatment (Mason et al. 2005, Dewan et al. 2009). Actually, the fractionation of radiation dosage is being re-evaluated to adjust the treatment protocols and to obtain better response in tumor cell cytotoxicity and in immuno-stimulation. Significant advantages have been reported in mouse adenocarcinomas treated with fraction size of locally delivered radiotherapy. After single doses, tumor control increased with the size of radiation dose, as did the number of tumor-reactive T cells. This was counterbalance at the highest dose by an increase in Treg representation. Fractionated treatment with medium-size radiation doses gave the best tumor control and tumor immunity while maintaining low Treg numbers (Kachikwu et al. 2011, Schaue et al. 2011).

Our results show that MHC radio-induction is caused by an elevated transcription of β_2-m, H-2 class I heavy chains and the APM components calreticulin, tapasin, LMP2, LMP7, TAP1 and TAP2 genes. This transcripcional up-regulation of the components of the proteasome and the peptide transporters is in concordance with the study of Reits and colleges describing an increase in the production and the degradation of proteins by radiation treatment of tumor cells (Reits et al. 2006). We also found an induction of several genes of the IFN-γ signaling pathway in irradiated tumor cells: STAT1, STAT2, IRF1, IRF7 and Kpna1. The induction STAT1, after radiation treatment, have been previously associated with the induction of the MHC-I expression on tumor cells, even in IFN-$\gamma^{-/-}$ mice (Lugade et al. 2008). More recent studies have demonstrated that *in vitro* radiation of breast cancer cells activated the secretion of IFN-β and increased MHC-I expression by an autocrine/paracrine signaling (Wan et al. 2012).

In the current experiments the radiation of all tumor cells produced an arrest of the *in vitro* cell proliferation. However, more positives MHC-I phenotype clones presented cytotoxic effects, whereas low or negative MHC-I clones presented more resistance. In fact, B11 and B9, which are the more MHC-I negatives clones, are the more resistant to the radiation treatment maintaining a slight proliferation of tumor cells when radiated at 22Gy, and at 44Gy in the case of B9 clone. All irradiated-cells presented a decrease in cyclin D1, cdk4 and metAP-2, and an increased transcription of p21. The over-expression of p21 may influence in the decrease of cyclin D1 and cdk4, since p21 has been associated with inhibitory effects in the assembly of cyclin D/cdk4 during cell cycle progression (Blundell et al. 2006). Consequently, the down-regulation of cyclin D1, cdk4, as well as of the protector of E2F1 inhibitory phosphorylation metAP-2, could promote a long cell cycle arrest in G1 phase of tumor cells (Kastan et al. 2004).The induction of p21 appeared to be independently of any change in p53. It has been also described that ATM and DNA-PK, critical intermediates of numerous cellular responses to radiation and direct regulators of p53, were dispensable for the radio-induction of cancer testing antigens or MHC-I expression in irradiated breast and melanoma cells (Sharma et al. 2011). These results suggest that MHC-I expression may be involve in the regulation of the expression of p21 gene. In fact, we have before showed that the transfection of Ando-2 melanoma cells with the HLA-A2 allele induced the transcription of p21 in a p53-independently way (Garrido et al. 2012). Furthermore effects of radiation in MHC-I surface expression and p21 gene expression could be useful in the development of anti-tumor vaccines. The expression of p21 is related with the entry in senescence of tumor cells (Romanov et al. 2012). Senescent tumor cells displayed an altered immuno-stimulatory senescence-associated secretory phenotype allowing the intercommunication with the surrounding cells, and perhaps promoting the mobilization of the immune system for their clearance (Rodier et al. 2009). The appearance of senescent tumor cells was correlated with a delay in melanoma tumor growth in mice after RT and veliparib treatment. These senescent cells displayed an altered immuno-stimulatory senescence-associated secretory phenotype characterized by expression of p21, IFN-β, CCL2, CCL5 and CXCL11; and an efficient immuno response mediated by mature dendritic cells, CD8+ and NK lymphocytes was triggered when tumor were radiated and when senescent cells were used as vaccine (Meng et al. 2012). The use of senescent tumor cells seems to be a promising resource in tumor vaccination (Brown et al. 2012). Also dendritic cells pulsed with irradiated tumor cells are being used as anti-tumor vaccine (Teitz-Tennenbaum et al. 2009). The synergistic effects of inducing TLR ligands and MHC-I expression in tumor cells by radiation would lead an effective maturation and activation of DCs, which could potentiate the effects of the DC-based vaccines (Roses et al. 2008).

Our findings establish that the surface expression of MHC-I molecules by tumor cells might have an important role in the effect of radiotherapy. MHC-I molecules may act as radio-sensitizing factors, influencing the ionizing radiation capacity to kill tumor cells by arresting their proliferation as well as the capacity of inducing MHC-I surface expression. Furthermore, the fact that radiation induces different genes involved in the surface expression of MHC-I molecules and IFN-γ signaling pathway genes could have further implications in the success of radiotherapy, which requires an immunological reaction, and could result beneficial in the combination of radiotherapy with immunotherapy. Immunotherapy treatment protocols try to potentiate anti-tumor immune response, which may require: the induction of specific CTLs,

correct surface expression of MHC-I molecules and antigen presentation, and activation and maturation DCs; whereas inhibition of immunosuppressive Treg, AAM, and MDSC cells. (Demaria et al. 2005, Hodge et al. 2009, Shiao et al. 2011). Combined with radiotherapy could help in the overcoming of some immunosuppressive effects found after radiation: as the lost of T memory response maintained by increasing of T regulatory cells population (McFarland et al. 2012); or the inhibition of NK lytic activity mediated by induction of HLA-E expression on macrovascular endothelial cells (Riederer et al. 2010). Indeed, promising results have been obtained combining RT with DC vaccines (Nikitina and Gabrilovich 2001), or with a mixture of plasmid that induce MHC class I and II molecules (Wang et al. 2005).

Concerning about immunotherapy, a variety of cancer immunotherapy protocols are widely used for the treatment of cancer patients, especially in patients with metastatic disease, since primary tumors are usually surgically removed (Rosenberg 2001, Schadendorf et al. 2009). Patients with metastatic disease have a very low survival rate, even though many of them can achieve substantial tumor reduction by surgical resection, radiation therapy or systemic therapy. An effective, nontoxic immunotherapy could benefit such patients (Lonser et al. 2011, Dillman et al. 2012). Immunotherapy treatments have promoted a favorable anticancer immunoresponse in the patients, in terms of polarization towards a Th1 dominated response potentially directed against tumor cells (Burgdorf 2010). However, a high percentage of patients receiving immunotherapy eventually develop metastatic progression. Wide efforts are being made to identify possible biomarkers of prognostic significance. In this context the expression of cancer-testis antigens or the circulating of T cells responding to tumor antigens were found to be important as diagnostic and prognostic markers in different clinical trials with cancer patients (Curioni-Fontecedro et al. 2011, Weide et al. 2012, Zou et al. 2012). The cancer-induced immunosuppression, which is mechanistically attributed to oncogenic signals frequently activated in cancer cells, is a major problem to restore the immune response in cancer patients and for the improvement of current immunotherapy (Yaguchi et al. 2011). Scientific evidences have been accumulating that tumors generate sophisticated escape variants to avoid the attack of different components of the immune system which could impair the effects of immunotherapy (Aptsiauri et al. 2008, Xu et al. 2010, Carretero et al. 2011b, Bernal et al. 2012a, Ghiringhelli et al. 2012, Iwami et al. 2012, Smith et al. 2012). The study of these immune escape mechanisms is crucial to optimize the application of these immunotherapy treatments in the clinic and to select the patients and to select the patients who can benefit from their application (Ferris et al. 2010).

Changes in the expression of MHC class I molecules (classical and non-classical) are frequently detected in primary tumors and metastatic lesions due to their role in antigen presentation to T lymphocytes and regulation of NK cell function (Rodriguez et al. 2005, Andrews et al. 2012, Bernal et al. 2012b , Coppage et al. 2012, Fernández-Messina et al. 2012, Madsen et al. 2012). Previous results from our group indicate that low expression and/or irreversible defects in MHC-I expression on tumor cells may play a major role in the appearance of progressing metastases after immunotherapy in melanoma patients, and similarly in bladder tumor relapses after BCG immunotherapy (Cabrera et al. 2007, Carretero et al. 2008, Carretero et al. 2011a). So thus, we favor the idea that the level of MHC-I expression in a particular cancer cell target is a crucial factor for immunotherapy success. Here, our findings show that MHC-I surface expression in the primary tumor and in subsequent

metastases could determine the success of immunotherapy treatments. Preclinical assays with antimetastatic therapeutic approaches showed that different immunotherapy treatments (CpG+TC-100Gy, PSK and PSK+docetaxel) successfully eradicated metastatic development from A7 tumor cells, a highly MHC-I positive subclone derived from GR9 fibrosarcoma (Garrido C. et al. 2011). These same treatments only reached a partial inhibition of metastatic spread for B7 tumor cells, a middle MHC-I positive subclone from the GR9 fibrosarcoma. These results indicate that MHC-I cell surface expression on primary tumor may be crucial for the success of immunotherapy. During spontaneous metastases progression these three tumor cell lines led to a new variability in the MHC-I phenotype. A7-metastases as well as GR9-metastases presented no-alterations or reversible alterations in their MHC-I surface expression. Nevertheless, B7 clone produced metastases with reversible MHC-I alterations, but also metastases with irreversible loss of the H-2L^{d} allele. These results show that the loss of MHC-I surface expression is a frequent event during metastatic progression. Indeed, clinical observations patients with gastric carcinoma have shown that the HLA class I expression was abrogated in tumor cells that had metastasized to lymph nodes, suggesting that loss of HLA class I was associated with lymph node metastasis (Yoshiipark et al. 2012). In cervical carcinomas patients, it was found a significant increase in the prevalence of HLA-A and HLA-B/C down-regulation in metastasized neoplastic cells compared with the primary tumour and in all cases this was accompanied by loss of TAP-1 expression (Cromme et al. 1994). Moreover it has been described that patients with MAGE-C1/CT7 or MAGE-C2/CT10 positive primary melanoma had significantly more lymph node metastases, which could suggest the use of these targets as predictor for lymph node metastases (Curioni-Fontecedro et al. 2011). Comparison of the MHC-I surface expression means, in the control groups, between the metastases derived from each tumor cell line, showed that GR9-metastases presented lower MHC-I expression than A7-metastases and B7-metastases. These differences in MHC-I expression between the metastases might be a factor that contributed to explain the inefficacy of immunotherapy as antimetastatic treatment against metastases derived from GR9 tumor cells.

Analysis of spleen lymphocytes subpopulations showed that tumor and metastatic progression produced immunosuppression in all cases, stronger in GR9-injected mice, follow by B7-injected mice and finally by A7-injected mice. This immunosuppression consisted in a decrease in CD3+ T population, CD8+ T cells, NK and NKT cells. GR9 and A7-injected mice also presented an increase of Treg cells. NK cells were very slightly affected in B7-injected mice. Immunotherapies completely reversed the immunosuppressive effect of A7 tumor cells, inducing in the hosts the CD3+ T population, CD8+ and CD4+ T cells even rising above the values founded in wild type non-injected mice whereas reducing the percentage of Treg cells. These mice were metastases-free at the end of the treatments (Garrido C. et al. 2011). In GR9 and B7-injected mice PSK and PSK+docetaxel therapies partially reversed the immunosuppression produced by the tumor progression, slightly increasing T cells. These mice presented a partial inhibition of metastatic development. CpG+TC-100Gy therapy did not produce induction of lymphocyte populations in GR9 or B7-injected mice, and the NK and NKT populations were down-regulated in these two groups. This therapy partially reduced metastases in GR9-injected mice, but B7-injected mice receiving this treatment presented higher metastatic development than control group. These results suggest that the efficacy of

immunotherapeutic antimetastatic treatments may inversely correlate with the immunosuppressive effect of the tumor progression and with the ability of the immunotherapeutic treatment in promoting immunostimulatory effects in the host.

In brief, comparing the two GR9 subclones, A7 and B7, we can observe a correlation between the MHC-I phenotype of primary tumors with the success of the immunotherapy, as well as with the capacity of the different immunotherapeutic treatments to produce an effective immunostimulation. Administered immunotherapies completely eradicated A7-metastases, and they got an effective immunostimulation of spleen lymphocyte populations. In contrast, B7-metastases could not be eradicated and they only were partially inhibited, and lymphocyte subpopulations in the hosts were also partially recovered. We suggest that the different MHC-I surface expression of these tumor clones, conditioned the results found. In the case of GR9 tumor cells, the strong immunosuppression originated in the hosts, joined to very low MHC-I surface expression on GR9-metastases could produce the inefficacy of the immunotherapies as antimetastatic treatment. Present results alongside the previous findings from human studies of our group (Cabrera et al. 2007, Carretero et al. 2008, Carretero et al. 2011a), suggest that the progression or regression of metastases after immunotherapy may depend not on the type of immunotherapy used but rather of the MHC-I expression on tumor cells and of the immune status of the host (Garrido et al. 2010b).

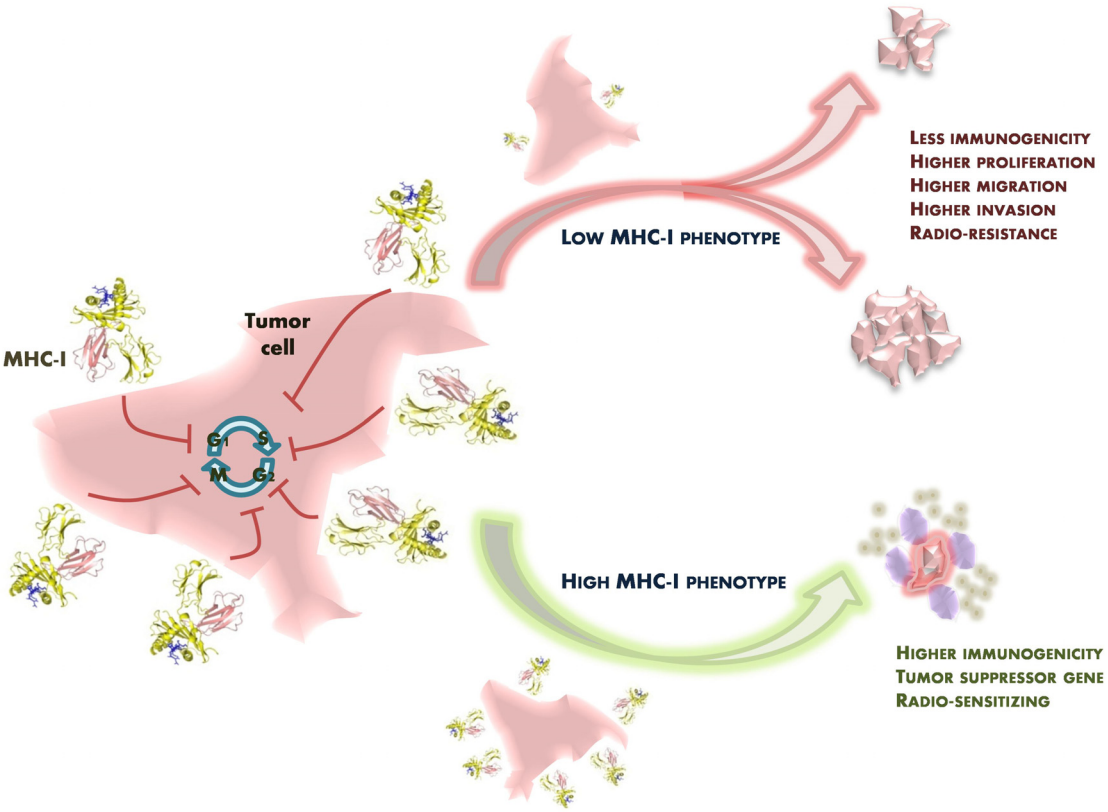
So that antitumor immunotherapy may have different effects over the different subclones that form a tumor depending on the genotype or phenotype alterations that each cancer cell would develop to overcome environmental and immunological pressures. But this is no exclusive for immunotherapy, chemotherapy tolerance, as well as tumor propagation potential and cancer growth, were different between the diverse subclones from ten human colorectal cancers (Kreso et al. 2012). Moreover, the MHC-I alterations that tumor cells accumulate during tumor progression could have a direct effect in the efficacy of chemotherapy treatments, not only as we mentioned previously by the role of MHC-I molecules as tumor supressor genes, but also by their immunological functions. It has been found that the effects of different chemotherapeutic protocols depend on the preservation of the immune system and on the treatment-mediated promotion of tumor-specific immunity. For example, observations of non-small cell lung cancer patients showed predictive significance of tumor-infiltrating lymphocytes for response to platinum-based chemotherapy (Liu et al. 2012). Other findings show how dose-dense regimen of combinatorial regimen of low-dose cisplatin and paclitaxel chemotherapies for ovarian cancer patients exerts their therapeutic effects by being low toxic to the immune system, reducing immunosuppression by the tumor microenvironment, and triggering recruitment of macrophages and tumor-specific CD8+ T cell responses to tumors (Chang et al. 2013). Other example showing the importance that the alterations of MHC-I expression could have over the efficacy of chemotherapy is that anti-tumor effects of cisplatin or gemcitabine for murine mesothelioma tumors were found to be mediated by amplifying the presentation of subdominant epitopes on MHC-I molecules and stimulating effective CTL specific response, which seem to be reinforced by the administration of IL-2 (Jackaman et al. 2012).

Anti-cancer strategies would require a strong control of their efficiency, i.e.: analyzing the immunological phenotype and oncologic behavior of the tumor and of the metastatic nodules during their evolution, and monitoring the immune response, as well as the evolution. It might be beneficial to design therapies more individualized and even more dynamics, considering a specific patient and tumor, and always based on the continuous evolution of the immune response and of the tumor cell genotype and phenotype during the treatment period.

A surprising result was the fact of that CpG+B7-100Gy was the unique one treatment that did not present any efficacy as antimetastatic immunotherapy against B7-metastases. Actually, the incidence of metastatic development was higher in mice receiving CpG+B7-100Gy than in B7-control group, and mice from that group showed a decrease in the population of T and CD4+ T lymphocytes, as well as of NK and NKT lymphocytes. To determine if this negative effect was due to CpG or irradiated-tumor autologous cells, new assays were performed using PSK or BCG (analog in immune effect produced to CpG) combined with autologous or non-autologous tumor cells. These assays showed that principally CpG or BCG were responsible of the worse results found. In this context, previously some research teams have reported that when tumor cells present a low expression of MHC-I molecules, CpG ODN 1826 did not get the expected results reducing tumor growth. Indeed, in mice injected with glioma, the intratumoral injection of CpG ODN 1826 had a very variable anti-tumor activity, that was explained by the increase of Treg cells in the tumor microenvironment after the treatment (Ginzkey et al. 2010). Other group showed using a murine model of cervical carcinoma that CpG ODN 1826 significantly reduced more efficiently the growth of MHC class I-proficient tumors, whereas that CpG ODN 1585, which preferably activates NK cells, induced regression of the MHC class I-deficient tumors (Reineis et al. 2006). Later experiments showed that peptide vaccine, based on a "short" peptide $E7_{49-57}$ harboring solely a CTL epitope, and co-administered with CpG ODN 1826 was effective against MHC class I-positive but not -deficient tumors; whereas a "longer" peptide $E7_{44-62}$ (harboring CTL and Th epitopes)-based vaccines were also effective against MHC class I-deficient tumors (Reinis et al. 2010). Furthermore, the combination of CpG ODN 1826 therapy with the DNA methyltransferase inhibitor 5-azacytidine, which can induce MHC-I surface expression, improved the results obtained against MHC class I-deficient tumors (Símová et al. 2011). The inefficacy of CpG ODN treatment against MHC class I-deficient tumors could be explained by the induction of the plasmocytoid DC population which exerts an immunoregultory role by increasing the influx of Treg cells as well as the expression of the immunosupressor factors in the tumor microenvironment (Sorrentino et al. 2010, Nierkens et al. 2011). All these results suggest that different strategies should be used for the treatment of tumor cells with low MHC-I expression.

An interesting preliminary finding was that after successful immunotherapy against A7-metastases, the immunodepletion of one host of CD4+ T cells promoted the apparition of metastatic nodules. Disseminated metastatic cells derived from A7 primary tumor were controlled in dormancy by immune system, and they wake up after immunodepletion of T-helper lymphocytes. These "waked up" metastases were all positive for MHC-I surface expression, and they had very low proliferation rate, presenting a strong transcriptional up-regulation of p21. Again, other time appears joined MHC-I molecules and p21. These results show that metastatic tumor cells could be maintained in dormant state after successful immunotherapy. We may think that the control of metastatic disease was due to the

convergence of: 1) high expression of MHC-I molecules, 2) a low proliferation rate of tumor cells due to the up-regulation of the cell cycle inhibitor p21 and 3) an immuno-stimulated anti-tumor immunosurveillance promoted by an effective immunotherapeutic schedule. According to these results, we might hypothesize that immunotherapy can be a real alternative as antimetastatic therapy, always that the treated metastatic nodules present high expression of MHC-I molecules and the immunotherapy get an effective stimulation of the immune response. However, after a successful antimetastatic treatment the change of immunological conditions in the host could favour the reappearance of metastases in the patient that could be maintained under control by the immune system. The gravity of metastatic recurrence highlights the importance of the development of treatments for metastatic prevention (Lollini et al. 2006). The role of the immune system in tumor dormancy and the mechanisms implicated to wake up tumor cells from dormancy are poorly understood, due principally to the extreme difficulty of isolating dormant tumor cells from patients (Quesnel 2008). An improved understanding of tumor dormancy is needed for better management of patients at risk for late-developing metastases (Hedley and Chambers 2009). Although some preclinical experimental models of tumor dormancy exist, we consider that they do not involve spontaneous metastases and usually are transgenic. This non-transgenic dormant metastatic model may be very useful to investigate mechanisms and cell implicated in dormancy. These last findings open new exciting research areas for the future.



CONCLUSIONS

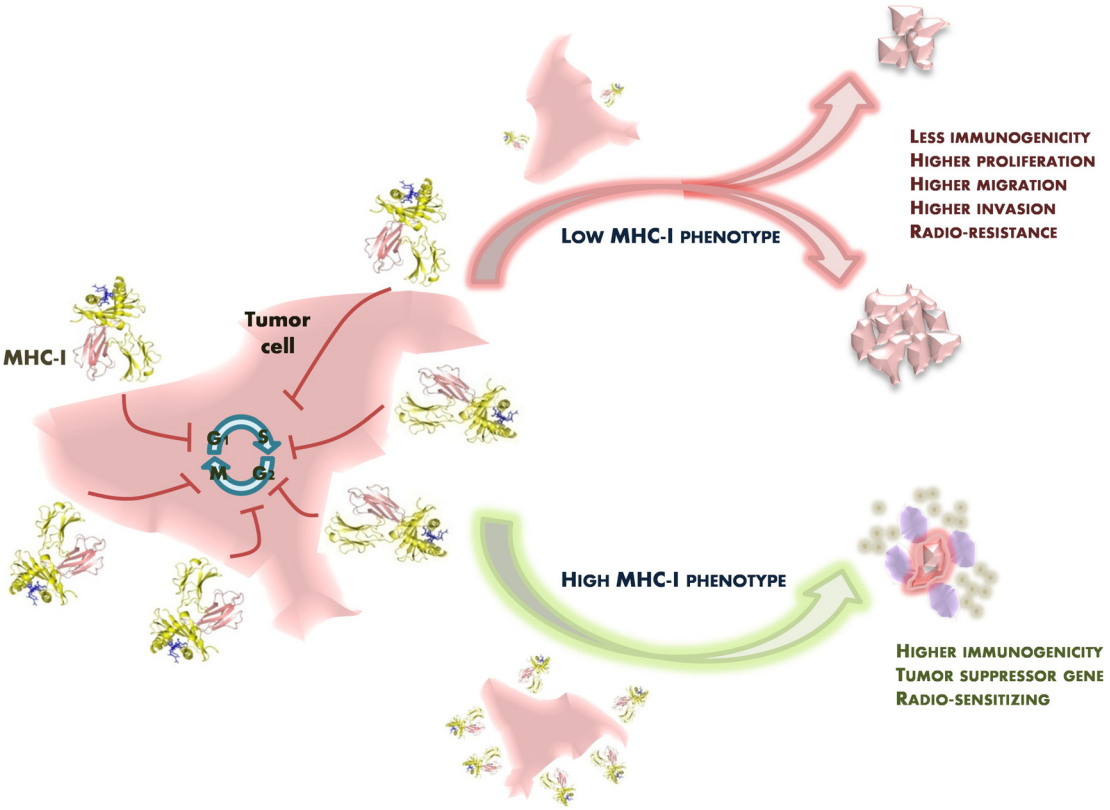
- Human tumor cell lines can suffer alterations in their HLA class I surface expression after tumor growth in immunodeficient mice. This is a frequent and reproducible phenomenon. The consequence is that tumour cells with altered HLA class I expression have greater malignant potential.

- MHC class I molecules exert a direct action as tumor suppressor genes. MHC class I defects play a direct role in cancer progression, increasing the growth, intrinsic oncogenicity, and migratory and invasive capacities of cancer cells. There is a direct correlation between MHC class I molecules and the expression of cyclin A1, AP-2α, and p21WAF1/CIP1 tumor suppressor genes.

- The success of immunotherapy as antimetastatic treatment depends on the MHC class I surface expression of the primary and metastatic tumor cells. Higher expression of MHC class I molecules may produce a greater stimulation of the immune response and may constitute a good prognostic marker for immunotherapy treatments.

- The immune response generated by immunotherapy treatments may maintain metastasis in a dormant state under immune control.

- The MHC class I phenotype of tumor cells may determine their radio-sensitivity: highly positive MHC-I tumor cells show stronger MHC class I radio-inducing and radio-cytotoxic effects. These effects decrease with the loss of surface expression of MHC-I molecules. Radiation of tumor cells induces MHC class I surface expression by increasing the transcriptional level of MHC class I heavy chains, β2-microglobulin, and antigen processing machinery components.



CONCLUSIONES

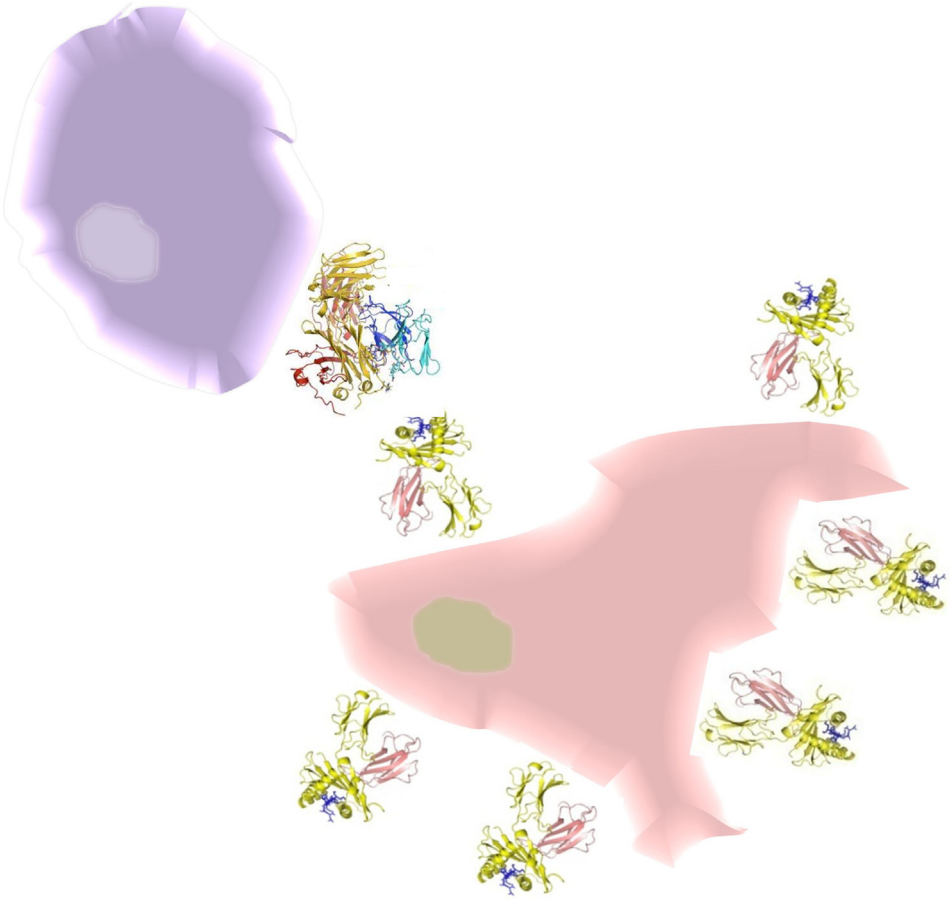
- Los tumores humanos pueden sufrir pérdidas en la expresión en superficie de moléculas HLA de clase I tras su crecimiento en ratones inmunodeficientes. Este fenómeno ocurre de una manera frecuente y reproducible. Como consecuencia, las células tumorales con pérdidas de expresión de moléculas HLA de clase I presentan una mayor capacidad oncogénica.

- Las moléculas MHC de clase I presentan una directa acción como genes supresores de tumores. Los defectos en la expresión MHC de clase I tienen una consecuencia directa en la progresión tumoral, produciendo un aumento del crecimiento tumoral y de la oncogenicidad, así como también de la capacidad de migrar e invadir de las células cancerígenas. Existe una relación directa entre la expresión MHC-I de las células tumorales y la expresión de los genes supresores de tumores ciclina A1, AP-2α y p21WAF1/CIP1.

- El éxito de inmunoterapia como tratamiento anti-metastásico depende de la expresión en superficie de moléculas MHC de clase I del tumor primario y de las células metastásicas. Una expresión más alta de moléculas MHC de clase I puede provocar una fuerte estimulación de la respuesta inmune y puede ser un factor de buen pronóstico para la respuesta a tratamientos de inmunoterapia.

- La respuesta inmune promovida por un tratamiento de inmunoterapia puede ser capaz de mantener metástasis en un estado de latencia.

- El fenotipo MHC de clase I de las células tumorales condiciona su respuesta a radiación ionizante: células tumorales con una alta expresión de moléculas MHC de clase I presentan una mayor inducción de la expresión de estas moléculas y una mayor sensibilidad a los efectos citotóxicos de la radiación. El aumento de expresión en superficie de moléculas MHC de clase I es debido a una inducción transcripcional y coordinada de la expresión de las cadenas pesadas, de la β2-microglobulina y de varios componentes de la APM.



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