

Review Article

Vaccine Immunotherapy for a Deadly Disease Like Malignant Melanoma: Where Do We Stand?*

Farid Saleh¹, Ghada Ibrahim², Hussein Dashti¹, Pedro Romero³, Abdullah Behbehani², Hilal Al-Sayer², Sami Asfar², Ali Dashti⁴, Waleed Renno¹, Suad Abdeen⁵, Ivo Klepacek¹

¹Faculty of Medicine, Health Sciences Centre, Department of Anatomy, Kuwait University, Jabriya, Kuwait

²Department of Surgery, Muabrak Al-Kabeer Hospital, Jabriya, Kuwait

³Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Hospital Orthopedique, Lausanne, Switzerland

⁴Faculty of Health Sciences, Kuwait University, Shuwaikh, Kuwait

⁵Department of Pathology, Kuwait University, Jabriya, Kuwait

Kuwait Medical Journal 2005, 37 (1): 4-17

ABSTRACT

The incidence of cancer and its related morbidity and mortality remain on the increase in both developing and developed countries. Cancer remains a huge burden on the health and social welfare sectors worldwide and its prevention and cure remain two golden goals that science strives to achieve.

Among the treatment options for cancer that have emerged in the past one hundred years, cancer vaccine immunotherapy seems to present a promising and relatively safer approach as compared to chemotherapy and radiotherapy. The identification of different tumor antigens in the last fifteen years using a variety of techniques, together with the molecular cloning of cytotoxic T lymphocytes (CTLs)-and tumor infiltrating lymphocytes (TILs)-defined tumor antigens allowed more refining of the cancer vaccines that are currently

used in different clinical trials. In a proportion of treated patients, some of these vaccines have resulted in partial or complete tumor regression, while they have increased the disease-free survival rate in others. These outcomes are more evident now in patients suffering from melanoma.

This review provides an update on melanoma vaccine immunotherapy. Different cancer antigens are reviewed with a detailed description of the melanoma antigens discovered so far. The review also summarizes clinical trials and individual clinical cases in which some of the old and current methods to vaccinate against or treat melanoma were used. These include vaccines made of autologous or allogenic melanoma tumor cells, melanoma peptides, recombinant bacterial or viral vectors, or dendritic cells.

INTRODUCTION

Cancer remains a major cause of mortality in developed and developing countries. It has huge physical, psychological, and social impacts on both the sufferer and the caregiver. This is in addition to its burden on the health care system. The current possible treatments for cancer include surgery, chemotherapy, radiotherapy, biotherapy, and immunotherapy including cancer vaccine. The last treatment option seems to be a promising one especially in melanoma.

Malignant melanoma is a skin cancer type that often metastasises. It has several predisposing factors including repetitive sun exposure, light skin complexion (freckles), light hair colour with blue, grey, or green eyes, a history of repetitive sunburn before the age of twenty, or a family history of melanoma or other skin cancer. A melanocytic lesion is often asymmetrical, has irregular border,

and varies in colour and diameter (ABCD's of malignant melanoma - Fig. 1). It may take many different forms. The most common is the "superficial spreading" melanoma which usually begins as a tan spot that may slowly change and grow. Another type of melanoma is called "nodular" melanoma and usually develops in a black, blue, or white mark that rapidly grows into a bump.

There were 65,177 new cases of melanoma worldwide in the year 2000. Among these cases, 19,990 resulted in deaths^[1]. The new cases were distributed as follows: 5702 in Australia and New Zealand, 26,106 in Europe, 5734 in Asia, 3790 in Africa, and 29,302 in America^[1]. Given the numbers listed above, it seems more evident now that melanoma is a common problem that becomes life threatening when it metastasises to vital organs and structures in the human body including the

* Simultaneously under publication with *Current Pharmaceutical Design Journal*

Address correspondence to:

Farid Saleh, Faculty of Medicine, Health Sciences Centre, Department of Anatomy, Kuwait University, Jabriya, P.O.Box: 24923, Safat, Kuwait, Postal Code 13110, Kuwait. Tel: 4986265, Fax: +965 5319478, E-mail: Fred@hsc.edu.kw or FaridSlh@netscape.net



Fig. 1: An asymmetrical melanocytic lesion showing irregular border and diameter.

brain, lungs, liver, bone, and lymph nodes. Such metastases are sometimes inoperable or are resistant to chemotherapy or radiotherapy or both. This is in addition to the fact that chemotherapy or radiotherapy does not often result in 100% eradication of the tumor and that the severe side effects often associated with such treatments could be devastating on both the patient and his/her family. Therefore, and all things considered, the ideal treatment option for melanoma, and for cancer in general, should be the one with which there would be a complete eradication of the tumor or a significant prolonged survival with minimum side effects. One way of achieving this goal could be through stimulating the patient's immune system to recognize the tumor as being a foreign body and thus to attack and eliminate it. This forms the basis for melanoma and cancer vaccine immunotherapy.

The idea of a possible immune reaction against cancer in humans is not new. In 1906, Paul Ehrlich suggested that our body is exposed to what he called "aberrant germs" on a daily basis. These germs, he added, do not develop into cancer because they are kept in check by our immune system^[2]. Ehrlich's idea was later developed into the phenomenon of immune surveillance against cancer^[3,4]. This phenomenon has received strong support based on a number of observations:

1. Tumor transplantation studies in animals have demonstrated that certain tumors could be rejected when the animal is repeatedly immunized against the tumor^[5].

2. The observation that some types of cancer develop in immuno-compromised patients. These include Kaposi's sarcoma, lymphoma, and squamous cell carcinoma^[6-8].

3. The medical literature has reported clinical

cases in which cancer spontaneously regressed partially or completely. Such regression was found to be associated with immune infiltration of the tumor^[9-11].

4. More recently, definitive support for the role of immune surveillance against cancer has come from studies on the incidence of chemically-induced tumors in mice genetically deficient in key components of the immune system such as INF-pathway^[12] or perforin^[13].

5. The continuous discovery of cytotoxic T lymphocyte (CTL)-and tumor-infiltrating lymphocyte (TIL)-defined tumor antigens in humans, which led to the development of cancer vaccines that are currently used in clinical trials.

AN OVERVIEW OF HUMAN CANCER ANTIGENS

Cancer antigens are divided into five different groups. Cancer testis (CT) antigens, which are found in melanomas and several other tumors but not in normal tissues except germinal cells in the gonads. They include antigens encoded by MAGE-1, MAGE-3, BAGE, GAGE, and PRAME genes, in addition to the more recently discovered NY-ESO-1 antigen^[14-26]. The second group of cancer antigens include the melanocyte differentiation antigens, which are expressed in melanomas and normal melanocytes. Examples of such antigens are Melan-A/MART-1, tyrosinase, gp100/Pmel17, TRP-1/gp75, and TRP-2^[27-40]. Mutation-based antigens represent the third group of cancer antigens and include p53, Ras, P15, gp100-in-4, MUM-1, Beta-Catenin, GnT-V, and CDK4^[41-47]. The last two groups of cancer antigens are the over-expressed normal "self" antigens such as HER-2/neu and the viral antigens including the Epstein-Barr virus (EBV) antigen, hepatitis B & C virus (HBV & HCV) antigens, human papilloma virus (HPV) antigen, human herpes virus 8 (HHV8) antigen, and human T lymphotropic virus (HTLV) antigen. The above viruses have been found to be associated with Burkitt's lymphoma (BL), hepatocellular carcinoma, cervical and anal carcinoma, Kaposi's sarcoma, and T-cell leukaemia respectively.

MELANOMA TUMOR ANTIGENS

MAGE

This family of genes was the first to be cloned using a CTL clone derived from the peripheral blood of a melanoma patient (MZ2) who received autologous tumor cells as a vaccine over a number of years^[48]. This clone was found to be HLA-A1 restricted and recognized an epitope derived from the MAGE-A1 encoded nonapeptide 161-169 with the amino acid sequence EADPTGHSY. An independent HLA-A1-restricted CTL clone from

the same patient recognized a highly homologous nonapeptide, EVDPIGHLY, derived from the MAGE-A3 gene. Yet, another CTL clone from the same patient recognized a different MAGE-A1 derived nonapeptide, SAYGEPKRL, in the context of the HLA-CW 16 molecule. Around forty percent of metastatic melanoma tumors express MAGE-1. It is also expressed in sarcomas, lung carcinoma, breast carcinoma, and laryngeal cancer. KWVELVHFL and YLQLVFGIEV are two epitopes derived from the MAGE gene family member MAGE-A2. CTLs recognizing both epitopes KWVELVHFL and YLQLVFGIEV were found to kill MAGE-2 expressing cells in a HLA-A2-restricted manner in transgenic mice^[26]. MAGE-B5, MAGE-B6, MAGE-C2, and MAGE-C3 are additional members of the MAGE gene family and are currently undergoing further investigation. The only normal tissue where the MAGE gene family is found to be expressed is the testis.

BAGE

An additional CTL clone derived from the MZ2 patients was used to identify another gene named as BAGE. BAGE encodes a HLA-Cw16-restricted antigen and its pattern of expression is similar to that of the MAGE gene. It is also expressed in the testis^[14].

GAGE

Melanoma cells cultured from the MZ2 patient also expressed a third gene that was named GAGE-1^[23]. Peptides-derived from GAGE-1 are HLA-CW-6-restricted and are expressed in melanomas, head and neck carcinoma, non-small cell lung carcinoma, bladder carcinoma as well as in normal testicular tissue.

PRAME

Melanoma cell lines derived from a melanoma patient (LB33) were used to identify a new melanoma antigen named PRAME^[19]. Melanoma tumors expressing PRAME were found to be lysed by CTLs in a HLA-A24-restricted manner. In addition to being expressed in melanoma, PRAME is also expressed in normal endometrial and testicular tissues.

NY-ESO-1

This antigen is a newly discovered CT antigen, and was found to be expressed in 23 of 67 melanoma specimen, 10 of 33 breast carcinomas, 4 of 16 prostate carcinomas, 4 of 5 bladder cancer specimen, and 2 of 11 cultured melanoma cell lines^[49]. Three HLA-A2 and two HLA-A31-restricted peptides have been identified so far from this antigen.

-catenin

-catenin is a cytoplasmic protein that interacts with the cellular adhesion molecule E-cadherin. A

Table 1

Melan-A/MART-1, gp100, and tyrosinase melanoma differentiation antigens: Summary of epitopes identified and HLA class restriction

Antigen	Epitope Identified	HLA Restriction
Melan-A/MART-1	32-40	A-2
	27-35	A-2
	26-35	A-2
gp100	280-288	A-2
	476-485	A-2
	17-25	A-3
	209-217	A-2
	154-162	A-2
	457-466	A-2
Tyrosinase	243-251	A-1
	369-377	A-2
	1-9	A-2
	206-214	A-24
	192-200	B-44

mutated peptide epitope has been isolated from the -catenin gene and was found to be recognized by melanoma-specific CTLs^[45]. Since -catenin plays a role in cell adhesion, it is suggested that it could be involved in cancer invasion and metastasis. In fact, loss of cell adhesion molecules such as E-cadherin has been observed in invasive carcinomas, which further support the belief in the role of such molecules in cancer progression^[50].

Melan-A/MART-1

The first cloning of the Melan-A or Melanoma Antigen A gene was performed by Coulie and his colleagues using autologous CTLs from melanoma patients^[51]. The same gene was cloned by Kawakami *et al* using TILs established in continuous in vitro culture^[31]. The authors designated it MART-1 or Melanoma Antigen Recognized by T cells 1. Melan-A/MART-1 has been found to be expressed in pigmented cells in normal skin and the retina but not in other normal tissues. Analysis of different melanoma and other cancer specimens revealed that the antigen is expressed with a frequency ranging from 87 to 100% in melanoma tumors and 0% in other types of cancer^[31,51-53]. Melan-A/MART-1 encodes a small transmembrane protein consisting of 119 amino acid residues and recognized by CTLs in a HLA-A2-restricted manner (Table 1).

gp100

The gene encoding gp100 was first identified as a melanocyte lineage-specific antigen. Analyses of different cancer cell lines including lung, breast, colon, and neuroectodermal carcinomas did not reveal any expression of gp100, which suggest that the latter is a melanoma-specific tumor antigen. This suggestion was subsequently confirmed through different studies including the ones where

gp100 was found to be secreted in supernatants of cultured melanoma cell lines, and where different histological types of melanoma tumors were found to stain positive immunohistochemically for gp100 using specific antibodies such as HMB45, NKI-beteb, and HMB50 as diagnostic markers^[54,55].

The 661- amino acid gp100 glycoprotein contains a signal peptide and a single transmembrane domain. It is located mainly in the membrane and filamentous matrix of premelanosomes and, therefore, could be associated with melanin synthesis. Six different epitopes of this antigen have been identified, five of which are recognized by CTLs in a HLA-A2 restricted manner (Table 1). The epitope 280-288 (YLEPGPVTA) was also found to be recognized by TILs^[56].

Tyrosinase

Tyrosinase was initially cloned and found to be a membrane-associated protein^[57]. It is an enzyme involved in the first step of melanin synthesis. CTLs recognizing five different epitopes in addition to a sixth unsequenced one have been isolated following in vitro stimulation with melanoma cells. These epitopes are capable of generating CTLs and sometimes TILs in different HLA subtypes-restricted manner (Table 1). Furthermore, HLA-A24-restricted CTLs were derived from a TIL 888 line that was associated with tumor regression when administered along with IL-2^[58]. This study suggests that tyrosinase could be identified as a tumor-regression antigen in HLA-A24 cancer patients.

TRP-1/gp75 and TRP-2

CTLs specific for TRP-1 or tyrosinase-related protein-1 were first identified in a melanoma TIL line 586 obtained from a melanoma patient following tumor regression^[59]. Analysis of the cDNA library of the TRP-1 gene revealed that it is similar to another gene that was identified earlier, namely the glycoprotein 75 or gp75. Northern blot analysis indicated that the TRP-1 gene is expressed in melanoma, normal melanocyte cell lines, and retina but not in other normal tissues tested. This was consistent with results obtained with monoclonal antibodies, which revealed that gp75 has similar expression and HLA restriction, namely HLA-A31. The identification of TRP-1 was subsequently followed by the discovery of another melanoma antigen, TRP-2, from the same cultured TIL line used to screen for TRP-1. TRP-2 was found to give rise to HLA-A31 and HLA-A33-restricted antigenic peptides.

P15

Northern blot analysis revealed that this melanoma antigen is also expressed in a range of normal tissues. The non-mutated T cell epitope

isolated from this antigen was sequenced as AYGLDFYIL^[46]. Cultured CTLs against this epitope were capable of lysing melanoma cell lines in a HLA-A24-restricted manner.

MUM-1 (melanoma ubiquitous mutated-1)

Similar to P15, MUM-1 is expressed in both melanoma and normal tissues. It was first identified in the melanoma cell line LB33-MEL^[41]. Over a third of the CTL clones established against such a cell line were directed against MUM-1, which suggests a high immunogenicity of this mutated epitope.

CDK4

CDK4 is a key protein involved in the regulation of cell cycle progression as part of the CD4-p16-RB pathway. Such a pathway is usually inactivated in human melanomas where CDK4 is found as a mutated antigen. This antigen was found to be recognized by three HLA-A2-restricted CTL clones isolated from the peripheral blood of the melanoma patient SK29^[47].

GnT-V

In addition to the MUM-1 epitope, a novel product of the N-acetylglucosaminyltransferase V (GnT-V) gene was shown to encode an epitope recognized by HLA-A2-restricted melanoma-reactive T cells^[42]. The antigenic epitope was identified as the decapeptide VLPDVFIRC or the nonapeptide VLPDVFIRC.

Gp100-in 4

A HLA-A24-restricted TIL1290 was found to recognize a variant of the gp100 gene that had retained the entire fourth intron of this gene, termed gp100-in4^[44]. The gp100-in 4 transcript is a relatively rare one since it could be detected by RT-PCR using melanoma RNA but not by Northern blots. Analysis of this transcript in the region corresponding to the fourth intron gave rise to an additional 35 amino acids not found in the normal gp100 protein. The peptide VYFFLPDHL found in this region could be recognized by a T cell subline derived from TIL1290.

MELANOMA VACCINES

The concept of vaccination against cancer was first initiated by the New York surgeon, Coley, during the early years of the century. He used bacterial vaccines in patients with advanced sarcomas following which some tumor regressions were observed.

Different types of vaccines have been used in melanoma. They include autologous or allogenic tumor cells, wild type or amino acid-substituted-synthetic peptides with or without adjuvants [incomplete Freund's adjuvant, QS21, cytokines

Table 2

Controlled-randomized trials to treat melanoma using vaccine immunotherapy

Vaccine	Disease Stage	Outcome
Allogeneic melanoma vaccine (processed melanoma peptides presented in a HLA-A2 & HLA-C3-restricted manner) ^[120]		83% of treated patients had 5-year relapse-free survival (RFS) as compared to 59% in controls (p= 0.0002).
HLA-A2 gp100 peptide (280) YLEPGPVTA ± modified T helper epitope from tetanus toxoid AQYIKANSKFIGITEL+ adjuvant Montanide ISA-51 or QS-2 ^[121]	IIB-IV	Overall survival (OS) was 75% at 4.7 years follow up.
Polyvalent-shed-antigen-vaccine ^[122]	IV	Median time to disease progression was 2.5 x longer (p= 0.03). OS was 40% longer.
Interferon -2b ^[123]	IIB-III	Better RFS and OS with a hazard ratio (HR)= 1.47, p= 0.0015; = 1.52, p= 0.09 respectively.
Vaccinia melanoma oncolysate (VMO) ^[124]	III	Better disease-free interval (DFI) and OS at 2, 3, and 5 year interval (P= 0.046).
BCG Pasteur ^[125]	I	Longer DFI (P= 0.05). Stronger antibody response. Stronger regional and systemic reactions to vaccination. Patients converted more frequently to positive PPD skin tests.
Vaccinia viral lysates of allogeneic melanoma cell (VMCL) ^[126]	III	Improved survival. Lower incidence of cutaneous metastases.
New polyvalent melanoma cell vaccine (MCV) ^[127]	IIIA& IV	Cell-mediated and humoral immune responses to common melanoma associated antigens. Increased activation of TILs. Increased DTH (P= 0.0066). Tumor regression in 23% of the cases (3 complete). Increased survival in patients with stage IIIA and IV by two and three folds respectively.
Levamisole adjuvant ^[128]	III	29% reduction in both death rate (p= 0.08) and recurrence rate (p= 0.09).
Imidazole carboxamide (DTIC) + BCG ^[129]		12.7% complete tumor regression. 9.3% partial tumor regression.
Oral BCG ^[130]		Increased survival
Intralymphatic methano-extracted residue of BCG ^[131]		Increased disease-free survival (DFS) (p= 0.015).
C.parvum adjuvant ^[132]	Stage I (melanoma > 3 mm in thickness)	Increased DFS.
Intralesional BCG ^[133]		Median survival of 21.1 months.
Intralesional BCG ^[134]	I	Destruction of ~90% of injected intradermal nodules.
DTIC + BCG ^[135]		0% recurrence or death.

such as GM-CSF, interleukin 2 (IL-2) or interleukin 12 (IL-12)], recombinant bacterial or viral vectors, naked DNA or purified recombinant proteins. They also include dendritic cells (DCs) pulsed with peptides, whole protein, or tumor cells, or transfected with a vector for better antigen presentation. Fifty-three controlled-randomized clinical trials aimed at treating melanoma using vaccine immunotherapy have been reported between 1973 and 2002 (Table 2). Some of these trials reported significant positive outcomes in the

treated melanoma patients. These outcomes included complete and partial regression of the melanoma tumors in up to 13% and 23% of the patients respectively. Melanoma regression was sometimes associated with loss of skin pigmentation or vitiligo, which provides further evidence that the immune reaction that resulted in the regression of the melanoma tumor is melanoma-specific since melanoma differentiation antigens are also shared by normal melanocytes. The above trials also included significant increase

in the disease/relapse-free survival and overall survival rates (p values ranging between 0.05 and 0.0002). Included in these trials were vaccines consisting of autologous dendritic cells infected with recombinant adenovirus encoding whole protein tyrosinase, and allogenic melanoma cells (Melacine) consisting of processed melanoma peptides such as gp100_{YLEPGPVTA} presented in a HLA-A2-restricted manner. Included also were polyvalent melanoma cells, adjuvants such as levamisole, vaccinia melanoma oncolysate (VMO), vaccinia viral lysates of allogeneic melanoma cells (VMCL), or BCG with or without GM2.

The idea of using recombinant viruses in melanoma and cancer vaccines is based on the tactic of improving antigen delivery to the immune system thus providing a strong immunostimulator. We know by now that melanoma, and cancer cells in general, may be poorly immunogenic and could induce apoptosis, which in turn inhibits cancer antigen-specific immune responses. This is in contrast to the results obtained in the presence of viral infection in which widespread cellular lysis and blocking of apoptosis are present. Both widespread cellular lysis and blocking of apoptosis are believed to be strong immunostimulators. Since live viruses could have severe side effects especially in immunocompromised patients such as those with AIDS, attenuated viruses have been used in melanoma vaccines as vectors for antigen delivery. The first successful experience using recombinant vaccinia (rVV) in melanoma took place in the mid-1980s when rVV expressing the melanoma antigen p97 was employed in treating murine melanoma^[60]. This was followed by successful construction of rVVs expressing the melanoma antigen gp100 and TRP-1 whose administration resulted in vitiligo in the murine melanoma model^[61, 62]. The vitiligo seen in the murine model was similar to the one we described earlier in humans whose melanomas underwent regression after successful vaccination. Moreover, the mice that received rVV-expressing TRP-1 became resistant to subsequent challenge with the syngeneic melanoma tumor line B16. The early 1990s were characterized by the development of two VV strains that are unable to replicate in human cells, namely MVA and NYVAC^[63, 64]. More importantly, MVA was found to lack immune evasion molecules, a characteristic that is very important in cancer immunology, and accordingly it was integrated in cancer vaccine clinical trials^[65]. It was demonstrated that human DCs infected with rVV expressing melanoma antigens were capable of inducing melanoma-specific CTLs, which in turn recognized the same HLA-restricted peptide antigens, as did CTLs derived from melanoma patients.

The role of DCs in melanoma and cancer vaccine immunotherapy has occupied a major part of clinical trials and individual experimental studies aiming at providing an effective method of treating such debilitating diseases. In fact, DCs are now regarded as "the future hope" in cancer treatment because of their unique ability to present cancer antigens to T cells, and it is strongly believed that a defect in cancer antigen presentation is among the main reasons, behind failure of the immune system to destroy the cancer cells. These statements are based on the results obtained from studies in which DCs pulsed with tumor RNA, autologous tumor antigens (10-100µg/ml), or tumor lysate, or fused with heat shock protein, liposomal antigen, or tumor cells demonstrated effective stimulation of CD4+ T cells, CD8+ T cells, and even Natural Killer (NK) cells and subsequent potent antitumor responses^[66-69]. The injection of dendritic cells derived from bone marrow and pulsed with associated-haplotype-specific peptides resulted in 80% eradication of established tumors as large as one cm² in size in naïve mice. It also resulted in the rejection of subsequent inoculation of tumors in up to 80% of the mice^[70]. More interestingly, the presentation of tumor antigens by DCs were found to override the immunological unresponsiveness / tolerance, a common problem that cancer immunotherapy currently faces. This was confirmed in studies where immunization with donor DCs overrode neonatal tolerance to cancer^[71]. This is considered to be true when tumor lysate is used as a source of tumor antigens loaded to DCs for the following reasons: First, the use of tumor lysate would provide a range of tumor antigens which would lead to the generation of a broad spectrum of CTLs. Second, it would decrease the chance of tumor escape from individual cancer antigens. Third, it would provide an opportunity for feeding both MHC class I and II antigens to DCs.

The positive effects of DC-based vaccination are becoming more compelling in melanoma patients. In a group of 16 patients with advanced melanoma, three partial and two complete objective antitumor responses were observed in the patients after vaccination with autologous DCs cultured with GM-CSF and IL-4 and pulsed with specific tumor peptides or with tumor lysate^[69]. There were minor side effects in the vaccinated patients above, and tumor regression was associated with a positive DTH response that was bigger than 10 mm in diameter. Biopsies taken from the DTH areas showed peptide-specific CTLs, which induced specific lysis against targets expressing the melanoma common tumor antigens gp100 and Melan A/MART-1. The biopsies also showed the

infiltration of the DTH areas with large number of CD8⁺ T cells. It is important to mention at this stage that for DCs to work as effective melanoma and cancer vaccines they have to: First, travel to draining lymph nodes in order to present MHC class I peptides to CD8⁺ T cells. CD4⁺ T cell activation is also required which in turn results in the interaction between CD40 ligand on the activated CD4⁺ T cell and its receptor on the DC. Second, go through a proper process of technical preparation and monitoring. Different techniques are currently used to isolate DCs for melanoma and cancer vaccine immunotherapy. These include leukopheresis, density gradient centrifugation, and in vitro expansion from purified monocyte precursor cells using a mixture of IL-4 and GM-CSF. Moreover, confocal laser scanning and phase contrast microscopy, interferon gamma (INF-) ELISOPT and chromium-release assays, MHC class I tetramers, as well as flow cytometry are used to characterize, phenotype, and clone generated DCs as means of monitoring DC-based vaccination^[72-74].

Tumor cell and peptide-based vaccination have also proven to be effective in treating and causing regression of different types of cancer including melanoma, and lung and breast carcinomas^[75-78]. Individual patients (SK-29 and MZ-2) with metastatic melanoma were vaccinated intradermally with irradiated autologous tumor cells which resulted in complete regression of their tumors and in a disease-free survival^[77,78]. Similar objective melanoma tumor regression was observed in individual studies in patients vaccinated with peptides derived from different melanoma antigens such as Melan A/MART-1 and gp100^[79,80]. We reported a patient with complete regression of subcutaneous melanoma metastases and partial regression of melanoma metastases to the lungs after vaccination with Melan A/MART-1^[73]. In cases where objective regression was not observed, we reported patients with stabilization of subcutaneous and visceral melanoma metastases for one year. Similar to the results obtained with vaccination with Melan A/MART-1, adoptive transfer of TILs recognizing gp100, gp-75, and tyrosinase melanoma antigen-derived peptides showed that these antigens could be used as tumor rejection antigens^[27,32,58]. These studies are paralleled by the ones in which vaccination with peptides derived from the MAGE-3 antigen resulted in tumor regression in 7 of 25 melanoma patients^[81], and where a three-month vaccination with a combination of MAGE-1 and MAGE-3 antigen-derived peptides resulted in partial regression of lung and liver metastases (E. Jager *et al* - unpublished data).

Recently, the melanoma common tumor antigen Melan A/MART-1 received further attention as a potent antigen for melanoma vaccine immunotherapy. We provided evidence of direct involvement of this antigen in tumor regression in patients with multiple primary melanoma^[10]. The analysis of the melanoma primary tumors from these patients revealed that the concept of immune surveillance against cancer is operative in humans. The last primary tumor in the multiple primary patients showed statistically higher percentage of spontaneous histopathological tumor regression compared to the first primary tumor of the same patients and to matched tumors from single primary melanoma patients. Such tumor regression was associated with significantly higher number of TILs and with significant loss of the melanoma common tumor antigen Melan A/MART-1 as measured by image analysis. Such loss of Melan A/MART-1 was also significantly associated with Melan A/MART-1 specific CTLs in the peripheral blood of the multiple primary patients as measured by ELISPOT. Similar findings with Melan A/MART-1 were seen in another group of patients where an immune reaction against the melanoma tumor could be more prominent. These patients are occult primary melanoma patients in whom the melanoma has spread to the lymph nodes and this was associated with complete regression of the initial primary cutaneous melanoma tumor. We believe that T lymphocytes in the melanoma-affected lymph nodes of these patients recognized Melan A/MART-1 in the initial primary cutaneous melanoma tumor, which led to its complete regression. Unlike Melan A/MART-1, no similar results were seen when the antigens gp100 and CD63 were analyzed in our study^[82].

IMPROVED MELANOMA VACCINES

There are two frequent questions that people from different background including clinicians, scientists, and cancer sufferers and their families usually ask: First, why do melanoma and cancer vaccines work on certain occasions and in certain people while they do not work in others? Second, what should be done to improve the current and future clinical trials aiming at providing the ultimate melanoma and cancer vaccine? The answers probably lie in the very basic foundation of tumor immunology and more specifically in preventing the different mechanisms used by tumors to escape a confrontation with the patient's immune system. These mechanisms include loss or down regulation of HLA/MHC class I expression, the absence of co-stimulatory molecules, the presence of immune suppressive factors, changes occurring in the tumor environment, and a

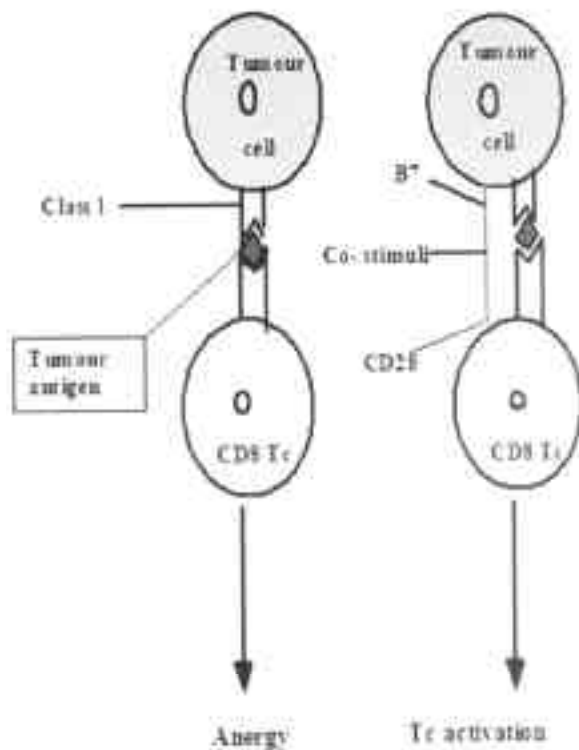


Fig. 2: Modified after Roitt *et al.* 1996^[136].

breakdown in the CD95 system. Although it is beyond the scope of this review to describe in details each of the above mechanisms, we will tackle the important issues that underlie such mechanisms.

MHC expression is needed for a proper presentation of tumor antigens to CTLs. Accordingly, if such expression becomes low (down regulated) or is lost, CTLs become incompetent in dealing with such antigens, and therefore the tumor will evade CTL attack. This issue has been confirmed in leukemia, lymphoma, and many solid tumors^[83-86]. This is even more evident in melanoma and prostate cancer where up to 35% of primary tumors and up to 80% of lymph node metastases have been found to totally lack expression of MHC class I^[84,87]. Such defects are caused by loss of chromosome six where HLA class I heavy chains are encoded, or by deletion of class I genes. Experimental studies have shown that melanoma cells lacking class I expression become resistant to CTL lysis, while others which have partial loss of MHC class I were partially resistant to such lysis^[84,88]. The latter condition was found to improve when exogenous melanoma antigen-derived peptides such as Melan A/MART-1 were added to the culture^[89].

Lack of co-stimulatory molecules, such as B7, can lead to a state of T cell anergy (Fig. 2^[136]). This state of anergy is characterized by lack of response

of T cells in terms of proliferation and IL-2 production upon exposure to cancer antigens even when the tumor cells express the proper MHC class I molecules. This was confirmed in experimental studies in which long-term survival of pancreatic islets transplantation was achieved after blocking of B7 by a high-affinity inhibitor CTLA4-4Ig^[90]. Another surface molecule that is needed as a ligand for the T cell activation molecules CD28/CTLA.4 is B7.2. It has been shown that B7.2 is an important co-stimulatory molecule for T cells early in the process of activation^[91]. The importance of the presence of co-stimulatory molecules stems from the fact that most neoplasms originate from mesenchymal or parenchymal cells that do not express B7 or B7.2, and accordingly become weak or non-immunogenic. This in turn could explain the lack of proper cancer immune surveillance in cancer patients as well as the ineffectiveness of a variety of cancer vaccines that are currently in use. This state of poor immunogenicity has been shown to improve following the insertion of B7 gene into squamous cell carcinomas of the head and neck in a phase I clinical trial conducted by Gleich *et al.*^[92].

Another important hurdle that cancer vaccines have to overcome is the presence of immune suppressive factors including tumor growth factor beta (TGF- β) and IL-10 in the tumor environment. These factors have been involved in inhibiting lymphokine production, macrophage recruitment, TIL and CTL proliferation, and secretion of IL-2, IL-4, IL-12, INF- γ , and TNF- α , which are often associated with immune responses against cancer. TGF- β and IL-10 have been demonstrated in culture supernatants of tumor cells derived from melanomas, basal cell carcinomas, endometrial, colorectal and breast carcinomas, neuroblastomas, and glioblastomas, and have been found to be associated with altered expression of IL-2 and MHC receptors^[93-99]. The list of immune suppressive factors is on the increase and includes insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2), and lymphocyte blastinogenesis-inhibiting factor^[100-103]. VEGF and PGE2 are associated with interfering with the maturation of cancer antigen-specific DC precursors and with down regulation of MHC class II expression respectively.

Extra-tumor factors that also play a role in resisting tumor destruction by the immune system and in enhancing tumor invasion and metastasis include decreased expression or total lack of adhesion molecules such as ICAM-1, ICAM-2, VCAM-1, N-cadherin, vitronectin, and fibronectin^[104,105]. This has been demonstrated in various types of cancer including melanoma, where resistance of cancer cells to lysis by monocytes was observed

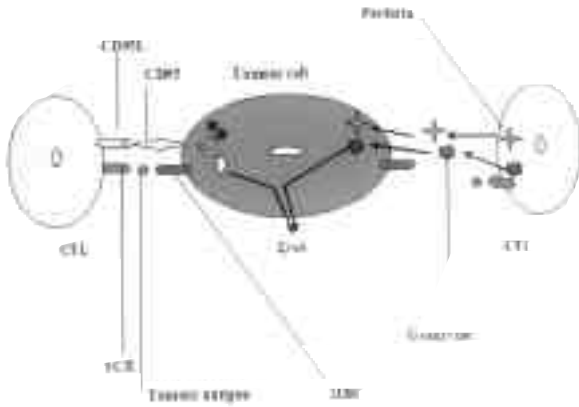


Fig. 3: Modified after Strand *et al.* 1996^[137].

following reduced expression of adhesion molecules^[105]. Such resistance was overcome by transfection of adhesion molecule genes into the cancer cells^[106]. Tumor invasion and metastasis has also been attributed to proteolysis of tissue barriers by enzymes degrading collagens, proteoglycans, or gelatin^[107]. A well-known proteolytic enzyme is metalloproteinase whose gene and the product of its gene (stromelysin-3 or ST3) are specifically expressed respectively in stromal cells and cells proper of invasive but not in situ breast carcinomas^[108]. Added to proteolytic enzymes, angiogenesis or new blood vessel formation remains an important tool for tumor invasion and metastasis. It depends on a variety of regulatory factors including fibroblast growth factor (FGF), TGF- β , and other cytokines, which are often secreted by malignant cells^[109, 110].

Lastly, another mechanism that is involved in tumor escape from immune destruction and that the scientists and clinicians should take into consideration when developing melanoma and cancer vaccines is the breakdown in the CD95 system. It has been demonstrated that CTLs can kill target cells through Fas/APO-1 to Fas Ligand interaction leading to lysis of the cells^[111]. This process is initiated through the binding of a death receptor, APO-1/Fas/CD95 present on the surface of the tumor cell, to its natural ligand APO-1L/FasL/CD95L on the surface of the T cell (Fig. 3^[137]). Accordingly, loss of CD95 would result in failure of tumor cell lysis, a condition demonstrated in solid and lymphoid tumors^[112]. Such failure is also demonstrated in conditions involving blocking of T cell CD95 ligands by soluble CD95 or defects in the CD95 signaling pathway^[113, 114].

DISCUSSION AND CONCLUSIONS

Fifty years ago, cancer was a taboo and people suffering from it were considered as being "sentenced to death" due to both lack of proper

understanding of the disease and the absence of proper and effective treatment options. Although there are nowadays people still dying from cancer, the journey that we have traveled aiming at finding a cure for such a debilitating disease has been quite impressive. The cancer treatment options that are currently available and which include surgery, chemotherapy, radiotherapy, and vaccine immunotherapy have saved so many lives. Nevertheless, our battle with cancer is yet to be conquered and cancer vaccine and immunotherapy will hopefully help in providing the means to achieve this goal, especially in melanoma.

Melanoma is a skin cancer that affects mostly people with fair skin, and it constitutes a serious problem in both developed and developing countries. Once a melanocytic lesion invades the dermis of the skin and the subcutaneous tissue, the chances of metastasizing to vital organs in the human body become very high. An important phenomenon seen in some types of melanoma is their tendency to spontaneously regress. Such regression was found to be associated with infiltration of the tumor by TILs, which implies that the tumor destruction is immunologically-mediated^[10, 11, 82]. This phenomenon resurrected the concept of immune surveillance against cancer that was introduced early in this century by Paul Ehrlich and followed up thereafter by Burnet^[2, 3]. Such a concept states that there are numerous cells in our body that undergo changes including DNA damage and repair and from which abnormal cells might develop. However, such abnormal cells, which have the tendency to develop into cancer cells, are recognized and destroyed by our immune system before they grow into tumors. In cases where these cells develop into tumors, the authors attributed this defect to either the cells being weakly immunogenic or they employ different immune-escape mechanisms or to both. Accordingly, boosting the immunogenicity of cancer tumors and/or suppressing the different escape mechanisms are currently the focus of research on melanoma and cancer vaccine immunotherapy.

What distinguishes melanoma from other cancer tumors is that melanoma antigens are differentiation antigens that are shared by most melanoma tumors, which implies that developing an "elixir" vaccine that could be used to treat different types of melanoma including the deadly metastatic ones might be possible in the near future or on the long run. Moreover, melanoma-associated antigens are now well identified, and CTL-defined cancer vaccines in melanoma and other epithelial cancers have provided the first clear evidence of useful induced tumor immunity. Two recent studies added more support to the existing evidence of

useful induced tumor immunity, and demonstrated that vaccination against melanoma, be it in patients with early stage or advanced melanoma, could be quite effective. In one study, vaccination with peptides derived from the melanoma common tumor antigen Melan A/MART-1 administered with incomplete Freund's adjuvant resulted in the generation of peptide-specific CTLs in 50% of melanoma patients recruited for a phase I clinical trial, with a tendency towards a significant increased patient survival^[115]. In the other study, DCs loaded with peptides derived from the melanoma antigen MAGE-3 and administered to patients with advanced stage IV melanoma produced significant CTL expansion in eight out of 11 patients which correlated with significant CD8+ T cell infiltration and regression of the melanoma tumors in six of the 11 patients^[116].

An important aspect for establishing a proper melanoma vaccine immunotherapy protocol is the efficient recruitment of T lymphocytes to infiltrate the metastatic tumor. It has been demonstrated that T cell memory has two subsets expressing differently the chemokine receptor, CCR7, which is involved in homing of lymphocytes to secondary organs^[117]. The first subset consists of CCR7-ve effector cells that express receptors for migration to inflamed tissues and that display immediate effector function. The second subset consists of CCR7+ve central memory T cells, which lack immediate effector function but which stimulate DCs efficiently and differentiate into CCR7-ve effector cells following secondary stimulation. Accordingly, any melanoma or other cancer vaccines should include activation of the central memory T cells in the draining lymph node to convert them into T cells capable of infiltrating the tumor. Such vaccines should also provide means to overcome factors involved in tumor escape from the different arms of the immune system which include poor immunogenicity of cancer cells due to lack of expression of co-stimulatory molecules, poor antigen processing and presentation, presence of immune suppressive factors such as TGF and IL-10, and/or the heterogeneity of cancer, as mentioned earlier.

Early in this century, opposers to cancer vaccine immunotherapy used to believe that using the immune system to eradicate cancer is as impossible as attempting to destroy the right ear while keeping the left one intact. Nevertheless, and since the seventies, clinical trials and experimental studies have shown that the outcomes from such therapy could be as effective as increasing the survival of cancer patients, partial regression of the cancer tumors, or complete regression/remission of the

neoplasm. This has been demonstrated in primary and metastatic melanoma as well as in other types of cancer including lymphoma, prostate, bladder, and uterine cervical carcinomas, to name a few. In addition, using vaccine as a preventive measure has proven to be very effective in preventing the occurrence of certain cancer types including hepatocellular carcinoma and uterine cervical neoplasia that are associated with infection with the hepatitis B and papilloma viruses respectively^[118,119].

REFERENCES

1. Ferlay F, Bray F, Pisani P, Parkin DM. Cancer Incidence, Mortality and Prevalence Worldwide, IARC Press: Lyon, 2001.
2. Ehrlich P. Arbeiten aus dem Koniglichen Institut fur Experimentelle Therapie, Fisher: Frankfurt, 1906.
3. Burnet F. The concept of immunological surveillance. *Prog Exp Tumor Res* 1970; 13:1-27.
4. Thomas L. Reactions to homologous tissue antigens in relation to hypersensitivity, Hoeber: New York, 1959.
5. Roberts L, Daynes R. Tumor Immunology, CRC Press: Florida, 1986.
6. Abu E, Reed M, Pathak R, Niazi M, Sivakumar M, Fernandes E, *et al.* Malignancy in HIV/AIDS: a single hospital experience. *J Surg Oncol* 2000; 75:11-18.
7. Fogla R, Biswas J, Kumar SK, Madhavan HN, Kumarasamy N, Solomon S. Squamous cell carcinoma of the conjunctiva as initial presenting sign in a patient with acquired immunodeficiency syndrome (AIDS) due to human immunodeficiency virus type-2. *Eye* 2000; 14:246-247.
8. Lazzi S, Ferrari F, Nyongo A, Palumbo N, de Milito A, Zazzi M, *et al.* HIV-associated malignant lymphomas in Kenya (Equatorial Africa). *Human Pathol* 1998; 29:1285-1289.
9. Challis GB, Stam HJ. The spontaneous regression of cancer. A review of cases from 1900 to 1987. *Acta Oncol* 1990; 29:545-550.
10. Saleh F, Crotty K, Hersey P, Menzies S. Primary melanoma tumor regression associated with an immune response to the tumor-associated antigen melan-A/MART-1. *Int J Cancer* 2001; 94:551-557.
11. Tefany FJ, Barnetson RS, Halliday GM, McCarthy SW, McCarthy WH. Immunocytochemical analysis of the cellular infiltrate in primary regressing and non-regressing malignant melanoma. *J Invest Dermatol* 1991; 97:197-202.
12. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, *et al.* IFN gamma and lymphocytes prevent primary tumor development and shape tumor immunogenicity. *Nature* 2001; 410:1107-1111.
13. Smyth MJ, Snook MB. Perforin-dependent cytolytic responses in beta2-microglobulin-deficient mice. *Cell Immunol* 1999; 196:51-59.
14. Boel P, Wildmann C, Sensi M.L, Brasseur R, Renaud JC, Coulie P, *et al.* BAGE, a new genetic encoding an antigen recognized on human melanomas by cytolytic lymphocytes. *Immunity* 1995; 2:167-175.
15. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora JP, De Smet C, *et al.* Structure, chromosomal localization and expression of twelve genes of the MAGE family. *Immunogenetics* 1994; 40:360-369.
16. Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, *et al.* Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med* 1994; 179:921-930.
17. Gaugler B, Brouwenstijn N, Vantomme V, Szikora JP, Van

- der Spek CW, Patard JJ, *et al.* A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma. *Immunogenetics* 1996; 44:323-330.
18. Herman J, van der Bruggen P, Luescher IF, Mandruzzato S, Romero P, Thonnard J, *et al.* A peptide encoded by the human MAGE3 gene and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE3. *Immunogenetics* 1996; 43:377-383.
19. Ikeda H, Lethe B, Lehmann F, van Baren N, Baurain JF, de Smet C, *et al.* Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL-expressing an NK inhibitory receptor. *Immunity* 1997; 6:199-208.
20. Jager E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jager D, *et al.* Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; 187:265-270.
21. Tanaka F, Fujie T, Tahara K, Mori M, Takesako K, Sette A, *et al.* Induction of antitumor cytotoxic T lymphocytes with a MAGE-3-encoded synthetic peptide presented by human leukocytes antigen-A24. *Cancer Res* 1997; 57:4465-4468.
22. Traversari C, van der Bruggen P, Luescher IF, Lurquin C, Chomez P, Van Pel A, *et al.* A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigens MZ2-E. *J Exp Med* 1992; 176:1453-1457.
23. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. *J Exp Med* 1995; 182:689-698.
24. van der Bruggen P, Bastin J, Gajewski T, Coulie PG, Boel P, De Smet C, *et al.* A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur J Immunol* 1994; 24:3038-3043.
25. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on human melanoma. *Science* 1991; 254:1643-1647.
26. Visseren MJ, van der Burg SH, van der Voort EI, Brandt RM, Schrier PI, van der Bruggen P, *et al.* Identification of HLA-A*0201-restricted CTL epitopes encoded by the tumor-specific MAGE-2 gene product. *Int J Cancer* 1997; 73:125-130.
27. Bakker AB, Schreurs MW, de Boer AJ, Kawakami Y, Rosenberg SA, Adema GJ, *et al.* Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J Exp Med* 1994; 179:1005-1009.
28. Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plaen E, Lethe B, *et al.* The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* 1993; 178:489-495.
29. Castelli C, Storkus WJ, Maeurer MJ, Martin DM, Huang EC, Pramanik BN, *et al.* Mass spectrometric identification of a naturally processed melanoma peptide recognized by CD8+ cytotoxic T lymphocytes. *J Exp Med* 1995; 181:363-368.
30. Cox AL, Skipper J, Chen Y, Henderson RA, Darrow TL, Shabanowitz J, *et al.* Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 1994; 264:716-719.
31. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, *et al.* Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc Natl Acad Sci USA* 1994; 91:3515-3519.
32. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, *et al.* Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci USA* 1994; 91:6458-6462.
33. Romero P, Gervois N, Schneider J, Escobar P, Valmori D, Pannetier C, Stein L, *et al.* Cytolytic T lymphocyte recognition of the immunodominant HLA-A*0201-restricted Melan-A/MART-1 antigenic peptide in melanoma. *J Immunol* 1997; 159:2366-2374.
34. Skipper JC, Hendrickson RC, Gulden PH, Brichard V, Van Pel A, Chen Y, *et al.* An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *J Exp Med* 1996; 183:527-534.
35. Skipper JC, Kittlesen DJ, Hendrickson RC, Deacon DD, Harthun NL, Wagner SN, *et al.* Shared epitopes for HLA-A3-restricted melanoma-reactive human CTL include a naturally processed epitope from Pmel-17/gp100. *J Immunol* 1996; 157:5027-5033.
36. Wang RF, Appella E, Kawakami Y, Kang X, Rosenberg SA. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *J Exp Med* 1996; 184:2207-2216.
37. Wang RF, Johnston SL, Southwood S, Sette A, Rosenberg SA. Recognition of an antigenic peptide derived from tyrosinase-related protein-2 by CTL in the context of HLA-A31 and -A33. *J Immunol* 1998; 160:890-897.
38. Wang RF, Parkhurst MR, Kawakami Y, Robbins PF, Rosenberg SA. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. *J Exp Med* 1996; 183:1131-1140.
39. Wang RF, Robbins PF, Kawakami Y, Kang XQ, Rosenberg SA. Identification of a gene encoding a melanoma tumor antigen recognized by HLA-A31-restricted tumor-infiltrating lymphocytes. *J Exp Med* 1995; 181:799-804.
40. Wolfel T, Van Pel A, Brichard V, Schneider J, Seliger B, Meyer zum Buschenfelde KH, *et al.* Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur J Immunol* 1994; 24:759-764.
41. Coulie PG, Lehmann F, Lethe B, Herman J, Lurquin C, Andrawiss M, *et al.* A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc Natl Acad Sci USA* 1995; 92:7976-7980.
42. Guilloux Y, Lucas S, Brichard VG, Van Pel A, Viret C, De Plaen E, *et al.* A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene. *J Exp Med* 1996; 183:1173-1183.
43. Kawakami Y. New cancer therapy by immunomanipulation. Development of Immunotherapy for human melanoma. *Cornea* 2000; 19:S2-S6.
44. Robbins PF, El-Gamil M, Li YF, Fitzgerald EB, Kawakami Y, Rosenberg SA. The intronic region of an incompletely spliced gp100 gene transcript encodes an epitope recognized by melanoma-reactive tumor-infiltrating lymphocytes. *J Immunol* 1997; 159:303-308.
45. Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, *et al.* A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med* 1996; 183:1185-1192.
46. Robbins PF, el-Gamil M, Li YF, Topalian SL, Rivoltini L, Sakaguchi K, *et al.* Cloning of a new gene encoding an antigen recognized by melanoma-specific HLA-A24-restricted tumor-infiltrating lymphocytes. *J Immunol* 1995; 154:5944-5950.
47. Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, *et al.* A p16INK4a-insensitive CDK4

- mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* 1995; 269:1281-1284.
48. Van den Eynde B, Hainaut P, Herin M, Knuth A, Lemoine C, Weynants P, *et al.* Presence on a human melanoma of multiple antigens recognised by autologous CTL. *Int J Cancer* 1989; 44:634.
 49. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, *et al.* A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; 94:1914-1918.
 50. Doki Y, Shiozaki H, Tahara H, Inoue M, Oka H, Iihara K, *et al.* Correlation between E-cadherin expression and invasiveness in vitro in a human esophageal cancer cell line. *Cancer Res* 1993; 53:3421-3426.
 51. Coulie PG, Brichard V, Van Pel A, Wolfel T, Schneider J, Traversari C, *et al.* A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* 1994; 180:35-42.
 52. Chen YT, Stockert E, Jungbluth A, Tsang S, Coplan K.A, Scanlan M.J, *et al.* Serological analysis of Melan-A (MART-1), a melanocyte-specific protein homogeneously expressed in human melanomas. *Proc Natl Acad Sci USA* 1996; 93:5915-5919.
 53. Sarantou T, Chi DD, Garrison DA, Conrad AJ, Schmid P, Morton DL, *et al.* Melanoma-associated antigens as messenger RNA detection markers for melanoma. *Cancer Res* 1997; 57:1371-1376.
 54. Colombari R, Bonetti F, Zamboni G, Scarpa A, Marino F, Tomezzoli A, *et al.* Distribution of melanoma specific antibody (HMB-45) in benign and malignant melanocytic tumors. An immunohistochemical study on paraffin sections. *Virch Archiv - A Pathol Anat & Histopathol* 1988; 413:17-24.
 55. Vogel AM, Esclamado RM. Identification of a secreted Mr 95,000 glycoprotein in human melanocytes and melanomas by a melanocyte specific monoclonal antibody. *Cancer Res* 1988; 48:1286-1294.
 56. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, *et al.* Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 1995; 154:3961-3968.
 57. Kwon BS, Haq AK, Pomerantz SH, Halaban R. Isolation and sequence of a cDNA for human tyrosinase that maps at the mouse *c*-albino locus. *Proc Natl Acad Sci USA* 1987; 84:7473.
 58. Robbins PF, el-Gamil M, Kawakami Y, Stevens E, Yannelli JR., Rosenberg SA. Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy. *Cancer Res* 1994; 54:3124-3126.
 59. Topalian SL, Solomon D, Avis FP, Chang AE, Freerksen DL, Linehan WM, *et al.* Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. *J Clin Oncol* 1988; 6:839.
 60. Estin CD, Stevenson US, Plowman GD, Hu SL, Sridhar P, Hellstrom I, *et al.* Recombinant vaccinia virus vaccine against the human melanoma antigen p97 for use in immunotherapy. *Proc Natl Acad Sci USA* 1988; 85:1052-1056.
 61. Overwijk WW, Tsung A, Irvine KR, Parkhurst MR, Goletz TJ, Tsung K, *et al.* gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J Exp Med* 1998; 188:277-286.
 62. Overwijk WW, Lee DS, Surman DR, Irvine KR, Touloukian CE, Chan CC, *et al.* Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *Proc Natl Acad Sci USA* 1999; 96:2982-2987.
 63. Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc Natl Acad Sci USA* 1992; 89:10847-10851.
 64. Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, *et al.* NYVAC: a highly attenuated strain of vaccinia virus. *Virology* 1992; 188:217-232.
 65. Antoine G, Scheiflinger F, Dorner F, Falkner FG. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* 1998; 244:365-396.
 66. Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J Exp Med* 1996; 184:465-472.
 67. Gong J, Chen D, Kashiwaba M, Kufe D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat Med* 1997; 3:558-561.
 68. Hsu FJ, Benike C, Fagnoni F, Liles TM, Czerwinski D, Taidi B, *et al.* Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 1996; 2:2-58.
 69. Nestle FO, Aljagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, *et al.* Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998; 4:328-332.
 70. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, *et al.* Bone marrow-derived dendritic cells pulsed with synthetic tumor peptides elicit protective and therapeutic antitumor immunity. *Nat Med* 1995; 1:1297-302.
 71. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 1996; 271:1723-1726.
 72. Dunbar PR, Ogg GS, Chen J, Rust N, van der Bruggen P, Cerundolo V. Direct isolation, phenotyping and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood. *Curr Biol* 1998; 8:413-416.
 73. Romero P, Valmori D, Pittet MJ, Zippelius A, Rimoldi D, Levy F, *et al.* Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunol Rev* 2002; 188:81-96.
 74. Schmitt A, Keilholz U, Scheibenbogen C. Evaluation of the interferon-gamma ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. *J Immunol Methods* 1997; 210:167-174.
 75. Berd D, Sato T, Cohn H, Maguire HC, Mastrangelo MJ. Treatment of metastatic melanoma with autologous, hapten-modified melanoma vaccine: regression of pulmonary metastases. *Int J Cancer* 2001; 94:531-539.
 76. Hadden JW. The immunology and immunotherapy of breast cancer: an update. *Int J Immunopharmacol* 1999; 21: 79-101.
 77. Knuth A, Danowski B, Oettgen HF, Old LJ. T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin 2-dependent T-cell cultures. *Proc Natl Acad Sci USA* 1984; 81:3511-3515.
 78. Knuth A, Wolfel T, Meyer zum Buschenfelde KH. T cell responses to human malignant tumors. *Cancer Surv* 1992; 13:39-52.
 79. Jager E, Ringhoffer M, Karbach J, Arand M, Oesch F, Knuth A. Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8+ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. *Int J Cancer* 1996; 6:470-476.
 80. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, *et al.* Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T

- lymphocytes associated with in vivo tumor regression. *J Immunol* 1995; 154:3961-3968.
81. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, *et al.* Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 1999; 80:219-230.
 82. Saleh F, Crotty KA, Hersey P, Menzies S, Rahman W. Autonomous histopathological regression of primary tumors associated with specific immune responses to cancer antigens. *J Pathol* 2003; 200:383-395.
 83. Chen L. Immunological ignorance of silent antigens as an explanation of tumor evasion. *Immunol Today* 1998; 19:27-30.
 84. Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today* 1995; 16:487-494.
 85. Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, Lopez-Botet M, Duggan-Keen, *et al.* Implications for immunosurveillance of altered HLA class I phenotypes in human tumors. *Immunol Today* 1997; 18:89-95.
 86. Packard B, Komoriya ZA. Tumor cell recognition by lymphocytes: is the MHC always essential? *Critical Rev Immunol* 1998; 18:139-144.
 87. Simons JW, Mikhak B, Chang JF, DeMarzo AM, Carducci MA, Lim M, *et al.* Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res* 1999; 59:5160-5168.
 88. Seliger B, Ritz U, Abele R, Bock M, Tampe R, Sutter G, *et al.* Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. *Cancer Res* 2001; 61:8647-8650.
 89. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* 2000; 74:181-273.
 90. Rivoltini L, Barracchini KC, Viggiano V, Kawakami Y, Smith A, Mixon A, *et al.* Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res* 1995; 55:3149-3157.
 91. Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG, *et al.* Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 1992; 257:789-792.
 92. Hathcock KS, Laszlo G, Dickler HB, Bradshaw J, Linsley P, Hodes RJ. Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. *Science* 1993; 262:905-907.
 93. Gleich LL, Gluckman JL, Armstrong S, Biddinger PW, Miller MA, Balakrishnan K, *et al.* Alloantigen gene therapy for squamous cell carcinoma of the head and neck: results of a phase-1 trial. *Arch Otolaryng — Head & Neck Surg* 1998; 124:1097-1104.
 94. Gastl GA, Abrams JS, Nanus DM, Oosterkamp R, Silver J, Liu F, *et al.* Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. *Int J Cancer* 1993; 55:96-101.
 95. Gold LI, Parekh TV. Loss of growth regulation by transforming growth factor-beta (TGF-beta) in human cancers: studies on endometrial carcinoma. *Sem Repro Endocrinol* 1999; 17:73-92.
 96. Moretti S, Pinzi C, Berti E, Spallanzani A, Chiarugi A, Boddì V, *et al.* In situ expression of transforming growth factor beta is associated with melanoma progression and correlates with Ki67, HLA-DR and beta 3 integrin expression. *Melanoma Res* 1997; 7:313-321.
 97. Schmid P, Itin P, Ruffli T. In situ analysis of transforming growth factors-beta (TGF-beta 1, TGF-beta 2, TGF-beta 3) and TGF-beta type II receptor expression in basal cell carcinomas. *Br J Dermatol* 1996; 134:1044-1051.
 98. Siepl C, Bodmer S, Frei K, MacDonald HR, De Martin R, Hofer E, *et al.* The glioblastoma-derived T cell suppressor factor/transforming growth factor-beta 2 inhibits T cell growth without affecting the interaction of interleukin 2 with its receptor. *Eur J Immunol* 1988; 18:593-600.
 99. Sulitzeanu D. Immunosuppressive factors in human cancer. *Adv Cancer Res* 1993; 60:247-267.
 100. Voorzanger N, Touitou R, Garcia E, Delecluse HJ, Rousset F, Joab I, *et al.* Interleukin (IL)-10 and IL-6 are produced in vivo by non-Hodgkin's lymphoma cells and act as cooperative growth factors. *Cancer Res* 1996; 56:5499-5505.
 101. Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, *et al.* Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nature Med* 1996; 2:1096-1103.
 102. Mishra L, Bass B, Ooi BS, Sidawy A, Korman L. Role of insulin-like growth factor-I (IGF-I) receptor, IGF-I, and IGF binding protein-2 in human colorectal cancers. *Growth Hormone & IGF Res* 1998; 8:4473-4779.
 103. Salazar-Onfray F. Interleukin 10: a cytokine used by tumors to escape immunosurveillance. *Med Oncol* 1999; 16:86-94.
 104. Sugimura K, Wada Y, Kimura T, Ohno T, Kobayashi S, Azuma I. Abnormal behavior of gamma-committed B lymphocytes probed by a lymphocyte blastogenesis inhibitory factor in autoimmune MRLmice. *Eur J Immunol* 1990; 20:1899-1904.
 105. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J Cell Biol* 2000; 148:779-790.
 106. Ohene-Abuakwa Y, Pignatelli M. Adhesion molecules in cancer biology. *Adv Exp Med & Biol* 2000; 465:115-126.
 107. Jonjic N, Alberti S, Bernasconi S, Peri G, Jilek P, Anichini A, *et al.* Heterogeneous susceptibility of human melanoma clones to monocyte cytotoxicity: role of ICAM-1 defined by antibody blocking and gene transfer. *Eur J Immunol* 1992; 22:2255-2260.
 108. Noel A. Tumor-host interactions in the progression of breast cancer. *Bullet Mem Acad Roy Med Belg* 1999; 154:347-353.
 109. Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, *et al.* A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* 1990; 348:699-704.
 110. Gasparini G. The rationale and future potential of angiogenesis inhibitors in neoplasia. *Drugs* 1999; 58:17-38.
 111. McLeskey SW, Zhang L, Kharbada S, Kurebayashi J, Lippman ME, Dickson RB, *et al.* Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. *Breast Cancer Res & Treat* 1996; 39:103-117.
 112. Traidl C, Sebastiani S, Albanesi C, Merk HF, Puddu P, Girolomoni G, *et al.* Disparate cytotoxic activity of nickel-specific CD8+ and CD4+ T cell subsets against keratinocytes. *Cell* 2000; 88:355-365.
 113. Muschen M, Warskulat U, Beckmann MW. Defining CD95 as a tumor suppressor gene. *J Mol Med* 2000; 78:312-325.
 114. Owen-Schaub LB, Angelo LS, Radinsky R, Ware CF, Gesner TG, Bartos DP. Soluble Fas/APO-1 in tumor cells: a potential regulator of apoptosis? *Cancer Letters* 1995; 94:1-8.
 115. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinel E, Neipel F, *et al.* Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997; 386:517-521.

116. Wang F, Bade E, Kuniyoshi C, Spears L, Jeffery G, Marty V, *et al.* Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. *Clin Cancer Res* 1999; 5:2756-2765.
117. Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, *et al.* Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999; 190:1669-1678.
118. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; 401:708-712.
119. Hill AVS. The immunogenetics of human infectious diseases. *Ann Rev Immunol* 1998; 16:593-617.
120. Schiller JT. Papillomavirus-like particle vaccines for cervical cancer. *Mol Med Today* 1999; 5:209-215.
121. Sosman JA, Unger JM, Liu PY, Flaherty LE, Park MS, Kempf RA, *et al.* Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumor vaccine: impact of HLA class I antigen expression on outcome. *J Clin Oncol* 2002; 20:2067-2075.
122. Slingluff CL, Yamshchikov G, Neese P, Galavotti H, Eastham S, Engelhard VH, *et al.* Phase I trial of a melanoma vaccine with gp100 (280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. *Clin Cancer Res* 2001; 7:3012-3024.
123. Bystryjn JC, Zeleniuch-Jacquotte A, Oratz R, Shapiro RL, Harris MN, Roses DF. Double-blind trial of a polyvalent, shed-antigen, melanoma vaccine. *Clin Cancer Res* 2001; 7:1882-1887.
124. Kirkwood JM, Ibrahim JG, Sosman JA, Sondak VK, Agarwala SS, Ernstoff MS, *et al.* High-dose interferon alfa-2b significantly prolongs relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIB-III melanoma: results of intergroup trial E1694/S9512/C509801. *J Clin Oncol* 2001; 19:2370-2380.
125. Wallack, MK, Sivanandham M, Balch CM, Urist MM, Bland KI, Murray D, *et al.* Surgical adjuvant active specific immunotherapy for patients with stage III melanoma: the final analysis of data from a phase III, randomized, double-blind, multicenter vaccinia melanoma oncolysate trial. *J Am Coll Surg* 1998; 187:69-77.
126. Henz BM, Macher E, Brocker EB, Suci S, Steerenberg PA, Jung E, *et al.* Prognostic value of tuberculin and BCG immunoreactivity in stage I high-risk malignant melanoma (EORTC protocol 18781). *Dermatology* 1996; 193:105-109.
127. Hersey P. Evaluation of vaccinia viral lysates as therapeutic vaccines in the treatment of melanoma. *Ann NY Acad Sci* 1993; 690:167-77.
128. Morton DL, Foshag LJ, Hoon DS, Nizze JA, Famatiga E, Wanek LA, *et al.* Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Ann Surg* 1992; 216: 463-482.
129. Quirt IC, Shelley WE, Pater JL, Bodurtha AJ, McCulloch PB, McPherson TA, *et al.* Improved survival in patients with poor-prognosis malignant melanoma treated with adjuvant levamisole: a phase III study by the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 1991; 9:729-735.
130. W.H.O. Controlled study with imidazole carboxamide (DTIC), DTIC + bacillus Calmette-Guerin (BCG), and DTIC + corynebacterium parvum in advanced malignant melanoma. W.H.O. Collaborating Centres for Evaluation of Methods of Diagnosis and Treatment of Melanoma. *Tumori* 1984; 70:41-48.
131. Falk RE, Makowka L, Ambus U, Falk JA, Bugala R, Landi S. Nonspecific and selective stimulation of the immune system in the treatment of carcinoma in humans. *Can Med Assoc J* 1983; 128:1385-1388.
132. Ariyan S, Kirkwood JM, Mitchell MS, Nordlund JJ, Lerner AB, Papac RJ. Intralymphatic and regional surgical adjuvant immunotherapy in high-risk melanoma of the extremities. *Surgery* 1982; 92:459-463.
133. Balch CM, Smalley RV, Bartolucci AA, Burns D, Presant CA, Durant JR. A randomized prospective clinical trial of adjuvant C. parvum immunotherapy in 260 patients with clinically localized melanoma (Stage I): prognostic factors analysis and preliminary results of immunotherapy. *Cancer* 1982; 49:1079-1084.
134. Nathanson L, Schoenfeld D, Regelson W, Colsky J, Mittelman A. Prospective comparison of intralesional and multipuncture BCG in recurrent intradermal melanoma. *Cancer* 1979; 43:1630-1635.
135. Cohen MH, Jessup JM, Felix EL, Weese JL, Herberman RB. Intralesional treatment of recurrent metastatic cutaneous malignant melanoma: a randomized prospective study of intralesional Bacillus Calmette-Guerin versus intralesional dinitrochlorobenzene. *Cancer* 1978; 41:2456-2463.
136. Roitt I. Tumour Immunology. In: *Essential Immunology*. Blackwell Scientific Publications, Oxford. 2000.
137. Strand S, Hofmann WJ, Hug H, Muller M, Otto G, Strand D, Mariani SM, Stremmel W, Krammer PH, Galle PR. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? *Nature Medicine* 1996; 2:1361-1366.