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# Immune-Therapy in Cutaneous Melanoma – Efficacy Immune Markers

Monica Neagu and Carolina Constantin
*"Victor Babes" National Institute of Pathology,*
*Splaiul Independentei, Bucharest,*
*Romania*

## 1. Introduction

The chapter presents updated results of immune-therapy in cutaneous melanoma in the light of the well-known resistance of this disease and the unrelenting efforts in overcoming it. The types of immune-therapy are correlated intrinsically with the efficacy immune-markers utilized for thorough monitoring of treated patients.

This life threatening disease has a recently reported increased incidence; in the United States, this continues to rise from 4% to 6% annually, despite steps towards primary prevention. Similar increases are being noted worldwide. It has been estimated that the current lifetime risk for developing invasive melanoma is 1 in 58 and it has been reported that over 8,000 Americans died of melanoma in 2009 (Rigel, 2010). These statistics highlight the need to find both new efficient therapies and improved markers for predicting disease evolution and therapy monitoring.

Immune markers related to therapy monitoring are an important field for at least two reasons: the lack of good clinical responses in immune-therapy and the need for accumulating knowledge regarding the molecular processes that governate tumour progression (Riker et al., 2006). The intent of this chapter is to describe the immune-therapies approaches in the intimate link with the immune markers as therapy efficacy monitoring. Immune monitoring is valuable not only for efficacy purposes but also for immune-tailored individualized therapy.

## 2. Immune therapy – The ultimate solution in cutaneous melanoma?

Up to date immunotherapy approaches that are in the stage of clinical development include: cytokines (IL-2, IFN, TNF, IL-7, IL-12, IL-21), cytokine-antibody fusion proteins or immunocytokines, whole tumour cell vaccines, genetically modified tumour cells, heat shock protein vaccines, peptide vaccines, dendritic cells pulsed with tumour antigens, tumour antigen-naked DNA vectors, recombinant viral vectors, adoptive transfer of cloned tumour antigen-specific T cells, Toll-like receptor ligands, antagonistic antibodies to

Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4), activating antibodies that target CD40 and CD137.

All the therapeutical approaches seek to induce cytotoxic T-cell responses to tumours ranging from mono-specific immunotherapy, targeting only one specific tumour antigen, to polyvalent immunotherapy, which attempts to induce immune responses to multiple antigenic components, to gene-based therapy, which manipulates the immunogenicity of the tumour. Each of these therapies' goal is to induce proliferation and differentiation of anti-tumoral antigen-specific memory T-cells.

The FDA approved immune therapies for cutaneous melanoma comprise: IL-2, IFN and the most recent anti-CTL-4.

## 2.1 Immune therapy and Immune markers

There are still no validated immune markers to be used in monitoring immune-therapy in cutaneous melanoma, although it is the domain that would benefit the most from immune monitoring biomarkers and that surrogate immunologic markers of efficacy have not been reported thus far.

One of the most used immune-marker in cutaneous melanoma is the "immune cell". As an established disease with a high immune-suppressive background, peripheral immune cells were quantified both in relation to the monitoring of the patients immune status, and in therapy efficacy monitoring. We have previously published that peripheral blood CD4+/CD8+ ratio can monitor a good therapeutic response and that monitoring the dynamics of this subpopulations ratio can detect a good therapeutical response (Neagu et al., 2010). In the first reported study of high-risk melanoma patients immunized with gp100 and tyrosinase peptides (Cassarino et al., 2006) no difference in nevi tissue was found regarding CD3, CD4, CD8, MHC-I, MHC-II, CD1a, HMB-45, MART-1, tyrosinase, but an increase in p53 and bcl-2 staining, in the nevi post-treatment has been found. Authors explain that activating melanoma-specific T cells for preventing melanoma recurrence a response mediated by p53 and bcl-2 is triggered in benign melanocytes (Cassarino et al., 2006). Another group showed that following transcutaneous delivery, gp100 vaccination activates Langerhans cell and antibody production, markers of definitely immune activation (Frankenburg et al., 2007).

In a phase I/II trial for melanoma vaccine comprising six melanoma-associated peptides (MAGE proteins, MART-1/MelanA, gp100, and tyrosinase), patients' follow-up was performed using the *in vitro* proliferation of CD4+ lymphocytes. After vaccination, the monitoring of a good response was marked by an increased proliferation of T cells to relevant peptides in over 80% of patients correlated with good clinical response as well (Slingluff et al., 2008). Another study enrolling stage III/IV melanoma patients showed the data regarding patients vaccinated with Melan-A/Mart-1 peptide and Klebsiella outer membrane protein p40 as an adjuvant. In this trial the therapy was monitored by *ex vivo* analysis of Melan-A/Mart-1 specific CD8 T cells. Increased percentages of T cells, memory/effector T cell differentiation, positive IFN-gamma and antibody responses to p40 were observed in all patients and positive clinical response in half of the treated patients (Lienard et al., 2009).

## 3. Immune-therapy – *Ups and downs*

Each of the immunosuppressive mechanisms that underlie cutaneous melanoma development can be a target for clinical manipulation and it is obvious that immune–related parameters are useful in immunotherapy monitoring of the melanoma.

### 3.1 Immunomodulatory antibodies

Therapeutic antibodies induce cellular/complement-dependent cytotoxicity against tumour cells, or can modify immune responses by blocking the inhibitory signal pathways or stimulating the excitatory signal pathways.

The surface molecules involved in this type of therapy are CD28, CTLA-4 (CD152), Toll-like receptor and many more. Both clinical and preclinical data indicate that CTLA-4 blockade using anti-CTL-4 antibodies results in direct activation of CD4+ and CD8+ effector cells in melanoma. Immune cells as parameters for immune-therapy efficacy should be evaluated in terms of progression-free survival and overall survival.

In a very comprehensive recent paper (Joel et al., 2010) it was demonstrated that the receptors for the Fc region of the antibodies Fc$\gamma$R (Fc$\gamma$RIIB) expressed by the tumour act as „decoy receptors", binding the IgGs that have an anti-melanoma action. Through this mechanism, the Fc recognition by the effector cells is hindered and the tumour escapes the immune effectors and can evolve toward metastasis. This group demonstrated that Fc$\gamma$RIIB inhibits *in vitro* antibody dependent cell cytotoxicity. It seems that Fc$\gamma$R suffer a selection process during the metastasis and that if the cutaneous melanoma evolves, their expression increase in the liver or in the lymph nodes. The FcR action is described in its dual mode as follows: Fc$\gamma$RIIB1 are not detected in melanocytes and have a low expression in primary tumours, while Fc$\gamma$RIIB1 is highly expressed in metastatic tumours in spleen and lymph nodes (Cassard et al., 2008). Knowing the potential of these receptors to modulate the immune response, therapeutic antibodies with an optimized Fc region can trigger an increased Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), Antibody Dependent Cell Phagocytosis (ADCP) and Complement Dependant Cytotoxicity (CDC). The authors propose (Joel et al., 2010) to lower the Fc binding capacity to the Fc$\gamma$RIIB and to increase Fc binding to Fc$\gamma$RIIIA and Fc$\gamma$RI. It is of high probability that these improvements could trigger an enhanced efficiency of therapeutical anti-melanoma antibodies.

### 3.1.1 Targeting cytotoxic T-lymphocyte antigen-4

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is expressed by activated T cells and a subset of regulatory T cells being a co-inhibitory molecule. Its physiological role is to maintain an immune homeostasis by limiting T cell responses and inducing tolerance to self (Yuan et al., 2008). CTLA-4 has binding affinity for the B7 surface molecules of antigen-presenting cell (APC), its affinity exceeding that of CD28. The binding induces T-cell anergy and inhibits secretion of mainly IL-2. When CD28 is bound, costimulatory pathways are triggered and T-cell proliferation and IL-2 production are enhanced (Weber, 2008). Therefore, this type of cell has a crucial negative role in the development of cutaneous melanoma.

Antibodies targeting CTLA-4, in their therapeutical form of ipilimumab and tremelimumab, have proven good clinical results. From early-phase clinical trials anti-CTLA-4 antibodies showed good results in melanoma and manageable toxicities. Then in phase II overall and long-term survival were increased in combined or in individual therapies (Tarhini et al., 2010). Recently published results from a phase III clinical trial (ClinicalTrials.gov number, NCT00324155) (Robert et al, 2011) performed in several centres and institutes showed that in untreated metastatic melanoma overall survival of patients receiving ipilimumab plus dacarbazine was significantly longer compared to the other arms of the clinical trial. Side effects did not occur in the ipilimumab-dacarbazine group. This year, FDA approved ipilimumab and it was considered the „major breakthrough" in cancer immunotherapy.

Side effects in anti-CTLA-4 therapy are named "immune-related adverse events" (irAEs). IrAEs mainly include digestive related negative effects, dermatitis, hepatitis and endocrinopathies, among other occasionally reported ones. It is clear that irAEs are the direct consequence of CTLA-4 blockade but can be managed by the physician (Phan et al., 2008; Di Giacomo et al., 2010).

The markers involved in this type of immunotherapy are several and compile both soluble/circulatory and tissue related groups. Therefore, anti-CTLA-4 treatment was monitored through Treg evaluation (Menard et al., 2008). After this therapy, the effector and memory CD4+ and CD8+ T-cell pool and TCR-dependent T-cell proliferation were restored. In this case, free survival and overall survival were directly correlated with the resistance of peripheral lymphocytes to Treg-inhibitory effects (Menard et al., 2008). The authors state that the biological activity marker of memory T-cell resistance to Treg resulting from anti-CTLA-4 treatment is a good efficacy marker (Langer et al., 2007). At the tissue level, after anti-CTL4 treatment diffuse intra-tumoral infiltrates of CD8+ T cells correlated with good clinical outcome of the patients. Moreover patients with regressing tumours had an increased frequency of CD8+ cells with/without a concomitant increase in CD4+ cells (Neagu et al., 2010).

Immunomodulatory therapy in melanoma needs thorough efficacy monitoring in both circulatory and tissue immune markers.

## 3.2 Dendritic cell therapy

Skin's immune system has several cellular components, mainly regulatory T cells, natural killer T cells (NKT), and distinct subsets of immature and mature dendritic cells (DCs). All these cellular components comprise the immunosuppressive network (Rabinovitch et al., 2007). DCs are more therapy tools than actual immune-markers. The reason is that DC release large quantities of dexosomes, their function being the transfer of antigen-loaded MHC class I / II and other associated molecules, to naive DC potentially leading to the amplification of the cellular immune response (Delcayre et al, 2005). Few years ago, using these DC "products" – dexosomes, two phases I clinical studies were published. In these studies, the monitoring was performed using T and NK cells as markers for cellular immune responses (Escudier et al., 2005; Hao et al., 2006). Dexosomes (Dex) are stable and carry defined proteins and lipids that can be standardized. The authors report that they could generate 10 vaccines of NK cell-stimulating Dexosomes. Using these dexosomes, melanoma patients can be boosted for DC-mediated T- and NK-cell responses (Viaud et al., 2010).

One of the first pilot trials using DC pulsed with autologous tumour lysate has shown that among all the advanced cancer patients entering the study one melanoma patient with extended metastases had a partial response lasting 8 months, while seven patients were stable for more than 3 months, and 7 had progression of the disease. Dendritic cells immune-therapy can induce a cell-mediated antitumour immune response in patients giving good clinical outcome (Mayordomo et al., 2007). In another study, isolated DCs were activated with CD40L, loaded with antigenic melanoma peptides and then injected into patients with resected melanoma. The patients were monitored for antigen-specific immune responses, namely skin reactions to peptides alone or peptide-pulsed DCs and circulating T-cells responses (Davis et al., 2006).

When vaccinated with DC, clinical investigators monitor the efficacy by the presence of vaccine-related tumour antigen-specific T cells in delayed type hypersensitivity (DTH) skin biopsies, in correlation to the clinical outcome. Punch biopsies taken from positive DTH sites proved clusters of CD2+ and CD3+ infiltrating cells, out of which over 50% were CD4+, the rest being CD8+ T cells (Aarntzen et al, 2008).

It seems that there is an effervescent period of publication on dendritic cell therapy in cutaneous melanoma, but all the papers underlie some crucial points in using DC therapies. When using *ex vivo* generated DC, the effective migration of DCs to the T-cell areas in the lymph node is necessary; therefore, adding inflammatory cytokines would be beneficial (Aarntzen et al, 2008). Moreover, the addition of synergistic immunomodulatory agents to enhance immunogenicity (Erdmann & Schuler-Thurner, 2008) can enhance the clinical response.

Monitoring the DC therapy comprises several steps to be taken. The inoculated DCs have to be monitored for their migratory potential to the tumour site. It is commonly agreed on the efficacy monitoring of the vaccine by detecting T-cell responses in vaccinated patients using biopsies derived from DTH sites in good correlation with the clinical outcome in melanoma patients (Aarntzen et al, 2008).

## 3.3 Vaccines with melanoma antigens

The processes that induce effective antitumour immunity by cancer vaccines implie several events: antigen delivery to antigen-presenting cells, migration of dendritic cells that carry the processed antigen to draining lymph nodes, antigen presentation to circulating T cells that enter the lymph nodes through the high endothelial venules, expansion of antigen reactive T-cells in the lymph nodes, dissemination of specific T-cells to tumour site, and tumour destruction by activated tumour-reactive T-cells (Slingluff Jr et al., 2008).

The vaccines that were built until now against melanoma are based on strategies for activating the protective immunity by Tregs depletion and blockade of T-cell inhibitory molecules such as CTLA-4. All these approaches have developed multiple new proteomic/genomic tools to monitor immune responses in vaccinated patients. Several published clinical trials emphasized that melanoma antigen vaccination should be monitored using peripheral immune cells as the principal parameter. Some types of vaccination with melanoma-associated antigens will be highlighted in this section.

Developing cancer vaccines implies complex monitoring of the immune responses. Firstly, the efficacy of the vaccine in inducing/augmenting a specific T-cell response has to be tested. Secondly, the spontaneous tumour-directed immune responses, the functional characteristics of T-cell responses and, last but not least, the relationship between immune monitoring assay results and clinical end points have to be evaluated (Neagu et al, 2010).

Giving a three decades view on cutaneous melanoma vaccination, important points have been highlighted (Sondak et al., 2006). Some of the vaccines, in late stages of clinical trials were discontinued due to regulatory and commercialization technicalities. Vaccines from autologous samples are reduced to patient groups still having accessible, surgically removable and sufficient tumour tissue for a complete treatment. Vaccines of allogeneic source can have common antigens, but they can lack the actual individual molecular particularities of an autologous tumour (Sondak et al., 2006).

In a review of the National Cancer Institute's experience with 440 patients (mostly melanoma patients) receiving 541 different vaccines, only 4 complete and 9 partial responses were seen, for an overall response rate of 3% (Rosenberg et al., 2004). A randomized comparison of a peptide-pulsed dendritic cell vaccine to the cytostatic agent dacarbazine (DTIC) in patients with advanced melanoma showed similarly low levels of objective response for the vaccine; the median time to progression and a median survival was relatively the same when DTIC alone was used (Rosenberg et al., 2004).

One of the largest randomized clinical trials involving vaccines for the adjuvant therapy of melanoma was published several years ago. The trial compared a GM2 ganglioside vaccine (GMK vaccine, Progenics, Tarrytown, NY) to high-dose IFN in the adjuvant therapy of patients with resected melanoma at high risk of recurrence. In this trial, antibody responses to the vaccinated ganglioside were achieved by many patients. Unfortunately, the overall results were far more better for IFN treatment in both relapse-free and overall survival (Kirkwood et al., 2001, 2004) and the clinical trial was stopped. Then two randomized trials of a polyvalent whole cell melanoma vaccine (Canvaxin, CancerVax, Carlsbad, CA) were performed on patients with resected stage III and IV melanoma (an even higher-risk population than in the aforementioned ganglioside study); they were also stopped early because of a poor vaccine efficacy (Cancer Vax Corporation, 2005).

A different polyvalent melanoma vaccine phase III trial (Melacine, Corixa, Seattle, WA) was conducted in patients with resected stage II melanoma. Analyzing the data, an effect of the vaccine on relapse-free survival (Sosman et al., 2002) and overall survival (Sondak et al., 2004) for vaccine-treated patients expressing HLA antigens (HLA-A2 or HLA-C3) was obtained. This subset of patients (over half of the enrolled patients) was intended to be followed in order to verify the results, probably a follow-up of several years. In the end, the manufacturer of this vaccine simply decided not to support the development of Melacine.

### 3.3.1 Autologous tumour vaccines

Autologous vaccines derived from the patient's own tumour have the main advantage of containing unique or rare tumour antigens that develop during mutational events. Autologous tumour vaccines are designed as appropriately HLA-matched for optimum antigen presentation to T lymphocytes host. Until recently, no autologous melanoma vaccine had ever been successfully tested in a phase III clinical trial. A phase III clinical trial

using heat shock proteins (HSP) extracted from autologous tumour (Oncophage, Antigenics, New York, NY) was developed. In the cell, these proteins act as ''chaperones'' for peptide antigens and have the potential to present tumour antigens to the immune system (Rivoltini et al., 2003; Lewis, 2004). Even though the preliminary results indicated no statistically significant effect for the heat shock vaccine compared with the physician's choice of therapy, an intriguing observation of this trial is that the group of patients with M1 stage of disease treated with the vaccine, lived longer than those receiving other therapy, although not significant (Antigenics press release, 2005).

### 3.3.2 Allogeneic vaccines

A different type of vaccine developed in parallel with the previously presented one, are the allogeneic vaccines. These are composed of intact or modified melanoma cells from other patients selected for the presence of shared antigens found on a large percentage of melanomas. Compared with autologous tumour vaccines, the allogeneic ones have significant advantages in terms of availability for patients in all stages of the disease, and providing the capability to administer multiple vaccinations over an extended period. They may also be more naturally recognizable by the patient's immune system than an autologous cell preparation. However, they may lack unique or rare antigens that could be important antigenic targets in any given patient's melanoma.

Canvaxin is a polyvalent irradiated melanoma auto/allo vaccine, originally developed by Dr. Donald Morton and commercially developed by CancerVax in partnership with Serono (Geneva, Switzerland) (Hsueh & Morton, 2003; Motl, 2004). This vaccine has been studied in two multi-centre randomized phase III trials in patients with resected stage III and IV melanoma. Recently, the Data Safety Monitoring Board overseeing these two studies determined that the trials were unlikely to provide efficacious results and the protocols were discontinued. The authors' state that there are multiple reasons for a vaccine to prove its usefulness in earlier stage patients compared with the ones diagnosed in later stage disease (Salazar & Disis, 2005). Firstly, increasing evidence suggests that tumour progression leads to increased immunosuppression mechanisms directly mediated by tumour cells and/or by their microenvironment. The immune suppression is also induced by the presence of increasing numbers of Tregs (Viguier et al., 2004). Moreover, increasing numbers of tumour cells are increasingly likely to express antigenic heterogeneity that would limit the ability of an induced immune response to completely eradicate the tumour (Riker et al., 1999). Finally, in a patient with a relatively high residual tumour burden (patients stage IV melanoma), tumour progression could readily occur during the period required for induction of an immune response post-vaccination. In that case, the vaccination "failure" conclusion is not true as it did not have the chance to even naturally develop. To overcome this, it has been proposed that measuring the T-cell response from lymph nodes draining the cutaneous sites of vaccination could be a sensitive assessment of immunogenicity developed by a melanoma vaccine administration (Slingluff Jr et al., 2008).

Surprisingly, in some studies, induction of T-cell responses is not reliably associated with clinical tumour regression. As an example, vaccination with a modified gp100 peptide led to detectable CTL responses in the peripheral blood in over 90% of patients, but no clinical tumour regressions were observed. In another section of the same study, in which patients were received in addition high-dose of IL-2, there was a noticeable clinical tumour

regressions in 41% of patients, but T-cell responses were registered in only a small minority of patients (Rosenberg et al., 1998). Nevertheless, optimization of cancer vaccines will benefit from complex immune monitoring in multiple lymphoid compartments. Critical compartments for immune monitoring are comprising the lymph node as the site of T-cell response induction, the circulating peripheral blood lymphocytes (PBL) and the sites of primary and metastatic tumour as well (Slingluff Jr et al., 2008). Cancer vaccine clinical trials meant to induce T cell - mediated immunity are evaluated routinely by measuring the immune response in the PBL but not in the other two compartments. Some murine and human studies suggest that, even after a single immunization, epidermal Langerhans cells migrate and mature to draining nodes within hours, with a peak of T-cell accumulation in the draining lymph nodes at day 5–10 (Macatonia et al., 1987; Rosato et al., 1996; Yoshizawa et al., 1991). Thus, it has been hypothesized that evaluation of T-cell responses in the draining lymph node would permit a more sensitive measure of immunogenicity than evaluation of T-cell responses in the peripheral blood alone (Slingluff Jr et al., 2008). From patients enrolled in clinical trials of experimental melanoma peptide vaccines, sentinel immunized nodes (SIN) were harvested a week after the third vaccine round, in order to identify the peak time of T-cell accumulation. T-cell responses to defined peptides were detected 25% more often in the SIN compared with PBL. Combining the evaluation of PBL and SIN leads to increased sensitivity in detecting immunogenicity to nearly 75%. Moreover, the T-cell responses in a SIN were detected at a high level, when they were either absent or at low level in both blood and the involved lymph node prior to vaccination (Yamshchikov et al., 2001). This finding supports the continued evaluation of the SIN for monitoring T-cell responses to melanoma peptide vaccination.

The challenges in melanoma vaccination are extensive, but they can be surmounted by a thorough understanding of immune mechanisms and proper guided clinical trials (Sondak et al., 2006). We can add to these relevant conclusions that careful monitoring of anti-tumour T-cell responses, directed toward tumour peptides/proteins/antigens, developing complex monitoring of the patient's immune responses and establishing an intricate relation with clinical end points would definitely add important value to vaccination treatment in melanoma.

### 3.4 Immune-therapy with cytokines

Using combinations of chemotherapy with cytokines, clinical parameters of patients were improved. All the promising new therapeutic agents have to be related to identification of predictors of response leading towards personalized therapy. IL-2, IFN, IL-12 and other therapeutical combinations were used and monitoring their efficacy was performed using immune markers.

Melanoma is resistant to standard chemotherapy, having a response rate for any single agent or combination of agents of 15% - 25%. Using combinations of chemotherapy, IFN and IL-2 the response rate was improved, but with no clear effect on overall survival (Chapman, 2007).

### 3.4.1 IL-2

Particularly IL-2 was involved in cutaneous melanoma immune-therapy in the last 20 years. Several years ago, it has been published that over the course of IL-2 based immunotherapy,

difficulties associated with the monitoring of anti-tumour immune responses arise (Andersen et al., 2003). A couple of years ago, despite promising phase II data, phase III studies have failed to show meaningful clinical benefit for the combination of cytokines with cytotoxic chemotherapy (Andersen et al., 2003) and now the effort is focused on identifying predictors of therapeutic response, therefore an increased efficacy in term of patients benefit (Atkins, 2006). Following high-dose IL-2 administration, the number and frequency of regulatory T cells (Tregs) were monitored in patients with progressive disease and the values returned to normal in patients with objective clinical responses (Cesana et al., 2006) or the monitoring was performed analyzing pSTAT5 in patient's PBMC (Varker et al., 2006). Investigating patients with intralesional IL-2 treatment authors pointed out an increase in the CD4+/CD8+ ratio and a rise in the percentage of CD25+ cells in the CD4+ population, the majority of this population being activated T cells. The local IL-2 is able to induce a systemic beneficial immunological effect (Green et al, 2008).

There are several studies that combine IL-2 with vaccines or growth factors. Thus when combining IL-2 with Melan-A-specific CTL, an anti-tumour response was elicited and monitored by an elevated frequency of circulating Melan-A + T cells, an increase in eosinophils and a selective loss of Melan-A expression in lymph node metastases without major adverse effects (Makensen et al., 2006). Another phase II clinical trial using a combination of IL-2 with GM-CSF (Elias et al., 2005), showed the DC activation, and an increased IL-2 receptor expression on T cells. The treatment was intended to high-risk melanoma patients and no significant side-effects were registered. Continuing their work (Elias et al., 2008) the same group reported the clinical benefit of using GM-CSF and IL-2 with or without autologous vaccine in patients with resected melanoma. Just recently published, a phase III clinical trial (Schwartzentruber et al., 2011) with melanoma patients diagnosed in stage III and IV (ClinicalTrials.gov number, NCT00019682) receiving IL-2 and gp100 peptide vaccine showed that the response rate was improved and progression-free survival increased when combining these two immune therapeutical agents in comparison to IL-2 administered individually.

### 3.4.2 IFN

IFN-alpha is one of the most used immune-therapy agent and IFN treatment was demonstrated to significantly prolong relapse-free survival of patients diagnosed in stage IIB-III melanoma. When these patients were subjected to IFN-alpha2b therapy, a significant decrease of serum levels of immunosuppressive / tumour angiogenic/growth stimulatory factors (Vascular Endothelial Cell Growth Factor - VEGF, Epidermal Growth Factor - EGF and hepatocyte growth factor- HGF), increased levels of anti-angiogenic IFN-gamma inducible protein 10 (IP-10) and IFN-alpha along with their good clinical outcome (Yurkovetski et al., 2007) have been noted. As therapeutic monitoring tools, gene microarray analysis of the transcriptional profile of peripheral T cells, NK, and monocytes as a response to IFN-alpha therapy was demonstrated. Authors point out that the transcriptional profiles of PBMCs from IFN-alpha treated patients may be a useful predictor of the *in vivo* response of immune cells to IFN-alpha immunotherapy (Zimmerer et al., 2008).

In a prospective neoadjuvant trial for IFNalpha2b, in which tissue samples were obtained before and after therapy, double immunohistochemistry for pSTAT1 and pSTAT3 was done. The pSTAT1/pSTAT3 ratios were augmented by IFNalpha2b both in melanoma cells and in

lymphocytes. Antigen peptide transporter 2, involved in the transport of various molecules across extra- and intra-cellular membranes (TAP2), was augmented by IFNalpha2b (but not TAP1 and MHC class I/II). The authors prove that IFNalpha2b significantly modulates the balance of STAT1/STAT3 in tumour cells and host lymphocytes, mechanisms that lead to the up-regulation of TAP2 and an augmented host antitumour response. The baseline of pSTAT1/pSTAT3 ratio in tumour cells may serve as a useful predictor of the therapeutic effect of IFN (Wang et al., 2007).

### 3.4.3 IL-12

IL-12 as therapy agent has both immunoregulatory function and anti-tumour activity mediated by stimulation of T and NK effector cells. Authors propose IL-12 as a therapeutical agent using a protocol to pre-screen melanoma patients for IL-12Rbeta2 expression to stratify the potential responders, administrate non-toxic doses and target IL-12R+ tumour cells, by local administration or injection of IL-12 fused to an antibody specific to tumour cells (Cocco et al., 2009). This interleukin is recently highly involved in cutaneous melanoma genetic therapy (see section).

Cytokines are the molecular messengers that control almost any physiological function of immune cells and are involved in the neoplastic transformation of normal cells. Cytokines are involved in immune recognition, proliferation and effector functions of immune cells. From the beginning of the research in the biomarkers discovery field, cytokines and their receptors were searched in association to diagnostic, prognostic and/or therapy. Some of the cytokines became therapeutic agents, such as IL-2 and all the recent papers on the clinical efficacy of cytokines state that this type of immune-therapy gets its best place in therapeutical combinations.

### 3.5 Targeting innate-immunity

Toll-like receptors (TLR) are involved in the regulation and activation of both innate and adaptive immunity (Takeda et al., 2003), activating therefore the anti-tumoural activity of lymphocytes. In a recently published experimental model, synthetic agonists for TLR 3 and 9 activated T cells improving their anti- melanoma activity (Amos et al., 2011) action that was supposed to take place *via* IFN-γ production. Moreover, TLR agonists can be good adjuvants in immune therapy.

In a phase I clinical study with synthetic agonists of TLR 9 in advanced stages melanoma, a complete regression was obtained in one of the 5 tested patients. The immune monitoring was performed testing the serum concentrations of IL-6, IP-10, IL-12p40, TNF-alpha. The immune treatment proved good clinical response metastatic melanoma (Hofmann et al., 2008). In a case study with a long period of treatment with TLR 9 agonist, it was reported that this compound induced strong tumour-specific immune response (Stoeter et al., 2008).

A phase II clinical study using TLR 7 agonist administered in patients with metastatic melanoma published its results several years ago and showed a prolonged stabilization of the disease. Monitoring was performed measuring IFN and IP-10 and peripheral blood immune cells. Out of 13 patients, 4 had disease stabilization and one patient had a partial remission. CD86 expression on monocytes, IFN and IP-10 increased in most patients upon therapy (Dummer et al., 2008).

Combining DTIC with the topical application of a TLR agonist in an experimental model decreased the rate of tumour evolution and enhanced animal survival. In this study authors stated that the topical application of TLR agonist is more efficient than intratumoural inoculation. Immune monitoring showed that the antitumour effect is both CD4+ and CD8+ dependent, the B220+CD8+ subset of dendritic cells and NK1.1+ CD11c+ cells within the tumours were enhanced, thus a more effective immune response against melanoma (Najar & Dutz, 2008). When using another combination, namely topical application of TLR agonist and intra-tumoural inoculation of DCs a good immune response was obtained in an experimental model. This therapeutical combination resulted in a significant tumour regression and a therapeutical approach to be considered (Lee et al., 2007).

In patients topically treated with TLR-7 agonists, the tumour infiltrate showed an increased population of CD3+, CD4+ and CD8+ T cells, cytotoxic T cells (TIA-1+, granzyme B+) and DC CD 123+ (Wolf et al., 2007).

Overall, agonists that address Toll-like receptors act through enhancing anti-tumoural cellular activity and systemically by an increased production of cytokines and chemokines.

## 3.6 Gene therapy

In cutaneous melanoma the genetic patterns can be the starting point for developing new molecular targets for therapeutic intervention and diagnostic biomarkers for melanoma (Su et al., 2009). In melanoma several altered tumour suppressor genes (p53, CDKN2A, Ras) are frequently found in primary and metastatic melanomas and are incriminated for the invasive and metastatic potential (Lalou et al., 2010). Gene therapy has been a rapidly expanding field in cancer in the last years and evermore in cutaneous melanoma. In this disease, the gene therapy tendency is the manipulation of interleukin genes. Thus, this year, in mouse experimental models, the results of an interesting attempt of gene therapy have been published. DCs genetically altered to express IL-12 can induce cytotoxic CD8+ T cell antitumoural response. The immune-intervention has good therapeutical potential as peptides from tumour-associated stromal antigens can be recognized by peripheral blood CD8+ T cells from melanoma patients after *in vitro* stimulation (Zhao et al., 2011). In addition, this year, in IL-10-knockout mouse model it was demonstrated that these mice are more resistant to melanoma development, IL-12 and IFN-γ being secreted in increased quantities (Marchi et al., 2011). The authors have constructed a plasmid containing the murine IL-10 receptor. When treating the animals with this plasmid, their survival time extended post-inoculation with melanoma cell lines. Associating this gene therapy with IL-12 gene therapy a beneficial therapeutical response could be obtained.

Several years ago, the first report on the clinical safety of *in vivo* particle-mediated epidermal delivery (PMED) as a cancer gene therapy was published (Ryan et al., 2007). PMED is transferring to specific location genes without viral carrier, the procedure has no viral potential risks, presents multipotency in cell transfections and multi gene transfer. For this therapy, authors have chosen the genes for gp100 and GM-CSF. Using PMED, they delivered these genes to patients with melanoma. The immune monitoring of treated patients was multifaceted: a complex array of auto-antibodies, rheumatoid factor, peripheral blood lymphocytes CD69+ expression, CD1a+ dendritic cells in biopsies and so on. The first reported clinical trial transferring cDNAs for gp100 and GM-CSF to patients concluded that the procedure is safe and that it can induce transgene expression in the treated skin.

Gene therapy's future and clinical benefit rely on the genotypic pattern of the patients. Therefore, several years ago it was reported on a large number of patients that the presence of chemokine receptor 5 gene (CCR5Delta32) polymorphism in patients diagnosed in advanced stages subjected to immunotherapy can indicate a decreased survival and, moreover, this gene pattern can be useful in selecting patients that have the best clinical outcome when subjected to immune therapy (Ugurel et al., 2007).

Gene therapy has still to overcome clinical compliances and prove its lack of deleterious side-effects in order to conquer the immune therapy field in melanoma.

## 3.7 Epigenetic therapy

The investigations regarding cancer alterations have gained, in the recently years, a new thrilling way for defining novel clinical settings and therapies, namely the epigenetic research (Selcuklu & Spillane, 2008). In contrast with genetic events involving irreversible changes in the genome (e.g. deletions), epigenetic changes imply alterations in gene expression without modifications of the primary DNA sequence. In addition, an epigenetic profile should be concurrently self-perpetuating, heritable and reversible (Bonasio et al., 2010; Howell et al., 2009). It is now accepted that malignant transformation of melanocytes occurring in cutaneous melanoma, is greatly accompanied by epigenetic modifications (Sigalotti et al., 2010). Such "epimutations" could be turned on in defining new therapies considering the high incidence, poor prognosis and resistance of metastatic melanoma to conventional therapies (Howell et al., 2009).

The epigenetic modifications of mammalian DNA involve at a glance cytosines methylation from CpG dinucleotide islands, deacetylation of DNA-binding histones and noncoding RNA profiles (miRNA) (Esteller, 2007). However, it is still under debate if post-translational modifications of histones and miRNAs signature are considered epigenetic events; in addition, some authors sustain that methylation of cytosines C-5 is the only clearly identified epigenetic modification of DNA in mammalian cells (Howell et al., 2009; Bird, 2002).

The *DNA methylation* process is under the balance of complex equipment consisting of DNA methyltransferases (DNMTs) responsible for genome-wide DNA methylation patterns, and methyl-CpG-binding proteins dedicated to deciphering the methylation profile. A normal cellular development is characterized by a properly specified DNA methylation outline but any deviation from this established pattern is one of the important characteristics for the majority of human cancers. As a general characteristic, neoplastic transformation is accompanied by complex disturbances of the genomic DNA methylation homeostasis. There is a variable and still relatively unknown extent of methylation range, (Howell et al., 2009) with both gene-specific hypermethylation and genome-wide hypomethylation as a consequence (Sigalotti et al., 2010; Jones & Baylin, 2007).

Histones are DNA-binding proteins mostly responsible for packaging of DNA into chromatin (Bird, 2002) and DNA segregation from the transcriptional machinery (Jones & Baylin, 2007). The acetylated histones at lysine residues are associated with transcriptionally active DNA whereas *histone deacetylation* means transcriptionally inactive DNA.

Histone deacetylases (HDACs) are involved in the development of various tumours through the alteration of normal gene expression and inhibition of DNA repair machinery (Hadnagy

et al., 2008) thus representing one of the main epigenetic targets in cancer therapy (Howell et al., 2009). Despite the substantial information regarding the DNA methylation pattern in cutaneous melanoma, the existing data for abnormal post-translational modifications of histones are limited and someway indirect; these resulting from subsequent observations post-therapy with pharmacologic inhibitors of histone-modifying enzymes (i.e., HDACi) (Sigalotti et al., 2010).

Although relatively new entry and still not fully accepted as steady players in the epigenetic research of cancer, *microRNAs* gained a special attention in melanoma studies. MicroRNAs (miRNAs) are endogenous small noncoding RNAs (≈22-nt) with a regulatory nature upon gene expression by inhibiting mRNA translation (Bartel, 2004) or by causing mRNA degradation (Sood et al., 2006). Nowadays there are hundreds of confirmed miRNAs in humans (Molnár et al., 2008) and current evidence is emerging to the important role of particular miRNAs in human cancer epigenetic pathogenesis (Howell et al., 2009). Thus, data regarding miRNA deregulation in melanoma is rather limited, the majority of studies about miRNA involvement in tumourigenesis are completed especially on melanoma cell lines. However, the altered pattern of miRNA in melanoma seems to be related with apoptosis (miR-15b), cell cycle (miR-193b) and invasion/metastasis (miR-182) (Satzger et al., 2009; Chen et al., 2010; Segura et al., 2009).

Taking into account the epigenetic deregulation imprinting described so far in cutaneous melanoma, the intervention therapy from this point of view is focused on the DNMT and/or HDAC inhibitors. DNMT inhibitors (Lyko & Brown, 2005) are consisting of cytosine analogues and thus mimic the substrate for DNMT. 5-azacytidine (Vidaza), 5-AZA-CdR (Dacogen), S110 and zebularine (Yoo et al., 2007) are among the most accepted cytosine analogues exploited in melanoma *epitherapeutics*. Such analogues are incorporated in genomic DNA during the S-phase of the cell cycle and upon methylation a covalent bond between modified DNA and enzyme are formed, resulting the inactivation of DNMT, cellular depletion of enzyme and, therefore, the demethylation of DNA (Schermelleh et al., 2005). Along with DNMT inhibitors, histone deacetylase inhibitors (HDACi) have been hailed as a powerful new class of anticancer drugs. One of these inhibitors, trichostatin A (TSA), is thought to promote immune responses against tumours by an epigenetic control of cell cycle progression in G1 and G2-M phase, leading the growth arrest, differentiation or apoptosis (Setiadi et al., 2008). TSA treatment enhance the expression of some components of the antigen processing equipment and of MHC class I molecules on the carcinoma cell surface, leading to an enhanced vulnerability to killing by antigen-specific CTLs. Thus, immunotherapeutic anti-tumour approaches for eliminate cell cancers could be perceived differently by these novel insights defined by epigenomics (Setiadi et al., 2008).

## 4. Summary

Immune-therapy in cutaneous melanoma has to evaluate antitumour efficacy, autoimmunity, and reconstitution of a functioning immune system. Clinical studies and experimental research have gathered significant results to conclude that complex vaccines, combination of different therapeutical approaches, dendritic cell–based therapies in conjunction with co-stimulatory molecules, are superior to conventional immunization protocols in the induction of tumour-specific immune responses.

| Target Agent | Immune markers therapy monitoring | Comments | Stage | Ref. |
|---|---|---|---|---|
| *Immunomodulatory antibodies* | | | | |
| CTL-4 | peripheral CD4+ CD8+ T, CD8+; tumour infiltrates | Progression-free and overall survival | FDA appr. | Robert et al., 2011 |
| *Anti-TLR synthetic agonists* | | | | |
| TLR 7 | IFN, IP-10, peripheral blood immune cells, intratumoral infiltrates | Stabilization of the disease | Phase II | Dummer et al., 2008 Wolf et al., 2007 |
| TLR 3 TLR 9 | anti-tumoral T cells, IFNγ | Good clinical response metastatic melanoma | Phase I | Amos et al., 2011 Hofmann et al., 2008 Stoeter et al., 2008 |
| *Dendritic cell* | | | | |
| Dex | T and NK cells | Activation of T-cells and NK cells | Phase I | Escudier et al., 2005 Hao et al., 2006 Viaud et al., 2010 |
| DC | Cell mediated anti-tumoral immune response DTH reaction | Good clinical outcome | Pilot Phase I Phase II | David et al., 2006 Mayordomo et al., 2007 |
| *Vaccines with melanoma antigens* | | | | |
| HSP | T cell stimulation | Longer survival | Phase III | Rivoltini et al., 2003, Lewis, 2004 |
| gp100 peptide | T cytotoxic activation | Tumour regression | Phase III | Schwartzentruber et al., 2011 |
| *Cytokines* | | | | |
| IL-2 | Number and frequency of Tregs | Good clinical responses | FDA appr. | Cesana et al., 2006 |
| IL-2 and Melan-A-specific CTL | Circulating % of Melan-A + T cells, eosinophils | Good clinical responses | Phase II | Makensen et al., 2006 |
| IL-2 and gp100 vaccine | CD4, CD8, Tregs | Response rate and progression-free survival increased | Phase III | Schwartzentruber et al., 2011 |

| IFN-alpha2b | IP-10, VEGF, EGF, HGF, peripheral NK, T and monocytes Tissue pSTAT1/pSTAT3 | Prolong relapse-free survival Adjuvant therapy Predictor of the therapeutic effect | FDA appr. | Yurkovetski et al., 2007 Wang et al., 2007 |
|---|---|---|---|---|
| IL-12 | Stimulation of T and NK | Marker for stratifying the potential responders | Phase I/II | Cocco et al., 2009 |
| *Gene therapy* | | | | |
| DC express IL-12 | CD8 anti-tumoral response | Extended survival time | Animal models | Zhao et al., 2011 |
| Plasmid - IL-10R gene | IL-12 and IFN secretion | Extended survival time | Animal models | Marchi et al., 2011 |
| *Epigenetic immune-therapy* | | | | |
| TSA | CTL antigen-specific | Decreased invasion and cell cycle arrest | Cellular models | Setiadi et al., 2008 |

Table 1. Immune-therapies and immune efficacy markers in cutaneous melanoma
(FDA appr. = FDA approval; Dex.= Dexosomes)

The immune parameters that efficiently monitor the evolution of the patients subjected to immune therapy comprise circulatory and tissue immune-related components. Hundreds of opened/approved clinical trials testing immune-therapy efficacy in cutaneous melanoma are using the immune monitoring of enrolled patients. All the authors agree that when using immuno-therapeutic agents in cutaneous melanoma, the systemic immune response is to be monitored (for a "at a glance" perspective see Table 1).

## 5. Conclusions and future perspectives

All the recent studies and research regarding melanoma immunotherapy have demonstrated that complex vaccines and the combination of different approaches, such as the use of dendritic cell vaccines in conjunction with co-stimulatory molecules, are superior to conventional immunization protocols in the induction of tumour-specific immune responses. Evaluation of clinical parameters and patient's-specific real-time immunological parameters may identify the most effective immunotherapy in melanoma. There are at least hundreds of approved clinical trials in immune-therapy related to cutaneous melanoma and the majority is foreseeing the utility, if not actively using, immune monitoring of enrolled patients.

Besides the approved cytokines in the immune-therapy of cutaneous melanoma, antibodies targeting CTLA-4 have demonstrated their feasibility, safety, and clinical activity and were approved as therapeutic agent in melanoma.

The failure of immune-therapies underlined in various sections probably reside in the immunological profile of the patient, thus the need of finding predictive markers is mandatory in order to detect treatment outcome in melanoma. Molecules that arise in the processes of neoplastic transformation, invasion and metastasis, are interrelated with the

components of the immune responses. Future molecular patterns of cutaneous melanoma will compulsory comprise immune-related molecules. Nowadays forces are gathered to search for immune markers that specifically indicate the disease stage and eventually develop new targets for personalized efficient therapy.

We expect melanoma vaccination to become clinical routine therapy in the very near future. In this respect, a thorough monitoring is foreseen and in the future-to-be biomarkers panel, immune markers will have their foremost role. As the pathophysiology of melanoma is complex it is more convincing that only a combination of markers from genomics, transcriptomics, metabolomics and proteomics can cover the array of processes comprising the development of cutaneous melanoma. In this future set of markers, MHC allele expression in the individual patient's tumour and T-cell mediated immune responses specific for autologous melanoma will be evaluated.

As the incidence of melanoma is steadily increasing, an individualized immunological profile of the patient can reorient/personalize immune-therapy and monitor the efficacy of the treatment approach and the molecular diagnostic of cutaneous melanoma will have important immune components. Although far from being exhaustive, this chapter was intended to overview the main immune therapy possibilities used in the management of cutaneous melanoma patient. In all the stages of cutaneous melanoma development, immune elements are involved; they are associated with tumour initiation, tumour evasion, suppressed immune response and probably many more unknown mechanisms.

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## 7. References

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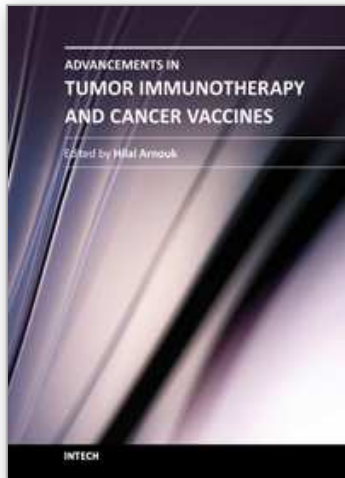
**Advancements in Tumor Immunotherapy and Cancer Vaccines**

Edited by Dr. Hilal Arnouk

Harnessing the potential of the human body's own immune system to attack malignant tumor cells has been the goal of many scientific investigators in recent years, with advances in cancer biology and immunology enabling cancer immunotherapy to become a reality. World-class bench and clinical researchers have joined forces to collaborate and review current developments and trends in cancer immunology for the purposes of this book, and the result is a promising review of contemporary clinical treatments. In each chapter the authors present the scientific basis behind such therapeutic approaches, including cancer vaccines with special focus on prostate cancer, melanoma and novel approaches utilizing both innate and adaptive immune responses.

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