Chapter 9

# New Insights in Cutaneous Melanoma Immune-Therapy — Tackling Immune-Suppression and Specific Anti-Tumoral Response

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Additional information is available at the end of the chapter

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## 1. Introduction

In this chapter the maturity of immune-therapy in cancer, with emphasis on melanoma will be discussed.

The heterogeneity of melanoma tumour regarding primary and metastatic variants will be argued. Therefore the mutational heterogeneity of this type of tumor triggers complex immune-therapy approach. Notions such as *Immune-therapy* will be tackled, meaning targeting immune elements like immune suppression and using immune drugs like monoclonal antibodies against targets that can or not be immune elements. The chapter will end with the importance of designing complex immune-therapies like abolishing the immune-suppression and enhancing the specific anti-tumoral effect.

Physiologically, the immune system can recognize cells that display an aberrant proliferation like neoplasia. The immune system is equipped with cells that can destroy cancer cells them during their early development. Years ago, when the theory of immunoediting was initiated [1], immunosurveillance was defined as a complex pathway that supervises and controls the elimination of transformed self cells/tissue. When the immune system cannot properly control these aberrantly proliferating cells, and the equilibrium is deregulated, tumour cells escape and form a clinically significant tumoral tissue [2].

In cancer immune-therapy, several approaches that aim to start and sustain the immune response and eventually elicit an immunological memory were lately tackled. The therapy armentarium that started the "bumpy road" from bench to bedside comprises cancer vaccines, adoptive T cell therapy, anti-tumor antibodies, immune checkpoint blockade and/or various immune combinations. Some of the conclusions of the last Congress of SITC (*Society for*

*Immunotherapy of Cancer*) is that combining these above mentioned immune approaches with other immunomodulators (e.g. cytokines, cyclic dinucleotides) and/or indoleamine 2,3-dioxygenase (IDO) inhibitors can increase the efficacy of immunotherapy [3] and hopefully replace in the future routine approaches such as chemotherapy/radiotherapy.

Early diagnosed stages of melanoma are resolved mainly by surgery and large margin excision, but for advanced stages, systemic therapies, whether chemotherapy, immune-therapy or combined ones have had very low efficacy. Advanced melanoma remains a continuous clinical provocation for the physicians who use therapeutical approaches with low response rates, unmanageable toxicities, and reduced efficacy.

One of the main *molecular hurdles* in cutaneous melanoma is the heterogeneity of tumors. A tumoral tissue has cells with different characteristics in terms of proliferation, invasiveness and pheno/genotype. In melanoma, aggressiveness has a distinct cellular genotype and in the tumor dynamic development, cells go through several phenotype switching [4]. Specific genetic expression studies have identified more than 100 genes over-expressed in cells with a higher proliferative capacity or in cells that were committed to invade tissues [5].

Recently, a study was published on single melanoma cells and the report shows that there are 114 genes expression that could distinguish the proliferative and invasive phenotype of cells. Among these genes, regulatory networks were found along with genes that encode for pluripotency factor (e.g. POUF51); all these genes were found associated with cell's tumorigenic potential. Authors report that among the regulatory network genes, MITF (microphthalmia-associated transcription factor) is one of the key players in the heterogeneous character of tumour cells populations. Moreover, the heterogeneity of cells depends on the 2D or 3D status of the cell cultures, thus TPBG (trophoblast glycoprotein) is expressed in a melanoma cell line, 501Mel, only in 3D cultures [4]. In the same way, in 1205Lu melanospheres, PI3K/AKT (phosphatidylinositol 3' –kinase/protein kinase B) signaling pathway is enhanced [4], while DAPK1 (death-associated protein kinase 1) expression is decreased in 501Mel experimental tumors, finding that is in line with the hypermethylated gene promoter associated to melanoma [6]. This study emphasizes that when transformed melanocytes start to organize in growing tumors, the heterogeneity of the cellular populations' increases, the tumoral tissue having MITF-low/negative cells [4, 7]. In experimental tumour spheres, cells that grow on the exterior layers have an active proliferation, while in the interior of the tumour, due to hypoxic conditions, cells are arrested in the G1 phase [8]. The overall mechanism is that 3D growth enhances tumour-initiating properties [9].

Cell's heterogeneity is important from the immunological point of view. Tumour heterogeneous tissues have different expression of tumor antigens, thus any type of therapy that addresses only one tumour epitope is proned to have low efficacy. This is the rationale to investigate tumor antigens and evaluate the patient's immune responses prior to any immune-therapy [10].

Extensive studies performed by large groups of researchers and extended networks like *Melanoma Research Networks* established in Europe, Canada, and New Zeeland have provided seminal scientific information regarding melanoma's immune biology. All these insights have

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led to an actual scientific leap by the development of the first immune-therapies that proved efficacy in advanced melanoma treatment. Therefore drugs that aim intracellular pathways such as mitogen-activated protein kinase (MAPK) pathway, or antibodies that aim CTLA4 (cytotoxic T lymphocyte-associated protein 4), or PD1/PD1-L (programmed cell death 1/ programmed cell death 1 ligand) have recently followed the bench to bedside path [11]. Building upon the early success of these therapies, trials involving new classes of drugs and combinations of these drugs are underway [12].

Starting from 2011, metastatic melanoma beneficiated from four new approved drugs, all these drugs proving in clinical trials the improvement of patient's survival. From this four drugs, one is a B-Raf enzyme inhibitor (vemurafenib), one is an inhibitor of the associated enzyme B-Raf (dabrafenib), and one is a MEK inhibitor (trametinib); the only one that is an actual immune-therapy, is an anti-CTLA-4 (ipilimumab) antibody [13]. Therefore searching new efficacious immune-therapies in this disease is still an open subject of intense research.

This chapter summarizes the main achievements gathered in the last 3 years regarding immune-therapy as the ultimate approach for melanoma treatment.

## 2. Reducing specific immune-suppression

Until recently, the approved therapy armentarium in advanced melanoma was comprising only dacarbazine (DTIC), hydroxyurea, while the only approved immune agent was high-dose interleukin-2 (IL-2) [14]. These drugs cannot provide satisfactory overall survival (OS) rates in advanced stages. After searching various combination of immune-therapies, from vaccination [15] to drugs that inhibit immune-signaling pathways [16], in 2011 FDA approved, ipilimumab and vemurafenib, agents that significantly increased OS and long-term improvement in advanced melanoma [17].

In this case, monoclonal antibodies (mAbs) as immune-therapy agents have the intrinsic role to establish an antineoplastic action through stimulation of a specific immune response. This action can be performed by inducing *de novo* primary response and/or by eliciting an already existing antitumor action, but repressed in these patients.

The most advanced, in terms of positive research results, were the immune-related drugs that abrogated the immune –suppression such as CTLA4 or PD-1. Other mAbs were designed to aim toward stimulation of co-stimulatory receptors, molecules that are expressed by antigen presenting cells (APC); the aimed molecules were CD40 or OX40 (member 4 tumor necrosis factor receptor superfamily) or GITR (TNFRSF18), expressed on activated T lymphocytes.

Tremelimumab, also an anti CTLA4 mAb, is being evaluated in solid tumors. Nivolumab, an anti PD-1 mAb is also in the evaluation phase. This year (2014) clinical trials assessing OX40- and GITR-activating mAbs were initiated as well [18].

### 2.1. Anti-CTLA-4 antibodies

Activated T lymphocytes express transiently CTLA-4 transmembrane protein, while on T regulatory lymphocytes (Tregs) this protein is expressed constitutively. Two possible mechanisms are known accounting for CTLA-4 immune-suppressive effect. One of the mechanisms is the competitive binding to B7-1 and B7-2 in the detriment of the normal binding to CD28, delivering thus an immune-suppressive signal [19]. Actually CTLA-4 competes with the binding of CD28 to B7, thus hindering a normal activation. The other possible mechanism is that cells expressing CTLA-4, endocytose the appropriate ligands of other cells, as such, CD28 cannot trigger activation [20].

Overall in cancer research, drugs that aim B7 family can enhance the therapeutic panel and thorough studies are further needed for elucidating these regulatory pathways [21].

The particular action of monoclonal anti-CTLA-4 antibody is to link to CTLA-4, blocking the inhibitory immune-suppressive signal, T cell can perform thus its activation pathways, proliferate while infiltrating the tumors, and in the end, set off tumour apoptosis. Designed to bind to CTLA-4, ipilimumab was approved by FDA in 2011 for advanced melanoma [22] and in the same year the European Commission issued a marketing authorization for ipilimumab [23].This type of immune-drug aims to downregulate the inhibitory activity of T-lymphocytes leading to the normal activation of T cells by allowing the binding of CD28 to B7, this costimulatory process participates to the main coupling of MHC (major histocompatibility complex) that presents the tumour antigen to TCR (T cell receptor). Re-establishing these physiological molecular interactions, T-cells can mount an efficient antitumor immune response (Figure 1) [24]. CTLA-4 expressed on T cells is omnipresent and is not dependent upon the tumour's particularities, thus the drug's action should not be dependent on tumor's characteristics.

EMA recommendation after analyzing the post-phase III results extended clinical trials [25,17] is a dose of intravenously 3 mg/kg ipilimumab over a 90 min period, this procedure needs to be repeated every 3 weeks; overall four doses should be administered [23]. If the patients are treated in combination with DTIC it is recommended that a dose of 10 mg/kg should be used [25,17].

The actual clinical results of using this immune-therapy showed that patients with advanced melanoma increased their median OS to 10 months when compared to the 6 months OS in gp100 melanoma vaccine. When comparing OS in patients treated with ipilimumab in combination with DTIC, OS was 11 months, while DTIC monotherapy just 9 months.

As stated above ipilimumab's action is not dependent on specific and individual tumor cell mutations, hence it can be efficient in different patients and stages [26, 27]. The beneficial clinical results regarding OS were registered as independent of various parameters such as age, gender, stage and/or previous therapy regimens.

Investigating the patients survival curves it has been shown that there are groups surviving more than 4 years [28], and that their clinical response was durable during the follow-up [29]. Durability is an important differentiating criterion when recommending first-line therapy with immunomodulators in comparison to the duration obtained in kinase inhibitors treatment.

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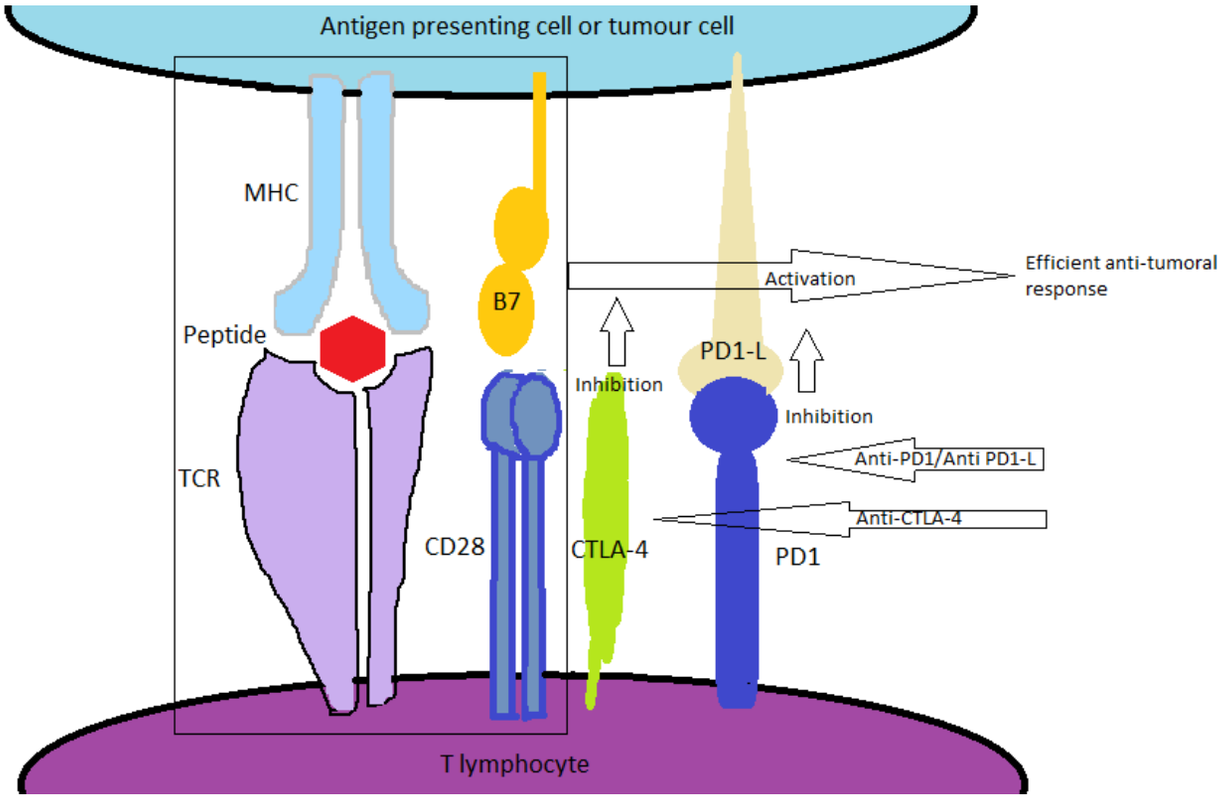


**Figure 1.** Main immune-suppression molecular mechanisms that can be overridden by therapeutical monoclonal antibodies targeting CTLA-4 and PD1.

As we are focusing on an immune therapy and as *immune memory* is a characteristic of this process, it was a logic approach to see what the effects are when ipilimumab gets another round of therapy. The National Comprehensive Cancer Network (NCCN) recommendation show that ipilimumab re-treatment can be done when the first round induced intolerable toxicity and/or for patients relapsing after the first therapy or proving at least 3 months stable disease [30]. Adverse reaction during re-treatment are similar to those for the first approach (see below) and no actual predisposition was noted regarding first encountered toxicity with the re-treatment one. Authors report that retreatment with ipilimumab is a feasible therapy and, currently a phase II trial (http://trialsunited.com/studies/NCT01709162) focuses on the immune response parameters in ipilimumab re-treatment [31].

Non-cutaneous melanomas were also therapeutic targets for ipilimumab therapy. Thus, in an Australian study published in 2014 over 100 patients were followed after ipilimumab therapy. Median OS for mucosal and uveal melanoma patients was half of that registered in cutaneous melanoma patients. This report underlines the severities of adverse effects, even death-related to therapy cases, thus administration and follow-up by an experienced clinical team is extremely necessary in this type of clinical trials [32].

Stage IV patients presenting brain metastasis were another therapy target group as blood-brain barrier is permeable to activated T lymphocytes, cells capable of inducing a local immune response [33]. In a large study comprising over 800 patients with brain metasta-

sis treated with ipilimumab, up to 25% survived at least 1 year, as the un-treated median OS is only 5 months [34].

Adverse reactions in ipilimumab therapy are associated with hyper-immune reactions, but these can be solved by the physician using additional classic therapies. Over 10% of patients experienced gastrointestinal deregulations (e.g. diarrhea, nausea, vomiting, decreased appetite and abdominal pain), rash, pruritus, fatigue. Side-effects are manageable by the physician and, seldom, the severity can lead to treatment discontinuation (www.ema.europa.eu). These immune-related adverse effects (irAEs) are mild to moderate toxicity and are experienced by around 60% of the patients, while around 15% developed grade 3 or 4 toxicity [25]. Endocrine system – related adverse effects were also reported in this therapy; in patients group receiving ipilimumab, 8% have experienced hypophysitis and 6% hypothyroidism/thyroiditis. Combined therapy, ipilimumab and nivolumab, induce in 22% of the patients thyroiditis or hypothyroidism and in 9% hypophysitis. Authors report hormone replacement as adjuvant therapy and immediate initiation of this therapy reverses symptoms [35]. If the ipilimumab therapy is combined with vemurafenib, important hepatotoxicity was reported, thus caution should be taken when combining immune-therapy with this B-Raf enzyme inhibitor [36].

### 2.2. Anti-PD-1 or PD-1L antibodies

#### *2.2.1. Nivolumab*

In July 2014, the first human monoclonal antibody against programmed death receptor-1 was announced as approved in Japan-*Nivolumab* [37]. As shown above in Figure 1, it targets a negative regulatory molecule that sustains immunosuppression. After accomplishing phase I and II clinical trials, around 25% of stage III and IV patients had a good clinical response when 2 mg/kg intravenous nivolumab was administered every 3 weeks. The clinical outcome was very optimistic in this study, patients did not progress in their disease for a median of 172 days, and at the time of publication (July 2014) median OS was still not achieved.

Nivolumab displayed a good tolerability profile; grade 3 or 4 adverse effects were reported in les than 18 % of patients, mainly an increased γ-glutamyl transferase [38].

Another group studying T lymphocyte interaction with tumour cell, interaction mediated by PD-1 receptor linking to PD-L 1, has shown that in phase I/II studies this antibody can lead to tumor regression and can enhance OS in various cancers including skin melanoma. Studying antibodies that target PD-1 or PD-1L (e.g. nivolumab, MK-3475, pidilizumab, MPDL3280A, BMS-936559, MEDI4736, MSB0010718C) the authors show that the positive clinical response goes to a maximum of 50% response rate when antibodies against PD-1 combined with anti-CTLA-4 were used. The clinical responses start early upon treatment and continue after the treatment is finished [39].

Another study published in 2014 searched to evaluate the survival of patients upon discontinuation of the therapy. After enrolling over 100 patients with advanced melanoma, the authors concluded that the median OS was 16.8 months [40].

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Melanoma patients along with other cancer diagnosed patients were treated with anti-PD-1 (nivolumab). This early-phase clinical trial published in 2014 aimed to elucidate the link between PD-1, PD-L1, and PD-L2 expression, immune cell infiltration and the clinical efficacy of this therapy. The degree of PD-L1 expression depicted on tumor cells was associated with its receptor PD-1 expressed by lymphocytes. The other ligand of PD-1, PD-L2 corroborated with PD-L1 expression. The expression of PD-L1 on the tumors was correlated with the clinical efficacy of the anti-PD-1 therapy, and the found best correlation when compared to other studied factors, such as PD-1 expression and/or TIL (tumor infiltrating lymphocytes). The study concludes that, when achieving maximum efficacy with novilumab, tumor PD-L1 expression is the base of anti-PD-1 therapeutical blockade [41].

In 2013, results from phase I clinical trials, of nivolumab and MK-3475 (anti-PD-1 and anti-PD-L1 antibodies) were released. For MK-3475 an objective response of 38 % with only 13 % of the patients reporting grade 3/4 toxicities was shown [42]. These results were probably the ground for further approval (see below).

Phase III clinical trails are on-going and, it seems that PD-1-PD-L1 triggers a sequence of intracellular signaling that brings important clinical benefits [43].

Adverse effects in this type of therapy are of low grade, the physician can impose a good patient management [39] and long-term safety is acceptable [40].

*2.2.2. Pembrolizumab*

In September 2014, FDA granted accelerated approval to pembrolizumab (formerly known as MK-3475), an antibody targeting PD-1, to be used following ipilimumab therapy. A recently published report showed the efficacy and safety results of this antibody at two doses (2 mg/kg and 10 mg/kg) given every 21 days. The enrolled patients that received the therapy were refractory to ipilimumab therapy. Similar safety profiles were reported whether patients were treated with 2 mg/kg or 10 mg/kg and authors show that no drug-related deaths were registered [44]. Another published study had similar results with the difference that the response rate between patients with or without prior ipilimumab treatment were not statistically different. Positive clinical outcome was registered with the overall median progression-free survival exceeding 7 months. Patients with advanced melanoma, prior refractory to ipilimumab, proved in this study a high rate of tumor regression [45].

Drug-related adverse effects were fatigue in one third of the patients and around 20% of them experienced pruritus and rash. Grade 3 fatigue was the single drug-related grade 3 to 4 adverse effect in 3% of the patients [44].

The positive results in a difficult to manage patients, like the ones refractory to ipilimumab, probably accelerated this drug authorization with two months ahead of its planned approval and the clinical study is still on going [46].

After ipilimumab approval, finding another antibody that could be used as immune-therapy for blocking an immune checkpoint like PD-1 and PD-1L gained an intense research frenzy in the last years.Therapeutical approaches that use immunomodulatory drugs have completely

different mode of action in comparison to the well-known chemotherapeutical procedures. From this point of view investigating the intimate mechanisms that underlie their effect is of outmost importance because it can reveal new signaling molecules, future to be drug targets. Moreover biomarkers that can clinically predict the patient response could optimize the approach and personalize the immune-therapy [47].

### 2.3. Biomarkers for clinical benefit prediction

In the last couple of years there is a less abundant literature focusing on predictive and/or prognostic biomarkers in the immune-therapy of cutaneous melanoma. Biomarkers that were published lately range from classic serum LDH, to membrane molecules and circulating cells without any *clear-cut biomarker* that could predict the immune-therapy efficacy.

In the last year researchers were focusing on biomarkers that can predict immune-therapy with ipilimumab outcome. Thus some studies show that ipilimumab therapy was correlated with an increase in peripheral blood absolute lymphocyte count when patients had a good clinical outcome in terms of OS. More specifically OS was 11.9 months for patients that had more than 1,000 lymphocyte count / μL peripheral blood in comparison to OS of 1.4 months in patients with lower counts [48, 49]. These results were confirmed this year when increased absolute lymphocyte count was associated with increased progression free survival (PFS) but not with OS. Any other parameters including classic serum LDH did not relate to OS or PFS [32].

Another cell biomarker, forkhead box P3 (FoxP3) expressed by T-regs, was correlated with positive clinical outcome in advanced melanoma patients [50].

Correlations were investigated regarding circulatory myeloid-derived suppressor cells (MDSC) in treated patients. Authors report that circulatory MDSC with Lin(-) CD14(+) HLA-DR(-) phenotype are increased in patients compared to normal. After surgically removing the tumour and subjecting patients' to ipilimumab treatment, this immune parameter did not change. Then again, an interesting finding was that patients could be stratified in the ipilimumab-responders and non-responders based on the lower and respectively higher concentration of circulatory MDSC, thus pinpointing these cells as possible predictive markers of response to ipilimumab. This candidate immune-marker did not correlate with baseline serum LDH, but showed higher values in severe metastasis compared to localized metastasis to skin and/or to lymph nodes [51].

As to the possible efficacy biomarkers for nivolumab therapy, in a phase I clinical trial, stage III or IV patients were followed after this therapy by several biomarkers evaluation. This recent study reports that high circulatory T lymphocytes with NY-ESO-1 and MART-1-specific CD8(+) phenotype are associated with disease progression. After therapy, increased circulatory Tregs and decreased antigen-specific T cells are the two immune biomarkers that were found associated with disease progression. The expression of PD-L1 on the tumor did not correlate with the clinical response [52].

### 2.4. Animal models studying immune-therapy mechanisms

There are few studies focusing on animal models that bring new data regarding the intimate cellular mechanisms in immune-therapy. Having in mind the fact that these recent immune-

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therapies are limited to certain groups of patients, the published animal models searched for the resistance mechanisms that could hinder this therapy.

In 2013, the role of IDO upon an experimental anti CTLA-4 blockade was shown. Authors used IDO knockout mice and showed that, upon treatment with anti-CTLA-4 antibody, B16 melanoma was growing more slowly and that, the animals' overall survival increased compared to normal mice expressing IDO. The mechanisms were similar when the animal model was treated with anti PD-1/ anti-PD-L1 and GITR. The authors show in this animal model that CTLA-4 and IDO inhibitors converge and that the inhibitory role of IDO can be the background mechanisms accounting for the resistance to anti-CTLA-4 therapy. Moreover, the process is T lymphocyte dependent and, if this resistance is overridden, effector T cells are found increased in tumour infiltration, the effector-to-regulatory T cell ratio increases as well [53]. The molecular mechanisms of IDO expression are intimately related to the immune response. Several years ago it was reported that IDO expression is controlled by T activated lymphocytes through their secreted cytokines. IL-13 can repress the induction of IDO mediated by IFN-gamma [54]. Regarding possible emerging therapies, authors report that fludarabine that hinders the up-regulation of IDO in a T lymphocytes dependent manner, can be tested as a pre-treatment drug for melanoma patients. These patients can receive afterwards immuno-therapies that would have been less efficient when IDO was over-expressed [55].

Using animal models, new emerging therapies can be discovered, overriding the resistance to immune-therapies in certain patients groups.

## 3. Dendritic cells pulsed with specific antigens as inductors of specific immune-response

Treatment paradigms aim to include naturally occurring dendritic cells subsets in a single vaccine. The studies that are in the pre-clinical phases show synergistic effects between various antigen-presenting cells. We will present different types of methodologies to pulse dendritic cells, starting with mere cultivation of dendritic cells in total tumour lysates and ending with newer technologies such as electroporation mRNA-pulsed dendritic cells. Recently, the first clinical trials released their results and showed increased survival rates and broader anti-cancer immune responses. These new clinical findings will be presented.

In just a short period of time, the cancer immunotherapy field has gained new combatants through sipuleucel-T FDA approval, first DC immunovaccine for metastatic prostate cancer patients, followed closely by ipilimumab, an antibody specific to CTLA-4 as major target in metastatic melanoma [56]. Due to its clear tumor immunogenicity, melanoma treatment could be handled from an immunotherapeutic viewpoint. However, an important issue in melanoma immunovaccine success is the highly heterogeneous composition of antigens expressed within tumor site along with different genetic patterns of melanoma patients [57]. Deciphering this complex (intra)tumor heterogeneity of melanoma is directly linked to the possibility for clearly identifying, targeting and manageing drug-resistant cell subpopulations from the tumour site [58]. The genetic profile of melanoma patient is one of the foremost rationales for an autologous

whole cell vaccine acting more efficiently in treating the micrometastasis than would an allogeneic designed one.

Revisiting the melanoma vaccines, it was pointed out that a preponderant cytokine-driven therapy activates a robust antitumoral T-cell mediated response representing a class of individualized auto-vaccination formula, surmounting thus the melanoma intratumoral heterogeneity [57]. Assembling a cancer vaccine aims to activate a specific anti-tumor immune response and/or to better access the tumor-associated antigens. In cutaneous melanoma, the pattern of tumor specific and tumor associated antigens is both large and heterogeneous, making melanoma immunogenicity exploitable in therapeutic approaches. Therefore, the plenty of discovered or yet hidden melanoma tumor antigens could open the way for vaccination of patients groups with the same vaccine type [59]. Being explored with synthetic peptides, whole tumor cells, cellular lysates or autologous immunovaccine, dendritic cells (DCs) take advantages in treatment or even in prevention approaches of cancer [60].

Dendritic cell in melanoma immunotherapy undergo a sequential number of actions. Thus, loading DCs with a tumor antigen and a specific adjuvant will induce the maturation state which involves antigens processing by proteasomal degradation and presenting the resulted peptides to T cells *via* MHC complex to stimulate further CD4+ cells, CD8+ cells as well as phagocytes and NK cells or, in certain activation conditions, induce Tregs that hamper antitumor responses [61].

### 3.1. Dendritic cells subsets

In malignant skin, the principal subsets of DC responsible for Ag-specific T cell immune response comprise *epidermal* (Langerhans cells) and *dermal* cell populations. The primary tumor site and the sentinel lymph nodes endure the immune-suppression generated by melanoma, this immune site being the field where T cells should start the fight against melanoma while being armed by activated DC to engender anti-melanoma immunity [62]. As in case of tumor associated macrophages, under the influence of tumor milieu DC are versatile players which could become *tumor-associated DC* enhancing Immune-suppression by sustaining T cell regulatory activity [63] (Table 1). Upon electrochemotherapy of tumor cells, for example, a relatively new approach to deliver better an antitumor drug, melanoma inflammatory infiltrate contains beside dermal DC, *plasmacytoid* DC cells for capturing tumor antigens to further elicit, along with dermal and Langerhans cells, a T cell antitumor response [64].

| DCs subsets | Uptake receptors evaluated for immunotherapy of DC | | |
|---|---|---|---|
| | **Antigen uptake receptors** | **Unique receptors** | **TLR receptors** |
| LC/dermal DC | FcR; DEC205; CD40; DCIR | Langerin | TLR-3 |
| pDC | FcR; DEC205; CD40; DCIR | CD303; CD123; BDCA-2 | TLR-7; TLR-9 specific to pDC |
| MoDC | FcR; DEC205; CD11c; CD40; DCIR | DC-SIGN; MR | TLR-4; 8; 3; 7. |

**Table 1.** DCs subsets and receptors for immunovaccine (LC – Langerhans cell; pDC - plasmacytoid-derived DC; MoDC – monocyte-derived DC; DEC205 – C-type lectin receptor on DC; BDCA-2 – blood dendritic cell antigen, specific to pDC subset; DCIR - dendritic cell immunoreceptor; DC-SIGN - dendritic cell specific ICAM-3 grabbing non-integrin; MR – mannose receptor; TLR – toll-like receptor )

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### 3.2. Exploring dendritic cells in melanoma immunovaccine

As skin is an abundant cellular immune network and hence an accessible portal for therapeutical approaches, DC cells remain an attractive target in melanoma therapy both as *ex-vivo*-generated or *in-vivo*-DC-targeting immunovaccine blueprint [65]. Peripheral blood and Langerhans cells are the main sources for DCs immunotherapy. Langerhans cells and monocyte-derived DCs elicit immune responses in comparable levels although the cytokine stimulation conditions are different. The initial therapeutical attempts with DCs vaccination was based on *ex-vivo* generated monocyte-derived DC pulsed with tumor lysates, peptide or tumor antigens which led to a tumor regression rate of 3-7%, having also a lower toxicity compared with standard therapeutical procedures [66]. In melanoma, DC derived from CD34+ progenitors prove better results compared with monocyte-DC vaccine in spite of the known heterogeneity of tumor antigens [10].

#### *3.2.1. Loading DC with tumor associated-antigens*

Loading effector immune cells with antigenic peptides or a whole tumor-associated antigen (TAA) was initially designed as an immune vaccination system with T lymphocytes *via* MHC molecules recognition. Due to possible issues related to molecule stability and/or delivery route, resulting in an ineffective antigen presentation, these approaches could fail in clinic due to a low response rate in patients. Using DC as tool for intracellular delivery of such tumor antigenic peptides, the process of antigen presentation to T cells could be improved [67]. Moreover, the Th1/Th2 balance could be regulated by such modified DC. Therefore, DCs loaded with MART-126-35 melanoma peptide were used in combination with anti-CTLA4 monoclonal antibody (tremelimumab) in advanced melanoma patients. Upon therapy, high levels of pro-inflammatory Th1 invariant natural killer T cells (iNKT) CD8(+) was associated with positive clinical responses, indicating that antitumor T cell activity could be immunomodulated *via* iNKT cells by peptide-pulsed DCs [68]. In another recent study, high-risk stage III melanoma patients with lymph node resection were vaccinated with DC loaded with MHC I melanoma peptides respective to the patient's haplotype. The peptide pulsed DCs were well tolerated and elicited immune specific responses to melanoma antigens or/and IFN-γ-producing CD8+ cell response to melanoma peptides in 15 of 22 patients. The three-year overall survival rate was 68.2% vs. 25.7% in the control patients group [69].

Monocyte-derived DCs could be loaded in different conditions with a mixture of peptides, tumor lysates or even with a single tumor peptide such as from Mage-3A1. For 8 of 11 patients enrolled in the study it was registered an increase of Mage-3A1-specific (CD8+) T cells, with regression of a few metastases for 6 advanced melanoma patients; a lack of Mage 3A1 expression was observed in some non-regressed areas of melanoma [70]. Even immature DCs could be exploited in vaccination, thus DCs generated from CD34+ progenitor cells were cytokines-stimulated and pulsed *in vitro* with a pool of melanoma derived peptides [71].

Engineered DCs loaded with peptides or antigens could be delivered by lymphatic nodes or intradermally; the last type being the optimum method for generating T cell antitumor immunity [72].

*3.2.2. DC electroporation with mRNA*

Cellular electroporation is a transfection method to efficiently introduce mRNA encoding for a certain biomolecule in order to express at high level that specific antigen. A main advantage of mRNA transfection is the prolongation of the exposure and an accurate antigens processing. This approach was translated for DCs as a promising opportunity to facilitate access to tumor antigens and thus priming the T cell specific antitumor melanoma response, being applicable even in advanced stages of melanoma [73].

In the last few years, mRNA was proposed as an innovative vehicle for antigen delivery appropriate for cancer vaccination purposes. DCs were evaluated as the most suitable immune cells for mRNA transfection due to their professional quality in processing and presenting antigens for inducing specific immune responses by T cells. mRNA as an antigen delivery tool could generate a whole antigenic protein with all epitopes ready to be viewed by MHC molecules; last but not least, the interest mRNA molecule could be produced in large quantities with high purity.

The successful use of *ex vivo* mRNA-modified DCs for melanoma immunotherapy rely on DCs cellular subtype involved, the proper cellular activation and path of delivery [74]. The DCs *subtype* electroporated with mRNA in cancer vaccination purposes accounts for vaccination efficiency. pDCs cells loaded with melanoma antigenic peptides and further activated at CD40L elicit T cytotoxic anti-melanoma responses [75]. The cellular *activation* method of DC counts for CTL full activity and subsequently inhibition of Tregs. Engagement of TLR in DC activation proves to be a good approach in immunovaccine. Moreover, transfection with mRNA and activation of DC cells is the fundament of the TriMix mRNA set encoding for CD40L, CD70 and active form of TLR4. Thus in one clinical study with TriMix-DCs intradermally administrated, the efficacy of procedure was registered by means of the skin infiltrating CD8+ lymphocytes monitored by IL-12p70 as a marker of successful inoculation of the DC product [76]. The *delivery way* of modified DCs was compared in several clinical studies. The best way to target the lymph nodes in melanoma site by modified mRNA DCs is intradermic inoculation associated with an increased number of T cells although intranodally delivery reveal a higher number of DC. One recent study refers to DC electroporated with mRNA for gp100/tyrosinase antigens and injected in regional lymph nodes of melanoma patients prior to local surgery. The mRNA for electroporated melanoma antigen was immunohistochemically detected in T cells populations from both the primary and the adjacent lymph node concomitantly with a CD8+ T lymphocyte responses registered in 7 of 11 patients subjected to immunovaccination [77]. Some promising results in terms of safety, feasibility and immunogenic properties were obtained in patients with advanced cutaneous melanoma in a pilot study were DC were co-electroporated with mRNA encoding for CD40L, TLR4 and CD70 as a autologous TriMix-DC formula combined with IFN-$\alpha$-2b sequential administration [76].

The foremost parameter to be monitored for immunovaccine efficacy is the induction or the enhancement of melanoma anti-tumor immune response. Several technologies like ELISPOT technique allows quantifying the precise number of active immune cells by counting antigen-specific cells that secrete a particular anti-tumor cytokine e.g. IFN-$\gamma$ [59]. Since 2006, clinical studies with tumor mRNA transfected DCs administrated usually intradermic in patients with

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metastatic melanoma, detect an immunological T cell response with promising results at least for disease stabilization. In addition, advanced melanoma patients were treated intradermic with DCs simultaneously co-electroporated for 4 tumor antigens – Mage A3, Mage C2, Tyrosinase and gp100/DC-LAMP followed by TriMix mRNA single or in combination with additional therapy like IFNa-2b. In both clinical set-ups, the CD4/CD8 T cell responses were enhanced [74].

Recently, a small study on seven melanoma patients involved DCs from peripheral blood mononuclear cells pulsed with gp100 peptides and maturated further *in vitro* with a cocktail of CD40L and INF-γ. It was noted a direct correlation between the clinical responses and levels of IL-12 produced by modified DCs. The highest levels of IL-12 were registered in one patient with complete remission. DCs from non-responding patients were unable to produce IL-12 without additional stimulation with TLR agonist, observation that induced a modification of the *in vitro* maturation protocol and thus contributed to the improvement of the clinical outcome [78].

## 4. Translating up-dated knowledge to clinics — Therapeutical future scenery

There is a recent trend that advises as first therapy a BRAF inhibitor in *advanced* stages, because, time-wise, these patients cannot build an effective immune response while in patients with less advanced stages, immune-therapy will lead to a better outcome whether the patient presents or not a BRAF mutation [79].

In the clinical management of melanoma it is certain that immune-therapy is one of the pillars. Among this new therapeutical approaches, MEK inhibitors can overcome the resistance induced by BRAF inhibitors [79, 80]. has shown in phase 3 clinical studies, an improved [81].

Combination of these antibodies with the new anti-CTLA4 drug induces an astonishing 80% or more tumor regression in patients (http://www.clinicaltrials.gov/show/NCT01783938; http://trialsunited.com/studies/NCT01024231/) [82]. Newer therapeutical combination of ipilimumab with oncolytic immunotherapy with GM-CSF-expressing engineered herpes simplex virus, have shown in phase 3 clinical studies an improved OS in advanced melanoma [83].

On-going studies seek to establish the clinical boundaries of NK adoptive transfer in melanoma. Last year a study was published searching to rationalize the clinical trial framework [84] as the prior published results of a pilot trial showed no tumor regression in spite of the increased concentration of circulating autologous NK cells persistence [85].

Immunotherapy is the only therapeutical "light" that can increase OS in advanced melanomas. Eliciting an efficient immune response takes a certain amount of time in order to have an efficient anti-tumoral response, but as *immunological memory* is installed, the effect of immune-therapy persists in the absence of further immune-treatment.

We strongly believe, taking into account our experience in melanoma patients follow-up [86, 87] that immune-therapy is a major therapy weapon, increasing survival in advanced melanomas. The only draw-back is that, in order to develop its full efficacy, immune-therapy takes time, and this time interval can be up to 2–4 months. Thus, an early diagnostic in the metastatic stage would make a huge difference for a positive clinical outcome [88].

Besides imaging-based follow-up, immune parameters should complete the panel of investigation. New data regarding circulatory MDSC can enhance this panel and prospective clinical trials should soon validate them. Resistance to immune-therapy, like IDO expression opens new therapeutical avenues aiming at immune checkpoints and combining specific antibodies with IDO inhibitors.

## 5. Conclusion

The immune system is still not fully explored in this type of skin cancer [89, 90], thus new insights in tumour microenvironment and the involvement of innate immunity cells could enhance the panel of new therapeutical targets.

Balancing antitumor efficacy and reconstitution of a proper functioning immune system are processes aimed by immune-therapy in cutaneous melanoma. Owing to cutaneous melanoma immunogenic outline, this disease treatment could be addressed from an immunotherapeutic viewpoint. As we are facing the great success of having the first immune-therapy approved drug in melanoma, there is an open research combat of targeting personalized/individual antigens or undifferentiating antigens-stem-like to tackle the aggressive character of this disease.

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