Cutaneous Melanoma: A Test Field for Immunotherapy and a Medical Challenge

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Abstract: Cutaneous melanoma (CM) is the fourth tumor in frequency, and that whose incidence increases faster. In half of the cases, CM arises from pre-existing nevi. CM is a highly heterogeneous tumor, whose degree of differentiation varies among different hosts, and such heterogeneity is probably based on the accumulation of mutations that determine transitions from normal melanocytic stem cells → mutated benign melanocytic stem cells → malignant melanocytic stem cells → clonogenic melanocytic cells. These populations may express different antigens and would therefore trigger the proliferation of different T and B cell clones. Early diagnosis is the clue for the cure of CM; when visceral metastatic disease is established, the prognosis is somber. This is especially so since CM is quite resistant to chemotherapy, and some of the reasons for such resistance will be discussed here. However, CM has proved to be sensitive to immunological effectors, although the mechanism of such sensitivity is still being investigated. These effectors range from therapeutic vaccines, *in vitro* expanded cytotoxic lymphocytes, cytokines, and monoclonal antibodies. Finally, we will discuss new therapeutic approaches that include the combination of immune modulators and vaccines which are being assayed in light of recent tumor immunology research.

Key Words: Melanoma development, chemotherapy and resistance, immunotherapy, cancer vaccines.

Cutaneous melanoma (CM) accounts for 4% of all neoplasia and it is the tumor with the fastest growing incidence worldwide [1]. Early diagnosed tumors (Breslow index <1mm) are cured with surgery alone in more than 95% of the cases. In contrast, some, but not all, patients with Breslow indexes higher than 2 mm metastasize to regional lymph nodes and eventually to distant metastatic sites, in which case only a minority of patients might be cured [2]. A peculiarity of melanoma is that it is highly resistant to chemotherapy, but more responsive to immunological treatments. In an attempt to understand these characteristics, we shall focus our attention on the origin of melanocytes, melanoma, and the factors that determine its resistance to drugs and its sensitivity to immune effectors.

THE DEVELOPMENT OF MELANOCYTES

Melanocytes are melanin-containing pigmented cells that are the main responsible for pigmentation in vertebrate organisms. In humans, they account for eye, hair and skin pigmentation, and they are critical for the tanning response, protecting skin cells from ultraviolet induced DNA damage, one of the risk factors for skin cancers such as melanoma.

In the vertebrate embryo, multiple cell types originate from a common structure, the neural crest (NC), which forms at the dorsal tips of the neural epithelium. The NC gives rise to migratory cells that colonize a wide range of embryonic tissues and later differentiate into neurons and glial cells of the peripheral nervous system (PNS), pigment cells (melanocytes) in the skin and endocrine cells in the adrenal and thyroid glands. In the head and neck, the NC also yields mesenchymal cells that form craniofacial cartilages, bones, dermis, adipose tissue, and vascular smooth muscle cells (Fig. **1a**) [3].

Neural crest formation and migration starts at around 6 weeks in human embryos. It is then when melanocytes precursors, the melanoblasts, migrate along characteristic pathways to various destinations such as the iris and the choroid of the eye, the inner ear, the dermis, and the epidermis. In addition, the precursors distribute into the bulged region of developing hair follicles, where they persist as self-renewing *stem cells* [4, 5]. One of the first studies of pigmentation in developing human skin was performed in preparations of epidermis from black fetuses at progressive stages of gestation, and the distribution of melanocytes was studied by silver staining of melanin, revealing the migration of melanocytes precursors from dermis to epidermis [6]. Later, the appearance, density and distribution of melanocytes in human embryonic and fetal skin by HMB-45/gp100 expression (a melanocyte differentiation antigen, MD-Ag) was analyzed [7]. Melanocytes with a dendritic phenotype were found among cells in the embryonic epidermis around 7 weeks (50 days Estimated Gestational Age, EGA), and in the skin even earlier (40-50 days EGA), being recognized at least two to three weeks prior to melanosome formation. Further studies of HMB45 (+) cells in human fetal skin preparations support previous evidence, where melanocytes were revealed either

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in the epidermal basal layer and hair follicles or among the mesenchymal cells of developing dermis [8]. MITF (microphthalmia-associated transcription factor, a key regulator of melanocyte development) and Mart-1 (another MD-Ag) expression in human skin samples at 6-24 weeks gestation also support melanocyte progression from intradermal to suprabasal intraepidermal, to basal intraepidermal followed by follicular colonization (Fig. **1b**) [9]. This migratory pattern is consistent with studies of melanoblast migration during murine embryonic development [10]. In human developing skin, by 6-8 weeks, most MITF and Mart-1 positive cells were intradermal with only rare positive cells in the epidermis. By 12-13 weeks, most of these cells had migrated to the suprabasal layers in the epidermis. Between 15-17 weeks, these cells are mainly localized to the basal layer and colonized developing hair follicles. At 18-24 weeks, MITF and Mart-1 positive cells were identified in the outer root sheath, bulge, and follicular bulge epithelium, in addition to the epidermis. Intriguingly, rare intradermal MITF and Mart-1 positive cells were found as late as week 20, often in a perivascular distribution. This may account for *de novo* melanocyte proliferation in the dermis, originating benign nevi or melanoma.

Regarding melanocytes distribution, the matricellular protein CCN3 and its receptor DDR1 would be critical mediators of the basal localization of human mature melanocytes [11]. It was found that overexpression of CCN3 in melanocytes increases adhesion to collagen type IV (the major component of the basement membrane) via DDR1 (discoidin domain receptor 1), a keratinocyte receptor that acts as a collagen IV adhesion receptor. Either CCN3 or DDR1 knockdown resulted in dissociated melanocytes migrating either upward to the suprabasal layers of the epidermis or downward into the dermis. Probably these genes might be upregulated by 13-15 weeks EGA, in the transition of melanocytes from the suprabasal to the basal layer of epidermis, accounting for the importance of accurate interactions between keratinocytes and melanocytes to sustain epidermal homeostasis.

MOLECULAR PATHWAYS IN MELANOCYTES DEVELOPMENT

One of the main questions is how melanocytes specify from the neural crest. Gene expression programs that direct the development of distinct cell lineages from unspecified precursor cells are the result of complex interactions between cell-extrinsic signals and transcription factors. This integration of signals was proposed to converge on a "nodal point", where MITF acts as a master regulator of melanocyte development, controlling activation of downstream differentiation genes, playing an essential role in survival, migration, proliferation, and differentiation [12, 13]. MITF targets genes including those involved in cell survival (Bcl2, Met) [14, 15], cell proliferation (p21, p16, CDK2) [16-18], and differentiation (Tyr, Tyrp1, Dct; gp100, Mart1, MC1R) [19-23].

WNT signaling is essential for NC induction and melanocytes development. In the best understood canonical WNT/β-catenin signaling pathway, when extracellular WNT ligand binds to its receptor (Frizzled), β-catenin accumulates, enters the nucleus, and subsequently interacts with members of the lymphoid enhancer binding factor 1/T-cell specific factor (Lef1/Tcf) family of transcription factors, which then modulate transcription of target genes. Along with Lef-1, β-catenin interacts directly with Mitf enhancer/promoter to activate MITF transcription [24, 25].

Both Neural Crest transcription factors Sox10 in synergy with PAX3, strongly activate MITF expression [26, 27]. In turn, Pax3 competes with MITF for occupancy of an enhancer required for expression of dopachrome tautomerase (Dct) until Pax3-mediated repression is relieved by activated beta-catenin. Thus, a stem cell transcription factor can both determine cell fate and simultaneously maintain an undifferentiated state, leaving a cell poised to differentiate in response to external stimuli [28]. Interestingly, this mechanism could contribute to the uncontrolled cell growth and loss of terminal differentiation in melanomas. In fact, it was found that PAX3 is commonly expressed in primary melanoma and several melanoma cell lines whereas it is significantly less frequently expressed in benign pigmented lesions [29]. Further analysis revealed that PAX3 expression was strongly correlated with younger patients with low or no evidence of sun damage, providing evidence for a genetic underlying rather than environmentally source in Pax3 (+) melanomas.

Although expression of many melanocytic/pigmentation markers is lost in human melanoma, MITF expression remains intact, even in unpigmented tumors, suggesting a role for MITF beyond its role in differentiation. β-catenin potently mediates melanoma cells growth in a MITF-dependent manner. Suppression of melanoma clonogenic growth by disruption of beta-catenin-T-cell transcription factor/LEF is rescued by constitutive MITF by a prosurvival mechanism. Thus, β-catenin regulation of MITF expression represents a tissue-restricted pathway for controlling melanoma growth [30].

Several pigmentation disorders are due to mutations in genes involved in melanocyte development, like Waardenburg syndrome (WS). To date there are at least four types of WS that are due to mutations in separate transcription factors, including SOX10, MITF, PAX3, and SNAI, and in signaling molecules, such as EDNRB (endothelin receptor type B) and EDN3 [31-35].

The differentiated state of melanocytes is characterized by their dendritic morphology and the accumulation and trafficking of melanin within melanosomes. Biosynthesis of melanin involves several enzymes from the tyrosinase related protein (Trp) family, including Trp1, dopachrome tautomerase (Dct/Trp2) and tyrosinase (Tyr). Melanosomes normally mature from undifferentiated and non-pigmented organelles (termed stage I melanosomes) to differentiated and highly pigmented organelles (termed stage IV melanosomes). Mature pigmented melanosomes bind to microtubules and undergo actin-dependent transport towards the cell periphery prior to their transfer to keratinocytes [36]. The donation of melanosomes from melanocytes to surrounding keratinocytes is critical in order to protect their DNA from UV radiation. In this process, melanocytes acquire a highly dendritic appearance, with many projections interacting with neighboring keratinocytes at a 1:40 ratio. This interaction accounts for the homeostasis of melanocytes. In fact, proteins related in promoting adhesion to basal membrane, such

as CCN3, are inversely correlated with melanoma progression [37].

Once differentiation from neural crest cells is achieved, the differentiated state of the melanocytic lineage must be maintained, mainly in response to environmental signals. Although EDNRB and KIT signal pathways are not required for the initial expression of MITF in melanocyte development, both pathways induce the phosphorylation of MITF in mature melanocytes. The KIT pathway is critical in the control of normal human melanocyte homeostasis. It was found that Stem Cell Factor injection on human xenografts increases the number, size and dendricity of melanocytes, whereas KIT inhibition showed the opposite effect [38]. Kit pathway is also necessary for pigmentation, as MITF requires functional KIT signaling for Tyrosinase expression in melanoblasts [39]. Furthermore, SCF-KIT signaling leads to MITF phosphorylation and subsequently to an increase in the antiapoptotic gene Bcl-2, critical for melanocytes survival [14]. Bcl-2 is involved in melanocyte stem cell maintenance, being responsible for the loss of hair pigmentation in mice and humans [4, 5]. Recently, it has been shown that endothelin (EDN) signaling leads to an increase in MITF phosphorylation and CREB phosphorylation in cultured human melanocytes. Then, EDN would stimulate expression of melanocytic pigmentation and proliferation markers in a MITF-dependent way [40]. Another pathway for mature melanocyte modulation is through cAMP. Melanocytes increase melanin production and dispersion in response to UV radiation and other types of stress [41]. One of the main determinants of pigment phenotype is the melanocortin 1 receptor (MC1R), a G protein-coupled receptor activated by α-melanocyte-stimulating hormone (αMSH) and adrenocorticotropic hormone (ACTH), regulating quantity and quality of melanin synthesis [42-44]. In human epidermal melanocytes, UVB stimulus increases cAMP levels, activating Sox-9 via PKA, which in turns binds to Mitf promoter activating its transcription, resulting in upregulation of Dct and Tyr and therefore to a major pigmentation in response to UV radiation [45].

In all, Sox E (Sox8/9/10), Pax 3/7 and Wnt genes are necessary both for initial neural crest induction and melanocytes differentiation. This regulatory network is evolutionary conserved, with founding elements in ancient species such lamprey and hagfish [46], leading to the hypothesis that the primitive role of the neural crest may have been the production of pigment cells and their dispersal [47].

MELANOMA DEVELOPMENT

Clinical data analyzing melanoma development pathways reports that, in half of the cases, Cutaneous Melanoma arises from nevi [48]. A nevus can be described as a benign clonal proliferation of cells expressing the melanocytic phenotype [49]. Nevi can develop congenitally or can be acquired during lifetime. It is accepted that in congenital nevi the precursor cell carries a mutation, which determines a migration from dermis to epidermis. Some of the excess melanocytes completing migration might be discarded as "pagetoid" cells in the epidermis. Cells within the nevus tend to differentiate towards the epidermis. In acquired nevi, there is increasing evidence supporting migration towards the skin surface, probably developing from a mutated dermal component [50, 51]. However, experiments with transformed cultured melanocytes showed that these cells can penetrate into the dermal component of the graft, indicating that migration from epidermis to dermis is also possible [52].

There are different patterns in nevi, and the growth pattern is considered to be a consequence of the underlying mutation and of local influences on cell growth and survival. N-Ras mutations are frequently found in congenital nevi, whereas B-raf mutations are found in acquired nevi [53, 54]. N-Ras activation leads to turn on several pathways, including PI3 kinase, Raf as well as Braf, leading to MAPK and ERK activation. In fact, culture of nevus cells requires less growth factors than normal melanocytes. This implies that although they are not malignant cells, they have more independence from homeostatic mechanisms [55].

According to the traditional Clark´s model for describing melanoma progression, the first phenotypic change in melanocytes is the development of benign nevi, which are composed of neval melanocytes. Additional mutations would originate dysplastic nevus aberrant growth, with random cytologic atypia. Then, malignant transforming mutations would give rise to melanoma cells, proliferating all through the epidermis (radial-growth phase), invading the dermis (vertical-growth phase) and from there to other organs (metastasis). One of the main questions is whether nevi are required for melanoma development. Clinical data analysis indicates that melanoma can indeed develop from normal skin, without a nevus precursor required [56]. However, cutaneous melanoma arising from a pre-existing nevus would have a greater Breslow thickness [48]. Nowadays, through *in vivo* imaging modalities, such as dermoscopy and reflectance confocal microscopy (RCM), subsurface dermoscopic structures can be visualized. These helped to establish a dermal origin for intradermal/compound nevi and an epidermal origin for junction nevi. Also, these contributed to describe morphological similarities between globular nevi and nodular melanoma (NM), and between reticular nevi and superficial spreading melanoma (SSM), allowing to propose a model for progression of these two types of melanoma (Fig. **1c**) [57].

It is not quite clear why some melanomas develop from nevi and some others do not. One possible explanation is that melanoma develops from a mutated melanocyte stem cell. Initially, mutations can be accumulated in a quiescent melanocyte stem cell. Eventually, appropriate environmental signaling may activate an aberrant cell proliferation due to previous mutations. Depending on the nature of initial mutations, there may be a benign nevus phase requiring additional transforming mutations, or a melanoma may develop *ab initio*. In this scenario, nevi would be a possible but not a necessary stage in melanoma development. Transformed cells would try to follow normal differentiation pathways, including migration into the epidermis. Epidermal growth factors would drive further proliferation and differentiation, whereas accumulating mutations may give rise to different subclones, more independent from substrate and growth factors, with aberrant patterns of migration into the dermis, including nerves and lymphatic vessels. Eventually, non-adherent clonogenic cells could be washed out into the lymph system and the systemic circulation, where under appropriate condi-

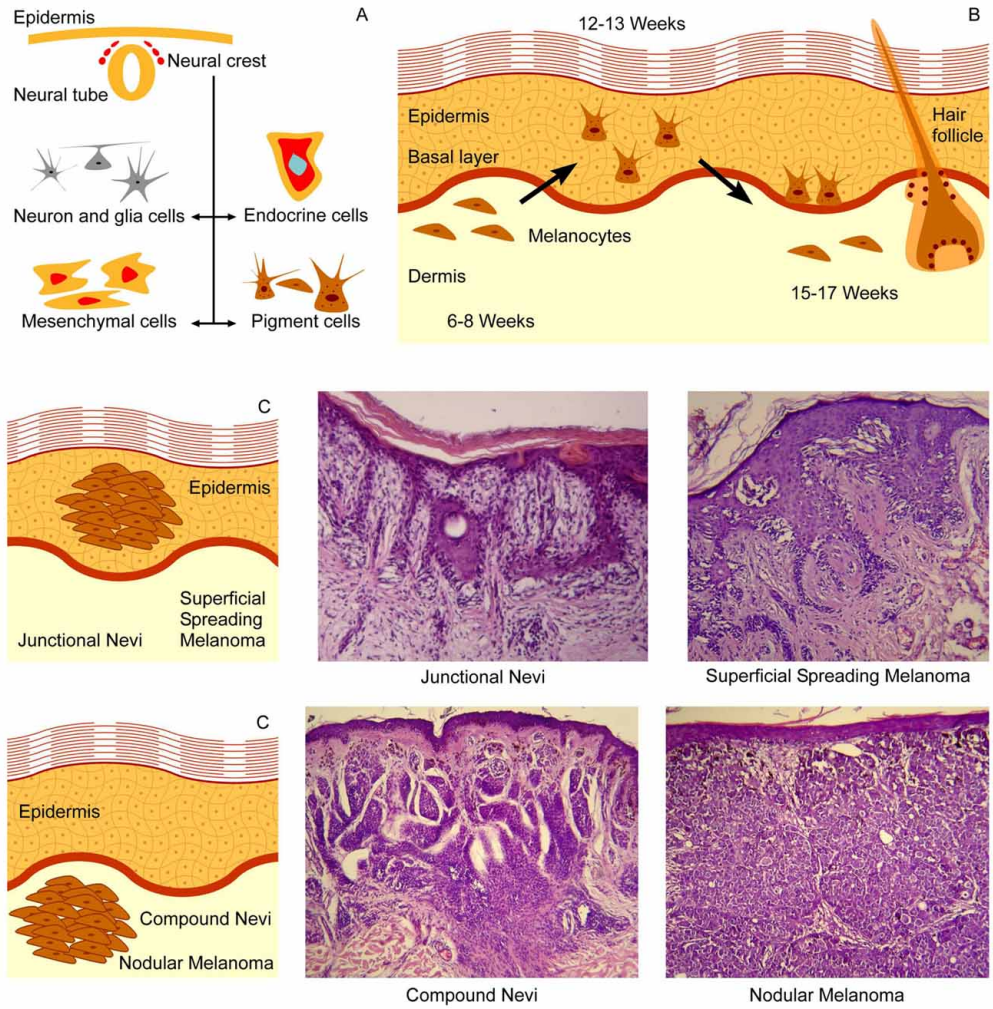

Fig. (1). On the origin of melanocytes, nevi and melanoma. a) Cells from the Neural Crest, sometimes called the fourth germ layer, migrate during development giving rise to multiple lineages, including peripheral neurons and glia cells, pigment melanocyte cells, endocrine cells from adrenal and thyroid glands and mesenchymal cells, like cartilage, bone and connective tissue. b) MITF and MART-1 positive melanocytes in human developing skin migrate from the dermis to suprabasal intraepidermal, to the basal layer of the epidermis and hair follicules. In developed tissue, intradermal melanocytes are also found. c) The presence of melanocytes both in the dermis and in the interphase of dermis/epiderms may account for the origin of compound and junctional nevi respectively. Regarding morphological similarities, a model for progression might be proposed from compound nevi to nodular melanoma, and from reticular nevi to superficial spreading melanoma. Original magnification: 200X.

tions can re-initiate the process in other organs. Whether melanoma develops migrating from or to the dermis is still under discussion. However, in either scenario, the deeper the tumor in the dermal tissue, the lower the requirement for epidermal factors, and the greater the risk that the tumor will be able to proliferate outside the skin surface and be potentially life threatening.

Stem cells or precursors accumulating mutations will decrease their capacity to differentiate along normal melanocytic pathways, unmasking a dedifferentiation process and accounting for tumor heterogeneity. This model proposes a step-based transformation from normal melanocyte stem cells → mutated benign melanocytic stem cells → malignant melanocytic stem cells → clonogenic melanocytic cells, anchorage-independent growth cells capable of migrating and giving rise to metastasis. These populations may express different antigens and could therefore trigger the proliferation of different T and B cell clones.

The stochastic model states that most cells within a tumor are able to proliferate extensively and give rise to new tumors [58]. Opposite, the cancer stem cell model for tumor heterogeneity proposes that only a minor subset of cells, called cancer stem cells (CSC), are able to extensively proliferate and give rise to new tumors. It proposes a hierarchical model of tumor heterogeneity where CSC, like normal stem cells, are able to self-renew and to give rise to a more differentiated and less proliferative population of cancer cells. In turn, they would be the only population capable of forming new tumors upon transplantation. Therefore, it would be crucial to address this population of CSCs, or tumor initiating cells, so as to efficiently eradicate a tumor.

Some attempts have been made to isolate and characterize melanoma stem cells (MSC). One of the first studies reported the enrichment in non-adherent human melanoma spheres in embryonic stem cell media [59], which were able to propagate *in vitro* for several passages (self-renew), dif-

ferentiate to different lineages, recapitulating neural crest stem cells plasticity, and were more tumorigenic than adherent cells in immunodefficient mice. Also, the $CD20^+$-positive fraction was enriched in spheres and had more potential of differentiation. Others isolated putative MSC that had the ability to highly efflux the Hoechst dye, like leukemia stem cells [60]. A different group studied CD133 in melanoma, a cancer stem cell marker in other malignancies like glioblastoma [61]. $CD133^+$ cells, a minor population in several melanoma samples, had enhanced tumorigenic potential in NOD/SCID mice [62]. The expression of the stem cell markers CD133, CD166 and nestin was also study by immunohistochemistry in melanoma biopsies [63]. Their expression was found to be increased in melanoma compared with nevi; specially nestin was overexpressed in metastatic melanoma.

The ABC family receptor ABCB5 was found to be expressed in a $CD133^+$ subset in melanoma biopsies and cell lines, and would be responsible for doxorubicin transport and resistance [64]. Later, it was showed that only $ABCB5^+$ cells obtained from melanoma samples were able to form tumors in NOD/SCID mice [65]. $ABCB5^+$ cells were proved to be the self-renewing and tumor differentiating population in a competitive tumor development model with $ABCB5^-$ cells. Furthermore, tumor establishment and growth could be inhibited by depleting $ABCB5^+$ tumor initiating cells with an anti-ABCB5 mAb, accounting for ABCB5 as a promising melanoma stem cell marker.

Recently, it was reported that by modifying xenotransplantation assay conditions, the frequency of tumorigenic melanoma cells could dramatically increase [66]. Using a more highly immunocompromised NOD/SCID interleukin-2 receptor gamma chain null mice ($Il2rg^{-/-}$) and matrigel as support, tumorigenic potential could increase several orders of magnitude. In all samples from 12 different patients, 25% of unselected cells gave tumors under these more permissive conditions. What is more, in single-cell transplant experiments, 27% of unselected cells caused tumor. In conclusion, tumorigenic cells were found to be common in all tumors, whether they were from primary cutaneous or metastatic melanomas. These data call for attention to xenotransplantation assay conditions to ascertain that a subpopulation has indeed a differential tumorigenic potential.

CHEMOTHERAPY OF MELANOMA: FAILURE AND RESISTANCE

Several therapeutic strategies like surgery, radiotherapy and chemotherapy are being used to treat cancer. However, in the case of melanoma, only surgery can be curative in a high percentage of patients at the very early stages of disease (stages I-II). Once melanoma has invaded profoundly the dermis, if skin lesions are ulcerated, or if lymph node metastases have appeared, the prognosis is poorer. The 5-year survival rate for patients with distant metastases is less than 15% [67]. Chemotherapeutic agents, such as dacarbazine (DTIC), fail to eliminate melanoma cells efficiently, since 7.5-12.1% response rates and 6.4-7.8 months median survival have been reported [68]. This might be due to primary unresponsiveness or to the development of acquired drug resistance by the tumor, commonly defined as multi-drug resistance (MDR).

The identification of specific genes indicates that there are three major mechanisms of drug resistance: a) decreased uptake of water-soluble drugs such as cisplatin, nucleoside analogs and folate antagonists, due to alterations in membrane transporters; b) alterations in the tumor cell that leads to the inability of a given drug to kill the cells, like defects in apoptotic pathways, and c) an increment in the active efflux of hydrophobic drugs that enter the cell by passive diffusion [69].

Most advanced melanoma patients receive systemic therapy. Single-agent cytotoxic chemotherapy with dacarbazine (DTIC) is the standard of care in medical practice, although the response rate is generally low and few patients attain complete remission [70]. Also, combination chemotherapies adding other drugs such as cisplatin, vinblastin or BCNU (Carmustin) to DTIC have not yielded better results, specially considering toxicity and overall survival [71]. Some combination regimens of DTIC with crosslinking agents (Cisplatin), microtubule destabilizators (Vinblastine, Vincristine, Vindesine) and topoisomerase inhibitors (Etoposide, Camptothecin, Irinotecan) have induced responses on metastatic lesions to the liver, bone and brain, commonly unresponsive to DTIC alone, but have failed to produce impact on patient survival [72-74].

The chemotherapeutic agents DTIC and Temozolomide (TMZ) are pro-drugs of the cytotoxic agent 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC). MTIC possesses alkylating activity and generates methyl-DNA-adducts. The most crucial methyl-DNA-adduct for the efficacy of TMZ/ DTIC treatment is O6-methylguanine (O6-meG). This lesion can be repaired by the enzyme O6-methylguanine-DNA-methyltransferase (MGMT) reversing the mispairing of the O6-meG adduct with thymine. Guanine-thymine (GT) mismatch replication errors are normally corrected by the mismatch repair system (MMR). In MMR the repair process initiates by activating an exonuclease-mediated degradation of DNA. Futile attempts to correct these mismatches lead to long-lived nicks in the DNA that finally results in apoptosis of the cell. Thus, the success of a DTIC/TMZ therapy may strongly depend on low MGMT and mismatch repair [75]. MGMT levels in melanomas and gliomas are relatively low, as compared to other cancers [76]. The contribution of MGMT to melanoma resistance to methylating and alkylating agents has yet to be defined. MGMT-induced resistance can be overcome by enzyme inhibition with O6-benzylguanine or by MGMT depletion due to exposure to a methylating agent such as streptozotocin [77]. Although preclinical studies indicated that melanoma cells pretreated to deplete MGMT increased their sensitivity to carmustine, a Phase II clinical trial failed to demonstrate improved survival of patients treated with this drug combination resulting in low response rates [78].

The base excision repair (BER) pathway also repairs lesions generated by methylating agents. N7-methylguanine adducts, although induced at a higher frequency than O6-meG, are efficiently repaired by this mechanism and are so of limited therapeutic significance. Inhibition of BER could be of clinical benefit in combination with methylating agents. PARP (poly (ADP-ribose) polymerase) is a central enzyme involved both in the reparation of DNA strand

breaks and in the recruitment of apoptosis. Since there is evidence that tumors express high levels of PARP and are thus chemoresistant, during the last years PARP inhibitors are being investigated to overcome tumor resistance to TMZ. AG014699 is a prodrug of AG014447, a potent inhibitor of PARP, which has been shown in preclinical models to potentiate cytotoxicity of TMZ and Irinotecan. Inhibition of PARP during TMZ exposure prevents the repair of the strandbreaks that are formed after base excision, thereby triggering apoptosis. Recently, AG014699 has been assayed in combination with TMZ in 33 patients with solid tumors, including melanoma, in a Phase I study. The drug was well tolerated, with no evidence of any significant toxicity, and all patients treated with AG014699 at 12mg/m2 showed high (>90%) inhibition of PARP activity at 5 hours both at the tumor sites and peripheral blood lymphocytes [79]. A Phase II study of AG014699 in combination with 200 mg/m2 TMZ in metastatic melanoma has preliminary encouraging results suggesting some clinical benefit of this chemopotentiation strategy in patients with metastatic melanoma [80]. Another PARP inhibitor, ABT-888 (2-(R)-2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide), has a structural modification including a cyclic amine in the benzimidazole ring system that confers a significant decrease in enzyme activity. This is translated to an *in vivo* improvement of cytotoxic agents such as TMZ, carboplatin and cyclophosphamide as determined in melanoma and breast cancer tumor models [81]. This compound is currently undergoing Phase I clinical trials in cancer patients.

Cisplatin is one of the most active and widely used anticancer drugs. It has proven efficacy against various cancers, including malignant melanoma. Cisplatin (cis-diamminedichloroplatinum) has been largely used alone or in different combinations and regimen schedules to treat melanoma, but all have failed to produce satisfactory results. The cisplatin analog carboplatin [1,1-cyclobutanedicarboxylate platinum (II)] has also been used increasingly in the last 2 decades. Therapeutic use of these platinum-containing compounds is limited by their dose-dependent side effects, including nephrotoxicity, myelosuppression, neurologic damage and strong nausea [82]. The efficiency of cisplatin and its analogs might be often unsatisfactory due to primary unresponsiveness or by acquired drug resistance by tumor cells, which develops rapidly both *in vitro* and *in vivo*, being the major reason for failure of platinum-based therapy. After the entry of cisplatin into the cell, bifunctional platinum adducts are formed with DNA bases. Although only about 1% of intracellular cisplatin reacts with nuclear DNA, this is presumably the critical event in cisplatin-mediated cytotoxicity. Formation of these platinum–DNA adducts *per se* may not be sufficient to cause cell death, whereby the exact cascade of the downstream events leading to cell death is not clear. However, it is generally accepted that formation of platinum–DNA adducts and the subsequent triggering of cellular signal-transduction pathways leading to apoptosis may be the primary cytotoxic mechanism of platinum- containing drugs [82]. In fact, testicular germ-cell cancer is one of the few cancers which may be cured by chemotherapy including cisplatin, and its tumor cells lack NER (Nucleotide Excission Repair) [83].

ATP Binding Casette (ABC)- transporters are a large family of molecules that are involved in active drug efflux mechanisms, and different members of this superfamily are specifically related to different cytotoxic drug resistance depending on the tumor type. In melanoma, the well known ABCB1 (MDR1/P-glycoprotein) is less implicated in drug resistance. ABCB5, a close structurally related member of the ABCB subfamily has been recently associated with a subset of melanoma cells with cancer stem properties demonstrated both in *in vitro* and *in vivo* experiments [65]. ABCB5 expressing cells are found in clinical samples of melanoma and increased expression of ABCB5 may confer doxorubicin resistance [64]. Also, of the two isoforms of ABCB5 (alfa and beta) only specific inhibition of ABCB5 beta at the mRNA level can induce sensitivity to camptothecin 10-OH, 5-FU and mitoxantrone in some melanoma cell lines [84].

Among the mechanisms conferring cellular resistance to platinum-based anticancer drugs in melanoma is the enhanced expression of ABCC2 (MRP2) [85], leading to platinum-compounds extrusion from the cells and consequently to a reduction of platinum-DNA adducts. Clinical use of compounds that inhibit MRP2 *in vitro*, e.g., MK-57114 or cyclosporine A, is not feasible since they are unspecific and intrinsically toxic at effective doses necessary for activity. To selectively block MRP2 mRNA, antisense oligonucleotides [86], siRNAs and hammerhead ribozymes [82] have been investigated *in vitro* in melanoma cells in order to revert cisplatin resistance with encouraging results but it will take a few years to reach the clinical setting with this class of compounds.

RESISTANCE DUE TO DEFECTS IN THE CELL DEATH PATHWAYS

As before mentioned, melanoma resistance to apoptosis can be in part explained by defects in tumor cells that, after cytotoxic chemotherapy, cannot trigger cell death by activation of apoptotic pathways. This final engagement of apoptosis can be impaired by several alterations of the multiple pathways involved in cell survival and apoptosis, like mutations in Ras and Braf oncogenes [87], loss of PTEN and its regulatory control of akt [88], upregulation of Bcl-2 [89] and methylation or allelic loss of Apaf-1 [90].

Most cytotoxic drugs trigger cancer cell death activating an apoptotic cascade that starts with the release of mitochondrial cytochrome C and caspase 9 activation. Chemotherapy resistance in melanoma has been attributed in part to the overexpression of Bcl-2, an antiapoptotic protein that blocks cytochrome C release. Both *in vitro* and *in vivo*, targeted reduction or inactivation of Bcl-2 has resulted in an incremented response to chemotherapy. An antisense strategy has been developed to specifically block Bcl-2 mRNA translation by means of Oblimersen, a 18-base phosphorothioate antisense oligonucleotide that, after binding to mRNA, mediates RNA cleavage by RNAse H. Downregulation at the Bcl-2 protein and increase in chemotherapy-induced apoptosis has been demonstrated *in vitro* and in human melanoma xenografts thus allowing Oblimersen to reach the clinical setting [91, 92]. In 2006, the results of a randomized, controlled trial (The Oblimersen Melanoma Study Group) conducted to examine whether pretreatment with Oblimersen

could enhance the efficacy of DTIC in 771 patients with advanced melanoma were published. After 24 months of minimal follow up, the authors reported that the addition of Oblimersen to dacarbazine resulted in a trend toward improved survival (P=0.077), compared to dacarbazine alone and significant increases in multiple clinical outcomes, with a significantly increased overall survival in patients without an elevated baseline serum LDH. Although neutropenia and thrombocytopenia were increased in the Oblimersen-dacarbazine group, no serious infections or bleeding events were seen [93].

During the last two years, Oblimersen has been clinically tested in combination with doxorubicin, docetaxel, adryamicin and cyclophosphamide for breast cancer [94], rituximab for recurrent B-cell non-Hodgkin lymphoma [95], carboplatin and etoposide for extensive-stage small-cell lung cancer [96]. In all these Phase I/II clinical trials only a modest effect was observed in the Oblimersen combined arm, and some of them failed to observe significant bcl-2 down-regulation in tumors. Further studies are needed to test if this lack of efficacy may be due to insufficient suppression of Bcl-2 *in vivo*.

A recent paper has proposed that melanoma resistance to the alkylating agent TMZ is due to defective apoptotic signaling resulting from activation of p53, but the nature of such defects in apoptotic signaling are still under investigation [97]. The authors found that melanoma cells, but not lymphoma cells, treated with TMZ (72h) reduced their viability in MTT assays but did not undergo apoptosis or necrosis. TMZ sensitivity was associated with a G2/M cell cycle arrest, with accumulation of wild type p53 and p21. Also, inhibition of MGMT resulted in further decrease of viability. TMZ induces more cellular senescence in melanoma cell lines with wild type p53 than in those with mutated p53, and the levels of MGMT also play a role in TMZ sensitivity. Another recently published paper demonstrated in a panel of melanoma cell lines that cells exposed to TMZ for longer periods of time, came to apoptosis. The apoptotic process started 72 hours after drug addition, and at longer times of TMZ exposure (120-144 h), DNA double stranded breaks (DSBs) were formed and these DSBs might act as a downstream trigger of O^6-meG –induced apoptosis. Resistance to TMZ was not related to p53 status but there was an inverse correlation between MGMT activity and TMZ-induced apoptosis. Inhibition of MGMT by O^6-benzylguanine sensitized MGMT-expressing cells and the transfection of MGMT caused TMZ apoptotic resistance [98].

BIOCHEMOTHERAPY SCHEMES: RATIONALE BASIS IS LACKING?

The combination of different drugs and immunotherapy (biochemotherapy) to treat metastatic melanoma has been explored in clinical trials over the last 20 years, but overall survival and complete durable response rates have not improved so far [99-101]. IL-2 promotes T-cell proliferation, the generation of CTLs, and the activation of T and B lymphocytes, enhancing activity of natural killer cells (NKs). High dose-IL-2 can achieve long-term control of melanoma since high dose IL-2 regimen was able to produce durable responses in 16% of patients, with 6% achieving complete responses [102]. Also, IFN- α2b, has proved to be active in combination with other agents, against melanoma with an overall response rate of 24% (range 10-46%) in untreated patients with metastatic disease [103]. In combination strategies, chemotherapy is given for debulking tumors while IL-2 is thought to increase lymphocyte and NK cell counts, improve the CD4+/CD8+ ratio and decrease the number of regulatory T cells. In the case of IFN-α2b, however, the molecular mechanism of action remains unclear. In 2008, Kirkwood *et al.* [104] reported that in patients treated with high-dose IFN α2b, MAPK signaling is regulated differentially in melanoma tumor cells and host lymphoid cells *in vivo*. They analyzed tumor and lymph node biopsies obtained pre- and post- treatment and found that high-dose IFN α2b down-regulated both MEK/ERK MAPK and pSTAT3 activation in tumor cells, but not the ERK MAPK in lymphoid cells. Thus, high-dose IFN α2b seems to disrupt the MEK/ERK MAPK pathway in melanoma cells *in vivo*. Activation of this pathway is important for tumor cell motility [105] and therefore its down-regulation may be correlated with inhibition of tumor metastasis.

Biochemotherapy combinations including cisplatin, vinblastine and DTIC (CVD) along with IL-2 and IFN- α2b, have shown some promising results in small, controlled phase II trials [106]. Recently, the results of the largest Phase III Trial comparing concurrent biochemotherapy with CVD plus IL-2 and IFN- α2b (BCT) to CVD alone in patients with metastatic melanoma (E3695) has been published with disappointing results. Although BCT produced slightly higher response rates and longer median progression-free survival than CVD alone, no statistically significant improvement in overall survival or durable responses was observed. Also, higher toxicity was observed in the BCT arm compared to the CVD arm (95% versus 20% grade 3 or worse toxicity) was seen, thus concluding that BCT should not be recommended for patients with metastatic melanoma [71].

TMZ has also been used in combination with IFN-α2b [107], thalidomide [108] and thalidomide plus radiotherapy [109] without a significant impact in overall survival.

Failure of these clinical studies make evident that there are still several issues that need to be investigated to set a rational basis to these combination regimens. It is possible that the concurrent administration of IL-2 and chemotherapy can abolish the durable immunotherapeutic effect of IL-2, since complete responses to high-dose IL-2 were reported in 15% of patients who failed to respond to BCT [110]. Also, progression-free and overall survival were longer with the use of maintenance IL-2 and granulocyte-macrofage colony-stimulating factor immunotherapy after similar concurrent BCT regimen [111].

Most cancer chemotherapy and radiotherapy research has been developed without considering any contribution from the immune system to the mechanisms of tumor regression, since the different strategies were validated in models of human xenografts in immnunodeficient mice. This issue is changing since there is increasing evidence indicating an agonistic interplay between cytotoxic drugs and the immune system, both contributing to tumor cell death (recently reviewed in [112]). Many therapeutic approaches currently used in the clinic can abrogate potential immune responses against tumors, such as surgical resection of tumor-draining

lymph nodes and/or chemotherapy-induced elimination of cytotoxic effectors. Since the majority of chemotherapeutic drugs exert their anticancer effects through the induction of apoptosis, it is believed that no inflammation is associated with tumor cell death and thus the process is not immunogenic. However, there is an increasing amount of evidence demonstrating that some chemotherapeutic and radiation schemes can render dead tumor "immunogenic" cells, by means of increased expression of MHC molecules, tumor Ags and death receptors. For example, HMGB1 (high-mobility group box 1 protein) is a nuclear protein that is released from dying tumor cells during late-stage apoptosis that binds to TLR4 on Dendritic cells (DCs), promoting antigen processing and presentation [113]. Besides, some drugs can impact in the immune system, either modulating immunosuppressive lymphocyte subsets, like cyclophosphamide [114], or stimulating immune effectors, like gemcitabine [115, 116]. Future treatments should consist in a more personalized strategy, in which tumor characteristics, immune status (activation capacity and/or immunosuppression) and intrinsic patient characteristics, such as polymorphisms and mutations that may influence therapeutic outcome, will be evaluated in an integrated form.

IMMUNOLOGICAL APPROACHES TO MELANOMA TREATMENT

Due to the failure of most chemotherapy regimes, attention has turned to the immunological treatment of melanoma. This is so since for still poorly understood reasons, T cell lymphocytes directed against melanoma differentiation antigens have not been fully submitted to central deletion, and may be therefore be activated. Two main approaches are being used to activate T cell clones directed against melanoma antigens: either *in vivo*, through the use of vaccines, or *ex vivo*, through the expansion of T cells in numbers sufficient to cope with growing tumor masses. We shall first refer to the use of vaccines.

VACCINATION WITH WHOLE CELLS, CELL LYSATES AND PURIFIED ANTIGENS

The concept that tumors express specific antigens was demonstrated in sarcomas in the 1950s [117, 118]. Thus, immunity to cancer might be acquired, and the use of vaccines against cancer begun. Pioneering work in melanoma vaccines was performed by Morton and colleagues, who detected specific melanoma antigens by immunofluorescence [119]. Morton, as well as Seigler´s group, used intralesional BCG, a potent nonspecific immune stimulant [120, 121]. Berd and Mastrangelo associated vaccination using autologous irradiated melanoma cells with cyclophosphamide in low doses (150 mg/sq.m) to diminish immune suppression [122, 123]. This immune-enhancing effect of cyclophosphamide was later confirmed in murine experimental systems [124]. After these pioneer works, several melanocytic differentiation antigens (MD-Ags) were discovered, such as MelanA/MART-1 (MART-1) [125, 126], gp100/PMEL17/silver (gp100) [127], tyrosinase [128]; tyrosinase-related protein-2 (trp-2) [129], MELOE-1 [130] and a group of cancer-testis Ags (CT-Ags), such as the MAGE super-family and NY-ESO-1 [131, 132]. However, vaccination with MD-Ags purified peptides has provided scarce clinical responses in melanoma patients [133, 134]. At that time, the still fully unanswered question was whether humoral or cellular immune response would be more convenient to eradicate tumors in general and melanoma in particular. Livingston *et al.* had observed that vaccination of melanoma patients with purified ganglioside GM2 coupled to KLH induced high IgM and IgG titers against GM2 [135]. However, in an ECOG randomized assay in 880 melanoma patients comparing this vaccine against high-dose interferon-alfa, the vaccinated patients arm did worse than the interferon arm, and the assay was interrupted before completion [136].

The advantage as vaccines consisting of irradiated whole tumor cells, is based on the belief that melanoma has some known, and probably many unknown antigens, and therefore the immune system should be given the opportunity to deal with all of them. The rationale assumption is that vaccination with a single or a few tumor antigens would not be sufficient to eradicate tumors, even if CD8+ and CD4+ cells are generated and are efficient in killing Ag+ tumor cells, since Ag-negative cells would soon repopulate the tumor [137]. However, the original approach of using autologous cells as vaccines has two major disadvantages: first, patients must have heavy metastatic disease to obtain enough cells after surgery to manufacture vaccines, and are therefore relatively advanced; second, reproducibility in vaccine preparation is difficult to attain, since different patients have different tumor masses and cell yields are quite diverging. Therefore, we and others have attempted vaccination with irradiated, whole allogeneic melanoma cells, which may be easily obtained from cell lines. A group that pioneered this approach was that of Morton *et al.*, who used a mixture of allogeneic irradiated melanoma cell lines (Canvaxin) to vaccinate stages III and IV melanoma patients with BCG [138]. However, in spite of encouraging results in non-randomized Phase II trials, a randomized Phase III clinical trial of Canvaxin versus BCG led to an early suspension of the trial due to worse results for the Canvaxin arm. A significant advance in the vaccination field was achieved when it was demonstrated that the addition of the cytokine GM-CSF to vaccines increased the immune response against tumors [139]. Several clinical trials have thereafter used GM-CSF to increase immunogenicity. Luiten *et al.* used autologous melanoma cells transduced with a GM-CSF-producing retrovirus, and observed in some patients that tumor sites became infiltrated with lymphoid cells [140]. However, the delay in producing the cells for vaccination took an average of ten weeks and circulating GM-CSF could not be detected. Also, Soiffer *et al.* [141] used as vaccines irradiated autologous melanoma cells transduced with an adenovirus containing the GM-CSF gene. In this trial, immmune reactivity at tumor sites was also observed, but all tested patients developed anti-adenovirus antibodies. We therefore decided to use as therapeutic vaccine a mixture of irradiated allogeneic melanoma cell lines, with BCG as adjuvant and injecting at the vaccination site GM-CSF coincidently with the vaccines; in this way we could exactly determine the amount of injected GM-CSF, and thus avoid the uncertainty of the amount and length of GM-CSF production by irradiated tumor cells. Using this approach in a Phase I clinical trial including 20 patients, stages III and IV, we obtained 70% of relapse–free survivors in stage III melanoma patients after five years of follow-up [142]. This

vaccine has now entered a randomized Phase II / III clinical study, where Vaccine CSF470 plus GM-CSF and BCG shall be compared with IFN-alfa during two-years in stages IIB, IIC and III melanoma patients.

Also, several clinical trials in stage IIB- IV melanoma patients utilized vaccination with multiple peptides derived from melanocytic differentiation antigens (Mart-1, gp100 and tyrosinase, and from cancer-testis antigens (MAGE), but success have been limited [143]. The overall evidence has been shifting to the belief that immunity against melanoma should be based more in the development of specific CD8 and CD4 cells rather than in antibodies. The rationale of vaccination with tumor antigens, either in the form of purified antigenic peptides or present in whole tumor cells or cell lysates, is that these antigens should be captured by Dendritic cells (DCs), which, to exert their function, should migrate to lymph nodes and prime naïve lymphocytes. Since it is difficult to evaluate the efficiency of this process *in vivo*, the idea seemed natural to produce DCs *ex vivo*, incubate them with tumor antigens and re-inject them to the patients.

Dendritic cells (DCs) were first described by Steinman and Cohn in murine lymphoid organs [144], and were later found to be potent stimulators of naïve lymphocytes [145]. Subsequent work demonstrated that DCs participate in the afferent and efferent limbs of the immune response, each requiring antigen presentation and MHC restriction. When studying Langerhans cells, a distinct type of DCs present in epidermis, Schuler and Steinman [146] demonstrated that DCs may be in two states: immature DCs, which are able to phagocytose antigens but are unable to present them to naïve lymphocytes, and mature DCs, which lose the ability to capture antigens but present processed antigens with great efficacy. The generally accepted pathways that DCs use to activate naïve lymphocytes involves several steps: i) capture of antigens in the periphery; ii) maturation and migration to draining lymph nodes; iii) establishment in the lymph nodes and activation of naïve lymphocytes. Some of these steps have been analyzed to some detail in mice, although evidence in humans is still lacking. Thus, Eggert *et al*. demonstrated that only about 1% of subcutaneously injected DCs migrate to lymph nodes [147], although resident Langerhans cells, after immunization *in vivo*, migrate in high numbers to lymph nodes and persist there for about two weeks [148]. It may be thus concluded that *in vivo* migration of CDs is substantially more efficient than migration after CDs production *in vitro* and subcutaneous injection, which could hamper vaccination attempts with CDs loaded with tumor antigens. After this scarce migration of injected DCs to regional lymph nodes, they must still overcome another difficulty: to find the appropriate T cells expressing the adequate TCR while maintaining bound to their HLA molecules the antigenic peptides long enough to induce long-lasting contacts [149]. In fact, a clinical study comparing DCs charged with tumor peptides or cell lysates demonstrated that only the later were capable of inducing immune responses [150]. An explanation for these results came recently, since it has been shown that antigenic peptides induced a quicker and stronger, but less prolonged response, than larger antigenic peptides that are taken up by DCs and degraded inside the cells [151].

Recently, several approaches have taken profit of the ability of DCs to capture foreign antigens, among them tumor antigens, and present them to naïve lymphocytes [152, 153]. In humans, Palucka *et al*. [154] demonstrated that autologous DCs were able to capture killed cells from an allogeneic tumor cell line and induce CD8+ T cell responses in 20 stage IV melanoma patients, leading to one complete and one partial response. von Euw *et al*. demonstrated that autologous DCs could capture a mixture of apoptotic and necrotic allogeneic melanoma cells, subsequently mature and cross-present melanoma differentiation antigens to CD8 T cell clones [155]. von Euw *et al*. also performed a clinical study in melanoma patients demonstrating that up to 1% anti-MART-1 and anti-gp100 CD8 T cell lymphocytes could be found in circulating blood after vaccination [156]. Although 80% of Stage III patients attained disease-free survival for more than four years, all stage IV patients relapsed. None of the approaches used so far could demonstrate that injected CDs charged with apoptotic/necrotic tumor cells were able to migrate efficiently to draining lymph nodes and establish a correct communication with naïve lymphocytes. The duration of the *in vivo* tumor antigenic exposure by DCs has also not been thoroughly studied in humans, as well as the number of CD8 cytolytic T cells formed.

TREATMENT WITH EXPANDED TUMOR SPECIFIC LYMPHOCYTES

The slow formation of CD8 T cells after immunization with vaccines would render this approach only effective when patients have a minimal tumor burden at the start of vaccination [156], but it would be ineffective for treating stage IV patients with big tumor masses. Moreover, melanoma duplicates fast, since 15-50% of the cells are Ki-67-positive (*Mordoh et al., unpublished results*). The concept thus emerged, pioneered by Steven Rosenberg and his colleagues at the NCI [157], that the low rate of effectors formation after vaccination could be overcome by effectors growth *in vitro*. Expansion of tumor infiltrating lymphocytes (TIL) was undertaken in the presence of IL-2, and the infusion of 10^9-10^{11} expanded TIL together with high dose IL-2 determined a total response rate of 30% in early studies in stage IV melanoma patients [158]. One of the relevant questions of this approach is if CD8 tumor-antigen specific lymphocytes act by themselves, or if their activity must be complemented by other effector cells, such as NK cells or polymorphonuclear neutrophils. Breart *et al*. [159] have provided an elegant answer to this question. They have studied with two photon microscopy in a murine model of solid tumor (EL4/EG7) if the ability of specific lymphocytes to attack the tumor is enough to kill tumor cells, or if accessory cells are needed to perform this task. They have concluded that CD8 T cells alone are sufficient to kill tumor cells, although a long time is required to kill a tumor cell, about six hours. However, how many tumor cells a single CD8 T cell may kill is not known. Besides, there are many circumstances that may diminish the number of lymphocytes targetting tumor cells. It has been found that functional CD8 lymphocytes directed against MART-1 are active in peripheral blood, but their activity against tumor cells within the tumor is considerably reduced [160, 161]. A possible explanation for this lack of functional activity of CD8 lymphocytes would be the release by tumor cells of immunosuppressive molecules,

such as galectin-1 [162] or galectin-3 [163]. Another important factor would be the inability of CD8 T cells to enter the tumor. In this sense, it has recently been demonstrated that tumor vasculature exhibits numerous defects which inhibit lymphocytes from entering into tumors, and that this defect could be reversed in murine models by knocking down the gene Rgs5 (regulator of G-protein signaling) [164]. The presence within the tumor of regulatory suppressor CD4+ CD25+ Fox P3+ cells could also dampen the immune response, and it is a predictive factor of lack of response to immune treatments [165].

Recent work has demonstrated that if infusion of *ex vivo* expanded CD8 lymphocytes is preceded by chemotherapy ablating patient´s lymphoid system, the chances of response are increased [166]. Also, the importance of infusing CD4 T cells has recently been highlighted [167], since their synthesis of IL-2 would allow the growth of CD8 T cells under more physiological and less toxic conditions than when high-dose IL-2 is infused.

The lack of expression by tumor cells of HLA-I and II carrying the appropriate tumor peptides could also be a determining factor of resistance to immunological treatments. In this sense, we have observed that even in primary tumors a large proportion of tumor cells do not express HLA-I molecules on their surface, thus rendering them unable to be attacked by CD8 T cells (*Mordoh et al., unpublished results*): Although tumor cells not expressing HLA-I at the surface could theoretically be a target for NK cells, we have recently determined that the non-classical HLA-E molecule inhibits NK activity, at least in colorectal cancer cells [168]. Also, we have demonstrated that in some melanoma cell lines the receptor MICA for the NKG2A effector molecule of NK cells may be downregulated and thus evade NK activity [169].

Analyzing the development of melanocytes has revealed their migratory nature allowing them to colonize both the dermis and the epidermis. Thus, transformed melanocytes can result in melanomas arising from both locations. Melanoma may arise from a mutated stem cell in the skin but it is still not known whether a subpopulation persists with a stem cell phenotype, the cancer stem cells, within the tumor. The discovery of the mechanisms involved in molecular transformation is generating specific new targets to improve therapy and to classify subsets of tumors.

Multiple evidences support that melanoma is essentially resistant to chemotherapy, both by intrinsic and acquired pathways. In terms of melanoma therapy, perhaps a sequential use of the currently employed agents could be more rationale and result in a more efficient outcome without the high expense of extreme toxicity seen in the latest trials. Chemotherapy and/or radiotherapy could be used to reduce tumor masses, resulting in a lower number of remaining tumor cells, but also in the generation of a systemic antigenic exposition of surface and intracellular epitopes, along with some necrosis-driven inflammation due to tumor destruction. In patients with high probability of relapse, immunotherapy with vaccines should be provided in the context of clinical trials as soon as possible, when the disease is still at its microscopic stage. Instead, when visceral metastatic disease develops, vaccines should render its place to treatment with effectors developed *ex vivo* to eliminate cells in the tumor sites as well as circulating tumor cells that will ultimately form additional metastases. For the time being, the complexity of this treatment and its still low rate of complete response precludes its use in patients with stages IIB, IIC and III, in whom a significant proportion may be already cured after surgery.

More research is needed to test these different combinations of therapeutic tools to control melanoma as well as to unravel the specific mechanisms of action and interactions between immune effectors and tumor cells *in vivo*.

NOTE

This review has been written using selected references which, in the opinion of the authors, serve to illustrate the changing directions of the field, and the literature is by no means exhaustive. We apologize for the great number of high-quality papers that could not be cited here.

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