

**Unraveling the crosstalk between melanoma and immune cells in the tumor  
microenvironment**

**Monica Marzagalli<sup>a</sup>, Nancy D. Ebel<sup>b</sup> and Edwin R. Manuel<sup>b,\*</sup>**

<sup>a</sup>Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano,  
Milano, Italy

<sup>b</sup>Department of Immuno-Oncology, Beckman Research Institute of City of Hope, Duarte,  
California, United States of America

\*Corresponding author:

Edwin R. Manuel  
City of Hope,  
1500 E. Duarte Rd  
Duarte, California, 91010  
USA  
+1-626-218-2452  
[emanuel@coh.org](mailto:emanuel@coh.org)

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## **Abstract**

Cutaneous melanoma is the most common skin cancer with an incidence that has been rapidly increasing in the past decades. Melanomas are among the most immunogenic tumors and, as such, have the greatest potential to respond favorably to immunotherapy. However, like many cancers, melanomas acquire various suppressive mechanisms, which generally act in concert, to escape innate and adaptive immune detection and destruction. Intense research into the cellular and molecular events associated with melanomagenesis, which ultimately lead to immune suppression, has resulted in the discovery of new therapeutic targets and synergistic combinations of immunotherapy, targeted therapy and chemotherapy. Tremendous effort to determine efficacy of single and combination therapies in pre-clinical and clinical phase I-III trials has led to FDA-approval of several immunotherapeutic agents that could potentially be beneficial for aggressive, highly refractory, advanced and metastatic melanomas. The increasing availability of approved combination therapies for melanoma and more rapid assessment of patient tumors has increased the feasibility of personalized treatment to overcome patient and tumor heterogeneity and to achieve greater clinical benefit. Here, we review the evolution of the immune system during melanomagenesis, mechanisms exploited by melanoma to suppress anti-tumor immunity and methods that have been developed to restore immunity. We emphasize that an effective therapeutic strategy will require coordinate activation of tumor-specific immunity as well as increased recognition and accessibility of melanoma cells in primary tumors and distal metastases. This review integrates available knowledge on melanoma-specific immunity, molecular signaling pathways and molecular targeting strategies that could be utilized to envision therapeutics with broader application and greater efficacy for early stage and advanced metastatic melanoma.

## 1. Introduction

The recent rise of immunotherapies has led to an old, but revolutionary, concept of cancer treatment based on the improved activation of the endogenous immune system against cancer cells (1-3). The relevance of the field is well represented by the joint contribution of the 2018 Nobel Prize recipients for Physiology or Medicine James P. Allison and Tasuku Honjo for their discovery of cancer therapy by inhibition of negative immune checkpoint regulation (4, 5). The first immune-checkpoint inhibitors, antibodies that specifically target the immunoregulatory molecules cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4, Ipilimumab) and programmed cell death protein 1 (anti-PD-1, Nivolumab), were approved by the US Food and Drug Administration (FDA) in 2011 and 2014, respectively, for the treatment of unresectable or metastatic melanoma, thus enormously improving the management of this aggressive cancer, and doubling the median survival for metastatic disease (6, 7).

Malignant melanoma represents one of the most immunogenic tumors, which means that it has incredibly high genomic mutational load and has the highest potential to elicit specific adaptive antitumor immune responses. It serves as an excellent model for the evaluation of innovative immunotherapies such as checkpoint inhibitors as well as anticancer vaccines and engineered chimeric antigen receptor T cells (CAR T cells) (8-10). Moreover, melanoma may be vulnerable to a newer cohort of checkpoint inhibitors targeting B- and T-lymphocyte attenuator (BTLA), T-cell immunoglobulin and mucin domain-3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3) that continue to be areas of intense research (11). Despite these major advances in cancer immunotherapy, a large subset of melanoma patients do not respond or relapse due to primary or acquired resistance, resulting in 40 to 65% treatment failure for patients treated with anti-PD-1, and treatment failure in over 70% of patients treated with anti-CTLA-4 (12).

The plasticity of melanoma cells leads to a phenomenon called “immune escape”, whereby cancer cells acquire a less immunogenic phenotype and the ability to suppress anti-tumor immune

cells within the tumor microenvironment (TME) (13, 14). While many factors contributing to immune escape have been elucidated, a therapeutic strategy to completely re-instill curative anti-tumor immunity has not yet been realized. This review will describe the immune landscape participating in the initial control of pre-malignant cells and then will highlight the molecular and cellular “crosstalk” exploited by melanoma to reprogram immune cells and the TME to cause immune escape and progression to advanced disease. Finally, this review will shed light on the innovative immunotherapies that are currently under investigation with the aim to rescue anti-tumor immunity.

## **2. Immune surveillance in melanoma**

### **2.1 Immunity and melanoma**

The immune system is generally thought to keep the body in a state of homeostasis by defending against infection and disease caused by bacteria, viruses, fungi, and parasites. However, it is now generally accepted that the immune system also functions to constantly survey and eliminate pre-cancerous cells to prevent progression to melanoma (15-19). In most cases, intracellular check points are engaged within a malfunctioning cell that leads to a process of self-destruction, or apoptosis, negating the need for immunity. However, in instances when pre-malignant cells do not properly undergo apoptosis, the immune system must quickly act to prevent further transformation and the potential for immune escape (20-22).

As in infection, both the innate and adaptive arms of immunity must work together to eliminate both pre-malignant and early stages of melanoma, and to provide long-term protection from potential relapse (23-26). For this to occur, the innate compartment must quickly eliminate tumor cells and act to recruit adaptive immune cells, present tumor antigens through major histocompatibility complexes (MHC) and provide the proper co-stimulatory signaling through surface receptors and/or cytokines to generate a long-term, tumor-specific memory population. Cytokines involved in this process include interleukins (IL), interferons (IFN) and colony

stimulating factors (CSF), which can be activating or suppressive in nature. Orchestrating an effective, anti-tumoral response, especially after negative selection in the thymus that leads to self-tolerance of tumor cells, seems to be a nearly impossible task. However, melanoma tends to be incredibly immunogenic, generating neoantigens through chromosomal rearrangements or genetic polymorphisms that can mimic “foreign” infection and thus potentially elicit cytotoxic responses (27-31). Many of the key innate and adaptive subsets capable of anti-tumor activity have been identified in independent studies of melanoma and other solid malignancies (23, 32). The most effective therapeutic strategy should integrate as many of these immune subsets, without causing significant toxicity, for the greatest clinical benefit (33). Here, we focus on the inherent anti-tumor functions of both innate and adaptive immune subsets that are postulated to control pre-malignant cells during early stages of melanomagenesis.

## **2.2 Innate immunity**

The fast and non-specific anti-tumor responses elicited by innate immunity are not only critical in preventing and controlling early stages of melanoma, but also in priming robust adaptive immunity to provide long-term, tumor-specific immune surveillance. Several therapeutic strategies to inhibit melanoma growth have specifically focused on the activation of the anti-tumor activities of naive or differentiated innate subsets found within tumors, which includes, but are not limited to, macrophages, polymorphonuclear neutrophils (PMN), natural killer (NK) cells and dendritic cells (DC) (33, 34). These innate cells comprise an immune system within the skin known as the skin-associated lymphoid tissue (SALT) (35). It is important to note that innate cells are incredibly plastic and can acquire both pro- and/or anti-tumor functions depending on cell-cell or tumor-cell engagement and soluble factors present in the microenvironment (36, 37). Here, we first discuss the anti-tumor activities that can be exerted by innate immune cells.

Tumor-resident macrophages, PMN, NK and DC are among the first to contribute to immediate, non-specific cytotoxicity against melanoma cells. Through activating NK receptors (NKG2D,

NKp30, NKp46, DNAM-1) and agonists present on the surface of melanoma cells, NK are independently activated to eliminate melanoma cells that have significantly downregulated their MHC class I molecules (23, 38). Indeed, previous studies have found that IL-15 stimulation of NK cells is sufficient to cause regression of MHC class I low melanomas in mice. It has been demonstrated that the expression of NK receptor ligands by melanoma cells can be both dependent on tumor progression (acute vs. late recognition) and localization (primary vs. metastatic and in different metastatic sites). For example, NKp44 and NKp46 are expressed on lymph node metastasis but in a lesser extent in skin metastasis. The expression of DNAM-1 ligands, such as nectin-2 (CD112) and PVR (poliovirus receptor), is independent of the anatomical site and tumor stage, and the disruption of their interaction with DNAM-1 is responsible of the loss of cytotoxicity and of failed tumor rejection. DNAM+ NK cells showed higher cytotoxicity with respect of DNAM- NK cells, despite the two populations shared the same positivity for both NKp46 and NKG2D, suggesting the relevance of DNAM-1 signaling in activating NK cells against melanomas, at least during early recognition and lymph node metastasis (38).

In addition to direct tumor interactions, NK cytolytic activity can be induced through DCs activated by soluble antigenic peptides present in the TME (23, 39). In general, NK cells poorly infiltrate primary cutaneous melanomas and mostly accumulate in the peritumoral space, however, during regression, they can be observed more dispersed throughout the tumor tissue (40-42). Intratumoral NK cells (activated) can then indirectly contribute to recruitment and maturation of antigen-presenting cells through the secretion of cytokines such as CXCL1 (CXC-motif ligand 1) and CCL5 (CC-motif ligand 5). Macrophages, PMN and DC that are recruited to tumor tissue can then phagocytose apoptotic or dead melanoma cells or debris and cross-present tumor antigens that drive secondary adaptive immune responses involving CD4 helper and CD8 effector T cells (CD4 Th and CD8 T<sub>eff</sub> cells, respectively) (43-45). Interestingly, several studies in pre-clinical melanoma models have shown that soluble factors secreted by activated T cells in turn induce anti-tumor activity of innate immune cells (macrophages and granulocytes) to assist in primary tumor growth control and minimize lung metastases (46).

As professional antigen-presenting cells (APCs), DCs are among the most efficient in eliciting cytotoxic T cell responses against infection and malignancy. DCs circulate and survey various tissues throughout the entire body, ultimately migrating to lymph nodes where interactions with naive or memory T cells occur (39). Mature DCs express a plethora of co-stimulatory markers, including CD80 and CD86 (cluster of differentiation 80 and 86, respectively), which are essential for activation of melanoma-specific T cells (47, 48). The T cell receptor (TCR)-MHC class I interaction, co-stimulatory markers and proper cytokines (IL-12, IFN- $\gamma$ ) produced by DCs and helper T cells are requisite for the proper development of melanoma-specific, cytotoxic T cells (49, 50). Ultimately, functional effector T cells must be recruited to melanomas through a chemokine gradient (CXCL9, CXCL10, CXCL11) generated by DCs or tumor-associated stroma (51, 52). Inefficiency in any or all of these steps can lead to compounding deficiencies in adaptive tumor-specific immunity. Overall, DCs exert a protective role against melanoma tumors as evidenced by high frequency of DCs in tumor-negative sentinel lymph nodes (53-55).

Anti-tumor macrophages and neutrophils, designated M1 and N1, respectively, have been studied extensively for their potential use as immunotherapy for melanoma (56-61). These innate subsets exert anti-tumoral effects through phagocytosis, secretion of tumoricidal agents (reactive oxygen species, nitric oxide, IFN- $\gamma$ , Fas ligand/FasL) or assemble other tumor-specific immune cells through secretion of chemotactic factors. Interestingly, while macrophages can stimulate adaptive T cell responses, a reciprocal relationship also exists whereby activated Th1 cells generate tumor-killing macrophages through the expression of IFN- $\gamma$ , CD40 ligand and lymphotoxin-alpha. Use of microbial agents (Bacillus Calmette-Guerin (BCG) and vaccinia virus) have been shown to be effective against melanoma by inducing the anti-microbial, cytotoxic functions of macrophages. Topical agents, such as ingenol-3-angelate, are known to recruit neutrophils in cutaneous melanomas and induce their N1 anti-tumor functions (62, 63). Similarly, we found that the primary immune population mediating tumor growth control of subcutaneous melanomas, following

intravenous administration of a tumor-colonizing, *Salmonella*-based therapy, were cytotoxic PMN responding to the bacterial vector (64, 65). In both cases of neutrophil-mediated killing, cytotoxic activity was contained within tumor tissue, minimizing adverse effects to healthy tissue that would normally be observed with radiotherapy or chemotherapy. Adoptively transferred M1- and N1-polarized cells generated *in vivo* with GM-CSF (granulocyte-macrophage colony stimulating factor) treatment have also been shown to eliminate melanoma in recipient mice (66-70). Overall, the innate immune system plays a critical role in first-line defense against melanoma and will likely be indispensable for generating effective anti-tumor immunity using immunotherapy.

### 2.3 Adaptive immunity

Long-term memory responses critical for life-long melanoma remission involve the activation and expansion of adaptive immune cells, namely helper CD4<sup>+</sup> T cells and cytotoxic or memory CD8<sup>+</sup> T cells. As previously mentioned, DC, and to some extent macrophages, are the most capable in priming adaptive immunity to incite cytotoxicity of CD8<sup>+</sup> effector T cells and also mediate generation of memory immune populations involved in long-term remission. DC are initially activated in the tumor bed in the presence of cytosolic melanoma DNA through the cGAS-STING pathway (71-74). Soluble tumor antigens from necrotic melanoma cells are engulfed by DC and macrophages and proteolytically processed for direct presentation or cross presentation to naïve T cells in tumor-draining lymph nodes (TDLNs) via MHC class I and II molecules. In some instances, neutrophils have also been shown to present antigen in TDLN, although their efficiency to elicit T cell responses is incredibly low (75). Cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), play pivotal roles in activation of melanoma-specific T cells during TCR-MHC interactions and are expressed by DCs and macrophages of the M1 phenotype.

While neo-antigens expressed by melanoma cells would be the most immunogenic, i.e. most likely to expand cytotoxic T cells, self-antigens expressed at high levels could potentially break tolerance and activate low-affinity CD8<sup>+</sup> T cells. It is becoming more apparent, however, that more



than just overexpression of wildtype peptide sequences from melanoma cells is needed to induce effective anti-tumor responses from tolerant or low-affinity T cells. Hence, the use of checkpoint inhibitors, such as anti-PD1/PD-L1 and anti-CTLA-4, and adjuvants in combination with self-antigen cancer vaccines is almost requisite (76, 77). Currently, identification of neo-antigens broadly expressed by melanomas is an area of intense research. Indeed, vaccines encoding neo-antigens expressed by a single melanoma can be therapeutically beneficial as determined in pre-clinical models of melanoma utilizing a single melanoma cell line or graft. However, neo-antigens are not identical from patient to patient or even from distal melanomas within the same individual, thus requiring development of highly personalized vaccines, which can be both cost- and labor-intensive with no guarantee of success (78, 79).

Once CD8<sup>+</sup> T cells are sufficiently primed and activated, naturally or through vaccination, they begin to seek out and induce apoptosis of melanoma cells through the release of perforin and granules, which then provides additional antigens for presentation and expansion of melanoma-specific T cells (80). Life-long remission can then be achieved with this repeating cycle (**Figure 1**), but in many cases, a select population of resistant melanomas subvert immune recognition and destruction by downregulating antigen presentation machinery, direct suppression of both innate and adaptive immune cells or expansion of immune-suppressive subsets.

### **3. Melanoma cross-talk leading to immune escape**

#### **3.1 Hallmark of cancer: Immune escape**

The existing relationship between cancer cells and immune cells can be described following the 3 E's rule (Elimination, Equilibrium, Escape). During early phases, transformed cells are actively eliminated by immune cells, thus impeding tumor initiation. Due to the high plasticity of tumor cells, and the eventual development of favorable mutations, a subset of transformed cells can acquire properties that lead to immune evasion. During "equilibrium", tumor initiation is achieved by selection and expansion of the "immune-resistant" clones, but the host's immune system is able

to control tumor outgrowth through continuous elimination of “immune-sensitive” clones. The last phase is characterized by immune escape: cells that are most fit to evade immunity are free to proliferate leading to tumor progression, analogous to Darwinian selection (9, 81).

Escape mechanisms are easily developed by cells with high plasticity. Malignant melanoma represents one of the most immunogenic tumors due to its high mutational burden, but plasticity of melanoma cells allows them to adapt to a hostile immune microenvironment. In this context, tumor cells can acquire different immunogenic features, as well as the ability to produce immunomodulatory molecules that can, in turn, affect immune cell activation or the composition of the immune infiltrate within the tumor (82). The immunogenicity of melanoma cells is due to the expression of tumor-associated antigens (TAAs), highly immunogenic proteins that can be divided into three classes:

1. Lineage-specific markers that are overexpressed in melanoma cells, such as MART-1 (Melanoma Antigen Recognized by T cells), tyrosinase and gp100 (glycoprotein 100);
2. Cancer-testis (CT) antigens, physiologically expressed (low levels) by adult germ cells and placenta, but aberrantly over-expressed by cancer cells;
3. Neoantigens, antigens originating from somatic mutations.

Melanoma cells can escape T cell recognition through: i) the downregulation of TAAs, ii) defects/deletions in antigen processing machinery that may include proteasome subunits or transporters associated with antigen processing (TAP) and/or iii) the downregulation of MHC molecules, typically through  $\beta$ 2-microglobulin mutations. By reducing antigen presentation alone, melanoma cells are capable of becoming virtually “invisible” to the immune system (9, 83-85). On the other hand, melanoma cells are not only restricted to a single avenue of immune escape: the high antigen load within the tumor microenvironment can contribute itself to T cell exhaustion and failed tumor control, thus adding to the complexity, and difficulty, of curative treatments (Figure 2). It is important to note that melanoma cells are able to affect the behavior of stromal cells, namely cancer-associated

fibroblasts (CAFs), in order to promote the recruitment of pro-tumor immune cells (86, 87). CAFs are known to promote cancer cell proliferation and invasion (88), and other findings demonstrated that these cells can participate to the evolution of an immunosuppressive environment, both within the tumor bulk and in metastatic niches, through the release of immunomodulatory factors. It has been recently demonstrated that melanoma-derived extracellular vesicles can induce a pro-inflammatory signature in lung fibroblasts (upregulation of cytokines and chemokines, including IL-1 $\alpha$ , IL-1 $\beta$ , CXCL10, CXCL1, CCL2, CCL3 and CCL5), thus enhancing the recruitment of myeloid-derived cells such as neutrophils (86). Moreover, pro-inflammatory pathways, eventually associated with BRAF mutations, can in turn induce the expression of PD-1 ligands and COX-2 on CAFs, contributing to the immune suppression (89). CAFs can favor immune escape through the secretion of MMPs and the secretion of Prostaglandin E2 (PGE<sub>2</sub>), affecting melanoma cell susceptibility to NK-mediated lysis (90), eventually mediated by a decreased surface expression of the activating receptor NKp44 (91). T cell functions can be also affected by CAFs: the secretion of cytokines (i.e., CXCL5) can induce the expression of PD1 on cancer cells (92), and the CAFs-mediated metabolic stress of CD8+ T cells limits their functions against tumor cells (93).

This section will highlight the mechanisms exploited by melanoma cells to communicate with cells of the tumor microenvironment in order to gain an immunosuppressing, tumor-promoting, setting.

### **3.2 Cell-cell contact**

In order to elicit an efficient antitumor immune response, T cells must be fully activated by two costimulatory signals. The first one is MHC-T cell receptor (TCR) interactions and depends on DC or tumor cell antigen presentation; if only this signal is present, T cells are not completely stimulated and become anergic. The complimentary signal is represented by the expression of co-stimulatory molecules on T cells (e.g. CD28), that bind to their cognate receptors on antigen presenting cells (e.g. CD80, CD86). Similarly, inhibitory receptors (e.g. CTLA-4, PD-1, PD-L1, B7-H2, B7-H3) trigger negative stimuli that lead to T cell anergy (94). It has been demonstrated that melanoma suppresses T cell activation through upregulation of co-inhibitory molecules such as

PD-L1 (95, 96) and that the overexpression of PD-1 in T cells continuously exposed to cancer antigens leads to T cell anergy (97). Indeed, it has been demonstrated that the high antigen load is involved in CD8+ cell dysfunction and exhaustion, so that despite melanomas are highly immunogenic, T cells are not able to restrain tumor growth. Dysfunctional T cells are characterized by high levels of clonal expansion together with the overexpression of checkpoint molecules (i.e., PD-1, TIM-3, LAG-3): thus, exhaustion might be induced by antigen-driven interactions with melanoma cells (98, 99). From a functional point of view, dysfunctional T cells lose their effector functions (i.e., cytotoxicity) while maintaining the proliferative capacity, at least early during the acquisition of the exhausted phenotype (100). In an elegant study, Schietinger A. et al were able to follow the activation state of T cells during tumor initiation and progression. The authors demonstrated that the chronic antigen presentation, eventually by non-professional presenting cells (such as tumor cells), in a non-inflammatory context, is responsible for the induction of a programmed, alternative, dysfunctional differentiation program in T cells. Such program is triggered early during tumor initiation, even in pre-malignant lesions, evolving to a “fixed” dysfunctional state as tumor progresses. In this context, a comparative whole-genome transcriptomic analysis showed that “early” and “late” dysfunctional T cells share a signature related to the inhibition of their effector functions (e.g. *Tbx21*, *Eomes*, *Id2*, *Gzmk*, *Ccr5*, *Cxcr3*). Conversely, the early phase is characterized by the downregulation of genes involved in activating T cell functions (e.g., *Foxo1*, *Foxp1*, *Tcf7*, *Klf2*) and the late phase by the upregulation of genes involved in reducing immune function (e.g., *Egr1*, *Batf*, *Blimp*, *Lag3*, *Ctla4*) (101). T cell dysfunction seems to be reversible during early phases, becoming irreversible over time. The transition from the dynamic and the fixed states is epigenetically imprinted: the two conditions are characterized by different chromatin assets, leading to a differential gene expression. For example, in early dysfunction, T cells showed low expression of CD38, CD101, CD30L and high expression of CD5, while late dysfunction is characterized by the opposite pattern (102). Otherwise, other studies demonstrated that PD-1-expressing T cells chronically stimulated by tumor antigens undergo fast proliferation followed by apoptosis due to a microenvironment-driven DNA damage. In this way, an equilibrium is reached between proliferation and death, impeding T cells expansion and leading to failed control of tumor outgrowth (103). The expression of immune-checkpoint molecules by melanoma cells, or by tumor-associated

immune or stromal cells, formed the rationale for the development of the immune-checkpoint inhibitor therapies (i.e., PD-1/PD-L1/CTLA-4 axis inhibitors), that have been approved for the treatment of unresectable, metastatic melanoma. However, most patients show primary or acquired resistance and, eventually, tumor relapse. Unfortunately, melanoma cells are known to exploit alternative mechanisms to suppress the immune system such as upregulation of TIM-3, LAG-3 and BTLA which can compensate for PD-1 or CTLA-4 axis inhibition, thus sustaining immunosuppression (104-108).

### 3.3 Pro-tumor cytokines and chemokines

Cancer cells are able to shape the local immune landscape through the recruitment of pro-tumor, and the suppression or exclusion of anti-tumor, immune subsets. In this context, the secretion of cytokines and chemokines by melanoma cells is often mediated through the hyperactivation of NF $\kappa$ B signaling pathways (109). Pro-inflammatory events initiated by melanomas result in the recruitment of innate immune cells, which include neutrophils, macrophages and DCs. As previously discussed, DCs can process tumor antigens and migrate to regional lymph nodes where they can prime effector T cells ( $T_{eff}$ ) (85), thus representing the functional bridge between innate and adaptive immunity. Melanoma cells, however, impair DC recruitment and maturation through vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)- $\beta$  secretion, which impedes T cell targeting of tumor cells (107, 110). Moreover, dysregulation of the Wnt/ $\beta$ -catenin signaling pathway in melanoma cells, often related to aggressive cancer cell subsets (i.e., cancer stem cells), leads to defective CCL4 production, which in turn impairs DC and T cell recruitment while also inducing resistance to anti-PD-1 therapies (107, 111). IL-37b is another factor involved in anti-tumor suppression that mediates the downregulation of costimulatory molecules CD80 and CD86 on APCs (12), resulting in suboptimal activation and dramatic impairment of  $T_{eff}$  cells.

In addition to the impairment of DC-mediated T cell recruitment, melanoma cells can redirect the production of cytokines to favor recruitment of pro-tumor T cells while rejecting T<sub>eff</sub> cells. Melanoma growth and progression have been correlated with the presence of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub>) within the tumor in B16F10 tumor-bearing mice and in melanoma patients (112). Physiologically, T<sub>reg</sub> functions are required for maintaining self-tolerance, thus providing suppressive control over antigen-specific T<sub>eff</sub> cells. Thus, it is not surprising that their recruitment by melanoma cells represents a strategy to evade elimination. T<sub>reg</sub> produce immunosuppressive cytokines and chemokines, such as IL-10, IL-35 and TGF- $\beta$ , and can also engage DC directly through CTLA-4 in order to inhibit antitumor immune responses (113, 114). Furthermore, they can inhibit effector functions of Natural Killer (NK) cells through expression of membrane bound TGF- $\beta$  which is responsible for NK cell downregulation of the Natural Killer Group 2D receptor (NKG2D) (115).

The ratio of T<sub>eff</sub>/T<sub>reg</sub> within the tumor has a predictive value in immunotherapeutic responses (116). A study by Shabaneh et al. demonstrated that T<sub>reg</sub> cells have an important role not only in late phases of anti-tumor suppression, but also in early phases of tumor development. In this study, an inducible PTEN/BRAF melanoma mouse model was utilized to demonstrate the importance of BRAF oncogenic signaling in the recruitment of regulatory cells, driven by CCL2, CCL17 and CCL22 (117). Moreover, defective production of cytokines and chemokines involved in T cell homing was observed, contributing to an imbalance between T<sub>eff</sub> and T<sub>reg</sub> cells. Among these molecules, IFN- $\gamma$  expression contributed to both a tumor-supportive and a tumor-suppressive immune environment. Aberrant IFN- $\gamma$  signaling has been associated with the downregulation of the Jak1/2 pathway in tumor cells and subsequent reduced production of CXCL9 and CXCL10 within the TME, two important chemotactic molecules responsible of T cell migration and infiltration (118). Epigenetic silencing of genes encoding chemotactic molecules is one mechanism that has been described in melanoma (85).

Melanoma cells recruit and modify the function of macrophages (M) and neutrophils (N) within the TME. These inflammatory, phagocytic cells can behave in an anti-tumor (M1 and N1) or in a pro-tumor (M2 and N2) fashion, depending on the signals received within the TME. Macrophages are recruited by secretion of CCL2 (MCP-1, monocyte-chemoattractant protein-1) and it has been reported that the expression levels of this chemokine is determinant for the induction of a pro-tumor or an anti-tumor setting, with high levels favoring tumor rejection and low-to-intermediate levels sustaining tumor growth (119). A similar biphasic effect is achieved by VEGF-C, involved in macrophage recruitment in addition to pro-angiogenic processes (120). Tumor-associated macrophages (TAM) are frequently polarized toward a M2 phenotype because of the secretion of TGF- $\beta$  (121). IL-10 plays a pivotal role in the ability of macrophage to modulate immune responses, which functions to downregulate MHC class II antigens and upregulate the costimulatory molecule B7, leading to poor antigen-presentation and the inhibition of T cells (121). Other M2 tumor-supportive features are related to the production of matrix metalloproteases (MMPs), involved in tumor invasion, and the production of proangiogenic molecules such as VEGF to mediate extravasation of tumor cells (122).

Together with DCs, neutrophils are among the first to respond to tumor-mediated inflammatory signals. Malignant melanomas produce chemokines that lead to neutrophil infiltration during tumor initiation and progression. Particularly, the mobilization of neutrophils is achieved by molecules that bind to CXCR2, which includes CXCL1, CXCL2, CXCL3, CXCL5 and CXCL8 (123). Interestingly, UVB radiation exposure, one of the well-known causes of melanoma, induces the production of CXCL1 and CXCL8 and the recruitment of anti-tumor neutrophils (124). Like macrophages, neutrophils can be polarized to become predominantly anti-tumor (N1) or pro-tumor (N2). The neutrophils role in tumor initiation and progression has been matter of debate: it seems that tumor initiation is characterized by the presence of N1 neutrophils that mediate melanoma cell killing, while in late stages N2 neutrophils are the most abundant phenotype and have a role in tumor progression (125, 126). To date, emerging clinical evidence support the finding that a high

neutrophil-to-lymphocyte ratio (NLR) represents poor prognostic outcomes and is a negative predictive indicator of immune checkpoint inhibitor therapy success (127, 128). The specific mechanisms that drive neutrophil phenotypic switching are not fully understood, but may be related to the complex network of soluble mediators within the TME. Tumor-derived IFN- $\beta$  has been demonstrated to be involved in the induction of the N1 phenotype (59), thus limiting the pro-angiogenic and pro-invasive properties of tumor-associated neutrophils (129). Moreover, it has been demonstrated that circulating tumor cells (CTCs) promote the establishment of metastasis through the secretion of G-CSF (granulocyte colony stimulating factor) and CXCL6, and subsequent recruitment of N2 neutrophils (130). Pro-tumor neutrophils can contribute to immune evasion orchestrated by melanoma cells through the expression of immune checkpoint proteins (i.e., PD-L1), the overexpression of other immunosuppressive molecules, such as IDO (indoleamine 2,3-dioxygenase) and iNOS (inducible nitric oxide synthase), or the secretion of molecules involved in the recruitment of T<sub>reg</sub> (i.e. IL-17) (131, 132).

Melanoma is also known to recruit myeloid-derived suppressor cells (MDSC). MDSC are comprised of immature precursors of DCs, macrophages and neutrophils, usually retained within the bone marrow, but that can be mobilized upon appropriate stimuli. Their expansion and migration can be induced by inflammatory molecules produced during chronic inflammation and cancer progression, such as GM-CSF, IL-6, IL-10, IFN- $\gamma$  and VEGF (103) and a central role seems to be played by CCR5 ligands in melanomas: CCL3, CCL4 and CCL5. Importantly, an enriched CCR5<sup>+</sup>MDSC infiltrate within the melanoma microenvironment has been observed in mouse models, and the administration of CCR5-Ig fusion protein leads to melanoma growth inhibition associated with impaired MDSC trafficking (104). The inhibitory role of MDSCs on anti-tumor immunity is due to: 1) the production of NO (nitric oxide) and Arg-1 (arginase 1), inducing T cell apoptosis and cell cycle arrest, 2) high expression of PD-L1, inducing T cell exhaustion, 3) upregulation of IDO, leading to T cell anergy and 4) secretion of IL-10 and TGF- $\beta$ , suppressing T cell trafficking (103). The presence of MDSC in cancer tissue is another potential prognostic



indicator for immune-checkpoint inhibitor therapy treatment, and has been reported as a predictive marker of response to ipilimumab in melanoma patients (105).

### 3.4 Metabolic mediators.

Cancer cells can escape immunosurveillance or evade immunotherapies *via* metabolic reprogramming. Metabolic mediators can drive immunosuppressive signaling and essential substrate consumption by cancer cells can cause metabolic depletion leading to anergy of immune cells.

Cancer cells require high nutrient consumption in order to support tumor growth. Their plastic phenotype allows for rapid reprogramming of cell metabolism for survival in hostile conditions such as hypoxia, as well as the ability to activate “unconventional” metabolic pathways, such as glycolysis, even in the presence of normal oxygen levels (Warburg effect). The increase of glucose consumption by melanoma cells leads to glucose deprivation for cells within the TME whose metabolism is strikingly glycolytic. In this context, glucose deprivation inhibits T cell proliferation and activation, dampening anti-tumor immune responses. CD28 is involved in multiple pathways related to T cell activation, such as the upregulation of the glucose transporter GLUT1, which when silenced, significantly impairs T cell functions (133). It has been demonstrated that the production of IFN- $\gamma$ , cytolytic activity and cell cycle progression of T cells are regulated by glucose consumption (134). Moreover, recent studies show that the increase of oxidative metabolism that can occur in melanoma cells consumes oxygen from the TME leading to oxygen deprivation for T cells (135).

Lactate derived from cancer cell glycolysis represents another metabolite involved in immune cell suppression. In particular, it has been involved in the reduction of antigen-presenting efficiency of DCs (136). Furthermore, as a consequence of excessive amounts of lactate, extracellular acidosis causes the inhibition of NK and T cells in mouse models of melanoma (137), and the neutralization of acidic conditions improves response to immune-checkpoint inhibitor

therapies (138). Amino acid availability within the TME is crucial for T cells that, similar to other immune cells, are unable to synthesize amino acids (i.e., tryptophan, glutamine, arginine). Malignant melanoma has been demonstrated to be highly dependent on glutamine metabolism that fuels oxidative phosphorylation by entering the TCA (tricarboxylic acid cycle) after conversion to glutamate and then  $\alpha$ -ketoglutarate (139). The glutamine addiction of melanoma cells restricts glutamine availability for T cells, preventing proper T cell activation (140).

L-arginine metabolism in melanoma has been associated with immunosuppression (141). Arginine uptake is crucial because its endogenous synthesis rate is not sufficient to sustain highly proliferative cells, and studies have demonstrated that downregulation of arginosuccinate synthetase in melanoma cells renders them unable to generate arginine (142, 143). High arginine uptake by cancer cells leaves little for T cells, leading to reduced proliferation and survival (141, 144). Importantly, L-arginine is the precursor for NO synthesis. NO is a crucial immunomodulatory factor that exerts its suppressive effects through inhibition of T cell proliferation and function. Moreover, reactive nitrogen species, such as peroxynitrite, are known to induce apoptosis of T cells (145, 146).

In contrast to glutamine and arginine, which are directly consumed by tumor cells, tryptophan deficiency within the TME is due to the upregulation of the catabolic enzyme IDO in tumor cells and MDSC (147, 148). Physiologically, this enzyme is involved in tolerance during pregnancy to prevent rejection of the fetus and it can thus be exploited by tumor cells as a mechanism of immune escape. In fact, it is frequently overexpressed by cancer cells and its upregulation has been associated with tumor progression and poor prognosis (149). Regarding melanoma, its expression has been correlated with Breslow thickness and PD-L1 expression, and it negatively correlates with progression-free survival (150, 151). Mechanistically, local tryptophan deprivation is signaled through glucokinase, an amino acid-sensing kinase that in turn triggers downstream pathways such as mTORC1 (mammalian target of rapamycin complex 1) inhibition

and the consequent activation of the autophagic process, leading to T cell anergy (152). Another mechanism implicated in T cell anergy due to tryptophan deficiency is the activation of stress sensors such as GCN2 (general control nonderepressible 2), which senses the lack of tryptophan-charged tRNAs and induces a stress response that limits protein translation. Moreover, GCN2 activation promotes T<sub>reg</sub> differentiation and activation (153). However, recent evidence has demonstrated that this sensor does not affect immunity in B16 melanoma tumors, and this could be due to the maintenance of adequate tryptophan levels within the TME despite its catabolism through IDO (154).

IDO catalyzes the conversion of tryptophan to kynurenine, that can directly act as an immunosuppressive molecule. Increased kynurenine in the TME directly inhibits NK cell cytolytic activity through the downregulation of activating receptors (NKp44, NKp30, and NKG2D) (155). In other cell types, kynurenine binds to the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor that, once activated, promotes the differentiation of T<sub>reg</sub> cells, reduces the immunogenicity of APCs and induces the upregulation of PD-1 expression on T<sub>eff</sub> cells (156). Another metabolic product that acts as an immunosuppressive mediator is adenosine obtained from ATP through the activity of the ectonucleotidases CD39 and CD73. Specifically, the release of intracellular ATP is followed by its conversion to AMP by CD39, and subsequently AMP undergoes dephosphorylation by CD73. Hypoxic conditions, as well as extracellular stresses, represent the driving events: the induction of the transcription factor HIF1 $\alpha$  (hypoxia inducible factor 1) in response to low oxygen levels promotes the expression of CD39 and CD73 both on cancerous and non-cancerous cells (i.e., endothelial cells and lymphocytes) (157, 158). The A2A high-affinity adenosine receptor has been implicated in the immunosuppressive effect of this molecule because of its high expression levels on immune cells. Specifically, adenosine has been shown to inhibit NK infiltration and function, to impair macrophage activation and to favor T<sub>reg</sub> cell maturation, while impairing T<sub>eff</sub> cell priming, proliferation and cytokine release (159-161). Furthermore, increased production of adenosine has been observed in melanoma progression during

immunotherapy (i.e., during adoptive T cell transfer or immune-checkpoint blockade), and it has been attributed to the phenotype switch of melanoma cells. Thus, adenosine can be implicated both in immune escape and in mechanisms leading to adaptive resistance (162).

### 3.5 MicroRNAs (miRNAs)

MicroRNAs are small, 20-25 nucleotide-long, non-coding RNAs that are involved in the attenuation or complete inhibition of protein translation. Their binding to a specific RNA is due to their complimentary nucleotide sequence: a fully complimentary sequence leads to mRNA degradation and inhibition of protein expression, whereas a partial complementarity is responsible for the attenuation of protein expression (163). Several miRNAs have been implicated in cancer progression and both oncogenic and tumor-suppressor miRNAs have been recognized. The overexpression of oncogenic miRNAs by cancer cells often leads to the inhibition tumor suppressor proteins (i.e., apoptotic proteins, proteins involved in cell differentiation or in cell cycle regulation), and the downregulation of tumor-suppressor miRNAs leads to aberrant expression of proteins involved in cancer progression (i.e., oncogenes, anti-apoptotic proteins or proteins involved in cell proliferation) (164). MiRNAs can modulate intracellular processes, as well as be transferred to nearby cells for cross-talk within the TME. In order to overcome degradation by RNAses in the extracellular space, miRNAs are carried by transporters, such as proteins (argonaute, ARG), high-density lipoproteins (HDL), or extracellular vesicles (exosomes) (163).

miRNAs are involved in the modulation of the immune microenvironment in malignant melanoma. The miR-30b/-30d cluster, upregulated in melanoma cells, has been associated with GalNAc transferase 7 (polypeptide N-acetylgalactosaminyltransferase 7) downregulation, which impairs recruitment of T<sub>eff</sub> cells and increases infiltration of T<sub>reg</sub> following increased IL-10 secretion (165). Recently, a panel of miRNAs (miR-146a, miR-155, miR-125b, miR-100, let-7e, miR-125a, miR-146b, miR-99b), carried from tumor cells by extracellular vesicles, have been implicated in monocyte conversion to MDSC and in immune-checkpoint inhibitor resistance in melanoma

patients (166). Among other miRNAs, miR-210 is upregulated by hypoxic conditions in melanoma cells and impairs the susceptibility of tumor cells to T cell-mediated lysis (167). MiR-21 and -29a are known to target anti-angiogenic pathways, thus promoting tumor angiogenesis, and genes involved in M1 macrophage polarization (168). The role of miR-155 is more controversial because it can exert both immune-promoting and immune-suppressive effects (169). It has been implicated in macrophage polarization toward the M1 phenotype to promote anti-tumor immunity, but in tumors with increased IL-1 $\beta$  signaling, it mediates the induction MDSCs (170, 171). Moreover, transgenic mice lacking miR-155 showed defective T cells and increased B16F10 melanoma growth (172).

Other studies have demonstrated that melanoma-derived miRNA can affect response to immune-checkpoint inhibitor therapies. MiR-146a has been implicated in supporting immune suppression during melanoma growth wherein mice lacking its expression showed lower metastasis and increased survival. Targeting miR-146a with a specific antagomir acted synergistically with anti-PD-1 to enhancing the antitumor immune response (173).

### **3.6 Exosomes**

Exosomes are small vesicles originating in the cytosol and derived from endosomal compartments. They are delimited by a phospholipid bilayer and can carry various molecules (proteins, lipids, nucleic acids). They are secreted into extracellular spaces and are exploited by tumor cells to deliver signals to the surrounding cells, allowing crosstalk with other tumor cells or cells of the TME.

T cell function is frequently affected by tumor-derived exosomes. They have been implicated in the expansion of T<sub>reg</sub> and in the promotion of their function (174), thus reinforcing immune evasion. Melanoma exosomes can also deliver membrane-bound ligands, such as PD-L1, that find their cognate receptors on T cells providing inhibitory signals (175). Moreover, exosomes may carry soluble factors such as Fas and TRAIL (TNF-related apoptosis-inducing ligand) which

can induce T<sub>eff</sub> cell apoptosis (176). As mentioned above, melanoma-derived exosomes can carry miRNAs that silence anti-apoptotic Bcl (B-cell lymphoma) proteins, such as Bcl-2, Bcl-xl, and Bcl-w, to induce mitochondrial-mediated apoptosis in CD4<sup>+</sup> T cells (177). In some cases, however, the exposure of MHC-I and melanoma-associated antigens (MART-1, gp100, tyrosinase) on exosome membranes can mimic antigen presentation processes, leading to T cell activation (178).

Several studies demonstrated the important role of tumor-derived exosomes in the development of metastatic niches. An elegant study performed by Peinado H. et al. demonstrated that melanoma-derived exosomes induce the acquisition of pro-angiogenic and pro-metastatic phenotypes by bone marrow cells leading to accelerated tumor growth and increased metastasis. This is due, at least partially, to the exosomal-mediated transfer of the Met receptor to bone marrow progenitor cells, inducing their mobilization *via* S6 induction and ERK phosphorylation (179).

The two-fold nature of melanoma-derived exosomes is further demonstrated in DCs where melanoma exosomes may carry TAAs to DCs to promote the activation and expansion of cytotoxic T cells, but they may also inhibit differentiation of DCs from monocytes due to high IL-6 content (178).

#### **4. Augmenting or rescuing immunity in melanoma**

##### **4.1 The era of cancer immunotherapy**

In recent years, several treatments have been approved by the United States Food and Drug Administration (FDA) for melanoma. Application of each treatment is dependent on the features of the cancer (stage, location and genetics) and can include combinations of surgery, photodynamic therapy, chemo/radiotherapy, targeted therapy or immunotherapy. Most melanomas of stage I-III are removed by surgery followed by adjuvant therapy (targeted or immunotherapy) (180). Metastases are treated with a combination of surgery (if solitary/localized) and adjuvant chemotherapy as well as radiotherapy for advanced metastases of the skin, bone and brain (181). Metastases are the main cause of death, thus requiring more effective strategies to target distal

metastases with greater efficacy and significantly less toxicity. To date, tumor infiltrating lymphocytes (TILs) have been associated with positive outcome and improved survival in patients with malignant melanomas (182). Thus, immunotherapeutic strategies have been a focal point for treatment of advanced stage, metastatic melanoma, with some having shown incredible success in a select cohort of melanoma patients.

The prognostic factors that determine the efficacy of a particular immunotherapy are still largely unknown. Despite successes, cancer relapse and variable response rates among patients of all stages are observed. This is likely exacerbated by the ability of melanoma to quickly adapt multiple immune suppressive pathways to escape immune attack as previously discussed. A better understanding of these escape mechanisms has led to predictions that targeting multiple suppressive pathways will be more efficacious than single immunotherapy treatment, and will be critical for maintaining long-term tumor surveillance.

#### **4.2 Administration of cytokines**

IFNs are cytokines normally secreted by leukocytes during infection and are instrumental in the development of anti-proliferative and anti-angiogenic activities against melanoma (183). IFNs act as agonists for the anti-tumor activity of both adaptive and innate immune cells and are antagonistic towards suppressive immune subsets such as MDSCs and T<sub>reg</sub>. IFN- $\alpha$  and IL-2 are cytokines generally given in combination with surgery and chemotherapy, radiotherapy or targeted therapy. IFN- $\alpha$  was FDA approved in 1995 as adjuvant therapy for resected stage IIB/III melanoma (184). IFN- $\alpha$  has been shown to induce upregulation of MHC class I on melanoma cells and immune cells to cause increases in cancer cell death and extension of survival (185). Only a small percentage of patients, however, respond to IFN- $\alpha$  adjuvant treatment, with ulceration of the primary melanoma being a predictive indicator of IFN sensitivity (186). A pegylated form of IFN- $\alpha$ , Peg-IFN has also been approved for stage III melanomas, with effects mimicking those of unpegylated IFN- $\alpha$  (187), but with a longer half-life in circulation leading to increased efficacy.

However, additional care must be taken to minimize adverse events. FDA-approved in 1998 for metastatic melanoma, IL-2 is known to act directly on T cells, which includes effector CD8 and regulatory CD4 cells (188). Due to potentially dangerous adverse events such as tachycardia and multisystem organ failure, patients are first screened for biomarkers such as VEGF and fibronectin (189).

#### **4.3 Expanding melanoma-specific T cells and targeting suppressive immune subsets**

Gp100 is a glycoprotein that is overexpressed by melanoma cells and has negligible expression in healthy tissues, making it an ideal melanoma-specific antigen for vaccine development. Monotherapy with gp100 peptides has shown little efficacy in preclinical melanoma models, however, combination treatment with gp100 peptides and IL-2 showed a dramatic increase in median progression-free survival (PFS) and complete responses of 5% (190). Most importantly, gp100 peptides are capable of inducing T cell responses in patients with advanced melanoma (191, 192). Gp100 is currently being evaluated in several clinical trials as monotherapy (NCT01744171, NCT0-117647) or in combination with immunotherapies (NCT00960752, NCT01176461, NCT02535078).

$T_{\text{regs}}$  suppress effector T cell responses and can be found circulating or infiltrating tumors in melanoma patients, ultimately contributing to poor clinical outcome (193). Strategies to target  $T_{\text{regs}}$  and increase T cell immunity are limited. Ontak, which was FDA-approved in 1999, is an IL-2 protein fused to diphtheria toxin, and is designed to target peripheral blood  $T_{\text{regs}}$  through their IL-2 receptor (194). A Phase II trial in late stage melanoma patients (stage IV) showed 17% partial response, 15% mixed responses and 5% stable disease (195). However, in another clinical study, no objective response, survival benefit, or depletion of  $T_{\text{regs}}$  was observed (196). Thus, while  $T_{\text{reg}}$  depletion could provide great benefit alone or combined with other immunotherapeutic treatments, more effective strategies must be developed.

#### **4.4 Checkpoint inhibitors**



Current FDA-approved checkpoint inhibitors consist of antibodies which bind checkpoint proteins or receptors to prevent signaling which causes T cell anergy. CTLA-4, also known as CD152, is an important negative regulator (checkpoint) in T lymphocyte activation (197, 198). CTLA-4 is upregulated on the surface of tumor-associated T cells and, in contrast to CD28, transmits an inhibitory signal when bound to co-stimulatory molecules B7-1 (CD80) or B7-2 (CD86) found on antigen presenting cells (199). CTLA-4 binds with much greater affinity and avidity to co-stimulatory molecules compared to CD28, thus favoring a suppressive phenotype in the TME (200). Furthermore, CTLA-4 engagement inhibits T cell cytokine production and proliferation (201). In CTLA-4<sup>-/-</sup> knockout mice, lethal hyperproliferative lymphocyte expansion occurs early in life and prevents survival past three weeks (202).

Given the role that CTLA-4 plays as a negative regulator of T cell activation, it was hypothesized that blocking engagement with CTLA-4 could boost immunity against tumor cells (203). Ipilimumab, FDA approved in 2011 for melanoma, is an antibody which binds and prevents signaling through CTLA-4 (197, 204). Anti-CTLA-4 antibodies act as antagonists, blocking inhibitory signaling, and increasing the potential for cytotoxic T cells in melanomas to become activated and expand. Ipilimumab has been combined with cytokine therapies, as discussed previously, with overall response rates of ~40% and median progression-free survival (PFS) of 6 months (205). In Phase III trials, ipilimumab monotherapy has been shown to be more effective than cancer vaccines alone (gp100 vaccine) in increasing median overall survival (OS) of metastatic melanoma patients (10.1 months vs. 6.4 months, respectively). Combination therapy did not increase median OS, suggesting that *in situ* vaccination may be occurring (206).

Another checkpoint receptor found on the surface of T cells, PD-1, binds its agonist PD-1 ligand (PD-L1) to also suppress T cell activation. PD-L1 is expressed by melanoma cells or tumor-associated stroma, and this expression is strongly correlated with efficacy of anti-PD-1 immunotherapy (207). PD-1 is also expressed on B and NK cells, and thus therapeutic blockade

could potentially affect these immune subsets as well (95). Nivolumab was the first anti-PD-1 antibody to be approved by the FDA in 2014 for the treatment of patients with metastatic melanoma. Nivolumab binds PD-1 to prevent the interaction between PD-1 receptor and its ligands in the TME, favoring a more active anti-tumor phenotype. Nivolumab treatment significantly increases median PFS to 6.9 months, compared to 2.9 and 2.2 for ipilimumab and chemotherapy monotherapies, respectively (205, 208). However, more impressive is the median PFS of 11.5 months with combination nivolumab/ipilimumab. Another anti-PD-1 antibody, pembrolizumab, was FDA-approved in 2015, for advanced melanomas (209, 210). Like nivolumab, pembrolizumab prolongs PFS and OS with less toxicity than ipilimumab. Currently, there are numerous clinical trials utilizing checkpoint inhibitors alone or in combination with chemotherapy, radiotherapy and other immunotherapies (NCT01103635, NCT0253078, NCT02643303, NCT03086174, NCT02608268). Similar studies are also being performed using anti-PD-L1 antibodies such as durvalumab, avelumab and atezolizumab (NCT02535078, NCT03167177, NCT03138889).

## **5. Concluding remarks**

Fully unraveling the crosstalk between the immune system and melanoma that causes loss or suppression of anti-tumor responses will be critical for the development of more effective and less toxic treatments. The identification of cytokines, immunosuppressive immune subsets and checkpoint pathways that are critical for melanoma progression has led to the development and FDA approval of numerous immunotherapeutic agents. This is important since the heterogeneity of advanced melanomas will undoubtedly require a combination of these agents to establish durable, life-long immunity. Also, one must take into consideration the optimal doses required to establish durable tumor control while minimizing adverse events. Since each patient would be unique in the features of their melanoma, it will be necessary to determine those features prior to treatment in order to select a balanced combination that will maximize PFS and minimize toxicity.

**Conflict of Interest:** The authors declare that there are no conflicts of interest.

## Figure Legends

**Figure 1. Melanoma Clearance by Functioning Immune Cells.** In the innate arm of immunity, natural killer (NK) cells bind tumor cells via receptor/ligand interactions and release cytolytic molecules causing tumor cell death. Phagocytes, such as polymorphonuclear neutrophils (PMN), macrophages (M $\phi$ ), and dendritic cells (DC) take up dead tumor cells and process and present tumor associated antigens (TAA). DCs are actively recruited by cytokines secreted from activated NK cells. Recruitment of T- and B-lymphocytes by chemokine gradients and presentation of TAAs to T- and B-cells activates the adaptive arm of immunity. Tumor specific CD8<sup>+</sup> T-cells bind tumor cells presenting TAA on MHC molecules via engagement of the T-cell receptor, leading to release of cytotoxic granules into tumor cells. Tumor specific CD4<sup>+</sup> T-cells engage B-lymphocytes via TAAs presented by MHC molecules leading to release of antibodies specific for TAAs whose binding causes tumor cell death through various mechanisms including NK cell killing. Adaptive immune cells also re-activate innate immunity through receptor/ligand interaction as well as cytokine release, and tumor cell killing by adaptive immune cells releases further TAAs to be endocytosed and processed by APCs.

**Figure 2. Mechanisms of Immune Escape in Melanoma.** Melanoma cells secrete proteins such as VEGF and TGF- $\beta$  to inhibit recruitment and function of APCs such as DCs. Immunosuppressive regulatory T-cells (Treg) are recruited by melanoma cells through chemokine secretion and these in further inhibit APCs through engagement of CD86 by CTLA-4 expressed on the Treg surface. Treg also release inhibitory cytokines to activated effector T-cells (Teff) which prohibits melanoma cell killing. Tumor cells directly inhibit Teff action through expression of the PD-L1 ligand which when binding the PD-1 receptor on T-cells induces anergy, as well as secretion of apoptosis –inducing factors such as Fas ligand within exosomes. Melanoma cells also recruit and convert myeloid-derived suppressor cells (MDSC) through secretion of GM-CSF or IL-6 as well as delivery of exosome loaded micro RNAs. MDSC inhibit Teff through multiple mechanisms such as expression

of IDO which depletes necessary tryptophan and converts it to suppressive kynurenine. Finally, melanoma cells deplete glucose and amino acids such as glutamine and arginine from the tumor microenvironment resulting in immune cell starvation.

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