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Melanoma Immunomodulation: A War of Attrition

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1. Introduction

Melanoma is an aggressive skin cancer that has an occurrence rate of about 1 in every 50 Americans (Giblin & Thomas, 2007). It is the 6th most common cancer, with a lifetime risk of 2.04% for men and 1.38% for women (Jemal et al., 2008; Jilaveanu et al., 2009). According to the American Cancer Society, in 2009 the number of new cases of melanoma rose to 68,720 with 8,650 new deaths attributed to the disease (ACS, 2010). There are many known factors that contribute to the formation of melanoma. One major factor influencing the increase in melanoma cases is increased exposure to ultra-violet radiation (UVR). Other risk factors include skin type, hair/eye color, the presence of dysplastic nevi and/or increased nevi, and a family history of melanoma (Jilaveanu et al., 2009). Also, mutations in BRAF, CDKN2A, and CDK4 genes have all been attributed to melanoma development. BRAF mutations have been found in 70% of all melanomas and greater than 90% of these mutations carry a single missense (single nucleotide change) mutation (Meyle & Guldberg, 2009). CDKN2A is involved in melanoma pathogenesis and is a germline mutation found in younger patients (Liu et al., 2007). CDK4 is involved in cell-cycle arrest and has been identified in 10% of melanomas (Bennett, 2008). These mutations are mostly detected in non-chronic sun-induced damage melanomas, while chronic sun-induced damage melanomas are more commonly observed.

When melanoma arises, early diagnosis is crucial to survival. With early diagnosis, more than 80% of cases can be treated successfully through surgery (Zhu et al., 2009). This surgery includes excision of the tumor and surrounding tissue; lymph nodes near the tumor may also be removed if evidence of metastasis is present. Other treatments for melanoma include radiation and chemotherapy, used particularly in cases of highly aggressive and metastatic disease (ACS, 2010). Side-effects from these treatments include fatigue, malaise, and an increased susceptibility to non-melanoma cancers (Kamposioras et al., 2010). Also, these therapies are severely toxic to the patients, suggesting a need for improved, less toxic treatment options that specifically target melanoma tumors, like immunotherapy. The multiple ways that melanoma alters the immune system locally, at the site of the tumor, and systemically makes the disease difficult to treat but ideal for the study of immunomodulation (Berinstein, 2009). Immunomodulation, the alteration of the immune system or its function, is exploited in multiple forms by melanoma tumors from changes in the cellular and sub-cellular makeup of the tumor to changes in the tumor microenvironment that suppress localized and systemic attempts at disease reduction.

At the cellular level, melanoma tumors differentially express cytokines, chemokines, and soluble molecules responsible for immunosuppression and tumor proliferation which will be discussed further in this chapter, particularly those with potential for targeting or with therapeutic benefits (Lazar-Molnar et al., 2000). Melanoma cells are also less efficient in antigen (Ag) presentation to CD4⁺ T cells, reducing immune detection of melanoma tumors and the effectiveness of some immunotherapy strategies (Norton & Haque, 2009). Multiple defects along the HLA class II pathway are present in melanoma cells, the alteration of which could prove useful in novel tumor targeting and immunotherapeutic vaccination strategies. These defects and the potential to overcome them will be further explained in this chapter. Costimulatory molecules are also altered in melanoma cells, reducing positive cellular interaction with T cells and professional antigen presenting cells (APCs), while promoting immunosuppressive interactions through CD28, CTLA-4, and the B7 family of immune inhibitors (Pardee et al., 2009; Wolchok & Saenger, 2008). Study focused on enhancing these secondary stimulation signals would promote complete T cell stimulation and activation of anti-tumor CD8⁺ T cells, a current goal of most immunotherapy strategies. Melanoma cells are also capable of modulating the surrounding immune cells including: suppression of tumor infiltrating lymphocytes (TILs), enhancement of CD4⁺CD25⁺FoxP3⁺ T regulatory cells (Tregs), increased immature myeloid suppressor cells, increased pro-tumorigenic m2 macrophages, and generation of melanoma-associated fibroblasts (Oble et al., 2009; Camisaschi et al., 2010; Balsamo et al., 2009; Ilkovitch & Lopez, 2008). These topics will be further dissected throughout the chapter as they relate to both immune suppression and multimodal treatment strategies. The course of tumor progression not only adds more problems to immune regulation of the disease, but also more potential targets for therapeutic intervention. Melanoma angiogenesis and metastasis aggravate immune suppression in distinct and specific ways increasing the morbidity and mortality of the disease, while reducing the effectiveness of current treatment options (Schadendorf et al., 2009; Zbytek et al., 2008; Mahabeleshwar & Byzova, 2007). Both of these topics will be further examined with specific emphasis on the relationship between Interleukin (IL)-8, vascular endothelial growth factor (VEGF), the matrix metalloproteinases (MMPs), and the distinct problems faced when tumors metastasize, particularly to lymph nodes, the lungs, liver, and brain (Sloan et al., 2009; Vahrmeijer et al., 2008; Huang et al., 2008; Yang et al., 2009).

Melanoma also represents one of the most widely studied tumors in terms of immunotherapy design, clinical evaluation, and therapeutic application (Jandus et al., 2009). This chapter will discuss the successes and failures of melanoma immunotherapy strategies like IL-2 therapy, melanoma vaccines like canvaxin, and adoptive cell transfer in terms of immunomodulation and its effect on treatment (Atkins, 2006; Goldman & DeFrancesco, 2009). This chapter will also discuss current strategies being developed and potential new directions in treatment that address the immunomodulatory nature of melanoma. These include combined chemoimmunotherapy, melanoma monoclonal antibodies, and multimodal therapy strategies (Kudo-Saito et al., 2005; Ascierto et al., 2010; Flaherty, 2006). The goal of this chapter is to summarize the multiple roadblocks in melanoma treatment associated with the immunomodulation instigated by melanoma tumors. By understanding these issues, novel targets for melanoma therapy can be developed and the shortcomings of current treatment modalities can be enhanced leading to improved patient care and patient outcomes. The fight against melanoma in many ways is a war of attrition: gradual gains can and must be made by making treatment more effective through improved knowledge of the immunomodulatory mechanisms that melanoma tumors employ.

2. The tumor microenvironment

When discussing the ability of melanoma tumors to induce both local and systemic immunomodulation, it is important to first understand the tumor microenvironment itself, and how the alterations at this level affect both tumor progression and limit the effectiveness of some treatment strategies. Melanoma arises through a complex process of cellular mutation and a loss of keratinocyte control over melanocyte growth and differentiation (Hsu et al., 2002; Shirakata, 2010). This imbalance leads to the formation of early stage nevi, appearing localized near the basement membrane of the skin. As malignant melanoma progresses, it develops through interaction between dysfunctional melanocytes and the tumor microenvironment. The progression from nevocellular nevi to dysplastic nevi is accompanied with changes in both keratinocytes and local adhesion molecules allowing for increased melanocyte-melanocyte interaction and the formation of nevocyte nests at the dermal-epidermal junction (Danen et al., 1996; Hsu et al., 2002). Following this development, melanocytes fail to respond to keratinocyte or epidermal cell signaling, they no longer form dendrites, and start to modulate the immune environment through the release of cytokines and immune activation factors which will be described in this chapter (Ilkovitch & Lopez, 2008). Tumors next proceed through two distinct growth phases, radial and vertical growth, accompanied by increased inflammation, immune modulation, and healthy cell destruction. In this sense, the abuse of the immune system drives the progression of disease to a more aggressive phenotype, again through shed factors which will be further explained in this chapter. Finally, following vertical growth through the basement membrane of the dermis following periods of high angiogenesis, melanoma cells are now free to metastasize to local and distant sites resulting in poor disease prognosis (Ria et al., 2010). This section will also focus on the distinct ways melanoma cells respond to the tumor microenvironment during the course of melanoma progression, and how these alterations could be exploited in developing novel melanoma therapies.

2.1 Shed molecules and immunosuppression

At the cellular level, melanoma tumors differentially express cytokines, chemokines, and soluble molecules responsible for immunosuppression and tumor proliferation. Initially, these molecules can have regulatory roles in the tumor microenvironment through growth inhibition, but these functions are lost as tumors slowly progress to a state of localized immune suppression (Lu & Kerbel, 1993). Table 1 is a brief summary of some of the cytokines and growth factors associated with melanoma progression and immunosuppression. A more thorough examination of these factors is expertly presented in a review by Ilkovitch et al (Ilkovitch & Lopez, 2008).

Some cytokines appear to have dual roles within the tumor microenvironment depending on the stage and advancement of disease. During initial tumor formation the inflammatory cytokine IL-6 shed by localized keratinocytes, epithelial, and immune cells inhibits tumor proliferation. IL-6-induced growth inhibitor during early stages of melanoma garnered some attention as an immunological target, but clinical application failed to show any benefit (Lu & Kerbel, 1993). During late stages of disease, IL-6's control of over-growth is lost and autocrine usage of IL-6 produced by melanoma cells actively enhances tumor progression through the STAT3 pathway, which can be further enhanced through interactions with IL-17 (Wang et al., 2004; Hodge et al., 2005; Wang et al., 2009). Elevated STAT3 activity regulates tumor oncogenic factors, cell survival, and cell proliferation

Molecule	Role
IL-1(α and β)	Melanoma derived IL-1 α and IL-1 β induce fibroblast and endothelial growth factors as well as surface adhesion molecules allowing for the growth and metastasis of melanoma cells, also can stimulate IL-6 production.
IL-6	Initial tumor suppression, then stimulates tumor growth through autocrine regulation by activating Stat3, IL-17 can also be induced in this system further stimulating Stat3.
IL-8	Highly involved in angiogenesis through chemoattraction of infiltrating lymphocytes and cell adhesion regulation.
IL-10	Anti-inflammatory, induces T cell and DC suppression, can be excreted by tumors and by tolerized or regulatory T cells.
IDO	Inhibits T lymphocyte mediated antigen-specific immune responses through suppression of tryptophan, also promotes immune tolerance.
FasL	Melanoma cells lack functional FasL, which prevent FasL interaction with Fas receptor on lymphocytes, and modulate apoptosis induction.
TGF- β	Multiple roles in immunosuppression, can be secreted by melanoma tumors acting in both autocrine and paracrine manner, converts immune cells to suppressive regulatory phenotype.
PGE2	Released from melanoma associated fibroblasts, inhibits NK T cell activity and adds to immunosuppression.

Table 1. Immunomodulatory Molecules Influencing Melanoma Growth

molecules resulting in angiogenesis, tumor growth, and in some cases (i.e. brain) metastasis (Xie et al., 2006). Similarly to IL-6, TGF- β also displays growth inhibitor paracrine function during early stages of disease, and autocrine tumor growth in later stages of progression (Ma et al., 2009; Pardali & Moustakas, 2007).

Anti-inflammatory cytokines and immunomodulatory molecules are often exploited by melanoma cells, most notably IL-10. Melanoma cells, melanoma recruited myeloid suppressor cells, and Tregs actively secrete IL-10 to induce tolerized T cells and dendritic cells (DC) (Huang et al., 1999; Polak et al., 2007). The chemokine IL-8 also plays a major role in melanoma progression, particularly in angiogenesis. Autocrine produced IL-8 can stimulate melanoma growth and induce expression of cellular adhesion molecules allowing for tumor cell migration. The chemoattractant nature of IL-8 also allows for the recruitment of monocytes and macrophages to the tumor site which release growth factors modulating vascular permeability contributing to cell migration. The tumorigenic properties of IL-8 are well summarized by Waugh and Williams (Waugh & Wilson, 2008). Based on these characteristics, targeting IL-8 could reduce the angiogenic nature of melanoma cells allowing for improved clearance of tumors, an area that should be further studied for its therapeutic potential. Additionally, two shed molecules indoleamine-2,3-deoxyginase (IDO) and prostaglandin E2 (PGE2) also contribute to melanoma induced immunosuppression and represent interesting potential targets in immunotherapy design. IDO is produced primarily by suppressive lymphocytes and immature myeloid/dendritic cells. IDO also acts as a tryptophan sink in the tumor microenvironment, severely inhibiting T cell activation (Polak et al., 2007; Honig et al., 2004). Recent study suggests that PGE2 is produced by melanoma-associated fibroblasts and immature myeloid cells and aids in the recruitment of a specific lineage of migratory DC with low cytokine expression profiles (Luft et al., 2002). PGE2 also inhibits NK T cell anti-tumor activity contributing to immunosuppression in the tumor microenvironment (Balsamo et al., 2009). Taken together, these molecules represent hurdles to

most immunotherapy strategies employed, which rely on the state of the immune system at the time of treatment to overcome the tumor burden. Steps should be taken to inhibit PGE2 and IDO, as well as the suppressive cytokines mentioned prior to immunotherapy, allowing for a more robust immune response. These cytokines are not solely responsible for the deficiency in current treatment strategies; the cellular makeup of TILs and the melanoma-associated neighboring cells may play major roles in immune subversion.

2.2 Immune cells

As seen in the description of shed molecules, there is dynamic crosstalk between tumors and surrounding tissues and infiltrating immune cells that result in both tumor challenge and in tumor progression. Often, autocrine and paracrine signaling pathways between melanoma cells and surrounding cells contribute to tumor progression and metastasis as reviewed by Lazar-Molnar et al (Lazar-Molnar et al., 2000). The shift from tumor suppression to tumor progression and metastasis results, in part, from the alteration in the type and characteristics of TILs. These changes include the enhancement of CD4⁺CD25⁺FoxP3⁺ Tregs, increased immature myeloid suppressor cells, increased pro-tumorigenic m2 macrophages, and the generation of melanoma-associated fibroblasts (Kalluri & Zeisberg, 2006; Mantovani et al., 2002; Almand et al., 2001; Bronte et al., 2001). From a treatment perspective, understanding the alterations in immune cells localized to the tumor site provides both the reason for the failures of some immunotherapy and some novel ways to treat the disease. As stated previously, the progression from healthy melanocyte to melanoma occurs through both mutations within the tumor and through alterations of the cellular environment around the melanoma. In the skin, tissue homeostasis is critical in cellular regulation as well as immune control, and melanoma tumors disrupt this regulation through multiple processes.

Melanoma cells release high levels of basic fibroblast growth factor (bFGF) which results in the generation and localization of melanoma-associated fibroblasts to the tumor microenvironment (Meier et al., 2000). These cells are unlike normal skin fibroblasts in that they proliferate rapidly and eventually outnumber most other cell types within the tumor microenvironment (Li et al., 2003; Lee et al., 2005). They also release molecules that support the growth and movement of melanoma tumors in the extracellular matrix (ECM) of surrounding tissue and affect the function of NK T cells, aiding in immune inhibition (Balsamo et al., 2009). Melanoma tumors also recruit immune suppressor cells such as immature dendritic cells, myeloid derived suppressor/immature myeloid cells, and M2 macrophages (Kusmartsev & Gabrilovich, 2006; Bronte et al., 2001; Hanson et al., 2009). These cells work together releasing immunosuppressive molecules like TGF- β , IL-1 α , IDO, IL-10, PGE2, and reactive oxygen species (ROS) (Kusmartsev & Gabrilovich, 2003; Almand et al., 2001; Valenti et al., 2006). Functionally, these cells are deficient in melanoma tumor antigen presentation, resulting in indirect tumor tolerance through interaction with CD8⁺ T cells (Almand et al., 2001). These factors work against the anti-tumor infiltrating lymphocytes creating a network of immune suppression surrounding the tumor, and through localized inflammation and dysregulation of cell adhesion molecules, they aid in tumor growth, movement, and angiogenesis (Brigati et al., 2002). Thus, targeting these cells in combination with melanoma tumors should improve the efficacy of immunotherapy strategies, such as targeting shared signaling pathways between tumors and surrounding tissue like STAT3 and BRAF/MAPK (Sumimoto et al., 2006; Inamdar et al., 2010).

Figure 1 illustrates the cells melanoma tumors induce for their benefit and the effect that they have on anti-tumor immunity. When discussing melanoma immunosuppression, one cannot leave out the role that T cells play in the tumor microenvironment, in particular the detrimental role of FoxP3+ Tregs, reviewed by Oble et al (Oble et al., 2009). Tregs are normally involved in regulating immune responses to avoid autoimmunity and in reigning in the cytolytic effects of effector CD8+ T cells. They represent the suppressive arm of CD4+ T cells, and are best identified by their high expression of CD25 and FoxP3 (Camisaschi et al., 2010; Vence et al., 2007). In melanoma, particularly in advanced disease states, Tregs are the primary infiltrating lymphocyte where they directly inhibit any cytotoxic antitumor activity through direct contact inhibition, and the release of high levels of IL-10 (Baumgartner et al., 2007). Study suggests that high serum concentrations of Tregs are associated with poor prognosis, poor treatment responses, and an increased risk of recurrence (Vence et al., 2007).

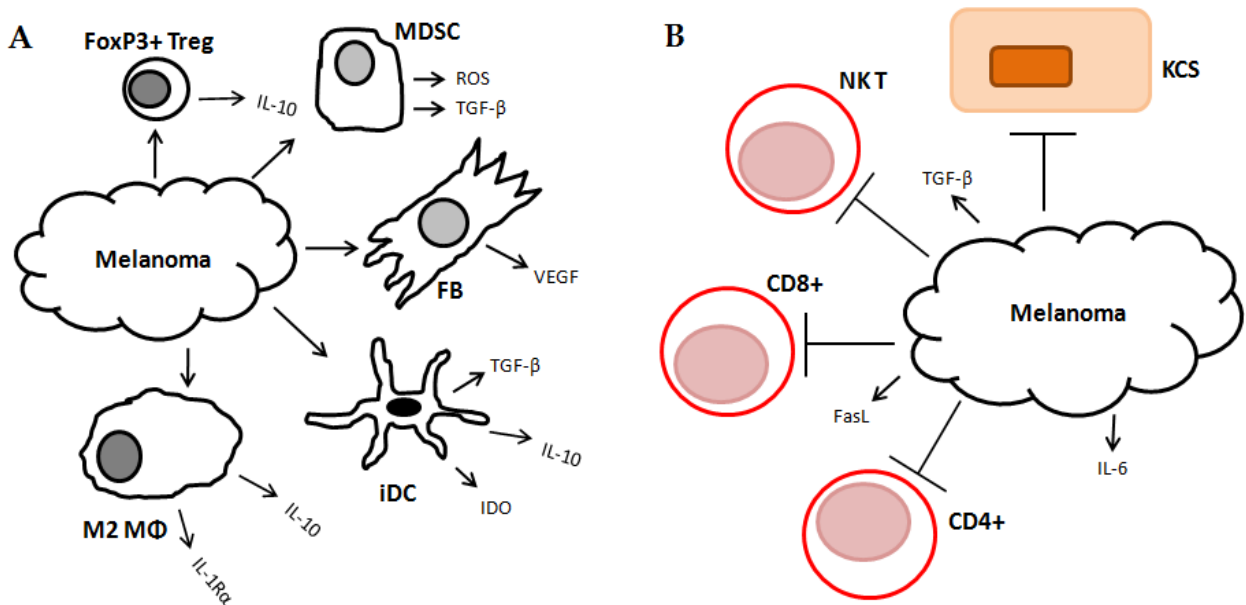


Fig. 1. Microenvironment and immune cell dysfunction. A. Melanoma cells recruit melanoma-associated epithelial and immune cells. These include fibroblasts (FB), immature dendritic cells (iDC), myeloid derived suppressor cells (MDSC), M2 macrophages (M2 MΦ). And CD4+CD25highFoxP3+ T cells (FoxP3+Treg) which release molecules like IL-10, IL-1Rα, IDO, TGF-β, VEGF, and ROS that can inhibit antitumor activity and promote tumor growth. B. Melanoma cells directly interfere with limiting endothelial and immune cells like keratinocytes (KCS), NK T cells (NK T), cytotoxic lymphocytes (CD8+), and CD+ effector cells (CD4+) through the expression of TGF-β, shed and surface FasL, and IL-6.

Tregs are also found in high numbers in sentinel lymph nodes and peripheral blood in cases of metastatic melanoma where they interfere with the expansion of CD8+ and CD4+ effector cells trough IL-2 suppression (Viguier et al., 2004). Study in mice revealed that Treg depletion resulted in the expansion of highly reactive CD8+ T cells resulting in tumor clearance, and in human studies, depleting lymphocytes prior to adoptive cell transfer (ACT) improved the effectiveness of treatment (Mahnke et al., 2007; Matsushita et al., 2008). These results could be associated with the deletion of the large pool of Tregs in the tumor microenvironment and represent an important immunotherapeutic option going forward.

The multiple ways of targeting these immunosuppressive cells in therapy design will be highlighted later in this chapter.

3. Melanoma antigen processing and presentation

In conjunction with these observed deficiencies in the tumor microenvironment leading to immune dysfunction, it is important to understand the direct interplay between melanoma tumors and the immune cells attempting to regulate and destroy tumors. Much focus has been paid to the suppressive nature of Tregs, yet melanoma cells have their own mechanisms of directly inhibiting CD8⁺ cytotoxic and CD4⁺ effector T cells (Viguier et al., 2004; Lampen & van Hall, 2011; Norton & Haque, 2009). CD8⁺ and CD4⁺ T cells interact with melanoma tumors through contact with HLA class I and HLA class II molecules on their cell surface, respectively. Multiple defects exist in melanoma cells ranging from complete loss of class I and II expression to subversive Ag generation attributed to defects in endosomal/lysosomal machinery. These issues and how they represent novel mechanisms for disease treatment and immunotherapy design will be discussed in this section.

3.1 Antigen processing and presentation

The general consensus when describing immunological strategies against melanoma is in the induction of a cytotoxic immune response mainly generated by the activation of anti-tumor CD8⁺ T cells. Though CD8⁺ T cells perform the bulk of the tumor destruction, by focusing solely on activating these cells and not CD4⁺ T cells, melanoma tumors are capable of devising strategies to avoid CD8⁺ T cell detection and activation. Clinical evidence supports this notion, as even in patients with advanced disease there are detectable CD8⁺ T cells specific for melanoma tumor Ag, yet the tumor remains unchallenged (Harlin et al., 2006). This occurs through multiple mechanisms from the immunosuppression described in the previous sections of this chapter, and also through flaws in melanoma Ag processing, presentation, and costimulation. Melanoma tumors have also been shown to downregulate HLA class I surface expression, preventing any T cell activation and tumor clearance (Lopez-Nevot et al., 1988; Cabrera et al., 2007). Tumors also differentially express costimulatory molecules required for complete T cell activation, which will be discussed further in the next section of this chapter. An additional reason for the inability of CD8⁺ T cells to clear a tumor completely is that there are few to no support signals driving the anti-tumor immune response further following the initial activation and a complete lack of potent antitumor immunological memory. The support signals needed are supposed to come from activated CD4⁺ effector T cells, differentiated from Tregs by their low CD25 expression and the lack of FoxP3 (Lizee et al., 2006). CD4⁺ T cells release immune stimulatory cytokines and can directly cross present Ag to professional antigen presenting cells (APCs), driving a complete immune response that can lead to the development of immunological memory. CD4⁺ T are also crucial as they interact with HLA class II on melanoma tumors which present self tumor Ags (Figure 2). However, melanoma cells are severely hindered in their ability to present endogenous tumor Ags (Goldstein et al., 2008). Although melanoma cells can reduce their HLA class II, studies have shown that detectable levels of surface class II are still present that could be exploited in immunotherapeutic vaccine design.

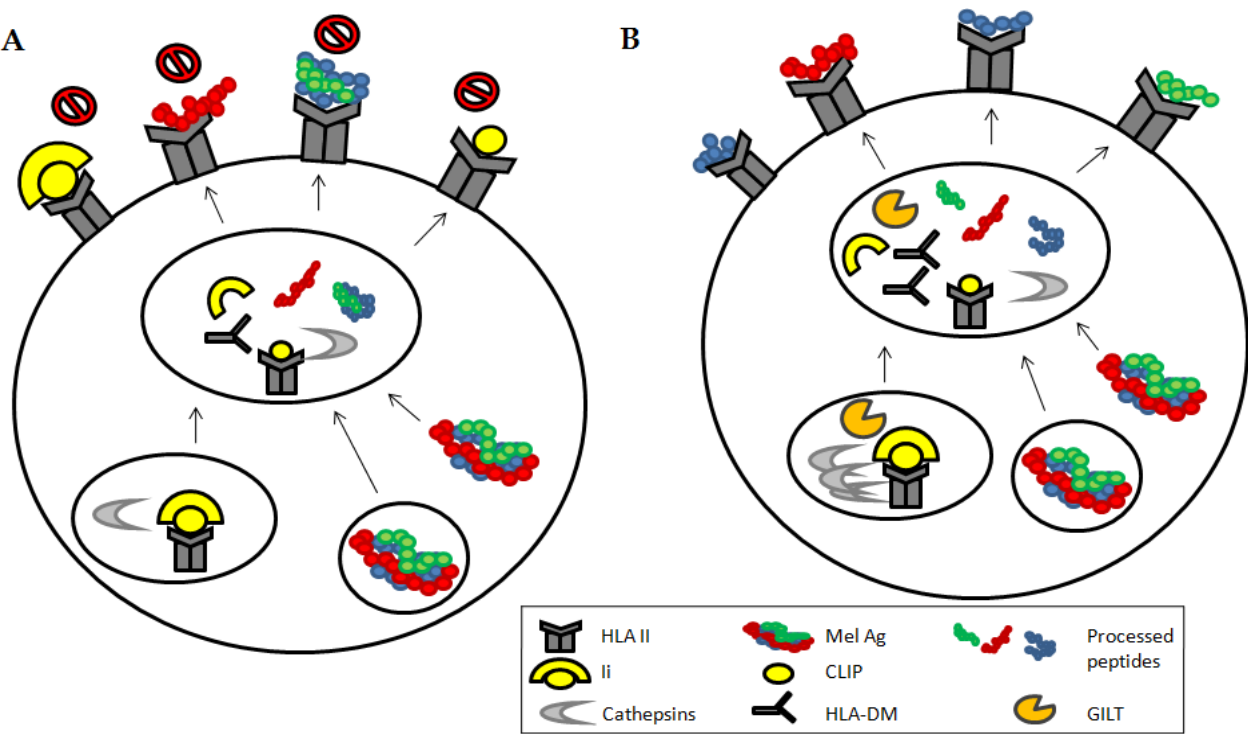


Fig. 2. Defects in HLA class II Ag Processing in Melanoma cells. A. Melanoma cells differentially process endogenous and exogenous Ags in endolysosomal compartments through deficiencies in Ii, HLA-DM, acidic cathepsin activity, and the failure to reduce oxidized peptides. This results in the presentation of a peptide milieu which fails to stimulate interacting CD4+ T cells, limiting the effects of CD8+ antitumor responses. B. The presence of GILT in endolysosomal compartments facilitates increase in both acidic cathepsin processing of tumor Ag and HLA class II components, and the functional processing of cysteinylated or oxidized peptides for improved CD4+ T cell activation.

Melanoma cells also differentially express acidic cathepsins which catalytically process endogenous and exogenous Ags in endolysosomal compartments. The lack of these enzymes or their limited activity results in poor Ag processing and the generation of nonfunctional antigenic determinant(s), that when presented are incapable of stimulating T cells (Goldstein et al., 2008). They also express low levels of HLA-DM, a nonclassical class II molecule responsible for peptide loading onto HLA class II molecules and the removal of the class II-associated invariant chain (Ii) peptide (CLIP) (Norton & Haque, 2009). Without active HLA-DM function, low affinity peptides are loaded onto class II proteins and in some cases CLIP is not removed, the result of which is again poor immune activation (Weber et al., 1996). Melanoma cells also lack an important enzyme, Gamma Interferon-inducible Lysosomal Thiol Reductase (GILT), which is required for the functional reduction of cysteinylated or oxidized proteins and peptides (Haque et al., 2002). Spontaneous cysteinylated or oxidized peptides and Ags occurs through the formation of disulfide bonds between cysteine residues or when cysteine residues bind to free floating cysteine in biological fluid (Haque et al., 2002). These peptides display high binding affinity for the HLA class II binding groove, yet they are nonfunctional at CD4+ T cell activation. Figure 2 illustrates some of the defects in HLA class II processing and presentation utilized by melanoma tumors and the ability for GILT induction to improve or reverse some of these deficiencies.

GILT is a lysosomal reductase that is highly expressed in professional APCs, but is absent or expressed at low levels in melanoma (Phan et al., 2002). GILT can be induced in melanoma cells and in other tumor cells when treated with interferon-gamma (IFN- γ) (Goldstein et al., 2008). The expression of GILT in melanoma cells upregulates active forms of cysteinyl and aspartyl cathepsins such as cathepsins S, B, and D; and GILT expression also upregulates the non-classical class II molecule DM (Goldstein et al., 2008). GILT also breaks disulfide bonds within Ags/peptides providing further access for loading and processing by cathepsins (Goldstein et al., 2008). Melanoma cells expressing GILT may be able to efficiently process and present peptides to CD4⁺ T cells for immunological recognition and elimination of tumors. Unfortunately, the numerous defects illustrated here do not form the complete picture of melanoma immunomodulation in terms of Ag processing. Once functional peptides are loaded into either HLA class I or class II compartments and CD8⁺ and CD4⁺ T cells recognize these molecules, a second signal received from costimulatory molecules on the surface of the tumor is needed to activate these immune cells.

3.2 Costimulatory molecules

Surprisingly, melanoma tumors represent immunogenic cancers with various activation of antitumor immunity, yet the natural immune responses are incapable of eradicating the tumor, the reason for which is not fully understood (Pandolfi et al., 2008). A contributing factor to this is the previously mentioned immunosuppressive microenvironment and the altered Ag processing and presentation in melanoma that can stimulate T cells, but not in the same way as APCs which would drive a strong immune response against the tumor. A second factor influencing this inhibition is the lack of, or inhibition of, costimulatory signals required for optimum T cell activation. Following Ag processing and the loading of tumor derived peptides into the HLA class II groove, this complex is translocated to the cell surface for presentation to T cells. CD4⁺ T cells recognize functional class II complexes with antigenic peptides and tight junction binding occurs between the T cell receptor (TcR) and the class II/Ag complex (Cochran et al., 2000). CD4 molecules on T cells then bind to a different site on the HLA class II molecule and T cells receive their first stimulation signal (Chambers, 2001). A second signal is then required for activation/regulation of the T cell. If the T cells receive a stimulatory signal from the tumor in the form of CD80/CD86 (B7-1/2) binding to T cell expressed CD28, then T cells become activated and mount an antitumor response (Figure 3). However, costimulatory molecules are often modified on melanoma tumor cells inhibiting T cell activation. Melanoma tumors have been shown to express high levels of CTLA-4, a cell surface receptor that also interacts with CD28 but in a regulatory role, inhibiting T cell activation (Weber, 2008). Naturally, this function may inhibit autoimmune conditions, but tumors exploit this process, functionally silencing CD4⁺ T cell activation and shifting the environment to a T-regulatory setting.

These issues are further compounded by the presence of death receptor ligands on the surface of melanoma tumors (Pilon-Thomas et al., 2010). T cells naturally express programmed death receptors (PD-1) on their cell surface as a limiting factor during T cell activation, sparing healthy “self” cells that may activate these T cells. TIL's have been specifically shown to express higher levels of PD-1 than circulating T cells, a paradigm that is not completely understood (Ahmadzadeh et al., 2009). Study shows that melanoma tumors express high levels of the ligand for PD-1, PD-L, which during TcR-HLA interaction sends a death signal to both CD4⁺ and CD8⁺ T cells causing them to undergo apoptosis

(Pilon-Thomas et al., 2010). Melanoma specific-myeloid suppressor cells also express PD-L, further accelerating immunosuppression. Thus, these molecules represent ideal targets in developing improved melanoma immunotherapy strategies which will be discussed further in the following sections, particularly the use of anti-CTLA-4 monoclonal Ab (mAb). An additional signal worth mentioning is the much debated role of tumor FasL (CD95) (Hallermalm et al., 2004). FasL is a transmembrane protein belonging to the TNF superfamily, which when bound to its receptor induces apoptosis (Hallermalm et al., 2004). Multiple studies have shown melanoma tumors express detectable surface FasL expression both in vivo and in vitro and that this ligand may act as a first line immunosuppressor through inhibiting CTL activity (Shukuwa et al., 2002; Andreola et al., 2002). High surface FasL expression also correlates with poor disease prognosis, but whether this is due to enhanced immune impairment or through an autocrine tolerization against FasL-FasR binding remains unknown (Hallermalm et al., 2004).

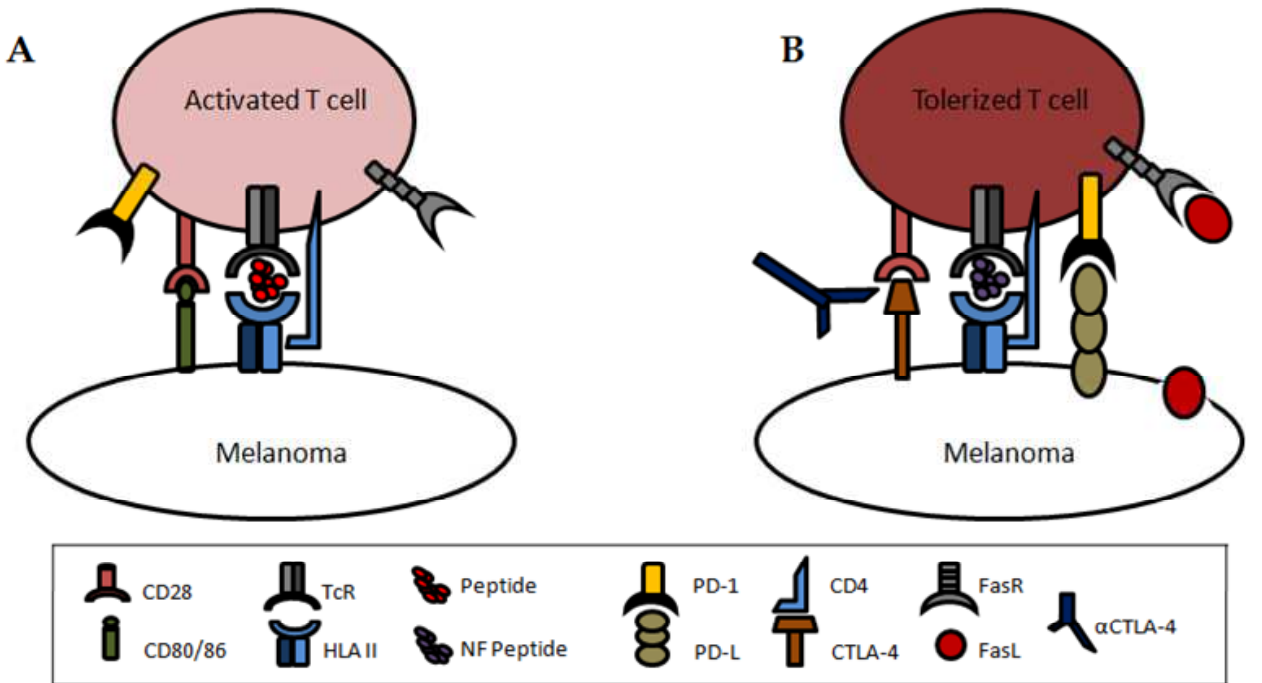


Fig. 3. Costimulatory Signals in Melanoma. A. Complete T cell activation requires binding between HLA class II-Ag complexes with T cell receptors on CD4+ T cells. A second signal between B7-1/2(CD80/86) molecules on melanoma tumors with CD28 on T cells activates CD4+ T cells initiating a robust immune response B. Melanoma cells differentially present oxidized peptides to the TcR which can inhibit T cell activation. They also express high levels of CTLA-4 which preferentially binds CD28 and suppresses T cell function. Blockade of CTLA-4 using mAb can restore immune recognition. Melanoma cells also express programmed death ligands (PD-L) which bind PD-1 on T cells inducing T cell tolerance. In addition, tumors also express surface and secretory FasL which bind FasR on T cells favoring apoptosis.

Study in uveal melanoma has also shown the potential for shed FasL encapsulated in a microvesicle that degranulates in the microenvironment and binds to FasR expression on lymphocytes (Andreola et al., 2002). More research is needed to fully develop this concept, but the targeting of surface FasL on melanoma tumor may improve the immune

environment allowing immunotherapy strategies to be more effective. More effective targeting of these factors is also paramount in limiting disease progression before tumors undergo angiogenesis and eventually metastasize, which pose their own issues in targeting the disease.

4. Issues with advanced disease

The previous sections have discussed the immunological concerns with melanoma in the tumor microenvironment and the interplay between the tumor and the immune system. However, the course of tumor progression further complicates this picture as tumors develop microvasculature, migrate to the blood stream, and metastasize to distant sites. Angiogenesis is tightly linked with the vertical growth phase of melanoma; a process which restructures the surrounding environment allowing tumor cells to migrate to the blood stream. This process is accompanied by a shift in the immune environment as infiltrating monocytes and macrophages converge at the site of angiogenesis and are misused by tumor cells to further proliferate. Following angiogenesis, melanoma tumors often metastasize to distant and local sites contributing to the great number of melanoma associated mortality. Once melanoma tumors invade the vasculature and colonize distant sites, new immunologic dysfunction is enacted at the site, further complicating and reducing the efficacy of therapeutic strategies, particularly in the lymph nodes and the brain. This section will further examine the immunological issues presented during angiogenesis and metastasis, and stress the need for new techniques at targeting these processes and how it will require specialized immunotherapy strategies.

4.1 Angiogenesis

As melanoma tumors progress, there is a distinct shift from the radial growth phase to the vertical growth phase which is accompanied by many changes to the cellular and immune environment. This change is also closely linked with angiogenesis. Angiogenesis is the formation of new blood vessels, and is abused and unregulated in advanced melanoma (Mahabeleshwar & Byzova, 2007). Figure 4A shows some of the molecules involved in angiogenesis, and in the switch from radial growth to vertical growth in melanoma tumors. With the formation of new blood vessels and the vertical growth of tumors, an influx of immune cells occurs as a result of shed molecules like IL-8 (Waugh & Wilson, 2008).

As previously discussed, IL-8 as well as IL-6 can act as circulating tumor cell attractants, which can accelerate tumor growth, angiogenesis, and recruitment of other chemo-attractants (Kim et al., 2009). IL-8 signaling has also been shown to increase the transcription of nuclear factor- κ B in melanoma, which may be increased through protein kinase C (PKC) activity (Wang & Richmond, 2001). Along with NF- κ B, STAT3, and β -catenin, IL-8 indirectly upregulates the activity of AP-1 and mTOR, which are both implicated in cell proliferation, invasion, and cell survival (Karst et al., 2009). VEGF is also linked with IL-8 signaling through its activation by the GPCR of the IL-8 receptor. VEGF is the primary molecule responsible for angiogenesis in both natural and melanoma settings (Srivastava et al., 2003). Melanoma cells have also been shown to utilize VEGF in an autocrine fashion to fuel progression and growth. Tumor-derived molecules like basic fibroblast growth factor (bFGF), placental growth factor (PGF), and platelet derived growth factor (PDGF) aid in melanoma angiogenesis, and may represent potential targets in immunotherapy design (Figure 4). Other angiogenic factors involved in melanoma are the matrix metalloproteinases

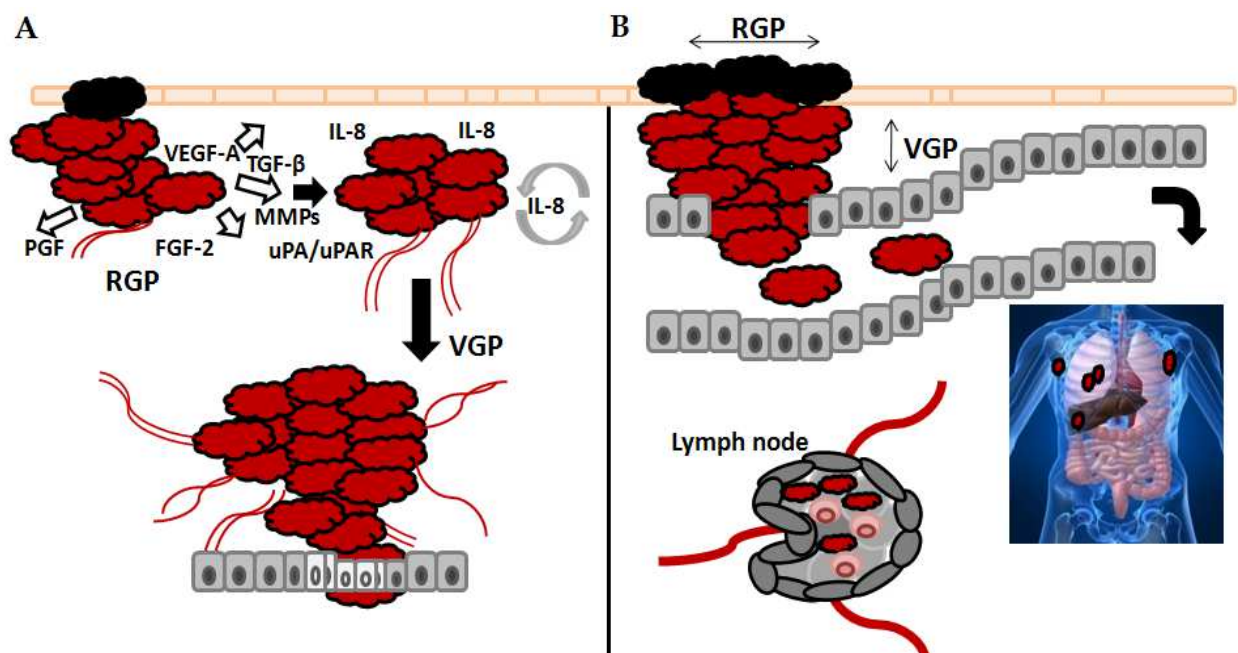


Fig. 4. Angiogenesis and Metastasis in Melanoma. A. Extracellular restructuring molecules are shed by both metastatic tumors and infiltrating bystander cells. These molecules (VEGF, PGF, FGF-2, and MMPs) lead to increased cell-cell contact, movement, and vascularization. The transition into vertical growth phase is then accompanied by increased autocrine IL-8 signaling which supports angiogenesis through recruitment of tissue destabilizing molecules and immune cells. Urokinase plasminogen activator and its receptor (uPA/uPAR) further angiogenesis by promoting cell movement and reorganization of endothelial cells into tube-like structure allowing tumors to reach the blood stream. B. Following RGP and VGP tumors metastasize to colonize distant sites such as sentinel lymph nodes, the lungs, liver and brain by entering the blood stream or through the lymphatic network. Within lymph nodes further immune suppression is induced through high levels of T regs, IL-10, and immature DC.

(MMPs). MMPs are a large group of secreted proteases that are involved in normal physiological and pathologic processes such as embryogenesis, wound healing, angiogenesis, tissue remodeling, tumor invasion, and metastasis (Kondratiev et al., 2008). Within this family of proteins, MMP-2, MMP-9, MMP-13, and MMP-14 have been found in melanoma, and are used as biomarkers for staging (Bosserhoff, 2006; Beshir et al., 2010). MMP-2 and MMP-9 degrade connective tissue and basement membrane collagen, and are believed to play an important role in skin and uveal melanoma progression (Kondratiev et al., 2008; Seftor et al., 2001). During the utilization of these molecules and others, there is localized inflammation occurring with the influx of lymphocytes, monocytes and macrophages. These cells are then activated and secrete $\text{TNF-}\alpha$ and $\text{IL-1 } \alpha$ in response to the shed VEGF and IL-8, which tumor cells then use in furthering their proliferation (Moldovan, 2002; Moldovan & Moldovan, 2005). In this system, the inhibition of macrophage/monocytes activation could aid in limiting tumor progression and angiogenesis. In an expert review of angiogenesis in melanoma, Ria et al describe the complete angiogenic process, the molecules involved, and the strategies designed to target these factors (Ria et al., 2010). The targeting of these molecules and the inhibition of the pro-tumorigenic influx of immune cells should aid in improving melanoma immunotherapy strategies.

4.2 Metastasis

Following melanoma angiogenesis and the vertical growth of melanoma into localized blood vessels, tumors metastasize to multiple distant sites including lymph nodes, the liver, lungs and brain (Streit & Detmar, 2003; Zbytek et al., 2008). This process has often occurred by the time melanoma is detected clinically, further complicating the treatment of the disease (Murakami et al., 2004). The first site of tumor metastasis is often the sentinel lymph node, which also serves as an important prognostic marker of melanoma progression (Takeuchi et al., 2004; Streit & Detmar, 2003). This progression to sentinel lymph nodes is closely related with angiogenesis, as these sites in metastatic melanoma patients have high expression of VEGF molecules (VEGF-C and VEGF-D) providing the link from tumor progression to metastasis. This process is also further driven by chemoattractive activity of tumor associated lymphatic endothelial cells which may act to draw melanoma cells to the lymph node (Kakinuma & Hwang, 2006; Streit & Detmar, 2003). This activity is a natural means of attracting mature dendritic cells to sentinel lymph nodes, but some melanoma tumors express the same surface receptor (CCR7) as dendritic cells, and are mistakenly drawn to the lymph nodes (Murakami et al., 2004). This is interesting, as targeting this surface receptor may aid in the prevention of melanoma metastasis.

The deregulated immune environment is also responsible for the spread of melanoma tumors from tissue to lymph nodes. The highly immunogenic nature of melanoma and the manipulation of immune cells by tumors, particularly in the development of Tregs, could contribute to the movement to sentinel lymph nodes. Within these lymph nodes, melanoma tumors further suppress the influx of anti-tumor immune cells through the tolerization of DC and the large number of Treg cells accompanying the tumor (Figure 4B) (Shu et al., 2006). As described in the immune cell section of this chapter, these Treg cells in the lymph are now capable of more widespread suppression allowing for the colonization of distant sites by travelling through the lymphoid network as veritable "body guards" surrounding the tumor. Tumors are also capable of traveling through the vascular network to colonize distant sites, which requires surviving the vascular environment, adhering to the desired organ, and invading the desired tissues. These processes are accomplished through upregulation of adhesion molecules on melanoma tumors like integrins allowing for adhesion and passage into distant tissue (Yoshimura et al., 2009).

Melanoma tumors now metastasize to distant sites including the liver, lungs, and brain. Single solitary tumors are effectively cleared through surgery, but multiple metastatic lesions limit surgeries effectiveness. The involvement of major organs represents a sharp decrease in the efficacy of treatment options, and with respect to brain metastasis, accounts for the overwhelming majority of melanoma deaths (Zbytek et al., 2008). Liver metastasis is often seen in cases of uveal melanoma and is treated through chemotherapy and surgery (Vahrmeijer et al., 2008). Unfortunately, unlike other cancers that metastasize to the liver, melanoma metastasis often cannot be resected due to the gross number of tumors and the location of the tumor (Vahrmeijer et al., 2008). Melanoma tumors which metastasize to the lungs often pose the same problems that liver metastases do, in which surgical resection is often impossible, and, to compound problems, brain metastases are often concurrent with lung metastasis, though the lungs are probably colonized first (Fidler et al., 1999). In these unique cases immunotherapy may be considered in place of or in conjunction with chemotherapy. A recent in vitro study on preferential liver metastasis of melanoma showed a correlation between the presence of high integrin 2α on invading tumor cells versus those that did not metastasize to the liver (Yoshimura et al., 2009). Targeting this surface molecule

through the use of a monoclonal antibody could then prove useful in inhibiting disease before it progresses to the liver. Lung cancer can pose additional concerns; airway passage epithelial cells, like those found in the lungs, can possess TLR2 surface expression, which in vitro study has shown to be upregulated in metastatic melanoma cell lines (Yang et al., 2009). TLR2 activation can promote an influx of lymphocytes and cytokine production through STAT3 regulated pathways, which we have already mentioned, are deregulated in cases of melanoma. This same study showed that inhibiting TLR2 through the use of an anti-TLR2 antibody inhibited the extent of pulmonary melanoma metastasis, a concept which could prove useful in devising novel immunotherapy strategies (Yang et al., 2009). Despite brain metastasis resulting in the majority of melanoma deaths, relatively little is known about the processes involved in brain metastasis (Sloan et al., 2009). Patients with multiple melanoma brain lesions have few treatment options. When 1-3 brain metastases are present, surgery and stereotactic radiosurgery are routinely performed, but when more metastases are present surgery loses efficacy and response rates to chemotherapy remain low (Sloan et al., 2009; Aboody et al., 2006). Unfortunately, the presence of brain metastasis was often used as an exclusion criterion for immunotherapy trials of metastatic melanoma. Some studies using both biological response modifiers like IL-2/IFN α and cellular immunotherapy employing MART-reactive TIL's have shown some success (Sloan et al., 2009). Given the ability for some immune passage through the blood brain barrier, immunotherapy should be on the forefront of treatment for melanoma brain metastases, yet clinical manifestations of successful immunotherapy remain limited (Sloan et al., 2009).

5. Issues with immunotherapy

In many ways, melanoma represents the gold standard in immunotherapy design and clinical application. Melanoma is a highly immunogenic tumor, and clinical evidence has shown spontaneous regression of primary lesions in a significant number of tumors (Komenaka et al., 2004). This should not be confused with the outlined immunomodulation and immune deficiencies outlined in this chapter, melanoma tumors still evade immune clearance and detection, particularly in advanced metastatic disease. The study of melanoma immunotherapy has existed in some form for the last 30 years, with some major breakthroughs to show for all this hard work (Weber, 2011). In general, immunotherapy refers to any therapeutic intervention designed to stimulate, inhibit, support, or alter the immune system as a means of inducing tumor destruction. Immunotherapy strategies include the use of immunostimulatory cytokine administration like IL-2 and IFN α , adoptive cell transfer (ACT), multiple cancer vaccination strategies, and the use of monoclonal antibodies which target tumor Ag or suppress melanoma derived inhibitory signals (Weber, 2011). Some of the strategies used in a clinical setting are highlighted in Table 2. Surprisingly, with all of this work done, only recently has an immunotherapy shown a survival advantage in patients with advanced disease (Hodi et al., 2010). This section will briefly highlight some of the successes and issues with current immunotherapy, and try to determine how these deficiencies may be overcome. A more complete review of melanoma immunotherapy strategies can also be found from Sivendran et al (Sivendran et al., 2010). The first immunotherapy strategies to show clinical promise were the immune stimulating cytokines IFN α and IL-2, both receiving FDA approval for the treatment of melanoma in the 1990's, IFN α in the treatment of stage III melanoma with Decarbazine and IL-2 in stage IV

Name	Action	Approval status	Clinical results	Issues
IFN-α (IFN-α2β, PEG-IFN)	Activates and stimulates DC and T cells, while cytotoxic to melanoma through STAT1 activation and STAT3 downregulation.	1995, As adjuvant with surgery or cytotoxic chemotherapy, most notably dacarbazine.	10-15% response rate, useful in cases of limited disease.	As single agent, high toxicity profile, adjuvant therapy has been linked with psychiatric issues like depression and mania.
IL-2 (Proleukin)	Expands and activates T cells, administered in bolus high dose.	1998, Stage IV melanoma.	15% response rate, some patients having durable responses to treatment, when in high dosage treatment regimen.	High toxicity profile. Best results only seen in a small inclusion group with limited disease progression and minimal organ involvement.
Canvaxin (onamelatucel-L)	Antigen-rich, allogeneic whole-cell vaccine.	Phase III trials ended in 2005 due to inefficacy.	Initial clinical trials were promising showing increased overall survival gains of 19 %and 12% in stage III and IV melanoma respectively.	During phase III clinical trails an independent safety reviewed board reveled Canvaxin was no more effective than control arm.
Adoptive cell therapy (ACT-CTL or ACT-TIL therapy)	Patient PBMCs are pulsed in vitro with melanoma peptide/ Ag or stimulatory cytokines and CTL clones are isolated, expanded and injected.	Ongoing clinical trials using pretreatment of lymphodepletion through irradiation or chemotherapy prior to ACT injection.	Response rates have been reported as high as 50%, particularly in large bulk tumor cases when following lymphodepletion .	Lymphodepletion required for efficacy, but all patients can't tolerate.
Ontak (DAB/IL-2, Denileukin Diftitox)	Recombinant IL-2/ diphtheria toxin conjugate binds to IL-2R expressing cells depleting these lymphocytes.	Currently in Phase II clinical trial as intervention.	Promising results from phase II trials where lymphodepletion was observed with the formation of anti-melanoma CD8+ T cells.	While it depletes detrimental T reg cells, CD8+ and CD4+ effector cells are also depleted limiting overall efficacy.

Table 2. Issues Associated with Various Melanoma Immunotherapy Strategies

disease (Fang et al., 2008). IL-2 therapy remains the only immunotherapy strategy for late stage melanoma but significant issues remain with its use. IL-2 therapy displays moderate to severe toxicity and relatively low efficacy in patients with non-cutaneous metastasis and metastatic organ involvement (Petrella et al., 2007). Thus, the key to improving IL-2's use in a clinical setting is in the selection of patients receiving treatment. Younger patients who are in good health, with little to no organ metastasis would see the most benefit from IL-2 therapy, and patients with stage II/III tumors may see even greater response to this treatment. Similarly, IFNα displays toxicity similar to cytotoxic drug therapy and this toxicity increases greatly when administered over longer periods of time. Some study also showed a correlation between IFNα and clinical manifestations of depression, mania, and suicidal tendencies (Greenberg et al., 2000). The cause behind these effects is poorly understood, but will remain a concern to monitor with IFNα therapy moving forward. However, the main concern with IFNα is the lack of improvement in overall survival, with only transient gains seen in relapse-free survival (RFS) (Kirkwood et al., 2004). Efforts to combine IFNα with a cancer vaccine strategy were disappointing, but seem to reflect issues in the design and selection of the melanoma vaccine (Kirkwood et al., 2004). The use of IFNα

to boost an antitumor response paired with an effective vaccine or with ACT could still prove beneficial and shouldn't be ruled out in therapeutic design.

Another very promising concept in immunotherapy design which has failed to aid in the clinical setting is melanoma cancer vaccines. These can come in the form of whole cell cancer vaccines, tumor cell lysates, protein/peptide vaccines, DC loaded vaccines, viral vectors, and DNA vaccines (Terando et al., 2007). To date, the largest phase III melanoma vaccine clinical trial involving late stage III and IV melanoma compared the use of CanVaxin with the nonspecific immune stimulator Bacillus Calmette-Guerin (BCG) or BCG alone (Morton et al., 2002). BCG is currently being evaluated in phase III clinical trial administered following surgery versus best standard medical care alone in patients with advanced metastatic disease. CanVaxin is an allogeneic whole cell cancer vaccine using three of the most widely studied melanoma cell lines which encompassed a vast pool of Ag targets and showed great promise during phase II clinical trials.

Unfortunately, an independent safety review board halted the phase III trial when evidence showed no detectable advantage in the treatment arm, virtually stopping CanVaxin in its tracks (Eggermont, 2009). A key issue with the design and application of CanVaxin could be the lack of host Ag presented in the vaccine, given differences between the tumor cell lines used versus the primary tumor. Studies utilizing host-derived irradiated tumor cells instead of cell lines, have shown immune reactivity and limited toxicity, but the time required for their generation remains a concern. These cancer vaccine strategies have been investigated in early clinical trials but the clinical manifestation of strong antitumor T cell activation remains elusive (Goldman & DeFrancesco, 2009). Some efforts to improve the design and application of these vaccines will be further discussed in the next section.

Currently, an immunotherapy strategy with the potential for success is adoptive cell therapy (ACT). As previously mentioned, the highly immunogenic nature of melanoma tumors generates large pools of melanoma reactive CD8⁺ T cells in vivo, which can be extracted and expanded in vitro. This expansion of patient lymphocytes can be done through stimulation with T cell growth factors like IL-2 or through stimulation with melanoma tumor Ags (Rosenberg et al., 2003). Following expansion, these T cells are then reinjected into patients to attack the tumor. This method has resulted in durable response rates, particularly in stage II/III patients; and in some cases, complete tumor reduction (Rosenberg et al., 2003). However, trials in late stage melanoma failed to show durable tumor clearance, most likely due to the high percentage of Treg cells and immunosuppressive cytokines in the microenvironment (Rosenberg et al., 2008). To combat this, the combination of lymphodepletion with chemotherapy or radiation prior to reinfusion of T cells can greatly improve the response to treatment (Dudley et al., 2008). Unfortunately, lymphodepletion in itself is hazardous to the patient as it destroys both the antitumor CD8⁺ and CD4⁺ effector cells along with the Treg cells, and leaves patients vulnerable to bacterial and viral infections (Dudley et al., 2008). Agents capable of specific Treg depletion would aid ACT therapy greatly, and a few molecules in clinical trials potentially fit this need. Ontak (Denileukin Diftitox), a recombinant fusion protein combining IL-2 with Diphtheria toxin, binds to IL-2R expressing cells and induces apoptosis through toxin release. It has been shown to deplete Treg cells, resulting in a CD8⁺ antitumor response in phase II clinical trials (Mahnke et al., 2007). However, CD4⁺ effector cells were also depleted following Ontak administration, a potentially limiting factor in the long term durable anti-tumor response. The combination then of Ontak with ACT could improve the efficacy of immunotherapy in advanced disease patients, but an important aspect of immune stimulation remains absent from this

therapeutic design: the activation of CD4⁺ effector T cells. As mentioned previously, CD4⁺ T cells play an important role in anti-tumor immunity particularly in the presentation of tumor Ags to professional APCs and CD8⁺ T cells, and in the induction of long-lasting anti-tumor immunological memory (Hung et al., 1998). Therefore, future strategies should aim to activate both CD4⁺ and CD8⁺ T cells in ACT therapy design, an idea which will be further explored in the following section.

6. Potential new directions

As highlighted in the previous section, our lack of success in melanoma immunotherapy is not for a lack of effort. With the last thirty years of immunotherapy design and clinical trials we can now apply what we've learned to novel ways of addressing the complex problem of metastatic melanoma. This may include revisiting some previously attempted ideas, but applied within a new context, particularly with what we know about the localized immune inhibition in melanoma patients. This section will discuss current strategies being developed and potential new directions in treatment that address the immunomodulatory nature of melanoma. These include novel chemoimmunotherapy ideas and techniques, the further use of monoclonal antibodies against melanoma Ags and T cell inhibitory factors and finally, on how combining multiple approaches in multimodal immunotherapy design represents a fight on multiple fronts with the potential for increased tumor destruction and disease free survival.

6.1 Chemoimmunotherapy

Therapeutic approaches combining cytotoxic chemotherapy with immunotherapy is not a new concept by any means (LoRusso et al., 1990). In fact, both IFN α and IL-2 have been extensively tested in combination with chemotherapeutics like decarbazine (DTIC), temozolamide, and cisplatin (Schadendorf et al., 2009). Yet, only the pegylated (PEG)-IFN α + DTIC or PEG-IFN α + temozolomide showed enhanced response rates, and all other trials failed to show any significant survival rates (Schadendorf et al., 2009). Moreover, the combination of these molecules increased overall toxicity greater than individual treatment, another limiting factor in the use of combined chemoimmunotherapy (Schadendorf et al., 2009). However, these results only indicate that the combination of two highly cytotoxic agents with limited efficacy in their own right are incapable of enhancing patient survival in highly advanced stages of diseases. Similar approaches should be carefully studied and considered in patients with diminished risk who demonstrate high tolerability to treatment, like the concession currently made for those receiving high dose IL-2 therapy. In selecting the right patient for this therapy, considerable gains may still be made in the combination of these agents. A second approach may be to limit the toxicity of the chemotherapeutic and select a drug with immunostimulatory properties. These molecules may include the highly en vogue antioxidant molecules like green tea extracts, holistic mushroom extracts, and flavinoids, each displaying cytotoxicity in tumor models in vitro with limited toxicity to healthy cells (Baliga & Katiyar, 2006; Harhaji Trajkovic et al., 2009; Craig, 1999). More importantly, these molecules may work synergistically with immune stimulating molecules through enhanced immune activation (Banerjee et al., 2008). Extensive research needs to be performed to ensure similar results in vivo as displayed in vitro, but the combination of these more tolerable antitumor agents with immunostimulatory cytokines may generate increased tumor clearance while reducing the toxic burden to individuals. Similarly, altering

the immune cytokine used could aid in the efficacy and reduced toxicity of chemoimmunotherapy.

Cytokines like IL-15, IL-7, and IL-21 could prove more beneficial in stimulating antitumor immune cells during chemotherapy, with reduced toxicity when compared to IL-2 or IFN α (Epardaud et al., 2008; Ribas, 2006). IL-21 has the added benefit of more selective T cell expansion versus IL-2, in which Tregs are not responsive to IL-21 stimulation (Sivendran et al., 2010). Currently IL-21 is being tested in phase I/II clinical trials displaying promising results (Sivendran et al., 2010). IL-15 shares similarity with IL-2 through a shared receptor subunit, but IL-15 has been shown to enhance both ACT and chemotherapy strategies in mouse models with almost no cytokine associated toxicity (Epardaud et al., 2008). Current research performed using IL-15 preconjugated with its receptor (IL-15 α) increased both the half life and activity of the cytokine, and improved its ability to destroy advanced solid tumors (Epardaud et al., 2008). Though preliminary, this discovery could prove beneficial in the use of cytokine therapy as both single agent, and in combination with chemotherapeutics to more effectively destroy melanoma. An additional strategy for combined chemoimmunotherapy use is in cancer vaccination design and application. In this case, chemotherapy prior to or concurrent with peptide or DC loaded vaccine techniques could allow for improved efficacy of a single treatment alone. Unfortunately, clinical trials have yet to show significant advantages to combining cytotoxic therapy with vaccine strategies over chemotherapy alone (Lens, 2008). To address this issue, study should incorporate drugs which target progression or metastasis of melanoma in conjunction with immunotherapy as a means of limiting tumor movement and increasing the chances of immune induced tumor clearance. As previously mentioned, Ria et al describe multiple anti-angiogenic drug candidates which could halt the progression of tumors through inhibiting VEGF, VEGFR, tyrosine kinase receptors, integrins, and MMPs (Ria et al., 2010). Combining these inhibitors with immune vaccines to stimulate antitumor immunity or with ACT could allow the reduction of both localized and metastatic tumors.

Currently, the most beneficial chemoimmunotherapy strategy uses lymphodepleting chemotherapeutics (cyclophosphamide and fludarabine) prior to reinjection with expanded TILs and high dose IL-2. This strategy vastly improves the response rate and efficacy of ACT through depletion of Tregs allowing for greater TIL expansion in vivo following reinjection. Unfortunately, lymphodepletion leaves the patient susceptible to both bacterial and viral infection, and is not specific for Tregs; all supportive anti-melanoma CD8+ and CD4+ T cells are also destroyed in the process. Treg-specific reduction prior to ACT represents the gold standard of improved treatment, yet as described previously our best efforts using drugs like Ontak are still shy of clinical relevance (Mahnke et al., 2007). Increased effort needs to be placed on developing Treg specific lymphodepleting chemotherapy, allowing for the full effect of ACT to show clinical benefit. These improvements to chemoimmunotherapy design are not the only potential new directions in melanoma therapy; the most promising option in the near future appears to be the use of monoclonal antibodies in the fight against advanced disease.

6.2 Monoclonal antibodies

Monoclonal antibodies (mAb) have been the focus of much research in cancer since their discovery some thirty plus years ago (Oldham & Dillman, 2008). Though some mAb have been approved to treat other cancers, most notably Trastuzumab (Herceptin) in the

treatment of breast cancer, there is only one FDA approved mAb for the treatment of advanced melanoma, ipilimumab (Dillman, 2011). Ipilimumab is one of a number of mAb designed to selectively block CTLA-4 activity in melanoma tumors allowing for the expansion and activation of CD8⁺ and CD4⁺ anti-melanoma T cells (Robert & Ghiringhelli, 2009). This chapter previously described the effect high CTLA-4 expression has on melanoma tumors by inhibiting the costimulatory signal needed for T cell activation, shifting the immune response from active to suppressive. Ipilimumab has been tested in clinical trials both as a single agent and in combination with chemotherapy and vaccine strategies (Wolchok et al., 2010; Weber et al., 2009). Recent phase III clinical trials of advanced disease showed, for the first time, a significant survival advantage of patients treated with ipilimumab versus a gp100 vaccine alone, representing a huge breakthrough in these advanced disease patients (Hodi et al., 2010). A larger phase III comparison study using DTIC ± ipilimumab involving more than 500 patients has been performed, the results of which led to the approval of Ipilimumab in the treatment of advanced melanoma on March 25, 2011. Ipilimumab, like any other treatment, is not without issue, as patients undergoing treatment have experienced immune-induced side effects including dermatitis, enterocolitis, hepatitis, and most significantly diarrhea (Sivendran et al., 2010). Some interesting treatment effects have also been observed in a number of patients treated with ipilimumab which will require close attention in determining the best treatment protocol. A number of patients receiving ipilimumab displayed delayed slow ongoing responses lasting a year or more, delayed responses taking up to 6-12 months, and surprisingly, tumor growth and progression followed by tumor regression (Weber, 2011). These interesting effects could lead to the withdrawal from treatment due to the appearance of tumor growth or the delayed response, but these patients need to remain in treatment as the patients who displayed these characteristics often had better disease prognosis than others (Weber, 2011). New immune criteria for the judgment of drug efficacy should be included in determining the best course of patient treatment to avoid issues like these, particularly as ipilimumab has great potential as a combined agent with vaccination and cytotoxic drug therapy, and most interestingly with ACT.

Ipilimumab is not the only mAb targeting advanced disease receiving clinical attention; anti-PD-1, anti-CD137, and anti-CD40 are all undergoing clinical investigation (Sivendran et al., 2010). Anti-PD-1 has shown promise in melanoma cell lines and small phase I/II clinical trials displayed immune activity without major drug associated toxicity, opening the door for its use in combination with other mAb and immunotherapeutic strategies (Brahmer et al., 2010). Clinical trials are ongoing, pairing anti-PD-1 with CTLA-4 blockade or with multiple melanoma peptide vaccine strategies (Curran et al., 2010). Anti-CD137 binds to 4-1BB expressed on activated immune cells sending a costimulatory signal promoting lymphocyte activation (Meseck et al., 2011). In trials using anti-CD137 with GM-CSF-secreting tumor cell immunotherapy induced complete rejection of tumor in a B16 mouse model, displaying proof of principal in melanoma immunotherapy use (Li et al., 2007). Results of a phase I clinical trial revealed antibody activity associated with minimal toxicity in patients with advanced solid-tumor malignancies (Sivendran et al., 2010).

Similarly, anti-CD40 binding with CD40 on the surface of immune cells results in the activation of T cells, and the upregulation of MHC class II complexes and costimulatory molecules, two characteristics we have previously described as being major roadblocks to immunotherapy of advanced melanoma (Law & Grewal, 2009). Anti-CD40 has been tested in advanced solid tumors, refractory Hodgkin lymphoma, and multiple myeloma (French et

al., 1999). In melanoma, anti-CD40 seems ideal for combination with protein/peptide vaccine strategies as upregulation of MHC class II complexes and costimulatory molecules would increase the amount of tumor-vaccine interaction and presentation to T cells where increased costimulatory molecules would promote T cell activation instead of suppression or T cell anergy. Future efforts in melanoma mAb design should include the targeting of immunosuppressive cytokines and molecules like those outlined at the start of the chapter as a novel mechanism of enhanced immune activation. These molecules include, but are not limited to, IL-10, TGF- β , PGE2, IDO, and VEGF. Through the targeting and inhibition of these molecules a more immunosupportive tumor microenvironment could increase the efficacy of current and future immunotherapy strategies. Along with cytokine therapy, the use of mAbs in the treatment of metastatic melanoma appear to have the fastest course to clinical use, but the ideal immunotherapeutic strategy would combine these techniques with ACT or vaccination strategies, promoting long-term, sustained immunological anti-tumor responses.

6.3 Multimodal therapy strategies

As outlined in this chapter, melanoma tumors deploy multiple immune evasion techniques to subvert both natural and therapy induced anti-melanoma immune responses, despite the highly immunogenic nature of melanoma tumors. It should then make logical sense that targeting one of these pathways or suppressive mechanisms would incompletely abrogate the problem, as different mechanisms would take over the immunomodulatory duties of melanoma tumors inhibiting the treatments efficacy. Thus, strategies to target melanoma should target multiple suppressive pathways preventing tumors from avoiding single agent strategies. This is accomplished by the administration of multimodal therapy design, in which the combination of therapies enhances the effectiveness of each individual therapy increasing the durability and intensity of the anti-tumor response (Kudo-Saito et al., 2005). Multiple in vitro and melanoma mouse models support the notion of multimodal therapy as a way to completely ablate tumor burden and prevent its recurrence (Ascierto et al., 2010). Clinically, the combination of strategies is not a new method by any means, and even current immunotherapy techniques often combine chemotherapeutic agents with immune cytokines and mAbs for improved treatment (Bhatia et al., 2009). However, in moving forward in the design of improved melanoma therapy, combining multiple immunotherapy strategies like, ACT, gene therapy, DC or peptide vaccines, cytokine therapy, mAb therapy, and novel tumor molecule targeting could represent the future standard of advanced melanoma therapy. Before this ideal can be realized, there are some issues with each technique individually that need to be fixed prior to their combination. Some of these issues we have already mentioned: the inherent toxicity of immune stimulatory cytokine like IL-2 and IFN α , the failures of ACT to fully stimulate long-term anti-tumor immunity, the time required in generating an expanded pool of TIL for ACT therapy, and the inefficacy of vaccine strategies in vivo like those seen with CanVaxin (Berinstein, 2009). The first two issues we have already addressed; the use of less toxic cytokines like IL-7, IL-15/IL-15R, and IL-21 could alleviate toxicity concerns while promoting the expansion of activated immune cells, particularly those which promote immunological memory, and through lymphodepletion prior to ACT expanded lymphocytes are capable of repopulating the immune environment with anti-tumor CD8 $^{+}$ T cells leading to longer efficacy (Dudley et al., 2008). The last two issues are more difficult to solve, but there is hope on the horizon.

The effect that melanoma has on the immune system both hinders our ability to treat the disease, and also allows for treatment like ACT to effectively kill tumors due to the immunogenic nature of melanoma. Unfortunately, only about 50% of melanoma patients have TILs capable of being expanded in vitro for reinjection, a number still much higher than in other cancers where the feat is near impossible (Rosenberg et al., 2008). Further complicating things, the generation of expanded activated TILs takes a few weeks, during which, valuable treatment time is lost for those patients with advanced disease, and with lymphodepletion the chances of an infection increase greatly (Rosenberg et al., 2003). Thus, the ability to expand activated patient TILs from all patients and the rapid generation of anti-tumor lymphocytes would further the effectiveness and clinical application of ACT (Rosenberg, 2001). To achieve this, rapid selection of CD8⁺ T cells with characteristics predetermined beneficial for anti-tumor immunity can be separated in culture and expanded with administration of anti-CD3 and the presence of feeder cells to increase the activation and expansion rate. Results from this treatment manipulation have shown benefit over previous strategies (Rosenberg et al., 2008). To combat the inability of all patients to generate active TIL, strategies which clone TcRs from activated TIL population into peripheral blood mononuclear cells (PBMCs) of melanoma patients allows for the reinjection of primed anti-tumor lymphocytes without the need for expanded TILs (Weber, 2011). An additional benefit of cloning in these TcRs is that they can be manufactured to express theoretically any melanoma Ag capable of synthetic production, improving the chances of T cell stimulation in vivo through cross-presentation (Robbins et al., 2011). Study using this method has shown benefit in a number of cancers, even in the absence of prior lymphodepletion, further reducing treatment associated toxicity (Robbins et al., 2011).

An additional strategy which should be explored is the expansion of CD4⁺ effector TIL in the same fashion as CD8⁺ CTL for combined ACT capable of a more prolonged interaction resulting in increased tumor clearance. These CD4⁺ TIL can be expanded and raised against patient derived or synthetically generated melanoma Ag, allowing for direct cross-presentation with patient lymphocytes in vivo. This could then lead to the development and support of CD8⁺ memory T cells, as long as the synthetic Ag is highly immunogenic and replicates the immunodominant HLA matched tumor epitopes. This last point, the ability to present synthesized melanoma Ag capable of stimulating patient immune cells in vivo is also a major issue in vaccine development. As previously mentioned, melanoma vaccine strategies are as varied as they are disappointing in the clinical setting facing issues of inefficacy, inferiority to chemotherapy, and a failure to work in collaboration with other techniques (Berinstein, 2009). Two key issues face melanoma vaccine strategies, the selection of immunogenic tumor associated Ags and the reliance on only CD8⁺ CTL stimulation in vaccine design, and in many ways these two problems go hand in hand (Sondak et al., 2006). In this chapter, we have highlighted the importance of stimulating CD4⁺ effector T cells in developing a robust immune response through cross-presentation to CD8⁺ T cells and other APC, but also for their release of immunostimulatory cytokines like IFN γ (Corthay et al., 2005). Yet, until recently, vaccine strategies (protein/peptide, whole cell, or DC loaded vaccines) have solely used tumor Ags designed for CD8⁺ CTL activation (Terando et al., 2007). Though these T cells are responsible for the bulk of tumor destruction, time and time again they fail clinically to eradicate host tumors, often due to induced T cell tolerance against these Ags propagated by tumors (Terando et al., 2007). Only through the discovery and application of HLA class II specific anti-tumor melanoma Ags, effector CD4⁺ T cells may get the much needed stimulation required for a complete T cell response.

Excellent work has been done in the field of HLA class II specific melanoma Ags in recent years, specifically in the identification of an HLA-DR4 restricted Melan-A/Mart-1₅₁₋₇₃ epitope (RNGYRALMDKSLHVGITQCALTRR) capable of stimulating CD4⁺ anti-tumor T cells in a number of DR matched patients (Zarour et al., 2000). The identification of Ags like these should be added to patient specific vaccine strategies matching both HLA class I and HLA class II allele specific immunogenic Ags in order to improve the efficacy of melanoma tumor vaccines. A second vaccine strategy which stimulates both CD8⁺ and CD4⁺ T cells utilized a natural process of HLA class II peptide presentation in a whole cell cancer vaccine design for enhanced Ag-T cell interaction (Bosch et al., 2010; Thompson et al., 2008). This class II vaccine uses tumor cells genetically modified to express surface class II and costimulatory molecules without expressing invariant chain (Ii). As previously described, Ii blocks the loading of antigenic peptides into the HLA class II peptide binding groove prior to presentation in the endolysosomal compartments (Thompson et al., 2008). By silencing Ii, numerous novel class II peptides are loaded onto the HLA binding groove which would have normally been blocked (Thompson et al., 2008). These peptides may or may not be functionally active, but they do represent peptides which T cells are not tolerant against, allowing for potential T cell activation. The presence of costimulatory molecules on the vaccine-cell surfaces furthers the chance of immune activation, as T cells receive the support signal needed for activation. A third way to generate novel immunogenic peptides for HLA class II presentation, which has been briefly stated, is the induction of GILT into whole cell vaccine strategies. GILT has been shown to increase HLA-DM expression, cysteinyl protease activity, and functionally reduces cysteinylated or oxidized Ags/peptides when transfected into melanoma cells (Goldstein et al., 2008; Rausch et al., 2010). In much the same fashion as the previously described vaccine design, GILT's presence could generate novel immunogenic tumor Ags capable of stimulating CD4⁺ T cells in vivo. Though early in discovery, techniques like these should be pursued in the improved design of melanoma cancer vaccines. The combination of these improved techniques, with those already described, in a multimodal immunotherapeutic design could then provide the most benefit in terms of long-term tumor free survival. A proposed outline of a multimodal immunotherapy regime is shown in Figure 5.

By first clearing the Tregs from the tumor microenvironment, any immunotherapy strategy will see improved efficacy, similar to those seen in ACT clinical trials (Dudley et al., 2008). ACT TIL therapy utilizing both CD4⁺ and CD8⁺ activated and expanded T cells or through the addition of TcR-Ag specific transfected PBMCs are then free to repopulate the immune environment. TIL responses against the tumor should then sustain for much longer than previously observed with the addition of activated CD4⁺ T cells using HLA class II specific tumor Ags and with concurrent stimulation with mAb against CTLA-4. The patient should then be monitored for response to treatment seen in tumor reduction and by extracting a sample of patient TILs checked for tumor reactivity. The addition of an improved DC loaded vaccine pulsed with processed tumor peptide-GM-CSF fusion protein, like those used in the successful prostate cancer therapeutic Sipuleucel-T (Provenge), could then act as a boost to the anti-tumor TILs present sustaining the development of immunological memory (Brower, 2010). Finally, using less toxic cytokine therapy through IL-15/IL-15R or with IL-7, IL-21 will support the continuation of CD8⁺ memory T cells and fuel the remaining anti-tumor lymphocytes into attacking the tumor. Though complex, the combination of these procedures would more effectively target the multiple

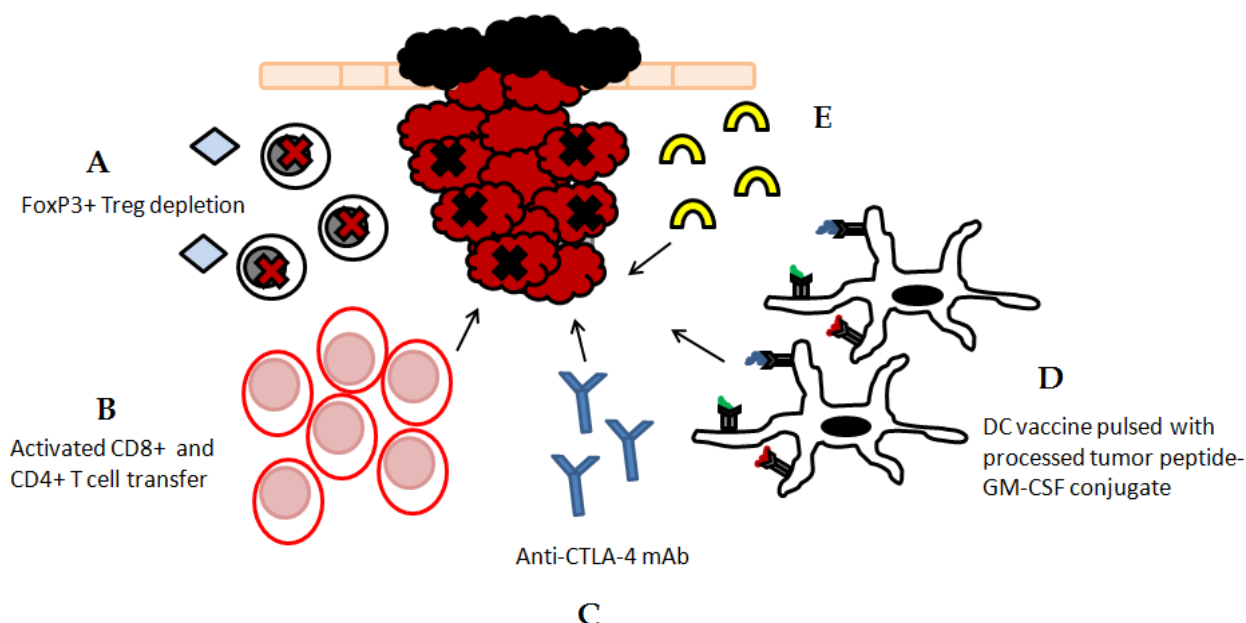


Fig. 5. Multimodal Therapy Design. A. Most of not all immunotherapy strategies should include a means of Treg depletion to prime antitumor immune responses. B. This may come in the form of an adoptive cell therapy (ACT) using autologous TIL's expanded in vitro and pulsed with tumor specific peptides targeting both CD8+ and CD4+ T cells. C. The addition of anti-CTLA-4 mAb could further support these TIL's life and prevent de novo generation of Treg cells D. Following a resting period to limit the chances of autoimmune reactions, concomitant challenge with a dendritic cell vaccine loaded processed tumor peptide and GM-CSF could then further expand the tumor specific immune response of the ACT. E. IL-15a therapy could also be administered during vaccination protocol to stimulate and expand T cell responsive with significantly less toxicity than through IL-2 addition.

immunomodulatory mechanism employed by tumors to evade the immune system. Much work will be needed to bring these theoretical ideals into clinical reality, but through improved understanding of melanoma tumors and their ability to modulate the immune environment, substantial gains can be made in both therapeutic design and patient care.

7. Conclusions

The future looks promising for melanoma immunotherapy, even with the multiple disadvantages that research scientists and clinicians face from the disease. This chapter attempted to summarize the multiple steps of immunomodulation that melanoma tumors employ to thwart even the most well thought out therapy strategies. By understanding both the mechanisms tumors employ to suppress and abuse the immune system and the ways that previous immunotherapy strategies have failed to meet our expectation, we can move forward in devising new strategies in improved immunotherapeutics and disease management. These improved concepts include combining chemotherapy and immunotherapy strategies in new ways to more effectively shift the balance of the tumor microenvironment from immunosuppressive to immunostimulatory prior to immunological intervention, and the targeting of protumorigenic pathways like the BRAF/MAPK and STAT3 pathways to aid in tumor destruction and promote immune responses. Improved understanding of the defective Ag processing and presentation pathways in melanoma

presents novel targeting strategies through genetic manipulation of tumors by induction of GILT capable of generating novel immunogenic Ags/peptides. The importance of incorporating strategies to expand and activate both CD8+ and CD4+ T cells will also help in the design and efficacy of ACT, whole-cell, and protein/peptide based vaccine therapy with improved durable immune responses and the development of immunological memory. New cytokine adjuvants like IL-15 and IL-7 may also expand the efficacy of these techniques and aid in the reduction of toxicity seen in IL-2 or IFN α strategies. Monoclonal antibodies like ipilimumab also represent the very near future in melanoma therapy following its recent FDA approval. More strategies combining these molecules with ACT and vaccine strategies should be explored as a means of enhancing the expansion and activation of immune cells and in avoiding immunosuppression induced by melanoma cells. More collaborative efforts need to be made between basic scientists, clinical investigators, and physicians to devise the best way to take discoveries made on the bench into the clinic, particularly in the immunomodulation of melanoma tumors. It appears that for every step we take forward in targeting melanoma, the tumors develop a new suppressive mechanism to avoid our efforts. These deficits must be understood and overcome for immunotherapy to become a true therapeutic option for late stage melanoma patients. As always our best weapon in the fight against melanoma is knowledge, and the collective knowledge gained from understanding melanoma immunomodulation will lead to the next generation of techniques capable of alleviating this deadly disease.

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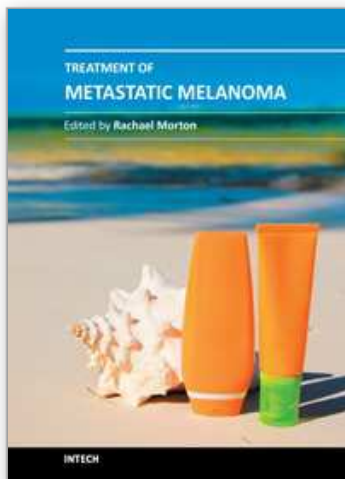
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Surgery continues to be the mainstay treatment for melanoma localized to the primary tumor and/or lymph nodes. Results from randomized controlled trials indicate that sentinel node biopsy for the treatment of cutaneous melanoma of intermediate thickness has a beneficial effect on recurrence rates, and adjuvant radiotherapy to regional lymph node fields following surgical resection reduces loco-regional recurrence in patients at high risk of relapse. Isolated limb perfusion, electrochemotherapy, and photodynamic therapy continue to be evaluated for treatment of stage IV disease. However, the greatest excitement in new treatment has been with targeted therapies for genetic mutations. In particular, the promising results of partial and complete tumor response in stage IV disease from early phase trials of the B-RAF kinase inhibitors. This book provides a contemporary insight into the therapeutic treatment options for patients with metastatic melanoma and is relevant to clinicians and researchers worldwide. In addition, an update on current clinical trials for melanoma treatment has been included, and two chapters have been reserved to discuss the treatment of oral and uveal melanoma.

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