Chapter 25

Immunomodulation

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1. Introduction

Immune treatment for melanoma has roots in clinical observations surrounding regression of normal nevi, the appearance of halo nevi, the correlation of vitiligo with outcome, the occurrence of spontaneous regression of primary melanomas, nodal metastases of unknown primary lesions and the occurrence of metastases many years after resection of a primary lesion. Coupling these observations with the observations on the power of the immune system to reject transplanted organs and control leukemia after allogeneic bone marrow transplant, the possibility of harnessing and directing this power has been a source of both excitement and disappointment.

In examining the natural history of normal nevi, it is noted that they undergo a life cycle in which growth occurs during childhood in the border of the epidermis and dermis (junctional nevi). With increasing age the melanocytes move deeper into the dermis. As adult life continues they regress in old age. It is not evident that this type of regression has an immune basis.

Another type of regression called halo nevi is more definitively tied to the immune system [1]. Akasu, et. al have described halo nevi regression in four stages characterized initially by pan-T lymphocytes in stage one and the addition of KP-1 positive cells as well as FX IIIa-positive cells in stage two. Stage three continues with increased numbers of FX IIIa-positive cells and the addition of Langerhans cells. Finally upon complete regression in stage four there is a moderate mononuclear infiltrate comprised predominantly of T cells [2]. The role of natural killer (NK) cells has been studied in normal and malignant melanocytic lesions. The highest concentration of NK cells was seen in regressing malignant lesions followed by regressing normal nevi [3]. This type of "spontaneous" regression is observed in other be-

nign skin lesions such as keratoachanthomas, and the pathologic studies give insight into the possibility of stimulating similar immune action against malignancies [1, 2].

The autoimmune condition of vitiligo has been associated with regression of metastases as well as better outcomes associated with immunotherapy. Vitiligo is mediated through autoantibodies. Antibodies against tyrosinase have been observed in patients with melanoma as well as vitiligo not associated with melanoma. Other autoimmune effects are well documented with immunotherapeutic treatments, indicating the ability of the therapy to break tolerance to self-antigens [4-7].

Spontaneous regression is observed in primary melanoma [8, 9]. Statistics vary on the incidence but may be as high as 20%, especially if cases of unknown primary are included. When remnants of primary lesions are found, they frequently are partially regressed and show histologic evidence of infiltration by lymphocytes. Although some have observed a worse clinical outcome with partially regressed primary lesions, patients with nodal metastases and unknown primaries tend to have a better outcome. The latter observation is attributed to improved immune surveillance compared to patients with intact primaries [10-14]. Regression of metastases is less common and has anecdotally been tied to infections or surgeries. Regression of metastases seems to predict a better overall outcome [15, 16].

The issue of late metastases from primary melanoma is well documented but much harder to explain. Issues within the tumor microenvironment remain incompletely explored, but high on the list of explanations is the possibility that the immune system is able to control proliferation until some as yet undocumented effect allows escape [17, 18].

Early studies with nonspecific therapies described below produced enough positive results to keep interest in melanoma immunotherapy alive. However, progress in the clinic has been slow until very recently. Many issues have presented challenges to progress. Among them is an incomplete understanding of the normal immune system, including immune tolerance and the effects of the tumor microenvironment on the immune response. Drug development required technology to produce biologic agents, and that capability has only recently been perfected. Issues of study design also need to be kept in mind. Subject selection can be difficult since these studies require immune competence but disease advanced enough to answer the question in a reasonable time frame with a reasonable number of subjects.

2. Biomarkers and endpoints

Biomarkers and surrogate endpoints are tools to obtain information about disease status or response to interventions. The mainstay of efficacy determination in cancer therapeutic clinical trials has been the regression of known tumor masses listed as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) along with various survival endpoints: overall survival (OS), disease free survival (DFS) and progression free survival (PFS). Each of these endpoints is defined by the study. The response criteria have been

standardized by various systems. The World Health Organization (WHO) developed the most commonly used system called response criteria for solid tumors (RECIST) [19]. Recent developments in assessing response during trials utilizing the drug, ipilimumab, have served to highlight differences in direct cytotoxic chemotherapy responses and those seen with immunotherapy. The responses in tumor measurement seen in the ipilimumab trials did not correspond to the survival endpoints. Subjects had prolonged survival with delayed or no tumor measurement changes. These observations have led to a new system for assessing immune based therapies called immune-related response criteria (irRC) [20].

Biomarkers can serve to assess efficacy, but it is difficult to find results that consistently correlate with clinical response. Some assays can serve as immunologic endpoints in addition to or instead of tumor regression, PFS and OS in clinical studies. The most frequent measurements include: antibody titers, delayed type hypersensitivity (DTH) skin tests, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot assay (ELISPOT), tetramer markers of antigen specific T cells and other assays indicating that the therapy induced a response toward its target [21-25].

All of these issues should be considered in assessing the past studies and in the planning of future studies. As we will discuss, progress has been made and enthusiasm is at an all-time high.

2.1. Patient selection

Predicting response to treatment is probably the best way to increase benefit from a variety of different immunotherapy agents that are currently approved or under investigation for the treatment of early stage (as adjuvant therapy) or advanced melanoma. Identifying patients that are likely to respond to these agents would spare patients unnecessary toxicities and encourage more research in the field.

Response to treatment may be determined by clinical characteristics or by the presence or level of biomarkers in the serum or tissue that predict a robust response to immunotherapy agents. Clinical characteristics such as ulcerated lesions appear to get more benefit from interferon (IFN) in the adjuvant setting [26]. In addition, Eastern Cooperative Oncology Group (ECOG) performance status, the number of involved organs and sites of metastases appear to be treatment factors predicting response to interleukin 2 (IL-2) in some studies [27].

The appearance of serum autoantibodies or clinical manifestations of autoimmunity during treatment with IFNα-2b was associated with improved outcomes in patients with melanoma [28]. In addition, multiplexed analysis of serum cytokines appeared to be potentially useful as a predictive marker of response to IFNα-2b in patients with high-risk operable melanoma [29]. A serum proteomic analysis has been found to possibly predict response to IL-2 treatment [30]. In this study, high pretreatment serum vascular endothelial growth factor (VEGF) and fibronectin levels were predictive of resistance to treatment. A marker of potential interest in ipilimumab therapy appears to be the absolute lymphocyte count [31]. Absolute lymphocyte counts that exceeded 1 x 10^9/L at the time of the third ipilimumab dose were

associated with a survival benefit at one year. Immune responses to NY-ESO-1, a cancer-testis antigen, also appear to correlate with clinical benefit from ipilimumab [32].

Gene signatures within the tumor have also shown some correlation with clinical benefit both for IL-2 and vaccination [33, 34]. Little is known about some of the newer drugs such as the CTLA-4 antagonists (ipilimumab) or other checkpoint inhibitors. It has been suggested that the presence of PD-L1 expression detected by immunohistochemistry may predict response to PD-1 antibody therapy [35].

Undoubtedly, utilizing serum and tissue biomarkers for response to treatment is much more challenging for immunotherapy than in the field of molecular targeted therapies. For example V600E BRAF mutations predict treatment response in patients who receive vemurafenib, a BRAF inhibitor. In melanoma immunotherapy, however, no serum or tissue biomarkers have yet been prospectively studied in the context of a clinical trial.

3. Immunoregulatory barriers, immune tolerance and tumor microenvironment

The immune system is designed to protect our bodies from foreign agents. This protection is selective, such that host tissues are recognized as self and preserved (termed immunologic tolerance), while other agents are recognized as foreign and targeted for killing. Cancer cells, however, present the immune system with a unique challenge. While some, such as virally transformed cells, express foreign viral proteins on their surface, most tumors express normal proteins and carbohydrates. Efforts to understand how tumors survive immunosurveillance versus how and when they are targeted for killing have preoccupied scientists for well over half a century. This section explores what we currently know about the complex interplay between the immune system and cancer cells as it relates to immunotherapy.

We know now that tumor cells are immunogenic but efficacy is limited due to the lack of robustness of the response. There are two primary reasons for this: 1) due to the nature of the immunogens (self antigens) and 2) the active role-played by tumors to suppress the response. The mammalian anti-tumor response engages both the humoral and cell-mediated arms of the immune system through both specific (adaptive) and non-specific (innate) effectors. While cytotoxic T lymphocytes (CTLs), NK cells and T helper (TH) cells are viewed as the most significant players in the anti-tumor response, they are not alone [36]. Antigen presenting cells (APCs - macrophages and dendritic cells) are absolutely essential to stimulate a variety of anti-tumor responses across tumor types, and anti-tumor antibodies are often easily found in patients with melanoma and many other solid tumors, indicating a strong humoral response following stimulation by antigen specific TH cells [37]. There is accumulating evidence that the CD4+ T cell population is far more involved in the anti-tumor response than previously thought [38, 39]. When APCs present antigen to TH cells in the context of a major histocompatibility complex (MHC) molecule on their surface, TH cells become activated and can stimulate B cells to result

in the proliferation of antibody-producing plasma cells and CTLs to result in direct killing of the tumor cells. NK cells and macrophages can also directly kill tumor cells alone or with the help of antibodies or complement.

3.1. Melanoma antigenicity

The goal of cancer immunotherapy is to provoke the immune system to generate a tumor cell rejection strength response and to prevent recurrence of cancer by establishing long-term effector cell memory. In order for the immune system to mount an attack against melanoma, it must first recognize the involved tumor cells as foreign or in need of clearing (a danger signal); it can then target them for killing. Tumor cells, like all cells, display a variety of proteins on their cell surface, and when antigen is presented in the context of MHC, the cell may be recognized by the T cell receptor (TCR) on an effector T lymphocyte. Tumor cells in general and melanoma tissues specifically, are antigenically diverse, and their ability to survive correlates with the ability of the tumor antigens to avoid detection by the immune system [40, 41]. Highly antigenic tumor cells are killed off rather quickly, due to the immune system's ability to recognize the tumor cells and mount an effective immune response, while poorly antigenic tumor cells thrive. Tumor specific transplantation antigens (TSTAs) generally convey strong immunogenicity. These are antigens expressed on the surface of tumor cells that are specific to that tumor or type of tumor. However, the majority of antigens associated with melanoma cells are tumor associated transplantation antigens, or TATAs. TATAs are antigens that are associated with tumor cells, but not unique to tumor cells. TATAs are far better at preserving a tumor cell under the radar of the immune system, because these antigens are not danger signals.

Within the tumor microenvironment, tolerance may be naturally overcome by antigen expression levels or the timing of antigen expression. Melanomas overexpress many antigens that are present in normal melanocytes but at lower levels, and expression of these antigens suggest a progression of differentiation from normal melanocytes to melanomas. For example, a melanoma expressing a mutant triosephosphate isomerase protein was discovered to bind MHC class II at five times greater affinity than the wild type oligopeptide, resulting in both a significant increase in surface expression and an increase in immunogenicity [42]. Some melanoma cells overexpress the transferrin receptor by a factor of 100 [43]. Some human melanomas overexpress the gangliosides relative to levels seen in normal melanocytes, illustrating that overexpression of carbohydrates can attract the attention of the immune system, similar to protein antigens [44].

Much of melanoma's antigenicity comes from the more than 100 identified melanoma TATAs. Melan-A/MART-1, gp100 and tyrosinase are well studied differentiation antigens expressed in both primary and metastatic melanoma [45-53].

Melanoma cells may also express oncofetal antigens which are normally displayed during embryogenesis but only expressed in select tissues, if at all, in adults. These include the cancer germ-line/cancer-testis (CT) antigens. MAGE-A family members and NY-ESO-1 are the most significant members of this group to date, and expression of MAGE-A1 and MAGE-A4 increases with tumor progression [47, 54, 55]. NY-ESO-1 is only

expressed in adults in testis and placenta tissue, however, it is expressed in up to 40% of late stage melanomas and is highly immunogenic [56]. MAGE-6 is expressed in more than 70% of metastatic melanomas [57].

Identification of melanoma TATAs is crucial as a key strategy for immunotherapy. Administration of vaccines that deliver TATAs can push the immune system into overcoming tolerance. T cells specific for TATAs have been identified in melanoma patients, and spontaneously occurring circulating T cells reactive to Melan-A and NY-ESO-1 were recently found to be predictive of better survival [58]. A recent study reported an analysis of the human leukocyte antigen 1 (HLA-I) peptidomes from melanomas in four patients, and while finding that melanoma antigenicity was highly variable, the investigators also found that the peptidomes were highly immunogenic, identifying new potential peptides for melanoma vaccines [41].

3.2. Immunoevasion strategies

Tumor cells increase their odds of survival by lowering their immunological profiles. TH cells, CTLs and antibodies specific for TATAs are readily detectable in the blood, lymph nodes and tumors of cancer patients. Despite tolerance, the immune system can mount an immune response to these antigens, but tumors and/or tumor cells may persist, so all the efforts of the immune system are not enough to clear tumor cells. The immunoevasion strategies utilized by melanomas are impressive and generally include down-regulation of TATAs on the tumor cell surface, secretion of immunosuppressive cytokines that affect APCs and shedding of material that promotes the stimulation of the inhibitory regulatory T cells (Tregs). Together, these strategies create a toleragenic tumor microenvironment that is both adaptive to immune pressures and predictive of clinical outcomes.

Many tumor cells stop displaying TATAs or TSTAs on their surface to escape immune recognition [59]. Expression of MART-1, gp100 and tyrosinase generally decreases as melanoma progresses [60]. Following immunization with gp100 or MART-1 peptides, melanoma metastases lost expression of the corresponding TATA, suggesting that TATAs can be downregulated in direct response to a specific CTL anti-tumor response [45]. This strategy is specifically adaptive to removing known CTL targets from the tumor population and selects for proliferation of tumor cells that do not bear antigens yet targeted by the CTL response.

Tumor cells may additionally repress expression of MHC class I proteins by repressing MHC I gene expression or posttranslational modifications [59]. In fact, many human tumors demonstrate a decreased expression of MHC I, and the loss of MHC I expression is often associated with more invasive and metastatic tumors [61, 62]. In melanoma, MHC I expression correlates with disease progression, and the lack of HLA I expression and lack of response to T cell based immunotherapy may be linked to acquired β2-microglobulin gene defects [63-65]. C-myc oncogene overexpression in melanoma also correlates with HLA I downregulation [66]. The one caveat of this strategy, however, is that a total lack of MHC expression invites attack by NK cells [67]. To circumvent this, tumor cells often only lower MHC I expression, retaining some minimal expression to protect themselves while not alarming the immune system.

Tumors in general create microenvironments with depressed immune activity such that few functional cytotoxic cells are found near the developing tumor. One strategy for this involves the poorly understood regulation of lymphocyte types within the tumor microenvironment. Moderate to large numbers of tumor infiltrating lymphocytes (TILs) have been associated with improved survival in melanoma patients; however this has not been observed consistently [68, 69]. While most patients with melanoma have TILs, the mere presence of TILs is obviously not sufficient to mount an effective anti-tumor response [70]. Tumor cells are able to attract a particular type of T cell that is immunosuppressive. Tregs can directly inhibit and kill CTLs and TH cells, and they functionally drive the tumor's T helper Type 2 (Th-2) immune environment by producing the immunosuppressive cytokines IL-10 and tumor growth factor beta (TGF-β) while suppressing CTL production of immunostimulatory T helper Type 1 (Th-1) cytokines interferon gamma (IFN γ) and IL-2 [71]. Tregs also negatively regulate effector dendritic cells and NK cells. In melanoma, depletion of Tregs prior to infusion with activated T lymphocytes (adoptive cell therapy) measurably improves response rates [72].

Melanoma cells may also create an immunosuppressed microenvironment through galectin expression [73]. Deregulation of galectins is common in human tumors. Expression of galectin 3 correlates with melanoma metastasis and poorer disease outcomes, perhaps through induction of TIL apoptosis. Galectin 1 may also induce apoptosis of T cells and this may be an important mechanism of tumor evasion for melanoma. Additionally, galectins 1 and 3 convey resistance to apoptosis in tumor cells, though this is less studied in melanoma.

Immunosuppression similar to that found in the tumor microenvironment can also be found in the sentinel lymph node [71, 74]. Tregs are found in higher numbers in metastatic melanoma sentinel lymph nodes, and as in the tumor microenvironment, this appears to be mediated by Th-2 cytokines IL-6, IL-8, IL-10 and TGF-β, among others. This locoregional immunosupporession is thought to be necessary for metastasis and prepares the lymphatic environment for the arrival and survival of metastastic cells [75]. While dendritic cells are detectable in melanoma sentinel lymph nodes, they may be present in lower number and/or contain a higher percentage of immature dendritic cells that lack the costimulatory molecules necessary for effective T cell activation [74, 76].

IL-10 and TGF-β are immunosuppressive cytokines utilized by melanoma to create an immunosuppressive microenvironment and progress disease toward metastasis [77, 78]. Both cytokines can induce T cells to undergo apoptosis; TGF-β can additionally induce apoptosis in dendritic cells and macrophages. Normal melanocytes are subject to TGF-β anti-proliferative regulation, and loss of this phenotype is thought to be a crucial step toward melanoma development [77]. Neutrophils from patients with melanoma constitutively and spontaneously synthesize IL-10 through activation by serum amyloid A 1 (SAA-1) which is enriched in melanoma tissue [79].

There are a variety of other general tumor microenvironment conditions and immunoevasion strategies that melanomas employ to ensure their survival. Hypoxia occurs in solid tumor masses and is well known to create an immunosuppressive tumor microenvironment [80]. Tumor cells can also alter the expression of stress proteins that bind NK cells for target-

ed killing. Melanoma cells predominantly express the MICA and ULBP2 stress proteins, and a correlation has been found between poor prognosis and expression of soluble ULBP2 that is competitive for NK cell binding [81, 82]. Heat shock proteins are well known to promote tumor growth, invasion and metastasis through a variety of mechanisms [83]. Expression of heat shock proteins 90 and 40 (hsp90 and hsp40) in melanoma tissue correlates with advanced disease and patient survival, in the case of hsp40 [84].

It is important to remember that while immune evasion and creation of an immunsuppressive tumor microenvironment is highly variable among melanomas, the ability of the tumor to effectively create a strong toleragenic microenvironment correlates with clinical outcome. Toleragenic tumor microenvironments are associated with sentinel lymph node involvement and more advanced disease [74]. Efforts to overcome this tolerance and re-capitulate the balance of immune system regulators to a state of anti-tumor effectiveness comprise the field of immunotherapy, and success in this therapeutic approach holds tremendous promise for not only halting tumor progression but for turning back the clock to ultimately result in tumor clearance.

3.3. Immunotherapy strategies

Most immunotherapy efforts strive to activate T cells and specifically CTLs. Therapeutic melanoma vaccines may enhance antigen presentation directly through peptides or DNA. A synthetic peptide vaccine targeted to the melanoma gp100 TATA, for example, has resulted in good objective clinical responses [85]. Vaccines may also rely on the assistance of dendritic cells, a key stimulator of immune cells [86-88]. The goal is to utilize TSTA or TATA antigens to provoke the development and proliferation of cytotoxic cells directed against tumor cells, thereby overcoming tolerance.

Adoptive cell transfer or therapy (ACT) is a passive immunotherapeutic approach in which a patient's antigen-specific cells are expanded and activated *ex vivo* and then reintroduced following radiation or chemotherapy [89]. TILs, autologous T cell clones, donor anti-tumor lymphocytes and genetically engineered lymphocytes have all been used in this strategy. Some encouraging results have been seen in melanoma patients with advanced disease [90]. In three separate trials, autologous TILs provided through ACT and administered with IL-2 to metastatic melanoma patients resulted in up to a 72% objective response rate, and 22% of the 93 subjects had complete tumor regression [91]. ACT employing autologous T cells targeting NY-ESO-1 resulted in objective responses in five of 11 metastatic melanoma patients and two complete regressions at one-year post-procedure [92]. While it is argued that ACT is more effective in metastatic melanoma than ipilimumab (see below), it is practically more complex to administer and less accessible for a majority of patients [90].

Cytokines have shown efficacy in high risk local and metastatic melanoma patients as well. IL-2 and IFNα-2b have been investigated the most. IL-2 alone produces a durable remission in some patients, though it is often associated with significant side effects, and better outcomes may be obtained by combining it with other therapeutic approaches [93]. A pooled analysis of nearly 2,000 stage IIB and III melanoma patients indicated that adjuvant high dose IFNα-2b prolongs relapse free survival in patients [94].

At present, the greatest promise for metastatic melanoma patients lies in immunomodulatory antibody therapy against immunological checkpoints. Immunotherapies that employ this targeting strategy are recent and have yielded some of the most promising clinical responses in decades. Immunological checkpoints are negative regulators of the immune system. Cytotoxic T lymphocyte antigen 4 (CTLA4) is found on naïve T cells and Tregs; upon activation it turns off TCR signaling and serves to stop activation of targeted T cells. Antibodies to CTLA4 prevent this from happening and prolong and intensify T cell activation [36]. Ipilimumab was approved by the U.S. Food and Drug Administration (FDA) in 2012 for the treatment of metastatic melanoma owing to the overall survival benefit observed in a phase III study that has now resulted in durable responses lasting 8 years and beyond [95]. Although ipilimumab is the only FDA-approved checkpoint inhibitor indicated for treatment of melanoma, there are others in the pipeline [96]. Tremelimumab is another CTLA4 antibody, and there are several programmed death ligand 1 (PD-1) antibodies undergoing clinical development as well [97, 98]. Though both CTLA4 and PD-1 antibodies have demonstrated significant improvement in clinical outcome for metastatic melanoma patients, they are not effective in all patients and cause a new and unique spectrum of side effects termed "immune-related adverse events."

4. Nonspecific immune therapy and adjuvants

The success of anti-tumor and antiviral vaccines often requires the use of an adjuvant, a substance that significantly enhances the immune response to a coadministered antigen. Only a handful of adjuvants have both sufficient potency and acceptable toxicity for clinical investigation. The critical roles of vaccine adjuvants lie in their ability to: (1) enable the use of otherwise impotent antigens; (2) extend the benefits of vaccination to poor responders (e.g., older or immune-compromised patients); and (3) effect dose-sparing of rare and expensive antigens in short supply (e.g., during an epidemic) [99]. Vaccine adjuvants for the most part can be evaluated as such only when they are associated with a vaccine. Early therapies were nonspecific and were thought to produce a general immune response. Many current vaccine trials utilize nonspecific immune stimulants as adjuncts.

4.1. BCG

Adjuvant therapy of melanoma assumes that treatment will be more effective when the tumor burden is small. Mycobacterium bovis bacillus Calmette-Guérin (BCG) is an old vaccine/adjuvant used in countries where tuberculosis is widespread. First used in humans in 1921, BCG is made from a strain of weakened bovine tuberculosis bacterium. The local and systemic effect of BCG has been known for decades and is an immunomodulating agent for melanoma. BCG therapy induces a massive local immune response characterized by the expression of multiple cytokines. A significant correlation between a reduced risk of melanoma and BCG and vaccinia vaccination in early childhood or infectious diseases later in life has already been reported from the FEBrile Infections and Melanoma (FEBIM) multicenter

case-control study [100]. Such observations suggest that BCG can augment immune responses and be used in adjuvant therapy strategies.

Phase II trials indicate that active specific immunotherapy can alter the natural course of American Joint Committee on Cancer [AJCC] Stage III and IV melanoma following surgical resection of nodal or distant metastases. Initial adjuvant immunotherapy trials demonstrated a greater disease-free interval in patients treated with BCG compared with historical controls [101]. In one study 149 patients at high risk of recurrence after surgical treatment of local or regional malignant melanoma were given BCG for 2 years and were followed up for a median of 28 months from the start of immunotherapy [101]. Studies such as these suggest that improved survival rates following recurrence might be explained by the pattern of recurrence; suggesting local or regional sites might be more responsive to treatment. Mechanistic studies suggest that tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is induced by BCG treatment. Subsequently, BCG and components of the mycobacterial cell wall can directly stimulate the release of soluble TRAIL through toll-like receptor-2 (TLR2) recognition that is augmented by IFN. Based on the need for a Th-1 cytokine response to BCG therapy for therapeutic results, it might be proposed that cells migrating in response to BCG treatment release TRAIL. In addition, IFN acts to augment and prolong the amount of TRAIL released by effector cells, resulting in an effective therapeutic outcome.

Early trials of BCG-based immunotherapy for melanoma consistently showed a trend toward improved clinical outcomes in patients treated with BCG compared with observation alone (reviewed by [102]). However, mature results of a phase III randomized trial of BCG versus observation and BCG plus dacarbazine versus BCG in the adjuvant therapy of AJCC stage I-III melanoma (E1673: an ECOG trial) ascribes no benefit for BCG for this patient population [103]. As early as 1976 BCG was tested as an immunotherapy systemic adjunct to surgery in malignant melanoma [104]. In 1984 a polyvalent BCG formulation along with Canvaxin began testing in phase II trials as postsurgical adjuvant therapy for stage III melanoma [105], with results from such trials summarized as no apparent benefit from vaccination [106, 107].

Although experiments with animals have demonstrated that BCG mediates anti-tumor activity, most randomized adjuvant clinical trials have failed to show significant benefit to patients with malignant melanoma. This may be because there is no accepted clinical technique for monitoring *in vivo* BCG activity. As a result the optimal route of administration and dose of BCG have not been truly determined as well as the optimal BCG strain. Attempts to improve the efficacy of BCG therapy have been made. One approach introduced the gene encoding the 65 kDa hsp of Mycobacterium tuberculosis into a mouse malignant melanoma cell line (B16) as proof of principle [108].The 65 kDa hsp was expressed after gene transduction and significantly enhanced the anti-tumor effect of BCG immunotherapy, further indicating that CD4+ T cells play an important role in this anti-tumor effect.

4.2. DETOX

Many adjuvants currently under evaluation for use in cancer vaccines activate relevant APCs, such as dendritic cells and macrophages, via TLRs and promote effective uptake,

processing and presentation of antigen to T-cells in draining lymph nodes. The Gram-negative bacterial cell constituent lipopolysaccharide (LPS) is known to possess strong immunostimulatory properties and has been evaluated as an adjuvant for promoting immune responses to minimally immunogenic antigens, including TATAs. The relatively recent discovery of TLRs and the identification of TLR4, in particular, as the signaling receptor for lipid A have allowed for a better understanding of how this immunostimulant functions with regard to induction of innate and adaptive immune responses. Local TLR stimulation is an attractive approach to induce anti-tumor immunity. Tumor cells respond to TLR ligands with an increase in MHC class I expression and induce IL-6 secretion *in vitro*. Melanoma cells are typically characterized as having low expression of MHC I. Consequently TLR ligands interacting with melanoma cells might enhance MHC class I expression, along with their targeting by melanoma specific CTLs. Although several lipid A species, including LPS and synthetic analogs, have been developed and tested as monotherapeutics for the treatment of cancer, monophosphoryl lipid A (MPL), a ligand for TLR4 has been evaluated as a cancer vaccine adjuvant in published human clinical trials. MPL comprises the lipid A portion of Salmonella minnesota LPS [109]. LPS and MPL induce similar cytokine profiles, but MPL is at least 100-fold less toxic.

DETOX, an adjuvant consisting of MPL and purified mycobacterial cell-wall skeleton (CWS) is another vaccine potentiating agent. MPL (Corixa Corp., Seattle, Washington, USA) adjuvant is a chemically modified LPS derivative that displays greatly reduced toxicity while maintaining most of the immunostimulatory activity of LPS [110] signaling through TLR4 to stimulate the innate immune system. MPL adjuvant has been used extensively in clinical trials as a component in prophylactic and therapeutic vaccines targeting infectious disease, cancer and allergies. MPL has been administered to more than 300,000 human subjects in studies of next-generation vaccines, emerging as a safe and effective vaccine adjuvant. In one study DETOX markedly potentiated antibody but had little effect on DTH responses to melanoma vaccine immunization. It did not appear to improve DFS in comparison to alum in this non-randomized study [111]. DETOX has been formulated into Melacine (Corixa Corp.), a vaccine prepared from the lysate of two melanoma cell lines adjuvanted with DETOX. In clinical trials with Melacine, tumor progression is delayed in the vaccine-treated patients, although this was only observed in patients with certain HLA phenotypes.

The potency of local TLR treatment in therapy demonstrates that local treatment with TLR adjuvants like MPL might effectively restore anti-tumor immunity. Melacine is available for sale in Canada.

4.3. QS-21

Saponins are natural glycosides of steroid or triterpene which exhibit many different biological and pharmacological activities [112]. Notably, saponins can activate the mammalian immune system, and this has led to significant interest in their potential as vaccine adjuvants. The most widely used saponin-based adjuvants are Quil A and its derivative QS-21, isolated from the bark of the Quillaja saponaria Molina (Chilean soap bark tree); these have been evaluated in numerous clinical trials [113]. Their unique capacity to

stimulate both the Th-1 immune response and the production of CTLs against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as cancer.

QS-21 possesses an ability to clinically augment significant antibody and T cell responses to vaccine antigens against a variety of infectious diseases, degenerative disorders and cancers. Currently, there exists no rapid *in vitro* biological screen for assessing the potential efficacy of saponin vaccine adjuvants, given that the mechanism by which saponins augment the immune response is unknown. As a result, evaluation of novel saponins as immunostimulants typically proceed directly to preclinical studies involving mouse vaccination with antigens [99, 112-114].

QS-21 appears to augment both Th-1 and Th-2 type responses and to favor the *in vivo* priming of antigen-specific CD8+ cytotoxic cells. QS-21 has been used in a variety of melanoma targeting vaccines [115]. QS-21 has been shown to be superior to some vaccine formulations such as GM2-KLH plus QS-21 vaccine compared to GM2/BCG vaccine [115]. Efforts to further advance QS-21 in the clinic, as well as to illuminate its unknown mechanism of action, require access to adjuvant-active samples of known composition [114]. The recent synthesis of active molecules of QS-21 has provided a robust method to produce this leading vaccine adjuvant in high purity as well as to produce novel synthetic QS-21 congeners designed to induce increased immune responsiveness and decreased toxicity [99, 112-114].

4.4. Montanide

Mineral oils are known to be very efficient adjuvants but can sometimes induce local reactions with reactive antigens. In contrast, non-mineral oils are well tolerated but less effective with poor immunogens. Mineral oils stay at the injection site and are progressively eliminated by competent cells like the macrophages. They can also be partially metabolized into fatty acids, triglycerides, phospholipids or sterols. Water in oil emulsions represent one of the new promising generations of adjuvants for immunotherapy [116-118]. In this class both Montanide ISA 51 and 720 have been tested in animals and thousands of individuals and found to be safe. Nevertheless, the proper antigen concentration has yet to be established [116-118]. Adverse effects usually depend on the concentration and nature of the antigen.

The mechanistic premise of emulsions is the “depot” effect, in which the adjuvant protects the antigen from both dilution and rapid degradation and elimination by the host. By localizing and slowly releasing intact antigen, the adjuvant permits a slow, prolonged exposure of the immune system cells to a low level of antigen. This prolonged exposure results in continued stimulation of antibody producing cells, resulting in the production of high levels of antibody by the host.

MONTANIDE™ ISA 51 VG has been used in Phase I and II clinical trials for vaccines against malaria, HIV, and various cancers. MONTANIDE™ ISA 51 VG, has been tested in AIDS and cancer vaccine trials which together represent more than 10,000 patients and around 100,000 injections. A survey of ongoing clinical trials listed in ClinicalTrials.gov revealed 36 trials currently accruing patients that are using the olive-derived Montanide ISA

51 IFA. The formulation is generally well-tolerated and induces transient local reactions. Some transient general reactions such as flu-like symptoms are also observed. The results suggest that numerous repeated vaccine doses can be safely administered. Immunization with tumor associated antigen peptides in combination with montanide expands tumor antigen-specific CD8+ T cells in melanoma patients [119-122].

4.5. Cyclophosphamide

High-dose cyclophosphamide (CY) has long been used as an anti-cancer agent, a conditioning regimen for hematopoietic stem cell transplantation and a potent immunosuppressive agent in autoimmune diseases including aplastic anemia. High-dose CY is highly toxic to lymphocytes but spares hematopoietic stem cells because of their abundant levels of aldehyde dehydrogenase, the major mechanism of CY inactivation. CY has emerged as a clinically feasible agent that can suppress Tregs and allow more effective induction of anti-tumor immune responses [123]. Tregs have become an important player in regulating anti-cancer immune responses, with poor prognoses often ascribed to their action [124].

Studies using low dose CY in combination with vaccine components and IL-12 continue to suggest that CY is a viable addition to affect immune responses [125]. Low-dose CY is found to selectively deplete CD4+CD25+ T cells (Tregs) and impede tolerance allowing for a more active immune response. CY preconditioning can enhance the CD8+ T cell response to peptide vaccination, thus leading to enhanced anti-tumor effects against pre-existing tumors [126]. CY markedly enhanced the magnitude of secondary but not primary CTL response induced by vaccines and synergized with vaccine in therapy but not in prophylaxis tumor models [127].

4.6. Conclusions

The major issues that need to be addressed are designing more effective melanoma vaccines with a mix of melanoma-associated antigens that can stimulate clinically beneficial anti-tumor immune responses and finding an adjuvant that can safely, easily and powerfully boost the frequency and magnitude of these responses.

5. Cytokine therapy

Cytokine therapy has had an important position in the treatment of melanoma in the adjuvant and metastatic settings. Various cytokines have been studied with variable success. The most important cytokines in melanoma treatment thus far have been IFN, IL-2, IL-21 and GM-CSF.

5.1. IFN

Interferon IFN is a pleotropic cytokine that exerts anti-tumor activity through numerous mechanisms. High-dose IFNα-2b was approved by the FDA in 1995 for adjuvant therapy of

resected stage IIB and III melanoma based on the results of ECOG E1684 [128]. This was a randomized controlled study of IFNα-2b administered at doses of 20 megaunits/m^2/d intravenously (IV) 5 days per week for 1 month and 10 megaunits/m^2 3 times per week subcutaneously (s.c.) for 48 weeks versus observation in 287 patients. Through this study IFNα-2b was the first agent to show a significant benefit in relapse-free survival (RFS) and OS of high-risk melanoma patients in a randomized controlled trial. A subsequent study [129] (E1690) with a total of 642 patients evaluated the efficacy of high-dose IFNα-2b for 1 year (20 megaunits/m^2/d IV 5 days/week for 4 weeks; 10 megaunits/m^2 s.c. TIW for 48 weeks) and low-dose IFNα-2b (3 megaunits/d TIW) for 2 years versus observation in high-risk (stage IIB and III) melanoma with RFS and OS as end points. The results of the intergroup E1690 trial demonstrated a RFS benefit of IFNα-2b that was dose-dependent and significant for the high-dose INFα-2b. Neither high-dose nor low dose IFNα-2b demonstrated an OS benefit compared with observation at the time.

Pooled data from E1684 and E1690 showed that RFS, but not OS, was significantly prolonged for patients treated with high dose IFN versus observation [94]. Long term OS data from E1684 also shows a diminishing level of statistical significance (P=.02 at 7 years, but P=. 09 at 12.6 years' median follow-up) [94]. Additional studies by the Eastern Organisation for Research and Treatment of Cancer (EORTC) [130] with adjuvant pegylated IFNα-2b (6 μg/kg per week for 8 weeks followed by 3 μg/kg per week for an intended duration of 5 years) showed a similarly significant and sustained effect on RFS.

The effect of IFNα on OS has been criticized strongly since only two studies (E1684 and E1694) have shown a survival benefit. In contrast, several other studies mentioned above have shown RFS as the only benefit. A recent meta-analysis [131] showed statistically significant improvement in both RFS and OS. The meta-analysis included 14 randomized controlled trials published between 1990 and 2008 and involved 8,122 patients, of which 4,362 were allocated to the IFNα arm. Subgroup analysis and meta-regression did not identify an optimal IFNα dose, the optimal treatment duration or a subset of patients more responsive than others to the adjuvant therapy. Therefore, the role of IFN in the adjuvant setting remains controversial by many. The National Comprehensive Cancer Network (NCCN) has a 2B recommendation for the use of IFN as an adjuvant treatment, and enrollment in clinical trials is encouraged.

Single-agent IFN has demonstrated modest activity in patients with metastatic malignant melanoma with response rates between 10%-20% [132]. Most of the responses were transient and usually restricted to cutaneous metastases [133]. Therefore, its use in the metastatic setting has been employed more frequently in combination with chemotherapy (biochemotherapy) with improved response rates but without a well documented survival benefit [134]. Other cytokines have been subsequently evaluated, and the interest in IFN has been shifted to the adjuvant setting as mentioned above.

5.2. IL-2

One of the most promising immune stimulating cytokines has been interleukin 2 (IL-2). High dose IL-2 produces not only PRs but also CRs. Overall objective response rates (ORR)

are approximately 16% with IL-2 with a 6% CR rate [93]. Importantly, some patients achieved durable CRs which led to the approval of high-dose IL-2 for patients with metastatic melanoma. Responses occurred with all sites of disease and in patients with large tumor burdens (unlike previously with IFN). Disease progression was not observed in any patient responding for longer than 30 months, and in some cases where disease progression was observed, durable disease free status was achieved with metastasectomy.

The use of high-dose IL-2, however, is limited by its severe toxicity; 2.2% of the patients in the National Cancer Institute (NCI) trial series died from treatment-related toxicities with bacterial sepsis being the predominant cause of death. No deaths were observed in the NCI series when antibiotic prophylaxis was implemented. However, the incidence of grade 3-4 toxicities remains high, ranging between 1-64%. Alternate regimens have been employed including low dose IL-2 alone or in combination with IFN-α or chemotherapy. However, there is evidence that suggests that high dose IL-2 is a more efficacious regimen. A phase II study showed durable CRs with high-dose bolus IL-2 in patients with metastatic melanoma who have experienced progression after biochemotherapy [135]. In addition, IL-2 based biochemotherapy regimens have not shown significantly better results than chemotherapy alone, presumptively due to the fact that high dose IL-2 is not utilized [136].

5.3. IL-21

The role of other cytokines has also been explored. IL-21 has recently emerged as a promising cytokine [137]. In an open-label, multicenter phase II study, IL-21 was given as a bolus injection on days 1 through 5 on alternate weeks using three different dosing regimens in 40 patients with malignant melanoma. Cohort 1 received 50 μg/kg per day by outpatient IV bolus injection for 5 days of each week during weeks 1, 3, and 5 of an 8-week cycle. Cohort 2 received 30 μg/kg per day on the same schedule, and cohort 3 received 50 μg/kg per day for 5 days of each week during weeks 1 and 3 of a 6-week cycle. The primary objective of the study was to assess efficacy (ORR and PFS) of IL-21 in this population. The ORR to IL-21 was 22.5%. The median PFS was 4.3 months and the median OS was 12.4 months, suggesting that this is an active agent that warrants further investigation. The 30 μg/kg per day dose and schedule was generally well tolerated as an outpatient regimen, with the most common adverse events being flu-like symptoms and rash, most of which were grade 1 or 2.

5.4. GM-CSF

Granulocyte macrophage-colony stimulating factor (GM-CSF) has also been studied mostly in the adjuvant setting. Forty-eight patients with stage III or IV melanoma were treated in a phase II trial with long-term, chronic, intermittent GM-CSF after complete surgical resection of disease [138]. The median survival duration was 37.5 months in the study patients versus 12.2 months in the matched controls. OS and DFS were significantly prolonged in patients who received GM-CSF compared with matched historical controls, and treatment was well tolerated with acceptable toxicity. A phase III prospective, randomized, placebo-controlled

study (E4697) failed to show an OS benefit but improved DFS in patients with completely resected high-risk melanoma with minimal toxicity [139].

5.5. Conclusions

Immune stimulating cytokines have historically been an important part of the therapeutic armamentarium for early stage and metastatic melanoma due to the importance of the immune system in this disease. The currently approved IFN and IL-2 treatments in the adjuvant and metastatic settings, respectively, provide modest but reproducible clinical benefits. Their use is limited by toxicity and the lack of clearly defined predictive-to-treatment tools. In the near future, the development of novel molecular and immune treatments might limit their role. However, the durable responses that we see in some patients should not be ignored, and the search for predictive biomarkers should continue.

6. Vaccine therapy

The purpose of cancer vaccines is to evoke an immune response against malignant cells. One of the earliest approaches was taken more than 100 years ago when Dr. William Coley treated patients with Coley's Toxin derived from bacteria [22]. Although clinical success in individual trials has been uncommon, a meta-analysis of 56 clinical trials showed that evidence of an immune response predicted a better outcome [140]. Vaccines are of various types, each with advantages and disadvantages. The following discussion will be divided by the vaccine type, and when available, clinical data in advanced and adjuvant settings.

6.1. Autologous whole cell vaccines

Vaccines derived from the patient's own cancer should have the advantage of presenting the complete array of tumor antigens, both internal and external. There should be less chance of the remaining tumor mutating sufficiently to avoid detection. These vaccines should be able to produce both humoral and cellular immunity [141]. Autologous vaccines are produced by irradiating resected tumors or by establishing cell lines from resected specimens [22]. This approach is difficult from a technical and regulatory standpoint. It is further hampered by limiting eligible patients to those with accessible cancer and those who can wait for the vaccine development [141]. These vaccines have used the cells themselves with adjuvants or cells modified to produce cytokines.

An autologous whole cell vaccine is exemplified in work done by Berd, et. al. Early reports demonstrated clinical response to an irradiated autologous tumor cell vaccine given with BCG as an adjuvant. Subsequently, low dose CY preceded vaccination. In that study there were 5 responses in the 40 subjects assessable for response. The responses were associated with DTH responses [142]. In the next series of studies the vaccine was modified by the hapten, dinitrophenyl (DNP), and BCG and CY were maintained. Sixty two subjects with resected nodal metastases were vaccinated and compared to historical controls. There was a perceived benefit in disease progression and survival, especially in subjects over the age of

50 years [143]. In an expansion of this initial trial to 214 subjects, there was an improvement in OS in patients who developed a positive DTH response (59.3% vs 29.3%; $p < 0.001$). Forty-seven percent of subjects had a DTH response [144].

Work done by the National Biotherapy Study Group utilizing patient specific autologous tumor cell lines in patients with melanoma has been summarized [145]. This series of studies utilized different adjuvants including BCG, IFNγ and GM-CSF. Once again, benefit was seen in groups developing a positive DTH response [145-147]. Additional non-randomized studies show positive outcomes as well, but without randomization, the results are difficult to weigh [148-151]. Some studies have genetically modified the tumor cells to secret cytokines. These trials have also been non-randomized but have shown positive results [152, 153]. A randomized trial using an autologous tumor vaccine processed to extract hsps compared to physician's choice was conducted in 322 patients with metastatic disease. There was no difference between the two groups overall, but subjects with M1a disease had longer survival when treated with vaccine [154].

6.2. Allogeneic whole cell vaccines

Allogeneic whole cell vaccines are produced by a cell line or cell lines. Other features are similar to the autologous cell vaccines. The advantage of this approach is that it is more readily available and would prevent delays in treatment that are necessitated by the autologous vaccines. However the antigens may not match those of the patient's melanoma [141].

A vaccine developed by Dr. Donald Morton beginning in 1984 from three irradiated melanoma cell lines has been studied the most extensively. It is named Canvaxin (CancerVax Corp., Carlsbad, California, USA). There were extensive phase II trials done in patients with stage IV disease demonstrating response to therapy. However, when tested in randomized multicenter trials in resected stage III and IV melanoma patients, there was no benefit noted. However, the trials were stopped prior to their planned accrual by the data safety monitoring board for futility [141, 155-157]. Other allogeneic vaccines including VACCIMEL (produced from three melanoma cell lines) have had less mature study and similar biomarker results [158].

6.3. Tumor lysate vaccines

Tumor lysate vaccines have similar advantages and disadvantages of allogeneic vaccines. The vaccinia melanoma oncolysate (VMO) vaccine is prepared from four allogeneic cell lines infected with the vaccinia virus to increase immunogenicity. The cells are then lysed by sonication prior to administration. VMO yielded encouraging phase II results, however, there was no statistically significant increase in DFS when studied in an adjuvant setting [157, 159].

A second tumor lysate vaccine, called Melacine (Corixa Corp., Seattle Washington, USA) is also a cell lysate vaccine which has been tested in two large randomized trials. One adjuvant trial conducted by the Southwest Oncology Group (SWOG) studied 600 eligible patients treated with Melacine along with the adjuvant DETOX versus observation. To be

eligible the subjects had to have intermediate thickness lesions and negative nodes; however, sentinel lymph node biopsy was not required, and therefore, the staging in this trial would be considered inadequate by today's standards. Survival in the overall analysis showed no difference between treatment and observation, however, a subgroup of patients expressing certain MHC classes showed a five-year survival rate of 83% compared to 59% in the observation group. This subset analysis was statistically significant [160-163]. An Ad Hoc Melanoma Working Group reported a separate study in stage III resected patients comparing high dose IFN for one year versus low lose IFN plus Melacine with DETOX for 2 years. Six hundred subjects were registered. There was no difference in outcome [164]. As described above, high dose IFN had been shown previously to be superior to observation.

6.4. Protein vaccines

Protein vaccines using purified proteins have the potential for a broader spectrum of antigens, but they can be more complex to manufacture and monitor for response [157]. The use of hsps, which have a normal function in chaperoning proteins as they are processed into peptides, has also been explored with peptide vaccines [154] and may have a role in identifying new antigenic targets [165]. A trial using NY-ESO was found to produce a strong immunologic response after vaccination. There were better clinical outcomes compared to placebo in those given the protein vaccine compared to placebo. The adjuvant used in the trial was ISCOMATRIX [166].

6.5. Ganglioside antigen vaccines

Gangliosides are non-protein antigens (glycosphingolipids containing sialic acids) that have been shown to elicit antibodies. They are present on melanoma cells (GM2, GD2, GD3) [157, 167]. The GM2 antigen plus BCG versus BCG alone was studied in stage III resected patients. There was no difference in DFS, but subjects with IgM antibodies against the antigen had better outcome [167]. GM2 conjugated to a keyhole limpet hemocyanin (KLH) and administered with the adjuvant QS-21 had better immunogenicity [168]. This same vaccine was compared to high dose IFN in an Intergroup adjuvant trial in patients with resected stage IIB or III melanoma. The trial ended when an interim analysis showed therapeutic inferiority in the vaccine arm [169].

6.6. Peptide vaccines

Peptide vaccines have the advantage of being easy to manufacture and have an excellent safety record. However, there are challenges that impact their effectiveness. These include the identification of epitopes that stimulate a T cell response, selecting an appropriate adjuvant, breaking tolerance without causing limiting autoimmunity, handling MHC restriction and assessing the need for multi-epitope vaccines [170]. Peptide vaccines have been reported to increase survival following resection of metastatic lesions [171] and have been shown to have increased immune efficacy with various adjuvants [172, 173]. Use of multiple peptides has been another strategy [121, 174, 175]. An ECOG study test-

ing a multi-epitope vaccine with GM-CSF and/or IFNα-2b showed that an immune response to the vaccine correlated with outcome but that the cytokines did not affect outcome [176].

One of the most studied peptide vaccines is a modified gp100 peptide antigen. This vaccine has been studied in locally advanced stage III or stage IV patients comparing IL-2 to IL-2 plus vaccine. The results showed a statistically significant improvement in response rate and PFS in the vaccine arm [177]. This is in contrast to a report from the Cytokine Working Group analysis of three phase II trials looking at a similar vaccine with IL-2 showing no benefit to the addition of the vaccine [178]. Another phase III study comparing ipilimumab plus gp100 vaccine versus gp100 plus placebo versus ipilimumab plus placebo failed to show a benefit to the addition of the vaccine, and the vaccine alone was inferior [95].

6.7. Monoclonal antibodies

Anti-idiotype vaccines consist of monoclonal antibodies that mimic an antigen. The theoretical hypothesis is sound [179-181], but trials have been limited [182-184]. These vaccines have not been tested prospectively.

6.8. Viral vaccines

Viral vectors can boost the immunogenicity of the vaccines they carry [141, 185]. However the presence of neutralizing antibodies in the host could play a role [186]. Novel methods of utilizing this mode of immune stimulation are still being explored [187, 188]. A randomized trial in stage III resected patients utilizing a vaccinia viral lysate vaccine failed to show benefit [189]. Transduction of cell lines to produce expression of B7-1and IL-2 have been accomplished and show promising immunostimulatory effects [190, 191]. These techniques are difficult to pursue from a technical and regulatory standpoint. Recombinant viral vaccines have also been used to prime dendritic cells [192].

6.9. DNA vaccines

DNA vaccines have the advantage of specificity for the target for which they encode, which can simplify monitoring, but in general they have not done well in breaking tolerance [141, 193-195]. Two groups have reported on vaccines that rely on production of GM-CSF. Dranoff has reported on a vaccine utilizing melanoma cell lines engineered to produce GM-CSF and has noted improved anti-tumor effects [196]. A different approach using an intralesional vaccination with an oncolytic herpesvirus encoding GM-CSF has been developed and has had initial positive results [197, 198]. Another agent called Allovectin-7 consists of a plasmid containing DNA encoding for the MHC class I gene, HLA-B7. It was administered by intralesional injection. Early studies showed evidence of biologic activity [199]. Subsequent phase II studies showed efficacy locally as well as systemically [200-203]. A phase III study has not yet been reported.

7. Cellular therapy

The transfer of immunologically competent white blood cells or their precursors into the host (cellular adoptive immunotherapy, adoptive cell treatment, adoptive cell therapy [ACT]) has been studied extensively in patients with melanoma over the last 30 years. Since it was thought that the effect of IL-2 is potentiated by this form of therapy, various studies examined the role of combination regimens with lymphokine-activated killer cells (LAK cells) or TILs with or without lymphodepletion with mixed results.

7.1. TILs

Earlier studies with LAK cells [204] showed promise, improving the responses with IL-2. A randomized study [205] with IL-2 and LAK cells compared to IL-2 alone failed to show significant improvement in survival which tempered the initial enthusiasm. Subsequent studies with Tumor Infiltrating Lymphocytes (TILs) [206] showed some response to treatment (overall ORR in these patients was 34%) when combined with IL-2. Interestingly, there was no significant difference in the ORR in patients whose therapy with high-dose IL-2 had failed (32%) compared with patients not previously treated with IL-2 (34%). However, the responses appeared to be short-lived, probably due to the transient persistence of the transferred TILs [207].

The addition of lymphodepletion has been thought to promote the persistence of the transferred TILs by eliminating the regulatory cells. Pooled data from three clinical trials employing three different lymphodepleting regimens [91] showed high responses between 49-72%. Ninety-five percent of these patients had progressive disease following a prior systemic treatment. Twenty of the 93 patients (22%) achieved complete tumor regression, and 19 have ongoing complete regressions beyond 3 years. The actuarial 3- and 5-year survival rates for the entire group were 36% and 29%, respectively, but for the 20 complete responders were 100% and 93%. Factors associated with objective response included longer telomeres of the infused cells, the number of CD8+ CD27+ cells infused and the persistence of the infused cells in the circulation at one month. This treatment appears to also be helpful in the treatment of intracranial disease [208].

At this point, there is reserved enthusiasm about the role of TILs with lymphodepletion regimens. There are several programs within the United States [209] outside of the NCI, where this work was pioneered, and internationally more groups are starting to employ similar strategies.

7.2. Dendritic cells

Another form of ACT is the infusion of dendritic cells. There is a lot of interest in developing dendritic cell based immunotherapy strategies since the approval of sipuleucel-T, an autologous dendritic cell based immunotherapy in hormone refractory prostate cancer. Dendritic cells are believed to induce a Th-1 response which activates CTLs through processing and presenting of peptides derived from the tumor protein antigens. In a

study that evaluated the role of dendritic cells pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin, 6 of 11 patients with advanced stage IV melanoma experienced significant regression of their metastases [210]. Resolution of skin metastases in two of the patients was accompanied by $CD8^+$ T cell infiltration, whereas nonregressing lesions lacked $CD8^+$ T cells.

In another trial [211] 16 patients with metastatic stage IV melanoma were treated with dendritic cells derived from incubation of peripheral blood mononuclear cells with IL-4 and GM-CSF and overnight pulsing with several peptides (tyrosinase, gp100 and MART-1). One patient had a complete remission of lung and pleural disease after two cycles of therapy. Two additional patients had SD, and two patients had mixed responses. In general, reviewing over 30 studies that employed dendritic cell-based treatments [212], it appears that clinical response (defined as CR, PR or SD) was significantly correlated with the use of peptide antigens, use of helper antigen or adjuvant and induction of tumor antigen specific T cells.

Although there appears to be a real effect of these treatments on tumor response in a subset of the treated patients, undoubtedly the success has not been universal and convincing [213]. This could be due to Tregs that counteract the effect of dendritic cells. In addition, melanoma can also mediate dendritic cell suppression possibly through the activation of the MEK1/2-p44/42 axis [214]. Finally, little is known about optimal dendritic cell generation, administration and immune monitoring which could hamper progress in this field.

8. Enhancement of cellular immunity

8.1. Checkpoint inhibitors

Monoclonal antibodies targeted against a number of regulatory immune system checkpoints are being evaluated in patients with advanced melanoma. The recently approved ipilimumab remains the prototype, but others are currently being evaluated in several trials.

8.2. Ipilimumab

Ipilimumab is a monoclonal antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4). In two phase III trials ipilimumab showed improved OS in patients with advanced melanoma. In the first one [95], 676 HLA-A*0201–positive patients with unresectable stage III or IV melanoma, whose disease had progressed while they were receiving therapy for metastatic disease were studied. More than 70% of the patients had M1c disease (presence of visceral metastases), and more than 36% had elevated lactate dehydrogenase levels. The patients were randomly assigned, in a 3:1:1 ratio, to receive ipilimumab plus gp100, ipilimumab alone or gp100 alone. Ipilimumab, at a dose of 3 mg/kg of body weight, was administered with or without gp100 every three weeks for up to four treatments. HLA-A*0201–positivity was required because of the use of the gp100 vaccine. Certain patients were allowed to have another course of treatment upon progression. The primary end point was OS.

The median OS was 10.0 months among patients receiving ipilimumab plus gp100, as compared with 6.4 months among patients receiving gp100 alone (hazard ratio for death, 0.68; P<0.001). The median OS with ipilimumab alone was 10.1 months (hazard ratio for death in comparison with gp100 alone, 0.66; P=0.003). No difference in OS was detected between the ipilimumab groups (hazard ratio with ipilimumab plus gp100, 1.04; P=0.76). The best OR or SD was seen in the ipilimumab-alone group (10.9%) and a disease control rate (the proportion of patients with a PR, CR or SD) of 28.5%. In the ipilimumab-alone group, 60.0% maintained an OR for at least two years. Responses to ipilimumab continued to improve beyond week 24: in the ipilimumab-alone group, two patients with SD improved to a PR, and three with a PR improved to a CR. Interestingly, among 31 patients given reinduction therapy with ipilimumab, a PR, CR or SD was achieved by 21 weeks. Grade 3 or 4 immune-related adverse events occurred in 10 to 15% of patients treated with ipilimumab and in 3% treated with gp100 alone. There were 14 deaths related to the study drugs (2.1%), and seven were associated with immune-related adverse events.

In the second phase III study [215], 502 patients with previously untreated metastatic melanoma were assigned in a 1:1 ratio to receive 10 mg/kg ipilimumab plus 850 mg/m^2 dacarbazine or 850 mg/m^2 dacarbazine plus placebo, given at weeks 1, 4, 7 and 10, followed by 850 mg/m^2 dacarbazine alone every three weeks through week 22. Patients with SD or an OR and no dose-limiting toxic effects received ipilimumab or placebo every 12 weeks thereafter as maintenance therapy. Similarly with the previous study a significant number of patients had poor prognosis based on the presence of visceral metastases and increased lactate dehydrogenase. The primary end point was OS.

OS was significantly longer in the group receiving ipilimumab plus dacarbazine than in the group receiving dacarbazine plus placebo (11.2 months vs. 9.1 months), with higher survival rates in the ipilimumab–dacarbazine group at one year (47.3% vs. 36.3%), two years (28.5% vs. 17.9%), and three years (20.8% vs. 12.2%) (hazard ratio for death, 0.72; P<0.001). The rate of disease control (PR, CR or SD) did not differ significantly between the two groups: 33.2% in the ipilimumab–dacarbazine group and 30.2% in the dacarbazine group (P=0.41). The rate of best OR (PR or CR) was 15.2% in the ipilimumab–dacarbazine group and 10.3% in the dacarbazine group (P=0.09). However, the median duration of response among all patients with a PR or CR was 19.3 months (95% CI, 12.1 to 26.1) in the ipilimumab–dacarbazine group and 8.1 months (95% CI, 5.19 to 19.8) in the dacarbazine group (P=0.03). In addition, some patients in the study who were receiving ipilimumab had an improvement from PR to CR after six months. Grade 3 or 4 adverse events occurred in 56.3% of patients treated with ipilimumab plus dacarbazine, as compared with 27.5% treated with dacarbazine and placebo (P<0.001). No drug-related deaths or gastrointestinal perforations occurred in the ipilimumab–dacarbazine group.

Patients with untreated brain metastases were excluded from both phase III studies. However, phase II data indicate that ipilimumab has activity in patients with brain metastases [216]. The currently approved dose is 3 mg/kg based on the registration trial [95], however other doses and schedules [217] have been used that do not appear to produce significantly different results but appear to increase toxicity.

The experience with ipilimumab has shown that a subgroup of patients may experience a late response and more interestingly, some patients exhibit apparent disease progression after 12 weeks of ipilimumab followed by subsequent disease regression [218]. Therefore, traditional criteria (e.g. RECIST) may not apply in the evaluation of patients who receive ipilimumab or similar treatments, and different criteria may need to be established in the interpretation of efficacy data in clinical trials [20].

Based on the favorable results from the ipilimumab studies, other anti-CTLA4 antibodies are currently being evaluated such as tremelimumab. Tremelimumab has a longer half-life than ipilimumab and is dosed less frequently. Phase II data showed results similar to ipilimumab with durable responses suggesting a potential role for tremelimumab in melanoma [219]. However, a phase III trial comparing tremelimumab and chemotherapy failed to demonstrate an improvement in OS [220].

8.3. Toxicity with ipilimumab

The toxicity of ipilimumab appears to be related to the increased activation of the immune system. A variety of immune mediated adverse events have been observed. Some of them are life-threatening and the most common are enterocolitis, hepatitis, dermatitis and endocrinopathies, but others such as neurologic complications, ocular symptoms, hematologic manifestations, vasculitis, et al are also observed.The prompt administration of corticosteroids is paramount when these are observed, and in some cases treatment interruption or permanent discontinuation is required. A relationship between the development of side effects and anti-tumor activity has been proposed by several investigators [218].

8.4. PD-1

Another regulatory checkpoint is Programmed Death-1 receptor (PD-1). Its inhibition is currently being evaluated in clinical trials. The PD-1 and PD ligand-1 (PD-L1) interaction is believed to affect T cell anti-tumor immunity. Many tumors express high levels of PD-L1. When PD-L1 interacts with the PD-1 receptor on T cells, T cell function is impaired through a variety of mechanisms including induction of apoptosis, suppression of proliferation and inhibition of T cell cytokine production [96]. There are several ways to target PD-1; one way is targeting the PD-1 receptor and another is targeting the PD-1 ligand. There are several molecules currently under investigation that target the PD-1 receptor directly (BMS-936558, CT-011, MK-3475). In a recent study [98], BMS-936558 showed significant anti-tumor activity in a variety of solid tumors. In melanoma patients response rates were 28% and appeared to be durable. Interestingly, of 17 patients with PD-L1–negative tumors, none had an OR while 9 of 25 patients (36%) with PD-L1–positive tumors had an OR (P=0.006). CT-011 has demonstrated favorable results in hematologic malignancies but has not been well studied in melanoma patients yet [221], although there is an ongoing phase I study being conducted at present. The role of MK-3475 in melanoma is also currently being evaluated in a phase I trial. Another molecule which is not probably directly targeting the PD-1 is AMP-224; it is a fusion protein of B7-DC and an antibody Fc portion. It is not a monoclonal antibody like the three checkpoint agents mentioned previously, and no data has been reported yet regarding

its efficacy in human trials. PD-L1 monoclonal antibodies have emerged as another strategy to affect the PD-1/PD L-1 pathway. A multi-center phase I study evaluating the role of BMS-936559 in patients with advanced cancers including melanoma showed a 17% response rate in melanoma patients with some of those being durable responses [97].

8.5. Co-stimulatory agonists

4-1BB or CD137 is a member of the TNF receptor (TNFR) family and provides a costimulatory signal important to the effective generation of many types of T cell responses. A completed phase I study in melanoma with BMS-663513, a fully human anti-CD137 agonist monoclonal antibody, showed that this agent was well tolerated and three PRs were seen [222].

OX-40 is another member of the TNFR family. An agonist molecule is also under investigation, but mature data are not yet available [223].

8.6. Conclusions

Checkpoint inhibitors and co-stimulatory agonists are improving anti-tumor cellular immunity. In a similar way to the more non-specific activation through cytokines, responses are durable but are not seen in all patients. This brings up issues such as appropriate patient selection, biomarker development and optimization of the dose, frequency and administration in order to optimize efficacy.

9. Combination approaches

Combination approaches have been traditionally used in the treatment of melanoma (for example different cytokines together or cytokines with chemotherapy). It is quite interesting that the results of this strategy have not been yet as successful as expected. While new agents are developed and we understand more about their function, these strategies may become more successful. At this point there is a lot of interest combining checkpoint inhibitors with other checkpoint blocking agents, co-stimulatory agonists, chemotherapy, targeted agents (such as B-type Raf kinas [BRAF] inhibitors) and radiotherapy. Interestingly, the BRAF inhibitors appear to improve T cell recognition of melanoma [224] which reinforces a rational combination of targeted therapy and immunotherapy. The results are not yet mature and studies are ongoing, but there is preclinical and retrospective data that support this model of treatment [224-227]. The following combination strategies have shown some recent benefit and promise.

9.1. Anti-CTLA4 and IFNa

A phase II study [227] combining tremelimumab and high dose IFN in patients with advanced melanoma showed an ORR of 24% with 4 patients obtaining a CR. Toxicity was acceptable and the median OS was 21 months. The University of Pittsburgh group is now

contemplating a neoadjuvant and adjuvant therapy that employs an anti-CTLA4 and IFN-α combination strategy.

9.2. Anti-CTLA4 and GM-CSF

A phase II study through ECOG combining ipilimumab and GM-CSF is currently ongoing. Previous studies [228] with periodic infusions of anti-CTLA-4 antibodies after vaccination with irradiated, autologous tumor cells engineered to secrete GM-CSF have yielded favorable results with acceptable toxicity.

9.3. IL-2 and gp-100

A phase III trial evaluated the combination of IL-2 and the gp100 peptide vaccine in advanced melanoma. This combination therapy increased the response rate (16% vs. 6%) with more CRs in the combination arm and a trend toward increased OS [177]. Interestingly, the single arm responses were lower than expected. A similarly designed study employing ipilimumab did not show significant benefit in the combination arm [95].

10. Conclusion

Immunotherapy holds the promise of "the cure" for cancer. Glimpses of this outcome have been seen throughout the past century but may be best exemplified in melanoma therapy. Although attempts in the past have not had satisfactory results, the knowledge gained along with the ability to develop biologically active drugs is bearing fruit in the current generation of clinical trials. The few positive results have kept the interest for cancer immunotherapy alive which has also helped us to achieve a better understanding of the immune system in relationship to cancer treatment. The improvement in outcome seen with gp100 plus IL-2 is impressive, and the power of the anti-cancer and autoimmune toxicities seen with ipilimumab is dramatic. The excitement stems not only from the clinical results that were obtained but also by our ability to successfully manipulate the complex immune system in a different way than before. Undoubtedly, there are a lot of unanswered questions including why are there still a large number of patients who do not achieve responses with immunotherapy and eventually die from advanced disease? However, unlike immunotherapy applied to other advanced setting solid tumors, immunotherapy in advanced melanoma has resulted in some durable responses and possibly cures.

The future of immunotherapy in melanoma and other tumor types would ideally involve research in a broad range of directions. An optimization of the IL-2 based treatments is needed to improve the number of durable responses. Results from the current work at NCI augmenting IL-2 treatment with lymphodepletion are quite encouraging. Identification of serum or tissue biomarkers is also urgently needed. Biomarker research is often complex, but this work should reveal a better understanding of the tumor itself as well as the host. The checkpoint inhibitors such as ipilimumab and tremelimumab have opened the door for research into other immunologic checkpoints such as PD-1 and co-stimulatory signals. The

need for a reliable melanoma biomarker is again paramount, and the significance of PD-L1 expression that is currently under investigation is anticipated with interest. Studying mechanisms of resistance in all cancer immunotherapeutics is equally important.

The combination of different immunotherapeutics with each other, with molecularly targeted agents or with conventional treatments such as chemotherapy or radiation therapy, may not be the simple process that combination strategies with vaccines were in the past, but this approach is quite attractive and rational and will help us further understand the role of the immune system in this disease. In a similar fashion, sequential therapy, using different agents at different times might improve clinical response and survival. Appropriate first line selection is also quite challenging since there are currently three approved agents in BRAF V600E mutated patients (high does IL-2, ipilimumab and vemurafenib) and two for BRAF wild type patients (high dose IL-2 and ipilimumab). A randomized clinical trial of ipilimumab followed by vemurafenib versus vemurafenib followed by ipilimumab is planned through the NCI Cooperative Group mechanisms [218]. Future clinical trials will hopefully provide some answers to these questions. In the design of clinical trials, the importance of refining the RECIST criteria for immunotherapy agents cannot be overemphasized. Overall, this is an exciting time for cancer immunologists and clinicians who treat patients with melanoma. The future for innovative trials of new agents and combinations is brighter than ever before.

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