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# Melanoma Vaccines: Clinical Status and Immune Endpoints

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## Introduction

It has been known for decades that the immune system can be spontaneously activated against melanoma. The presence of tumor infiltrating lymphocytes (TIL) in tumor deposits is a positive prognostic factor [1]. Cancer vaccination includes approaches to generate, amplify, or skew antitumor immunity. To accomplish this goal, tested approaches involve administration of tumor antigens, antigen presenting cells (APCs) or other immune modulators, or direct modulation of the tumor. Because the success of checkpoint blockade can depend in part on an existing antitumor response, cancer vaccination may play an important role in future combination therapies. In this review, we discuss a variety of melanoma vaccine approaches and methods to determine the biological impact of vaccination.

### Historical perspective

Active immunotherapies are not a novel concept. Over a century ago, William B. Coley injected live streptococci into sarcoma patients to promote erysipelas and induce immune system-mediated tumor rejection. While therapies such as these have shown limited clinical benefit and high degree of systemic toxicity, they did usher in an era of cancer immunotherapy [2]. Some of the first melanoma cancer vaccine trials occurred in the 1970s. These included testing tumor lysate injections and pathogen adjuvants like Bacillus Calmette-Guerin (BCG) [3,4]; vaccinia virus oncolysate [5] or Corynebacterium parvum [6]. The results were promising, with some patients displaying clinical responses that were often transient. In the 1980s, there were studies focused on allogeneic melanoma cells [7], and in the early 1990s, gangloside (GM2, GD3)-based vaccines with adjuvants and immunogenic conjugates [8]. Cytokines were also tested at this time, both as systemically delivered agents (IL-2, IFNa) or as cytokine-secreting genetically modified tumor cell vaccines (especially GM-CSF) [9,10]. During the 1990s, the identification of the tumor antigens expressed by melanoma tumors which were recognized by the cytotoxic T cells (CTL) infiltrating tumors were beginning to be published which had a major impact on the field [11-13].

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The most common approach to cancer vaccination in the last two decades involves immunization with shared tumor antigens expressed by many different patients' tumors. The earliest tumor associated antigens (TAAs) identified were proteins that were overexpressed in tumor cells but minimally expressed in untransformed normal tissues [11,12,14,15]. TAAs were also identified after cloning the genes that encoded proteins that included epitopes recognized by tumor reactive TILs. Other types of TAA tested in melanoma include cancer testis antigens and mutated antigens (tumor specific or private antigens).

## Melanoma antigens

Melanoma antigens can be segregated into four categories: overexpressed antigens, cancer testis antigens, mutated oncogenes, and patient-specific mutated neoantigens [16]. Overexpressed antigens include melanoma lineage antigens such as MART-1/Melan-A, tyrosinase, and gp100 (expressed in >90% of melanoma tumors [17]). All have shown efficacy as targets in vitro and in murine models, and have been tested in clinical trials with some objective tumors) clinical responses [18-20]. A potential explanation for the limited clinical activity seen when targeting such TAAs is that the highest avidity T cells specific to these normal "self" antigens may have been deleted or exhausted by chronic antigen stimulation, leaving only less effective, lower avidity T cells to be activated.

Cancer testis antigens are expressed in a proportion of most tumor tissue types and in germ cells that, because of their physiologic location, are generally ignored by the immune system. Such antigens include the large MAGE-A (expressed in 9-51% of melanomas [17]), MAGE-B, and MAGE-C families, and NY-ESO-1 (expressed in 45%of malanomas [17]). These antigens have been tested in human clinical trials and implicated in therapeutic responses [21].

Mutated oncogenes have been known for decades, and commonly occurring shared mutations in the RAS family of oncogenes (NRAS is mutated in 15-25% of melanomas [22-23]) have been identified. However, earlier it was thought unlikely that such mutations would be present in a processed, presented, and immunogenic MHC restricted epitope in a characterized MHC class I or II molecules, making shared mutations seemingly impossible to target by vaccination in a clinical trial [24]. Neoantigens are those antigens that arise from random somatic mutations in individual tumors [25]. This group of antigens will be discussed in greater detail below.

## Cancer vaccine platforms

Most cancer vaccines are designed to activate tumor specific CD8+ CTL because studies in mice reproducibly support the key therapeutic role played by these cells. The most common vaccination strategies used have been based on MHC class I restricted peptide epitopes from TAAs. These have been delivered in a variety of adjuvant formulations (including cytokines and toll-like receptor (TLR) ligands) to promote in vivo presentation by endogenous APC. Peptide based vaccines take advantage of the existing data on MHC class I peptide binding motifs for the most common HLA types, and the algorithms which can screen protein amino acid sequences for peptide epitopes derived from TAAs. Data from animal models support the potential for such vaccines to have a substantial therapeutic effect [26-28].

## Peptide-based strategies

Peptides formulated in adjuvants (such as Montanide, which is analogous to incomplete Freund's adjuvant (IFA)), with or without cytokines, such as GM-CSF and interferon. (IFN.), or TLR agonists, have shown clinical benefit (partial responses, complete responses, and durable disease stabilization) in small and large scale clinical trials [29-32]. In smaller trials, peptides loaded onto APCs, such as dendritic cells, have also resulted in positive immune and clinical effects [33-35].

As peptide-based vaccines are tested, optimal adjuvants and formulations of these vaccines are still being identified. Clinical trials of peptide-based vaccines were recently reviewed [36]. A benefit of peptide-based approaches is that 9 to 10 amino acid long peptides are simple and inexpensive to manufacture. Large scale manufacture is possible and the peptides are stable when stored and shipped. While enthusiasm for peptide vaccines remains high due to their immunogenicity, data demonstrating clinical efficacy of vaccine peptide-specific T cells remains rare.

Because of HLA restriction, those who do not express common HLA types cannot be treated with this type of vaccine. In addition, the usual MHC class I binding short peptides do not activate CD4+ helper T cells, which may limit the functionality of CD8+ cytotoxic T cells. This problem has been overcome by the addition of non-tumor specific help (inclusion of keyhole limpet hemocyanin (KLH), tetanus, or (pan-DR binding synthetic helper (PADRE) peptides), although data are limited over the nature of the "help" provided by heterologous helper peptides. Shared melanoma antigen helper peptides have been tested in several trials, with data showing improved survival in vaccinated patients [16].

Another strategy that has shown significant clinical efficacy in the setting of cervical cancer is the use of synthetic peptides that are long enough to include multiple MHC class I and II epitopes [38,39]. These 23-45 amino acid long peptides, delivered subcutaneously, have been shown to be especially effective, possibly because they utilize a more efficient processing and presentation pathway, which leads to superior T cell activation [40]. We have observed in vitro that a 16 a.a. long MAGE-A6-derived peptide, MAGE-A6-172-187, that has been reported to be promiscuously presented by multiple HLA-DR alleles, can also induce HLA-A2-restricted CD8+ T cell responses against the MAGE-A6-176-185 epitope [16,41]. These results indicate that long peptides derived from melanoma antigens could also be implemented in future melanoma vaccine modalities.

Multiple peptides can be given at the same time, targeting several T cell clones and antigens at once [42-44]. A trial combining pre-vaccine cyclophosphamide with multiple peptides and GM-CSF showed that improved survival was associated with antigenic breadth of response and reduced suppressive circulating regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) [44]. The Slingluff group has previously generated a cocktail of 6 MHC class II-restricted peptides (6MHP; derived from MAGE, MART-1, gp100, and tyrosinase (TAAs)) which has been tested in two phase I/II trials. Survival outcomes of patients treated with the 6MHP vaccine are superior to those of matched institutional controls [46,47]. Currently, the vaccine is being tested in a new phase I/II study in combination with ipilimumab (NCT02385669).

To enhance the efficacy of cancer vaccines, “wild type” tumor antigen-derived epitopes can be modified in order to activate cross-reactive T-cell clones, resulting in activation of higher avidity T cell clones capable of superior tumor recognition and killing. One or two amino acid substitutions, normally within the predicted epitope anchor residues, have been shown to lead to higher binding affinity that resulted in induction of higher avidity, tumor-specific T cells [47,48]. The design of heteroclitic peptides can be difficult and labor-intensive. Recently, Cristian Capasso et al. have developed The Epitope Discovery and Improvement System (EDIS), an automated algorithm-driven platform to speed up the design of heteroclitic peptides [49].

Another potential strategy to enhance tumor antigen-derived epitope immunogenicity is to utilize highly homologous and cross-reactive “mimicking” peptides derived from proteins found in common microbial pathogens to which many individuals have pre-existing immunity. We have shown that a peptide derived from the Mycoplasma penetrans HF-2 permease protein, HF-2-216-229, shares a high degree of structural and functional homology with the aforementioned MAGE-A6-172-187 epitope. The functional avidities of CD4+ and CD8+ T cells primed with HF-2-216-229 are 100 and 1000x greater than those of CD4+ and CD8+ T cells primed with MAGE-A6172-187, respectively. Consequently, HF-2-216-229-stimulated T cells are superior at recognizing APCs pulsed with MAGE-A6-172-187 or recombinant MAGE-A6, as well as HLA-matched MAGE-A6+ melanoma cell lines [16,41].

## APC based strategies

Many types of APCs have been investigated, including peripheral blood mononuclear cells (PBMC), activated B cells, and, more commonly, dendritic cells (DC). DC are a heterogeneous population of APCs that can efficiently take up antigens and sample their environment. They then process and present these antigens to CD4+ and CD8+ T cells and incorporate immune response modulating cues (including the secretion of cytokines such as interleukin 12 (IL-12) p70, which skews the immune response towards type 1 to modulate the type of response. A type 1 response involves IFN., IL-2, and tumor necrosis factor (TNF) and it promotes the activation of cytotoxic CD8+ T cells. Several recent reviews summarize the history, biology, and clinical application of these cells [50-52].

Clinical trials of autologous DC vaccines involve individualized patient vaccination approaches and single clinical trial arms. It is difficult to compare trials and draw firm conclusions about the efficacy or different approaches [53-55]. Natural CD1c+ DC as well as DC generated from monocytes (Figure 1) [56-58] and CD34+ progenitor cells have been tested with various antigen formats, including complex tumor lysates that contain normal, TAA and tumor specific antigens, or synthetic MHC class I restricted peptides. Vaccines have been injected into the blood, skin (subcutaneously or intradermally), and lymph nodes. The early lessons learned were that DC vaccines are safe, feasible and immunogenic and can promote clinically significant tumor regression in 4.2-7.1% of patients [35,59-65].

One therapeutic cancer vaccine—Sipuleucel-T manufactured by Dendreon—was approved by the Food and Drug Administration in 2010. It consists of autologous APC loaded with the TAA prostatic acid phosphatase plus GM-CSF and is approved for metastatic prostate

cancer on the basis of a 4.1 month improvement in overall survival seen in data from large scale phase III clinical trials [66].

## Tumor based strategies

Early cancer vaccine studies found that mice could be immunized with tumor cells that were killed and engineered to express immune stimulatory cytokines [67,68] including GM-CSF. The data supporting syngeneic, autologous, or allogeneic tumor cells transfected to express high amounts of GM-CSF supported clinical testing, with some immune and clinical responses [69-73]. Other strategies that use the personalized approach of harnessing autologous tumor antigens include using tumor lysates to load APCs ex vivo and fusion of tumor cells and autologous APCs. Immunity to undefined tumor lysates and foreign helper proteins has been demonstrated in some cases [74-76]. Autologous tumor cells can also be used to load APCs (autologous or derived from allogeneic cell lines) with tumor genomic DNA [77]. This allows uncharacterized mutated gene products specific to the tumor to be processed and presented for immune activation (Figure 1).

## Oncolytic virus-based strategies

As mentioned above, the inclusion of pathogens in cancer vaccines can greatly increase immune stimulation in the context of presenting tumor antigens. Pathogens have complex arrays of molecules that can trigger multiple immune activation pathways. Oncolytic viruses have two anti-tumor mechanisms. First, they can infect and replicate inside the tumor cell, which directly mediates tumor lysis and further release of new viral particles into the tumor surrounding, which leads to additive tumor destruction. Second, tumor lysis leads to the release of tumor antigens, danger-associated molecular patterns, cytokines and chemokines into the tumor microenvironment, which leads to induction and/or enhancement of anti-tumor adaptive immunity [78].

Herpesviruses have been used as oncolytic viruses for cancer vaccination. A promising strategy has tested GM-CSF as an adjuvant or APC growth factor engineered into replicating herpesvirus vectors. One such vector, T-VEC (Talimogene Laherparepvec), was successfully tested in patients with melanoma in a randomized phase III trial [79]. The trial found an objective response rate of 26% and a complete clinical response in 11% of patients with stage IIIB-IV melanoma [80]. This virus was FDA approved in 2015 for these unresectable patients and has been shown to result in increased tumor PD-L1 expression and CD8+ T cell infiltration [81].

## Neoantigen-based Cancer Vaccines

Personalized immunogenic cancer vaccines loaded with neoantigens, or tumor specific antigens (TSA), have now been tested in the clinic. Data suggests neoantigen cancer vaccines may potentially generate a stronger anti-tumor immune response, compared to classical DC based cancer vaccines loaded with TAAs[82]. Neoantigens are different from TAAs because they are absent from normal tissues and are completely specific to the cancer cells[83]. These antigens have been reported to originate from viral proteins, post-translational modifications, and somatic mutations[84]. Moreover, research studies have revealed that neoepitopes can either be classified as “shared” or “personalized”. Shared

neoepitopes, or mutated oncogenes, are antigens that are found in a specific cancer type, where personalized neoepitopes, otherwise referred to as patient specific mutated neoantigens, are specific to an individual [85]. Examples of shared neoepitopes include BRAF, NRAS, and p53 mutations. Approximately 50% of melanomas harbor BRAF mutations at position V600 of the protein chain, the majority of which involve the substitution of glutamic acid for valine (V600E), resulting in constitutively active BRAF [86]. As stated above, NRAS mutations are found in approximately 15-25% of melanomas and occur at codons 12,13, and 61 [22,23]. It has been reported that p53 mutations are present in approximately 19% of melanomas. Additionally, wildtype p53 is reported to not function properly in melanomas. The literature indicates that wildtype p53 in melanomas fails to act as a tumor suppressor [87,88].

Neoantigen cancer vaccines are identified by the use of whole exome sequencing to identify mutations expressed in RNA from a tumor biopsy, compared to healthy tissues. Each mutation is screened using one of many algorithms to predict MHC binding and tested for immunogenicity (Figure 2). The mutations predicted to be processed and presented in the patients' MHC molecules sufficiently have the best anti-tumor immune response are used to generate personalized DC cancer vaccines[89-92]. The cost of manufacturing a personalized, single neoantigen vaccine is estimated to be $60,000, which includes the sequencing and analysis of tumor and matched healthy tissue DNA samples [93,94].

Murine studies have indicated the immunogenicity of neoantigens since the early 1990s. In 1916, Ernest Tyzzer reported the "acquisition of new immunogenic characteristics by cancer cells" [95]. Then, in 1943, Gross et al. showed mice were immune to re-challenge with tumor cells [96], suggesting that tumors could be immunogenic. In the early 2000s, Castle et al. and Kreitzer et al. each independently showed that nonsynonymous mutations are immunogenic and can produce CD4+ helper T cell responses [97,98]. Additionally, Castle et al. showed that mutated peptides can also be recognized by cytotoxic T lymphocytes in a B16F10 melanoma mouse, generating CD8+ T cell immune responses [97].

The pre-clinical data from murine studies indicate the use of neoantigen cancer vaccines in human clinical trials might lead to superior clinical response rates. Ott et al. were one of the first groups to propose a phase I study of a neoantigen cancer vaccine on the patient's own melanoma [90]. In the study, Ott et al. designed a vaccine that targeted approximately 20 predicted personal tumor neoantigen peptides. The results from the study revealed the vaccine aided in the generation of CD4+ and CD8+ T cell responses specific to various predicted neoantigens. Additionally, four out of six patients had no reoccurrence of the tumor at 25 months after vaccination [90]. Importantly, the two patients that had progressed achieved complete tumor regression after subsequent treatment with anti-PD-1. After anti-PD-1 treatment, it was reported that these patients had an expansion in neo-antigen-specific T cell responses [90]. Additionally, it has recently been shown that neoantigen cancer vaccines also can lead to determinant spreading, resulting in a more diverse immune response. Carreno et al. reported that a DC based neoantigen vaccine not only resulted in neoantigen specific T cell responses, but also resulted in new human leukocyte antigen (HLA) class I - neoantigens post vaccination in late stage melanoma patients [89].

The above studies were done based on the generation of peptides in adjuvant or peptide-pulsed DC cancer vaccines (strategy summarized in Figure 2). Recently, investigators have also been generating personalized RNA mutanome vaccines for the treatment of advanced melanoma [92]. Sahin et al. were the first group to generate this type of vaccine for advanced melanoma. Non-synonymous mutations were detected in 13 late staged melanoma patients; five mutations were selected per vaccine, per patient. All of the patients generated neoantigen-specific T cell responses and 2 patients had an increase in neoantigen specific infiltrating T cells post vaccination [92]. They also reported that 1 patient achieved a complete response to the vacation when used in combination with anti-PD-1 [92]. Arguably, the above research studies suggest neoantigen cancer vaccines are capable of generating a potent and clinically effective immune response. Yet, neoantigen cancer vaccines may generate the best immune response when used in combination with checkpoint blockade treatments (anti-PD-1 and anti-CTLA-4).

Single and combination checkpoint blockade therapies have been very successful in a large subset of patients with advanced melanoma. Research studies have shown that ant-CTLA-4 treatment can broaden CD8+ T cell responses in advanced melanoma patients [99-101]. For example, Kvistborg et al. observed a significant expansion of melanoma-specific T cell responses post-treatment with anti-CTLA-4 in 40 melanoma patients [97]. Even though checkpoint blockade treatments have shown promise in the clinic, clinical data has suggested that only approximately 20-40% of patients respond to anti-PD-1/anti-CTLA-4, and the ones that do respond already have a mounted anti-tumor immune response [102].

In advanced melanoma patients, checkpoint blockade treatments have been reported to enhance overall survival in responding patients [100,103,104]. However, the molecular mechanisms correlating with clinical benefit from these therapies are largely unknown. Whole exome sequencing of melanoma tumors (pre and post anti-CTLA-4 treatment) and matched normal tissue have indicated that patients who clinically benefit from anti-CTLA-4 treatment have a high mutational load [99-101]. Therefore, these patients likely have a high neoantigen load that can be recognized by the immune system. Snyder et al. examined the tumor neoantigen landscape in late stage melanoma patients [100]. Using computational methods, Snyder et al. identified neoantigens in tumors from patients with clinical benefit to anti-CTLA-4; importantly, these neoantigens were absent in tumors from non-responding patients. Presence of these neoepitopes were not only associated with clinical benefit, but also were associated with overall survival. Follow-up in-vitro studies revealed that some of the predicted neoantigens were able to efficiently prime an activated T cell response [100]. These studies suggest that checkpoint blockade treatment, in combination with neoantigen cancer vaccines, might result in an optimal anti-tumor immune response that correlates with clinical benefit (Figure 2).

Research is now exploring combination therapies of vaccines and checkpoint inhibitors in the clinic (Figure 2). A new phase 1B trial for advanced melanoma, bladder, and lung cancer, is exploring the safety and therapeutic benefit of a new neoantigen vaccine (NEO-PV-01) in combination with ipilimumab (NCT02950766) or nivolumab (NCT02897765). The vaccine includes up to 20 distinct neoantigen peptides [105].

In all, neoantigen cancer vaccines have shown promising results in early clinical trials. Clinical data has indicated that these vaccines are capable of generating anti-tumor immune responses and determinant spreading. Moreover, predicted neoantigens have been shown to correlate with clinical outcome, and when used in combination with checkpoint blockade treatments, a subset of patients have experienced a complete response.

An area for further study is the stability of neoantigen-specific T cell responses. A recent study has shown neoantigen expression can be lost from the tumor cell population overtime [106]. The loss of neoantigen expression on tumor cells correlated with neoantigen-specific T cell responses, therefore, tumor resistance may occur in a personalized vaccine. To avoid such resistance, a broad neoantigen landscape and induction of determinant spreading should be considered for vaccine development, which might lead to improved clinical outcomes for advanced melanoma patients.

## Determinant spreading

Although antigen choice is critical for generating antitumor immunity, the spread of the immune response from one antigen to another antigen expressed in the same tissue ("determinant spreading" [107] or "epitope spreading") has been linked to superior clinical outcome [33,108-113] in multiple tumor types and vaccination settings. The phenomenon of in vivo cross presentation of tumor derived antigens released in one wave of T cell attack to promote subsequent waves of anti-tumor T cells directed against different antigens may be an important mechanism for tumor rejection [111]. A vaccine that targets shared antigens may set the stage for subsequent rounds of immunity to mutated neoantigens. The most important role of vaccines containing TAAs may be to induce determinant spread to tumor specific antigens that activate higher avidity T cells, which more effectively mediate tumor rejection. The in vivo mechanism of cross priming may also result in autoimmunity [114], which has been found to be a biomarker of clinical response to interferon in patients with melanoma [115].

## Other recent promising vaccine strategies

**Adjuvant and agonistic antibody immunization—**Sagiv-Barfi, I. et al have shown in in multiple murine models that intratumoral (in situ) vaccination with CpG-enriched oligodeoxynucleotide (CpG; a Toll-like receptor 9 ligand) and agonistic anti-OX40 antibody triggers a potent systemic anti-tumor T cell immune response that mediates rejection of distant tumors and enhanced long-term survival of the animals [116]. This strategy is currently being tested in patients with low-grade B-cell non-Hodgkin lymphomas in a phase I trial (NCT03410901).

## Oncolytic vectors in combination with checkpoint blockade

TBI-1401 (HF10) is a spontaneously mutated HSV-1 vector that has attenuated neuroinvasiveness. It has previously been tested in a small phase I study in patients with recurrent breast cancer, where tumor shrinkage was reported [117]. HF10 is currently being tested for efficacy and safety with repeated administration of intratumoral injections in combination with intravenous infusions ipilimumab in patients with melanoma (NCT03153085).

### mRNA vaccines

The use of mRNA vaccines has shown significant potential as a vaccine modality due to its innate immunogenic properties. mRNA is a non-infectious, non-integrating platform, that gets degraded by normal cellular processes. It is an easily modifiable, stable and highly translatable vaccine platform [118]. BioNTech have demonstrated that DCs can be effectively targeted in vivo using intravenously administered RNA-lipoplexes (RNA-LPX). The LPX protects RNA from extracellular ribonucleases and mediates its efficient uptake and expression of the transgene in APCs. Most importantly, they have shown that RNA-LPX encoding tumor antigens induce strong antigen-specific effector and memory T cell responses in 3 melanoma patients [119]. The same company has also demonstrated in a phase I trial that its IVAC® MUTANOME, an individualized RNA vaccine based on patient-specific mutations, can induce anti-tumor activity in high-risk patients with late-stage melanoma [93].

### Strategies to optimize future immunotherapies

As evidenced over the years, vaccines, oncoviral and immunomodulating modalities used to treat cancer patients have displayed limited clinical effectiveness when used as single agents. Aforementioned pre-clinical and clinical data indicate that combining two or more immunotherapeutic strategies is the logical evolution of the field. However, in order to fully maximize the potential of such approaches, we need carefully perform optimization tests to determine the optimal sequence of drug deliveries. Additionally, novel strategies are needed to abrogate immune suppression in the tumor microenvironment that can hamper the efficacy of anti-tumor vaccines.

### Timing of future vaccine delivery strategies

A pre-clinical study by Messenheimer has indicated that the timing of anti-PD-1 delivery in combination immunotherapy may be crucial in the induction of effective anti-tumor immunity. Using agonistic anti-OX40 in combination with anti-PD-1, they have shown that concurrent addition of the two agents had a detrimental therapeutic effect when compared to anti-OX40 alone. Sequential delivery of anti-OX40 followed by anti-PD-1 (but not in the opposite order) had resulted in significantly improved therapeutic efficacy [120]. This study highlights the potential significance of the order in which combination immunotherapies are delivered.

### Patient selection in vaccine trials

Currently, only a minority of patients benefit from active and passive immunotherapies. However, patients that do respond frequently display durable benefit. Consequently, selection of patients that would most likely benefit from a specific immunotherapy based on specific predictive biomarkers of clinical benefit is a priority. It would allow for timely, personalized and cost-effective way of treating individuals. Future clinical trials need to implement robust and rational immunomonitoring strategies to evaluate immune cell phenotypes and function in circulation as well as tumors, serum levels of various circulating protein and DNA factors (i.e. liquid biopsies) and tumor profile. Consequently, a multidisciplinary approach that integrates phenotypic and functional analysis of immune

cells (e.g. multi-color flow cytometry; CyTOF; multi-color ELISpot assays), immunohistology (e.g. quantitative multi-color immunofluorescence), transcriptome analysis (e.g. NanoString, RNA-seq), cell metabolism analysis, liquid biopsy assessment (circulating tumor DNA, circulating proteins) and tumor mutational analysis (whole-exome sequencing) needs to be implemented in order to identify biomarkers predictive of optimal therapeutic responsiveness. There are currently many important signals (total mutation burden, CD8+ T cell infiltrate, PD-L1 expression, IFN-. expression signature) but no clear predictive biomarkers to act upon.

### Abrogation of MDSC-mediated immunosuppression

Accumulation of MDSC, immature myeloid cells, in peripheral blood and within tumor lesions correlates with poor prognosis and therapy resistance [121-123]. Additionally, MDSC have a multitude of immunosuppressive functions that hamper the development and maintenance of anti-tumor immunity [123]. In vivo studies have shown that depletion of MDSCs improves the therapeutic effectiveness of vaccination in a murine lung cancer model [124]. Multiple anti-MDSC treatments have been developed target different aspects of MDSC biology (accumulation, function, recruitment or viability) [123,125]. One such modulator is INB03 (INmune Bio, Inc.), a dominant-negative TNF biologic that selectively neutralizes the soluble form of TNF (solTNF) without affecting the transmembrane variant (tmTNF). INB03 has been shown to decrease the frequency of splenic PMN- and M-MDSCs and macrophages, and inhibit MDSC-mediated immunosuppression [126]. Other suppressors of MDSC include Tadalafil [127], all-trans retinoic acid [128] and TRAIL-R2 antibody [129].

## Conclusions

Cancer vaccination has a long history in melanoma, with success in driving immune responses, although objective clinical responses have only ever been detected in a minority of patients. This active area of research continues to investigate optimal vaccination platforms, and more recently, neoantigen-based vaccination and oncolytic virus combinations.

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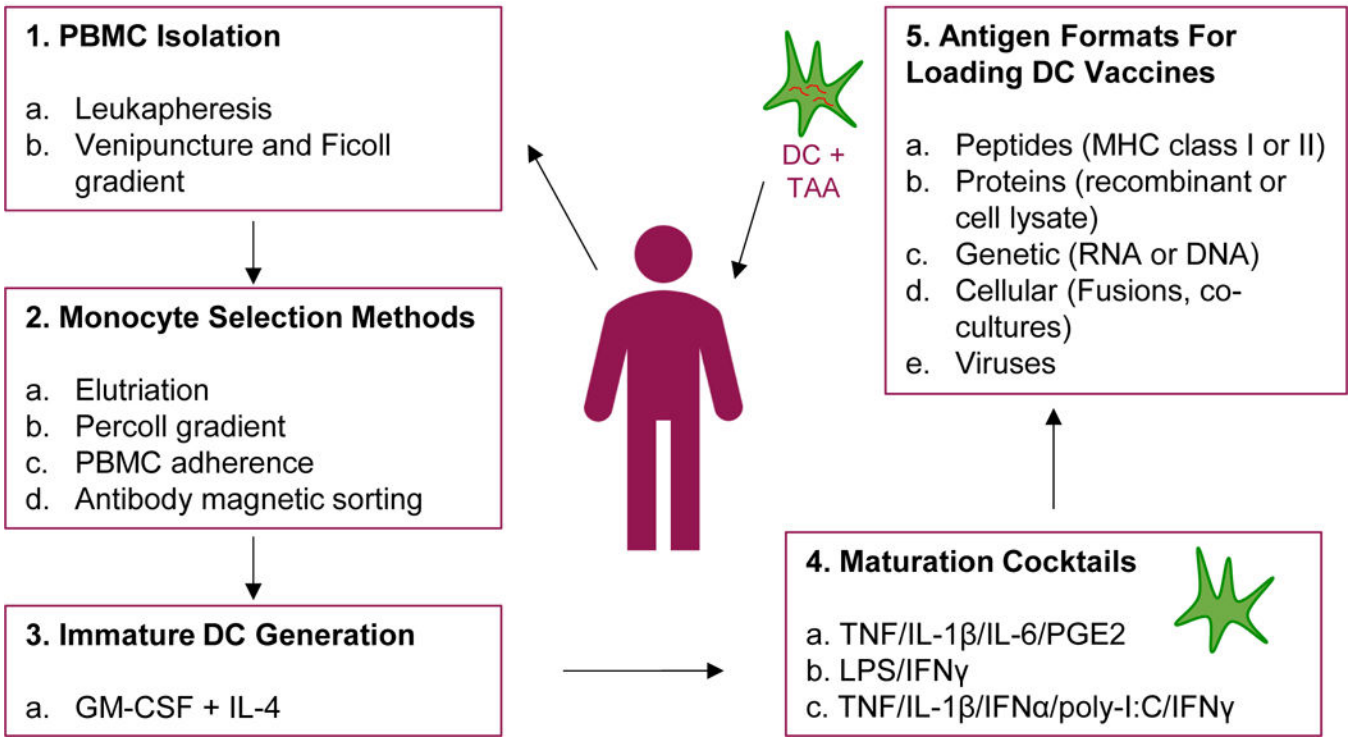


**Figure 1. Basic monocyte-derived DC-based immunization protocol.**

DC-based vaccines are generated by the selection of CD14+ monocytes from isolated PBMC. Monocytes are cultured with GM-CSF and IL-4, often for 5 days, to obtain immature DC. Immature DC are matured using various maturation cocktails as previously described by Kaka et al. (2009), Vujanovic et al. (2010), and Kalinski et al. (2005). After 24-48 hr maturation, matured DC are delivered to patients. DC are loaded with antigens either before, during, or after maturation

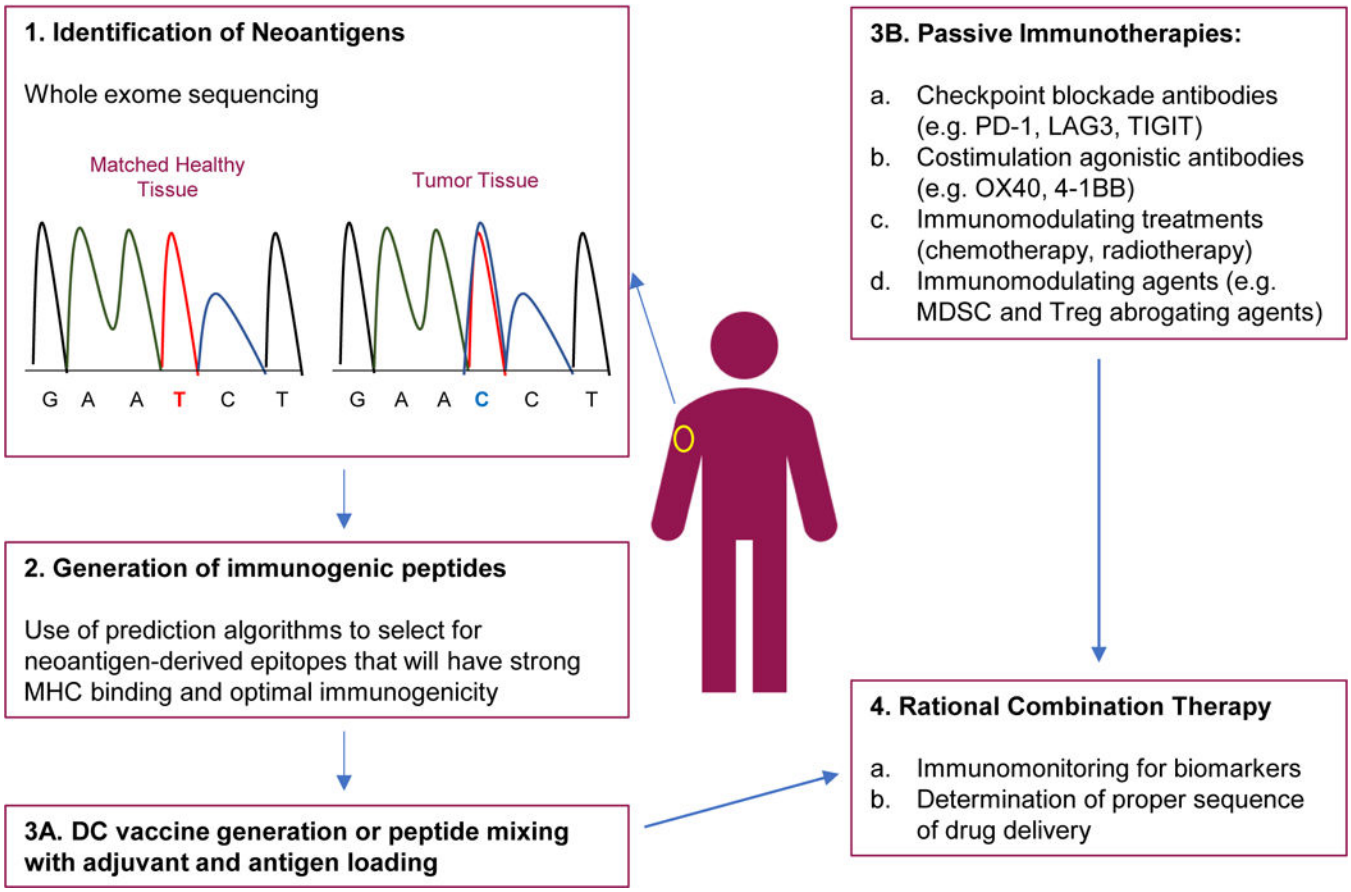


**Figure 2. Checkpoint + neoantigen vaccine combination therapies.**

**1-3A** Neoantigen based vaccines are generated by the identification of neoepitopes through whole exome sequencing of tumor and matched donor tissues. The neoepitopes are tested for immunogenicity and strong MHC binding through the use of computational algorithms and *in vitro* testing. Selected neoantigens are then used for vaccine production. **3B**. Other immunotherapies are in the clinic for the treatment of melanoma. These include numerous checkpoint inhibitors and agonistic therapies, standard chemotherapy and radiation, and immunomodulating agents. **4.** Combinations of neoantigen vaccines and other immunotherapies might lead to an optimal anti-tumor immune response.