Melanoma Peptide Vaccines: From Preclinical Background to Clinical Trials

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The discovery that immune T-cells recognize intracellularly processed peptides associated with major histocompatibility locus molecules has revolutionized the cancer vaccine field by providing new reagents for the generation of immune responses against cancer. The cloning of tumor antigen genes has proceeded most rapidly in melanoma because of the ease with which melanoma-specific T-cells can be propagated in vitro. The cloning and identification of tumor regression antigens and data from the initial clinical trials with peptide vaccines derived from those antigens are presented here.

Introduction

Eukaryotic cells express thousands of proteins which are degraded intracellularly into peptides via the multisubunit proteasome and presented on the surface of antigen-presenting cells as antigens for recognition by T-cells in association with class I and II major histocompatibility locus (MHC) molecules. The generation and detection of antigen-specific antitumor immune responses in humans has been revolutionized by the discovery that peptides are the ultimate target for T-cells [1–3]. A number of clinical trials of peptide vaccines with and without adjuvants in patients with metastatic and resected melanoma have been facilitated by the cloning and identification of several classes of tumor-associated and tumor-specific antigens on melanomas. A number of different classes of defined melanoma antigens are summarized herein. Also provided are descriptions of the available published and unpublished data concerning recent attempts to boost immunity directed against defined melanoma antigens using peptides, along with discussion of their clinical significance. Finally, a suggestion for an “optimal” cancer vaccine design is provided in the context of current peptide vaccine efforts in melanoma and other histologies.

Antigens Present on Human Melanomas

Cancer vaccine development has progressed most rapidly for patients with malignant melanoma, for which compel-

ling evidence of immunogenicity exists in humans. Spontaneous regression of primary melanomas is common and can be observed pathologically as well as clinically, and the prognosis of cutaneous melanomas varies directly with the level of lymphocytic infiltrate [4–5]. Vitiligo is an autoimmune destruction of melanocytes mediated by serologic and T-cell responses that commonly occurs in melanoma patients, especially those who have been treated with interferon- α or interleukin-2 (IL-2), and it is known to correlate with a favorable response to immunotherapy with high-dose IL-2 or chemobiotherapy [6–8]. Data from murine experiments and in melanoma patients suggests that both humoral and cellular immune responses to TRP-2, a component of the melanosome, is responsible for vitiligo. Tumor-reactive lymphocytes derived from patient peripheral blood or found to infiltrate metastatic melanoma lesions can be grown in vitro as oligoclonal T-cell lines or clones. The ease with which such melanoma specific T-cell clones can be grown in vitro allowed investigators in 1991 to describe the cloning of an antigen derived from a mutagenized melanoma cell line which was recognized by T-cells [12•,13]. This antigen was called MAGE (melanoma antigen-E), and it defined a family of antigens that had not previously been identified. MAGE-1 and several members of its multigene family were present on a significant proportion of melanoma cell lines and fresh tumors but were also found on a variety of tumors of epithelial and neuroectodermal origin as well as normal testis and placental tissue, but on no other normal tissue [14]. MAGE, GAGE, BAGE, and RAGE-defined families of genes linked to the X-chromosome that were found respectively on melanoma, gastrointestinal, breast, and renal-cell tumors, many of which encoded antigens that were recognized by T-cells and defined the “cancer/testis” class of tumor antigens. Between 20% and 80% of melanoma lines and fresh tumors were shown to express cancer/testis antigens from these four families.

After the cloning of the first cancer/testis antigens, several groups defined members of a second group of differentiation antigens that were melanosome-related “neo-antigens” derived from gene products produced in normal cells. The first melanosome-related antigen cloned was MART-1/Melan A, defined via recognition by cytolytic T-cell (CTL) clones from the peripheral blood of melanoma patients and by lymphocytes infiltrating tumors (TIL). The TIL that recognized MART-1 as well as TIL from a number of

other melanoma patients reacted with virtually all melanoma cell lines that expressed HLA-A2, and transfection of the A2 gene into other non-A2 expressing melanoma lines increased their sensitivity to TIL lysis [15]. These data suggested that MART-1 was a common HLA-A2 restricted melanoma antigen recognized by CTL. MART-1 was expressed by virtually all metastatic melanoma lesions, a majority of cell lines derived from metastatic melanomas, and also by melanocytes, but not by any other normal tissue. The nonamer sequence AAGIGILTV and decamer GAAGIGILTV, representing residues 27-35 and 26-35 of MART-1, respectively, bound most strongly to HLA-A2 [16]. These peptides stimulated the growth of specific CTL from peripheral blood mononuclear cells (PBMC) of melanoma patients and in normal tissue [17]. Multiple re-stimulations of PBMC with MART-1:27-35 peptide in the presence of IL-2 resulted in MART-1-specific CTL derived from 11 of 12 melanoma patients [18,19]. These CTL lysed fresh uncultured melanoma cells and were 100-fold more lytically active against melanoma cells than TIL grown in high-dose IL-2. The majority of TIL grown from patients with melanoma are capable of recognizing the MART-1:27-35 and 26-35 peptides, and a minority of those TIL cultures induced regression of metastatic melanoma after adoptive transfer with high-dose IL-2 [20]. The repertoire of V β T-cell receptor molecules from TIL and peripheral blood-derived CTL lines that are MART-1-specific is quite skewed [21–22].

Peptides derived from MART-1 were eluted from melanoma cells, suggesting that MART-1 is a naturally occurring antigen on fresh tumors [23]. A protein database analysis demonstrated that sequences conforming to the MART-1 A2 binding motif and possessing features important for CTL recognition occurred frequently in normal and viral proteins [24,25], and that a peptide derived from glycoprotein C of herpes simplex virus could sensitize target cells to lysis by MART-1-specific CTL [26]. These data suggest that epitope mimicry by normal or other commonly occurring proteins may account for the frequency of CTL detected against melanoma antigens like MART-1.

Greater MART-1 reactive CTL activity was demonstrated in the peripheral blood of melanoma patients compared with normal subjects, suggesting that a tumor related “priming” effect had occurred [27]. In a clinical study of patients with metastatic melanoma who had lymph nodal disease that was resected, a novel technology using peptide-bound class I tetramers was employed to detect MART-1 and tyrosinase-specific CTL in tumor-infiltrating lymph nodal tissue. From 0.1% to 3% of CD8+ lymphocytes infiltrating tumor-involved lymph nodes were MART-1 specific; in contrast, the proportion of MART-1-specific cells in non-tumor-containing lymph nodes was not different from background [28•]. The infiltrating tumor antigen-specific CTL were functional, and they secreted cytokines or could lyse peptide-pulsed targets or antigen-expressing tumor cells. Two subsequent studies using melanoma peptide tetramers for the detection and enumeration of tumor-

antigen specific CD8 CTL have suggested that fresh peripheral blood-derived T-cells are deficient in recognition of peptide-bearing target cells and neither upregulate CD69 nor secrete cytokines after exposure to peptide-bearing target cells, whereas viral-specific CTL from the same patient were highly active in the same assays. MART-1-specific CD8+ cells in the peripheral blood were shown to be predominantly CD45RA-naïve cells, not CD45RO-activated or memory cells.

The overlapping MART-1 26-35 peptide has been shown to be more immunogenic than the 27-35 epitope, and a single amino acid modification to the 26-35 peptide rendered it a stronger binder to A2.1 and even more immunogenic [28•]. This peptide is a candidate for future clinical vaccine trials. The MART-1 26-35 and 27-35 peptides have been shown to bind to multiple other A2 subtypes with a significant degree of heterogeneity [29,30], as well as allele A45 [31], but no other MART-1 specific peptides have yet been shown to elicit specific immune responses in patients bearing other HLA class I alleles [32].

Antigen pMel17/gp100 was defined through recognition by CTL clones from melanoma patient peripheral blood and by TIL. gp100 is a transmembrane glycoprotein of approximately 100 kilodaltons recognized by the HMB-45 and NK1-beteb monoclonal antibodies on melanocytes and melanoma cells [33,34]. CTL clones derived from melanoma patients and TIL grown from a melanoma patient recognized melanoma cells that expressed gp100 in association with HLA-A2.1, but not gp100 negative lines [35•]. Multiple peptides derived from the gp100 sequence that fit the consensus motif for binding to HLA-A2 can be recognized by TIL from melanoma patients, including gp100 209-217 (ILDQVPSFV), gp100 154-162 (TKTWGQYWQV), and gp100 457-466 (LLDGTATLRL) [36]. A naturally processed gp100 peptide 280-288 (YLEPGPVTA) was defined by elution of peptides from melanoma cells and purification by tandem mass spectroscopy. This peptide redirected HLA-A2 restricted lysis by CTL lines from a melanoma patient [37]. TIL from a melanoma patient also recognized gp100 280-288. All three gp100 peptides are recognized by self-reactive T-cells, and they bind with moderate or low affinity to HLA A2. This suggests that the gp100-specific TIL or CTL may have escaped from tolerance induction because of their low affinity for TcR (T-cell receptor), whereas the autoreactive CTL that bear high-affinity TcR may be deleted in the thymus. It is of some interest that, in melanoma patients, gp100-specific TIL have been reported to induce regression of metastatic melanoma in patients receiving TIL and IL-2 therapy. Four of a group of 14 TIL that were analyzed were reactive with one or more epitopes of gp100; all four induced tumor regression [35]. Peptides derived from gp100 that are recognized by CTL in association with members of the HLA-A3 superfamily have also been defined, suggesting that the repertoire of HLA haplotypes eligible for vaccination trials with gp100 may be expanded [38,39]. These data also suggest that gp100 may

be a promising target for immunotherapy using peptide vaccination strategies and/or adoptive therapy of antigen-specific cytolytic T-cells.

Tyrosinase is a membrane-bound protein involved in the melanin synthesis pathway [40]. Tyrosinase is expressed by virtually all primary cutaneous melanomas and by up to 90% of metastatic lesions [40]. It encodes several epitope peptides that are presented by HLA-A2 to CTL-reactive human melanomas [41]. A peptide derived from tyrosinase, aa 368-376, YMNGTMSQV, was shown to be posttranslationally modified by deamidation of asparagine to aspartic acid resulting in a sequence recognized by human CTL, YMDGTMSQV, known as tyrosinase 368-376 (370D) [42]. The tyrosinase 368-376 (370D) peptide is thought to encode a biologically important epitope and can induce CTL in vitro (Unpublished data, Weber *et al*).

TRP-1 (gp75) and TRP-2 are tyrosinase-related proteins that are integral parts of the melanosome apparatus, and they have been shown to be recognized by CTL from patients with melanoma, although these neo-antigens have not yet reached the clinical trial stage [43-45].

Recently, a "cancer-testis" antigen distinct from the MAGE, GAGE, BAGE, and RAGE families, called NY-ESO-1, was discovered. It has the property of eliciting both a strong humoral response but also encodes epitope peptides recognized by CTL clones and TIL clones from patients with melanoma and breast cancer [46•,47]. The antigen was found to be expressed by a variety of squamous and adenocarcinomas as well as by melanoma, suggesting a wide potential utility.

A final group of melanoma antigens are mutated normal genes that are uniquely present on individual tumors; *p16*, *β-catenin*, and *mum-1* are examples [48-50]. Because these genes are unique to individual tumors, their clinical utility for vaccines is limited and will not be pursued in this review.

Clinical Trials with Peptide Vaccines

Early trials of peptide vaccines for melanoma were performed in patients with metastatic disease to test the toxicity of and immune responses to the vaccine. The peptide vaccines showed few toxicities other than local pain and granuloma formation. Escalating doses of peptides in aqueous solution or combined, as in prior mouse experiments, with incomplete Freund's adjuvant (IFA or Montanide ISA 51, an oil-in-water emulsion) at doses up to 10 mg showed no serious side effects. Initial experiments with the HLA-A1-restricted MAGE-3 peptide EVDPIGHLY given in aqueous solution at low (100 to 300 μg/injection) doses to 12 patients with metastatic melanoma indicated that six patients were able to complete three injections at monthly intervals, and three of the six had objective clinical responses [51•]. No evidence of boosted immunity was detected in the peripheral blood. When the same epitope peptide was combined with IFA and administered to

patients with resected melanoma, immune responses were detected by CTL assays in five of 16 patients tested at our institution, and delayed-type hypersensitivity (DTH) skin responses to peptide were also observed [52]. No clear correlation was observed in this small trial between immune response by specific cytokine or chromium release after exposure to peptide-pulsed T2-A1 cells and time to relapse.

The MART-1 27-35 and 26-35, tyrosinase 1-9 and 368-376, as well as several gp100 peptides (280-288 and 457-467), have been tested alone and in combination in patients with metastatic or resected melanoma. Jaeger *et al*. [53] immunized six metastatic melanoma patients intradermally with multiple peptides from MART-1, gp100, and tyrosinase in aqueous solution at 100 μg each on a weekly basis for four immunizations. These authors observed augmented cytotoxicity of antigen-restimulated PBMC directed against one, but not necessarily all three, antigens [53]. Three of six patients showed augmented cytotoxicity to MART-1, and two of six to a tyrosinase peptide, but none of six demonstrated augmented reactivity to gp100 peptides. When granulocyte-macrophage colony stimulating factor (GM-CSF) was injected at 75 micrograms per dose subcutaneously as an adjuvant treatment for 3 days prior to and 2 days after immunization with multiple antigen peptides, significant boosting of immune reactivity was seen compared with vaccination using peptides alone [54]. Three of three patients showed increased immune reactivity to tyrosinase peptide 1-9, and one of three had increased reactivity to MART-1 26-35. Three of three objective clinical responses were seen, including two partial regressions in involved lymph nodal, cutaneous, and liver lesions, and one complete regression in a patient with subcutaneous disease. An infiltrate of CD8(+) T-cells was observed at vaccination sites after GM-CSF injections.

In subsequent peptide vaccine trials in melanoma, it was shown that six of 26 patients treated with MAGE-1, MAGE-3, MART-1/Melan A and tyrosinase peptides exhibited tumor regression [55]. Disease was stabilized in 22 of 26 evaluable patients with metastatic melanoma. A correlation was seen between strong MHC class I staining and response; there was also a correlation between skin/subcutaneous disease, low tumor burden, immune response, and clinical response. The authors argue that the strength of the correlation between clinical response and MHC expression would suggest that MHC staining of tumor by immunohistochemistry should be an eligibility criteria for peptide vaccine trials, which is consistent with other published data. Surprisingly, there was little loss of tumor antigen expression, although one of 11 patients had cytolytic T-cell responses to MAGE-1/3, eight of 30 to MART-1/Melan A, and six of 30 to tyrosinase peptides.

Cebon *et al*. [56] immunized patients with metastatic melanoma using intradermally administered MART-1₂₆₋₃₅ peptide with IL-12 in increasing doses given either subcutaneously or intravenously. The main toxicity of IL-12 by either route was flu-like symptoms. Of the first 15 patients, one complete

regression (CR), one partial regression (PR), and one mixed response were noted. Immune assays for T-cell generation included delayed type hypersensitivity, which was seen in patients with or without IL-12. Positive CTL assays were seen in patients with evidence of clinical benefit, but not in patients without regression. Scheibenbogen *et al.* [57] immunized 18 patients with four HLA class I restricted tyrosinase peptides, 16 with metastatic melanoma, and two adjuvant patients without evidence of disease. Increased CTL activity was observed in four of 15 patients, two adjuvant patients, and two patients with evidence of tumor regression. As in the trial by Cebon *et al.* [56] and in other trials, patients with progressive disease did not show boosted antigen-specific immunity.

A number of small pilot studies have been conducted at the National Cancer Institute of the US National Institutes of Health in which patients with metastatic melanoma received multiple subcutaneous injections of a single peptide emulsified with IFA at 3-week intervals. MART-1 27-35, gp100 209-217, 154-162, and 280-288 have been used in these trials [58,59]. In one study, escalating doses of the gp100 209 (ITDQVPFSY), 280 (YLEPGPVT), or 154 (KTWGQYWQV) peptides at doses from 1 to 10 mg were administered subcutaneously every 3 weeks with IFA [60]. Immune assays were performed using the above "native" peptides for antigenic stimulation and substituted 209-2M (IMDQVPFSY) and 280-9V (YLEPGPVT) peptides. Ninety percent to 100% of patients had strong evidence of boosted immune reactivity postvaccination as shown by an assay in which release of interferon- γ from PBMC restimulated one to three times in the presence of IL-2 and peptide antigen was measured by enzyme-linked immunosorbent assay (ELISA). Seven of seven patients had boosted gp100 reactivity postvaccine after only one restimulation with the 209-2M peptide, and five of six were boosted with one restimulation with the 280-9V peptide. Higher release of cytokine was seen after four immunizations than in two immunizations in most patients, and a greater level of reactivity was observed when substituted peptides were used in assays, in contrast with "native" peptides. Boosted cytokine release was shown to correlate with cytolytic responses. When tumor cell lines expressing the correct MHC restriction element and antigen were used as a stimulator in cytokine release assays, lower levels of cytokine were observed compared with T2 cells pulsed with the relevant peptide. This suggests that peptide density on the target was important for recognition by effector cells in PBMC. Objective partial and complete remissions were uncommon, with one of 20 patients having a complete regression. No clear correlation was observed between the level of immune response and the rare clinical responses, so that no statement about clinical benefit could be made. In a second study of escalating doses of the MART-1 27-35 peptide administered subcutaneously every 3 weeks with IFA, 15 of 16 patients had evidence of boosted immunity directed against the "native" MART-1 27-35 peptide, with increased reactivity after four vaccinations compared with fewer vaccinations [61]. No objective clinical responses were seen.

At the University of Southern California/Norris (UC/Norris), we have treated 25 melanoma patients with high-risk resected stage III/IV disease with increasing doses of the MART-1 27-35 peptide emulsified with IFA every 3 weeks subcutaneously [62]. Toxicity consisted of local inflammation and granuloma formation, and low-grade fevers, fatigue, headache, and myalgias were common. Ten of 22 patients had evidence of boosted immunity by cytokine release assays, and a correlation was observed between the absolute level of interferon- γ released after multiple restimulations of peptide-pulsed patient PBMC postvaccination and time to relapse, with a *P*-value of 0.003. The correlation of immune reactivity with time to relapse suggests that a positive response to peptide vaccination may incur clinical benefit. The data suggest that patients mount an immune response to flutamide (FLU-M) before and after vaccination and that the development of MART-1-specific CTL is antigen-specific, because MART-1-stimulated CTL do not react with FLU targets, and vice versa. All nine of the relapses and all three deaths have occurred in the minimal response or no response group thus far, supporting the idea that development of an immune response directed against MART-1 is clinically beneficial.

The anchor amino acids that form hydrogen bonds between epitope peptides and class I MHC molecules can be modified to strengthen their binding, resulting in greater immunogenicity in vitro and in vivo. When such heteroclitic peptides derived from gp100 were used to restimulate PBMC from patients with metastatic melanoma immunized with a wild-type gp100 peptide, immune reactivity was detected with greater frequency and sensitivity [60]. Patients immunized with the heteroclitic gp100 peptide demonstrated a higher level of immune reactivity compared with those immunized with the wild-type peptide. The MART-1 27-35 peptide has been shown to be "naturally" processed and is immunodominant; however, the MART-1 26-35 peptide has been shown to be a better MHC binder and to be more immunogenic in vitro [63] and was more effective at detection and quantitation of MART-1-specific CTL in a flow cytometry assay when used for the generation of MHC/peptide tetramers [28•]. Heteroclitic gp100 peptides combined with IFA have been used to immunize patients with metastatic melanoma. More than 90% of the patients in one vaccine study had evidence of boosted immunity detected after one restimulation in vitro, suggesting that the substituted peptide was more immunogenic than "natural" peptide in vivo [64•]. When the same substituted gp100 peptide was injected and followed within several days by high-dose intravenous IL-2, surprisingly, no detectable augmented immune response was observed, but 13 of 31 patients had an antitumor response, including 12 PRs and one CR, for an overall response rate of 42%, significantly greater than rates observed with IL-2 alone (15% to 20%) [60,64•,65,66].

The utility of a peptide vaccination added to high-dose IL-2 as an "adjuvant" is currently being explored in two

Table 1. Immune Response to gp100/tyrosinase Peptides with Incomplete Freund's Adjuvant and Interleukin-12

Patients, n /PRE/POST	Cytokine release/ FLU stim*			Cytokine release/ gp100 stim			Cytokine release/ tyr stim		
	T2 FLU	T2 gp100	T2 tyr	T2 FLU	T2 gp100	T2 tyr	T2 FLU	T2 gp100	T2 tyr
10236 PRE	860	0	222	0	0	40	12	0	370
10313 POST	346	96	14	0	830	0	0	190	1181

FLU—flutamide; Stim—stimulation; Tyr—tyrosinase.
*Antigen-specific responses are indicated by **boldface type**.

large multicenter trials. At our institution, the heteroclitic gp100 209-217 (210M) and tyrosinase 368-376 (370D) peptides emulsified in IFA with or without IL-12 as an adjuvant are being examined in a randomized phase II trial in patients with high-risk resected stage III/IV melanoma. Immune response is one end point, and time to relapse is the clinical end point. Strong DTH skin test responses to gp100 but not to tyrosinase have been observed in 20 of 24 patients, and augmented immune responses to both gp100 and tyrosinase have been observed in 19 of 24 patients, suggesting that multiple melanoma peptides injected simultaneously with adjuvant result in boosted immunity to both antigens in virtually all patients with resected high-risk melanoma (Unpublished data, Weber *et al.*). Whether IL-12 administration results in augmented skin test reactivity or increased antigen-specific immune responses measured by cytokine release or tetramer assays awaits a more detailed analysis (Table 1).

In one study, an HLA-A2.1 restricted tyrosinase peptide combined with adjuvant QS-21 was used to vaccinate patients with metastatic melanoma, and no tumor responses were seen, although in two of nine patients there was evidence of boosted tyrosinase-specific immunity [67]. Toxicity was modest, with fevers and local injection site pain as the only side effects.

Peripheral blood mononuclear antigen-presenting cells prepared by purifying adherent cells and exposing them to 1000 U/mL of GM-CSF for 7 days were pulsed with the MAGE-1 A1 restricted peptide EVDPIGHLY and injected into HLA-A1 patients with metastatic melanoma [68]. In a small pilot trial, T lymphocytes were grown from the vaccination site of two patients and found to be MAGE-1 reactive. No significant toxicity was observed in this trial. In one patient, T lymphocytes grown from a peripheral tumor were found to be MAGE-1-specific and could lyse autologous tumor cells. When a DTH assay was performed with peptide-pulsed, antigen-presenting cells (APCs) injected intradermally, a CD8 and CD4 infiltrate was seen, but no patients had an antitumor response. It was suggested that growth of vaccine site infiltrating lymphocytes (VIL) and the demonstration that they were tumor specific constituted a surrogate assay for immune response.

Gajewski *et al.* [69] took a different approach to peptide-based therapy, and treated 15 metastatic melanoma patients

with subcutaneous Melan A/MART-1 peptide-pulsed peripheral blood cells with rhuIL-12 in escalating doses subcutaneously every other day for three doses after each cell injection. One PR, two MRs, and three patients with stable disease were observed, with minimal toxicity. Antigen-specific immune reactivity was augmented in eight of 13 patients by the peptide-pulsed cell injections. These responses were directed against MAGE-3 and MelanA/MART-1.

Rosenberg *et al.* [70] used adenoviral vectors encoding the cDNAs for gp100 and MART-1 to immunize patients with metastatic melanoma. They performed a phase I trial of escalating doses of recombinant virus from 10^7 to 10^{11} particles per injection, given either intradermally or subcutaneously, either alone or with the addition of high-dose IL-2. Thirty-six patients received the MART-1 adenoviral vector, and 18 received the gp100 construct. Immunizations were given four times at monthly intervals. Mild and transient erythema at injection sites was the only side effect observed. One of 16 patients who were given the MART-1 vector had a complete response, but none of six patients who received the gp100 adenovirus alone responded. Of the 20 patients who were given IL-2 plus the MART-1 vector, there were two PRs and two CRS. No consistent augmentation of antigen-specific T-cell reactivity was observed in any subgroup of patients, in contrast with the significant reactivity seen with the use of peptides with IFA by the same investigators. Only five of 23 patients had augmented MART-1 immunity detected by release of cytokines by restimulated PBMC, and none of 6 had gp100 boosting; however, 34 of 45 patients sera-tested for adenoviral antibodies showed significant increases in antiviral IgG. These data suggested to the investigators that adenoviral serologic reactivity might limit the utility of engineered adenoviral vectors as vaccines, and they concluded that current recombinant adenoviral vaccines were not superior to peptide-based immunizations.

Dendritic cells (DC) serve as "nature's adjuvant." They are antigen-presenting cells that can prime naïve T-cells, and they are potent stimulators of cytolytic and helper T-cell immunity [71–75]. Dendritic cells pulsed with melanoma antigen peptides can stimulate the development of antigen-specific CTL in vitro more potently than peptide-pulsed PBMC or macrophages [76]. DC have been prepared from the adherent peripheral blood monocytes of patients by

Table 2. Immune Response to gp100/tyrosinase Peptide-pulsed Dendritic Cell Therapy

Patients, n/PRE/POST	Cytokine release/ FLU stim			Cytokine release/ gp100 stim			Cytokine release/ tyr stim		
	T2 FLU	T2 gp100	T2 tyr	T2 FLU	T2 gp100	T2 tyr	T2 FLU	T2 gp100	T2 tyr
10236 PRE	670	0	0	0	88	0	0	0	0
10313 POST	1148	0	0	0	1822	0	0	0	919

FLU—flutamide; Stim—stimulation; Tyr—tyrosinase.
 *Antigen-specific responses are indicated by **boldface type**.

growth in IL-4 and GM-CSF [77,78] and pulsed with multiple melanoma peptides. In vitro, DC pulsed with melanoma peptides can stimulate the development of potent antigen-specific CTL responses [79]. These DC have been injected subcutaneously and intra-lymph-nodally in patients with metastatic melanoma, and they have been shown to induce antigen-specific immune reactivity and clinical responses. In one published study, one complete remission and three partial remissions were observed in patients treated with multiple peptide-pulsed monocyte-derived DC without evidence of significant toxicity [80•]. At our institution, we have treated 16 melanoma patients with intravenous injections of monocyte-derived DC pulsed with multiple heteroclitic melanoma antigen peptides from gp100 and tyrosinase, and we have seen objective clinical responses and boosting of immunity in responding patients, as shown by the immune response assay data summarized in Table 2. These findings indicate that tyrosinase and gp100-specific CTL cytokine release were observed from a responding patient's PBMC that were restimulated once with relevant peptides after immunization with peptide-pulsed DC. In contrast with patients who receive peptides with IFA, no peptide-related DTH skin test reactivity has been observed in patients treated with peptide-pulsed DC.

The MART-1, gp100, and tyrosinase antigens are all expressed by normal melanocytes [73–75], and the use of neo-antigen peptide vaccines has the potential to induce autoimmune reactions. It is not known whether normal melanocytes effectively present the MART-1, gp100, or tyrosinase epitope peptides to T-cells in vivo. Previous clinical experience with the adoptive transfer of large numbers of CTL that were highly MART-1–or gp100–reactive and mediated regression of tumor did not indicate the onset of any autoimmune damage to skin, brain, inner ear, and retina, where melanocyte lineage cells are located. Patients with metastatic or resected melanoma who received MART-1, gp100, or tyrosinase peptide vaccines at our institution or in studies described in the published literature did not demonstrate any evidence of ocular or other toxicity (Unpublished data, Weber *et al.*) [60,63]. One patient of 75 who received a gp100/tyrosinase vaccine at USC/Norris showed evidence of vitiligo. No ocular problems were observed, nor was there any evidence of autoimmune pathology occurring in any patients on our MART-1,

gp100, or tyrosinase peptide trials with median follow-ups of greater than 16 months.

One potential pitfall of any antigen-specific immunization strategy is immunoselection in vivo after peptide vaccination [81–89]. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis has shown that MART-1 mRNA is present in virtually 100% of metastatic melanoma lesions, yet immunohistochemical staining has shown that there is considerable heterogeneity in MART-1, with 60% to 90% of all cells staining positively [83–89]. In one study, deletion of MART-1 expression and of TAP transporter expression rendered cells transparent to CTL recognition, suggesting that loss of MART-1 may be a mechanism for immune evasion [88]. Two patients who were repeatedly immunized with MART-1, gp100, and tyrosinase peptides in aqueous solution intradermally had tumors biopsied prior to and after vaccination. One patient developed MART-1 reactive T-cells in the peripheral blood, but a biopsy of a progressing lesion showed loss of MART-1 expression. Another patient had an initial biopsy showing expression of all three antigens, but after immunization and development of tyrosinase-specific reactivity, a progressing tumor expressed gp100/MART-1 but not tyrosinase [89]. A significant level of antigenic heterogeneity occurs within lesions, as well as complete loss of antigen expression on individual lesions [72–79,80•,81–87]. These data suggest that a successful vaccine would require multiple antigenic peptides to minimize the consequence of loss of any individual antigen.

Functional Analyses of Antigen-specific T-cells in Melanoma Patients

Functional defects in melanoma-specific T-cells from peripheral blood and tumor-bearing lymph nodes of patients with metastatic melanoma may render them ineffective. Romero *et al.* [28•] showed by tetramer staining that MART-1–specific antigen-experienced cells were found in tumor-infiltrated lymph nodes, but not in normal lymph nodes from the same patients. In a subsequent study, these authors demonstrated that peripheral blood-derived and lymph node–derived MART-1–specific T-cells were predominantly CD45 RA–positive, suggesting that they retained a naïve phenotype [90]. Lee *et al.* [91•] iso-

lated tyrosinase and MART-1-specific tetramer-positive cells from patients with metastatic melanoma and showed that antigen-specific activation of CD69 was deficient on those cells. Sorted antigen-specific tetramer-positive cells from fresh blood did not lyse antigen-expressing targets, nor did they release cytokines unless cultured in the presence of IL-2. These data support and are consistent with experiments performed by several groups, including Finke *et al.* [92], who showed that circulating total peripheral blood T-cells from patients with melanoma were deficient in TcR transduction pathways [93,94]. These results indicate that peptide and antigen-specific vaccine therapy may certainly augment T-cell responses to high levels, but that functional T-cell assays should be part of any measure of immune response in melanoma vaccine trials. Further efforts to develop strategies for overcoming immune suppression in melanoma are critically important for the successful development of melanoma vaccines.

Conclusions

One of the biggest problems in the cancer vaccine field today is the abundance of good ideas compared with a modest number of patients who would be appropriate candidates for vaccine trials. Good-prognosis melanoma patients with metastatic stage IV disease (M1a or M1b) or resected stage IV disease who are rendered disease-free would be logical candidates for vaccine trials because the clinical end points of tumor response and survival or time to relapse would be short. Such patients, however, are few. A second critical issue is the lack of a good surrogate laboratory assay for a successful immune response. Limiting dilution analyses using chromium release assays have been the immunologic "gold standard" for the enumeration of CTL precursors, but this intervention is labor intensive and difficult to perform. Bulk CTL cytokine release assays, enzyme-linked immunospot (ELISPOT) assays, and staining for intracellular cytokines are simpler and more reproducible, but they generally require restimulation of T-cells in vitro for 1 to 7 days, obscuring and artificially amplifying T-cell responses. Tetramer assays can be performed on fresh blood cells and may allow a functional analysis of tumor antigen-specific T-cells from fresh blood without the complications of in vitro restimulation and exposure to cytokines. Tetramer assays could be combined with intracellular cytokine staining and T-cell receptor variable chain analysis to yield important information about the specificity and function of antigen-specific CTL. Peptides with adjuvants or those that are pulsed onto dendritic cells, DNA plasmids encoding peptide mini-genes, or whole antigens and viral vectors encoding peptides/whole antigens are all promising candidates that must be tested.

Dose, schedule, route, and type of adjuvant are all variables that must be worked out in patients in small phase II pilot trials that should be devised based on the best information that we have from mouse models and using the best available immune assay.

Although achieving a clinical response to a particular vaccine regimen in patients with measurable metastatic melanoma is a desirable standard, the significant tumor burden and well-documented immune suppression present in many patients with bulk metastatic melanoma suggest that this group is not the ideal population in which to test new vaccines. However, it should be accepted that any phase II cancer vaccine trials must incorporate an end point of clinical benefit that should be correlated with assays of immune response. I propose that future vaccine trials be performed in patients with resected high-risk melanoma who have a greater than 50% chance of relapse and death from metastatic disease, but a microscopic disease burden. The clinical end point would be time to relapse, which should be correlated with any immune response assay. Although immune assays with restimulated PBMC have been used, new assays employing intracellular cytokine release and class I peptide tetramer binding combined with spectra-type TcR analysis and followed by multicolor flow cytometry may offer a more sensitive and accurate assessment of antigen-specific reactivity with fresh peripheral blood cells.

An "optimal" peptide vaccine trial that I would propose would include HLA-A2.1 patients who have resected stage IV melanoma and are rendered free of disease. Patients with stage III disease and two or more palpable lymph nodes, extracapsular extension, or in-transit metastases would also be included because of their poor outcome with surgery alone and the modest clinical benefit shown with interferon- α . Multiple heteroclitic peptides from tyrosinase, gp100, MART-1, and gp75, at a dose of 300 to 1000 μ g for each peptide, would be included with a pan-HLA-DR peptide and IFA as the adjuvant. Treatment with Flt-3 ligand for 7 days in the limb to be immunized would precede each injection, and a single dose of IL-12 would be given intradermally after vaccination. Several days later, low doses of IL-2 at "immune reconstituting" doses of 1–3 mU/M² would be given subcutaneously twice daily for 5 days after each vaccination. A total of eight to ten injections would be given over a period of 6 to 12 months at increasing intervals of time. The clinical end points would be time to relapse and overall survival. The immune end points would be tetramer and cytokine release assays on PBMC isolated from fresh blood and propagated for 24 to 48 hours ex vivo in the presence of cytokines but without antigen. A similar trial has been proposed to begin at our institution in the near future.

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