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A Randomized Phase II Trial of Multi-epitope Vaccination with Melanoma Peptides for Cytotoxic T-Cells and Helper T-Cells for Patients with Metastatic Melanoma (E1602)

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Abstract

Purpose—This multicenter randomized trial was designed to evaluate whether melanoma helper peptides augment cytotoxic T-lymphocyte (CTL) responses to a melanoma vaccine and improve clinical outcome in patients with advanced melanoma.

Patients and Methods—One hundred seventy-five patients with measurable stage IV melanoma were enrolled into 4 treatment groups, vaccinated with 12 MHC Class I-restricted melanoma peptides (12MP) to stimulate CTL (group A), plus a tetanus peptide (group B) or a mixture of 6 melanoma helper peptides (6MHP, group C) to stimulate helper T lymphocytes (HTL), or with 6MHP alone (group D), in incomplete Freund's adjuvant (IFA) plus GM-CSF. CTL responses were assessed using an in vitro stimulated IFN-gamma ELISpot assay, and HTL responses using proliferation assay.

Results—In groups A–D, respectively, CTL response rates to 12MP were 43%, 47%, 28%, and 5%, and HTL response rates to 6MHP were in 3%, 0%, 40% and 41%. Best clinical response was partial response (PR) in 7/148 evaluable patients (4.7%) without significant difference among

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Conflicts of interest:

Craig Slingluff:

- is an inventor for four of the MHC Class I-restricted peptides used in the E1602 clinical trial; those patents are held by the University of Virginia Licensing and Ventures Group.
- Is on the scientific advisory board for Immatics.
- Is PI for the Polynoma sponsored trial of a cancer vaccine POL-103A
- Is PI of an investigator initiated clinical trial of a cancer vaccine, funded by Glaxo-Smith Kline

There are no other potential conflicts of interest.

study arms. Median overall survival (OS) was 11.8 months. Immune response to 6MHP was significantly associated with both clinical response ($p=0.036$) and OS ($p=0.004$).

Conclusion—Each vaccine regimen was immunogenic, but melanoma helper peptides did not augment CTL responses to 12MP. The association of survival and immune response to 6MHP supports further investigation of helper peptide vaccines. For patients with advanced melanoma, multipptide vaccines should be studied in combination with other potentially synergistic active therapies.

Keywords

melanoma; cancer vaccines; peptides; survival; GM-CSF; human; multicenter phase II trial

INTRODUCTION

Therapeutic cancer vaccines using defined tumor antigens show promise for melanoma and other cancers [1–5]. Melanoma cells express peptides presented by MHC Class I molecules, recognized by CD8⁺ cytotoxic T lymphocytes (CTL), or by MHC Class II molecules, recognized by CD4⁺ helper T lymphocytes (HTL). Common melanoma peptides are from melanocytic differentiation proteins (MDPs) and cancer-testis antigens (CTAs) [6–13]. MDPs are expressed in 70–90% of metastatic melanomas and may be downregulated with progression [14–17]; several CTAs are expressed in 30–60% of melanomas, and their expression may increase in more advanced melanomas [18, 19]. Thus, there is rationale for vaccines targeting both MDPs and CTAs [20].

Most peptide vaccines have targeted only CTL; however, natural immune responses to pathogens induce both HTL and CTL. The present trial tests vaccines targeting both. A mixture of 12 melanoma peptides (12MP) restricted by HLA-A1, A2, or A3 has induced CTL in up to 70–100% of patients [20–22]. A mixture of 6 melanoma helper peptides (6MHP) has been immunogenic in 80% of patients and associated with clinical activity [22, 23], and a tetanus helper peptide has been immunogenic and safe. We proposed to test, in advanced melanoma, whether the immunogenicity and immune protection of a 12MP melanoma vaccine would be augmented by addition of melanoma-specific helper epitopes (6MHP).

PATIENTS AND METHODS

Patients

Eligible patients had histologically proven AJCC Stage IV melanoma with measurable disease, HLA-A1, A2, or A3 expression, age 18 years or older, ECOG performance status 0–1, adequate organ function, and LDH $2\times$ upper limits of normal (ULN). Exclusions included: pregnancy; other therapy within 4 weeks; allergy to vaccine components; other cancers within 5 years, untreated or progressive brain metastases; steroids; immunosuppression; or severe autoimmune disease. Patients provided informed consent, and the study was approved by the institutional review boards and the US Food and Drug Administration and registered at ClinicalTrials.gov (NCT00071981).

Vaccines

Patients received vaccines comprised of multiple peptides from MDP and CTA, including 12 melanoma peptides (12MP [20], Arms A–C) restricted by HLA-A1, A2, or A3, to stimulate CTL, 6 melanoma helper peptides (6MHP [23], Arms C and D), or a tetanus helper peptide (tet [24], Arm B) to stimulate HTL. Peptide sequences and details of GMP preparation and compliance [25] are in Supplemental Text.

Each vaccine was administered as a 2 ml stable water-in-oil emulsion, prepared by the two-syringe method, containing 100 mcg (12MP) and/or 200 mcg (6MHP, tet) of each peptide, plus 110 mcg GM-CSF (Berlex/Amgen) plus 1 ml Montanide ISA-51 or ISA-51 VG (Seppic, Inc., Paris, France/ Fairfield, NJ). On days 1, 8, and 15, vaccines were administered (half-intradermally and half-subcutaneously) at 2 injection sites (primary and replicate sites), on extremities uninvolved with tumor. On day 22, an optional biopsy was performed of a lymph node draining the replicate vaccine site (sentinel immunized node, SIN [26]). On days 29, 36, and 43, one vaccine was administered at the primary vaccine site only. Patients were reassessed at weeks 8 and 12 for evidence and confirmation of clinical response via Response Evaluation Criteria in Solid Tumor (RECIST) v1.0. Patients without progression were eligible for 6 cycles of 3 weekly booster vaccines, until progression or for 2 years.

Trial Design

This was an open-label, multicenter phase II study, with randomization equally to vaccination with 12MP, 12MP/Tet, 12MP/6MHP, or 6MHP (Supplemental Figure 1), through the ECOG web-registration program, using permuted blocks within strata with dynamic balancing within main institutions and affiliate networks. Patients were stratified by HLA status (HLA-A1⁺, HLA-A2⁺, HLA-A1⁺ and HLA-A2⁺, other) and sentinel immunized node (SIN) biopsy to be performed (yes, no).

The primary objective was to determine the CTL response to 12MP. Secondary objectives were: i) to determine the HTL response to 6MHP; ii) To estimate whether addition of 6MHP to 12MP augments CTL responses to 12MP; iii) to obtain preliminary data on whether booster vaccination may maintain immune responses, iv) to estimate rates of clinical response and survival, and v) to obtain preliminary data on whether cellular immune responses may correlate with clinical outcome.

The primary endpoint was the CTL response, with the main comparisons of CTL response rates in arms A, B, and C vs. D. We expected CTL response rates less than 10% in arm D and at least 40% in arms A–C. The sample size of 40 evaluable patients in each arm provided at least 85% power to detect a difference of at least 30% in CTL response rate, based on a two-sided type I error of 0.05 using Fisher's exact test. Secondary endpoints included HTL responses in peripheral blood mononuclear cells (PBMC), clinical response, and outcome. We expected HTL responses to be higher in arms B–D (at least 30%) than A (5%), with the main comparisons between HTL immune response rates in arms B, C, and D vs. A. There would be at least 82% power to detect a difference of at least 25% using Fisher's exact test with a two-sided type I error of 0.05.

Clinical endpoints

Tumor response was evaluated per RECIST v1.0 at weeks 8 and 12, then every 12 weeks, with survival followup thereafter. Objective response rate (ORR) was defined as complete response (CR) + partial response (PR). Disease control rate (DCR) was defined as CR+PR +SD (stable disease at 8 weeks). Overall survival (OS) the time from randomization to death from any cause. Patients still alive were censored at the date last known alive. Progression-free survival (PFS) was defined as time from randomization to disease progression or death, censoring cases without progression at the date of last disease assessment.

Correlative Studies

Peripheral blood (100ml) was collected pretreatment, at weeks 1, 3, 5, 7, and 11, and at the 1st and 3rd of each set of booster vaccines. PBMC were isolated from 90ml of blood and viably cryopreserved; serum was collected from 10 ml blood. Lymphocytes were isolated from the SIN [26]. CTL response was determined by interferon (IFN)-gamma ELISpot assay

performed 14d after one in vitro sensitization, as described (in vitro stimulated ELISpot assay) [20]. CTL response required at least a two-fold increase over background, and 30 responding cells per 100,000 (details in Supplemental Text) HTL response was evaluated in a 5d tritiated thymidine proliferation assay, as described [23] and in Supplemental Text, and required at least a 4-fold increase in proliferation Cytokine (IL-12 p70, IL-1b, IL-2, IL-5, IL-6, IL-10, IL-17, TNF, IFN, GM-CSF) and chemokine (CCL3, CCL4, CCL5, CXCL9, CXCL10) production was measured by Luminex in supernatants collected from PBMC after 2d or 5d culture with helper peptides or mitogens (phytohaemagglutinin (PHA), phorbol myristate acetate (PMA)). Treatment samples (week 3–7) were compared to pretreatment samples with paired student's T tests.

Toxicity Assessment and Stopping Rules

The trial was monitored continuously by the ECOG Data Monitoring Committee (DMC) for treatment-related adverse events, using NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Protocol treatment was to be discontinued for unexpected treatment-related grade 4 hematologic, grade 3 non-hematologic, grade 2 allergic, or grade 1 ocular events, or for disease progression requiring other therapy. If 3 of the first 9 patients in any arm experienced dose-limiting toxicities (DLT), we would consider closing that arm. If the true DLT rate was 5%, the probability of observing 3 patients experiencing DLT events out of 9 patients was 0.008. Interim analyses were performed for immune response and clinical outcome, as detailed in Supplemental Text.

Statistical Analysis

Immune response rate, objective clinical response rate, disease control rate and toxicity incidence were compared between treatment arms using the Fisher's exact test [27]. Distributions of OS and PFS were estimated using the Kaplan-Meier method [28], with 95% confidence intervals calculated using Greenwood's formula. Treatment effect on survival was tested using log rank tests. Associations between immune response and clinical response were explored using Fisher's exact test and logistic regression. Logistic regression models for rare events were also fit for testing association between immune response and clinical response as sensitivity analysis [29]. Multivariable Cox proportional-hazards models [30] were built to explore associations between immune response and overall survival using the landmark method [31], with 2 months from randomization as a landmark time point. In the landmark analysis, OS was defined from landmark to the event (death); patients with OS events before the landmark were excluded from that analysis. All reported p-values were for two-sided tests. Significance level was set at 0.05. Given the exploratory nature of this study, no adjustments were made for multiple comparisons.

RESULTS

Patients

One hundred seventy-five participants were enrolled from March 2005 to January 2009 (Table 2, Consort Diagram Figure 1). At interim analysis, Arm C met protocol criteria for stopping based on clinical outcome, after accruing 37 patients. On eligibility review, 21 patients were ineligible (19 treated). Reasons for ineligibility are in Supplemental Text. Among the 154 eligible participants, 6 never started assigned therapy; thus, 148 eligible and treated patients were used for clinical outcome analysis. Toxicity analysis was based on the 167 participants who received protocol therapy and had toxicity reported, regardless of eligibility. Immune response analyses were based on 140 eligible participants with CTL data, and 128 with HTL data. Forty-five patients (30%) received booster vaccines (16, 8, 7, and 14, on arms A–D, respectively); seven completed all 6 cycles. Study arms were well matched for known prognostic factors (Supplemental Table 1), but prior immunotherapy

was less frequent for arm B than others ($p=0.01$). Overall, serum LDH was elevated in 28%; 80% had 2 or more metastatic sites; M-stage was 7% M1a, 22% M1b, and 72% M1c.

Immune response

CTL response rate—CTL response rates to 12MP for Arms A–D were 43%, 47%, 28%, and 5%, respectively (Figure 2A, Supplemental Table 2), and differed among treatment arms ($p<0.001$) and for arms A–C vs. D ($p<0.05$), but not for A vs. C or B vs. C. In multivariable logistic analysis, the odds of CTL response was lower for patients on arm D, patients with more metastases, and increased age (Table 1). Responses to each peptide and responses to booster vaccines are summarized in Supplemental Text and Supplemental Table 3.

HTL responses—HTL response rates to 6MHP for Arms A–D, respectively, were 3%, 0%, 40%, and 41% ($p<0.001$); for tetanus peptide, they were 11%, 59%, 4%, and 0%, respectively ($p<0.001$, Figure 2A, Supplemental Table 2).

Cytokine and chemokine production—Antigen-specific cytokine and chemokine production by PBMC was evaluated in the 47 analyzable patients with HTL responses to 6MHP (26), tet (20), or both (1). Among arm B patients, there were treatment-related increases in tetanus-specific production of IL-2 (d2, $p=0.0383$), IL-5 (d2, $p=0.0149$; d5, $p=0.0088$), IFN-gamma (d2, $p=0.006$; d5, $p=0.0118$), GM-CSF (d5, $p=0.0377$), CXCL9 (d2, $p=0.0144$; d5, $p=0.0280$), and CXCL10 (d2, $p=0.0064$; d5, $p=0.0055$). Among 29 arm C+D patients, there were significant treatment-related increases in 6MHP-specific production of IL-2 (d2, $p=0.021$), IL-5 (d2, $p=0.0212$; d5, $p=0.0072$), IL-12p70 (d5, $p=0.0359$), CXCL9 (d2, $p=0.0176$; d5, $p=0.0434$), and CXCL10 (d5, $p=0.0076$). These data for IL-2, IL-5, CXCL9, and CXCL10 are shown in Figure 3. Within groups C+D, for patients with PR or SD ($n=9$), after stimulation with 6MHP, there were treatment-related increases in CCL3 (d2, $p=0.0212$) and CCL4 (d2, $p=0.0125$); for patients with PD ($n=19$), there were increases in IL-2 (d2, $p=0.0413$) and IL-5 (d2, $p=0.0292$; d5, $p=0.0096$), GM-CSF (d5, $p=0.0238$), and CXCL10 (d5, $p=0.0151$, data not shown).

Clinical Outcome

Seven patients had PR (4.7%, 95% CI: 1.9–9.5), and 27% stable disease at 8 weeks (SD, Table 2). There were no CR. Objective response rates (CR+PR, ORR) did not differ significantly across study arms ($p=0.74$). The disease control rate (CR+PR+SD, DCR) was 32% (47/148, 95% CI: 24–40) overall. For arms A–D, PR rates were 2, 3, 6, and 7%, respectively. DCRs were 46%, 15%, 25%, and 36%, respectively, differing among arms ($p=0.026$), with A > B ($p=0.006$). Stable disease persisted to 6 months in 5, 2, 3, 2 patients on arms A–D, respectively; thus, PR or durable stable disease were observed in 15%, 9%, 16%, and 12%, respectively.

Of the 148 analyzable patients, median OS was 11.8 (10.2–14.0) months. For arms A–D, median OS (and 95% CI) were 14.9 (10.1–18.6), 10.2 (6.7–12.2), 12.4 (4.8–16.8), and 11.1 (8.8–14.2) months, respectively. The respective 1-year OS probabilities were 59% (42–72), 38% (21–54), 53% (35–69), and 49% (33–63) [50% (42–58) overall], and were assessed in the context of prior outcomes in multicenter cooperative group trials [32]: correcting for cited variables (gender, visceral disease, performance status), the observed 1 year OS rate exceeds prediction by at least two-fold, for each study arm (Supplemental Tables 4 and 5) and the observed values fall above the 95th percentile curves for arms A, C, D, and overall (Figure 4D). Progression-free survival (PFS) was not a designated endpoint of the study and did not differ significantly among study arms (Supplemental text).

Association between Immune Response and Clinical Outcomes

For eligible and analyzable patients with and without CTL responses to 12MP, ORRs were 9.8% and 3.0% ($p=0.194$), and DCRs 37% and 32% ($p=0.695$), respectively (Figure 2B). Among patients vaccinated with 12MP (groups A–C), ORRs were 10.3% and 0% ($p=0.022$), and DCRs 36% and 30% ($p=0.661$), respectively. Landmark analysis showed no significant association between OS and CTL response ($p=0.253$, Figure 4B). In multivariable regression analyses for groups A–D, CTL response was not associated with clinical response (OR=5.9, 95% CI: 0.7, 48.6, $p=0.102$) or overall survival (HR=0.87, $p=0.557$, Table 3, **Model 1**). The landmark analysis data are comparable if eligible and ineligible patients were assessed ($p=0.312$ for 12MP; data not shown)

Among 128 analyzable patients with or without HTL response to 6MHP, ORRs were 15% (4/27), and 3% (3/101; $p=0.036$), respectively (Figure 2B). Evaluating only the 64 patients on arms C and D, ORRs were similar: 15.4% and 2.6%, respectively (**$p=0.149$ due to the smaller sample size**). In contrast, response to tetanus peptide was not associated with clinical response ($p=1.00$). Landmark analyses showed overall survival, for 64 patients on arms C and D, was strongly and significantly associated with HTL response to 6MHP ($p=0.0045$), with 1 year survival rate of 65% and 24%, and median OS of 14.7 and 7.9 months, respectively for patients with or without HTL response (Figures 2C and 4C). This association remained significant in multivariable Cox landmark analysis for arms C+D (HR=0.50, 95% CI: 0.26–0.96, $p=0.038$) (Table 3, **Model 2**). In contrast, of 27 analyzable patients on arm B, landmark analysis showed no significant association of survival with response to tetanus peptide ($p=0.153$, Figure 2C). The landmark analysis data are comparable if eligible and ineligible patients were assessed ($p=0.0183$ for 6MHP; data not shown); and the effect is in the same direction when groups C and D are assessed individually, though not significant for Arm D given the smaller sample size (Arm C: $p=0.009$, Arm D: $p=0.127$, Supplemental Figure 2).

Of the 128 analyzable patients with data on both CTL response and HTL response, patients without response to 12MP or 6MHP had an ORR of 1.5%; corresponding rates were 6.9% with response to either, and 50% for response to both ($p=0.005$, Figure 2B).

Toxicity

Treatment-related toxicities are summarized in Supplemental Table 6. Rates of grade 3 or higher toxicity were 13%, 24%, 19%, and 8% on arms A–D, respectively. There was no significant difference in toxicity rates among treatment arms ($p>0.05$). There were 6 grade 4 toxicities in 4 patients (neutrophils, troponin elevation, lung infection, lymphopenia, CNS ischemia, AST elevation) and no treatment-related mortality. The most common toxicities of grade 3 or higher were fatigue and injection site reaction.

DISCUSSION

The CTL response rates in arms A and B met primary study objectives by exceeding 40%; however, the study failed to demonstrate increased immunogenicity of a 12MP melanoma vaccine by addition of melanoma-specific helper epitopes (6MHP), since only 28% had CTL responses in arm C. This negative result is consistent with a parallel study performed in the adjuvant setting [22]. Possible explanations may include expansion of antigen-specific regulatory T cells, suboptimal cytokine induction, homing of T cells to tumor deposits, or sequestration of T cells at vaccine sites [33]. Prior experience with 6MHP vaccines argues against a Th2 dominant cytokine response (Dillon et al., manuscript in preparation) and against expansion of total regulatory T cells [23]. However, effects on melanoma-specific regulatory T cells and T cell trafficking warrant further study.

Data from the HIV literature show that induction of HTL can restore function in CTL [34]. However, combining epitopes for HTL and CTL into one colinear peptide may be preferable to using them separately in vaccines [35–37]. Short peptides (9–11 residues) can bind directly to Class I MHC molecules of cells in the vaccine-site microenvironment (VSME) [38]. On the other hand, longer peptides require uptake and intracellular processing prior to presentation on nascent MHC molecules in professional APC, which migrate from the VSME to draining lymph nodes after vaccination [38]. It is possible that the intermediate length helper peptides in 6MHP (14–23 amino acids) may be presented primarily by professional APC in the vaccine-draining lymph node (VDLN). If peptides for CTL and HTL are presented in different compartments (VSME and VDLN), intended synergy may not be achieved.

Despite a body of literature that supports use of GM-CSF as a vaccine adjuvant [4, 39, 40], two prospective randomized phase II trials have shown negative effects of GM-CSF in combination with other vaccine adjuvants [21, 41]. The overall CTL response rates (43 and 47% in Arms A and B) are based on ELISpot assays performed after in vitro stimulation; which has been required for other studies when GM-CSF was used in the adjuvant [4, 20, 21]; we expect that the magnitude of these responses would be greater using IFA without GM-CSF. It is also notable that the CTL response rates of 43–47% fall short of the circulating CTL response rate of 83% to the same 12 Class I MHC-restricted peptides in a prior study [20]; this may be due to effects of shipping blood from many centers or to cryopreservation methods (manuscript in preparation), or to the more advanced stage of the patients in the present study. However, this CTL response rate is at least as high as observed with a prior ECOG multi-peptide vaccine trial [42].

Although the melanoma helper peptides did not increase circulating CTL responses in this study, the HTL response rates to 6MHP in arm C (40%) and D (41%) were similar and were comparable to prior studies [22, 23]. Thus, HTL responses to 6MHP are not impacted negatively by addition of 12MP. The response to tetanus peptide and to 6MHP includes specific induction of low levels of Th1 associated cytokine IL-2, , but also significant production of the Th2 cytokine IL-5. In prior studies, HTL responses to tetanus peptide were primarily Th1-dominant [24], as were responses to 6MHP (Dillon P et al, manuscript in preparation). Regardless, it is anticipated that improved vaccine adjuvants are needed to induce T cells with stronger Th1 bias. Also, Th1-associated chemokines CXCL9–10, implicated in T cell homing to tumor [43, 44], were produced (Figure 3); so if these vaccine-induced CD4⁺ T cells infiltrate melanoma metastases, they may be helpful in recruiting melanoma-reactive CD8⁺ T cells to the tumor. Notably, there was a strong association between immune response to the 6MHP and clinical response and patient survival, unlike tetanus peptide. These findings suggest a more important clinical role of HTL responses to melanoma antigens than previously appreciated.

The overall OR rate of 4.7% is consistent with prior experience with peptide vaccines [45]; however, one-year survival for all study groups exceeded prior experience for advanced melanoma in cooperative group trials [32], even after correcting for multiple risk factors. The encouraging 1-year survival data may be impacted by patient selection, by fact that 6 untreated patients were not included, and by capping LDH in the eligibility; however, 72% of analyzable patients had M1c disease, 28% had elevated LDH, 80% had two or more metastatic sites, 43% had PS 1, and prior systemic therapy included immunotherapy in 44% and chemotherapy in 49%; these features may be compared to patients treated with carboplatin and taxol on the recent E2603 trial, for which 57% had M1c disease, 39% had elevated LDH, 39% had PS1, and 58% were treatment-naïve; overall median survival with carboplatin and taxol was 11.1 months [46]; overall median survival for the present trial E1602 was 11.8 months. Despite modestly encouraging early survival data with these

multi-peptide vaccines, most patients still died of their melanoma within 2 years. Thus, continued investigation of multi-peptide vaccines in patients with advanced melanoma should be in combination with other active agents. In particular, BRAF inhibitors may transiently improve antigen expression by melanoma cells and T cell infiltration into melanoma metastases [47–49]; so combination with vaccines may be considered as an approach to improve clinical activity of both agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF TRANSLATIONAL RELEVANCE

This manuscript reports results of a 4-arm randomized multicenter phase II clinical trial testing whether multi-peptide melanoma vaccines that stimulate CD4+ helper T lymphocytes (HTL) would augment CD8+ cytotoxic T lymphocyte (CTL) responses, for patients with advanced melanoma. Each vaccine regimen was immunogenic, but melanoma helper peptides did not augment CTL responses to 12MP. However, there was a strong and significant association between survival and HTL responses to vaccines containing a mixture of 6 melanoma helper peptides (6MHP), while a similar association was not observed with HTL responses to a tetanus helper peptide. These data suggest that HTL responses to melanoma antigens have clinical relevance and deserve further investigation, but require consideration of different vaccine preparations or adjuvants. The low rates of objective clinical responses may be enhanced by combination with immune checkpoint blockade or other combination immune therapies.

Figure 1.
CONSORT Diagram for E1602 trial.

Figure 2. Immune responses to 12MP and 6MHP

A) The proportions of analyzable patients with CTL response to 12MP (blue bars), HTL response to tetanus peptide (yellow bars), and HTL response to 6MHP (maroon bars) are shown for each study arm. These are based on 140 eligible participants with CTL data (40, 30, 29, and 41 on arms A–D, respectively), and 128 eligible participants had HTL response data (40, 30, 29, and 41 on arms A–D, respectively). **B)** The objective clinical response rate is shown for patients based on the presence or absence of CTL or HTL response to peptides in the vaccines ($n = 140$ for response to 12MP, and $n = 128$ for response to 6MHP or tetanus peptides; patients in each category are listed under the X axis); **C)** median survival and 1-

year survival based on 2 month Landmark analyses, by CTL response to 12MP, HTL response to 6MHP or tetanus, and considering just patients vaccinated with the respective peptides.

A) Group B (Tetanus vaccine) Day 2

B) Groups C+D (6MHP vaccines) Day 2

C) Group B (Tetanus vaccine): Day 5

D) Groups C+D (6MHP vaccines): Day 5

Figure 3. Cytokine/chemokine produced by HTLs

From 47 patients with proliferative responses to tetanus peptide or 6MHP, 2-day (A, B) and 5-day (C, D) supernatants of PBMC from patients on arm B (A, C) or arms C+D (B, D) stimulated in vitro with 6MHP (6MHP-0, 6MHP +) or tetanus peptide (Tet-0 or Tet +) were assayed by Luminex for 10 cytokines and 5 chemokines, with increases in IL-2, IL-5, CXCL9 and CXCL10, shown here. Each line represents data from PBMC taken pre-treatment (6MHP-0 or Tet-0) or after 3–6 vaccines (6MHP + or Tet +). The median concentrations induced by PHA for IL-2, IL-5, CXCL9, and CXCL10 were 4600, 198, 2248, and 760 pg/ml respectively.

Figure 4. Survival outcomes

Kaplan-Meier estimates of overall survival, for analyzable patients ($n = 148$) by study arm treatment (**A**, $n = 148$), by CTL immune response (**B**, $n=139$, landmark method), and by HTL immune response to 6MHP in arms C and D (**C**, $n = 64$, landmark method). (**D**) 1 year survival for each treatment arm and for the full study population is shown on the plot of 1 year OS for all melanoma trials.

Table 1

Multivariable logistic regression for CTL response to 12MP (N=140)

Baseline Characteristics	Levels	Odds Ratio	P value
Treatment	Arm A vs Arm D	11.9	0.009
	Arm B vs Arm D	33.1	<0.001
	Arm C vs Arm D	30.2	<0.001
Age	>65 vs ≤65	0.2	0.015
Sex	Female vs Male	1.3	0.657
ECOG performance status	1 vs 0	0.6	0.289
Number of sites	2–3 vs 1*	0.9	0.856
	≥4 vs 1*	0.1	0.005
Ulceration	Yes vs No	0.8	0.782
	Unknown vs No	0.6	0.469
Clark level	IV vs V	0.6	0.486
	Other vs V	0.4	0.432
	Unknown vs V	1.3	0.736
Serum LDH	Elevated vs Normal	2.4	0.160
Prior chemotherapy	Yes vs No	0.4	0.115
Prior radiotherapy	Yes vs No	0.3	0.063
Prior surgery	Yes vs No	0.6	0.288
Chronic disease pre-registration	Yes vs No	2.9	0.086

Note: ECOG = Eastern Cooperative Oncology Group; LDH= Lactate dehydrogenase

Table 2

Clinical response by Treatment Arm.

Best overall response	Arm A n (%)	Arm B n (%)	Arm C n (%)	Arm D n (%)	Total n (%)	P value
Analyzable (eligible and treated) patients	n = 41	n = 33	n = 32	n = 42	n = 148	
CR	0	0	0	0	0	
PR	1 (2.4)	1 (3.0)	2 (6.3)	3 (7.1)	7 (4.7)	
SD (at 8 weeks)	18 (43.9)	4 (12.1)	6 (18.8)	12 (28.6)	40 (27.0)	
PD	22 (53.7)	27 (81.8)	23 (71.9)	27 (64.3)	99 (66.9)	
Un-evaluable	0	1 (3.0)	1 (3.1)	0	2 (1.4)	
ORR (CR+PR)	1 (2.4)	1 (3.0)	2 (6.3)	3 (7.1)	7 (4.7)	0.741
DCR (CR+PR+SD)	19 (46.3)	5 (15.1)	8 (25.1)	15 (35.7)	47 (31.7)	0.026
All patients	n = 47	n = 43	n = 37	n = 48	n = 175	
CR	0	0	0	0	0	
PR	1 (2.1)	1 (2.3)	2 (5.4)	3 (6.2)	7 (4.0)	
SD (at 8 weeks)	19 (40.4)	4 (9.3)	7 (18.9)	13 (27.1)	43 (24.6)	
PD	24 (51.1)	31 (72.1)	26 (70.3)	29 (60.4)	110 (62.9)	
Un-evaluable	3 (6.4)	7 (16.3)	2 (5.4)	3 (6.2)	15 (8.6)	
ORR (CR+PR)	1 (2.1)	1 (2.3)	2 (5.4)	3 (6.2)	7 (4.0)	0.662
DCR (CR+PR+SD)	20 (42.5)	5 (11.6)	9 (24.3)	16 (33.3)	50 (28.6)	0.008

Notes: P values were from Fisher's exact test. CR: complete response, PR: partial response, SD: stable disease at 8 weeks, PD: progressive disease, ORR: objective response rate, DCR: disease control rate. Among analyzable patients, stable disease at 3 months was observed in 10, 4, 5, 5 patients on arms A–D, respectively; stable disease at 6 months was observed in 5, 2, 3, 2 on arms A–D, respectively.

Table 3

Multivariable Cox landmark regression analysis for overall survival by immune response and clinical variables

Variables	Level	Model 1 Overall survival by CTL response to 12MP, n = 139, Arms A-D		Model 2 Overall survival by helper T cell response to 6MHP, n = 64, Arms C, D	
		Hazard ratio	P value	Hazard ratio	P value
CTL response	Yes vs No	0.87	0.557	---	---
Helper T cell response to 6MHP	Yes vs No	---	---	0.50	0.038
Treatment	Arm B vs Arm A	1.57	0.124	---	---
	Arm C vs Arm A	1.05	0.863	---	---
	Arm D vs Arm A	1.42	0.198	---	---
	Arm D vs Arm C	---	---	1.36	0.305
Age	Years (continuous)	1.02	0.041	1.00	0.830
Sex	Female vs Male	.92	0.684	0.62	0.164
ECOG performance status	1 vs 0	.95	0.810	0.63	0.174
Number of sites	2-3 vs 1	1.74	0.033	2.32	0.037
	>=4 vs 1	2.66	0.003	2.55	0.046
Ulceration	Yes vs No	0.81	0.390	0.75	0.525
	Unknown vs No	0.75	0.310	0.58	0.209
Clark level	IV vs V	0.75	0.335	1.07	0.877
	Other vs V	0.43	0.027	0.28	0.036
	Unknown vs V	1.06	0.859	1.04	0.932
Serum LDH	Elevated vs normal	1.45	0.107	1.45	0.334

Notes: Two models for overall survival (OS) are summarized in the table: Model 1 includes all patients and includes the measures of CTL response; Model 2 includes patients vaccinated with 6MHP (arms C,D) and includes measures of the immune response to 6MHP.

1 patient died within 2 months and was not included in landmark analysis for OS. CTL: cytotoxic T lymphocytes, 6MHP: 6 melanoma helper peptides, ECOG: Eastern Cooperative Oncology Group, LDH: Lactate dehydrogenase