

Defining the effects of age and gender on immune response and outcomes to melanoma vaccination: a retrospective analysis of a single-institution clinical trials' experience

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Abstract

Background The impacts of patient age and gender on immune response (IR) and clinical outcome after cancer vaccines are not known. We hypothesized younger and female patients would have higher IR rates and better survival.

Methods Patients with resected stage IIB–IV melanoma in three clinical trials (Mel43, Mel44, Mel48) were vaccinated with 12 melanoma-associated peptides restricted by class I MHC. The cumulative incidence rate of CD8⁺ T cell responses (direct interferon-gamma ELISpot assay) by week 7 was compared by age and gender. Overall survival (OS) and disease-free survival (DFS) landmark analyses were compared by Kaplan–Meier estimates and in multivariate analyses.

Results T cell responses were evaluated in 327 patients and detected in 50 % of males and 48 % of females,

with no difference in IR by gender or menopausal status. Males had trends toward longer DFS ($p = 0.12$) and OS ($p = 0.09$). Cumulative incidence of IR was higher in patients <64 years of age versus older patients ($p = 0.03$). OS and DFS were similar by age group ($p > 0.50$). In multivariate modeling, younger age was associated with better IR (OR 0.40, p value 0.003), without an impact of age or gender on clinical outcomes.

Conclusion These data support the hypothesis that older patients are less likely to develop T cell responses to a cancer vaccine. Nonetheless, significant proportions of older patients mount immune responses with comparable survival outcomes. Thus, these data support including older patients in cancer vaccine trials, but suggest value in stratifying patients by age </>64 years.

Keywords Age · Gender · Peptide vaccines · Melanoma · Clinical trials · Outcomes

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Abbreviations

6MHP	6 Melanoma helper peptides
12MP	12 class I MHC-restricted peptides
DFS	Disease-free survival
FDA	Food and Drug Administration
IR	Immune response

Introduction

Vaccination as a strategy for eradication of disease has been validated since Jenner's cowpox vaccines in 1796. In recent decades, comparable approaches have been explored for eradication of cancer leading up to the US Food and Drug Administration (FDA) approval of a vaccine for prostate cancer [1, 2]. In addition, a peptide vaccine has improved

progression-free survival of melanoma patients treated with high-dose interleukin-2 [3]. Many other cancer vaccines have been evaluated, without clear success, but with provocative and encouraging findings for several vaccine approaches [4–7]. Recent advances in checkpoint blockade therapies also offer promise to improve the efficacy of cancer vaccines by blocking cancer-associated immune dysfunction and have been shown to be safe when administered in combination with cancer vaccines [8, 9].

The first goal of most cancer vaccines is to induce robust T cell responses against MHC-associated peptides derived from cancer-associated proteins. However, immune responses to currently available cancer vaccines are commonly of low magnitude and may be transient [10, 11]. In addition, patient factors may interfere with the immune response (IR) to vaccines. Potential limitations may include patient age and gender; however, their impact on cancer vaccine efficacy is poorly understood. Both age and gender have significant effects on immune function in a wide variety of other clinical settings. It is generally accepted that with increasing age, the T cell repertoire declines, and there is defective induction of T cell memory; however, memory T cell responses induced during youth can persist even in old age [12, 13]. Some of these changes, interestingly, have been associated with chronic cytomegalovirus infection [14, 15]. Other potential contributing factors may include thymic involution and age-related changes in bone marrow function [12]. Some clinical trials of cancer vaccines have excluded older patients because of concern about immune senescence, but the impact of age on T cell responses to a defined-antigen vaccine has not been well studied.

There is less consensus on the effect of gender on immune function. However, numerous studies highlight the potential impact. Preclinical data implicate sex-related differences in proportions of regulatory T cells, and in T cell trafficking [15]. T cells have also been implicated in the induction and progression of atherosclerotic disease, with recent data showing strong protection against aortic aneurysms in women, apparently mediated in part by differences in immune responses related to estrogens or androgen receptors [16, 17]. Also, females tend to mount a more robust and protective immune response to infection, critical illness, and vaccinations [18–21]. Similarly, females also tend to develop immune-mediated diseases more frequently and have overall worse outcomes.

We have performed a series of clinical trials of melanoma vaccines using a mixture of 12 class I MHC-restricted peptides (12MP). In the first study with these peptides, T cell responses were induced to one or more peptides in 100 % of 25 patients enrolled, as assessed by ELISpot assay after one *in vitro* stimulation [22]. In several larger subsequent studies, a more stringent measure of T cell response was employed: Immune responses were

measured by a direct *ex vivo* ELISpot assay. The same assay was performed in over 300 patients on three separate clinical trials, providing a significant dataset with comparable immunologic assessment. Melanoma patients range widely in age and have large proportions of both male and female patients, making it feasible to assess differences in immune response as a function of both age and gender. Thus, the objectives of the present study were to study these questions in hope that the findings may aid the design and interpretation of future clinical trials of cancer vaccines and combination immunotherapies. Our hypothesis was that younger age and female gender would be associated with higher rates of immune response to a multipptide cancer vaccine.

Materials and methods

This study included patients with resected stage IIB–IV melanoma who were clinically free of disease. Patients received a vaccine containing 12MP—a multivalent vaccine derived from melanocytic differentiation antigens and cancer testis antigens—in one of three prospective phase II clinical trials. All patients received 6–10 vaccines, with vaccines 1–6 completed in the first 7 weeks. T cell responses were detected using a direct interferon-gamma ELISpot assay and measured weekly for the first 7 weeks of the study period. Clinical trial design and assay protocols have been reported in detail in prior publications [23, 24]. In brief, all three trials assessed the CD8⁺ T lymphocyte response to 12MP administered in Montanide ISA-51 adjuvant (a form of incomplete Freund's adjuvant, purchased from Seppic, Inc., Paris, France). For each trial, patients were randomized to treatment with that vaccine with or without other immunologic agents:

For the Mel43 trial (NCT00089193), the vaccines included 12MP plus one tetanus toxoid helper peptide (MELITAC 12.1), and patients were randomized to receive granulocyte–macrophage colony-stimulating factor (GM-CSF, arms B, D) or not (arms A, C) as part of the vaccine adjuvant, and to be vaccinated in 1 (arms A, B) or 2 (arms C, D) vaccine sites [25].

For the Mel44 trial (NCT00118274), the four treatment arms included 12MP, but differed in the helper peptides included in the vaccines (tetanus peptide, MELITAC 12.1, arms A, B; or 6 melanoma helper peptides (6MHP), MELITAC 12.6, arms C, D), and also differed in whether the patients were pretreated once with one low dose of cyclophosphamide (arms B, D) [23].

For the Mel48 trial (NCT00705640), all patients received the same vaccine of 12MP plus tetanus helper peptide (MELITAC 12.1), but differed in whether the vaccine peptides were administered at one or two sites [26, 27].

For all trials, T cell response was assessed on peripheral blood mononuclear cell samples using direct ex vivo ELISPOT assay. Requirements for a positive response included at least a doubling of CD8⁺ T cell response over background and over any preexisting response as well as an increase of at least 20 per 100,000 CD8⁺ T cells. More specifically, the following definitions were used: N_{vax} = number of T cells responding to vaccine peptide; N_{neg} = number of T cells responding to maximum negative control; and $R_{\text{vax}} = N_{\text{vax}}/N_{\text{neg}}$. An immune response to the 12MP vaccine was present if all of the following conditions were met: (1) $N_{\text{vax}} - N_{\text{neg}} >$ or equal to 20 cells/100,000 CD8⁺ T cells, (2) $R_{\text{vax}} >$ or equal to 2, (3) $(N_{\text{vax}} - 1\text{SD}) >$ or equal to $(N_{\text{neg}} + 1\text{SD})$, and (4) R_{vax} after vaccination $>$ or equal to $2 \times R_{\text{vax}}$ before vaccination.

After the vaccination period, patients underwent scheduled clinical follow-up over a maximum of 10 years based on previously described protocols [23, 24]. Disease progression during or after therapy was recorded. This was categorized by gender and age. The primary endpoint was the cumulative incidence of immune response, which was calculated as the proportion of participants mounting an immune response by ELISPOT criteria over the first 8 weeks. Secondary endpoints included overall survival (OS) and disease-free survival (DFS).

Statistical analysis

Clinical data were collected from the clinical trials database and medical record, including stage (IIB–IV), clinical trial and trial arm, CD8⁺ T cell immune response, age, and gender. A histogram was generated to assess for normality of data distributions. At ages higher than 64 years, the decreasing sample size of older patients limited the power to adequately detect differences based on age. Thus, on the basis of our histogram, we defined groups based on a cutoff of 64 years or older in the analyses.

Immune response was assessed weekly and graphed over the first 7 weeks of the study period to yield cumulative incidence plots. Time 0 refers to the administration of the first vaccine. The 7-week cumulative incidence rates of immune response were used to compare groups based on age and gender. The two age groups were defined using the 64 years of age cutoff. A subgroup analysis of female participants was performed by separating younger and older females with a cutoff at 50 years of age or older to approximate the impact of menopause on immune response. Differences in the cumulative incidence of immune response among the groups were assessed using Gray's test [28].

Kaplan–Meier survival estimates and the log-rank test were used to perform time-to-event analyses of OS

and DFS combining all study participants. Study arms were divided into five groups: Mel43+ GM-CSF, Mel 43 without GM-CSF, Mel44+ tetanus, Mel44+ 6MHP, and Mel48. A subgroup analysis was also performed of the study arms previously found to have the highest proportions of patients with immune response (Mel 43 arms without GM-CSF [25] and Mel 44 arms with tetanus helper peptide [23]). OS was assessed based on a landmark analysis, calculated as the time elapsed from the 8 weeks post-registration date to either death or loss to follow-up, while DFS was calculated as the time elapsed to death, recurrence/progression of disease, or loss to follow-up. Patients experiencing the outcome of interest (IR, OS or DFS) before the 8-week window were excluded from landmark analysis.

A multivariate logistic regression model was developed to study the association of immune response with factors of interest. The factors assessed in the immune response model included gender (female/male), stage (II, III, IV), age ($</\geq 64$ years of age), and cohort group (high- vs. low-responding study arms). The inclusion of cohort group in this analysis was to account for this known confounder, and it should be noted that the aforementioned correlation between immune response and cohort is known to be independent of the factors: age, gender and stage.

Cox proportional hazard modeling (PROC PHREG in SAS 9.4; SAS Institute, Cary, NC) was used to perform landmark time-to-event analyses of OS and DFS. Landmark analysis included predictors—gender, stage, and age as well as immune response—captured in the first 8 weeks of the study period. Significance was determined using a Wald test. The model was performed using all study participants, and then on a subset focusing on the participants corresponding to the high-responder arms.

In summary, the following outcomes with corresponding baseline predictors were used:

- (1) Outcome: IR Predictors: stage, age, gender, cohort group (high- vs. low-responder arms)
- (2a) Outcomes: OS, DFS Predictors: stage, age, gender, immune response (all participants)
- (2b) Outcomes: OS, DFS Predictors: stage, age, gender, immune response (only participants from high-responder arms (Mel43 A & C) and (Mel44 A & B))

Assumptions of the Cox proportional hazard models were assessed using graphical methods. Differences were considered significant for p values (two-sided) ≤ 0.05 .

Results

A total of 332 participants were enrolled in the trials. A total of 327 patients met the inclusion criteria for the study and were evaluated for immune response measurable by week 8. Six subjects were excluded: Two did not have ELISpot assay data, and four were omitted due to HLA type mismatch. One individual was a participant in both the Mel43 and Mel48 trials. However, this individual's ELISpot data were complete and included in this analysis only for Mel48.

The median age of the study participants was 56 years (range 15–87). Histogram representation shows a slightly left-skewed distribution of ages (Fig. 1). The number of participants decreases significantly prior to age 40 and after 70 years of age. Fifteen percent (49 of 327) were less than or equal to 39 years of age; 10 % (34 of 327) were 71 years of age or older. Based on our defined age cutoff of 64 years of age, 249 of the 327 evaluable participants were in the

younger age group, while 78 participants were in the older age group. Thirty-one percent (103 of 327) of patients were women, a roughly 2:1 ratio of males to females. Stage of disease at enrollment included 44 patients with stage IIB/IIC, 45 with stage IIIA, 176 with stage IIIB/IIIC, and 62 with stage IV. Median follow-up was 3.0 years, with a maximum of 5.5 years. Overall, immune responses were detected in 49 % of study participants by previously specified ELISpot criteria. Two cohort groups had immune response rates exceeding 70 %: Mel 43 arms without GM-CSF [25] and Mel 44 arms with tetanus helper peptide [23]. Patients in these two cohorts were combined to form the high-responder group in the subset analysis (Table 1).

The 7-week cumulative incidence rate for immune response was 53 % (130/249) in patients <64 years of age, but only 38 % (30/78) in older patients ($p = 0.03$; Fig. 2a). Subset analysis of the high-responder group showed an even more pronounced difference between age groups, $p = 0.01$ (Fig. 2b), which suggests a consistent pattern

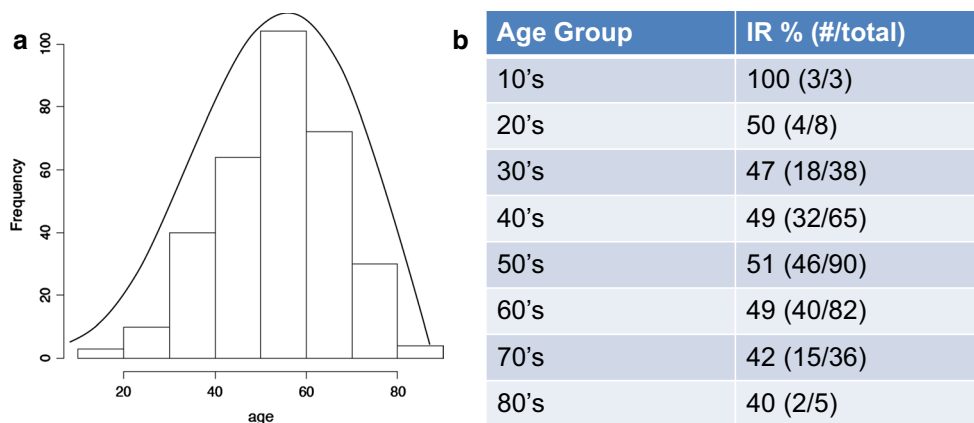


Fig. 1 Representation of age distribution of study participants. **a** Histogram of age range with superimposed line graph, **b** immune response by decade and total number of study participants per age group

Table 1 Class I MHC-restricted vaccine (12MP) trial protocols for patients with high-risk melanoma

Trial	Peptide	Adjuvant	N	Gender, M	Age, <64 years	IR+ (%)
Mel43 ^a	12MP; tetanus	IFA+ GM-CSF	58	37	47	20 (34)
		IFA	60	45	46	44 (73)
Mel44 ^a	12MP; tetanus	IFA ± cyclophos	82	53	57	58 (71)
		IFA ± cyclophos	85	59	64	18 (21)
Mel48 ^{a,b}	12MP; tetanus	IFA	42	30	35	20 (48)
Total			327 ^c	M 224	<64 years 249	+ (49)
				F 103	≥64 years 78	– (51)

MHC major histocompatibility complex, *12MP* 12 class I MHC-restricted melanoma peptides, *IFA* incomplete Freund's adjuvant, *GM-CSF* granulocyte–macrophage colony-stimulating factor, *6MHP* 6 class II MHC-restricted melanoma helper peptides, *IR* immune response

^a Ten vaccinations were administered for Mel43 and Mel44 and 6 for Mel48

^b Patients with nonmeasurable disease or equivocal findings may be enrolled in Mel48

^c One participant belonged to both trials 43 and 48

Fig. 2 Cumulative incidence of immune response, or the measure of immune response over time, over first 8 weeks of the trial period. **a** Includes all study participants by age grouping participants into either younger or older than 64 years, **b** uses only participants within the high-responder arms (Mel 43 without GM-CSF and Mel 44 with 6MHP) stratifying by age, **c** all study participants by gender, **d** includes only female patients and further stratifies them into younger or older than 50 years of age to approximate for menopause

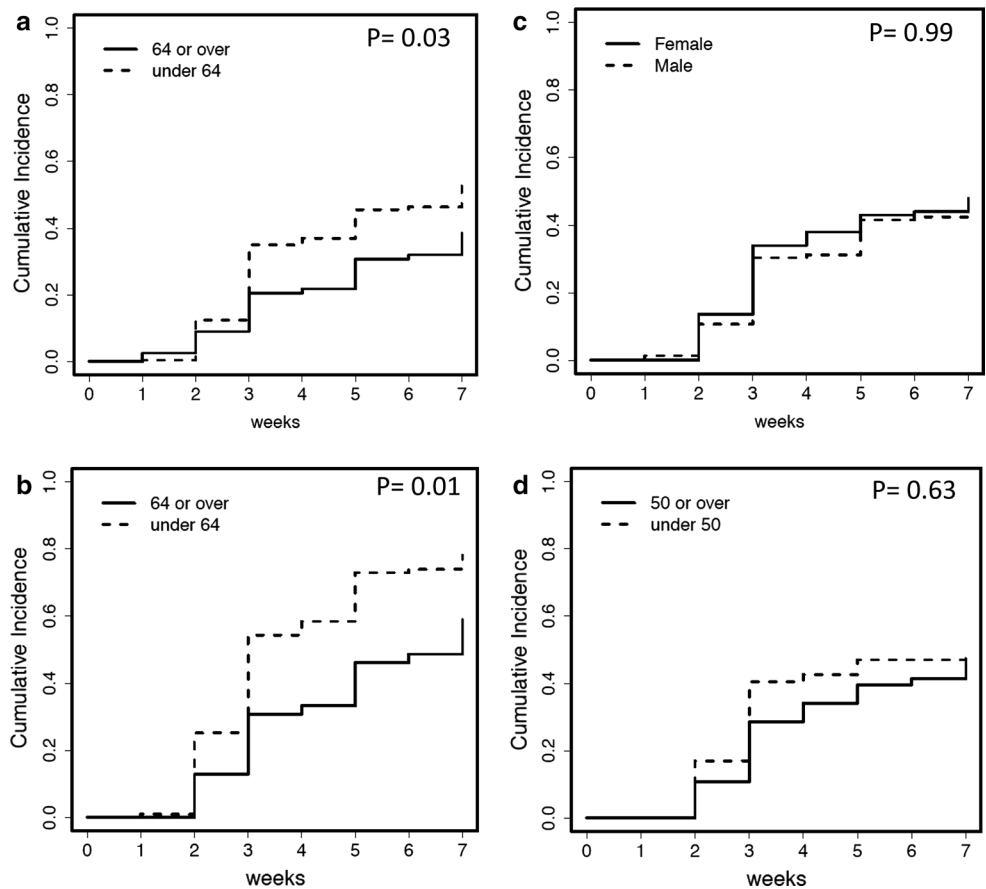


Table 2 Multivariate logistic regression modeling as a function of immune response

Parameter	Odds ratio	Confidence intervals	<i>p</i> value
Stage ^a			0.14 ^b
III	1.1	0.54–2.2	0.8
IV	0.56	0.24–1.3	0.2
III versus IV	0.52	0.27–0.99	0.047
Gender	1.2	0.7–2.0	0.47
Age	0.4	0.22–0.73	0.003
Cohort	6.6	4.0–11.1	<0.001

^a The reference group is patients with stage II melanoma unless otherwise stated

^b Cumulative *p* value for all stages

between younger patients and higher cumulative incidence of immune response regardless of clinical trial or clinical arm. The 7-week cumulative incidence rate for immune response was 50 % (111/224) in males and 48 % (49/103) in females, with no difference by gender (*p* = 0.99; Fig. 2c). Age greater than or equal to 50 years, in female patients, was used as a proxy for menopausal status. While

younger females responded earlier, the difference equilibrated over time and was not associated with a difference in the cumulative incidence for immune response (*p* = 0.63; Fig. 2d).

In a multivariate logistic regression model, younger age was again associated with a higher proportion of study participants with immune response to vaccination (OR 0.40, *p* value 0.003) while gender and stage did not predict immune response (Table 2). Cohort group referred to high-versus low-responder groups. Previously published data from our group showed a significant correlation between immune response and adjuvants and helper peptides administered with the 12MP peptides.

Associations of age and gender with OS and DFS were assessed with Kaplan–Meier estimates. No difference was detected in OS or DFS by age group (log rank *p* = 0.63 and 0.98, respectively). There was a nonsignificant trend toward improved DFS (*p* = 0.12) and OS (*p* = 0.09) for males (Fig. 3a–d). A subgroup analysis assessed low and high immune response arms for age and gender differences in OS and DFS (Supplementary Fig. 1–2). No association was shown among the subgroups. However, the individual clinical trials included in this study were not initially powered

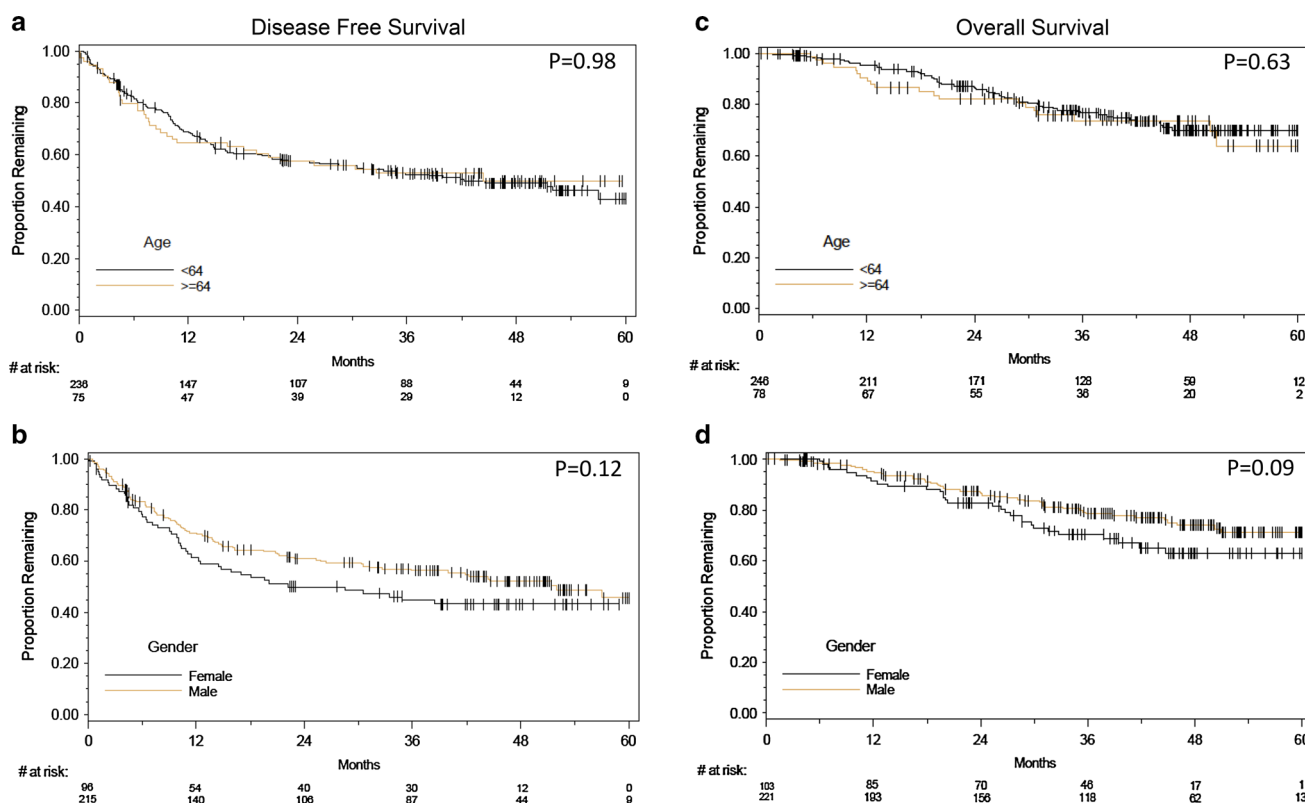


Fig. 3 Kaplan–Meier survival graphs of age (*top*) and gender (*bottom*) versus disease-free survival (DFS) and overall survival (OS) starting at 8 weeks to maximum of 60 months follow-up period. DFS

is shown on the *left column* and OS on the *right column*. **a** Association between age and DFS, **b** effect of gender on DFS, **c** age versus overall survival, **d** gender versus overall survival

Table 3 Multivariate analysis of key parameters and clinical outcomes

Parameter	Hazard ratio (DFS)	<i>p</i> value (DFS)	Hazard ratio (OS)	<i>p</i> value (OS)
Stage ^a		0.58 ^b		0.21 ^b
III	1.3	0.32	1.4	0.3
IV	1.3	0.35	0.87	0.75
III versus IV	1.0	0.91	0.6	0.12
Gender	0.76	0.12	0.66	0.07
Age	1.03	0.90	1.26	0.39
Immune response	0.93	0.69	1.05	0.83

^a The reference group is patients with stage II melanoma unless otherwise stated

^b Cumulative *p* value for all stages

with the intention of demonstrating a relationship between these variables if present.

Cox proportional hazard modeling of factors associated with DFS or OS indicated that there was no association between age and disease-free survival (HR 1.03, *p* = 0.90) or overall survival (HR 1.26, *p* = 0.39). There was a trend toward significance between male gender and improved OS (HR 0.66, *p* = 0.07). However, ultimately no difference was shown in DFS (HR 0.76, *p* = 0.12). In this dataset, immune response to the multipptide melanoma vaccine was not associated with DFS or OS (Table 3).

Discussion

As immune therapies are providing durable benefit for some cancer patients, there is an increasing need for new immune therapy combinations that may increase the proportion of patients who benefit from therapy. Further, there is a need for criteria to select the best therapeutic options for each patient. Cancer vaccines may expand the proportion of patients who benefit from checkpoint blockade therapy. Checkpoint blockade therapy typically works for patients who already have antigen-specific T cell responses;

new cancer vaccines targeting relevant differentiation antigens or neoantigens may induce or expand T cell responses in patients who otherwise would not benefit from checkpoint blockade alone.

Elderly patients are sometimes excluded from vaccine trials because of concern that they may be unable to mount an immune response; however, systematic analysis of CD8 T cell response to cancer vaccines as a function of age has been lacking. Our data support the hypothesis that a decreased proportion of older patients are able to elicit immune responses to cancer vaccines; however, it is noteworthy that immune responses were still detected in a significant subset of older patients (38 %, compared to 52 % for younger patients). Also, for those patients enrolled in study arms that had higher overall immunogenicity, the fraction of immune response rates seen in older patients approached 59 %, compared to 77 % for younger patients as shown in Fig. 2b.

Interestingly, after adjusting for common confounders, older patients did not have poorer clinical outcomes in this study. Despite overall improvements in outcome measurements in the last two decades, the elderly population has not benefited from technological advancements at the same rate as their younger counterparts. This discrepancy has been replicated in nearly all of the most common cancers [29, 30]. There are several explanations for this well-accepted notion and our study findings. Most studies have been based largely on administrative databases offering little clinical context and ability for risk adjustment. This significantly limits the analysis as the elderly have higher risk-adjusted morbidity and mortality. AlHilli et al. [31] argue that the effect of age is inconsequential once co-morbidities and other known risk factors are adjusted for in patients with endometrial cancer. Furthermore, the authors allude to varying cancer treatment regimens among younger and elderly patients, a practice that is not restricted to endometrial cancer alone [32, 33]. Studies that directly measure the effects of aggressive cancer therapies on clinical outcomes in the elderly are limited. This, combined with common hesitation to risk higher rates of complications underscores the need to include this patient group in clinical trials to improve patient selection and to form better predictive models. For the present study, enrollment on the clinical trials required meeting inclusion and exclusion criteria that ensured performance status of 0–1, absence of severe heart disease, and good organ function. Thus, elderly patients with declining functional status were not included. Thus, the conclusions of this report are relevant to elderly patients with good functional status, but may not apply to debilitated older patients.

Our results suggest that it may be reasonable to stratify patients on vaccine trials based on an age cutoff of about 64 years. However, even among older patients, there is a

significant probability of CD8⁺ T cell response to a multi-peptide vaccine. Thus, there does not appear to be evidence at this time to limit eligibility of study participants based on age alone. Further studies are necessary to determine the factors that predict an immune response in the elderly to guide patient selection and improve rates of response. The cutoff of 64 years in our study, between younger and older patients, also does not address whether there may be an older age beyond which immunogenicity falls off precipitously. However, there were immune responses in patients of all decades of life evaluated from teenage years into the 80 s. We were not able to identify an age beyond 64 years for which immune responses were not detected, but acknowledge that if there had been larger numbers of patients over age 70, the immune response rate estimate may have been precise enough to detect a decrement in immunogenicity for the more elderly group.

Female gender is commonly thought to be immunoprotective [19, 20, 34]. However, our study found that gender did not significantly impact immune response and was not associated with improved clinical outcome. The trend toward improved survival in males is an unexpected finding, as males with melanoma generally have significantly worse outcomes than females, stage-for-stage [35, 36]. Gender differences in melanoma histology have been reported, with males having higher rates of ulceration and a more frequent absence of tumor-infiltrating lymphocytes [36]. Those findings suggest a more aggressive tumor biology in males [37]. There is also evidence of gender differences in T lymphocyte infiltration in other human tissues, primarily in the cardiovascular literature involving hypertension, vascular/endothelial injury, oxidative stress and shock [38]. Thus, there may be complex and interacting gender-related differences in immune function and tumor biology that explain the trend toward improved overall survival without a concurrent difference in immune response rate after vaccination. Ongoing investigations are pursuing differences in sex hormones and the effect on the cytokine profile and microenvironment [16, 21]. However, the summary findings of this study are that there were not significant differences in immune response or clinical outcome between males and females receiving a multi-peptide vaccine. Thus, these data support evaluation of immune responses to cancer vaccines independent of patient gender.

The study has both strengths and limitations. Data were derived in a prospective manner despite being reviewed retrospectively. To our knowledge, the combined data from the participants of three clinical trials lead to the largest patient volume in humans assessing gender and age differences to a multi-peptide melanoma vaccine. All patients in these three studies were vaccinated with 12MP, and the immune responses were all evaluated in the same manner by the same laboratory. However, the vaccine adjuvants

and helper peptides used varied between the trials and study arms. They included GM-CSF, cyclophosphamide, and 6MHP versus tetanus peptide. Subsequent analysis revealed 6MHP and GM-CSF, but not cyclophosphamide, to have independent effects on CD8⁺ T cell responses to the 12MP vaccine. It is possible that these variables may have diluted our results; however, the higher fraction of immune response rates seen in younger patients were observed consistently across all studies and study arm subsets, and there was no detectable trend to an impact of gender on CD8⁺ T cell response in study subsets. Additional host factors could have also contributed to results, such as co-morbidities, homeopathic medicine use, and nutritional status; however, the entry criteria excluded patients with major co-morbidities.

Conclusions

In summary, a multipeptide vaccine can be administered to patients regardless of gender or age, with expectation of immunogenicity in all patient subsets. We do recommend considering stratification of patients by age when immune response is the primary endpoint. The ability to mount a response was observed at age 64 suggesting this to be a reasonable age cutoff. As cancer vaccines are optimized and combined effectively with other immune therapies, these findings support study of such combination immunotherapies, even in older patients, whose tumors commonly lack BRAF mutations, and thus lack good targeted therapy options.

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Compliance with ethical standards

Conflict of interest Craig Slingluff is an inventor of several peptides included in the vaccine that was administered during the clinical trials studied within this paper. The University of Virginia Licensing and Ventures Group holds the patents for those peptides, which have been licensed through the Ludwig Institute for Cancer Research to Glaxo Smith Kline. The remaining authors have nothing to disclose or competing interests in association with this study.

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