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Limited Induction of Tumor Cross-Reactive T Cells without a Measurable Clinical Benefit in Early Melanoma Patients Vaccinated with Human Leukocyte Antigen Class I–Modified Peptides

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Abstract

Purpose: The progressive immune dysfunctions that occur in patients with advanced melanoma make them unlikely to efficiently respond to cancer vaccines. A multicenter randomized phase II trial was conducted to test whether immunization with modified HLA class I tumor peptides in the context of adjuvant therapy results in better immunologic responses and improved clinical outcomes in patients with early melanoma (stages IIB/C-III).

Experimental Design: Forty-three patients were enrolled to undergo vaccination ($n = 22$) or observation ($n = 21$). The vaccine included four HLA-A*0201–restricted modified peptides (Melan-A/MART-$1_{[27L]}$, $gp100_{[210M]}$, NY-ESO-$1_{[165V]}$, and $Survivin_{[97M]}$) emulsified in Montanide ISA51 and injected subcutaneously in combination with cyclophosphamide (300 mg/m^2) and low-dose IL-2 (3×10^6 IU). The immune responses were monitored using *ex vivo* IFN-γ–ELISpot, HLA/multimer staining, and *in vitro* short-term peptide sensitization assays.

Results: Vaccination induced a rapid and persistent increase in specific effector memory CD8$^+$ T cells in 75% of the patients. However, this immunization was not associated with any significant increase in disease-free or overall survival as compared with the observation group. An extensive immunologic analysis revealed a significantly reduced cross-recognition of the corresponding native peptides and, most importantly, a limited ability to react to melanoma cells.

Conclusions: Adjuvant setting is an appealing approach for testing cancer vaccines because specific CD8$^+$ T cells can be efficiently induced in most vaccinated patients. However, the marginal antitumor activity of the T cells induced by modified peptides in this study largely accounts for the observed lack of benefit of vaccination. These findings suggest reconsidering this immunization strategy, particularly in early disease. *Clin Cancer Res; 18(23); 6485–96. ©2012 AACR.*

Introduction

Evidence suggests that only a small subset of patients entering cancer vaccine trials may actually benefit from treatment (1, 2). This remains a major limitation and underscores the need for more effective immunization strategies. The limited success of vaccine trials has been

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Translational Relevance

Cancer vaccines are frequently based on modified peptides, or altered peptide ligands (APL), to enhance their immunogenicity. Despite the large clinical application, this strategy has rarely shown convincing clinical efficacy in patients with advanced cancer. To assess whether modified tumor peptides could be more successful in early disease, we designed a randomized phase II trial to test adjuvant vaccination in patients with stages IIB/C-III melanoma. These patients showed no significant improvement in disease-free survival or overall survival when undergoing immunization compared with controls. An extensive immunomonitoring revealed that patients' T cells that had been efficiently primed by the modified peptides were only marginally reactive in the presence of cognate native epitopes and showed low or absent cross-recognition of melanoma cells. Our results underline the importance of monitoring true antitumor activity in vaccine-induced immune responses and suggest that APL-based vaccines require serious and definitive reconsideration as a treatment strategy.

associated with mechanisms that interfere with immunization as tumors progress, implying that tumor vaccines cannot reach their maximal potency in the presence of metastatic/bulky cancers (3). Indeed, patients with advanced melanoma display systemic accumulation of $CD14^{+}CD11b^{+}HLA\text{-}DR^{neg/low}$ myeloid-derived suppressive cells (MDSC) that are associated with impaired immune responses to cancer vaccines (4, 5). We previously reported that regulatory T cells (Tregs) expressing LAG-3, which potentiates immune suppressive activity, also accumulate in the peripheral blood and tumor lesions of patients with metastatic melanoma (6). Correspondingly, patients with earlier forms of cancer may be more likely to display proper immune responses, resulting in more effective tumor control upon vaccination. Thus, we designed a phase II randomized trial comparing multipeptide vaccination to observation alone in patients with stage IIB/C and stage III melanoma to determine whether a cancer vaccine could induce T-cell responses capable of preventing or delaying recurrence after radical surgery. To maximize the effect of vaccination, different immunologic features of the vaccine schedule were carefully addressed. Because immunization with multiple epitopes is more effective than single-epitope vaccines (7) and reduces the risk of antigen loss due to tumor immunoselection (8, 9), we chose a cocktail of 4 human leukocyte antigen (HLA)-A*0201–restricted peptides that were derived from highly expressed tumor antigens (e.g., MelanA/MART-1, gp100, and NY-ESO-1), including molecules involved in neoplastic transformation (e.g., survivin; ref. 10). Because of the poor immunogenicity of tumor self proteins due to immune tolerance (8), the peptides were used as altered peptide ligands (APL), which contain single amino acid substitutions that improve the affinity of the peptides for the HLA peptide-binding site and increase immunogenicity (11–13). The vaccine was administered in combination with low-dose cyclophosphamide to negatively affect Treg cell function (14, 15) and a later low-dose IL-2 to promote T-cell expansion (16).

This study reports the end-of-study immunologic and clinical data showing that the administration of an APL-based vaccine to patients with early melanoma induces rapid and persistent specific T-cell responses in vaccinated individuals compared with those of controls but does not result in any measurable clinical benefit. Systematic and detailed analyses of vaccine-induced immune responses showed that only a small number of T lymphocytes effectively cross-recognized melanoma cells. This limitation is likely the major factor responsible for the lack of benefit from APL vaccination.

Patients and Methods

Study design and endpoints

This report describes an open-label phase II-randomized study of a multipeptide vaccination in HLA-A*0201 patients with early melanoma (stages IIB/C and stage III) that compared APL vaccination with observation alone. The treatment schedule is shown in Supplementary Fig. S1. The enrollment of 160 patients was calculated to provide more than 80% power (83%) to detect a gain in 3-year disease-free survival (DFS) from 60% in the observation arm to 80% in the experimental arm (a difference that reflects a HR of 0.43). However, the trial was formally closed on July 21, 2008, because the manufacturer (Clinalfa Merck Biosciences AG) that initially provided the peptide batches with a 3-year validity GMP certificate pending yearly retesting was sold and discontinued any further certificate release.

Patients were selected after their primary tumor removal and were enrolled at the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan ($n = 37$), Azienda Ospedaliera Universitaria Senese ($n = 4$), and the Istituto Oncologico Veneto of Padova ($n = 2$). The protocol was conducted in compliance with the Declaration of Helsinki and was approved by the ethical committees of each institution. Written informed consent was obtained. The primary endpoint was to evaluate specific T-cell immunity against vaccine peptides and against HLA-A*0201 allogeneic melanoma cell lines expressing vaccine antigens. Secondary endpoints were to evaluate DFS and overall survival (OS) at 2 years and the potential association between immune responses and clinical outcome.

Patients and inclusion criteria

Patients with histologically confirmed (American Joint Committee on Cancer) stage IIB/C and stage III resectable melanoma (Eastern Cooperative Oncology Group score 0–1) and normal hematopoietic, liver, and renal function were eligible. Patients were checked for HLA-A*0201 expression using the OLERUP SSP HLA Kit (Qiagen S.p.A). The patient demographic and clinical data are shown in

Supplementary Table S1. Of the 22 patients randomized to the vaccine arm, 20 completed the 2 treatment cycles, whereas 1 patient (INT-030) dropped out due to noncompliance, and the second patient (INT-021) died before the second vaccination. These latter patients were included in the DFS and OS evaluations but were excluded from the immunologic analyses.

Vaccine preparation and administration

The APLs (MelanA/MART-$1_{[27L]}$, $gp100_{[210M]}$, NY-ESO-$1_{[165V]}$, and $Survivin_{[97M]}$; 250 μg of each peptide) were emulsified in Montanide ISA51 (0.5 mL, Seppic) and injected subcutaneously close to the inguinal or axillary lymph nodes. The peptides (>95% pure) were synthesized under GMP conditions by Merck Biosciences AG Clinalfa. The vaccine, cyclophosphamide, and IL-2 schedules are detailed in Supplementary Fig. S1. Toxicity was documented according to the World Health Organization common toxicity criteria. The vaccine was generally well tolerated (17). Mild nausea was observed upon cyclophosphamide administration, whereas IL-2 administration was occasionally associated with transient fever (invariably of low grade), headache, fatigue, and arthralgia.

Peripheral blood mononuclear cells and immunomonitoring

Blood (50 mL) was obtained from each patient at the time points indicated in Supplementary Fig. S1. Peripheral blood mononuclear cells (PBMC) were isolated within 3 hours after blood drawn by Ficoll gradient centrifugation using 50 mL Leucosep tubes (Greiner Bio-One GmbH) without whole-blood dilution followed by cryopreservation. The cells were stored in the vapor phase of a liquid nitrogen vessel until further use.

Immunomonitoring was conducted at the Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy) using an *ex vivo* INF-γ–ELISpot assay (1-D1K, Mabtech AB) according to our standard operating procedure (SOP;

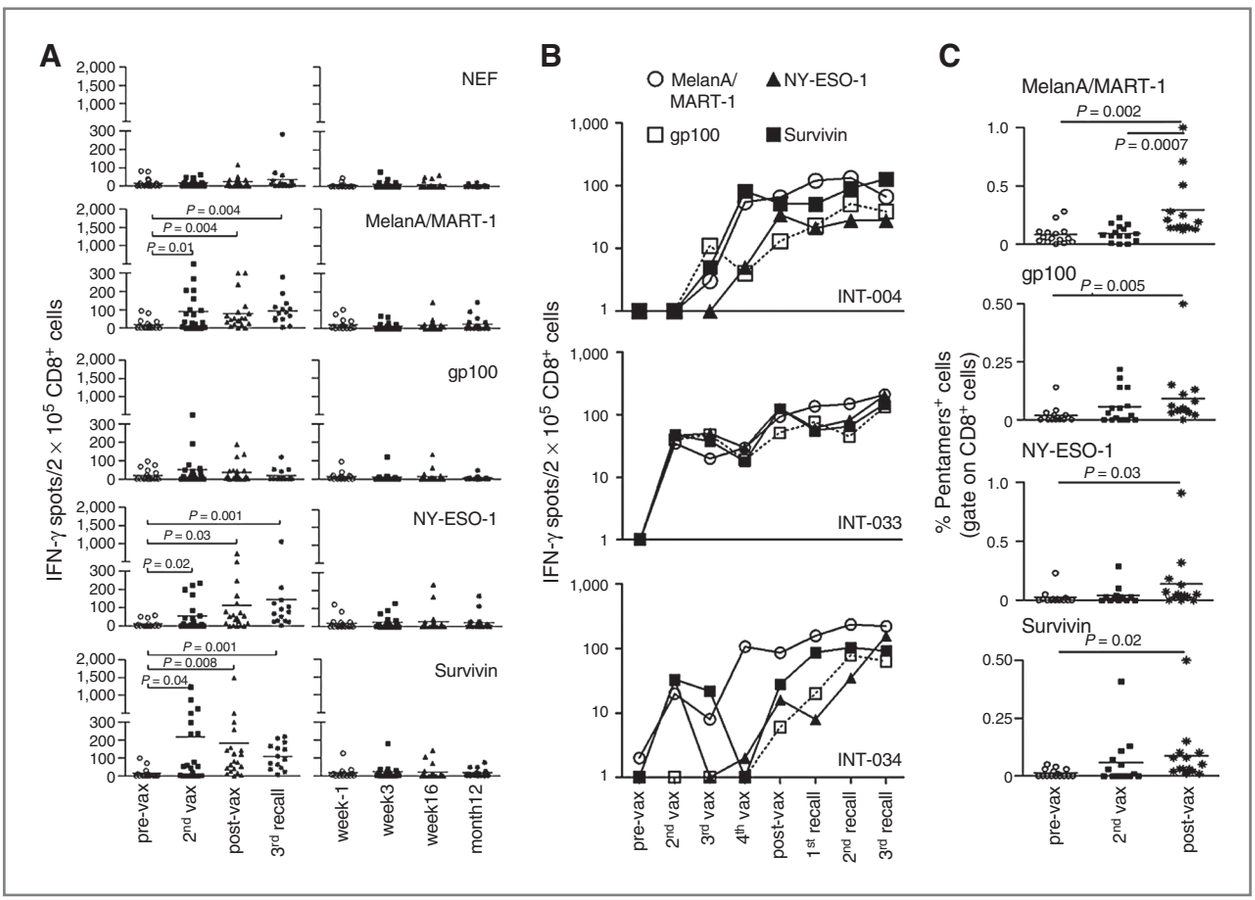

Figure 1. *Ex vivo* evaluation of immune responses. A, unfractionated PBMCs from patients in both arms of the study (left, vaccination, $n = 20$; right, observation, $n = 21$) were tested at the indicated times by ELISpot for IFN-γ release in response to vaccine peptides or the $NEF_{[180–189]}$ peptide as a negative control. Dots represent the results from individual patients (horizontal line, mean value). B, immune response kinetics in 3 representative vaccinated patients as detected by IFN-γ–ELISpot assay. C, freshly thawed PBMCs originally obtained at the indicated times were tested *ex vivo* for binding to HLA-A*0201/peptide multimers and analyzed by flow cytometry. Background results with the HLA-A*0201/HIVgag24 multimer were below 0.01% (not shown). Dots represent results from 20 individual patients (horizontal line, mean value). The nonparametric 2-tailed Wilcoxon-matched pairs test was used to calculate the *P* values (95% CI).

ref. 18). Briefly, after incubating thawed PBMCs overnight at 37°C, the cells were washed, resuspended in complete medium (RPMI + 10% FCS), and seeded in 4 replicate wells at a density of 3×10^5 cells per well in a Multiscreen 96-well plate (MAIPSWU10, Millipore) coated with the anti-IFN-γ antibody (1-D1K, Mabtech). Furthermore, antibody incubations and development of the ELISpot assay were conducted according to the manufacturer's instructions (Mabtech). As a positive control, the PBMCs were incubated with an HLA-1–restricted CEF peptide pool (Cytomegalovirus, Epstein-Barr virus and Flu virus; 3615-1, Mabtech).

To assess interassay variability, the melanoma-specific T-cell clone A42 (18) was cultured on a large scale, aliquoted (1,000 cells/vial), and stored in liquid nitrogen. An aliquot was thawed and tested in each ELISpot plate. The number of spots per well obtained in the presence of the autologous melanoma cells was highly reproducible (102 ± 11). In addition, all of the ELISpot reagents used for the entire immunomonitoring analysis belonged to the same production lots. The spots were counted with an ELISpot Reader Instrument (Aelvis-Tema, Germany). Results are presented as the number of APL-reactive cells per 2×10^5 $CD8^+$ cells (measured by double CD3/CD8 immunostaining on the same aliquot of cryopreserved cells). The ELISpot assay was considered positive when 3 criteria were met: (i) the number of APL-reactive $CD8^+$ T cells in the postvaccine PBMC population was more than 10; (ii) the number of spots was at least 2 times higher than the number of spots detected using an HIV-derived control peptide ($NEF_{[180–189]}$); and (iii) statistically significant (Student *t* test; $P \leq 0.05$).

HLA-A*0201 multimers, including the irrelevant HLA-A*0201/HIV-p24 peptide (HIVgag24; TLNAWVKVV), were provided by Proimmune Ltd. Fluorochrome-conjugated anti-CD3, anti-CD8, anti-CD45RA, anti-CCR7, anti-CXCR3, and anti-CXCR1 monoclonal antibodies (mAb) were from BD Biosciences. The staining was conducted according to the manufacturer's instructions. Cell samples were acquired (range, 2–3 × 10^5 $CD8^+$ cells) on a BD FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc.).

In vitro short-term peptide sensitization was carried out by stimulating the thawed PBMCs with vaccine APLs (2 μmol/L) plus 60 IU/mL IL-2 (Proleukin). The cells were tested after 10 to 14 days using an IFN-γ–ELISpot assay in the presence of APL-pulsed (2 μmol/L) T2 cells, HLA-A*0201$^+$ melanoma lines (MelanA/MART-1$^+$gp100$^+$NY-ESO-1$^+$Survivin$^+$), or C1R-A2-survivin$^+$ cells (an HLA-A/B-deficient B-lymphoblastoid cell line transfected with HLA-A*0201 and survivin). HLA-blocking experiments required target preincubation with anti-HLA-ABC (immunoglobulin-M) or anti-HLA-DR (L243) mAbs (hybridomas from American Type Culture Collection). Postvaccination, T-cell cultures were enriched for $CD8^+$HLA-A*0201/multimer$^+$ cells using positive immunomagnetic selection (CD8 MicroBeads, Miltenyi Biotec GmbH) or with a BD FACSAria Cell Sorting System (BD Biosciences). Granzyme B secretion levels were determined using a PeliSPOT human Granzyme B kit (Sanquin Reagents). The laboratory of the Unit of Immunotherapy of Human Tumors at the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan is a research laboratory in which the assays are conducted by well-trained personnel according to the SOP that include predefined criteria for positive responses. The MDSCs were identified according to the phenotype recently described (4), whereas the frequency and function of the Tregs were also monitored and will be described elsewhere (Camisaschi and colleagues, submitted for publication).

Confocal microscopy analysis

T2 target cells were pulsed with 2 μmol/L of either APL or wild-type peptides (NY-ESO-1 and survivin) for 1 hour at 37°C and were washed twice in PBS (BioWhittaker). Sorted $CD8^+$NY-ESO-$1^+_{[165V]}$ and $CD8^+$Survivin$^+_{[97M]}$ T-cell cultures were mixed 1:1 or 5:1 with their target cell lines: T2 and 624.38mel, respectively. After 1 hour at 37°C, the cells were treated with Brefeldin-A (10 μg/mL; Sigma-Aldrich), cultured for an additional 4 hours, plated on glass multiwell slides (Lab-Tek II CC^2, Nalgene Nunc International), and fixed with 4% paraformaldehyde for 14 minutes at room temperature. The cells were then treated with 0.1 mol/L glycine in PBS (pH 7.4) followed by 0.3% Triton X-100 buffer and double labeled overnight at 4°C with rabbit anticathepsin D Ab (1:200; Calbiochem, San Diego) and mouse anti-IFN-γ Ab (1:50; Genzyme Co.). Binding of the primary antibodies was monitored by the addition of Alexa Fluor 488-conjugated donkey antimouse (1:200; Invitrogen) and rhodamine-conjugated donkey antirabbit (1:100; Rockland) Abs. TOTO-3 iodide 642/660 (Invitrogen) was used for nuclei staining. The slides were mounted in PBS containing 95% glycerol. The samples were examined using a Radiance 2100 laser scanning confocal microscope (Bio-rad Laboratories) equipped with a krypton/argon laser and a red laser diode. To reduce bleed through, the confocal images were acquired sequentially. Noise reduction was achieved by using a Kalman filter during acquisition.

Bead-based multiplex immunoassay

In some experiments (see Supplementary data), the supernatants were tested for cytokine release (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, TNFα, and TNFβ) using a bead-based multiplex immunoassay for the flow cytometer (Bender MedSystems), which allows for the simultaneous detection of multiple analytes in a single sample.

Statistics

Differences between the ELISpot and HLA-multimer assay values obtained at the indicated times were compared using the nonparametric 2-tailed Wilcoxon-matched pairs test [confidence interval (CI) 95%]. Statistical analyses for the other *in vitro* functional assays (Figs. 3–5) were conducted using an unpaired Student *t* test. The probabilities of DFS and OS were estimated using the Kaplan–Meier method and tested for differences by the log-rank test.

Values of $P \leq 0.05$ were considered statistically significant. Statistical calculations were carried out using Prism5 (GraphPad Software) and SAS software.

Results

Patients with early melanoma develop rapid and persistent T-cell responses to the APL vaccine

This multicenter phase II randomized study enrolled 43 patients with HLA-A*0201$^+$ stage IIB/C and stage III melanoma (Supplementary Table S1) who underwent vaccination ($n = 22$) or observation ($n = 21$; Supplementary Fig. S1).

First-line monitoring of vaccine-induced T cells was carried out *ex vivo* using IFN-γ–ELISpot assays and HLA-A*0201/peptide multimer (HLA-multimer) staining. As shown in Fig. 1A (left), the initial mean prevaccination frequency of CD8$^+$ T cells responding to each APL was below the detection threshold but progressively increased after immunization (for details, see Supplementary Table S2). The mean spot numbers per 2×10^5 CD8$^+$ T cells achieved after 6 vaccinations were 78 ± 92 for MelanA/MART-1, 37 ± 53 for gp100, 144 ± 268 for NY-ESO-1, and 184 ± 332 for survivin. This increase was lower for the gp100 peptide than for the other peptides. No change in the recognition of the irrelevant peptide NEF$_{[180–189]}$ was observed, showing the specificity of the immune responses induced by APL immunization. The frequency of APL-specific T cells in the PBMCs from control patients did not change (Fig. 1A, right). As shown in Fig. 1B, T-cell responses developed rapidly in the vaccinated patients (beginning after the second or third injection) and were detectable in circulation throughout the treatment period (12 months as the latest time-point tested in the majority of the patients; see Supplementary Table S2).

Although between-patient variability was observed in the frequency and magnitude of the immune response, an estimated 75% of the patients responded to at least one epitope, whereas 65% responded to 2 or more peptides when evaluated according to the response criteria described in Patients and Methods. Expansion of APL-specific CD8$^+$ T cells in vaccinated subjects was confirmed by HLA-multimer staining, and the frequencies after 6 vaccine injections were 0.29 ± 0.26% for MelanA/MART-1, 0.1 ± 0.12% for gp100, 0.13 ± 0.24 for NY-ESO-1, and 0.08 ± 0.09 for survivin (Fig. 1C). The baseline values (all below the assay detection limit of ≤0.01%) observed in the control patients remained the same (data not shown).

The remarkable immune responses induced by APL immunization indicated that patients with early melanoma may be more suitable for immunization compared with patients with advanced disease (2, 19). Indeed, parallel analyses conducted during the study showed that the baseline frequencies of CD4$^+$CD25$^+$Foxp3$^+$ Tregs and MDSCs, identified according to the CD14$^+$CD11b$^+$HLA-DR$^{neg/low}$ phenotype recently described (4), were significantly lower among the PBMCs of patients with stage IIB/C and stage III than the frequencies detected in patients with stage IV recruited for a separate trial (ref. 20; data not shown).

The frequency of either Treg cells or MDSCs did not change significantly upon the administration of low-dose cyclophosphamide. As expected, the late administration of low-dose IL-2 caused a rapid and transient increase in Treg frequencies that did not interfere with the ability of the vaccine to induce peptide-specific CD8$^+$ T cells (Camisaschi and colleagues, manuscript submitted for publication).

APL immunization does not affect DFS or OS in vaccinated patients compared with control patients

As of December 2010, the median follow-up duration was 46 months. Of the 22 patients in the vaccine arm, 10 showed disease recurrence, and 5 eventually died. In the control arm, 10 of the 21 patients showed disease recurrence, and 3 eventually died.

The DFS and OS curves are shown in Fig. 2. For both endpoints, the log-rank test failed to show any significant difference between the 2 arms ($P = 0.7877$ for DFS and $P = 0.4803$ for OS). A mild survival advantage was

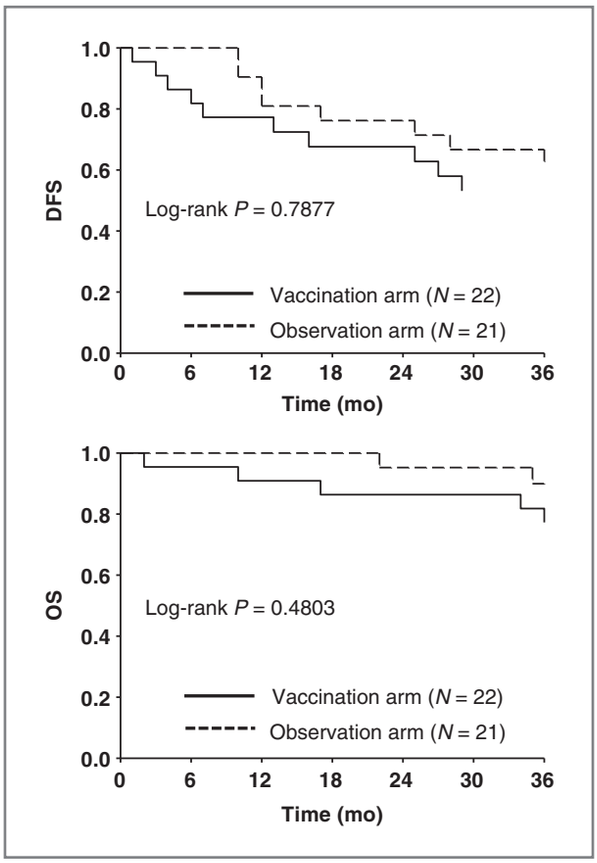

Figure 2. Survival outcomes for patients with melanoma in the study. Kaplan–Meier DFS and OS curves for the 43 patients with stage IIB/C and stage III melanoma enrolled in the study (solid line, vaccine, $n = 22$; dotted line, observation $n = 21$).

apparent for the control arm of up to approximately 40 months but was no longer evident beyond that time. At 60 months, the DFS and OS estimates were 50% and 77%, respectively, in the vaccination arm and 42% and 79%, respectively, in the control arm. The HR estimate (95% CI) for comparing the 2 arms was 1.25 (0.53–2.94); notably, the lower confidence limit excluded the target value (0.43) around which the study was designed, as described in Patients and Methods.

Despite the small number of enrolled patients, we assessed whether the course of the disease could have been influenced by immune-related parameters. As depicted in Supplementary Fig. S2, no significant correlation was detected at baseline between DFS and the frequencies of MDSC, Treg, or anti-CEF T cells (in both vaccinated and control patients), although patients with increased MDSCs and Tregs or decreased CEF-specific $CD8^+$ T cells tended to have a worse disease course. Interestingly, a more favorable DFS trend was evident in the vaccinated patients who mounted a more pronounced immune response, as defined by a response against at least 3 antigens. These data suggest that the ability to respond to self and foreign antigens represents a potential prognostic factor in early melanoma and might play a key role in disease control.

Peptide vaccination promotes *in vivo* expansion of effector memory T cells with limited affinity for wild-type epitopes and poor melanoma cross-recognition

Because the lack of clinical benefit may be due to functional defects in the vaccine-induced immune response, phenotypic analysis of postvaccine PBMCs was conducted, and the analysis showed that the APL-specific T cells were mostly confined to the effector memory compartment, with $CD45RA^-CCR7^-$ cells representing about 40% of the MelanA/MART-1^+ and $gp100^+$ T cells, 70% of the NY-ESO-1^+ T cells, and 60% of the $Survivin^+$ T cells (Fig. 3A). These cells were also able to infiltrate the tumor lesion *in vivo*, as suggested by the expression of the CXCR1 and CXCR3 chemokine receptors (Supplementary Fig. S3). In addition, the postvaccine PBMCs behaved *in vitro* as secondary immune responsive cells with prompt expansion following short-term exposure to APLs. No significant peptide specificity was detected in either the T-cell cultures from prevaccine PBMCs or control patient samples obtained at equivalent time-points (Supplementary Fig. S4), showing that the detected APL-immune memory was specifically induced by immunization.

To systematically address the ability of the APL-sensitized T lymphocytes to actually recognize tumor cells, the $CD8^+$ T cells were sorted from postvaccine APL-sensitized PBMCs and first tested for wild-type peptide cross-reaction. The MelanA/MART-1- and gp100-specific $CD8^+$ T cells secreted comparable IFN-γ levels upon exposure to APLs and wild-type peptides saturating doses (>1 μg/mL), although significantly lower levels of IFN-γ were produced upon exposure to wild-type epitopes at medium and low concentrations (<100 ng/mL; mean EC_{50} APL vs. wild-type: 3.98 ± 6.84 vs. 150 ± 254 and 0.06 ± 0.04 vs. 1.77 ± 0.92 for MelanA/MART-1 and gp100, respectively; Fig. 3B, A and B). NY-ESO-1- and survivin-specific $CD8^+$ T cells efficiently recognized the cognate APLs (mean EC_{50} values: 9.3 ± 5.8 and 25 ± 38.7, respectively) but displayed reduced affinity for the wild-type epitopes, even at saturating doses (mean EC_{50} values: 521 ± 518 and 426 ± 387, respectively; Fig. 3B, C and D).

The limited cross-recognition of wild-type peptides implied that the APL vaccine-induced T cells displayed poor affinity for tumor cells expressing the cognate antigens. Because no melanoma recognition was detected in the *ex vivo* postvaccine PBMCs (data not shown), APL-sensitized T cells were used as effectors in the presence of a melanoma cell line that expressed high levels of HLA-A*0201 and all of the relevant melanoma antigens (624.38mel; data not shown). MelanA/MART-1- and gp100-specific T cells produced detectable levels of IFN-γ in response to 624.38mel, but this reactivity represented only a small percentage of the cytokines secreted in the presence of the APLs (Fig. 4A). The limited cross-reactivity with tumor cells was even more pronounced with the anti-NY-ESO-1 and antisurvivin T lymphocytes, which did not react to 624.38mel (Fig. 4A) or to a large panel of melanoma lines or HLA-A*0201 Ag-transfected cells (Fig. 4B). In agreement with these results, none of the APL-specific T cells released IL-2 or TNFα in the presence of 624.38mel unless previously pulsed with exogenous APL (Supplementary Fig. S5). These findings, in addition to confirming the lack of tumor cross-recognition by APL-specific T cells, also rule out any potential immunosuppressive effect exerted by the 624.38mel cells on the T lymphocytes.

To further examine the potential melanoma recognition by the anti-NY-ESO-1 and antisurvivin $CD8^+$ T cells, these effectors were sorted for their HLA/multimer binding to reach more than 95% purity. However, no substantial improvement of the wild-type epitope or melanoma cell recognition was achieved (Fig. 5A). In contrast, the melanoma cells triggered IFN-γ secretion when pulsed with saturating doses of exogenous wild-type peptides, confirming the intrinsic low affinity of the T-cell receptors (TCR) expressed by the APL-specific T cells for naturally expressed tumor epitopes. As shown in Fig. 5B and C, comparable data were obtained when anti-NY-ESO-1 and antisurvivin $CD8^+$ T lymphocytes were assessed for granzyme B secretion and for intracellular localization of cytotoxic granules and IFN-γ in T-cell–APC conjugates. Indeed, when APL-pulsed T2 cells were coincubated with anti-NY-ESO-1 and antisurvivin $CD8^+$ T-cell cultures, lytic granules and IFN-γ polarized at the immunologic synapse in the majority of the detected T-cell/target conjugates (63% and 68%, respectively). In contrast, the ability of, anti-NY-ESO-1 and antisurvivin $CD8^+$ T-cell cultures to polarize at the contact zone with tumor cells was drastically reduced (11.3% and 13.3%, respectively).

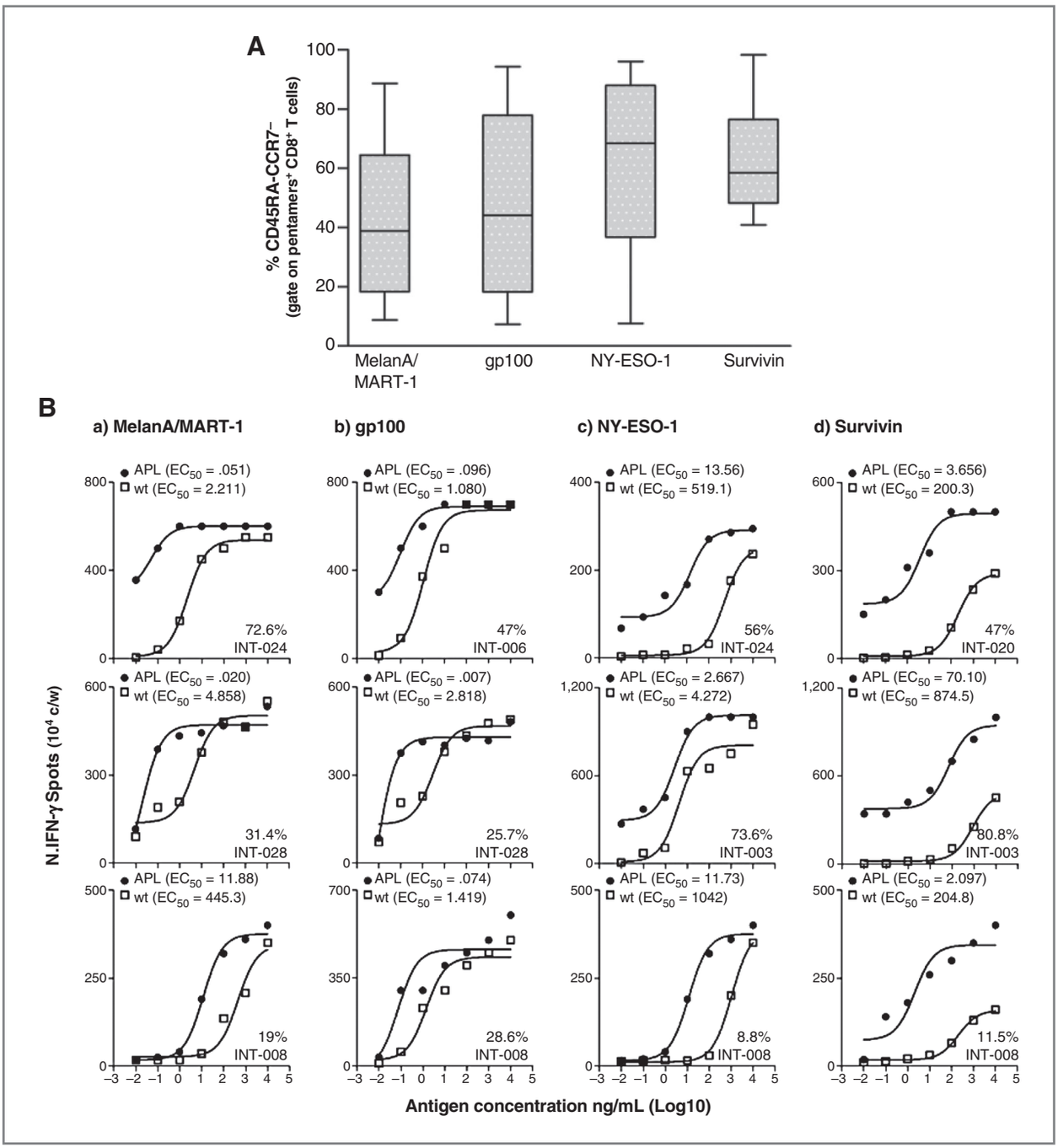

Figure 3. The APL vaccine mediates *in vivo* expansion of effector memory T cells with poor affinity for wild-type epitopes. A, freshly thawed postvaccine (sixth vaccination) PBMCs were gated for $CD8^+$/HLA-multimer$^+$ T cells and further investigated for CCR7 and CD45RA expression. Bars, box-and-whisker diagrams ($n = 13$). Horizontal line, median. B, the TCR affinity of postvaccine APL-sensitized PBMCs was evaluated by IFN-γ–ELISpot assay in the presence of titrated doses of APLs or wild-type peptides. Three representative cases are shown for each vaccine APL (MelanA/MART-1: INT-024, INT-028, and INT-008; gp100: INT-006, INT-028, and INT-008; NY-ESO-1: INT-024, INT-003, and INT-008; survivin: INT-020, INT-003, and INT-008). Numbers refer to the percentages of HLA-A*0201/multimer$^+$$CD8^+$ T cells in the tested T-cell cultures. Nonlinear curve fitting analysis and EC_{50} (index of functional affinity) calculations were carried out using Prism5 Graphics software.

Discussion

The multicenter randomized phase II trial presented here was designed to test whether a vaccine consisting of a cocktail of APLs from MelanA/MART-1, gp100, NY-ESO-1, and survivin antigens could reduce recurrence risk in patients with stage IIB/C and stage III surgically resected melanoma. Modified peptides were chosen to maximize

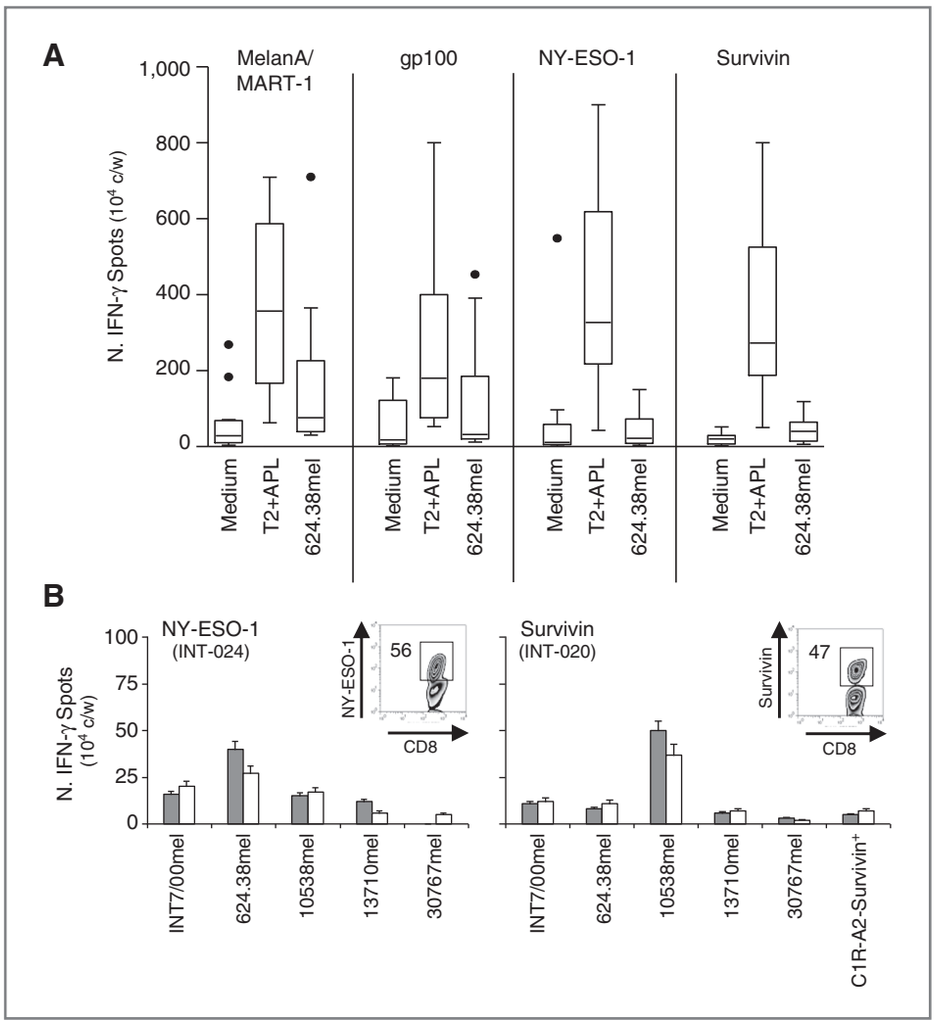

Figure 4. Antitumor activity of APL-specific T-cell cultures from postvaccine PBMCs. A, fourteen-day APL-sensitized cultures ($n = 12$ for each peptide included in the vaccine) obtained from postvaccine (sixth vaccination) PBMCs were tested by IFN-γ–ELISpot assay for the recognition of the HLA-A*0201$^+$MelanA/MART-1$^+$gp100$^+$NY-ESO-1$^+$Survivin$^+$ melanoma cell line 624.38mel. Bars, box-and-whisker diagrams. Horizontal line, median. B, anti-NY-ESO-1- and anti-Survivin-specific T-cell cultures enriched for CD8$^+$ cells by immunomagnetic selection were tested for IFN-γ release (ELISpot) in response to 4 different HLA-A*0201$^+$MelanA/MART-1$^+$gp100$^+$NY-ESO-1$^+$Survivin$^+$ melanoma cell lines or C1R-A2$^+$Survivin$^+$ cells. The HLA-A*0201$^-$ melanoma cell line INT07/00mel was used as a negative control. Targets were pretreated (open histograms) or untreated (filled histograms) with an anti-HLA class I mAb to test for HLA restriction. Dot plots with the percentage of CD8$^+$HLA-multimer$^+$ T cells are shown. Two representative cases are shown (NY-ESO-1: INT-024; survivin: INT-020).

the immunogenicity and clinical efficacy of vaccination, according to a strategy commonly applied in clinical trials using HLA class I–restricted tumor peptides (13, 21–22).

In this patient setting, we observed that APLs emulsified in Montanide and administered in association with low-dose cyclophosphamide and IL-2 promoted a consistent and selective increase in circulating antigen-specific CD8$^+$ T cells in most immunized patients compared with controls. The immunologic responses achieved were significantly superior to those observed in our previous experience with antitumor vaccines in patients with stage IV melanoma (2, 19), thus showing that individuals with early disease have more suitable immunologic conditions and that the APL strategy shows stronger immunogenicity. However, despite the remarkable induction of peptide-specific T-cell responses, no evident improvement in DFS or OS was observed in the vaccinated arm compared with the control patients as evaluated during the end-of-study analysis.

Although the study is based on a relatively small number of patients, these clinically negative results suggest the need to conduct detailed immunomonitoring and related functional profiling to identify the potential defects in the vaccine-induced immune responses. In terms of the kinetics, APL-specific T-cell induction in the peripheral blood was prompt and persistent (up to 12 months) in the majority of the immunized patients. Anti-APL T lymphocytes in postvaccine PBMCs showed effector memory features, expression of the tumor-homing receptors CXCR1 and CXCR3, polyfunctional properties in terms of cytokine release (IL-2 and TNFα), and no functional or phenotypic signs of exhaustion, such as upregulated expression of PD-1, Tim-3, or LAG-3 coinhibitory receptors (23, 24; data not shown). However, when analyzed for their direct antitumor potential, APL-specific CD8$^+$ T cells recognized their respective wild-type epitopes in a suboptimal fashion and reacted poorly in the presence of melanoma cells, despite the expression of high levels of HLA-A2 and cognate antigens. The degree of cross-reactivity varied with the different APLs and even reached undetectable values for NY-ESO-1 and survivin. This immunologic scenario clearly suggests that APL-primed T cells were endowed with low-affinity

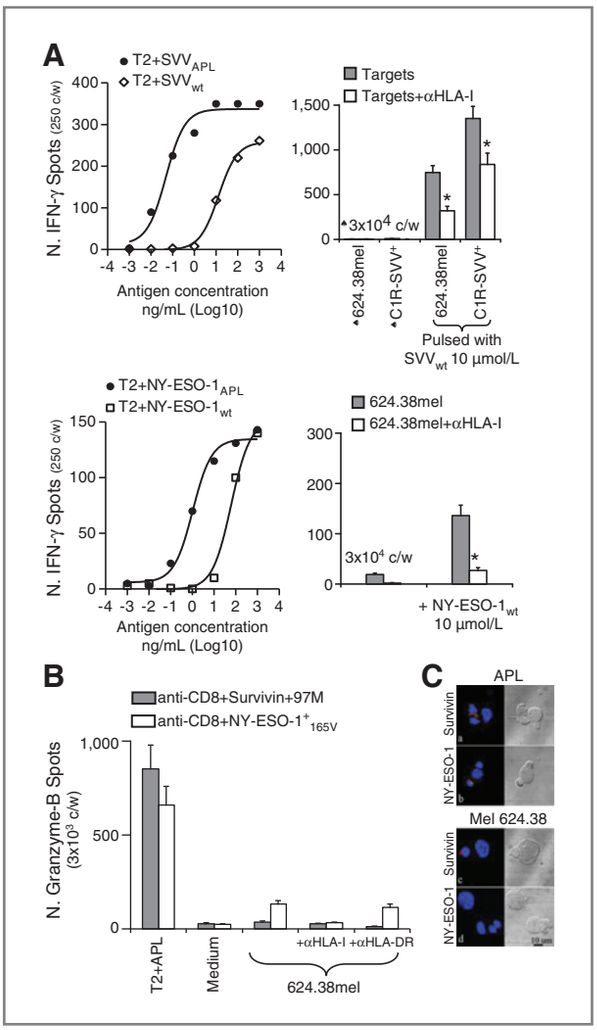

Figure 5. Lack of tumor recognition by NY-ESO-1$^+_{[165V]}$- and Survivin$^+_{[97M]}$-specific T cells. A, anti-Survivin$^+_{[97M]}$- or anti-NY-ESO-1$^+_{[165V]}$-specific CD8$^+$ T cells generated from postvaccine (sixth vaccination) PBMCs were sorted using a BD FACSAria Cell Sorting System and monitored for TCR affinity by IFN-γ–ELISpot assay using T2 cells pulsed with decreasing APL or wild-type peptide concentrations (left) as targets. Recognition of the HLA-A*0201$^+$ melanoma cell line 624.38mel (MelanA/MART-1$^+$gp100$^+$NY-ESO-1$^+$Survivin$^+$) or C1R-A2$^+$Survivin$^+$ cells pulsed or not pulsed with saturating doses of wild-type peptide was also evaluated (right). B and C, assessment of cytolytic function by granzyme B ELISpot assay and confocal analysis. Postvaccine HLA-multimer$^+$-sorted Survivin$^+_{[97M]}$ (gray bars) or NY-ESO-1$^+_{[165V]}$ (white bars) CD8$^+$ T cells were incubated with 624.38mel or APL-pulsed (2 μmol/L) T2 cells as a positive control. HLA-blocking experiments were conducted by preincubating the targets (624.38mel) with anti-HLA class I or anti-HLA-DR mAbs. Data represent the mean ± SD of quadruplicates. *, $P < 0.05$ (unpaired Student t test). In the confocal images, cells were double-stained with Abs specific for the cytolytic granule marker cathepsin D (red) and IFN-γ (green). TOTO-3 iodide 642/660 was used for nuclei staining. Bar, 10 μm. Magnification, × 30. Two representative cases are shown (NY-ESO-1: INT-024; survivin: INT-003).

TCRs that were likely unable to efficiently engage the low levels of HLA-peptide complex usually expressed on the tumor cell surface (25, 26). The finding that the tumor cells could instead trigger T-cell reactivity when exogenously preloaded with excess wild-type peptides supports this hypothesis.

The use of APLs is widely considered a suitable and effective strategy for peptide-based cancer vaccines, and it is one of the most prevalent approaches in clinical trials (27–30). Indeed, as the time of their discovery by Allen and colleagues in studies focused on TCR flexibility (i.e., the ability of the same TCR to cross-recognize diverse but structurally similar HLA-peptide ligands; ref. 31), APLs have been extensively exploited to improve the immunogenicity of tumor peptides by modifying TCR- (32, 13) or HLA-contact residues (33–35). Studies conducted *in vitro*, often involving *ex vivo* analyses of representative T-cell clones, or those conducted in HLA-A2 transgenic mice globally confirmed the immunologic advantage of APLs over native peptides. However, inconclusive evidence has been previously reported for the *in vivo* expansion of T cells with heterogeneous levels of cross-reactivity for the natural epitopes in patients with melanoma vaccinated with APLs from the MelanA/MART-1, gp100, and NY-ESO-1 antigens (36, 37). Yet, to our knowledge, no systematic analysis of the actual tumor recognition has ever been introduced or considered mandatory in the immunomonitoring of APL-based vaccine trials. For this reason, the comprehensive analysis of the antitumor potential we applied in our study provides crucial insights into the functional properties of immunization with APLs, potentially elucidating why this vaccine strategy has often failed to show major clinical benefits (27–29, 37). Because *in vitro* comparable data were generated with the APLs from different tumor peptides, such as those derived from the colon cancer-related carcinoembryonic antigen (38), the activation of T cells with poor antitumor potential might not be the prerogative of the melanoma-derived APLs included in this study.

The findings from our monitoring suggest that the use of APLs as an antigenic source for cancer vaccines should be seriously reconsidered in the clinical setting. Recently, Schwartzentruber and colleagues reported that the gp100 APL (209-2M) improves survival in patients with stage IV melanoma when administered in combination with high-dose IL-2 (22). Although no analysis of the antitumor potential of gp-100 (209-2M)-induced T cells was included in the study, the clinical benefit of the APL+IL-2 combination may be attributed in part to the presence of potent proliferative stimuli and an altered cytokine/chemokine milieu (39). Together, these may have favored the expansion of the gp100-specific tumor-reactive T cells and/or promoted the antigen-spreading phenomena with local activation of truly antitumor T cells (40).

Nevertheless, under the schedule applied in our adjuvant vaccine trial, the APLs did not support the sufficient generation of tumor cross-reactive T cells, which is a defect that in our opinion represents the principal reason for the undetectable clinical benefit. However, some specific characteristics of the patients or the treatment settings may have influenced the immunologic or clinical outcomes of the study.

For example, our analysis was confined to circulating T cells and did not permit any evaluation of the immune processes ongoing at the tumor site because of the lack of macroscopic disease in these patients. However, the subcutaneous lesions recurring during or after vaccination, once surgically removed and histologically analyzed, displayed no signs of immune selection (such as downregulation or loss of antigen and HLA expression) or significant changes in T-cell infiltration compared with prevaccine lesions (Carbone A.; personal communication). Such findings have shown the poor affinity of the APL-specific T lymphocytes for melanoma cells. In addition, we cannot exclude the possibility that the administration of low-dose cyclophosphamide and IL-2 may have influenced, for unknown reasons, the TCR affinity of the vaccine-induced T-cell responses and their subsequent clinical efficacy.

In contrast to these results, a parallel trial in patients with prostate carcinoma using the same vaccine schedule but with HLA class I wild-type peptides, found T cells that recognized tumor cells and successfully generated clinical benefits (Rivoltini and colleagues, manuscript in preparation). In addition, several preclinical studies have shown that Tregs might help to maintain immune tolerance and restrain vaccine-induced tumor immunity by controlling suboptimal rather than high-affinity TCR-bearing T cells (41, 42). Together with a recent report on the ability of cyclophosphamide to enhance the trafficking of high-avidity CTLs to tumor sites (43), this evidence suggests that cyclophosphamide should favor the expansion of high-avidity CTLs in a peptide-vaccine setting. Regarding the use of low-dose IL-2, we deliberately chose the "late" administration schedule (i.e., 3 months after the beginning of vaccination) based on a previous report by Slingluff and colleagues that showed a beneficial effect with such a schedule compared with that of early IL-2 administration for the induction of T-cell responses by a multipeptide melanoma vaccine (16). Nevertheless, extended and dedicated Treg monitoring to test the impact of cyclophosphamide and low-dose IL-2 was also conducted in our vaccinated melanoma patients. Although these data will be described in detail elsewhere (Camisaschi and colleagues, submitted for publication), we note here that cyclophosphamide had a markedly limited and transient effect on the function and frequency of circulating Tregs, whereas low-dose IL-2, although expanding these cells, played a crucial role in sustaining the development of vaccine-induced $CD8^+$ T effectors.

Tumor vaccines continue to represent a potential therapeutic tool for patients with cancer (21, 22, 44), although their use is still far from clinical practice. Because of the prevalence of immune inhibitory pathways, the high tumor burden and the onset of multiple escape mechanisms characterizing advanced cancer patients, the field is presently moving toward the use of vaccination as an adjuvant treatment for the prevention of disease relapse after surgery (45). However, unlike conventional cancer therapies, the identification of proper conditions to effectively trigger tumor immunity is a complex and not yet validated process. The present trial indicates several of the issues that need to be considered in this line of inquiry, such as the need to routinely monitor the antitumor properties of the vaccine-induced T cells, which was recently emphasized at the iSBTc/FDA/NCI Workshop on Immunotherapy Biomarkers (46, 47). In addition, we now consider modified peptides as a potential "false ally" in cancer vaccines because their increased immunogenicity does not necessarily result in enhanced antitumor T-cell reactivity. Although the level of cross-recognition may vary among peptides, the unpredictability of T-cell function (48, 49) makes this approach suboptimal for the effective enhancement of tumor immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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