Clinical Studies

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Skin-Test Infiltrating Lymphocytes Early Predict Clinical Outcome of Dendritic Cell–Based Vaccination in Metastatic Melanoma

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Abstract

The identification of responding patients early during treatment would improve the capability to develop effective new immunotherapies more rapidly. Here, we describe a bioassay that may link early T-cell–mediated immune responses to later clinical benefits. This bioassay rests upon the tenet of immunotherapy that tumor-specific effector T cells capable of invading peripheral tissue can recognize tumor antigens and exert cytotoxic functions there. To show its utility, we conducted a retrospective study of a large cohort of metastatic melanoma patients ($n = 91$) enrolled in dendritic cell (DC)-based vaccination protocols to examine a hypothesized correlation of posttreatment skin-infiltrating lymphocytes (SKIL) with overall survival (OS). Stringent immunologic criteria were defined to identify long-term survivors. The presence of tumor-associated antigen (TAA)-specific $CD8^+$ T cell populations within SKILs (criterion I) was highly predictive for long-term survival. Further restriction by selecting for the presence of TAA-specific $CD8^+$ T cells specifically recognizing tumor peptide (criterion II) was also associated with improved OS. Recognition of naturally processed antigen (criterion III) maximized the accuracy of the test, with a median OS of 24.1 versus 9.9 months ($P = 0.001$). Our results show that detailed characterization of SKILs can permit an accurate selection of metastatic melanoma patients who benefit most from DC-based vaccination. This simple and robust bioassay integrates multiple aspects of cellular functions that mediate effective immune responses, thereby offering an effective tool to rapidly identify patients who are responding to immunotherapy at an early stage of treatment. *Cancer Res; 72(23); 6102–10. ©2012 AACR.*

Introduction

The focus of treatment for metastatic cancer patients is shifting from a generalized approach based on population markers, to a personalized approach based on individual tumor and host characteristics (1). Immunotherapy is intrinsically a personalized treatment modality, as it acts via the patients' own immune system to induce anticancer immunity. Recent trials have underscored the potential of immunotherapy in metastatic cancers, especially in melanoma (2). Owing to their unique immune stimulatory properties, dendritic cells (DC) are an essential target for anticancer immunotherapy. We and others have explored DC-based therapy to induce tumor-associated antigen (TAA)-specific immune responses in this population (3, 4). Interestingly, the reported rates of long-lasting responses in immunotherapy trials are generally low, but remarkably constant, regardless of the chosen regimen (5). This long-standing observation hints at a subgroup of "immune reactive" patients. Identification of responding patients early during treatment would, therefore, greatly improve clinical efficacy of these novel and costly therapies. Thus, bioassays that accurately link immune responses to clinical outcome are warranted (1).

The mainstay of immunotherapy is to induce, enhance, or sustain TAA-specific effector T-cell immunity. Consequently, currently used bioassays focus on cellular immune responses at different time points after immunotherapeutic intervention. For example, most vaccination studies include a control antigen such as keyhole limpet hemocyanin (KLH) or tetanus toxoid as a surrogate marker for immune competence (6). However, the high immunogenicity of these control antigens often induces profound cellular and humoral responses, which do not accurately model the less abundant, and often self-antigens, TAA-specific cellular responses. Another widely used approach is ELIspot, which determines the production of a single cytokine upon antigenic stimulation, for example, IFN-γ ELIspots. Typically, ELIspots are conducted on cell samples

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obtained from peripheral blood, whereas antitumor effects can only be expected from immune cells capable of leaving the circulation and invading peripheral tissues. Although ELIspots are rather sensitive, they need careful standardization (7, 8). Moreover, they evaluate a single cytokine, whereas cytokine profiles better reveal the functional programming of effector cells. Imaging results from previous trials using adoptive cell transfer show that the migratory capacity of effector cells is positively correlated to clinical outcome (9), suggesting that this functionality should be incorporated in bioassays as well (10). Novel techniques have recently been developed that allow high throughput assessment of individual variations in many functional processes, for example, differences in signaling pathways in immune cells (11). However, as of now these techniques lack validation and are not yet applicable in the evaluation for therapy-induced immune responses.

Previously, we reported that screening cultures from delayed-type hypersensitivity (DTH) skin-test biopsies, by using tetrameric MHC-peptide complexes, provides a valuable tool to link immune responses to clinical outcome in metastatic melanoma patients who underwent DC-based vaccinations (12). In this study, we extend these findings in a large cohort and show that detailed analysis of skin-test infiltrating lymphocyte (SKIL) cultures is a solid bioassay to predict survival in metastatic melanoma patients. This bioassay is simple, feasible, and integrates multiple aspects of effector cell functions needed for effective immune responses.

Materials and Methods

Patient characteristics

We retrospectively analyzed a cohort of 91 patients with irresectable locoregional or distant metastatic melanoma, who were enrolled in our vaccination studies between June 1999 and June 2008 (Table 1). Eligibility criteria included melanoma patients with irresectable locoregional or distant metastatic disease, according to the 2001 American Joint Committee on Cancer Staging criteria (13). Other inclusion criteria include HLA-A*02:01 phenotype, known HLA-DRB*01*04 status, melanoma expressing the melanoma-associated antigens gp100 and tyrosinase and World Health Organization performance status 0 or 1. Patients with symptomatic brain metastases, serious concomitant disease, or a history of second malignancy were excluded. The studies were approved by our Institutional Review Board and written informed consent was obtained from all patients.

Treatment schedule

All patients were vaccinated with cytokine-matured monocyte-derived autologous DCs loaded with TAA of gp100 and tyrosinase according to a schedule of 3 biweekly vaccinations followed in week 7 to 8 by a DTH skin test (Supplementary Fig. S1). Differences in protocols included the route of administration (intranodal, intradermal, or combined intradermal/intravenously), method of antigen loading (detailed information below) and pretreatment with anti-CD25 antibody; described in Table 1. Unless progressive disease was documented, patients received a maximum of 3 vaccination cycles, each with a 6-month interval. For the exact details about the vaccination protocols, we refer to these individual studies (14–18).

Dendritic cell vaccine

Monocytes were enriched from leukapheresis products by counterflow centrifugation using Elutra-cell separator

Table 1. Vaccination protocols and characteristics of SKIL cultures

Protocol	Number of evaluable patients (total)	Method of antigen loading	Route of administration	Anti-CD25 mAb[a]	Number of cultures with outgrowth, pos/total, (%)	Mean, $\times 10^6$	Range, $\times 10^6$
1	1 (1)	Class I wt[b]	id	No	4/4 (100)	0.15	0.09–0.2
2	13 (17)	Class I wt	in	No	48/48 (100)	0.50	0.03–5.6
3	17 (22)	mRNA[c]	in	No	115/126 (86)	0.53	0.01–7.0
4	13 (15)	Class I wt	iv/id	No	44/63 (70)	0.59	0.01–1.8
5	9 (10)	Class I mod[d]	iv/id	No	21/48 (44)	0.16	0.01–1.3
6	11 (11)	Class I/II wt[e]	in	No	65/73 (89)	0.53	0.01–3.1
7	13 (15)	Class I wt	iv/id	Yes	42/54 (78)	0.44	0.01–3.3
Total	**77 (91)**				**339/416 (82)**		

Abbreviations: mAb, monoclonal antibody; wt, wild-type; mod, modified, id, intradermal; in, intranodal; iv, intravenous.

[a]Four or 8 days before the first vaccination, anti-CD25 antibody 0.5 mg/kg was administered intravenously.

[b]Class I wt, HLA class I-restricted wild-type gp100-derived peptides 154 to 162 and 280 to 288 and HLA class I-restricted tyrosinase-derived peptide 369 to 377.

[c]mRNA, messenger RNA encoding full-length gp100 and tyrosinase.

[d]Class I mod, HLA class I-restricted modified gp100-derived peptides 154 to 162 Q→A and 280 to 288 A→V and HLA class I-restricted tyrosinase-derived peptide 369 to 377.

[e]Class II, HLA class II-restricted gp100-derived peptide 44 to 59 and tyrosinase-derived peptide 448 to 462 analog.

(Gambro BCT, Inc.) and single-use, functionally sealed disposable Elutra sets, as described before (19) and according to the manufacturer. Monocytes were cultured in the presence of interleukin (IL)-4 (500 U/mL) GM-CSF (800 U/mL; both Cellgenix) and KLH (10 μg/mL, Calbiochem). DCs were matured with autologous monocyte-conditioned medium (30%, v/v) supplemented with prostaglandin E_2 (10 μg/mL, Pharmacia & Upjohn) and 10 ng/mL tumor necrosis factor-α (Cellgenix) for 48 hours as described previously (20). This procedure gave rise to mature DC meeting the release criteria described previously (3). DC were pulsed with the HLA class I gp100-derived peptides gp100:154–162, gp100:280–288, and the tyrosinase-derived peptide tyrosinase:369–377. DC from HLA-DRB*01*04–positive patients were also pulsed with HLA-DRB*01*04–binding peptides of both gp100 and tyrosinase (gp100:44–59 and tyro:448–462 analog (21, 22). Peptide pulsing was conducted as described previously (16). In the other protocols, mature DCs were electroporated with mRNA encoding gp100 or tyrosinase as described previously (23) and cells were resuspended in 0.1 mL for injection.

KLH-specific proliferation and IFN-γ production

Peripheral blood mononuclear cell (PBMC) were isolated from heparinized blood by Ficoll-Paque density centrifugation, stimulated with KLH (4 μg/2 × 10^5 PBMC) in X-VIVO with 2% HS. After 3 days, cells were incubated with ^{3}H-thymidine for 8 hours; incorporation was measured with a β-counter. Experiments were conducted in triplicate, nonspecific proliferation upon stimulation with ovalbumin (OVA) was used as control. IFN-γ production was measured in the supernatants after 24 hours by ELISA. Human IFN-γ monoclonal antibody 2G1 was used for coating (0.75 μg/mL), human IFN-γ biotin-labeled mAb M701B (0.05 mg/mL) was used for detection (all Thermo Scientific Inc.). Recombinant human IFN-γ RIFNG100 was used as standard. At least a 2-fold increase compared with OVA was considered positive.

Skin-test infiltrating lymphocyte analyses

Skin tests were conducted within 1 to 2 weeks after each vaccination cycle (Supplementary Fig. S1; ref. 24). Briefly, 2 to 10 × 10^5 DC pulsed with either gp100, tyrosinase or both epitopes or transfected with mRNA encoding either gp100 or tyrosinase or both (specifically indicated in the relevant text and figures) were injected intradermally in the skin of the back of the patient at different sites, 4 cm apart from each other. After 48 hours, the maximum diameter of induration was measured by palpation and punch biopsies (6 mm) were taken. Half of the biopsy was cryopreserved by snap freezing and the other part was manually cut and cultured or 2 to 4 weeks in RPMI-1640 containing 7% HS and IL-2 (100 U/mL), every 7 days half of the medium was replaced by fresh IL-2 containing RPMI-1640 7%HS.

Tetramer staining of SKILs

SKIL cultures were stained with tetrameric-MHC complexes containing the MHC-I epitopes gp100:154–162, gp100:280—288, or tyrosinase:369–377 (Sanquin) as described previously (12). Tetrameric-MHC complexes recognizing HIV were used as correction for background binding. Tetramer positivity was defined as at least 2-fold increase in the double positive population.

Cytotoxic activity of SKILs

Cytotoxic activity by SKILs in response to T2 cells pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA-A*02:01 and no endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, or with gp100 or with tyrosinase, or an allogenic HLA-A*02:01-positive, gp100-positive, and tyrosinase-positive tumor cell line (Mel624) were measured. Target cells were incubated with 100 μCi $Na_2[^{51}Cr]O_4$ (Amersham) and, after washing, added to SKILs (1 × 10^5 cells) and unlabeled K562 cells (1 × 10^4 cells) in triplicate wells of a round bottom microtiter plate (E/T ratio 10/1). After 4 hours, supernatants were harvested and radioactivity was measured. The specific percentage of cytotoxicity was defined by the following formula:

$$\text{specific cytotoxicity} = \frac{(\text{experimental release [cpm]} - \text{spontaneous release [cpm]})}{(\text{maximum release [cpm]} - \text{spontaneous release [cpm]})} \times 100\%$$

Positive and specific cytotoxic activity was defined as a 2-fold increase compared with stimulation with the same cell-lines pulsed with an irrelevant peptide.

Cytokine production profiles by SKILs

The production of IFN-γ, TNF-α, IL-10, IL-5, IL-4, and IL-2 by SKILs was measured in supernatants after 16 hours of coculture with different target cells to obtain a cytokine profile of postvaccination SKILs. Target cells include T2 cells pulsed with the indicated peptides or BLM (a melanoma cell line expressing HLA-A*02:01 and no endogenous expression of gp100 and tyrosinase), transfected with control antigen G250, or with gp100 or with tyrosinase, or an allogenic HLA-A*02:01-positive, gp100-positive, and tyrosinase-positive tumor cell line (Mel624) with SKILs, using the cytometric bead array [Thelper 1/Thelper 2 (Th1/Th2) Cytokine CBA 1; BD Pharmingen], according to the manufacturer instructions. Positive and specific cytotoxic activity was defined as a 2-fold increase compared with stimulation with the same cell lines pulsed with an irrelevant peptide.

Skin-test infiltrating lymphocyte culture evaluation

SKILs were evaluated according to increasingly stringent criteria; the presence of TAA-specific $CD8^+$ T cells by tetrameric MHC-peptide complexes (criterion I); peptide-recognition by specific production of Th1 cytokines (e.g., IFN-γ and/or IL-2) or cytotoxicity and no Th2 cytokines (criterion II); or tumor recognition of naturally processed TAA by specific production of Th1 cytokines or cytotoxicity and no Th2 cytokines (criterion III), an example is provided in Supplementary Fig. S2. The best overall TAA-specific responses was used for analyses, regardless of the time point at which the SKILs were obtained within the study, as this reflects

the individual competence to generate a specific immune response.

Statistical analysis

Overall survival (OS) was calculated from the date of apheresis to date of death and analyzed by Kaplan–Meier estimation using SPSS19.0 (SPSS Inc.). Statistical significance was evaluated using the log-rank test. Cox proportional hazard model was used to calculate hazard ratio (HR) for survival.

Results

Vaccination protocols and SKIL cultures

Seventy-seven patients completed at least 1 scheduled cycle and were thus evaluable for immunologic response (Table 1). Fourteen patients did not complete 1 scheduled cycle because of rapid progressive disease. The patients were vaccinated according to the various vaccination protocols, including intranodal, intradermal, intravenous/intradermal vaccinations; mode of antigen loading of DC was either pulsing with MHC Class I or MHC Class I and II defined epitopes derived from gp100 and tyrosinase (submitted), or electroporation with mRNA encoding these tumor associated antigens (25). In 1 protocol, patients received a single infusion of anti-CD25 antibody therapy before the first vaccination (14).

To show that this procedure is feasible for large-scale clinical studies, we determined the rate of successful SKIL cultures and their yield (Table 1). 329 out of 410 (80%) cultures yielded sufficient numbers of cells to allow further analysis, which results appear to be independent of the vaccination protocol (Table 1). On average, 4 successful cultures with different specificities were obtained per patient during complete course of 3 vaccination cycles.

KLH-specific T-cell responses do not predict overall survival

Peripheral blood samples during and after completion of each vaccination cycle were available for 70 patients for testing of KLH-specific $CD4^+$ T-cell proliferation. The maximum standard index (SI) compared with OVA was recorded and designated strong if more than 40. No significant difference in median OS was observed for patients with or without strong KLH-specific $CD4^+$ T-cell response; being 6.9 and 9.0 months respectively ($P = 0.95$, Fig. 1A, Table 2). We analyzed the cytokines produced upon KLH encounter and 9 of 40 tested patients showed a clear IFN-γ dominant response. However, this parameter did not significantly discriminate between patients with good and poor prognosis; median OS 16.7 and 11.0 months respectively, p = 0.40 (Fig. 1B, Table 2).

Induration at the injection site is not predictive for clinical outcome

After completing the first cycle of 3 vaccinations, the induration in 70 evaluable patients upon intradermal challenge with DC expressing gp100 or tyrosinase was median 13 mm (range 0–36 mm) and 13 mm (0–34 mm) respectively. The

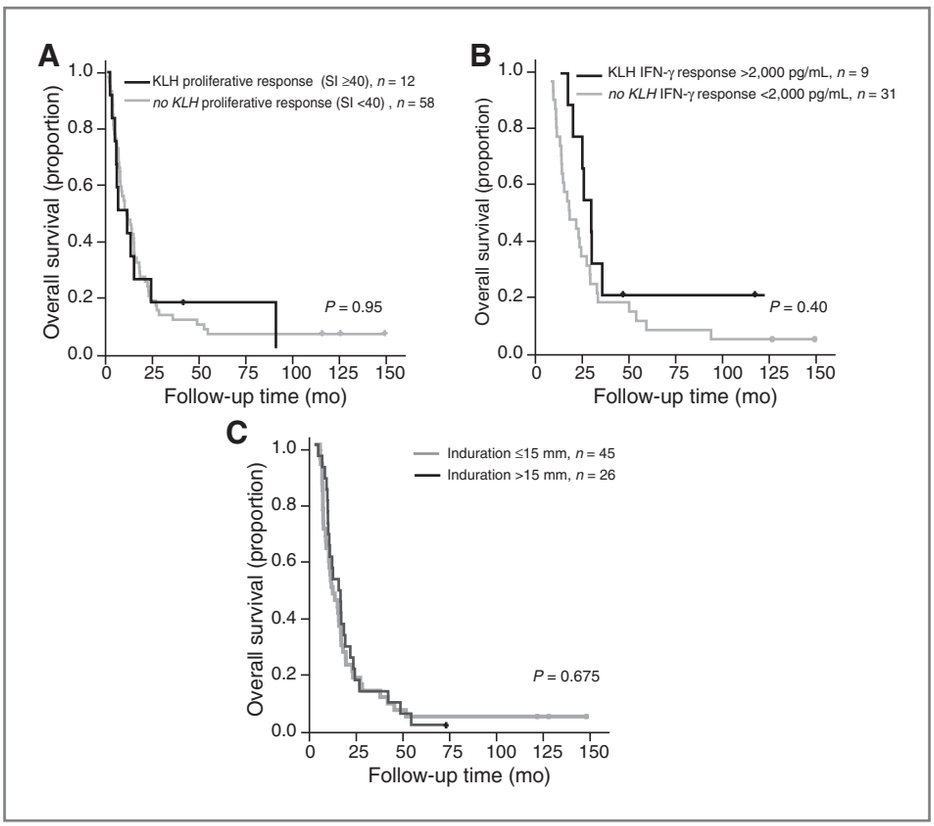
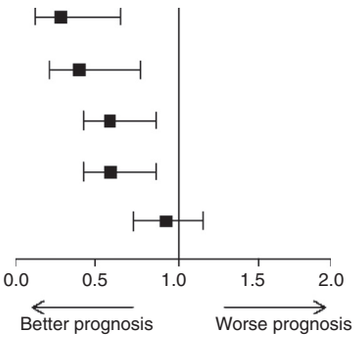


Figure 1. KLH-specific T-cell responses do not predict clinical outcome. Kaplan–Meier analyses of OS according to KLH-specific T-cell responses during DC-based vaccination in melanoma patients. A, the levels of KLH-specific proliferation, expressed as SI compared with proliferative responses to the irrelevant protein, were not predictive for OS. B, the production of significant levels of IFN-γ upon KLH stimulation was not associated with improved OS. C, the maximum diameter of induration of the skin test was not associated with OS.

Table 2. Hazard ratios associated with criteria for response

Response criterion	HR (95% CI)	*P*
Criterion III	0.30 (0.14–0.65)	0.002
Criterion II	0.42 (0.23–0.77)	0.005
Criterion I	0.60 (0.42–0.86)	0.005
No. of epitopes (0, 1, 2, or 3)	0.60 (0.42–0.85)	0.004
KLH proliferation (SI >40)	0.93 (0.73–1.20)	0.583



NOTE: A graphical representation of the hazard ratios (HR) associated with different response criteria were estimated using Cox proportional-hazard models. HR less than 1 defines a positive correlation with OS if the criterion is met. Horizontal lines represent 95% CIs. Abbreviation: KLH, keyhole limpet hemocyanin.

degree of induration was not predictive for clinical outcome with a maximum induration more than 15 mm corresponding with a median OS of 9.8 months and a maximum induration 15 mm or more corresponding with 13.0 months, $P = 0.675$ (Fig. 1C).

SKIL procedure does not require the presence of defined epitopes

In some protocols, we exploited DC transfected with mRNA encoding gp100 and tyrosinase, thus expressing multiple undefined epitopes. To investigate whether the results of tetramer screening of SKIL cultures depend on the presence of defined TAA epitopes, we conducted parallel skin-test challenges within individual patients. In 1 cohort ($n = 17$), we conducted the DTH procedure with intradermal injections of DC pulsed with defined epitopes (gp100:154–168, gp100:280–288, and tyrosinase:369–377) and DC transfected with mRNA encoding the same tumor antigens (full-length gp100 and tyrosinase) within individual patients, who were vaccinated with mRNA-transfected DC. Cultures from SKILs obtained from DTH sites containing DC loaded with defined epitopes or mRNA-transfected DC, yielded equal numbers of cells and successful cultures. In total, 126 DTH challenges were conducted in parallel, of these, 59 of 63 mRNA-DC-challenged and 54 of 63 peptide-DC–challenged skin-test biopsies yielded successful cultures. Comparable average yields per culture were obtained, 0.53 (range 0.01–7.0) $\times$ 10^6 cells with mRNA-transfected DC and 0.40 (range 0.01–2.8) $\times$ 10^6 cells for peptide pulsed DC. Furthermore, tetramer screening for TAA-specific T cells showed that challenge with DC pulsed with defined epitopes or DC transfected with mRNA have similar sensitivity and specificity (data not shown). TAA-specific $CD8^+$ T cells directed against all 3 defined epitopes were detected in single SKIL cultures obtained from intradermal challenge with DC transfected with mRNA encoding both gp100 and tyrosinase (data not shown).

SKIL culture evaluation accurately predicts clinical outcome

To investigate the role of the breadth of response, we analyzed the clinical outcome in relation to the number of epitopes that were recognized. The presence of 1 TAA-specific $CD8^+$ T-cell population in SKIL cultures was shown in 14 patients, who had a median OS of 9.8 months [95% confidence interval (CI), 7.3–12.3] compared with 10.9 months (95%CI, 5.7–16.0) in 52 patients in whom no TAA-specific $CD8^+$ T-cell populations were detected (Fig. 2A). Two or 3 TAA-specific $CD8^+$ T-cell populations were detected in 8 and 3 patients, respectively, corresponding with a median OS of 14.2 months (95%CI, 0.4–30.7) and median not reached, respectively (Fig. 2A). Overall, the presence of TAA-specific $CD8^+$ T cells in SKIL cultures (criterion I) was shown in 25 patients and was associated with improved survival; median OS 14.1 months compared with 10.9 months in patients without TAA-specific $CD8^+$ T cells (Fig. 2B) and HR 0.60 (95%CI, 0.42–0.86, $P = 0.005$, Table 2). As patients were vaccinated with DC loaded with either multiple melanoma peptides or mRNA-transfected DC presenting multiple undefined antigens, T-cell populations with different specificities could have been induced. In 18 patients with TAA-specific $CD8^+$ T cells we also showed vaccine-specific peptide-recognition (criterion II), which was strongly associated with improved survival (median OS 14.2 months versus 10.2 months in patients without vaccine-specific peptide recognition, Fig. 2C and HR 0.42 (95%CI, 0.23–0.77, $P = 0.005$, Table 2). To even better identify immune responsive patients, we selected for SKILs responding to naturally processed antigen by producing Th1 cytokines, predominantly IFN-γ. This was observed in 12 of these 18 patients who had a median OS of 24.1 versus 9.9 months in patients without SKILs responding to naturally processed antigen (Fig. 2D) and HR 0.30 (0.14–0.65), $P = 0.002$ (Table 2). As multiple cytokines were measured in SKIL cultures, we were able to identify functionally different cytokine profiles. As mentioned above and Supplementary Fig. S2, in the majority of cases, we detected IFN-γ

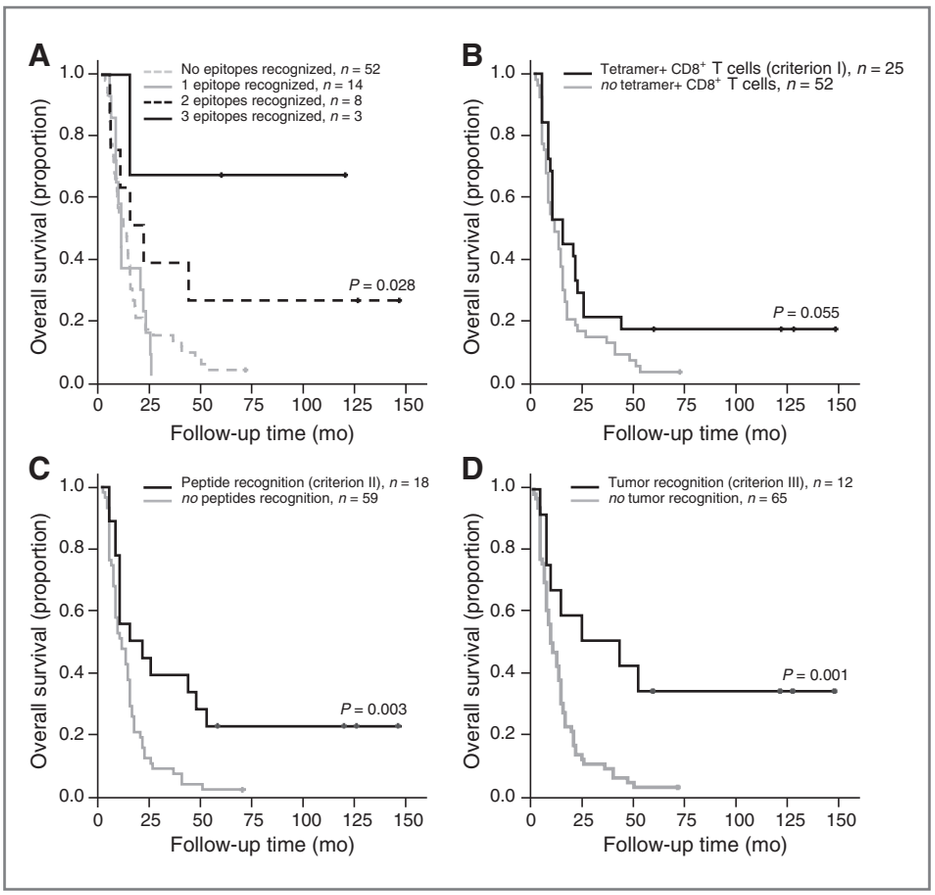


Figure 2. Analyses of SKIL cultures predict clinical outcome of DC-based therapy in metastatic melanoma patients. Kaplan–Meier analyses of OS according to different criteria for immune response in SKIL cultures obtained during DC-based vaccination in melanoma patients. A, the breadth of the vaccine-specific immune responses, measured as the number of vaccine-specific tetramer-positive populations in SKIL cultures, correlates with OS. B, SKIL cultures were sampled with tetrameric MHC-peptide complexes for the presence of TAA-specific $CD8^+$ T cells (criterion I). C, next, SKIL cultures were evaluated for recognition of tumor peptides by the production of IFN-γ or cytotoxicity, but no IL-5 production (criterion II), improving the accuracy of this bioassay to select patients with a favorable clinical outcome. D, lastly, SKIL cultures were evaluated according to the most stringent criterion III, specific IFN-γ production or cytotoxicity, but no IL-5 production upon recognition of naturally processed tumor antigen, which was highly associated with improved OS.

dominated cytokine production. However, in 2 patients, we detected predominantly IL-5 production by SKILs responding to peptide-pulsed target cells, indicative of Th2 skewing of the immune response. The detection of this response coincided with rapid progressive disease in both patients (Fig. 3).

Discussion

Although the presence of tumor-specific cytotoxic T cells (CTL) in cancer patients has been reported to associate with favorable clinical outcome (26), bioassays that accurately link vaccine-specific immune responses to survival are lacking. Although tetramer analysis or ELIspot assays of TAA-specific responses in peripheral blood are available, the low prevalence of TAA-specific T cells in peripheral blood makes this procedure less suitable for routine monitoring. Moreover, besides antigen specificity and effector activity, antitumor $CD8^+$ T cells must be able to extravasate and migrate into peripheral target tissues. We addressed this issue by evaluating SKIL cultures. This bioassay integrates multiple facets of an effective immune response. First, it serves as a fitness test; the *in vivo* intradermal challenge selects TAA-specific T cells that possess the migratory capacities to leave the circulation and penetrate peripheral tissue. Secondly, tetrameric MHC-peptide complexes allow sampling of different specificities (criterion I) within the SKIL population. As SKILs are expanded *in vitro* in the absence of antigen, the composition of different specificities closely parallels the *in vivo* situation. Thirdly, more stringent recognition can be assessed by challenging SKILs either with target cells loaded with defined antigenic peptides (criterion II) or by target cells that express naturally processed TAA (criterion III). Finally, measuring cytokine profiles upon antigenic challenge reflects the *in vivo* programming of TAA-specific T cells either towards Th1 or Th2 immune responses. The latter is of crucial importance to interpret antitumor responses and eventual clinical outcome.

In most clinical studies a highly immunogenic nontumor antigen is included in the vaccine, such as KLH or tetanus toxoid. The rationale for this approach is 2-fold; it is used as a surrogate marker to which excessive humoral and proliferative responses will be induced, in that respect is serves to evaluate immune competence per individual. Secondly, these immunogenic proteins contain a multitude of predominantly T helper cell epitopes; ergo, it functions as a nontumor specific adjuvant. We have previously investigated the magnitude and dynamics of humoral responses in this cohort of metastatic melanoma patients who underwent DC-based therapy (27). Our findings show that humoral anti-KLH responses are dictated by different vaccination parameters, such as route of administration and anti-CD25 mAb pretreatment. $CD8^+$ CTLs, as the endpoint effectors, represent a critical population for anticancer immunity. However, cellular responses to KLH are invariantly induced in the vast majority of patients,

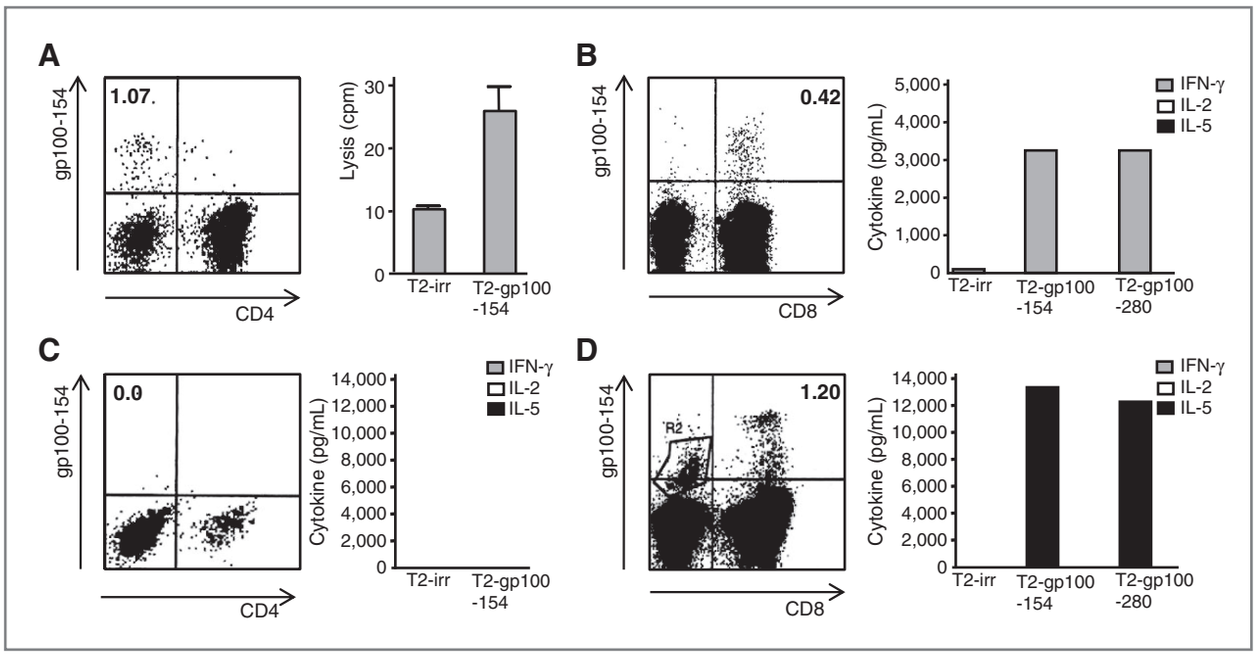


Figure 3. Immunologic response closely relates to the individual clinical course of disease. Two patients are shown in whom the immunologic evaluation predicted the course of their disease. A, data of IV-A-01 is depicted in whom TAA-specific $CD8^+$ T cells with specific cytotoxic capacity were detected after the first cycle of vaccinations. B, after completing the third cycle of vaccinations, IFN-γ–producing TAA-specific $CD8^+$ T cells were detected in SKIL cultures and no IL-5 was produced. As of May 2012, this patient survived 120+ months from start of vaccinations. C, data of IV-A-05 is shown in whom no TAA-specific $CD8^+$ T cells were detected after the first cycle of vaccinations. D, however, SKIL culture evaluation upon the third cycle of vaccinations revealed TAA-specific $CD8^+$ T cells that produced high levels of IL-5 upon antigen encounter. Planned evaluation showed rapid progression of disease; the patient died shortly after the third vaccination series, 20 months after start of vaccination.

regardless of the vaccination protocol (14–18, 28). In this study, we show that neither the magnitude of KLH-specific T-cell responses, nor the quality of the KLH-specific responses in terms of IFN-γ production is predictive for clinical outcome. This shows that loading *ex vivo* generated DC with KLH does not provide adequate means to assess antitumor cellular immune competence. This notion is further supported by the lack of correlation between the quality of vaccine-specific immune responses, as determined by the above-mentioned SKIL criteria, and the magnitude of KLH-specific cellular responses (Supplementary Fig. S2).

It has been reported in some studies that the degree of induration at the injection site would reflect the individual capacity to mount a vaccine-specific immune response, and therefore, correlate to therapy response. If so, the degree of induration would provide an easy and accessible measure for therapy evaluation. In general, induration is regarded as a typical $CD4^+$ T-cell reaction. On the contrary, others have shown that intradermal injection site can be briskly infiltrated by $CD8^+$ T cells, suggesting that the level of induration would in fact show Th1-type immune induction. Our data clearly illustrate that induration is not associated with OS or with TAA-specific $CD8^+$ T-cell responses (Supplementary Fig. S3). Furthermore, the measurement of induration is subject to high inter-test variability that complicates standardization.

Our increasing understanding of the complex interaction of an individuals' immune system and cancer and the development of immunotherapeutic interventions with clinical benefit, have drastically influenced the way we design and conduct clinical studies on immunotherapy (29). From small proof-of-principal studies focusing on a single parameter (3), we now focus on large randomized prospective studies with adjusted endpoints, designed to evaluate potential biomarkers and specific clinical response patterns (1, 30). In this respect, we evaluated SKIL culture analyses for its feasibility in large studies. With notion of its invasive nature, this procedure is relatively easy to conduct, does not need specialized personnel and is not labor intensive and is acceptable to the large majority of patients. Importantly, this procedure consistently yields sufficient numbers of SKILs to address the vaccine-specific immune response, which is independent of the vaccination protocol or intradermal challenge conditions. Even in patients vaccinated with DC loaded with modified tumor peptides to enhance MHC-binding efficacy, we detected CD8+ SKILs directed against the tumor to similar extend as in other vaccination protocols. This is in line with our previous observation that modified peptides efficiently elicit responses to wild-type peptides (31), resulting in comparable immunologic responses *in vivo* as vaccination with wild-type peptide loaded DC (15).

The development of tools to monitor immune responses during immunotherapy is complicated by the evaluation of different effector T-cell populations over time in response to vaccination and tumor changes. So far, it is not clear which time window after start of treatment reflects the vaccine-induced responses best and might be predictive of clinical

outcome. Furthermore, the relevant effector populations are distributed over several body compartments, such as draining lymph nodes, tumor tissue, bone marrow, and peripheral blood; and it is debatable what compartment is best suitable to monitor vaccine-specific immune responses. The patients displayed in Fig. 3 illustrate that the evaluation of both the kinetics and functional status of vaccine-specific responses is critical for correct correlation with clinical outcome. In the first patient, if only the percentage of vaccine-specific SKILs would have been assessed, it would have been a decline in vaccine-specific SKILs, However, the specific Th1 type responses after 1 and 3 cycles classifies this response as favorable. In the second patient, the appearance of vaccine-specific SKILs could have been interpreted as a favorable response. However, as we measured a typical Th2 skewed functional status, this response was correctly classified as nonfavorable. We acknowledge that further studies, incorporating multiple facets of tumor-specific immune responses at multiple time points after treatment, are warranted to elucidate the optimal time window to conduct immune monitoring.

In conclusion, by evaluating the migratory, antigen recognition, as well as the effector function of SKILs, we are able to select for multifunctional $CD8^+$ T cells with high tumor recognition efficacy. We showed that analyzing SKILs is a simple and robust, bioassay to predict OS in metastatic melanoma patients. Therefore, it represents an ideal candidate for immune monitoring in upcoming immunotherapy trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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