Autologous Melanoma Vaccine Induces Antitumor and Self-Reactive Immune Responses That Affect Patient Survival and Depend on MHC Class II Expression on Vaccine Cells

Michal Lotem,1 Arthur Machlenkin,1 Tamar Hamburger,1 Aviram Nissan,2 Luna Kadouri,1 Shoshana Frankenburg,1 Zvi Gimmon,2 Orit Elias,1 Inna Ben David,1 Anna Kuznetz,1 Eitan Shiloni,3 and Tamar Peretz 1

Abstract

Purpose: Autologous melanoma cells display a broad variety of tumor antigens and were used for treatment of American Joint Committee on Cancer stages III and IV melanoma as an adjuvant or active therapy. Survival data and immune response were evaluated in vaccinated patients.

Experimental Design: Forty-seven patients received 2,4-dinitrophenyl–conjugated autologous melanoma vaccine as an adjuvant (23 patients) or therapy (24 patients). CD4 and CD8 T-cell response in blood sampled before vaccination and after five or eight vaccine doses was evaluated against melanoma cells and autologous peripheral blood mononuclear cells using IFNγ enzyme–linked immunospot. Serum levels of antilivin, an inhibitor of apoptosis, and anti-gp100 IgG were determined.

Results: The immunologic effect of the vaccine differed between the two groups of patients. In the adjuvant group, there was a significant increase in CD8 melanoma-reactive T cells (P = 0.035) after vaccination and an increase in antimelanoma CD4 T cells correlating with improved overall survival (P = 0.04). In the therapeutic group, there was no objective tumor regression; antimelanoma T-cell reactivity increased by a small amount, stayed the same, or in some cases decreased. In all patients, a significant increase was noted in CD4 T-cell reactivity against autologous peripheral blood mononuclear cells (P = 0.02), which did not affect survival. Increased antilivin IgG was associated with improved survival. Expression of MHC class II on melanoma cells was vital for the immunogenicity of the vaccine.

Conclusion: Autologous melanoma cell vaccine is capable of inducing effective antitemelanoma CD4 T-cell activity associated with improved survival. Patients with active metastatic disease generally displayed reduced immune response and gained little from active immunization.
Currently available immune modulators that lack specificity but boost immune responses can be combined with vaccines to offer a new direction for combination therapies in a clinical setting (12, 13).

To estimate the benefit of any cancer vaccine, a monitoring study of immune effector cells against cancer antigens before and after therapy is essential. A practical approach is to select a specific peptide or full-length antigen and measure the frequency of antigen-specific T cells. Tetramer staining for specific TCR-bearing T cells is a reproducible technique most appropriate for defined vaccines (14). In whole-cell vaccines, tetramer choice focuses on known tumor-associated antigens already linked to clinical tumor regression. Many other antigenic moieties derived from specific tumor antigens or normal cellular components are ignored. Unfortunately, there is no specific reagent with high detection sensitivity that can be used for multiple antigens and in the context of a variety of HLA types. Thus, when it comes to monitoring the immune effect of complex vaccines, their advantageous targeting of multiple antigens actually proves to be a disadvantage.

In this study, we aimed to characterize immune responses in patients receiving autologous melanoma vaccines. Immunologic evaluation was done in prevaccination and postvaccination blood samples drawn from patients who received a melanoma autologous vaccine in two different settings: as a postoperative adjuvant protocol (23 patients) and as a therapeutic protocol for metastatic disease (24 patients). Because this patient population was vaccinated without any a priori knowledge of their HLA type or the antigenic composition of their tumor cells, we chose to test reactivity against the autologous melanoma and against autologous peripheral blood mononuclear cells (PBMC) as a normal tissue control using enzyme-linked immunoassay (ELISPOT) assay.

We found increased CD8 and CD4 T-cell responses against melanoma cells induced by the autologous vaccine, as well as an increase in humoral response. The capacity to increase CD4 T-cell reactivity against melanoma significantly correlated with improved survival. Another outcome of the vaccination process was increased self-reactivity against the PBMC non-tumor targets. Although the melanoma cell vaccine increases reactivity to both tumor and self antigens, survival benefit was seen only for the tumor-directed immune responses.

Translational Relevance

There are few published data regarding the immunologic effects of autologous melanoma vaccines, and surrogate markers have mostly been used to assess their efficacy. The objective clinical response to whole-cell vaccines is generally low, but evidence hints that its immunogenicity is real. With newly developed immune modulators like anti-CTLA4 and anti-PD1, it is possible that we will be able to augment the subclinical immunologic effects of autologous vaccines. We found that autologous melanoma vaccine is capable of inducing antitumor CD4 T cells, which affect survival, and causes a significant increase in antitumor CD8 T cells. The HLA class II expression on tumor cells is critical for their immunogenicity. The emergence of tumor-specific T-cell responses may be further enhanced by combination therapies, may be exploited for adoptive transfer, and could lead to tumor regression.

Materials and Methods

Patients. Forty-seven patients were included in this study, composed of two groups who received autologous melanoma cell vaccine either as (a) postoperative adjuvant protocol for American Joint Committee on Cancer (AJCC) stages III and IV totally resected metastatic malignant melanoma (23 patients) or (b) therapeutic vaccine for active metastatic melanoma (24 patients). The adjuvant group was nested in a larger group of 102 patients accrued between 1996 and 2007; to be included, patients had to undergo complete removal of their metastatic disease and have a normal computed tomography scan not more than 30 d before vaccination. Patients with active metastatic melanoma were offered the autologous vaccine after failing one line of treatment [DTIC-containing (II–2)–containing regimens] if they had a life expectancy of >3 mo. Patient data are included in Table 1.

 Patients were chosen for the immunomonitoring study by availability of prevaccination and postvaccination blood samples and delayed-type hypersensitivity (DTH) reactions to injected, unmodified autologous melanoma cells in the course of vaccination. Comparable numbers of patients with strong (>15 mm) or weak (<15 mm) DTH reactivity were allocated in each group. Clinical data were recorded for all patients participating in the vaccine trials, including overall survival (OS) and disease-free survival (DFS) measured from the day of the first vaccine.

Patients’ blood samples were taken before immunization and 1 wk after vaccine doses 5 and 8. PBMCs were cryopreserved at -180°C after Ficoll-Hypaque separation. Sera were divided into aliquots and stored at -80°C. In some cases, there was not enough blood to complete all tests; consequently, there are some missing data (Table 1).

Melanoma cell lines and vaccine preparation. Melanoma tumor specimens were taken from resected metastases for the preparation of autologous vaccine. All patients gave their informed consent to be included in the vaccination studies. The melanoma cell lines were established as described (15). To assure melanocytic progeny, the expression of S100, MART-1, and gp100 were evaluated by immunostaining using polyclonal antibodies (Dako). Positive staining of >50% of cells with at least one of these antibodies was required. HMC expression on tumor lines was determined using anti-HLA class I and HLA class II monoclonal W6/32 and CR3/43 antibodies, respectively (Dako). All cell lines were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mmol/L l-glutamine, and combined antibiotics (Invitrogen Life Technologies). Human lymphocytes were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated human serum, recombiant IL-2 (300 IU/mL; Chiron B.V.), 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, 25 mmol/L HEPES buffer, 50 μmol/L L-mercaptoethanol, and combined antibiotics (Invitrogen).

Vaccine preparation and vaccination procedure. Melanoma cell lines were expanded to the required number necessary for preparation of at least eight vaccine doses of 10 to 25 × 10⁶ cells each. On the day of treatment, the cells were thawed, washed, and irradiated to 170 Gy. A cell sample was stained with trypan blue and counted after irradiation. Conjugation of melanoma cells with 2,4-dinitrophenyl (DNP) was done as described (16). Briefly, melanoma cells were washed with Hank’s balanced solution, resuspended to a concentration of 5 × 10⁶/mL, mixed, incubated for 30 min, and washed again. Before vaccination, an appropriate amount of Bacillus Calmette-Guerin was added, starting with a dilution of 1:50 and reaching 1:500, according to the resulting granuloma at the injection site. The vaccination procedure was carried out as described (15, 17). On day 1, patients received 300 mg/m² of i.v. cyclophosphamide. On days 4 and 5, patients were sensitized to
Table 1. Clinical data and IFN-γ-ELISPOT results of melanoma patients participating in (A) the adjuvant protocol (23 patients) and (B) the therapeutic protocol (24 patients)

### A

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<th>Stage</th>
<th>DTH Status</th>
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<th>Antimelanoma (CD8 T cells) Pre</th>
<th>Post</th>
<th>Anti-PBMC Pre</th>
<th>Post</th>
<th>HLA class II</th>
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### B

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NOTE: Clinical data include AJCC stage at time of vaccination, DTH to unmodified autologous melanoma cells at vaccine 5 or vaccine 8 if not identical to 5, and current status (months from first vaccination to February 2008 or to time of death). CD8 and CD4 T-cell IFN-γ-ELISPOT (number of spots per 100,000 cells) is shown for pretreatment blood samples taken on day 1 of the vaccination protocol (pre) and on day of last vaccine (post). Separated CD8 and CD4 T cells were incubated ex vivo for 12 d with the autologous melanoma, and IFN-γ-ELISPOT was done using autologous tumor and autologous PBMC as targets. ELISPOT results represent the mean of two duplicates. HLA class II expression on tumor cell cultures is shown in the last column. The number of post-immunization values showing ≥2-fold increase are in bold and underlined if larger than 100. In some patients, blood samples did not allow full assessment. Abbreviations: NED, no evidence of disease; AWD, alive with disease; DOD, died of disease; DOC, died of other cause; P, progression; LFU, lost to follow up.
Kaplan-Meier analysis of OS. Patients received a second dose of 300 mg/m² cyclophosphamide. On day 21, the prepared vaccine was injected into three adjacent sites on the upper arm or thigh, avoiding limbs where lymph node dissection had been done previously. Seven additional doses of the vaccine were given at intervals of 21 to 28 d. Before injecting the second dose of the vaccine, a third dose of 300 mg/m² of cyclophosphamide was given i.v. Patients who responded received additional doses of vaccine at intervals of 3, 3, 6, and 6 mo.

The early administration of cyclophosphamide before vaccination can reduce the proportion of regulatory T cells (18). The adjuvant and costimulatory agents admixed with a vaccine determine the direction of emerging immunity (19). Bacillus Calmette-Guerin was reported to trigger dendritic cell maturation and to aid in diverting CD4 T-cell response toward a Th1 phenotype (20).

Patients were evaluated periodically every 3 mo and had a total body computed tomography scan done every 6 mo in the adjuvant group and every 4 mo for the therapeutic group, or as required according to the symptoms of the patient.

**DTH reaction.** Evaluation of DTH to autologous melanoma cells was done on the day of vaccine doses 5 and 8 by intradermal injection of 1 to 3 × 10⁶ irradiated (170 Gy) autologous melanoma cells at a different site. The DTH response was measured as the erythema diameter 48 h after the injection. The DTH response was defined as negative when the maximum erythema diameter was smaller than 5 mm. We arbitrarily chose 10 mm of erythema as definite positive and 15 mm as powerful DTH.

**Evaluation of melanoma-specific and non–melanoma-specific reactivity of prevaccination and postvaccination lymphocytes.** For all experiments, prevaccination and postvaccination cryopreserved peripheral blood lymphocytes from a given patient were tested concurrently. In vitro sensitization of peripheral blood lymphocytes. Twelve-day in vitro stimulation with autologous melanoma preceded evaluation of lymphocyte reactivity. Cryopreserved PBMCs from melanoma patients were thawed the day before stimulation and cultured in complete medium. The next day, peripheral blood CD8 and CD4 lymphocytes were purified from the PBMCs with magnetic beads (BD Biosciences), according to the manufacturer’s instructions. The cells were seeded in 24-well plates (1.5 × 10⁶ cells/well, 2 mL/well) in complete medium supplemented with 100 µmol/L L-1-methyl-1-tryptophan (Sigma), 10 ng/mL recombinant IL-4, and 5 ng/mL recombinant IL-7 (R&D), as described (21). Lymphocytes were stimulated with irradiated (100 Gy) IFN-γ-treated (50 units/mL; Prospeck) autologous tumor cells (3 × 10⁵ per well). Two days later, 300 IU/mL recombinant IL-2 (R&D Systems) was added to the cultures and renewed every 2 d. On day 12, the stimulated T-cell cultures were harvested, washed in complete medium, and used in immunologic assays.

**ELISPOT.** ELISPOT was done using the standard DuoSet kit (R&D Systems) according to the manufacturer’s protocol. In brief, 1 × 10⁵ CD8 or CD4 purified lymphocytes were incubated for 20 h with 1 × 10⁶ autologous tumor cells or PBMC in anti-IFNγ antibody (R&D Systems)—coated 96-well plates (polyvinylidene difluoride membranes, Millipore) followed by development and staining. The number of tumor- and nontumor-specific spot-forming cells was counted using a Bioreader 3000 system (Biosys). Results were presented as number of spots per 100,000 lymphocytes. An immunologically significant response was defined as a ≥2-fold increase in the number of preimmunization spots, as long as the postvaccination value was >100 spots per 100,000 lymphocytes.

Evaluation of humoral response. Maxisorp Immuno plates (Nunc) were coated with 5 µg/mL recombinant gp100 (a generous gift from J. Pitkovsky; ref. 22) or 5 µg/mL livin (generously donated by D. Ben-Yehuda; ref. 23) in PBS by overnight incubation at 4°C, followed by blocking with 2% nonfat dried milk diluted in PBS + 0.05% Tween for 2 h at 4°C. Sera from healthy volunteers and from prevaccination and postvaccination melanoma patients were diluted 1:100 to 1:800 in PBS + 0.05% Tween and added for 2 h at room temperature. After extensive washing with PBS + 0.05% Tween, polyclonal goat anti-human IgG (H+L) conjugated to horseradish peroxidase (Jackson ImmunoResearch, 1:20,000 dilution) in 1% nonfat dried milk/PBS + 0.05% Tween was applied for 45′ at room temperature. The plates were washed and developed with TMB reagent (DAKO). Antitumor antibodies to antinuclear factor were determined using mouse liver/kidney/stomach slides. Antithyroid antibodies were determined with primate thyroid slides (IMMCO Diagnostics). Readout was done with fluorescein antihuman IgG antibodies.

**Statistical methods.** To test the difference between paired preimmunization and postimmunization measurements, paired t tests and nonparametric Wilcoxon signed-rank tests were applied. The Pearson χ² test and Fisher’s exact test were applied to determine the association between two qualitative variables. The comparison of survival curves between groups was carried out using the Kaplan-Meier survival analysis with the log-rank test.

All tests applied were two-tailed, and a P value of 5% or less was considered statistically significant.

**Results**

**Clinical data**

Patients’ survival in the larger adjuvant group (102 patients) correlates with intensity of evolving DTH response to unmoldified tumor cells. Patient enrollment was initiated in October 1996. Patients’ OS and DFS were measured from the day of the first vaccine until February 2008, when analysis was done. A total of

![Disease free survival](image)

**Fig. 1.** Kaplan-Meier analysis of OS (right) and DFS (left) of 102 AJCC stages III and VI melanoma patients receiving autologous melanoma vaccine as postoperative adjuvant treatment. Patients’ survival time is categorized according to the diameter of skin response to injected unmoldified autologous melanoma cells. DTH, DTH reaction.
limits due to the low frequency of tumor-specific T cells, we used to expose melanoma-reactive T cells.

### Table 2. In vitro stimulation of peripheral blood lymphocytes to detect tumor reactive T cells

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<th>CD4</th>
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<tr>
<td></td>
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<td>M171 PBMC</td>
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<tr>
<td>0 d</td>
<td>IL2 72 17</td>
<td>IL2 51 19</td>
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<tr>
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NOTE: IFN-γ–ELISPOT results of CD8 and CD4 T cells derived from prevaccination blood of patient M-171 are displayed. T cells were incubated with the autologous melanoma for 3 or 12 d either with IL-2 (150 IU/mL) or with IL-15 (10 ng/mL) and IL-21 (10 ng/mL). IFN-γ secretion was analyzed against autologous melanoma target (M171) and autologous PBMC.

102 patients are included with a median follow up of 72 months (range, 7-135). DFS and OS were analyzed for a DTH of <15 mm (weak positive DTH) versus a DTH of >15 mm (strong positive DTH). Thirty-four percent of patients attained strong positive DTH, whereas 66% had weak DTH responses. Patients with a strong DTH response had a mean OS of 101 months (median not reached); the 5-year OS was 66% and DFS was 48%. In contrast, patients with weak DTH had a median OS of 38 months (mean, 56.5), 5-year OS of 41%, and DFS of 28%, which were significantly lower (P = 0.003 for OS and 0.015 for DFS). Using the Kaplan-Meier method and the log-rank test, the single parameter that was most strongly correlated in a univariate analysis with OS and DFS was the DTH response (Fig. 1). Patients who failed to attain strong DTH had an increased risk of 15 (95% confidence interval, 3.76; P = 0.001) to die of their disease and a relative risk of 4.5 (95% confidence interval, 1.8-11; P = 0.001) to experience metastatic recurrence. Age, gender, and depth of invasion of the primary melanoma had no effect on survival. When we analyzed survival according to a cutoff of 10 mm erythema of DTH, the same trend was noted, but with a smaller P value of 0.07.

Thus, the acquisition of powerful skin reactivity against non-modified autologous melanoma cells correlates favorably with survival.

**Patients’ survival in the metastatic group did not correlate with DTH response to unmodified tumor cells.** Twenty-four patients who received autologous melanoma vaccine as treatment for active metastatic disease were included in the immunomonitoring evaluation. Their median OS was 15 months. There was no significant difference in OS found in patients who did or did not attain a positive DTH response. No objective tumor regression after the vaccine alone was noted in patients with disseminated malignant melanoma.

**Immunologic evaluation**

**Twelve-day in vitro stimulation with tumor cells is required to expose melanoma-reactive T cells.** To overcome detection limits due to the low frequency of tumor-specific T cells, we used an in vitro stimulation procedure, which gave preference to T-cell subsets, which recognize autologous tumor and expanded their numbers to detectable levels. Postimmunization samples were compared with preimmunization samples to ensure that there was no bias with respect to overrepresentation of specific T-cell subsets. ELISPOT dynamics reflect the direction and relative strength of the evolving immunity induced by the vaccine.

Table 2 shows ELISPOT results of CD8 and CD4 T cells derived from prevaccination blood of patient M171. An initial experiment was designed to evaluate the effect of length of coculture and of adding cytokines on the expansion of lymphocytes in the presence of tumor or autologous PBMC. T cells were incubated with the autologous melanoma for 3 or 12 days either with 150 IU/mL IL-2 or with IL-15 and IL-21 at 10 ng/mL each (24). ELISPOT results after incubation were compared with those of unmanipulated T cells. It is shown that IL-2 was more supportive than IL-15 and IL-21 in maintaining cultures of antitumor T cells and that this effect was not due to increased background reactivity. Although on day 3 specific antitumor lymphocytes were already detectable, their dominance over anti-PBMC responding cells was marked more after 12 days. The stimulation of the cells that triggers amplification of response to detectable levels was important to expose interpatient variations to allow for clinical correlations.

**ELISPOT results of preimmunization and postimmunization samples.** CD8 and CD4 T cells from patients were cocultured with the autologous tumor for 12 days in complete medium supplemented with 150 IU/mL IL-2. In melanoma cells lines taken from 42 of the patients in this study, MHC staining revealed that 83% of cell lines expressed HLA class I and 48% expressed HLA class II. Positivity was determined based on expression in >10% of cells. Because melanomas express HLA-class II, it was important to use nontumor autologous targets that similarly express HLA class II as controls. Autologous PBMC were chosen for that purpose because they contain B cells and adherent monocytes that constitutively express HLA class II–restricted self antigens (25).

Collected ELISPOT results of the adjuvant and metastatic patients are represented by dot box plots of preimmune and postimmune scores (Fig. 2). Individual patients’ scores are presented in Table 1.

**Antimelanoma CD8 T-cell responses.** Overall for the whole group, there was an increase in IFN-γ production by CD8 T cells in response to autologous melanoma from a mean value of 283 spots per 100,000 CD8 T cells to 347 (medians 133 for preimmunization and 180 for postimmunization) and 10 of 45 patients (22%) had more than a 2-fold increase in their antimelanoma CD8 T-cell responses. Among the patients with active metastases, there was a general decrease in antimelanoma reactivity, with four patients showing a decrease of >2-fold (range, 2.5-10).

**Antimelanoma CD4 T-cell responses.** The number of IFN-γ-producing antimelanoma CD4 T cells was higher compared with CD8 T cells, with ELISPOT values for all patients in the study increasing from 442 spots per 100,000 cells before vaccination to 492 postimmunization. This change was not...
statistically significant. Here again, most of the increase in CD4 T cells was observed in the adjuvant patients. For this group, IFNγ ELISPOT rose from 392 to 485, and of the 20 evaluable patients, five increased the number of their CD4 antimelanoma T cells by >2-fold. In contrast, in the metastatic patients, there was a decrease in antimelanoma CD4 response, from 530 to 450, which was statistically nonsignificant.

**HLA class II expression determines autologous melanoma and antimelanoma T-cell responses.** A clear connection was evident between the emergence of CD8 and CD4 responses against melanoma and the class II status of tumor cells (Fig. 3). In the 20 patients with class II–expressing melanomas, pretreatment ELISPOT results were higher (CD4 and CD8). In adjuvant patients whose tumors (and vaccines) expressed class II, the increase in their CD4 and even more pronouncedly in their CD8 antimelanoma T cells was higher (CD4 T cells increased from 460 to 580 and CD8 T cells from 430 to 617), whereas patients with class II–negative tumors had a smaller increase (296-390 CD4 and 177-210 CD8 cells). In a similar manner, metastatic patients with class II–expressing tumors had higher numbers of IFNγ-secreting cells in the prevaccination and postvaccination blood samples compared with patients with class II–negative tumors. However, in the therapeutic group, the tumor’s class II expression did not prevent a decrease in CD4 and CD8 T cells after a vaccination that was very pronounced in this sub group.
As for HLA class I expression, 26 of 30 tumor lines (86.7%) stained positively. Three of four lines, which did not express HLA class I, lacked class II expression as well. Thus, we saw that vaccination with an autologous tumor that did not express HLA class II did not generate an increase in the number of melanoma-responsive CD4 and CD8 T cells. The antimalanoma immunity was dependent on the capacity of the vaccine to present HLA class II-restricted antigens. This observation suggests that, in this vaccination technique, it is the melanoma cells of the vaccine that trigger class II-restricted responses and HLA class II expression is not only necessary to increase the number of CD4 T cells but also to maintain CD8 lymphocytes. In spite of the clear effect of HLA class II expression on vaccine-induced immunity, we did not observe improved survival for patients with HLA class II–expressing melanomas.

**Anti-PBMC CD8 and CD4 T-cell responses.** High increase in postimmunization reactivity was found against PBMC targets. CD8 and CD4 T cells both showed a significant increase in IFN-γ production against autologous PBMC. This trend was observed in both groups of patients but was more pronounced in the adjuvant group where CD8 anti-PBMC rose from 358 to 451 (P = 0.04) and CD4 anti-PBMC rose from 380 to 546 (P = 0.001).

**Correlation of ELISPOT results and survival.** Using the Cox regression model, the single parameter that was found to correlate with OS was the increase in antimelanoma CD4 T cells in patients of the adjuvant group (P = 0.04). The effect of the vaccination procedure on anti-PBMC responses did not correlate with improved outcome nor did IFN-γ production against tumor targets before or after vaccination when taken as an independent single value. Thus, spontaneous pretreatment immunity had no significant effect on survival.

The correlation between OS and an increase in antimalanoma CD4 responses, together with the observation that tumor lines which lacked HLA class II expression failed to evoke CD4 and CD8 antimelanoma reactivity, emphasizes the major role of CD4+ T cells in the acquisition of protective immunity.

**Humoral responses**

**Vaccine-induced antilivin humoral response.** Livin is a member of the inhibitor of apoptosis protein family capable of incongruous antiapoptotic or proapoptotic activity, depending on its molecular configuration in a native or truncated form (26). We tested changes in titers of circulating antibodies against the native protein as an indicator of humoral immunity to a self antigen that is also a determinant of the malignant phenotype.

Serum samples were taken from a cohort of 32 patients for evaluation of circulating antilivin antibodies before and after immunization. Only 10 of these patients were also analyzed for T-cell responses. The ELISA results show that the autologous melanoma vaccine induced a significant increase in serum levels of antilivin IgG response (P = 0.001). Before vaccination, only three patients had antilivin IgG levels above the average for the normal population, whereas after vaccination, 14 had higher levels (Fig. 4). Using the Kaplan-Meier curve, a correlation was found between OS and a 1.5 and higher increase in postimmunization antilivin IgG levels. Patients with this increase had a mean OS of 5 years, whereas patients who had a lesser or no increase in serum levels had a mean OS of 27 months (P = 0.038).

As antibody production is linked to CD4 T-cell activation, these results support the correlation between OS and antimelanoma CD4 responses described above.

**Autologous melanoma vaccine decreases the anti-gp100 humoral response.** In contrast to the observation regarding an increase in antilivin antibodies after vaccination, an inverse correlation was found between serum levels of anti-gp100 IgG and vaccination. A consistent significant decrease in circulating anti-gp100 IgG was observed after the immunization protocol (data not shown). No correlation was found with OS.

**Discussion**

The basic goal of therapeutic vaccines against cancer is to generate a CD4 and CD8 T-cell effector immunity that will mediate tumor regression either directly or in combination with other immunomodulators (27, 28). In the present study, we enrolled two populations of melanoma patients to participate in an autologous vaccine protocol: (a) patients who had complete resection of their metastases and had no evidence of disease and (b) patients with active metastatic disease. We show here that DNP-modified autologous melanoma cells are capable of inducing CD8 and CD4 T-cell responses against autologous melanoma

![Fig. 4. A, antilivin IgG level in preimmunization and postimmunization sera of melanoma patients. B, OS of patients with ≥1.5-fold increase in postimmunization antilivin IgG levels (top curve) versus patients with smaller or no increase.](image-url)
in vaccinated patients and that this response occurs primarily in the adjuvant patients. Together with the antimelanoma reactivity, an autoreactive CD4 and CD8 response was found, assessed by IFNγ secretion against autologous PBMC. The capacity of the vaccine to mount an antimelanoma CD4 T-cell response was the most significant immunologic change that affected OS in the adjuvant patients (P = 0.04). In the metastatic group, none of the patients tested had an immunologically meaningful increase in CD4 melanoma-reactive T cells defined as 2-fold increase and a postvaccination value of >100. The increase in antimelanoma CD8 T-cell reactivity was more common than CD4 responses and reached statistical significance, but when ELISPOT results were analyzed, the CD8 T-cell reactivity did not translate into a survival benefit. Discordance between the emergence of peptide-specific T cells and clinical outcome has been shown in the past (29–31). It is possible, though, that the size of our patient group was not large enough to show the survival benefit conferred by vaccine-induced CD8+ T cells. Larger series and case studies linked melanoma-specific CD8+ T cells with favorable clinical outcome (32–34). Two of our own stage IV patients with the highest CD8 postimmunization scores are still alive 42 and 48 months after diagnosis, suggesting that vaccine-induced CTLs played a protective role.

This immunomonitoring study points toward a central role of vaccine-induced CD4 T-cell responses in improved survival of melanoma cancer patients. Successful induction of CD4 T cells has been shown in the past for full-length antigen vaccines and was correlated with antibody production (33, 35). Whole-cell vaccines were rarely analyzed for their effect on T-cell populations.

Although the critical effector that carries out tumor destruction is the CD8 T cell, the importance of CD4 T cells in melanoma immunotherapy was recently emphasized when adoptive transfer of an NY-ESO-1 antigen-specific CD4 T-cell clone induced complete tumor regression (36). In addition, the increased success rates of adoptive cell therapy of tumor-infiltrating lymphocytes may be attributed, at least in part, to CD4 T cells present in the transferred population (37). The interaction between CD4 and CD8 T cells triggers and sustains effective antitumor immunity (38).

Induction of CD4 T-cell responses to autologous melanoma vaccine may be linked to the capacity of melanoma cells to express HLA class II molecule on their cell surfaces. We have shown in the present study that tumors expressing HLA class II induced a larger increase in melanoma-targeting CD4 T cells than tumors lacking class II. The link between class II expression and T-cell responses implies that melanoma cells in the vaccine directly present antigens and stimulate T cells without requiring cross-presentation by professional APCs. This trait is unique to melanoma cells and may be one of the reasons why this tumor can undergo spontaneous regression (39, 40).

Other researches have shown an untoward effect of CD25+CD4+ regulatory T cells in suppressing vaccine-induced immunity, but this aspect was not addressed in this study.

One of the most surprising outcomes of this immunomonitoring study was the discovery of a prominent CD8 and CD4 anti-PBMC reactivity resulting from vaccination. A search for autoreactive T cells against self antigen is generally not done in vaccination studies, and the extent of vaccine-induced immunologic autoreactivity has not been reported. The processing of the prevaccination and postvaccination blood samples was identical and simultaneous, and the increase in autoreactive T cells was consistently seen only in the postvaccination samples. Therefore, we consider this increase to be a real change, which occurred in vivo, and not an assay bias. There is no basis to assume that the assay is detrimental to low-frequency antitumor T cells, because Karbach and colleagues have shown in the past that in vitro stimulation with tumor cells actually favors the selective expansion of low-frequency tumor-reactive T cells (41). In spite of its magnitude, the increase in autoreactive T cells did not correlate with improved survival and did not confer any therapeutic benefit to the metastatic patients. Reactivity to self antigens is viewed as an undesirable outcome of vaccination, and a deliberate attempt to induce it would generally be avoided. Nevertheless, self-reactive T cells were shown to be involved in cancer regression. Reuben and Ribas have shown a close connection between immunorelated adverse events and the response to the monoclonal anti CTLA4 antibody in patients with metastatic malignant melanoma (42, 43). Gogas and colleagues (44) showed that the preventive efficacy of IFN-α in high-risk melanoma patients was significantly correlated with an increase in serum levels of antithyroid, antinuclear, anti-DNA, and anticardiolipin autoantibodies. Although those authors regard the humoral response as a prognostic marker, it is possible that the survival benefit was gained by immune targeting of biologically significant antigens shared by normal and tumor cells (45). This is possibly the case for the significant correlation we found between survival and the increase in the antibody level against livin. In its native form, livin is linked to a malignant phenotype. Here, we show that livin possesses immunologic properties and that a consistent increase in antilivin IgG is detected in postvaccination sera and correlates with improved survival (Fig. 4). Our data on antibody response to livin correlate with previous reports on CTL response to livin-derived epitopes (46, 47), and the correlation of improved survival in patients with high antilivin IgG level may reflect a simultaneous livin-specific CD8+ T-cell response. This molecule deserves further attention for its value in targeted therapy.

Generation of humoral immune responses is a corollary of vaccine-induced CD4 T cells (48). The clinical relevance of the antibody response per se is not clear. For example, vaccine-induced IgM response against melanoma antigen TA-99 correlated with improved survival to an allogeneic melanoma vaccine, whereas IgG did not (49, 50). Similarly, our antibody data against gp100, in contrast to livin, showed decreased antibody levels after vaccination. Gp100 also possesses an endosomal targeting motif that facilitates its presentation in conjunction with MHc class II molecules (50). The effect of the autologous vaccine in a cohort of 32 patients was of a consistent and significant decrease in the level of anti-gp100 antibody levels.

Whereas the increase in antilivin antibody is encouraging, the decrease in anti-gp100 antibodies raises the question of a possible tolerogenic effect of vaccine-induced CD4+CD25+ regulatory T cells—a claim made against whole-cell vaccines, that they may worsen patients’ prognosis (51). However, the correlation between the development of strong DTH response to melanoma cells and improved survival of vaccinated melanoma patients as seen by us, as well as several other whole-cell vaccine studies, implies the opposite (52–56). Furthermore, DTH response is a reliable clinical parameter that reflects the overall immune
response to other vaccines, including peptides (32) and dendritic cell–based vaccines (53), and can be applied in vaccination protocol as an efficient and available immunologic tool.

Finally, the immunomonitoring data confirm the widely held view that patients with active metastatic disease are at the worst stage with respect to initiating a time-consuming process of active immunization. The antimelanoma responses of CD8 and CD4 T cells in the metastatic patients were inferior to those of the adjuvant group. No patient acquired a significant increase of melanoma-cognate CD4 T cells, and the mean response was decreased. The immune suppressive effects of metastatic disease have previously been thoroughly reviewed, and findings include a significant increase in Tregs, secretion of immune suppressive cytokines, and induction of apoptosis of tumor-reactive lymphocytes (57, 58) in accordance with our findings.

In summary, the use of autologous melanoma cells conjugated to DNP with Bacillus Calmette-Guerin adjuvant conferred protective immunity to a subset of patients who attained strong skin reactivity to unmodified melanoma cells. In adjuvant patients who were immunized in the absence of detectable disease, the vaccine generated melanoma-cognate CD4 and CD8 T cells and the relative increase in the number of CD4 T cells significantly correlated with OS. Expression of MHC class II antigens on tumor cells was vital for their immunogenicity.

In the metastatic patients, the capacity of the vaccine to induce effective immunity against melanoma was reduced, no clinical regression was noted, and no correlation was found with OS. A by-product of the use of autologous tumor cells was the increase in CD4 and CD8 T cells reactive against autologous PBMC. This anti-self-reactivity did not confer a protective effect.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Immune Response to Autologous Melanoma Vaccine

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Autologous Melanoma Vaccine Induces Antitumor and Self-Reactive Immune Responses That Affect Patient Survival and Depend on MHC Class II Expression on Vaccine Cells

Michal Lotem, Arthur Machlenkin, Tamar Hamburger, et al.


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