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Chemotherapy enhances vaccine-induced antitumor immunity in melanoma patients

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Combination of chemotherapy with cancer vaccines is currently regarded as a potentially valuable therapeutic approach for the treatment of some metastatic tumors, but optimal modalities remain unknown. We designed a phase I/II pilot study for evaluating the effects of dacarbazine (DTIC) on the immune response in HLA-A2$^+$ disease-free melanoma patients who received anticancer vaccination 1 day following chemotherapy (800 mg/mq i.v.). The vaccine, consisting of a combination of HLA-A2 restricted melanoma antigen A (Melan-A/MART-1) and gp100 analog peptides (250 μg each, i.d.), was administered in combination or not with DTIC to 2 patient groups. The combined treatment is nontoxic. The comparative immune monitoring demonstrates that patients receiving DTIC 1 day before the vaccination have a significantly improved long-lasting memory CD8$^+$ T cell response. Of relevance, these CD8$^+$ T cells recognize and lyse HLA-A2$^+$/Melan-A$^+$ tumor cell lines. Global transcriptional analysis of peripheral blood mononuclear cells (PBMC) revealed a DTIC-induced activation of genes involved in cytokine production, leukocyte activation, immune response and cell motility that can favorably condition tumor antigen-specific CD8$^+$ T cell responses. This study represents a proof in humans of a chemotherapy-induced enhancement of CD8$^+$ memory T cell response to cancer vaccines, which opens new opportunities to design novel effective combined therapies improving cancer vaccination effectiveness.

Key words: chemoimmunotherapy; dacarbazine; human melanoma; peptide vaccination

Melanoma is a life threatening skin cancer whose incidence is increasing in western countries.[1] In spite of a wealth of information regarding the molecular basis of melanoma progression, its clinical management remains a major challenge. In fact, surgery is curative only for localized minimally invasive tumors, whereas advanced disease withstands conventional chemotherapy regimens.[2] Dacarbazine (DTIC), a DNA alkylating agent exerting cytotoxic effects on actively growing cells, represents the first-line treatment in melanoma.[3]

Immunotherapy is an attractive and potentially effective alternative treatment strategy for the management of metastatic melanoma whose rationale relies on the molecular characterization of an increasing number of defined tumor-associated antigens.[4] This resulted in the development of specific immunization trials of patients with metastatic melanoma with a variety of vaccine formulations.[5] Although the overall clinical outcome of these studies have been disappointing, they provided, together with preclinical data, novel insights on the biology of immune recognition and the obstacles that should be overcome for successful immunotherapy.[6] A major goal of therapeutic cancer vaccines is the induction of functional long-lasting T cell memory against tumor antigens, to prevent tumor recurrence and metastasis.

Recently, it is becoming increasingly accepted that, in order to induce a clinically effective antitumor response, immunotherapy needs to be combined with chemotherapy.[7–10] Thus, the traditional perception that chemotherapy and immunotherapy act through unrelated mechanisms, which may be antagonistic, is challenged on the premises that a selected panel of drugs can induce an immunogenic cell death producing specific danger signals.[11,12] Furthermore, chemotherapy combined to immunotherapy may affect antigen cross-presentation,[13] induce a "cytokine storm,"[14] reduce the number of regulatory T cells[15] and activate homeostatic lymphoid proliferation.[16,17]

On the basis of the results obtained in a mouse model,[14,16] demonstrating that drug-induced cytokines can favor antitumor immunity, in our study we explored whether the administration of DTIC in disease-free melanoma patients 24 hr prior to peptide vaccination could result in an improved cellular immune response to vaccine and in an early upregulation of immune response-related genes. A remarkable *ex vivo* expansion of blood-derived peptide-specific CD8$^+$ T cells displaying a long-lasting effector memory phenotype and ability to specifically lyse HLA-A2$^+$/Melan-A$^+$ tumor cell lines was demonstrated only in immunized patients pretreated with DTIC. Notably, analysis of peripheral blood mononuclear cells (PBMC) gene expression profiles revealed an increased expression of immunoregulatory factors 1 day after chemotherapy. These results open new perspectives for the design of effective combination protocols of chemotherapy with cancer vaccines.

Material and methods

Study design and patient selection

This was a phase I/II pilot clinical study that was approved by the Ethical Committees of the Regina Elena Cancer Institute of Rome and the University of Rome "Tor Vergata" and designed to

Additional Supporting Information may be found in the online version of this article.

Abbreviations: APCs, antigen presenting cells; aRNA, antisense RNA; DTIC, dacarbazine; GMP, good manufacturing practice; IFN-α, interferon-α; Melan-A, melanoma antigen A; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; pts, patients.

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assess the toxicity and immunogenicity of combined chemoimmunotherapy. Two arms of treatment were designed: Arm 1, vaccine alone: patients received i.d. injections of Melan-A: 26–35 (A27L) and gp100: 209–217 (210M) peptides (250 μg each) formulated in Montanide ISA-51 plus s.c. injection of 3MU interferon-α (IFN-α), as an adjuvant on day 1 and 8 every 21 days for a total of 5 courses (10 vaccinations). Both peptides and IFN-α were injected in close but separate sites next to local lymph nodes. Arm 2, DTIC and vaccine: the same vaccination schedule was combined with DTIC (800 mg/mq i.v.) administered 1 day before each vaccination course according to the standard treatment. Treatment schedules are illustrated in Figure 1*a*. DTIC dose adjustments were foreseen, based on worst toxicity occurring in the previous cycle with a 25% dose-reduction in the event of grade 4 neutropenia with fever and/or platelet count below 25×10^9 per liter and any grade $\geq$3 nonhematological toxicity.

Thirty-six stage II–IV (AJCC, 2001) melanoma patients, with no evidence of disease following tumor resection, were screened for HLA expression after a signed consent, and 12 HLA-A0201 eligible patients were enrolled. Criteria for eligibility included histologically proven diagnosis of melanoma stage II, III and IV without clinical/radiological evidence of disease, life expectancy of more than 6 months, Eastern Cooperative Oncology Group performance status of 0–2, adequate blood cell counts and kidney-liver function, use of adequate contraceptive methods, written informed consent. Patients were excluded if they had a concomitant or previous history of malignant disease, except for *in situ* cervical carcinoma or nonmelanomatous skin cancer, a severe cardiovascular disease, clinically active infections and/or significant autoimmune diseases, concomitant or previous (within 6 weeks) treatment with immunosuppressive drugs, previous treatments with chemotherapy and/or interferon alpha or beta within 4 weeks and/or radiotherapy within 6 weeks and/or biological therapy within 8 weeks before starting vaccination, psychiatric illness interfering with patient compliance, pregnancy or lactation. Eligible patients were enrolled at the Regina Elena Cancer Institute of Rome ($n = 11$) and the University of Rome "Tor Vergata" ($n = 1$). Two patients were excluded due to the evidence of metastatic disease before the beginning of treatment; therefore, 10 out of the 12 HLA*0201 positive were assigned to 2 treatment arms by alternate allocation following the order of enrollment. All eligible patients receiving at least 4 vaccination cycles were considered assessable. Previous treatments were: biotherapy for patients 02, 10, 36; chemotherapy and radiotherapy for patient 18. Primary end points of the study were the evaluation of safety and tolerability of the treatment as well as the effects of DTIC administration on vaccine-induced $CD8^+$ T-cell response.

Clinical monitoring

All patients included in the study were confirmed to be without clinical/radiological evidence of disease as documented by physical examination and total body CT scan.

Patients were followed for disease progression with a total body CT scan every 4 or 6 months according to stage of disease (stage IV or stages II, III, respectively) and locoregional lymph nodes ultrasound every 4–6 months. Complete blood count and full chemistry panel were done before initiation and before each cycle of vaccination. Ophthalmic examination was performed before the enrollment, after 2 cycles and at the end of treatment. The study design foresees that after the first 5 years, patients were to be evaluated by physical examinations, and total body CT scan alternated to chest X-ray and abdomen-pelvis and locoregional lymph nodes ultrasound every 6 months.

Toxicity assessment

Safety and tolerability end points were determined throughout the duration of the study. WHO-CTC toxicity grading has been applied to report acute and late toxicities. Ophthalmologic/dermatologic examinations and hematological determinations of circulating autoimmune antibodies were carried out at baseline and at week 7 and 15 to evaluate possible autoimmune reactions caused by melanoma/retina cross-reacting differentiation antigens. A 1-week treatment delay has been applied in case of lack of recovery from intercourses toxicities.

Clinical outcome

The clinical outcome, included as a secondary end point of the study, was evaluated by the assessment of relapse-free survival and overall survival. At the end of study treatment, the patients were subjected to clinical follow up according to recommended guidelines. Relapse-free and overall survival were calculated from the time of the 1st chemotherapy/vaccination injection to the 1st evidence of relapse of disease or disease-related death, respectively (RECIST). Patients in clinical remission were contacted every 6 months to ascertain disease status.

Peptides and IFN-α

Two melanoma-associated peptides were utilized in the vaccine: Melan-A: 26–35 (A27L), ELAGIGILTV; gp100: 209–217 (210M), IMDQVPFSV. The peptides were prepared under good manufacturing practice (GMP) conditions by Clinalfa (Laufelfingen, Switzerland) and were supplied as a water soluble white powder. IFN-α (Human leukocyte interferon alpha, Alfaferone®) was kindly supplied by Dr. G. Viscomi (Bologna, Italy). The native Melan-A: 26–35 (EAAGIGILTV) and gp100: 209–217 (ITDQVPFSV) peptides (Primm) were used for *in vitro* studies in comparison with the respective modified peptides (data not shown). Peptides derived from Flu A matrix M1 (GILGFVFTL) and HIV-1 polymerase (ILKEPVHGV) proteins (Primm, Milan, Italy) were also used for *in vitro* studies.

Blood cells, HLA-A2/peptide tetramers and flow cytometry

For immunological monitoring, 30 ml of heparinized blood was obtained from each patient prior to vaccination (Pre) and 42, 84 and 105 (T42, T84, T105) days after the 1st vaccination. For some patients, samples from 9 to 16 months after the end of the treatment were also available. PBMC were isolated and cryopreserved as described previously.[17] Collected cryopreserved samples were simultaneously thawed and tested as described later.

Phycoerythrin (PE)-labeled HLA-A*0201/peptide (Melan-A A27L, ELAGIGILTV; gp100 210M, IMDQVPFSV) tetramers were purchased from Beckman Coulter (San Diego, CA). FITC-conjugated anti-CD8 monoclonal antibody was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany); FITC-conjugated CD45RA and PE-Cy7-conjugated CCR7 monoclonal antibodies were purchased from BD Biosciences (San Josè, CA). Briefly, $CD8^+$ T cells were positively enriched using anti-CD8-coated magnetic microbeads (Miltenyi Biotec), resulting in more than 90% $CD3^+CD8^+$ cells. Cells were first incubated with tetramers (1 μg/10^6 cells, 30 min, room temperature) and then with the appropriate Abs (30 min, 4°C). As negative control, we utilized iTAgTM HLA class I Human Negative Tetramers SA-PE (Beckman Coulter), developed for assessing the level of background PE fluorescence (0.01%). They were loaded with a peptide, which was shown to tightly bind to HLA-A2, and was proven not be recognized by any T cells from HLA-$A2^+$ individuals. Cells were immediately analyzed using FACScan and CellQuest software (Becton Dickinson).

IFN-γ ELISPOT assay

$CD8^+$ T cells were tested in ELISPOT assays for IFN-γ production in response to stimulation to specific or control peptides (Flu A matrix M1 as positive and HIV-1 as negative control) (10 μg/ml), as described.[18] Briefly, 5×10^4 *ex vivo* or 1×10^4 short-term sensitized $CD8^+$ T lymphocyte were seeded in each well. The number of spots was counted in quadruplicate by the use of Computer-assisted Video Image Analysis (Axioplan 2, Zeiss, Germany). In the *ex vivo* ELISPOT assay, positive was defined as

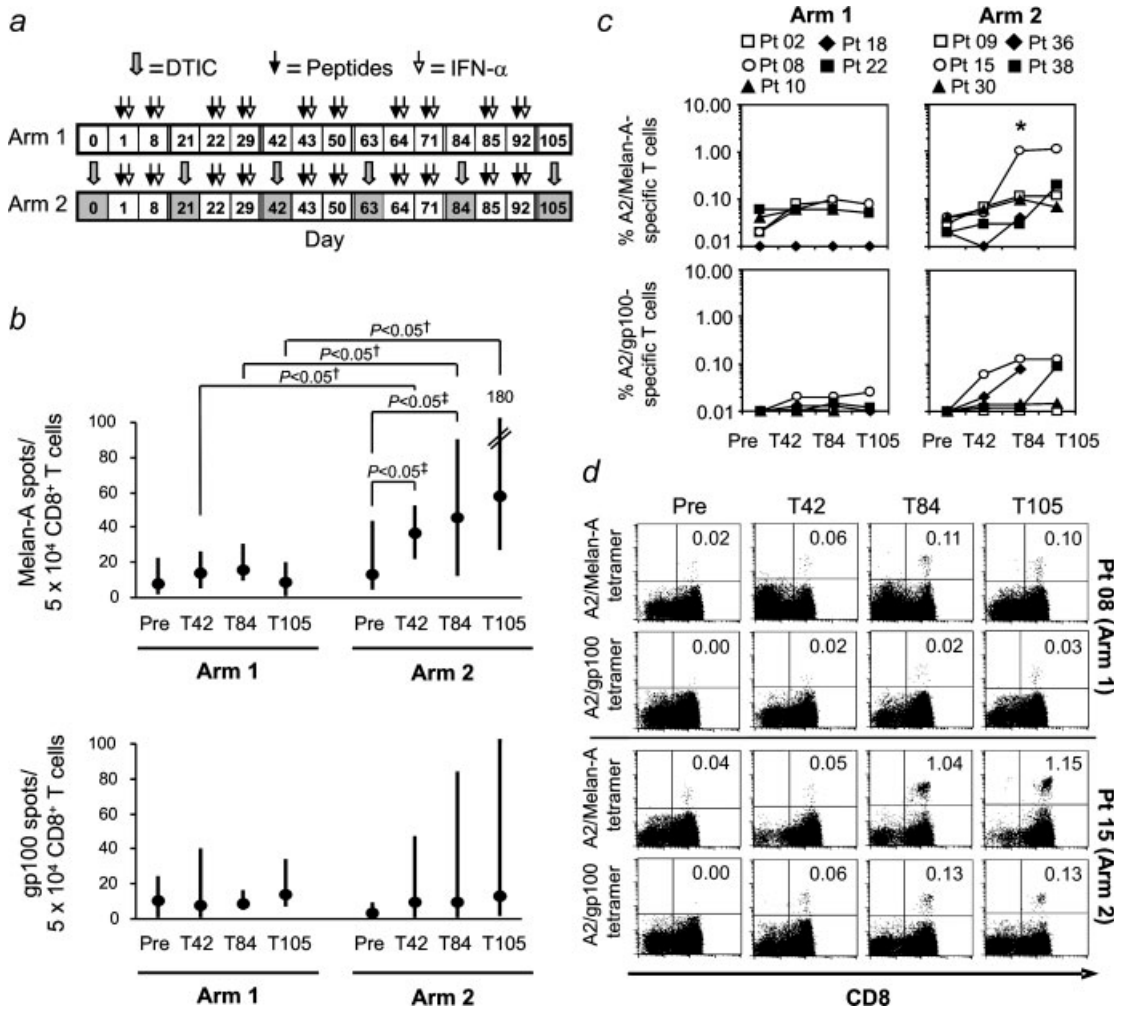


FIGURE 1 – Treatment schedule and *ex vivo* $CD8^+$ T cell response to peptide vaccination. (*a*) Ten patients were immunized with 5 cycles of 2 vaccinations with Melan-A (A27L, ELAGIGILTV) and gp100 (210M, IMDQVPFSV) peptides emulsified in Montanide ISA-51 (black arrows) in combination with IFN-α (white arrows). An infusion of DTIC 1 day before vaccination (gray arrows) was administered in the 5 patients of arm 2. (*b*) PBMC were collected before (Pre) as well as at different times during vaccination. Measurement of *ex vivo* $CD8^+$ T cell reactivity against Melan-A (A27L, top) and gp100 (210M, bottom) by IFN-γ ELISPOT is shown. Bars represent the range, with the geometric mean, of specific spot numbers, after subtraction of nonspecific spots (as determined by incubation with T2 cells alone or HIV-peptide pulsed target cells). Positive in the ELISPOT assay was defined as $\geq$10 spots per 5×10^4 $CD8^+$ cells and more or equal to twice background. Each bar relates to the 5 patients enrolled in arm 1 and to the 5 patients enrolled in arm 2. †, statistical analysis performed by Mann–Whitney two-sample test; ‡, statistical analysis performed by paired Wilcoxon ranks test. (*c*) $CD8^+$ T cells were double-stained with FITC-CD8 antibody and PE-HLA-A*0201 Melan-A (top) or gp100 (bottom) tetramers and analyzed by flow cytometry. Percentage of tetramer-specific $CD8^+$ T cells is shown. As negative control, iTAgTM HLA class I human negative tetramer SA-PE was used, showing no background of staining (0.01%; data not shown). Data are representative of 2 or more independent experiments with similar results. * Pre *vs.* T84, $p < 0.05$ (paired Wilcoxon ranks test) in the arm 2 group. (*d*) Representative dot plots from PBMC of patient 08 from arm 1 (top) and patient 15 from arm 2 (bottom), showing the percentage of Melan-A- and gp100-specific cells among $CD8^+$ T cells.

$\geq$ 10 spots per 5×10^4 $CD8^+$ cells and more or equal to twice background. For statistical evaluation, a *t*-test for unpaired samples was applied using the Inplot Software System (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were considered significant.

Short-term Ag-specific T cell lines

$CD8^+$ T cells were seeded at a concentration of 2×10^5 cells per well in RPMI-1640 medium supplemented with 10% human serum. Autologous $CD8^+$-depleted PBMC were used as an antigen presenting cells (APCs). APCs were irradiated, pulsed with Melan-A A27L or gp100 210M peptides (10 μg/ml) for 2 hr at 37°C in 5% CO_2 and plated with $CD8^+$ T cells at a 1:3 ratio. After 24 hr, human recombinant IL-2 (Roche Diagnostics GmbH, Mannheim, Germany) and IL-7 (PeproTech, Rocky Hill, NJ) (25 U/ml and 5 ng/ml, respectively) were added to the culture wells. Cells were restimulated after 1 week with the soluble peptide (1 μg/ml) for an additional 7 days before functional analysis.

Cytotoxicity assay

Lytic activity and antigen recognition were functionally assessed in a standard 4-hr ^{51}Cr release assay. Target cells were Mel 1 ($A2^-$/Melan-A^+), Mel 2 ($A2^+$Melan-A^-), Mel 3 ($A2^+$/Melan-A^+) melanoma cell lines (a kind gift of A. Anichini, Istituto Nazionale Tumori, Milan, Italy) and K562, an NK-sensitive erythroleukemia cell line. Cytotoxicity assays were performed by incubating ^{51}Cr-labeled target cells with effector cells at different effector/target ratios. The specificity of the assay was evaluated by preincubating target cells with the anti-MHC class I W6/32[19] mAb (IgG2a) (10 μg/ml). The IgG2a mAb W6/100[18] was used as

TABLE I – CHARACTERISTICS OF PATIENTS AND CLINICAL SUMMARY

Patient no	Arm[1]	Age	Sex	Stage	Disease sites[2]	Clinical response		
						RFS (mo)[3]	Site of relapse	OS (mo)[4]
02	1	47	M	IV	Lu	3	Brain	30+
08	1	56	F	II	–	36+	–	36+
10	1	39	M	IV	Lu	2	Lu, LN	7
18	1	61	M	IV	LN	6	LN	28
22	1	22	M	III	–	8	Brain	24
09	2	46	F	III	–	37+	–	37+
15	2	42	M	IV	ST	35+	–	35+
30	2	23	F	III	–	11	Lu[5]	31+
36	2	60	F	IV	Lu	2	Bone	9
38	2	45	F	III	–	4	Liver	9

No grade (G) 3/4 WHO criteria hematological and no hematological toxicities were observed.
[1]Arm 1, vaccine alone; Arm 2, dacarbazine and vaccine.–[2]Lu, lung; LN, lymph nodes; ST, soft tissue.–[3]RFS, relapse-free survival.–[4]OS, overall survival.–[5]The patient is presently disease-free following second line chemotherapy.

an irrelevant antibody for monitoring the specificity of the inhibition assay (not shown). The percentage of specific lysis was calculated as follows: 100 × (experimental release−spontaneous release)/(total release−spontaneous release).

Statistical analysis

The nonparametric Wilcoxon matched-pairs signed-ranks test was applied to compare prevalues *vs.* postvalues. The nonparametric Mann–Whitney two-sample test was used to evaluate differences between the 2 independent groups. A further comparison was performed by the Analysis of Variance (ANOVA) for longitudinal data, using group as factor between subjects and time as factor within subjects. Values of $p < 0.05$ were considered statistically significant.

mRNA amplification and hybridization to microarrays

Total RNA was isolated using RNeasy mini kits (Qiagen, Valencia, CA). Amplified antisense RNA (aRNA) was prepared from total RNA (0.5–3 μg) according to the protocol previously described.[20] For hybridization to the microarrays, test samples were labeled with Cy5-dUTP (Amersham, Piscataway, NJ), and reference samples (pooled normal donor PBMC) were labeled with Cy3-UTP. Test-reference sample pairs were mixed and cohybridized overnight to microarray slides in humidifying chambers. Microarrays were printed in house at the Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, NIH, with a configuration previously described.[21] Following hybridization, the slides were washed and dried in preparation for scanning.

Microarrays and statistical analyses

Hybridized arrays were scanned at 10-μm resolution on a GenePix 4000 scanner (Axon Instruments, Downingtown, PA) at variable PMT voltage to obtain maximal signal intensities with less than 1% signal saturation. Resulting jpeg and data files were analyzed via mAdb Gateway Analysis tool [http://nciarray.nci.nih.gov]. The raw data set were filtered according to standard procedure to exclude spots with minimum intensity (arbitrarily set to <200 in both fluorescence channels) or with diameters <25 μm. The filtered data (consisting of 16,154 genes) were then normalized using a median ratio equivalent to 1 over the entire array. All statistical analyses were done using the log2-based ratios. Validation and reproducibility were measured using an internal reference concordance system based on the expectation that results obtained through the hybridization of the same test and reference material in different experiments should perfectly collimate. The global gene-expression profiling of patient PBMC consisted of 10 experimental samples. Samples were obtained by 4 different patients at different times during the treatment, before and 1 day after DTIC administration: patient 15, T42/T43; patient 30, T0/T1; patient 36, T63/T64; patient 38, T0/T1, T42/T43. Supervised class comparison between before and after DTIC treatment samples was performed with the BRB ArrayTool [http://linus.nci.nih.gov/BRB-ArrayTools.html]. Paired samples were tested by two-tailed Student *t*-test for a univariate significance threshold set at $p \leq 0.005$. Significantly, differentially expressed genes (314 genes) were further analyzed using Cluster and TreeView software.[22] Gene ratios were average corrected across experimental samples and displayed according to the central method for display using a normalization factor as recommended by Ross *et al.*[23] Gene function was assigned by means of mAdb Gateway Analysis tool [http://nciarray.nci.nih.gov].

Results

Patient characteristics, treatment and toxicity

Thirty-six resected, disease-free melanoma patients were screened for their compliance to the study inclusion/exclusion criteria, and 10 of them were enrolled and assigned to 2 treatment arms either receiving tumor antigen-specific vaccination with Melan-A and gp100 analog peptides alone (arm 1) or in combination with DTIC pretreatment (arm 2) according to the schedule illustrated in Figure 1*a*. The characteristics of the patients and their clinical outcome are summarized in Table I. All patients were disease-free at the time of enrollment. The median age of the patients was 45.5 years (range between 22 and 61 years), with no difference in the median age of the 2 arms (arm 1, 47 years and arm 2, 45 years).

The vaccination therapy as well as the combined DTIC treatment were safe and well tolerated. No grade 3/4 WHO criteria hematological and nonhematological toxicities have been observed during the treatment. Nine patients developed inflammatory signs at subcutaneous injection sites, with a peak of symptoms (erythema and induration) around 2–3 days after injection. In arm 2, G 1/2 nausea and vomiting were observed in 3 patients, and in 1 patient (09) a G2 leukoneutropenia determined a 1 week-treatment delay. Other symptoms observed 1 day after vaccine administration were as follows: fever (6 patients), arthromyalgia (1 patient) and headache (1 patient). No signs of autoimmunity (*i.e.*, vitiligo and/or uveitis) were observed as assessed by dermatological and ophthalmic examination. During the treatment, patient 09 reported a clinically asymptomatic diffuse bilateral hyperplasia of ocular pigmented epithelium which improved 6 months after the end of the study. Immunohistochemical analysis of the tumors tissues, available before the treatment, showed a consistent, although heterogeneous expression of both differentiation antigens and varying $CD3^+$ peri- and intratumoral infiltrate that were equally distributed in patients from both arms (data not shown).

DTIC enhances peptide-specific $CD8^+$ T cells induced in vivo by the peptide vaccination

PBMC were collected at different times and analyzed *ex vivo*, without any *in vitro* manipulation, by IFN-γ ELISPOT assays and

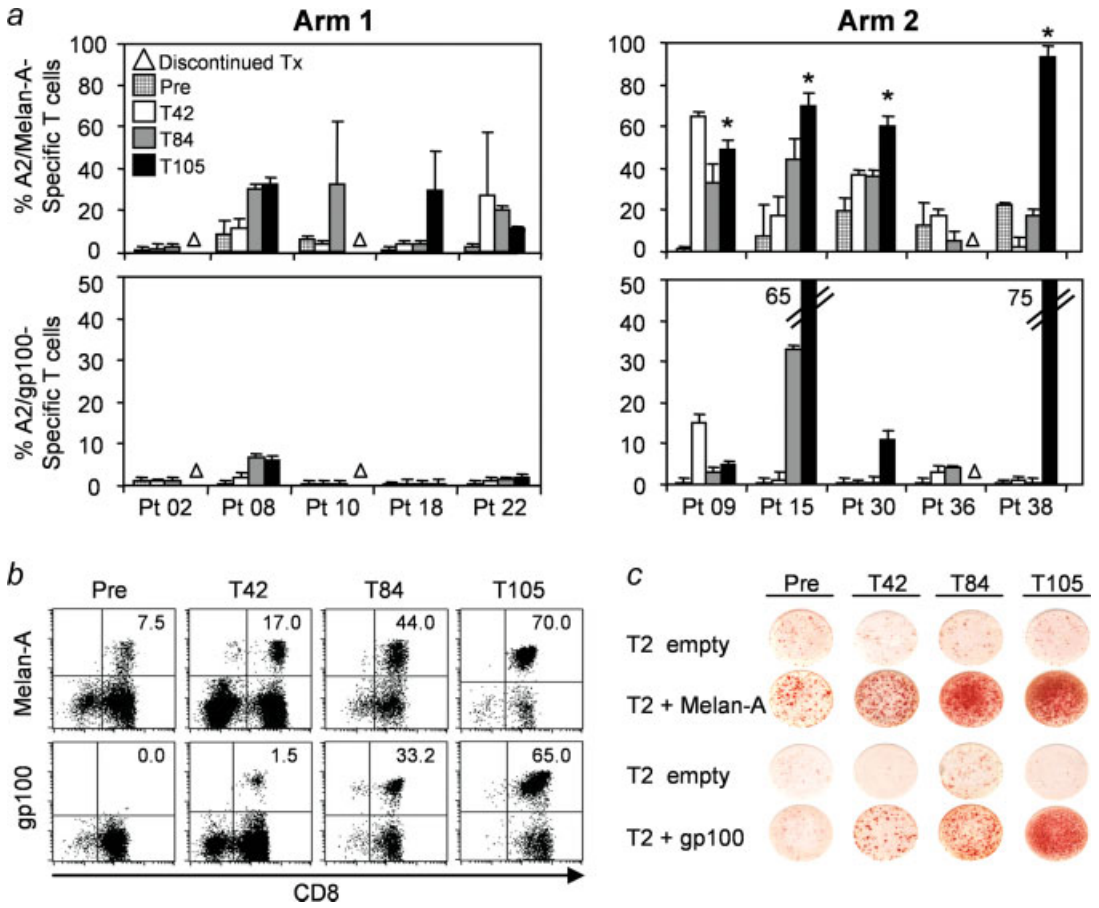


FIGURE 2 – Kinetics of *in vitro* expansion of peptide-specific $CD8^+$ T cells in response to vaccination. PBMC collected at different times during vaccination were magnetically sorted for $CD8^+$ T cells and short term sensitized with Melan-A or gp100 peptides. (*a*) Percentage of peptide-specific $CD8^+$ T cells as evaluated by the use of A2/Melan-A- (top) and A2/gp100- (bottom) tetramers. Data represent the mean ± s.d. ($n = 3$ experiments). As negative control, iTAgTM HLA class I human negative tetramer SA-PE was used, showing no background of staining (0.01%; data not shown). *, $p = 0.03$, performed by Mann–Whitney two-sample test. (*b*) Representative dot plots from patient 15 (arm 2) with percentage of Melan-A- and gp100-specific cells among $CD8^+$ T cells. (*c*) Representative IFN-γ ELISPOT assay (performed in quadruplicate) from Melan-A- and gp100- $CD8^+$ T cell lines from patient 15 at different times during vaccination.

by HLA-A2/Melan-A or HLA-A2/gp100 tetramers staining. Before treatment (Pre), a low frequency of Melan-A-specific $CD8^+$ T lymphocytes was detectable in almost all patients (Figs. 1*b* and 1*c*), with no significant differences among the two arms of treatment ($p \geq 0.5$). As depicted in Figure 1*b*, a progressive increase of vaccine-specific $CD8^+$ T cell precursors was evidenced by IFN-γ ELISPOT only in the patients treated with DTIC before vaccination (arm 2). Pre *vs.* post increase was statistically significant for Melan-A after 2 (T42) and 3 (T84) cycles of vaccination ($p = 0.04$, paired Wilcoxon ranks test). A Mann–Whitney two-sample test was performed to compare the 2 arms of treatment; this analysis showed that the strength of anti-Melan-A T cell response was statistically significantly higher in arm 2 *vs.* arm 1 patients along all the course of vaccination (Fig. 1*b*). Of note, the kinetics of anti-FLU peptide response did not change during the course of treatment and monitoring (data not shown).

In Figure 1*c*, the kinetic of frequency of peptide-specific $CD8^+$ T cells, analyzed by tetramer assay in the 10 patients enrolled, is shown. After 2 vaccination cycles (T42), an increase of Melan-A-specific $CD8^+$ T cells was observed only in 2 out of 5 patients in arm 1, whereas an enhancement was detected in 4 out of 5 DTIC-treated patients. This increase plateau in the 2 patients treated with vaccine alone but underwent a time-related increment, peaking at T84 and persisting at T105, in the 4 DTIC-treated patients. The frequency of vaccine-specific T cells ranged between 0.01 and 0.11% ($p = 0.09$) at T84 in arm 1, whereas in arm 2 it ranged from 0.04 to 1.04% ($p = 0.04$) (Figs. 1*c* and 1*d*). gp100-specific T lymphocytes were barely detectable before vaccination and their frequency did not significantly increase in arm 1 ($p = 0.32$), whereas it increased in 3 out of 5 DTIC-treated patients ($p = 0.05$) (Figs. 1*c* and 1*d*).

Kinetics of the proliferative response of vaccine-specific $CD8^+$ T cells to in vitro stimulation

To further analyze peptide-specific $CD8^+$ T lymphocytes and to determine their proliferative potential, lymphocytes were short-term *in vitro* sensitized with Melan-A or gp100 peptides as reported in Mat. and Meth. and analyzed by tetramer staining (Figs. 2*a* and 2*b*) and IFN-γ ELISPOT assay (a representative case is shown in Fig. 2*c*). Consistently with *ex vivo* results, patients treated with vaccine alone showed moderate levels of Melan-A-specific T lymphocytes and not related to the boost of vaccination, with the exception of patient 08 (Fig. 2*a*). In contrast, in DTIC-treated patients, the percentages of specifically expanded T lymphocytes dramatically increased already after 2 vaccination cycles, reaching values ranging from 50 to 90% at the end of vaccination treatment in 4 out of 5 patients ($p = 0.03$). No significant differences in the proliferative response of vaccine-specific $CD8^+$ T cells between the 2 arms were evident before treatment, thus

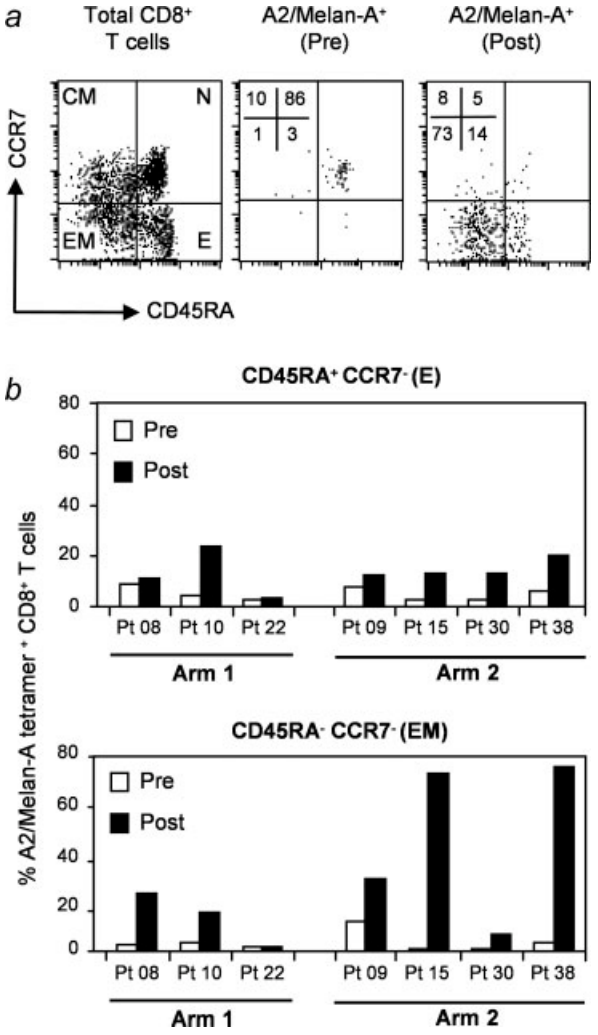


FIGURE 3 – Differential expression of CD45RA and CCR7 in tetramer-positive T cells of vaccinated patients. Tricolor cytometric analysis performed with A2/Melan-A tetramers and antibodies specific for CD45RA and CCR7 in *ex vivo* sorted $CD8^+$ T cells obtained before (Pre) and 3 months after the end of vaccination (Post). (*a*) Representative dot plots from patient 15. CM, central memory; N, naïve; EM, effector memory; E, effector. Pattern of expression of CCR7/CD45RA in total circulating $CD8^+$ T cells (left) and in Melan-A^+ gated cells Pre (middle) and Post (right) vaccination. (*b*) Histograms represent the percentage of circulating Melan-A^+ effector cells (top) and Melan-A^+ effector memory cells (bottom) present in $CD8^+$ T cells obtained before and after vaccination. Data are representative of 2 or more independent experiments with similar results.

indicating that the dramatic expansion observed in the DTIC plus vaccine arm was induced by the treatment. The overall results of ANOVA model, comparing the 2 treatment arms, showed a clearly significant improvement in the proliferative potential of Melan-A-specific $CD8^+$ T lymphocytes derived from arm 2 *vs.* arm 1 patients ($p = 0.001$). The overall proliferative responses to gp100 peptide were lower than those observed to Melan-A. Nevertheless, although no responses were detectable in subjects treated with vaccine alone, a trend of increase was evident in DTIC-treated patients. Figure 2*b* shows the percentage of $CD8^+$ and Melan-A^+ or $gp100^+$ double-positive T cells, as assessed by tetramer staining, in a representative DTIC-treated patient. Figure 2*c* shows the results obtained by ELISPOT assay in the same patient, indicating that both methods showed a similar time-related increase of peptide-specific T cells.

DTIC enhances the in vivo generation of peptide-specific effector memory $CD8^+$ T cells

As Melan-A-specific T cells in melanoma patients comprise both naïve and activated T cells,[24] we characterized the expression of relevant markers (CD45RA and CCR7) on the cell surface of Melan-A-specific T cells. Figure 3*a* shows a representative staining of *ex vivo* sorted $CD8^+$ T lymphocytes from a DTIC-treated patient. As expected, the majority (86%) of Melan-A-specific T cells were naïve $CD45RA^+$ and $CCR7^+$ cells before vaccination. Interestingly, 3 months after the end of the treatment, the majority (*i.e.*, 73%) of Melan-A-specific T cells displayed a $CD45RA^-$ $CCR7^-$ phenotype (effector memory cells), whereas a minor percentage of the $CD8^+$ T cells (*i.e.*, 14%) showed the $CD45RA^+$ $CCR7^-$ phenotype (terminally differentiated effector cells). Overall, this phenotype characterization in the different patients demonstrated that the percentage of Melan-A-specific $CD45RA^+$ $CCR7^-$ (effector cells) was slightly increased after vaccination by a similar extent in both treatment arms. On the contrary, the percentage of $CD45RA^-$ $CCR7^-$ (effector memory) T cells from DTIC-treated patients was consistently higher with respect to patients treated with vaccine alone (Fig. 3*b*). This increase was evident only in the Melan-A-specific $CD8^+$ T cell fraction and not in the total $CD8^+$ T cells population.

The absence of gp100-specific T lymphocyte in all patients at this time did not allow the analysis of gp100-specific memory phenotype.

DTIC treatment induces a long-lasting persistence of peptide-specific $CD8^+$ T cells recognizing naturally processed Melan-A

$CD8^+$ T cells isolated from PBMC collected 9–16 months after the last vaccine administration were stained with HLA-A2/Melan-A or HLA-A2/gp100 tetramers *ex vivo* and after a short *in vitro* expansion. *Ex vivo* Melan-A specific $CD8^+$ T lymphocytes ranged between 0.03 and 0.05% with no significant difference in the 2 arms, with the exception of patient 15 (arm 2) who showed a significant percentage of Melan-A^+ cells (0.2%) still detectable by *ex vivo* analysis 16 months after the end of the treatment (data not shown). Although a low frequency of *ex vivo* Melan-A^+ cells was depicted, these cells showed a high capability of *in vitro* proliferation in response to the cognate peptide only in the patients from arm 2. In fact, a strong expansion of Melan-A specific T cells, ranging between 40 and 90%, was evidenced in the DTIC-treated patients, whereas the percentage of these cells from arm 1 patients never exceeded 20% (Fig. 4*a*). Notably, peptide-specific T cells from DTIC-treated patients could be expanded *in vitro* at the same levels of those collected at the end of the vaccination cycle (T105). gp100-specific $CD8^+$ T cells did not show any proliferative ability with the exception of the DTIC-treated patient 15 (Fig. 4*a*). To test whether vaccine-specific T cells could specifically recognize and lyse tumor cells expressing and presenting the native Melan-A peptide, the *in vitro* expanded T cells were tested by cytotoxicity assays. As shown in Figure 4*b*, the representative results obtained with cells from 3 different patients (08 from arm 1, 09 and 15 from arm 2) demonstrated that none of the $CD8^+$ T cell lines from pre-vaccination blood samples was able to lyse any target tumor cell line, whereas T cell lines derived from samples collected 9–16 months after the end of treatment (post) specifically recognized and lysed HLA-$A2^+$/Melan-A^+ tumor cell line. Reactivity was not observed against HLA-$A2^+$/Melan-A^- cells, HLA-$A2^-$/Melan-A^+ cells or K562 cells. Of note, T lymphocytes from patients 09 and 15 (arm 2) showed a specific lytic activity already at T42, whereas T lymphocytes from patients 08 (arm 1) showed a weak specific lytic activity in the sample collected after the last vaccine administration.

Effects of DTIC on PBMC gene expression profiles at the time of vaccine administration

To determine whether DTIC treatment induced any systemic immunological effect, we tested the gene expression profiles of

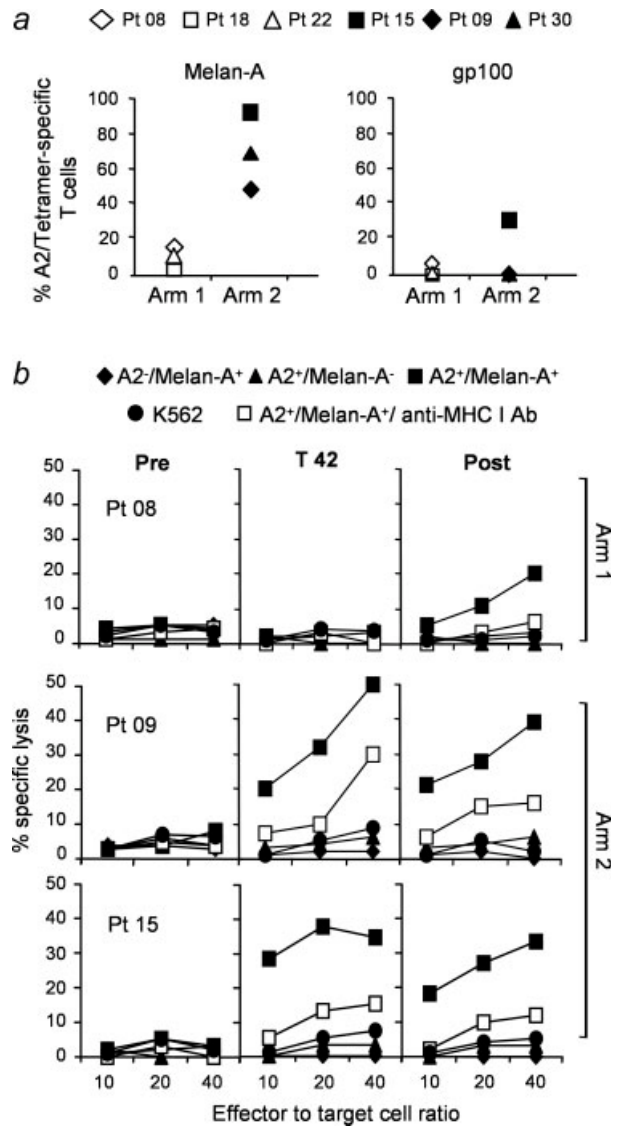


Figure 4 – Long-term maintenance of peptide-specific $CD8^+$ T cells and tumor-specific lytic activity of Melan-A specific T cells. (*a*) PBMC, collected 9–16 months after the end of the treatment from available patient blood samples, were stimulated and analyzed as reported in Figure 2. (*b*) Shortly expanded Melan-A-specific $CD8^+$ T cell lines from patient 08 (arm 1) and patients 09 and 15 (arm 2) were assayed for their lytic activity against melanoma cell lines in a 4-hr ^{51}Cr-release assay. Target cells were ◆ $A2^-$/Melan-A^+ (Mel 1), ▲ $A2^+$Melan-A^- (Mel 2), ■ $A2^+$/Melan-A^+ (Mel 3) melanoma cell lines and ● K562, an NK-sensitive erythroleukemia cell line. Blocking experiments were performed by preincubating the $A2^+$/Melan-A^+ melanoma cell line with the anti-MHC class I W6/32 mAb as described.[17] The percentage of $tetramer^+$ cells in the effector cell lines were patient 08: Pre 1.5%, T42 11%, Post 35%; patient 09: Pre 2%, T42 60%, Post 34%; patient 15: Pre 3%, T42 38%, Post 70%. Values represent the mean percent lysis from triplicate wells. Data are representative of 2 or more independent experiments with similar results.

PBMC of 4 DTIC-treated patients before and 1 day after chemotherapy, when available at the beginning or at different times during the treatment. To assess the consistent effects of DTIC administration, we performed class comparison analysis between the groups of samples collected before DTIC and after DTIC regardless of cycle of therapy. This statistical approach was intended to identify constant changes occurring in the first 24 hr following chemotherapy and was supported by the finding that no significant differences were observed among all the samples collected before chemotherapy (data not shown). A paired *t*-test revealed that 314 genes were significantly ($p < 0.005$) differentially expressed between the 2 classes. Hierarchical clustering of these genes perfectly segregated before- and after-DTIC treatment samples, confirming the consistency of gene expression within the classes. Treeview visualization showed that 138 genes were upregulated, whereas 176 genes were downregulated after DTIC treatment (Fig. 5*a*). The complete list of the differentially expressed genes and the fold difference in the expression levels between the 2 classes are reported as supporting material (Supp. Info. Table).

In Figure 5*b*, a table summarizing the functional classification (based on Gene Ontology) of upregulated and downregulated genes is shown. Biological process classes were ranked according to the abundance of genes fitting each class in proportion to the number of genes expected to be in each class by chance, which was calculated on the global composition of the array. The most represented classes of upregulated genes, partially overlapping, included genes involved in cytokine production and leukocyte activation (such as SYK and LCP2), immune response (such as IL-6 and IL-15) and cell motility (such as CXCL10, CXCL3, CXCL16), strongly supporting the notion that the primary systemic effects of DTIC administration, at the time of vaccination, is a modulation of immunoregulatory factors that may facilitate the induction of antigen-specific T cell expansion. Functional classification of genes downregulated by DTIC administration showed a dampening of biosynthetic and metabolic processes probably related to a mild hematological toxicity induced by the chemotherapy. The modulation of the expression of some selected genes (IL-6, IL-15 CXCL10) was confirmed by real-time PCR (data not shown).

Clinical outcome

Patients were evaluated for disease progression as reported in Mat. and Meth. with a median follow-up of 29 months. As reported in Table I, among the patients enrolled in arm 1, only patient 08 remained disease-free. Four patients (02, 10, 18 and 22) underwent progression, and patient 02 who discontinued the vaccination, due to a brain metastasis, was surgically resected.

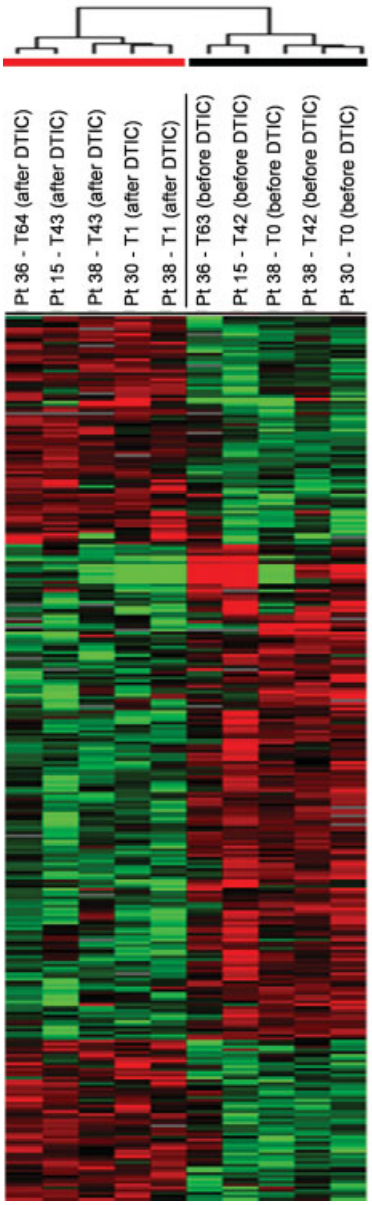
In arm 2, 3 patients are at present without any evidences of disease: patients 15 and 09 remained disease-free after the treatment; patient 30 relapsed in the lung but showed a complete remission following second line chemotherapy. Patients 36 and 38 underwent progression. Notably, the 3 disease-free patients in arm 2 were stage III/IV, whereas in arm 1 the only patient who remained disease-free after treatment was a stage II melanoma subject (Table I).

Discussion

The biological information and overall results stemming so far from clinical trials underline that immunotherapy of advanced metastatic disease, even though resulting in some clinical response, is unlikely to be curative. Thus, one of today's major challenges is the identification of valuable combination strategies for the enhancement of the efficacy of currently tested cancer vaccines, with the aim of inducing long-lasting tumor specific memory T cells, which may in turn efficiently prevent tumor relapse.[25]

Although the combination of chemotherapy and immunotherapy for cancer patients is generally considered unfeasible, based on the assumption that chemotherapeutic agents may negatively affect the function of T cells, recent results obtained in animal models provided evidence that chemotherapy can, under certain conditions, modulate biological processes that may result in enhanced response to immunotherapy.[7,8,14,16,25,26] The mechanisms underlying this phenomenon are complex, and an emerging view implies that reduction of cells of the lymphohematopoietic compartment, induced by certain antineoplastic agents, is followed by "homeostatic proliferation," which may favor the effectiveness of both active and adoptive immunotherapy strategies. This may

a



b

Biological Process of Up-regulated genes			Obs/ Exp
Cytokine production			5.4
IL6	LCP2	SYK	
TLR2			
Leukocyte activation			2.9
IL6	LCP2	SYK	
TNFSF13			
Immune response			2.9
BST1	CXCL3	IL6	
CD97	GAB2	LCP2	
CLEC4A	IFIT2	LILRA4	
CXCL10	IL15	SYK	
TNFSF13	TLR2		
Cell motility			2.6
C3AR1	CXCL10	PLAUR	
CD97	CXCL16	SPINT2	
STAT3	SYK		
Generation of precursor metabolites and energy			2.6
ATP5B	CYP27A1	IL15	
ATP6V0B	GPD2	INSR	
ATP6V0D1	GAK	PYGL	
COX5B	IBRDC2	SYK	
ZAK			
Response to external stimulus			2.4
AIF1	CXCL10	IL6	
C3AR1	CXCL16	PLAUR	
CD97	CXCL3	STAT3	
SYK	TLR2		
Defense response			2.4
AIF1	CXCL10	IL6	
C3AR1	CXCL3	STAT3	
CD97	TCIRG1	TLR2	
Circulation			2.3
C3AR1	CXCL10	SOAT1	

Biological Process of Down-regulated genes			Obs/ Exp
Secondary metabolic process			3.3
ALAS2	PPAT		
Cell homeostasis			2.6
BCL2L1	GCLC		
S100B	XCL1		
CCL5	LOC400986		
Immune response			2.4
HLA-C	CCL5	FYB	
S100B	TCF7	LTB	
TNFRSF7	XCL1	DEFA1	
IGHG1	IGLL1	IL24	
IGJ			
Cytokine production			2.2
S100B	TNFRSF7		
Biosynthetic process			2.1
RPS20	RPS21	RPS29	
RPS7	S100B	TMEM1	
ALAS2	LARS	RPL27A	
EIF5B	PPAT	RPL34	
FUT1	RPL13	RPL36A	
GCLC	RPL13A	RPL41	
HBB	RPL23	RPS13	
HSD17B2	RPL24	RPS16	
TNFRSF7			

FIGURE 5 – Genes differentially expressed between Pre and Post DTIC in patient PBMC. PBMC were obtained from 4 patients before (black bar) and 1 day after DTIC treatment (red bar) at the indicated time points during the treatment. Only for patient 38, samples from 2 different cycles were available. Antisense RNAs were obtained, labeled and hybridized to home-made cDNA arrays containing 17,500 genes, as described in Material and methods. (*a*) The 314 genes found to be differentially expressed between the 2 classes using paired *t*-test comparison ($p < 0.005$) were analyzed by unsupervised Eisen hierarchical clustering. Gene ratios of Cy-5-labeled test *vs*. Cy-3-labeled reference samples (pooled normal donor PBMC) were average corrected across experimental samples before Treeview visualization. (*b*) The 138 upregulated and 176 downregulated genes were functionally classified by means of mAdb Gateway Analysis tool into different biological process classes. For each class, the ratio between the number of genes observed in our analysis (Obs) and the amount of genes expected to be in each class by chance (Exp), calculated on the global composition of the array, are reported in the table (an arbitrary cutoff of 2-fold was chosen for Obs/Exp).

be driven by the release of an array of soluble factors (cytokine storm)[14] acting on different cell compartments, including those involved in the priming of the immune response. Animal observations suggest that several immunoregulatory cytokines are induced early after chemotherapy administration[14] and optimal vaccine-induced antitumor responses are observed when the vaccine is administered at a corresponding early time following chemotherapy (Bracci *et al*., unpublished results). Thus, we designed a pilot study combining DTIC with a peptide melanoma vaccine administered as early as 1 day after chemotherapy to treat disease-free melanoma patients.

In our study, we have shown that a marked expansion of blood-derived peptide-specific $CD8^+$ T cells displaying a long-lasting effector memory phenotype and ability to specifically lyse HLA-$A2^+$/Melan-A^+ tumor cell lines occurred only in DTIC-pretreated patients and not in subjects treated with the peptide vaccine alone. This expansion may not be attributable to a chemotherapic lymphopenic effect, because none of the DTIC treated patients evidenced a blood count reduction during the treatment. In particular, the combination of DTIC with peptide vaccination resulted in 3 out of 5 patients in a significant rise of T cell frequency that progressed till the end of the treatment. Notably, T cell responses

were detectable in these patients by *ex vivo* analysis, whereas in the majority of clinical studies reported so far antitumor T cell responses can only be detected after *in vitro* T cell stimulation.[27] Of interest, a remarkable expansion of the effector memory cell pool ($CD45RA^-$ $CCR7^-$) was particularly evident in 3 out of 4 DTIC-treated patients. Consistently, only in patients treated with DTIC before vaccination, we obtained a strong *in vitro* expansion of $CD8^+$ lymphocytes that are able to proliferate in response to the cognate peptide, thus suggesting that a different "quality" of these cells respect those stimulated by vaccination alone was obtained. In this line, we have studies in progress defining the avidity of Melan-A-specific $CD8^+$ T cell clones obtained either from patients treated with vaccine alone or with DTIC before vaccination to characterize their functional differences. Furthermore, although vaccination was based on modified melanoma antigen peptides, patient-derived Melan-A-specific T cell lines from DTIC-treated patients were capable of efficiently lysing melanoma cell lines expressing the native Melan-A antigen. These data suggest that the combination of DTIC and vaccine induces antitumor immune responses functionally different from those obtained by vaccination alone. This was observed in patients with similar pretreatment spontaneous immune response and tumor phenotype. Notably, 3 out of 5 patients are free from progression of disease (including patient 30 who showed complete remission from lung relapse following second line chemotherapy). Interestingly, the most impressive expansion of long-lasting antitumor $CD8^+$ T cells expressing an effector memory phenotype was observed in the DITC-treated subjects who did not show tumor recurrence, although the limited number of patients did not allow statistical evaluation of this correlation.

To get insights into the potential molecular mechanisms underlying DTIC-induced enhancement of the immune response, we performed global gene expression profiling of PBMC collected before and 24 hr after chemotherapy. This analysis revealed that DTIC administration markedly affects the global transcription profile of PBMC inducing primarily transient immune conditioning potentially leading to the enhancement of long-term vaccine-induced antitumor immune response. In particular, the downmodulation of several genes encoding for ribosomal proteins and for several hemoglobin, granzyme and immunoglobulin family members may suggest a general defect in translation processes and a selective decrease of blood cell subsets, indicative of a mild and transient hematological drug-induced toxicity. The large majority of genes induced by DTIC reflect a primary stimulation of the immune response, which could be interpreted as an early activation of homeostatic mechanisms counteracting drug toxicity effects. The increased expression of several chemokines, such as CXCL10, CXCL3 and CXCL16 (Fig. 5*b*), strongly suggests a general drift toward leukocyte activation and enhanced motility.[28] The possible role of DTIC in immune cell mobilization and activation is also supported by the observed induction of some proinflammatory cytokines, such as TNF and IL-6 (Supp. Info. Table and Fig. 5*b*). Of interest, similarly to the early effect of cyclophosphamide in a mouse model,[14] DTIC administration induced the expression of cytokines involved in lymphocyte expansion and activation, such as IL-2 as observed by real-time PCR (data not shown) and IL-15 (Fig. 5*b*). Notably, IL-15 plays a pivotal role in the maintenance of long-lasting, high avidity T-cell responses by supporting the survival and proliferation of $CD8^+$ memory T cells.[29,30] We speculate that the increased expression of the homeostatic cytokines IL-2 and IL-15, and the simultaneous upregulation of 3 genes encoding factors involved in their signaling pathways (SYK, STAT3 and GAB2)[31,32] (Fig. 5*b*), may account for the expansion of peptide-specific effector memory cells observed after DTIC-vaccine administration (Fig. 3).

It has been shown that IL-15 expression can be induced by type I IFN.[29,33,34] Of note, several of the genes found upregulated after DTIC administration (Supp. Info. Table) are known to be induced by IFN-α and are indicative of an IFN-α signature in different settings[35] (Aricò *et al.*, in preparation). Notably, evidence obtained in a mouse model showed that the therapeutic efficacy of cyclophosphamide combined with an adoptive cell immunotherapy is mediated by type I IFN.[16] Moreover, it has been suggested that IFN-α may play a relevant role in the magnitude and durability of $CD8^+$ T cell response induced in melanoma patients by peptide vaccination in combination with IFN-α[36] or with type I IFN-inducing adjuvant such as CpG oligodeoxynucleotides.[37]

Several mouse tumor models suggest the advantage of combining cancer vaccines or other immunotherapy strategies with chemotherapy.[8,14,26,38,39] Information stemming from these models may provide important insights on how to exploit early chemotherapy-induced events, such as drug-induced cell death, danger signals and cytokine storm, for enhancing the efficacy of active or adoptive immunotherapy.

The results reported in our article showed that DTIC administration before peptides vaccination was safe, well tolerated and able to induce a long-lasting enhancement of memory $CD8^+$ T cell responses to cancer vaccines. Although further studies with more patients and longer follow-up need to be carried out, this study open new perspectives for designing clinically effective combination therapies for metastatic melanoma as well as for other human malignancies.

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