

# Analysis and Characterization of Antitumor T-cell Response After Administration of Dendritic Cells Loaded With Allogeneic Tumor Lysate to Metastatic Melanoma Patients

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**Summary:** The primary goal of cancer vaccines is to induce CD8<sup>+</sup> T cells specific for tumor-associated antigens (TAA) but the characterization of these cells has been difficult because of the low sensitivity of ex vivo assays. Here, we focused on TAA-specific CD8<sup>+</sup> T-cell responses in melanoma patients after vaccination with autologous dendritic cells loaded with lysates derived from allogeneic tumor-cell lines (Lysate-DC). Out of 40 patients treated, 16 patients developed immune response to tumor-cell lysate and/or CD8<sup>+</sup> T cells specific for differentiation and cancer-testis antigens. TAA-specific CD8<sup>+</sup> T-cell responses were detected by interferon (IFN)- $\gamma$  enzyme-linked immunospot after in vitro sensitization and were, either transient during the treatment period or delayed, that is, observed after completion of all vaccinations. We could not correlate these immune

responses to clinical data as none of the patients achieved an overall objective response according to Response Evaluation Criteria in Solid Tumors criteria. Three patients were reported as stable disease and 10 patients presented evidence of antitumor activity. We found that TAA-specific T cells characterized in 4 patients produced perforin ex vivo, but no IFN- $\gamma$  in enzyme-linked immunospot. Differential expression of IFN- $\gamma$  and perforin was also observed for viral-specific T cells. Altogether, our results show that Lysate-DC therapy elicited tumor-specific CD8<sup>+</sup> T cells nonlimited to human leukocyte antigen-A2<sup>+</sup> patients, with some T cells secreting perforin ex vivo and IFN- $\gamma$  only after restimulation. The differential expression of perforin and IFN- $\gamma$  by antitumor and antiviral CD8<sup>+</sup> T cells supports that the sole use of IFN- $\gamma$  production to monitor T cells overlooks functional T-cell subpopulations triggered by vaccines.

**Key Words:** immunotherapy, dendritic cell, tumor lysate, ELISPOT, perforin

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Dendritic cells (DC) play a central role in the presentation of antigens to naive T cells and the induction of primary immune T-cell responses. They are also important in launching humoral and innate immunity through their capacity to activate naive and memory B cells,<sup>1,2</sup> natural killer (NK),<sup>3</sup> and NK-T cells.<sup>4</sup> Numerous studies in mice have shown that DC loaded with tumor antigens can induce therapeutic and protective antitumor immunity.<sup>5–7</sup> In humans, clinical studies using healthy recipients also proved the immunogenicity and safety of DC, and demonstrated that a single injection of a small number of DC is sufficient to rapidly expand T-cell immunity for both naive and recall Ags.<sup>8</sup> Clinical and immunological responses with minimal side effects have been reported after DC therapy.<sup>9–14</sup> A recent meta-analysis of a substantial number of clinical studies showed that vaccination of cancer patients with DC loaded with melanoma antigens resulted in more patients with tumor regression compared with patients treated

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with other vaccine protocols.<sup>15</sup> Recently, 2 large phase III trials demonstrated clinical benefit in subsets of melanoma and prostate cancer patients receiving DC therapy.<sup>16,17</sup> In the trial with stage IV melanoma patients, although vaccination with DC pulsed with tumor peptides was not more effective than dacarbazine chemotherapy in term of overall clinical responses, a human leukocyte antigen (HLA)-A2<sup>+</sup>/B44<sup>-</sup> subset of melanoma patients survived significantly longer when treated with DC as compared with dacarbazine therapy.<sup>16</sup> In another double-blind placebo-controlled phase III study, asymptomatic metastatic hormone refractory prostate cancer patients were treated or not treated with antigen presenting cells pulsed with a fusion protein combining the prostatic acid phosphatase tumor antigen to granulocyte macrophage colony-stimulating factor (GM-CSF). The median time to disease progression did not differ between the 2 groups of patients. However, the patients who received the vaccine had a longer median survival compared with those in the placebo group (25.9 mo vs. 21.4 mo, respectively). This difference remained significant after adjusting for other prognostic factors.<sup>17</sup>

These 2 studies used the loading of DC with defined peptides or recombinant proteins. As there is still debate about which known tumor-associated antigens (TAA) really represent rejection antigens in vivo and because of increased risk of tumor escape when a narrow antitumor T-cell repertoire is elicited,<sup>18</sup> other groups have performed clinical trials using DC sensitized with complex mixtures of tumor antigens, including apoptotic cells<sup>19</sup> and autologous or allogeneic tumor-cell lysates.<sup>20–27</sup> Preclinical studies have shown that vaccination of mice with irradiated allogeneic melanoma cells mixed with syngenic DC led to subsequent tumor rejection of syngenic melanoma in immunized mice.<sup>28</sup> DC loaded with killed allogeneic human melanoma cells have also been shown to prime naive T cells to differentiate into cytotoxic T lymphocytes that are specific for a broad spectrum of shared melanoma antigens and are able to kill autologous melanoma cell lines.<sup>29</sup> They also allow the proliferation of autologous antitumor CD4<sup>+</sup> T cells.<sup>29</sup> Hence, the use of allogeneic tumor cells should provide both major histocompatibility complex class I and II epitopes leading to a diverse immune response involving CTL and CD4<sup>+</sup> T cells.

Few clinical trials using DC pulsed with allogeneic lysates have been reported.<sup>24,26,30,31</sup> Anecdotal immune and clinical responses were observed but it was difficult to discriminate allogeneic immune response from antitumor immune response. We recently reported the results of a pilot study using DC pulsed with allogeneic tumor-cell lysate to vaccinate 15 melanoma patients that showed antitumor CD8<sup>+</sup> responses in HLA-A2<sup>+</sup> patients.<sup>31</sup> Here, we describe the T-cell immune responses induced by Lysate-DC therapy in 40 HLA-A2<sup>+</sup> and non-HLA-A2<sup>+</sup> melanoma patients evaluated in a phase I/II study. The antitumor CD8<sup>+</sup> T-cell response was detected with synthetic short peptides derived from tumor-associated

T-cell epitopes and was characterized in terms of intensity, kinetics, cytokine, and perforin production. Our results show that vaccination with DC loaded with allogeneic tumor-cell lysate can induce antitumor CD8<sup>+</sup> T-cell response, with some T cells producing perforin *ex vivo* not associated with interferon (IFN)- $\gamma$  detection.

## PATIENTS AND METHODS

### Melanoma Patients and Healthy Donors

Forty patients with stage IV malignant disease were included in a multicentric phase I/II study conducted in Europe and Australia. The study was designed to evaluate the safety and immunologic responses induced by therapeutic vaccination with allogeneic tumor-cell lysates loaded on autologous DC (Uvidem). The safety and detailed clinical data will be reported in a separate manuscript. Uvidem is a vaccine codeveloped by IDM and Sanofi-Aventis. Blood from healthy donors was obtained from the Etablissement Français du sang (Rungis, France).

### Preparation of Vaccine and Treatment Schedule

Autologous monocyte-derived DC were differentiated with GM-CSF and interleukin-13 (IL-13) as previously described.<sup>31</sup> The cell lines used to prepare the tumor-cell lysate were M44 (F. Jotereau INSERM U 463, Nantes, France), COLO829 (American Type Culture Collection, ATCC) and SK-MEL28 (ATCC) maintained under good manufacturing practices. The Master cell banks and tumor-cell lysate were manufactured by BioReliance Cooperation (Rockville, MD). DC were pulsed overnight with the 3 melanoma cell line lysates and for some patients were further matured for 6 hours with IFN- $\gamma$  (Boehringer Ingelheim, Vienne) and FMKp (Pierre Fabre, St Julien en Genevois, France). Characterization of the vaccine manufactured for patients showed an average purity of 94% with a viability of 83%. Patients received a maximum of 6 immunizations (subcutaneous and intradermal) with an average of  $20 \times 10^6$  Lysate-DC that were administered at 4-week intervals.

### Processing of Samples for Immunologic Tests

Peripheral blood mononuclear cells (PBMC), isolated by ficoll gradient centrifugation (lymphoprep, Axis Shield, Oslo, Norway), were cryopreserved in fetal calf serum (Cambrex, Belgium) with 10% dimethylsulfoxide (Sigma Aldrich, St Louis, MO) and stored in liquid nitrogen. PBMC were collected at baseline (W0) and 4 weeks after each injection: W4 (postvaccination no. 1), W8 (postvaccination no. 2), W12 (postvaccination no. 3), W16 (postvaccination no. 4), W20 (postvaccination no. 5), and W24 (postvaccination no. 6).

### Proliferation Assay

PBMC collected before and during treatment were thawed and tested in the same experiment for proliferative responses to tumor-cell lysates. PBMC ( $2 \times 10^5$  cells/well) were incubated with autologous DC ( $1 \times 10^4$  DC/well) loaded with tumor-cell Lysates (0.5 tumor cell

equivalents per DC) in triplicates in 96-well round bottom-tissue culture plates, in AIMV medium supplemented with 5% human AB serum. PBMC cultured with unloaded DC were used as negative control. For some patients, autologous DC were not available and PBMC were directly loaded with lysate ( $0.5 \times 10^4$  tumor cell equivalents). On day 5,  $1 \mu\text{Ci}$ /well of  $^3\text{H}$ -thymidine was added and plates were incubated for an additional 6 to 8 hours. Results are expressed as stimulation index (SI = mean cpm with antigen/mean cpm without antigen). SI was calculated when the mean cpm of the replicates with antigen was significantly different from the mean cpm without antigen (Student test,  $P < 0.05$ ). The proliferative response was considered to be increased by the vaccination when a significant increase of SI at any time point postvaccination compared with SI at baseline was observed (rank test,  $P < 0.05$ ).

**Peptides**

The list of melanoma and viral peptides used to detect specific CTL are detailed in Table 1. They were purchased from NeoMPS (Strasbourg, France) or Epytop (Nîmes, France) and were > 90% pure.

**ENZYME-LINKED IMMUNOSPOT**

**Enzyme-linked Immunospot for IFN- $\gamma$**

ELISPOT assays were performed as previously described.<sup>32</sup> Briefly, 96-well polyvinylidene difluoride plates (Millipore, Molsheim, France) were coated with 100  $\mu\text{L}$  capture antihuman IFN- $\gamma$  mAb (Diacclone, Besançon, France) and incubated overnight at 4°C. The plates were then saturated with 2% skimmed milk and incubated for 2 hours at room temperature. Cells directly pulsed with or without peptides were added to triplicate wells at  $10^5$  cells/well in AIMV medium for 20 hours at 37°C in 5% CO<sub>2</sub>. At the end of incubation, cells were washed and the second biotinylated anti-IFN- $\gamma$  mAb (Diacclone) was added to the plate for 90 minutes at 37°C, followed by streptavidin-alkaline phosphatase conjugate

(Diacclone) for 1 hour at 37°C. The chromogen substrates used to develop spot color consisted of a nitroblu tetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidine mix (Diacclone). Color development was stopped by washing under running tap water. After drying at room temperature, spots were counted using an automated stereomicroscope (Carl Zeiss, France). The number of specific T cells expressed as spot forming cells (SFC)/ $10^5$  cells were calculated after subtracting negative control values (background).

Cells incubated with medium alone or phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) (Sigma, St Louis, MO) and ionomycin (10  $\mu\text{M}$ ) (Sigma) were used as negative and positive controls, respectively.

We used 2 different criteria to define a positive response. The first criteria (referred as conventional criteria) considered a response positive if the number of spots in the wells stimulated with peptides was 1.5-fold higher than the number of spots in the wells without peptide with a cutoff of 5 SFC/ $10^5$  cells above mean background. These criteria have been proposed by various groups in different studies.<sup>32-36</sup> A second statistical criteria relied on the combination of a cutoff of 10 SFC/ $10^5$  cells and a Student *t* test, which was used to determine significant differences between control and positive sample with the level of significance set at  $P < 0.05$  as previously described.<sup>37,38</sup>

**ELISPOT Perforin**

ELISPOT for perforin was purchased from Diacclone and performed as recommended by the manufacturer. Positive controls included cells stimulated by PMA-Ionomycin or the use of the YT-cell line. Negative controls included unstimulated cells.

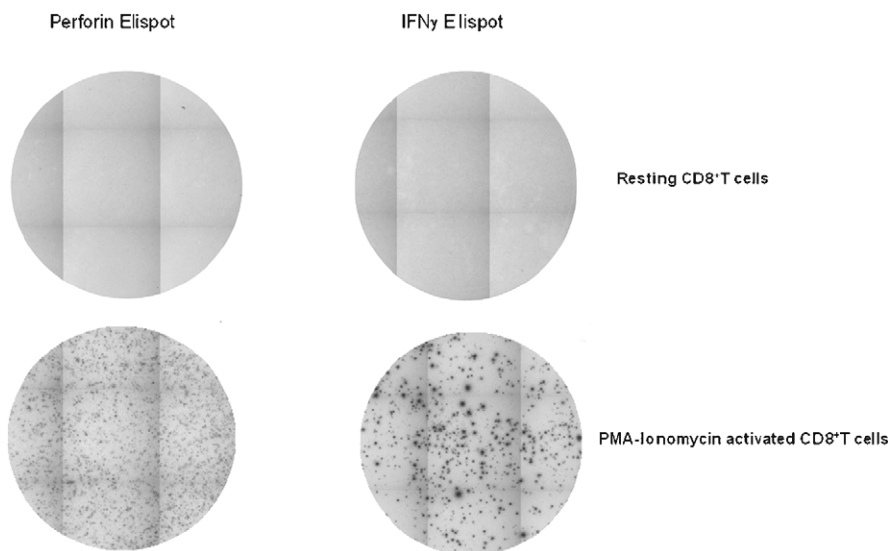
The human perforin-ELISPOT assay reproducibility was performed with the NK like cell line (YT) that constitutively expresses perforin.

- Intra-assay variability: all measurements were performed in triplicate. Calculated coefficient of variation (CV) obtained with the YT cell line was 9.2%.
- Interassay variability: assay to assay reproducibility was evaluated within the same laboratory by 2 technicians in 3 independent experiments. The Human perforin-ELISPOT assay was performed with 250 YT cells/well according to manufacturer's instructions. Calculated CV obtained with the YT cell line was 14.5%.

The in vitro sensitization step or the use of bulk PBMC sometimes led to some background. Indeed, after the step of in vitro sensitisation, 4 out of 6 purified CD8<sup>+</sup> T cells derived from 6 PBMC exhibited high background (> 20 SFC/ $10^5$  cells), whereas only 1 out of 6 CD8<sup>+</sup> T cells (derived from the same PBMC) tested directly ex vivo displayed some background. We could also mention that after the step of in vitro amplification, the same background occurred with the perforin ELISPOT, whether we tested directly the PBMC amplified or the purified in vitro amplified CD8<sup>+</sup> T cells. By contrast, after the in vitro amplification step, this background only occurred in 1 of the 6 purified CD8<sup>+</sup> T cells from the

**TABLE 1.** Listing of Melanoma and Viral Peptides

Peptide	Position	Sequence
CMV/pp65 <sub>HLA-A1</sub>	363-373	YSEHPTFTSQY
CMV/pp65 <sub>HLA-A2</sub>	495-504	NLVPMVATVQ
CMV/pp65 <sub>HLA-B7</sub>	417-426	TPRVTGGGAM
EBV/BMLF1 <sub>HLA-A2</sub>	259-267	GLCTLVAML
EBV/EBNA-3A <sub>HLA-A3</sub>	603-611	RLRAEAQVK
EBV/EBNA-4 <sub>HLA-A11</sub>	416-424	IVTDFSVIK
MelanA/Mart1 <sub>HLA-A2</sub>	26-35	EAAGIGILTV
NY-ESO-1 <sub>HLA-A2</sub>	157-165	SLLMWITQA
gp100 <sub>HLA-A2</sub>	209-217	IMDQVPFSV
gp100 <sub>HLA-A3</sub>	608-617	AVVLASLIYR
Tyrosinase <sub>HLA-A3</sub>	425-434	YMVFPPILYR
gp100 <sub>HLA-A24</sub>	Intron 4 170-178	VYFFLPDHL
MAGE 1 <sub>HLA-B7</sub>	289-297	RVRFFFPSSL
MAGE 3 <sub>HLA-A24</sub>	195-203	IMPKAGLLI
MAGE 3 <sub>HLA-B44</sub>	167-176	MEVDPIGHLY
Tyrosinase <sub>HLA-B7</sub>	208-216	LPWHRLFLL
gp100 <sub>HLA-B35</sub>	630-638	LPHSSSHWL
Tyrosinase <sub>HLA-B44</sub>	192-200	SEIWRDIDF



**FIGURE 1.** Comparison of the IFN- $\gamma$  and perforin ELISPOT assays. Resting CD8<sup>+</sup> T cells (Top) or PMA-Ionomycin activated CD8<sup>+</sup> T cells (bottom) were revealed using either a direct perforin (left) or IFN- $\gamma$  (right) ELISPOT. Note the smaller size of spot with the perforin ELISPOT assay.

identical patients described above and tested with the IFN- $\gamma$  ELISPOT.

Thus, the perforin ELISPOT assay was always performed on purified CD8<sup>+</sup> T cells without in vitro amplification of the cells. It is noteworthy that the spots obtained with this perforin ELISPOT assay were smaller than the ones observed with the IFN- $\gamma$  ELISPOT assay (Fig. 1). The criteria of 1.5-fold higher than the background with a cutoff of 5 SFC/10<sup>5</sup> cells was used as for the ELISPOT IFN- $\gamma$  (see above).

### CD8<sup>+</sup> T-cell Purification

CD8<sup>+</sup> T lymphocytes were purified from PBMC by magnetic beads using a MiniMACS device (Miltenyi Biotec, Paris, France). PBMC (10<sup>7</sup>) were incubated for 15 minutes with 20  $\mu$ L of anti-CD8 coupled beads. The cells were then washed and placed on the magnetic column. CD8<sup>+</sup> T lymphocytes bound to the column were recovered after removing the magnet followed by 2 washes. The purity of recovered cells ranged between 92% and 98%.

### In Vitro Sensitization Step for Indirect ELISPOT

Cells were either used directly after thawing for the ELISPOT assay (direct ELISPOT) or after an in vitro sensitization step (indirect ELISPOT). For this purpose, thawed cells were cultured at  $2 \times 10^6$  cells/mL in 24-well plates in RPMI medium supplemented with 10% fetal calf serum. In each well, a pool of peptides was added at a concentration of 10  $\mu$ g/mL. At day 2 after the beginning of the culture, IL-2 (Chiron, Emeryville, USA) was added at 20 IU/mL. After 6 days of stimulation, ELISPOT assay was performed as described above.

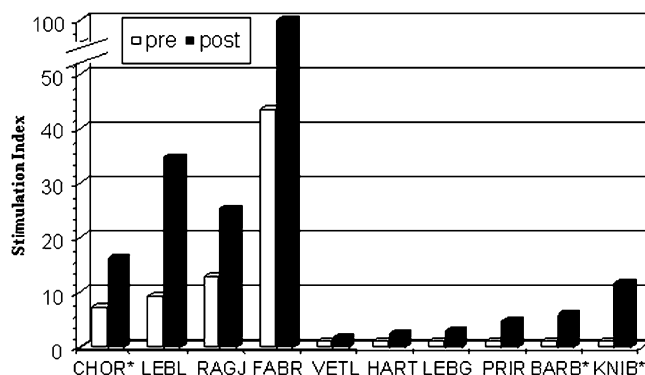
## RESULTS

### Analysis of Antitumor T-cell Responses

In this study, a total of 40 patients were analyzed for immune response to Lysate-DC vaccination: 37 patients

were evaluated for reactivity to tumor-cell lysate, 34 patients evaluated with TAA-derived peptides. For some patients, samples could only be assessed for Lysate (n = 6) or peptide (n = 3) reactivity.

Immune responses to tumor-cell lysate was measured by proliferation assay in 37 patients. As shown in Figure 2, 10 patients showed increased proliferation after vaccination, with 4 patients having already some reactivity at baseline. The patients CHOR, BARB, and KNIB received matured DC, whereas other responding patients received nonmatured DC. However, we did not see any difference between the 2 groups in the magnitude of the proliferative responses induced, and in the number of patients showing a positive response to the lysate after



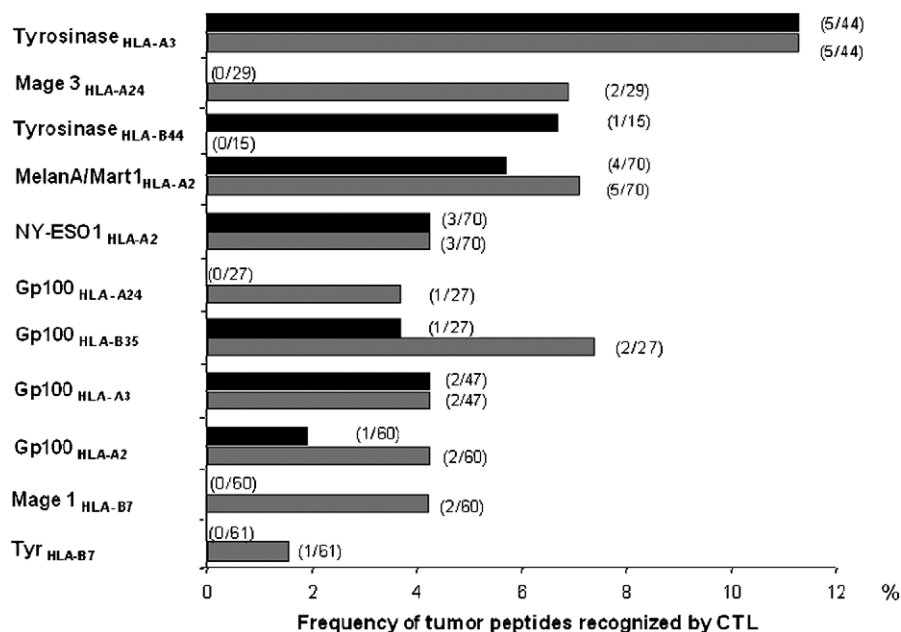
**FIGURE 2.** Patients with increased proliferative response to tumor-cell lysate after Lysate-DC therapy. PBMC from melanoma patients collected before and after vaccination were stimulated in parallel with Lysate-DC (or lysate directly in case of patients BARB and BORM) and T-cell proliferation was measured as described in Material and Methods. Patients with increased immune response postvaccination are shown [stimulation index (SI) postvaccination significantly different from SI at baseline, Rank test,  $P < 0.05$ ]. The maximum immune reactivity is shown as postvaccination time point. \*Patients vaccinated with matured DC.

vaccination. As the respective role of CD4<sup>+</sup> or CD8<sup>+</sup> T cells could not be discriminated with this proliferation assay and it is believed that CD8<sup>+</sup> T cells play a pivotal role in tumor immunity, we focused on the detection of antigen-specific CD8<sup>+</sup> T cells elicited by the vaccine. We used 9-mers of known TAA-derived peptides to stimulate PBMC of vaccinated patients and looked at the production of IFN- $\gamma$  by indirect ELISPOT assay. Peptides were derived from differentiation antigens (MelanA/Mart1, tyrosinase, gp100) or cancer-testis antigens (Mage-1, Mage-3, NY-ESO1), known to be expressed by the melanoma cell lines used to prepare the vaccine. As shown in Figure 3, CD8<sup>+</sup> T-cell reactivity was found against various peptides and restricted by various HLA molecules, including HLA-A3, A24, B44, B35, and B7. As different criteria of positive responses for the ELISPOT assay have been reported, we compared 2 of them: 1 criteria, referred as “conventional criteria” (the number of spots in the wells stimulated with peptides being 1.5-fold higher than the background and > 5 spots/10<sup>5</sup> cells after subtraction of the background) reported by different groups in the literature<sup>33,34,36,39</sup> and another 1 more stringent, combining a statistical criteria, the Student *t* test, and a number of spots  $\geq$  10 (referred later as “statistical criteria”). The Figure 3 shows that the results are in the same range whatever the test used, because a total of 4.9% (25/510) of positive ELISPOT responses against the different melanoma peptides were recorded with the conventional criteria, whereas 3.3% (17/510) of

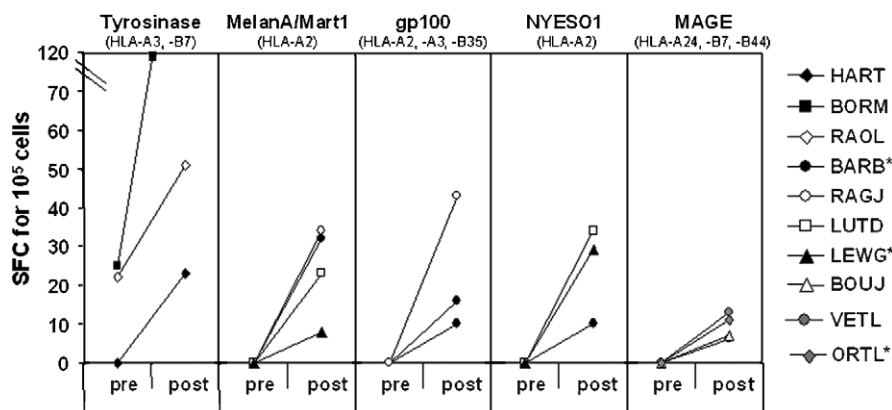
positive tests were found using the statistical criteria. The latter was more stringent than the conventional criteria and except in 1 case, all positive tests found with the statistical criteria were included in those recorded by the conventional criteria. To make these results comparable with previous studies, we applied the conventional criteria to define positive responses.

Using the conventional criteria, 10 patients out of 34 had increased reactivity to TAA-derived peptides after treatment (Fig. 4): 8 patients presented an induction of CD8<sup>+</sup> T cells directed against tumor peptides after DC therapy and 2 other patients had preexisting responses boosted by the vaccination. Overall, 17 CTL responses could be observed among the 10 responding patients and the CD8<sup>+</sup> T-cell reactivity was increased against 10 different melanoma peptides (Fig. 4). Few patients showed basal immune response to melanoma peptides (5 out of 34 patients tested). Preexisting T-cell response could be amplified in 2 of these 5 patients, compared with 8 responding patients in the remaining 29 patients who did not have reactivity at baseline. This indicates that patients with preexisting T-cell response against tumor antigen were not more likely to respond to the vaccine.

Among patients with increased peptide responses, 3 were vaccinated with matured DC and 7 with non-matured DC. Interestingly, the diversity of T-cell responses induced by matured DC seems more important than in the group treated with nonmatured DC. Indeed, responses against multiple melanoma epitopes were seen in



**FIGURE 3.** Frequency of CTL directed against different tumor-derived peptides in vaccinated melanoma patients. PBMC from melanoma patients were tested in an indirect IFN- $\gamma$  ELISPOT assay with pools of peptides (Table 1) at different time points during Lysate-DC therapy. Results were considered positive using the criteria based on Student *t* test (black bars) or conventional criteria (gray bars) as described in Material and Methods. Brackets indicate the number of positive samples/number of total samples tested. The difference in the number of samples depends on the HLA type of patients and the number of time points analyzed for each patients. The distribution of the main HLA of patients tested in this study was as follows: HLA-A2 (n = 17), HLA-B7 (n = 12) HLA-A3 (n = 11), HLA-A24 (n = 11), HLA-B44 (n = 9), HLA-B35 (n = 8).



**FIGURE 4.** CTL responses against TAA-derived peptides in vaccinated patients. PBMC from vaccinated melanoma patients were tested in an indirect IFN- $\gamma$  ELISPOT assay. Data represent all patients displaying an induction or increase of CTL specific for melanoma peptides derived from tyrosinase (Tyr<sub>HLA-A3</sub> and Tyr<sub>HLA-B7</sub> peptides), MelanA/Mart1 (MelanA/Mart1<sub>HLA-A2</sub> peptide), gp100 (gp100<sub>HLA-A2</sub>, gp100<sub>HLA-A3</sub>, and gp100<sub>HLA-B35</sub> peptides), NYESO1 (NYESO1<sub>HLA-A2</sub>), or Mage (Mage3<sub>HLA-A24</sub>, Mage3<sub>HLA-B44</sub>, Mage1<sub>HLA-B7</sub> peptides) antigens. Results correspond to positive values determined with the conventional criteria. The maximum immune reactivity is shown as postvaccination time point. The symbol assigned to each patients is depicted on the right of the figure. The name of antigen recognized by the CTL is shown on the top of each column. \*Patients vaccinated with matured DC. The number of SFC per 10<sup>5</sup> cells represent the data obtained after subtracting the background. Data are the mean of triplicate wells. SD for all measurement was always <10%.

2 patients (BARB, LEWG) out of 3 vaccinated with matured-DC whereas only 2 patients (RAGJ, LUTD) out of 7 responded to more than 1 peptide in the nonmatured group. Out of the 40 patients evaluated for immune responses in this study, 31 were tested for response to both tumor-cell lysate and TAA peptides. In 4 patients with induced TAA-specific CD8<sup>+</sup> T cells (VETL, HART, BARB, and RAGJ), an increased proliferation to the tumor-cell lysate was observed after vaccination (Fig. 2). Six other patients had lysate-specific response but without the associated induction of antitumor specific CD8<sup>+</sup> T-cell response. A summary of immune reactivity detected in responding patients is listed in Table 2.

### Duration and Kinetics of Antitumor CD8<sup>+</sup> T-cell Response

Among the 10 patients showing an induction of specific antimelanoma CD8<sup>+</sup> T-cell responses, 3 patients (33%), depicted in Figure 5A, had positive responses detected at 2 occasions postvaccination, whereas other CD8 responses were detected only at 1 of the time points tested. One possible explanation for the detection of specific CD8<sup>+</sup> T-cell response at only 1 time point could be the late appearance of some of these responses at the last time point of follow up (after 6 immunizations), which made further analysis not possible. Indeed, 5 CTL responses from 4 patients were only detected 24 weeks after the initiation of Lysate-DC therapy (Fig. 5B). For the patient BARB, CD8<sup>+</sup> T cells were induced only at 24 weeks for both MelanA/Mart1 and NY-ES0-1 HLA-A2 binding peptides (Fig. 5B), whereas CD8<sup>+</sup> T cells against gp100 were detected at 2 occasions (Fig. 5A). In general, induced CTL were detected after the patients received at least 3 vaccinations (evaluated at the time of W12). Overall, it may be concluded that 3 CTL responses lasted

at least for 2 time points, 9 were transient and 5 occurred late, after 6 vaccinations.

To address whether these kinetics and duration of CD8<sup>+</sup> T-cell responses were only observed with tumor antigens, the same analysis was performed with antiviral CTL. Twenty of the 34 patients exhibited CTL response against at least 1 viral peptide. In 13 of these 20 patients (65%), positive responses were observed at 2 consecutive time points or more, indicating that in contrast to antitumor CD8<sup>+</sup> T cells these antiviral CD8<sup>+</sup> T-cell responses were not transiently detected (data not shown).

### CTL Induced After DC Therapy Produced Perforin Ex Vivo

Previous results about CTL detection were obtained with an ELISPOT assay performed after 1 week of in vitro stimulation (indirect ELISPOT). Ex vivo assays, however, may better quantify the ongoing immune response in vivo. To examine this, we selected 4 patients for whom a CTL induction was clearly demonstrated by an indirect IFN- $\gamma$  ELISPOT and frozen postvaccination samples were available. We could not detect specific CD8<sup>+</sup> T cells against the different melanoma peptides in any of the 4 patients by an ex vivo IFN- $\gamma$  ELISPOT assay (Fig. 6). In contrast, 3 (LUTD, RAGJ, and BARB) of the 4 patients presented CTL with the ability to specifically produce perforin at detectable levels ex vivo in the presence of defined melanoma peptides (Fig. 6). As CD8<sup>+</sup> T cells from 2 of these 3 perforin positive patients were available from the baseline time point before vaccination, they were tested in an ex vivo IFN- $\gamma$  and perforin ELISPOT assay. No IFN- $\gamma$  or perforin producing antimelanoma peptide specific CD8<sup>+</sup> T cells could be detected in these patients before vaccination (data not shown). Therefore, at least for these 2 melanoma patients,

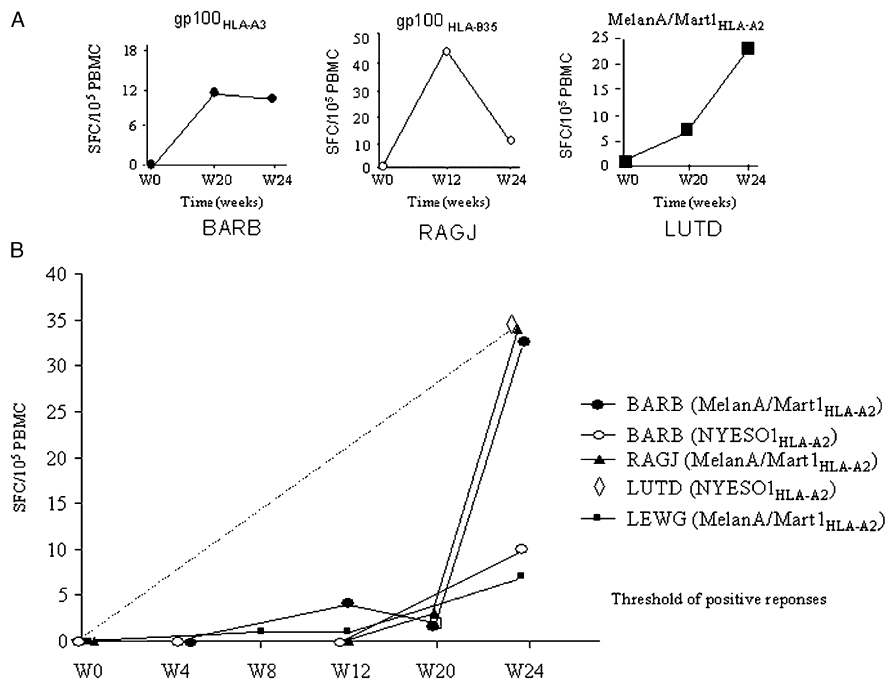
**TABLE 2.** Immune Responses in Vaccinated Melanoma Patients

Patient ID	No. Vaccination	Tumor-cell Lysate		Increased Response to	
				TAA-derived Peptides	
VETL	3	+	+	MAGE 1 <sub>HLA-B7</sub>	
HART	6	+	+	Tyrosinase <sub>HLA-B7</sub>	
LEBG	6	+	-		
PRIR	6	+	-		
BARB	6	+	+	gp100 <sub>HLA-A2</sub>	gp100 <sub>HLA-A3</sub> MelanA/Mart1 <sub>HLA-A2</sub> NY-ESO-1 <sub>HLA-A2</sub> MAGE 3 <sub>HLA-B44</sub>
KNIB	6	+	-		
CHOR	4	+	-		
LEBL	3	+	-		
RAGJ	6	+	+	gp100 <sub>HLA-B35</sub>	MelanA/Mart1 <sub>HLA-A2</sub>
FABR	2	+	-		
LUTD	6	-	+	MelanA/Mart1 <sub>HLA-A2</sub> NY-ESO-1 <sub>HLA-A2</sub>	
LEWG	6	-	+	MelanA/Mart1 <sub>HLA-A2</sub> NY-ESO-1 <sub>HLA-A2</sub>	
BORM	6	-	+	Tyrosinase <sub>HLA-A3</sub>	
RAOL	6	-	+	Tyrosinase*	
BOUJ	5	-	+	MAGE 3 <sub>HLA-A24</sub>	
ORTL	4	-	+	MAGE 1 <sub>HLA-B7</sub>	

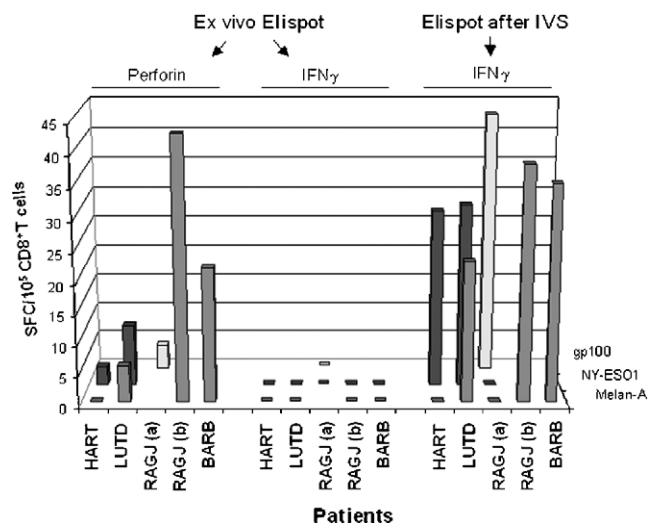
\*Peptide tyrosinase<sub>HLA-A3</sub> and tyrosinase<sub>HLA-B7</sub> were tested in pool for this patient.

the DC therapy was able to induce CD8<sup>+</sup> T cells able to produce perforin ex vivo. For patient RAGJ, the indirect IFN-γ ELISPOT assay detected CD8<sup>+</sup> T cells directed against HLA-A2 restricted gp100 and MelanA/Mart1

peptides. Using the direct ex vivo perforin ELISPOT assay, these CD8<sup>+</sup> T cells produced 40 spots after pulsing with the MelanA/Mart1 peptide, whereas only 4 spots were detected with the gp100 peptides sensitization



**FIGURE 5.** Analysis of the lasting and kinetics of antitumor CTL reponse induced after DC therapy. PBMC derived from metastatic melanoma patients were stimulated with melanoma-derived peptides (A, B) at different time points after Lysate-DC therapy. CTL responses were detected using an IFN-γ ELISPOT assay. The name of viral or melanoma-peptides recognized by CTL is indicated within each patient graph. A, Using the conventional criteria, 10 patients exhibited CTL response against tumor peptides after DC therapy. In 3 (shown) out of these 10 patients, these positive responses were observed 2 times or more. B, Patients with late induction (post-6 vaccinations or W24) of CTL responses against tumor peptides. White or black symbols represent positive CTL response against MelanA/Mart1<sub>HLA-A2</sub> or NYESO1<sub>HLA-A2</sub> peptides, respectively. The number of SFC per 10<sup>5</sup> cells represent the data obtained after subtracting the background. Data are the mean of triplicate wells. SD for all measurement was always <10%.



**FIGURE 6.** Antitumor CTLs induced by Lysate-DC therapy produce perforin *ex vivo*. Purified CD8<sup>+</sup> T cells from 4 vaccinated melanoma patients showing antitumor CD8<sup>+</sup> T cells after Lysate-DC therapy were tested in an *ex vivo* ELISPOT assay for either perforin (left) or IFN-γ production in the presence of the indicated peptides. PBMC collected at the same time points were stimulated 6 days *in vitro* with the specific indicated peptides and tested in an IFN-γ ELISPOT assay (right). For the patient RAGJ, cells corresponded to samples collected at 2 times postvaccination (samples a and b). The number of SFC per 10<sup>5</sup> cells represent the data obtained after subtracting the background. Data are the mean of triplicate wells. SD for all measurement was always <10%.

(Fig. 6). For this patient, only CD8<sup>+</sup> T cells directed against MelanA/Mart1 could therefore be considered to be significantly detectable with the *ex vivo* perforin ELISPOT assay.

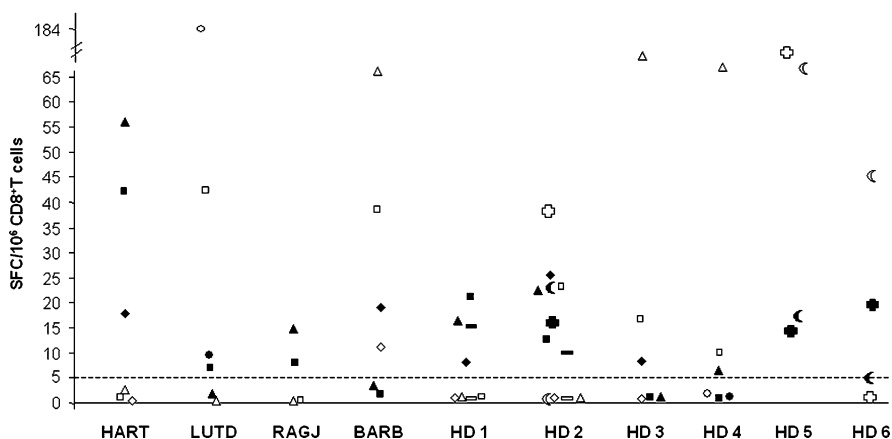
### Dichotomy Between the IFN-γ and Perforin *Ex Vivo* ELISPOT Assay Also Occurred for Antiviral CD8<sup>+</sup> T Cells

Because, we identified a discrepancy for some patients in the IFN-γ and perforin *ex vivo* assay to detect antitumor CTL, we wonder whether such discordance will also apply for the detection of antiviral CTL. Four melanoma patients and 6 healthy donors (HD) were tested for the presence of CD8<sup>+</sup> T cells specific for different viral-peptides derived from Epstein-Barr virus, cytomegalovirus, or influenzae virus using either the *ex vivo* perforin or IFN-γ-ELISPOT assay. It seemed that the same heterogeneity existed within the population of responding antiviral CD8<sup>+</sup> T cells, as for patients HART, RAGJ, HD1, HD3, and HD6 (Fig. 7), a specific production of perforin by some antiviral CD8<sup>+</sup> T cells not associated with cells producing IFN-γ was observed. Conversely, some antiviral CD8<sup>+</sup> T cells derived from patient BARB, HD3, and HD4 secreted IFN-γ without perforin. Production of both IFN-γ and perforin were also observed for some antiviral CD8<sup>+</sup> T cells (patients BARB, LUTD, HD2, HD5) (Fig. 7). All these results were observed after direct *ex vivo* ELISPOT. The perforin ELISPOT may provide complementary information to the IFN-γ ELISPOT when applied on *ex vivo* assay on purified CD8<sup>+</sup> T cells. The usefulness of this test compared with IFN-γ ELISPOT may be questioned when T cells were tested after a step of *in vitro* sensitization mostly owing to high background observed with the perforin ELISPOT.

### Correlation Between Immune Responses and Clinical Data

We could not correlate these immune responses to clinical data as none of the patients achieved an overall objective response according to Response Evaluation

- (Δ, ▲) EBV/LMP2A<sub>HLA-A2</sub>
- (□, ■) EBV/BMLF-1<sub>HLA-A2</sub>
- (◇, ◆) CMV/pp65<sub>HLA-A2</sub>
- (○, ●) CMV/pp65<sub>HLA-A1</sub>
- (◻, ◼) Influenza/MP<sub>HLA-A2</sub>
- (⊖, ⊗) EBV/EBNA3A<sub>HLA-B7</sub>
- (⊕, ⊙) CMV/pp65<sub>HLA-B7</sub>



**FIGURE 7.** Discordances between the *ex vivo* perforin and IFN-γ ELISPOT also occur for the detection of CTL directed against viral peptides. Purified CD8<sup>+</sup> T cells from 4 melanoma patients and 6 healthy donors (HD 1 to 6) were tested in an *ex vivo* ELISPOT assay for perforin (black symbols) or IFN-γ (white symbols). The viral-peptides derived from cytomegalovirus, Epstein-Barr virus, or influenzae virus are indicated in the figure.

Criteria in Solid Tumors criteria. Three patients were reported as stable disease. Some other patients ( $n = 10$ ) presented evidence of antitumor activity not meeting Response Evaluation Criteria in Solid Tumors criteria (isolated tumour target regression and stabilization in the growth of previously progressing lesions). The 3 patients with stable disease were treated with matured DC and 2 out of these 3 patients had responses to multiple peptides.

## DISCUSSION

This study shows, in a large cohort of melanoma patients, that administration of DC pulsed with allogeneic tumor-cell lysates induced specific antitumor CD8<sup>+</sup> T cells directed against well-defined melanoma derived peptides. We and others have shown previously in small cohorts of melanoma patients that such CD8<sup>+</sup> T-cell responses can be detected in HLA-A2<sup>+</sup> patients treated by similar DC therapy protocols.<sup>30,31,40,41</sup> Here, we have monitored a larger group of patients, better characterized the immune response and extended the analysis to non-HLA-A2 melanoma patients. We show that reactivity to HLA-A3, A24, B44, B35, and B7-binding TAA peptides can be increased after Lysate-DC therapy. An induction of specific antitumor CD8<sup>+</sup> T cells in 8 melanoma patients and an increased of preexisting tumor-specific CD8<sup>+</sup> T cell in 2 out of 34 patients tested were observed. Four patients showed responses to the tumor-cell lysate in addition to TAA-specific CD8 cells. Six other patients had lysate-specific responses but without the associated induction of antitumor specific CD8<sup>+</sup> T-cell response. These antilylate responses may reflect an antiallogeneic reaction and responses to nonidentified or nontested TAA-derived peptides. This underlines the difficulty of monitoring a global immune response to such vaccine and our choice to focus on the analysis of antimelanoma peptide CD8<sup>+</sup> T-cell responses. The frequency and magnitude of T-cell responses were in the same range than those observed after autologous Lysate-DC therapy.<sup>20,21,23,26,27,41</sup> This further supports the use of lysates derived from allogeneic tumor cell lines to standardize the Ag-loading strategy of such vaccines.

In contrast to various models demonstrating the superiority of mature DC compared with nonmatured DC to trigger CD8<sup>+</sup> T-cell response,<sup>42–45</sup> we did not find correlation between the induction of antitumor T-cell responses and the maturation state of the DC when administered by intradermal and subcutaneous routes to melanoma patients. Most previous studies were carried out using DC generated with GM-CSF and IL-4, whereas GM-CSF and IL-13 were selected in study. Prince et al<sup>46</sup> have shown that GM-CSF/IL-13 generated DC can migrate in vivo to lymph node when injected as nonmatured DC. The use of the intradermal route of administration in our study might favor a spontaneous maturation of DC during the course of migration to the draining lymph node as Barratt-Boyes et al<sup>47</sup> reported previously. This contrasts with the study conducted by Jonuleit and colleagues<sup>43</sup> where the comparison was

conducted in the same patient after intra nodal injection of the DC (ie, bypassing the migration step). In addition, other clinical studies have shown induction of immune responses after injection of nonmatured DC.<sup>9,48–50</sup> Although, it has been reported that immature DC led to the induction of IL-10 producing specific CD8<sup>+</sup> T cells,<sup>42,51,52</sup> we did not detect the production of IL-10 by the CD8<sup>+</sup> T cells stimulated with the melanoma-derived peptides tested (not shown). We have observed that T-cell responses against multiple melanoma peptides tend to be more frequent in the 3 patients vaccinated with matured DC compared with the nonmatured group. It is possible that the administration of matured DC favors the induction of polyvalent responses compared with nonmatured DC but more patients should be analyzed in a systematic fashion to evaluate this hypothesis.

From the 10 patients with increased TAA-peptide reactivity in this study, only 3 out of 17 CTL responses were detected at more than 1 time point. In 5 cases, specific CD8<sup>+</sup> T cells responses were delayed and observed only after 6 vaccinations, that is, at the last point of planned follow-up and therefore, their further persistence could not be assessed. Variability in the kinetics of immune response in melanoma patients after DC vaccination has already been reported with some patients requiring 3 or 4 vaccines for an increase of melanoma specific T cells to be detected.<sup>53</sup> Similarly, although the majority of patients responded quickly to melanoma peptides mixed with incomplete Freund's adjuvant, in some cases specific T cells were induced against some peptides only after 8 vaccinations.<sup>54</sup> This may be correlated with the fact that clinical tumor regression often appears late after vaccination with tumor antigens.<sup>55</sup>

Compared with antiviral CTL that were more stable, transient detection of antitumor CTL may relate to their low frequency in peripheral blood. In mice, it was shown that the expansion and activation of CTL by DC were transient and diminished after repeated immunizations.<sup>56</sup> In human, except in rare instances,<sup>57,58</sup> DC vaccines have generally led to transient immune responses with a drop in the number of melanoma peptide-specific T cells after repeated vaccination.<sup>30,53,59–62</sup> This rapid disappearance of specific CTL may be because of their migration to the tumor site or other tissues. This possibility is consistent with data from 2 groups who recently reported an absence of tumor-reactive T cells in the blood after vaccination with tumor antigens, they could be easily detected in skin or lymph node biopsies.<sup>63,64</sup> Alternatively, restimulation of T cells at the peak of their expansion/activation may result in activation-induced cell death.<sup>65</sup> As adoptive T-cell immunotherapy showed a correlation between tumor regression and the degree of persistence of adoptively transferred T cell in peripheral blood,<sup>66</sup> it is likely that protective immunity will require detectable long-lasting immune activity. Future DC clinical trials will therefore have to improve the durability of the induced immune response.

Although we are aware that ex vivo assays are now recommended, the detection of specific T cells presented

here was performed after an in vitro step of amplification, which can modify the phenotype and function of T cells and may not adequately reflect their status in vivo.<sup>35</sup> Indeed, we did not find immune responses without the in vitro stimulation step. In line with our experience, except in few clinical trials with defined peptides combined with adjuvants,<sup>67–70</sup> vaccination with DC and complex mixture of antigens (such as lysates) has not resulted in high frequencies of CTL in peripheral blood making the ex vivo assays difficult and not adapted. Therefore, immunomonitoring has generally been performed after an in vitro stimulation step to increase the frequency of CTL.<sup>11,31,33,43,59,60</sup> It also should be mentioned that short term in vitro expansion with PBMC (no more than 1 wk) does not generate specific T cells from naive precursors and thus the CTL detected by this assay represent memory antitumor CTL already present in the patient.<sup>32,71</sup> To further explore other ex vivo tests allowing the detection of the specific CD8<sup>+</sup> T cells induced by DC therapy, we used an ELISPOT for perforin which has been shown to correlate with cytotoxicity assays.<sup>72</sup> Four treated patients with available samples and antimelanoma peptide response were selected for this investigation. Surprisingly, some vaccinated patients had CD8<sup>+</sup> T cells that specifically released perforin ex vivo after contact with melanoma peptides whereas T cells secreting IFN- $\gamma$  were not observed. These perforin producing T cells were not present before vaccination, strongly suggesting that they were induced by the vaccine. These observations suggest a reinterpretation of previous studies in cancer patients where it was reported that T cells are in an anergic state because they could be detected by tetramer assay but they did not produce IFN- $\gamma$  or other cytokines.<sup>53,73–75</sup> Our study showed that in some cases the absence of IFN- $\gamma$  production may not mean that these cells are not functional, as they can produce perforin. It is known that IFN- $\gamma$  and cytotoxicity are regulated independently in CD8<sup>+</sup> T cells.<sup>76</sup> CTLs with lytic activity do not always secrete IFN- $\gamma$  and noncytotoxic cells are able to secrete IFN- $\gamma$ .<sup>77–79</sup>

Two activation thresholds have been described for CD8<sup>+</sup> T cells: for polarized granule secretion (lytic synapse formation), which can be triggered by low antigen concentration and requires a short time; and another for cytokine production at higher antigen concentrations.<sup>80–83</sup> As CTL detected in cancer patients are most often of low avidity, they may favor the formation of lytic synapse with production of perforin without associated cytokine.<sup>84</sup>

Other studies in cancer patients have shown that vaccine induced T cells can belong to an effector subtype, which lacked perforin but can produce IFN- $\gamma$ .<sup>74,85</sup> However, these results were obtained in patients vaccinated with tumor-derived peptides and not with DC-based or lysate/protein-based therapy. In addition, our results do not exclude that such effector cells exist. Indeed, we found antiviral CD8<sup>+</sup> T cells producing IFN- $\gamma$  without perforin. It is clear that a functional heterogeneity is a hallmark of CD8<sup>+</sup> T cells. Studies in mice at

the mRNA level showed that IFN- $\gamma$ , granzyme A and B, and perforin may all be expressed by a single CD8<sup>+</sup> T cell; however, CD8<sup>+</sup> T cells can also selectively express either of these molecules.<sup>86</sup> The proportion of cells expressing granzyme B, perforin, and IFN- $\gamma$  increased progressively after activation. Using a granzyme B ELISPOT, which may be compared with our perforin ELISPOT, Kleen et al<sup>87</sup> reported that 20% of anti-HIV CD8<sup>+</sup> T cells released simultaneously granzyme B and IFN- $\gamma$ . The majority of these specific CD8<sup>+</sup> T cells fell into a single positive category with induction of either granzyme B or IFN- $\gamma$  alone.<sup>87</sup> Similar conclusions were also drawn with a dual color IFN- $\gamma$ /granzyme ELISPOT.<sup>88</sup> We are developing a dual color fluorospot assay to detect T cells simultaneously producing perforin and other cytokines, which will provide a useful tool to further characterize the heterogeneity of the CD8<sup>+</sup> T-cell population.<sup>89</sup>

Overall, study demonstrated the ability of DC loaded with allogeneic tumor lysates to elicit specific antimelanoma derived peptides CD8<sup>+</sup> T cells with some of them producing perforin ex vivo. A differential expression of perforin and IFN- $\gamma$  production by some specific CD8<sup>+</sup> T cells found in this study also supports the notion that different parameters should be monitored to better characterize T-cell responses elicited by candidate vaccines.

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