Immunologic and Clinical Effects of Injecting Mature Peptide-Loaded Dendritic Cells by Intralymphatic and Intranodal Routes in Metastatic Melanoma Patients

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Abstract

Purpose: A phase I/II trial was conducted to evaluate clinical and immunologic responses after intralymphatic and intranodal injections of mature dendritic cells.

Experimental Design: Fourteen patients with a metastatic melanoma received matured dendritic cells, loaded with Melan-A/MART-1 and/or NA17-A peptides and keyhole limpet hemocyanin. The cells were matured overnight with Ribomunyl, a toll-like receptor ligand, and IFN-γ, which ensured the production of high levels of interleukin-12p70. Dendritic cells were injected at monthly intervals, first into an afferent lymphatic and then twice intranodally. Immunologic responses were monitored by tetramer staining of circulating CD8+ lymphocytes and delayed-type hypersensitivity tests.

Results: Dendritic cell vaccination induced delayed-type hypersensitivity reactivity toward NA17-A-pulsed, keyhole limpet hemocyanin-pulsed, and Melan-A-pulsed dendritic cells in 6 of 10, 4 of 11, and 3 of 9 patients, respectively. Four of the 12 patients analyzed by tetramer staining showed a significantly increased frequency of Melan-A-specific T cells, including one patient vaccinated only with NA17-A-pulsed dendritic cells. Furthermore, 2 of the 12 analyzed patients had a significant increase of NA17-A-specific T cells, including one immunized after an optional additional treatment course. No objective clinical response was observed. Two patients were stabilized at 4 and 10 months and three patients are still alive at 30, 39, and 48 months.

Conclusions: Injections into the lymphatic system of mature peptide-loaded dendritic cells with potential TH1 polarization capacities did not result in marked clinical results, despite immunologic responses in some patients. This highlights the need to improve our understanding of dendritic cell physiology.

Metastatic malignant melanoma is a cancer with a poor prognosis because none of the conventional treatments have shown any convincing increase in median survival (1). Currently, there is no evidence that vaccination with dendritic cells provides a therapeutic benefit, but the field is relatively new and many clinical variables remain to be investigated (2).

One unresolved problem is the best way of administering dendritic cells (i.e., i.d., s.c., intralymphatically, intranodally, i.v., or intratumorally). Assuming that the main function of dendritic cells is the transport of peripheral antigens to lymph nodes, where the primary immune response takes place, we should favor routes that allow dendritic cells to colonize lymph nodes with high efficacy. By injecting 111In-labeled macrophages into an afferent lymphatic vessel (intralymphatic), we have shown that this route allows the reproducible transfer of cells into at least 5 to 10 lymph nodes (3). In the present study, we focused on the intralymphatic route of vaccine injection: dendritic cells were first injected intralymphatically and then intranodally.

It is particularly important to consider the functional stage of dendritic cells when they are directed immediately toward lymph nodes. Dendritic cells may be functionally immature, specialized in antigen capture and processing, or mature and involved in T-cell activation. Mature dendritic cells are strongly immunogenic, whereas immature dendritic cells are considered as weak immunogens, if not actually tolerogens (4). After i.d. or s.c. injection, immature dendritic cells are expected to be weakly migratory, which prevents them, to any significant extent, from reaching lymph nodes where they might exert their tolerogenic effects. Conversely, intralymphatic injections do...
not allow such a physiologic dendritic cell selection, so care must be taken to inject dendritic cells that are ready to induce a strong, and preferentially TH1-polarized, immune response.

Many maturation products have been tested with this aim in view. One of the most commonly used cocktails includes several proinflammatory cytokines [i.e., interleukin (IL)-1β, tumor necrosis factor-α, IL-6, and prostaglandin E2; ref. 5]. This mixture is known to favor an effective dendritic cell migration toward lymph nodes but not the induction of TH1-type lymphocyte responses (6). An interesting alternative is the use of Toll-like receptor ligands. Dendritic cells are known to recognize microbial products through Toll-like receptor, which triggers a process of maturation (7). In this study, we used Ribomunyl (Pierre Fabre Médicaments, Boulogne, France), a multidimensional extract, as Toll-like receptor ligand. IFN-γ was added to Ribomunyl to favor IL-12p70 secretion (8).

In the present study, we report clinical and immunologic results for a dendritic cell–based vaccine, where mature dendritic cells with TH1 polarization capacities were pulsed with Melan-A/MART-1 and/or NA17-A peptides and injected by intralymphatic and intranodal routes.

**Patients and Methods**

**Patients.** The patients enrolled in this study had to provide a written informed consent and meet the following criteria: histologically confirmed stage M1a, or M1b, metastatic melanoma with measurable lesions according to WHO criteria (9); age ≥18 years; WHO performance status, ≤2; life expectancy, ≥4 months; hemoglobin, >10 g/dL; absolute neutrophil count, ≥1,000/μL; platelet count, ≥100,000/μL; serum bilirubin, ≤1.5 × the upper limit of normal; serum alanine aminotransferase and serum aspartate aminotransferase, ≤2.5 upper limit of normal; alkaline phosphatase, ≤5 upper limit of normal; and serum creatinine, ≤1.5 × the upper limit of normal. The patients had to be HLA-A*0201 and their tumors had to express Melan-A and/or NA17-A antigens. Previous adjuvant immunotherapy with IFN-α and/or previous chemotherapy or immunotherapy for metastatic disease was permitted but had to be completed at least 4 weeks before entering the trial (12 weeks for cell-mediated immunotherapy).

Patients were ineligible in cases of visceral metastases, except with pulmonary and/or lactate dehydrogenase elevation (stage M1c). Patients were also excluded if they presented with concurrent malignancy, psychiatric disorders, or any medical condition that could interfere with treatment administration. Pregnant or lactating women were ineligible. Hepatitis B and HIV infection were taken as exclusion criteria as well as prolonged corticotherapy.

**Study design and treatment.** Dendritic cells were prepared using the VaCell Processor device according to good manufacturing practices (Immuno-Designed Molecules S.A., Paris, France; ref. 10). In brief, the patients’ leukocytes were collected by leukapheresis and cultured in nonadherent ethyl vinyl acetate bags, in AIMV medium containing nonadherent ethyl vinyl acetate bags, in AIMV medium containing 500 units/mL granulocyte macrophage colony-stimulating factor (Leukomax, Shering-Plough, Nevallois-Perret, France) and 50 ng/mL IL-13 (Sanofi-Aventis, Labège, France), with a new addition of IL-13 on day 4 of culture. On the 7th day, dendritic cells were purified by elutriation. Five aliquots were made up. One of them was immediately suspended in AIMV at a concentration of 2 × 10^6 dendritic cells/mL and incubated for 2 h with 50 μg/mL keyhole limpet hemocyanin (KLH; Immucoltech, Biosyn, Felbach, Germany). After washing, dendritic cells were matured overnight in AIMV medium, in the presence of 1,000 units/mL IFN-γ (Imukin, Boehringer Ingelheim, Paris, France) and 1 μg/mL Ribomunyl. The cells were then washed and pulsed separately for 2 h, with 50 μmol/L Melan-A/MART-1(12-26)-H2L (ELAGIGILTV) and/or NA17-A(1-9) (VLDPDVHRC; Cinalfa, Laufelfingen, Switzerland), depending on the expression found by reverse transcript-
teratrazers were titered and used at the lowest concentration that showed a clearly distinguishable positive population in antigen-specific T lymphocytes among irrelevant T cells. At weeks 0, 4, 8, and 12, before treatment, samples of peripheral blood mononuclear cells were harvested and frozen. Peripheral blood mononuclear cells from each patient were thawed simultaneously and coincubated for 1 h at 4°C in the dark with Melan-A or NA17-A tetramers (10 μg/mL) and CD8-FITC monoclonal antibody (5 μg/mL). After washing in PBS-0.1% BSA, cells were resuspended in PBS and analyzed on a FACScan. Compensation was checked before each acquisition. Events (10^3 among CD8+ T cells) were collected using a FACScan set at a maximal flow rate of 1,000 events per second. For analysis, tetramer-positive events were evaluated within a Boolean gate, including cells within an extended lymphoid light scatter gate and a CD8 gate. The frequency of circulating antigen-specific CTLs is presented as a percentage of antigen tetramer-positive T cells among CD8 lymphocytes. A control of background labeling was done with an irrelevant tetramer [A2/NE-ESO157-165] for each sample. Analysis was also done on a tumor resected at the end of the vaccination. Briefly, short-term cultured tumor-infiltrating lymphocytes (TIL) were isolated by culturing fragments of tumors into two 12-well tissue culture plates with X-Vivo 15 serum-free medium (BioWhittaker, Walkersville, MD) containing 150 units/mL recombinant IL-2 (Eurocetus, Rueil-Malmaison, France) and 1 nmol/L glutamine (BioWhittaker) for 10 to 14 days. Expanded TILs were collected using a FACScan set at a maximal flow rate of 1,000 events per second. For analysis, tetramer-positive events were evaluated within a Boolean gate, including cells within an extended lymphoid light scatter gate and a CD8 gate. The frequency of circulating antigen-specific CTLs is presented as a percentage of antigen tetramer-positive T cells among CD8 lymphocytes. A control of background labeling was done with an irrelevant tetramer [A2/NE-ESO157-165] for each sample. Analysis was also done on a tumor resected at the end of the vaccination. Briefly, short-term cultured tumor-infiltrating lymphocytes (TIL) were isolated by culturing fragments of tumors into two 12-well tissue culture plates with X-Vivo 15 serum-free medium (BioWhittaker, Walkersville, MD) containing 150 units/mL recombinant IL-2 (Eurocetus, Rueil-Malmaison, France) and 1 nmol/L glutamine (BioWhittaker) for 10 to 14 days. Expanded TILs were derived as follows: 1.8 \times 10^6 short-term cultured TILs were plated at 300 viable lymphocytes per well with irradiated feeder cells (allogenic peripheral blood lymphocytes and B-epstein barr virus cells) into U-bottomed microplates in 200 μL of recombinant IL-2 medium, containing 15 μg/mL PHA-P (Difco, Detroit, MI). The presence of antigen-specific T cells among amplified TIL was tested after 14 days of amplification.

**Results**

**Patients’ characteristics**

A total of 14 patients were enrolled between September 2001 and March 2004 in two centers, Rennes and Lyon. All were included for efficacy and safety determinations. Patient baseline and disease characteristics are shown in Table 1. The 12 males and 2 females included were in excellent general status (WHO, 0). Four patients had received a prior adjuvant IFN-β treatment and six had received chemotherapy for systemic disease. The median time from diagnosis to metastasis was 40 months. Eight patients were at stage M_1a and four were at stage M_1b. Two patients were initially enrolled at stage M_1a and M_1b, respectively, but secondarily reclassified as stage M_1c because of an isolated elevation of lactate dehydrogenase (protocol deviation).

All patients, except for three, received the scheduled injections: two patients did not receive the second intranodal injection because of a major progressive disease, with degradation of performance status, and brain metastasis with seizures, and one patient received intranodal instead of intralymphatic injection because it proved impossible to catheterize a foot lymphatic vessel.

In addition to the scheduled treatment, the two stabilized patients received nine i.d. and five intranodal injections, respectively. For all these patients, a second apheresis was required.

**Analysis of vaccination product**

The dendritic cells prepared according to the Immuno-Designed Molecules S.A. technique displayed an immature phenotype pattern (10, 15). Monocyte-derived dendritic cells, incubated overnight in the presence of Ribomunyl and IFN-γ, displayed phenotypes of matured dendritic cells with upregulations of CD40, CD80, CD86, and neoexpression of CD83 (Fig. 1C). Cell viability at trypan blue exclusion was ~70% at the time of injection. During maturation, dendritic cells secreted IL-12p70 (mean, 28,000 pg/mL; median, 8,000 pg/mL) and IL-10 (mean, 331 pg/mL; median, 92 pg/mL) in culture medium, with an IL-12/IL-10 ratio above 1 in all preparations. For 13 preparations, an aliquot of cells was washed after maturation and further incubated, for 24 h, in medium alone, to check that dendritic cells continued to secrete cytokines, in the absence of maturing agents. Interestingly, as shown in Fig. 1, cells were not exhausted after maturation and kept on producing cytokines. The levels of IL-12p70 secreted during the 24 h following the end of maturation were lower than those produced during maturation (mean, 6,423 versus 16,433 pg/mL), but the IL-12p70/IL-10 ratio remained appreciably above 1. The release of kynurenine, a tryptophan catabolic product, was assessed in 24 culture supernatants. The mean level of kynurenin accumulation was 9.05 μmol/L (median, 7.11 μmol/L), which indicates a

| Table 1. Patient demographics and baseline disease characteristics |
|-------------------|-----------------|-----------------|-----------------|
| Median age, y (range) | 52 (27-83) | Male/female ratio | 12:2 |
| Type of tumor | Cutaneous melanoma | Melanoma with unknown primary site | Median of Breslow, mm (range) | 2.3 (1.1-4.6) |
| Presence of ulceration (cutaneous melanoma) | 3 | Prior adjuvant therapy with IFN | 4 |
| Time between initial diagnosis and metastasis, mo (range) | 40 (0-84) | Prior metastatic therapies | 8 |
| None | 8 | One line of chemotherapy with dacarbazine | 4 |
| Two lines of chemotherapy/immunotherapy, then vaccination | 2 | Metastatic sites at the inclusion* | |
| Cutaneous | 10 | Subcutaneous | 8 |
| Lymph nodes | 6 | Lung | 4 |
| Stage | M_1a | M_1b | M_1c |
| M_1a | 8 | 4 | 2 |
| M_1b | 9 | 3 | 2 |
| NA17-A alone | 9 | Melan-A alone | 3 |
| NA17-A alone | 2 |

*More than one site possible.

1 Isolated elevation of lactate dehydrogenase without visceral metastases apart from lung involvement.

**Statistical methods.**

Patients were accrued in this study using the Gehan design (14). According to this model, if no objective response is observed in the 14 first patients included, the probability of efficacy in >20% of the patients is considered <5% (β risk). In this case, the treatment should be considered as inefficient and stopped. If at least one objective response is observed among the first 14 patients, inclusion of new patients would be justified.

The statistical analyses presented here are descriptive for baseline demographics, response rates, adverse events, and immunologic response. Survival rates are estimated using the Kaplan-Meier method.
functional indoleamine 2,3 dioxygenase enzymatic activity. Indoleamine 2,3 dioxygenase activity was higher during the 24 h following the end of maturation, as shown in Fig. 1F (mean, 18.11 versus 8.61 μmol/L kynurenine).

For intralymphatic injections, a mean of 83 × 10^6 dendritic cells (range, 41 × 10^6 to 224 × 10^6) were injected. For intranodal injections, a mean of 50 × 10^6 dendritic cells (range, 11 × 10^6 to 53 × 10^6) were injected.

Response to treatment and survival

Neither complete response nor partial response was observed. In two patients, cutaneous (one patient) and s.c. (one patient) lesions remained stable, for 4 and 10 months, respectively. Median progression-free survival was 2 months (range, 1-10 months). The median overall survival for the 14 patients was 10.5 months (range, 7-48+ months). Three patients are still alive after follow-up of 30, 39, and 48 months, respectively.

Safety

Table 2 reports the adverse effects supposedly related to the treatment. We observed no related grade 4 toxicities and only three grade 3 toxicities. Although a digestive hemorrhage was reported, it was clearly not related to the treatment. No biological toxicity occurred, except for one grade 3 lymphopenia. No ocular toxicity was noted.

The 11 systemic reactions listed in Table 2 were observed in seven patients. They consisted of fever and chills and, in four cases, also included skin reactions. The 11 grade 2 skin reactions were observed in six patients, more frequently after intralymphatic (n = 8 events) than intranodal (n = 3 events) injections: these reactions involved various associations of erythema multiforme (n = 6), peripheral inflammation of skin tumor lesions (n = 5), urticaria (n = 4), halo nevus-like lesions (n = 3), and/or edema (n = 2). A skin biopsy done on two patients showed a graft-versus-host-like reaction with necrosis of keratinocytes and melanophage infiltration.

One grade 2 injection site reaction was noted after the initial i.d. injections. During additional i.d. injections in one stabilized patient, we recorded one skin reaction with grade 2 erythema and three grade 2 episodes of headaches.

Finally, some events were noted after dendritic cell injection that could have an immunologic relevance: inflammation, pain at the level of s.c. nodules, as transitory tumor regressions, and loss of pigmentation.

Fig. 1. Analysis of injected dendritic cells. Example of graphs obtained for patient 8 before (A) and after (B) maturation. C, the surface expression of CD80, CD86, CD83, and CD40 was analyzed by flow cytometry on immature dendritic cells (dotted line) and mature dendritic cells (bold line). Filled histograms, corresponding isotype controls. Numbers, the mean fluorescence intensities for immature (upper value, in italics) and mature (lower value) dendritic cells. Assays for IL-12p70 (D), IL-10 (E), and kynurenine (a catabolic product of tryptophan; F) were carried out in the supernatant (T) after an overnight maturation. Cell aliquots were further incubated for 24 h in fresh medium without maturing agents, and the tests were repeated once again (T + 24H).
**Immune responses**

**Skin tests.** Eleven patients could be evaluated for skin tests before and after treatment. Reactivity to dendritic cell control was observed in two patients before treatment (patients 4 and 12) and in two other patients only after treatment (patients 3 and 11). Four positive reactions against the antigens were observed before any treatment: two positive tests to dendritic cells-KLH (patients 2 and 14), one to dendritic cells-Melan-A (patient 2), and one to dendritic cells-NA17-A (patient 11). By excluding patients with reactions against autologous mature dendritic cells, and only considering those patients who became positive on treatment, we obtain the following results:

6 of 10 positive responses against NA17-A, 4 of 11 against KLH, and 3 of 9 against Melan-A (Table 3).

**immune lymphocyte response.** Twelve of 14 patients were analyzed for antigen-specific responses. Peripheral blood mononuclear cells were obtained before (week 0) and 4 weeks after each vaccine injection (weeks 4, 8, and 12). The mean frequencies of CD8+ T cells directed to Melan-A/MART-1(26-35L) and to NA17-A(1-9) before treatment were, respectively, 0.14% (range, 0.02-0.51%) and 0.21% (range, 0.02-0.76%). Patients were considered as responders when they showed a >2-fold increase in T-cell frequency above the baseline, with a frequency of at least 0.1% and the presence.

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**Table 2.** Summary of related-adverse events probably or suspected to be related to the treatment (by severity)

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Intralymphatic injection (n = 13)</th>
<th>Intranodal injection (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 2</td>
<td>Grade 3</td>
</tr>
<tr>
<td>Drug Fever</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Skin*</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>Headache</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Injection site reaction</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
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<td>—</td>
</tr>
<tr>
<td>Abdominal Pain</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phlebitis</td>
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</tr>
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</table>

*See text for explanation.

**Table 3.** Summary of immunologic and clinical data

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Skintests (mm of induration)</th>
<th>Immune lymphocyte responses (increase of specific T cells)</th>
<th>Clinical outcome</th>
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<td></td>
<td>Before treatment DC/K/DC-K/DC-M/DC-N</td>
<td>After treatment DC/K/DC-K/DC-M/DC-N</td>
<td>Melan-A(26-35L)* specific T cells</td>
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</tr>
<tr>
<td></td>
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<td>0/11/12/10/6</td>
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</tr>
<tr>
<td>2</td>
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</tr>
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<tr>
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<tr>
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</tr>
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</table>

NOTE: A, patients vaccinated with Melan-A(26-35L)*-pulsed and NA17-A(1-9)*-pulsed dendritic cells; B, patients vaccinated with Melan-A(26-35L)* pulsed dendritic cells; C, patients vaccinated with NA17-A(1-9)*-pulsed dendritic cells. Responses considered as positive are in bold.

Abbreviation: DC/K/DC-K/DC-M/DC-N, dendritic cells alone/KLH alone/dendritic cells pulsed with KLH/dendritic cells pulsed with Melan-A(26-35L)/dendritic cells pulsed with NA17-A(1-9); SD, stable disease; PD, progressive disease; ND, not done.
of a distinct population on flow cytometry dot blots. The results were as follows:

(a) Among the 10 patients vaccinated with Melan-\(\text{A}_{26-35}\)-pulsed dendritic cells, 3 patients (patients 2, 7, and 10) showed a significant increase in the frequencies of Melan-\(\text{A}_{26-35}\)-specific T cells, 4 or 8 weeks after the beginning of treatment (Fig. 2A and B).

(b) Among the nine patients vaccinated with NA17-A\(_{(1,9)}\)-pulsed dendritic cells, two patients showed a significant increase in the frequencies of NA17-A\(_{(1,9)}\)-specific T cells. For one patient (patient 12), this increase was observed 4, 8, and 12 weeks after the beginning of treatment (Fig. 2C), whereas, for patient 6, it occurred during a second course of treatment, rising from 0.19% to 0.90% after a first intranodal injection and then to 0.83% and 0.78% after a second and a third injection (data not shown).

(c) Interestingly, one patient (patient 11) vaccinated with NA17-A\(_{(1,9)}\)-pulsed dendritic cells showed increased frequencies of Melan-\(\text{A}_{26-35}\)-specific T cells (Fig. 2C).

Tumor of patient 1 was analyzed 2 months after the end of i.d. extra injections of loaded dendritic cells. As shown in Fig. 3, the fractions of Melan-A-specific and NA17-A-specific T cells were 0.51% and 1.29% of CD8 TIL, respectively. It is noteworthy that this patient had a high percentage of NA17-A-specific T cells before vaccination (0.65%); this percentage did not increase during the first course of treatment (Fig. 2A).

Discussion

The present study reports the clinical and immunologic outcomes of an investigation on 14 stage IV melanoma patients...
treated with a peptide-pulsed dendritic cell vaccine. The biological properties and the biodistribution of radiolabeled dendritic cells injected by intralymphatic, intranodal, and i.d. routes have been reported and discussed in a companion article (12).

In the present clinical trial, we took care to optimize various aspects of antigen-loaded dendritic cell vaccination. First, to include patients in excellent general condition, all M1c were discarded, except for two patients with elevated lactate dehydrogenase who had no visceral metastases. Second, the maturation of dendritic cells with Ribomunyl and IFN-γ ensured the production of high levels of IL-12p70. This cytokine is known to play a critical role in driving CD4+ T cells to TH1 polarization, a property essential for the development of T-cell–mediated antitumor response. As a matter of fact, dendritic cells matured with Ribomunyl and IFN-γ and cocultured with T cells are known to induce TH1-characteristic cytokines (12, 16, 17). Third, to minimize the risk of poor migration of dendritic cells toward lymph nodes, the first cell injection was done into a lymphatic vessel. As this procedure was judged uncomfortable for the patients and difficult to repeat, it was replaced by intranodal injections in the subsequent administrations.

Indeed, intralymphatic injection enabled the dendritic cells to reach 8 to 10 inguinal, crural, and iliac nodes, as reported previously (12). Intranodal injected dendritic cells probably reached the target lymph node in ~50% of cases, if we refer to recent studies with magnetically labeled dendritic cells and magnetic resonance tracking (18). The choice of these injection routes also aimed at attenuating antigen loss. Indeed, dendritic cells pulsed with peptides and set in culture are known to lose rapidly their ability to activate antigen-reacting T-cell clones (19). Likewise, the detection of HLA class I peptide complexes with a T-cell receptor–like antibody against MAGE-1.A1/HLA-A1 indicates a complete loss of staining 24 h after the pulse (20). Effective migration of dendritic cells to lymph nodes from the skin may require 24 to 48 h, a time interval sufficient for antigen to disappear. Intralymphatic or intranodal injections thus seem as good options for circumventing this risk.

Despite these optimization attempts, no objective clinical response was observed because only two patients were stabilized for >3 months. Currently, some argue that conventional oncologic criteria for clinical tumour response should be expanded for immunotherapy and in particular disease stabilization could be sign of clinical benefit. We also observed transitory tumor shrinkage or disappearance of cancer nodules during the treatment course. These temporary reactions were synchronous with skin reactions in two patients. Skin reactions were also frequently associated with the inflammation of skin tumor and/or halo nevus-like lesions, a phenomenon interpreted as a reaction against melanocytes. In fact, skin biopsies done on two patients showed an inflammatory reaction with melanophages. It is noteworthy that such cutaneous reactions have thus far never been reported. They were possibly linked with the intralymphatic route because ~50% of patients experienced such a reaction after intralymphatic injection, when only three grade 2 skin reactions were observed after 27 intranodal injections. The other side effects, such as fever and chills, were those commonly reported after dendritic cell vaccination.

Did the vaccination immunize against melanoma antigens? Immune response was assessed by delayed-type hypersensitivity reactivity and tetramer assays. Surprisingly, only 4 responders to KLH were found among 11 patients, whereas significant delayed-type hypersensitivity reactivity to KLH has been reported in 90% of patients, in two distinct dendritic cell vaccination protocols (21, 22). Similarly, responses against Melan-A were not as high in our study as those reported previously (22, 23). The highest rates of response were observed against NA17-A-pulsed dendritic cells, with 6 of 11 patients becoming positive. An expansion of Melan-A26-35 specific and NA17-A1-9 specific T cells occurred in 4 and 2 of 12 patients, respectively. These results are close to those reported by other authors (23–25). For three patients, the induction of Melan-A26-35 specific T cells was transient. This has already been reported (24) and is of unknown significance. Interestingly, one patient (patient 11) vaccinated with NA17-A-pulsed dendritic cells developed lymphocytes directed against Melan-A/MART-126-35. This could be explained by the phenomenon of antigen spreading, in which the CTL induced by the vaccine can interact with the tumor, thus enabling the stimulation of other antigen CTL (26–28). In this patient, although no increase in anti-NA17-A T cells was observed in blood, delayed-type hypersensitivity response against NA17-A was considered as positive (Table 3), indicating an immunologic response against this antigen. The tetramer assay was applied to one patient’s tumor and showed the presence of anti-Melan-A26-35 and NA17-A1-9 T cells. This result is difficult to interpret due to the lack of control before vaccination and because such frequencies in Melan-A T cells have already been reported in TIL established from untreated melanoma lesions (29). By contrast, the frequency of anti NA17-A1-9 T cells (1.29%) was particularly elevated. Indeed, in a series of 22 TIL...
preparations made up from tumors of HLA-A2 patients, we were unable to detect the presence of NA17-A(1-9) T cells.7

Thus, despite injections of mature dendritic cells into lymphoid tissue by intralymphatic and/or intranodal routes, no objective response was achieved and the immunologic responses were not higher than those reported previously. This raises the question as to whether one of the options taken in the protocol was not as optimal as first believed.

The particular type of dendritic cells injected may be questioned. Dendritic cells are usually prepared from separated monocytes cultured in the presence of granulocyte macrophage colony-stimulating factor and IL-4. In the process designed by Immuno-Designed Molecules S.A., monocytes are not separat-
ed from lymphocytes and are differentiated into dendritic cells in the presence of IL-13, instead of IL-4. Nevertheless, such dendritic cells have been shown to induce in vivo immune responses after s.c. and intranodal injections (15).

The mode of dendritic cell maturation should be reexamined. Besides the nature of the maturation stimulus, the kinetics of maturation is also a critical variable. It has been reported that dendritic cells matured in vitro for 6 h in presence of Ribomunyl and IFN-γ were able to generate high frequencies of Melan-A-specific CTL, contrary to dendritic cells matured for 20 h (30). Injection of less mature dendritic cells, secreting larger amount of IL-12p70, would perhaps have given better results. Furthermore, IL-10 and indoleamine 2,3 dioxygenase were released during the process of maturation (Fig. 1) and, contrary to IL-12p70, an increased level of release was generally observed after the overnight maturation. Until recently, the strongly immunogenic nature of mature dendritic cells was contrasted with immature dendritic cells that were known to be able to promote T-cell tolerance. However, this concept is currently being revisited and dendritic cell maturation is no longer regarded as a distinguishing feature of immunogenicity because semimature dendritic cells as well as fully mature dendritic cells can induce tolerance (31). As release of IL-10 and indoleamine 2,3 dioxygenase expression are two key factors in such an induction process, we wondered about a potential tolerogenic function of our dendritic cells after injection. Because one of the features of tolerogenic dendritic cells is their ability to expand regulatory T cells, we assessed the number of CD4+CD25+CD152+ T cells in four patients. These cells have been shown previously to represent a homogeneous population of regulatory T cells (32). Our study revealed no increased frequency in untreated patients (data not shown), in comparison with healthy controls (32, 33), and the count of CD4+CD25+CD152+ T cells remained constant throughout the trial (data not shown). Thus, our vaccination protocol did not appear to expand regulatory T cells, a possibility recently underlined in an animal model (34).

We should also question our route of administration. Millions of dendritic cells were injected per node, which is much higher than the number of dendritic cells expected after peripheral delivery, considering that <5% of mature dendritic cells reach the draining lymph node after i.d. delivery (35). In mouse studies, increasing the number of dendritic cells in nodes improves dendritic cell–based vaccination (36), which prompts to favor direct delivery to lymph nodes, as we did. The disadvantage of this technique is the lack of selection of dendritic cells transported to lymph nodes, in contrast to i.d. route. Furthermore, the optimal number of dendritic cells needed to induce a potent immune response is not known. In a study comparing intranodal, i.d., and i.v. injections of dendritic cells matured in the presence of calcium ionophore, as well as of IL-2 and IL-12, intranodal injection resulted in superior T-cell sensitization as measured by de novo target cell recognition and delayed-type hypersensitivity priming, but similar immune responses were observed after nodal injection of 2.5 million dendritic cells per node compared with 25 million dendritic cells (25). The injection of not so many dendritic cells, as previously done in a pioneer clinical trial (37), may therefore be sufficient while preventing a potential overcrowding into nodes. Alternative promising strategies of pretreatment of the vaccine injection site with inflammatory cytokines, such as tumor necrosis factor-α, or with preinjection of dendritic cells could also allow an efficient migration closer to the physiologic entry of dendritic cells into the lymph nodes (36).

All these results highlight the need to improve our understanding of dendritic cell physiology, considering the remarkable functional plasticity of these cells. As in vivo studies can only provide rather poor predictions of the in vivo behavior of dendritic cells, clinical trials should be continued in the light of the knowledge acquired from basic research.

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