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Pilot Clinical Trial of Type 1 Dendritic Cells Loaded with Autologous Tumor Lysates Combined with GM-CSF, Pegylated IFN, and Cyclophosphamide for Metastatic Cancer Patients

Carlos Alfaro,*† Jose L. Perez-Gracia,‡ Natalia Suarez,*‡ Javier Rodriguez,‡ Miguel Fernandez de Sanmamed,*§ Bruno Sangro,* Salvador Martin-Algarra,* Alfonso Calvo,* Miriam Redrado,‖ Alice Agliano,‖ Alvaro Gonzalez,‖ Inmaculada Rodriguez,* Elixabet Bolaños,*,† Sandra Hervás-Stubs,* Javier Perez-Calvo,† Alberto Benito,‡ Ivan Peñuelas,** Carmen Vigil,** José Richter,** Ivan Martinez-Forero,* and Ignacio Melero*†‡

Twenty-four patients with metastatic cancer received two cycles of four daily immunizations with monocyte-derived dendritic cells (DC). DC were incubated with preheated autologous tumor lysate and subsequently with IFN-α, TNF-α, and polyinosinic-polycytidylic acid to attain type 1 maturation. One DC dose was delivered intranodally, under ultrasound control, and the rest intradermally in the opposite thigh. Cyclophosphamide (day −7), GM-CSF (days 1–4), and pegIFN alpha-2a (days 1 and 8) completed each treatment cycle. Pretreatment with cyclophosphamide decreased regulatory T cells to levels observed in healthy subjects both in terms of percentage and in absolute counts in peripheral blood. Treatment induced sustained elevations of IL-12 in serum that correlated with the output of IL-12p70 from cultured DC from each individual. NK activity in peripheral blood was increased and also correlated with the serum concentration of IL-12p70 in each patient. Circulating endothelial cells decreased in 17 of 18 patients, and circulating tumor cells markedly dropped in 6 of 19 cases. IFN-γ-ELISPOT responses to DC plus tumor lysate were observed in 4 of 11 evaluated cases. Tracing DC migration with [111In] scintigraphy showed that intranodal injections reached deeper lymphatic chains in 61% of patients, whereas with intradermal injections a small fraction of injected DC was almost constantly shown to reach draining inguinal lymph nodes. Five patients experienced disease stabilization, but no objective responses were documented. This combinatorial immunotherapy strategy is safe and feasible, and its immunobiological effects suggest potential activity in patients with minimal residual disease. A randomized trial exploring this hypothesis is currently ongoing. The Journal of Immunology, 2011, 187: 000–000.

Dendritic cells (DC) present Ags to naive and memory T lymphocytes. DC that artificially present tumor Ags are efficacious antitumor vaccines for mouse-transplanted tumors (1–3). Many DC-based clinical trials have been performed in cancer patients with evidence of increased immune responses and clinical activity (1, 3–8), but efficacy is unfortunately lower than that observed in mouse models (9, 10). A key difference might be that cancer patients with bulky disease present multiple immunosuppressive regulatory mechanisms (11), such as the augmentation of CD4+ CD25+ FOXP3+ regulatory T cells (Tregs) (12, 13). DC for clinical trials are most often differentiated in cultures from monocytes with GM-CSF and IL-4 (14). Other protocols have substituted IL-4 for IFN-α (15) or IL-15 (16) with encouraging preclinical results (2). DC become highly immunogenic, as opposed to their steady state tolerogenic mode (17), when they sense in their microenvironment inflammatory cytokines and/or the presence of moieties denoting microbial infection such as viral nucleic acids (1).

DC can be artificially manipulated to present tumor Ags either in the form of defined protein sequences (18, 19) or as antigenic

*Gene Therapy and Hepatology Unit, Center for Applied Medical Research, University of Navarra, 31008 Pamplona, Spain; †Cell Therapy Unit, University Clinic of Navarra, University of Navarra, 31008 Pamplona, Spain; ‡Oncology Department, University Clinic of Navarra, University of Navarra, 31008 Pamplona, Spain; §Biochemistry Department, University Clinic of Navarra, University of Navarra, 31008 Pamplona, Spain; ‖Chemistry Department, University Clinic of Navarra, University of Navarra, 31008 Pamplona, Spain; ||Oncology Division, Center for Applied Medical Research, University of Navarra, 31008 Pamplona, Spain; ⬧Radiology Department, University Clinic of Navarra, University of Navarra, 31008 Pamplona, Spain; and **Nuclear Medicine Department, University Clinic of Navarra, University of Navarra, 31008 Pamplona, Spain

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Address correspondence and reprint requests to Prof. Ignacio Melero, Center for Applied Medical Research, Avenida de Pio XII, 55, 31008 Pamplona, Spain. E-mail address: imelero@unav.es

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Abbreviations used in this article: CEC, circulating endothelial cell; CTC, circulating tumor cell; DC, dendritic cell; PegIFN, pegIFN alpha-2a; poly(I:C), polyinosinic-polycytidylic acid; SPEICT, single photon emission computed tomography; Treg, regulatory T cell.

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material obtained from autologous tumor cells (20). Defined tu-
ror antigenic sequences facilitate experimental assessment of
tumor immunity (21), but most likely miss tumor-specific muta-
tions that ought to be drivers of the malignant phenotype.

DC are also important regulators of the activity of NK cells (22, 23). NK cells lyse tumor target cells in an Ag- and MHC-
unrestricted fashion, produce proinflammatory mediators, and regu-
late angiogenesis (24, 25). NK cells also play an important role in
the orchestration of the adaptive immune response (24, 26, 27).

IL-12 (28) and some surface-attached receptor-ligand pairs have been
found to be involved in the DC–NK interplay (22, 23).

Although autologous tumor lysates have been used as a source of
Ag (29, 30), the abundance of immunosuppressive factors (31–33)
and lysosomal proteases is a potential drawback of freeze/thaw tumor
lysates as Ag sources. Some of these factors should be ther-
mo labile, and, therefore, preheating the lysates to boiling
temperatures (34) might deactivate such moieties, while preserving
the primary amino acid sequences of the polypeptide Ags.

Kalinski and Okada (35) have reported that the optimal DC
to induce cellular immunity are those that have been activated by
IFN-α and that have sensed viral RNA or viral RNA analogs. Such
DC were termed type 1 DC (35) because they are powerful producers
of IL-12, migrate to lymph nodes guided by CCR7, and induce
immune responses dominated by Th1 and cytotoxic T lymphocytes
(7, 36). Type 1 DC are also potent activators of NK cells (23).

We have tested the safety and biological activity of immuno-
therapy based on type 1 DC in advanced cancer patients. Our
combination strategy was based on preclinical data that we de-
veloped in mouse models. Innovative elements include the stimuli
used to induce type 1 DC maturation and a step in which the
autologous tumor lysates are preheated to denature thermolabile
proteases and immunosuppressive factors that otherwise reduce the
suitability of tumor lysates for loading tumor Ags into DC. In-
tensive daily dosing to achieve sustained arrival of Ag to lymph
nodes and sequential combination with other immunomodulating
agents were implemented in a combinatorial fashion. In this regard,
pretreatments with cyclophosphamide were intended to reduce
the expression of the tumor microenvironment and the per-
formance of the immune system cells as if fighting an acute viral
infection. Indeed, the overall objective of this combinatorial im-
munotherapy strategy was to imitate the strong cellular immunity
that typically occurs following an acute viral infection as a result of
sustained Ag presentation in lymphoid tissue by type 1 DC.

Materials and Methods

DC production

Monocytes from leukopheresis products were selected by CD14 immu-
nomagnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany) (37).
Monocytes were cultured in cell-cultured flasks (175 cm2; Corning, Sigma-
Aldrich, St. Louis, MO) for 7 d in AIM-V serum-free media (Life
Technologies-BRL, Gaithersburg, MD) supplemented with GM-CSF (1000
U/ml; Leukine, Berlex, Richmond, CA) and IL-4 (500 U/ml; R&D Sys-
tems, Minneapolis, MN). Cytokines were added every 2 d. Purified mono-
cytes were differentiated to DC using GMP standard procedures (37). DC
were exposed to autologous tumor lysate generated by five rounds of
freeze/thawing and 10 Gy irradiation with a 5-min–long heating step at
100°C during the first thawing step. Tumor lysates were generated from
needle tumor biopsies or surgical samples. Tumor tissue disruption was
performed with the GentleMacs dissociator device (Miltenyi Biotec),
followed by the freezing/thawing and irradiation procedures, to be sub-
sequently cryopreserved at −80°C until used. DC loading with lysate was
carried out with 100–200 μg/ml protein during 2 h. DC were then matured
with clinical-grade TNF-α (50 ng/ml; Boehringer Ingelheim, Ingelheim,
Germany), IFN-α (1000 IU/ml; Schering-Plough, Kenilworth, NJ), and
polyinosinic:polycytidylic acid [poly(I:C)] (20 μg/ml; Amigen, Bi-
oclonies, Tokai, South Africa) for 24–48 h. Freezing and thawing of matured
and Ag-loaded DC were performed, as described previously, in aliquots
of 30 × 106 cells (37). DC were slowly frozen to −120°C in autologous
serum with 5% v/v DMSO by using a computer-assisted step down freezer
(CM-25; Carboeus Metalicos). The first two treatments were performed with
cultured cells without any previous freezing step, whereas the rest of
the treatments were prepared with thawed DC.

Maturation was confirmed by increasing the immunofluores-
ence of CD80, CD86, CCR7, B7-H1, and CD40 (BD Biosciences, Erembodegem,
Belgium) (35) have reported that the optimal DC
to induce cellular immunity are those that have been activated by
IFN-α and that have sensed viral RNA or viral RNA analogs. Such
DC were termed type 1 DC (35) because they are powerful producers
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tensive daily dosing to achieve sustained arrival of Ag to lymph
nodes and sequential combination with other immunomodulating
agents were implemented in a combinatorial fashion. In this regard,
pretreatments with cyclophosphamide were intended to reduce
Tregs and GM-CSF to ensure a better survival of the DC once
injected. PegIFN-α-2a (PegIFN) should lead to an enhancement of
Ag presentation in the tumor microenvironment and the per-
formance of the immune system cells as if fighting an acute viral
infection. Indeed, the overall objective of this combinatorial im-
munotherapy strategy was to imitate the strong cellular immunity
that typically occurs following an acute viral infection as a result of
sustained Ag presentation in lymphoid tissue by type 1 DC.

Study design and statistical considerations

Patients were required to have a diagnosis of metastatic cancer noname-
nable to standard treatment, adequate hematological and hepatic function,
Eastern Cooperative Oncology Group status ≤2, and adequate access to
tumor tissue for lysate production. Patients were excluded in the event of
relevant concomitant diseases, including other tumors, infections, or the need
to receive immunosuppressant treatment. All patients signed informed
consent. The trial was approved by the ethics committees of our institution
and by the Spanish regulatory boards (Agencia Española del Medicamento
y Productos Sanitarios), and was registered at http://www.clinicaltrials.
gov (NCT00610389).

The treatment schedule is presented in Fig. 2. Patients underwent aphe-
resis to obtain peripheral blood leukocytes and received a single dose of
600 mg/m² cyclophosphamide (day −7). DC were administered in two
cycles of four daily immunizations of 10⁷ cells per dose, separated by 3–6
wk. The first dose was delivered inside an inguinal lymph node, under
ultrasound control, and the rest intradermally in the opposite upper thigh.
GM-CSF, 100 μg/24 h (days 1–4), and PegIFN, 80 mg (days 1 and 8), were
injected s.c. in the upper thigh region. Patients could receive additional DC
doses contingent to the presence of clinical benefit and DC availability
according to investigators’ criteria. At baseline, complete blood tests and
imaging studies were performed. Clinical evaluation and blood tests were
repeated on days 15 and 29 of each cycle during the first two cycles and
every 6 wk thereafter. Imaging tests were repeated every 12 wk during
treatment. Follow-up after treatment discontinuation was performed every
3 mo. Informed consents and the approved clinical protocol included the
collection of biological samples and the performance of imaging techniques
used to study the immunological parameters used to assess clinical bene-
fit.

The main objective was response rate assessed by Response Evaluation
Criteria in Solid Tumors. Secondary clinical objectives were progression-
free survival, overall survival, and toxicity, according to circulating tumor
cell (CTC) criteria version 3.0. Sample size was calculated using Simon’s
Minimax two-stage method for P0 = 0.05 and P1 = 0.25, using error
probability limits of α = 0.05 and β = 0.10. It was estimated that at least 25 evaluable patients were required. Under these conditions, observation of at
least three responses was required to confirm a 25% response rate (P1).

Immunological monitoring

For immunological monitoring, sequential samples of PBMC and serum
were collected. PBMC from patient samples were isolated by Ficoll-Paque
Plus (GE Healthcare, Piscataway, NJ) gradient centrifugation. The cells
were slowly frozen to −80°C in FBS (Invitrogen, Paisley, U.K.) with 5% v/v
DMSO by using a Cryo 1°C Freezing Container (Nalgene; Thermo Fisher
Scientific, Roskilde, Denmark). The immediate yield of viable thawed
cells was >80%.

FACS analyses and ELISAs

Before immune staining, cells were incubated with PBS/human IgG (50 μg/
ml; Beriglobina P; Behring, Barcelona, Spain) for 10 min on ice, to block
Fc receptors. Subsequently, DC (10⁶) were washed in cold PBS and incu-
bated 15 min at 4°C with specific FITC- and PE-labeled mAb for CD80, CD83,
CD86, CCR7, B7-H1, and CD40 (BD Biosciences, Erembodegem,
Belgium). Tregs were analyzed by intracellular FOXP3 staining following CD4 and CD25 surface immunostaining (BD Biosciences), and absolute numbers were estimated with total lymphocyte counts in peripheral blood. NK cells were identified as CD3− CD16+ CD56+ lymphocytes. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Concentrations of IL-12p70 and IFN-γ were assessed by commercial sandwich ELISA kits (BD Biosciences).

NK cell cytotoxicity assays

Cytotoxic activity of NK cells against K562 cells was measured by the standard 5-h sodium [3H]Cr release assay. Target cells (104) were labeled with 50 μCi [3H]Cr (PerkinElmer, Boston, MA) for 1 h at 37°C, and labeled cells were then washed and resuspended in RPMI 1640 (Invitrogen) containing 10% FBS from Invitrogen. Isolated PBMC from different days before and after the treatments were used as effector cells. These cells were resuspended in the same medium and placed at various E/T ratios. Labeled target cells were added to each well at a concentration of 3 × 103 cells/well for a total volume of 0.2 ml/well. After 5-h incubation, release of [3H]Cr was stopped with tap water, and spots were analyzed with a CTL-ImmunoSpot S5 Micro Analyzer (CTL-Europe, Bonn, Germany). To calculate the number of T cells responding to a particular Ag, the mean spontaneous release was subtracted from total release (Spots/well). Each experiment was performed in triplicate.

IFN-γ ELISPOT assay

Multiscreen hemagglutinin plates (Millipore, Bedford, MA) were coated with 15 μg/ml monoclonal anti-human IFN-γ (1-D1K; Mabtech, Stockholm, Sweden) in PBS overnight at 4°C. Unbound Ab was removed by washing with PBS, and plates were blocked with RPMI 1640 (Invitrogen) containing 1% FCS and 0.05% Tween 20 for 1 h at 37°C. The medium was aspirated, and effector cells (2 × 105) were seeded in triplicates in RPMI 1640 with 10% heat-inactivated human serum type AB (Biowhittaker Lonza, Basel, Switzerland). PBMC, from 1 d before each treatment and 3 d after each immunization cycle, were used as effector cells. Stimulator cells were autologous DC loaded with tumor lysates (100–250 μg/ml) or autologous tumor lysates (5 μg/ml). Merck (Whitehouse Station, NJ) as a positive control and matured with TNF-α (50 ng/ml), IFN-α (1000 U/ml), and poly(I:C) (20 μg/ml) for 48 h in AIM-V medium (Biowhittaker Lonza). DC (2 × 105) were cultured with the effector cells. Negative control wells contained equally treated unloaded DC, and as a technical positive control effector cells alone stimulated with Con A (20 μg/ml; Sigma-Aldrich). Cells were incubated at 37°C in 5% CO2 in a water-saturated atmosphere. After a culture period of 20 h, cells were removed by six washings with PBS/0.05% Tween 20. Captured cytokine was detected by incubation for 3 h at 37°C with biotinylated mAb anti-human IFN-γ (7-B6-1; Mabtech) at 1 μg/ml in PBS with 0.5% FCS (PBS-0.5% FCS). After washing the cells six times with PBS/Tween, streptavidin-alkaline phosphatase (1/1000; Mabtech) was added for 2 h at room temperature. Unbound complex was removed by washing plates before, and IFN-γ spots were detected at sites of secretion with 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate (Sigma-Aldrich). The reaction was stopped with tap water, and spots were analyzed with a CTL-ImmunoSpot S5 Micro Analyzer (CTL-Europe, Bonn, Germany). To calculate the number of T cells responding to a particular Ag, the mean numbers of spots induced by DC alone were subtracted from mean spot numbers induced by Ag-loaded DC.

Assessment of circulating endothelial cells and CTC

Blood samples (10 ml) were collected before treatment (baseline sample) and after each cycle of DC injections (post 1 and post 2). A total of 1.5 ml was used for FACS analysis and the rest of the blood for RNA isolation. The response was measured by flow cytometry by enumerating circulating endothelial cells (CEC) and circulating endothelial progenitors. Complete blood was incubated with 10% goat normal serum diluted in PBS with 2% BSA and 5% EDTA for 30 min at 4°C to block unspecific signal. Samples were incubated for 30 min at 4°C with anti-CD31 FITC (BD Pharmingen, Franklin Lakes, NJ), anti-VEGFR-2 PE (R&D Systems, Minneapolis, MN), anti-CD133 alkaliphycocyanin (Miltenyi Biotech), and anti-CD45 PerCP (BD Pharmingen), or 7-aminocantoximycin D (Sigma-Aldrich). Anti-CD45 was used to exclude hematopoietic cells, whereas 7-aminocantoximycin D was used to exclude apoptotic and dead cells. After Ab incubation and red cell lysis, at least 1 × 106 cells/sample were acquired with a FACSCalibur flow cytometer (BD Biosciences). Following acquisition, appropriate gating was used to exclude dead cells, platelets, and debris, and analyses were considered as informative when adequate numbers of events (i.e., >50), typically 100–200) were collected in the relevant gates. Percentages of stained cells were determined and compared with appropriate negative controls defined as nonspecific background staining.

For RNA isolation, dDNA synthesis, and real-time PCR, the blood samples were centrifuged at 2500 rpm for 8 min. RNA from nucleated cells was isolated with the QIAamp RNA blood kit (Qiagen, Dusseldorf, Germany) following the manufacturer’s instructions. Reverse transcription of 1 μg RNA was performed with Superscript II (Invitrogen), according to the manufacturer’s instructions. RT-PCR was performed in a 7300 Real Time PCR system (Applied Biosystems, Carlsbad, CA) using the SYBR Green PCR Master Mix (Applied Biosystems). The relative expression of each gene was normalized with GAPDH. The list of primers used is shown in Supplemental Table 1.

Statistics

Descriptive statistics were used to present response rate, clinical characteristics, and toxicity. For comparisons, unpaired Student t tests or Mantel–Cox tests were performed. Correlations were assessed by R2 and Pearson’s correlation coefficient. Calculations were made using Prism software (GraphPad Software, La Jolla, CA).

Results

Rationale for the design of the vaccination strategy

The vaccination protocol was based on preclinical work developed by our group (Fig. 1) and on previous clinical studies (37). Boiling lysates from mouse and human tumors changed the protein patterns as visualized by silver-stained SDS-PAGE (Fig. 1A). Experiments in mice bearing transplanted CT26 colon carcinomas for 7 d (Fig. 1B) indicated that series of four daily immunizations with tumor lysate-loaded DC achieved complete tumor rejections in about half of the cases (up to 7 of 14 in the experiment shown in Fig. 1C). Freezing and heating the tumor lysates used to load DC to boiling point for 5 min provided a survival advantage to the treated mice (Fig. 1C) if compared with mice treated with DC-loaded nonheated tumor lysates. The maturation stimuli used for the mouse DC was murine TNF-α, murine IFN-α, and poly(I:C).

Based on these preclinical models, DC from patients were cultured with preheated autologous tumor lysates and matured with TNF-α, IFN-α, and poly(I:C) to induce differentiation of type 1 DC (35). The scheme of treatment is presented in Fig. 2. Cyclophosphamide was administered to decrease Tregs and their immunosuppressive effects. Daily DC vaccinations were planned to ensure sufficient and sustained bioavailability of Ags at lymph nodes. The first dose of DC in each cycle was given intranodally under ultrasound guidance in an inguinal lymph node, whereas the rest were given intradermally in the opposite thigh. This permitted independent observations on both routes of administration and maximized the number of responding lymph nodes. Patients received GM-CSF and PegIFN to potentiate DC functions and survival and to enhance overall immunity (30). GM-CSF and PegIFN were administered in the territory draining to the same lymph nodes to enhance and sustain DC performance.

Patient characteristics and treatment administration

From May 2008 to September 2010, 31 patients were included. Twenty-two patients received two cycles of vaccination, and two received just one cycle of vaccination (performed as described in Fig. 2). Seven patients did not complete the planned treatment because of patient decision or death. Therefore, treatment was
administered as planned in 24 patients. Three patients received additional vaccinations.

Patient characteristics are presented in Table I. Patients are color and number coded in the table to facilitate their individual follow-up in some of the figures. Histological diagnosis was as follows: colorectal cancer (9 patients, 38%), melanoma (5 patients, 21%), hepatocellular carcinoma (4 patients, 17%), renal cell carcinoma (3 patients, 13%), cholangiocarcinoma (2 patients, 8%), and carcinoid tumor (1 patient, 4%). Fifteen patients were male (62%), and nine were female (38%). Patients were followed for a range of 1–19 mo with a mean of survival of 11.7 mo (95% confidence interval: 8.9–14.5) and a median of 12 mo (95% confidence interval: 5–19).

Clinical efficacy and toxicity

Five patients presented disease stabilization (21%), including a patient with metastatic colon cancer and a patient with a surgically resected brain metastasis from melanoma, who were stable for 13 mo following treatment. Systemic toxicity included grades 1 and 2 fever (17 patients, 71%), asthenia (10 patients, 29%), and pain at the injection site (6 patients, 25%) (Table I). All toxicities lasted few days, and no grade 3 or 4 events were recorded. These mild side effects onset immediately (6–48 h) following DC injections and were attributed to the effects of the vaccines or the cytokines concurrently given rather than to cyclophosphamide. One patient presented a perineal abscess that evolved to a Fournier’s gangrene.

FIGURE 1. Heating tumor lysates and preclinical efficacy. A, Silver-stained SDS-PAGE analyses of tumor lysates obtained from CT26 mouse tumors and tumors from two representative cancer patients obtained following five freezing/thawing cycles with or without heating to 100°C in the first thawing cycle. Overlay profiles show the differences in protein bands. Green and red arrows mark the highest differences between the electrophoresis profiles with and without heating to 100°C. Bands enriched with heating are marked with red arrows, whereas those that predominate in the nonheated samples are marked with green arrows. B, Scheme of treatment with DC vaccines of BALB/c mice bearing established s.c. CT26 tumors. C, Follow-up of tumor growth (left) in BALB/c mice s.c. grafted with CT26 tumors for 7 d and then treated with DC pulsed with tumor lysate, as indicated in the scheme shown in B, and matured with poly(I:C), TNF-α, and IFN-α. In some cases, DC had been loaded with tumor freeze and thaw conventional tumor lysate, whereas in others lysates had been boiled for 5 min in the first thawing procedure. Four graphs represent individual tumor growth in each experimental condition, as indicated, and the fraction of animals completely rejecting their tumors is provided within the graph. Survival curves corresponding to each experimental group are provided (right). Asterisk indicates statistical significance, p < 0.05, in Mantel–Cox U test.
3 wk after receiving the first cycle and died as a result. Although the patient did not present neutropenia and the doses of cyclophosphamide administered were relatively low, a potential relationship of the event with treatment-induced immunosuppression could not be fully excluded. No clinical autoimmunity or changes in a battery of autoantibodies were observed (data not shown).

**Effect of cyclophosphamide on the number of Tregs**

Patients showed a sustained decrease in the percentage of CD4+ CD25+ FOXP3+ Tregs in peripheral blood (Fig. 3A). Interestingly, the decline was more evident in patients with the highest pre-treatment levels and reached levels routinely observed in healthy individuals. Frequencies of Tregs approximately ≤5% of total PBMC were maintained toward the second cycle (Fig. 3A). The FACS strategy of three-color immunostaining to identify and enumerate Tregs is presented in Fig. 3B. The effect on Tregs does not seem solely related to cyclophosphamide-induced leukopenia, because the decrease of total circulating lymphocytes was slight (Fig. 3C). The decrease in circulating Tregs was also observed in terms of absolute numbers (Fig. 3D).

**Type I DC produce IL-12 and show bright expression of costimulatory molecules**

Subcultures of the DC that were used for vaccinations indicated that maturation markedly induced IL-12p70 secretion (Fig. 4A). The DC maturation protocol also clearly enhanced the surface expression of CD80, CD83, CD86, CD40, and the chemokine receptor CCR7 (Fig. 4B), which is crucial to guide migration toward draining lymph nodes. Results of mature DC used for treatment are shown in comparison with DC without maturation. All these features of mature DC are related to the induction of type 1 immunity. It is of note that DC from patients tended to show a less costimulatory phenotype than those from healthy volunteers. B7-H1 (PD-L1), a receptor that has been ascribed to inhibit T lymphocytes, was also readily present on the surface of DC used for treatment (Fig. 4B).

**Increased circulating IL-12 and NK activity in the patients**

All patients experienced an important increase in circulating IL-12p70 levels that was sustained during each of the two immunotherapy cycles (Fig. 5A). Of note, circulating IL-12p70 in pre-treatment serum samples or in healthy volunteers was virtually undetectable. IL-12p70 serum levels of first cycle ranged from 92 to 1154 pg/ml (mean, 410 pg/ml), and levels of second cycle ranged from 76 to 1237 pg/ml (mean, 445 pg/ml). At least one of the sources for circulating IL-12p70 are the exogenously administered mature DC. This is supported by the correlation found between IL-12p70 output in the cultured DC and the serum concentrations of IL-12p70 in each patient (Fig. 5A). However, we cannot exclude other endogenous sources triggered by the combined immunotherapy strategy.

### Table I. Patient characteristics (n = 24)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Tumor Type</th>
<th>Age/Sex</th>
<th>Metastases</th>
<th>Previous Therapy</th>
<th>Cycles</th>
<th>Adverse Events</th>
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<tr>
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1 and II refer to degree of toxicity according to Common Toxicity Criteria.

A, anemia; AP, articular pain; As, asthenia; B, bone; BR, best clinical response; Carc, carcinoid tumor; ChC, cholangiocarcinoma; CRC, colorectal carcinoma; ECT, electrochemotherapy; F, fever; FG, Fourier’s gangrene; GA, gluteal abscess; HCC, hepatocellular carcinoma; K, kidney; L, liver; LAK, lymphokine-activated killer cells; LN, lymph node; Lu, lung; M, melanoma; Me, mediastinum; NA, not available; NED, no evidence of disease; NED(*), without macroscopy disease at the beginning of the vaccines; P, pancreas; PD, progressive disease; Pe, peritoneum; PSI, pain at site of injection; QT, chemotherapy; RCC, renal cell carcinoma; RE, radioembolization; RPLN, retroperitoneal lymph node; RT, radiotherapy; S, spleen; s.c., s.c. tissue; SD, stable disease; st, suprarenal; st, soft tissue; Surg, surgery; T, thrombocytopenia; TACE, transarterial chemoembolization; TKI, tyrosine kinase inhibitors; TTP, time to progression; V, vomiting.
NK activity was enhanced in 11 of 17 patients following the first cycle and in 8 of 17 after the second cycle (data summarized in Fig. 5B). NK activity correlated ($R^2 = 0.73$) with the concentration of IL-12p70 on day 1 of the first cycle (Fig. 5C). This is taken as an indication that IL-12 is the most likely factor accounting for NK activation (Fig. 5C). However, the percentages of circulating NK cells increased only modestly in 6 of 17 of the patients (Fig. 5D). Therefore, the percentages of NK cells (CD3$^-$ CD56$^+$) in Fig. 5D do not explain the drastically enhanced NK activity that should be ascribed to enhanced killing capability on per cell basis (Fig. 5B).

No Abs directed to surface proteins of a panel of eight tumor cell lines encompassing the histological origins of the patients’ neoplasia were observed (data not shown), indicating lack of induction of humoral responses to shared tumor surface Ags. This is
unfortunate because enhanced NK activity in the presence of Abs to Ags on the surface of tumor cells might have resulted in Ab-dependent cytotoxicity.

Evaluation of the tumor-specific cellular immunity when tumor lysates are used as immunogens is challenging from a methodological point of view. Sufficient material for these experiments was available in 11 cases, and increases in IFN-\(\gamma\)-ELISPOT reactivity from cryopreserved isolated PBMC to DC loaded with autologous tumor lysate (without any Ag-driven preculture) were observed in 4 of those 11 cases (Fig. 5E). No correlation was observed between DC phenotypes, IL-12p70 production, IL-12p70 serum concentrations, NK activity or ELISPOT counts, and clinical benefit in terms of disease stabilization (Supplemental Fig. 1).

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Treatment effects on CEC and CTC

The well-known antiangiogenic effects of IL-12 and type 1 immunity (38, 39) supported the evaluation of the number of CEC as a surrogate marker for angiogenesis and vasculogenesis (40). CEC were evaluated in 18 patients, and a dramatic decrease in circulating CD45\(^-\)CD31\(^+\)VEGFR-2\(^+\) endothelial cells was noted in most cases (Fig. 6A). At least two factors might underlie this effect on endothelial cells, as follows: the IL-12–IFN-\(\gamma\) axis and the described antiangiogenic effects of cyclophosphamide (41).

The increase in NK activity that we observed could also result in lysis of CTC. In samples from 19 patients, quantitative RT-PCR tests with primers for mRNAs selectively expressed by tumor cells were performed, to compare the presence of CTC before and after treatment. In 6 of the 19 cases (32\%), marked decreases in CTC were observed, including two hepatocellular carcinoma patients (Fig. 6B). Results are summarized in Supplemental Table 2.

DC migration following intranodal and intradermal injections

With the first intranodal injection and the first s.c. DC dose, 18 patients received a tracing dose of 10\(^7\) DC labeled with \([^{111}\text{In}]\) oxinate (42). This allowed us to use scintigraphy to monitor the anatomic biodistribution of the tracing dose (Fig. 7A). SPECT permitted clear identification of lymph node anatomy (Fig. 7B). The biodistribution from intranodal and s.c. injections is
FIGURE 5. Immunotherapy treatment increases circulating IL-12p70, NK activity, and T cell reactivity. 

A. Concentrations of serum IL-12p70 on the indicated days of each immunotherapy cycle. Data are provided as mean ± SD values. Asterisks indicate statistical significance, $p < 0.001$ (Student $t$ test). On the right, a correlation between DC in vitro production of IL-12p70 and the serum concentration of IL-12p70 on day 1 of the first cycle is presented. Correlations were assessed by $R^2$ and Pearson’s correlation coefficient. 

B. Follow-up before and after the first immunotherapy cycle of NK cytotoxicity assessed at 5:1 E:T ratio on cryopreserved isolated peripheral blood PBMC in [$^{51}$Cr] release assays against K562 target cells (bar graph represents the mean ± SD). For NK cytotoxicity, PBMC from 17 patients were analyzed. Asterisks indicate statistical significance, $p < 0.01$ (Student $t$ test).

C. Correlation of IL-12p70 serum concentration on day 1 of the first cycle with NK activity on day 4. Correlations were assessed by $R^2$ and Pearson’s correlation coefficient, as indicated in the figure.

D. Percentages of CD3$^+$ CD56$^+$ NK cells in PBMC obtained following Ficoll gradient separations before and after the first and second immunotherapy cycle. Samples from 17 patients were analyzed. Data are provided as the mean ± SD.

E. ELISPOT activity of cryopreserved PBMC from the indicated color-coded patients obtained at the given time points before and after each immunotherapy cycle. PBMC were exposed to mature DC that had been loaded with autologous tumor lysate. The center graph represents the mean ± SD. For ELISPOT activity, monitoring in 11 patients were analyzed. Asterisks indicate statistical significance, $p < 0.01$ (Student $t$ test). The pictures of ELISPOT microwells on the right show analyses from a representative case.
summarized in Supplemental Table 3. In 11 of 18 patients (61%),
the intranodal injections reached deeper lymph node chains. A
small fraction of the radioactive tracing isotope from the s.c.
injections was observed to reach draining lymph nodes in 89% of
cases.

Deeper biodistribution from intranodal injections did not cor-
relate with stable disease cases. In 10 cases, IFN-γ–ELISPOT
results and sequential scintigraphy images were both available.
Interestingly, our data reveal that those cases in which DC reached
deeper lymphatic chains in the first intranodal injection had higher
numbers of responsive T lymphocytes producing IFN-γ (Fig. 7C).

Discussion
Careful observation of biological effects induced by immunolog-
ical-based strategies is required to improve current paradigms
and to develop the most promising combination regimens for
immunotherapy (43, 44). This DC-based clinical trial tested a
number of innovative features such as the maturation mixture,
the daily administration, cyclophosphamide pretreatment, and the
accompanying cytokines. The rationale to design our treatment
strategy was based on our findings in preclinical mouse models
(as those shown in Fig. 1) and on previously reported clinical
data (37).
Our results indicate that pretreatment with a single 600 mg/m² dose of cyclophosphamide decreased Tregs, mainly in patients with higher baseline numbers. There is controversy regarding the optimal dose and schedule to achieve this effect, and metronomic oral administration of cyclophosphamide could be more convenient (45). The selective Treg-decreasing effects of cyclophosphamide are related to the selective low content of ATP in this lymphocyte subset (46). The drops in circulating Tregs, which we observed in relative and absolute numbers, may not accurately reflect what is going on inside the malignant tissue. The decreasing effects on Tregs are due to cyclophosphamide because they were observed before immunizations were started. However, later on, Treg decreases may also be related to IL-12, as described (47). Even if our procedure normalized percentages of Tregs, a transient but more drastic reduction of such subpopulation is likely to be required for efficacy.

Autologous tumor lysates were chosen as a source of tumor Ags to load DC because they contain tumor Ags from individual mutations and also because of technical feasibility under GMP conditions. Lysates were preheated at 100°C to counteract immunosuppressive compounds and denature lysosomal proteases. However, some thermoresistant components with immunosuppressive effects may certainly persist (31). Heating the lysates clearly changed the patterns of protein bands in SDS-PAGE, suggesting an effect on proteolysis. A feasible alternative to tumor lysates is to transfect mRNA-encoding tumor Ags (4, 8), a procedure that offers the possibility of adding mRNAs or small interfering RNAs that upregulate DC immunostimulatory functions (48). No direct clinical comparison between RNA transfection and preincubation with tumor lysates is available.

Repeated daily immunizations were chosen to secure Ag delivery to lymph nodes over a period of several days. When we designed our trial, we thought of the evidence suggesting that exogenous DC need to transfer the Ag to lymph node-resident DC (49), which are hypothesized to be the main actual performers of Ag presentation (50, 51). In addition, the incoming DC, such as those that we injected into the patients, would stimulate lymph node-resident DC with secreted proinflammatory cytokines, including IL-12. GM-CSF was administered at a site near each DC injection to sustain DC viability and enhance cross-presentation (52). Patients received IFN-α to promote Ag presentation at the tumor microenvironment and to foster cytotoxic lymphocyte responses (7, 30, 53). Alternatively, other groups have used systemic injections of Hiltonol [poly(I:C)] to induce the endogenous release of a cascade of these proinflammatory mediators (7).

**FIGURE 7.** Biodistribution of type 1 DC from intranodal and intradermal injections. A, Scintigraphic images of 111In-labeled tracing doses of DC at different time points following intranodal injection (time 0) and intradermal injection in the opposite thigh given 24 h later. The images correspond to patient 17. Red arrows show the intranodal injection site, and green arrows show the intradermal injection site. B, SPECT anatomical distribution of the radioactive signal. A representative case is shown (patient 17). Supplemental Table 3 summarizes results in the 18 patients in whom the scintigraphic techniques were performed. C, Comparison of IFN-γ-ELISPOT reactivity between patients in whom following intranodal injection DC reached or did not reach deeper pelvic/iliac lymph node chains. Asterisk indicates statistical significance, p < 0.05 (Student t test).
No objective radiological clinical responses in terms of Response Evaluation Criteria in Solid Tumors were observed. However, five patients showed disease stabilization, which lasted for 13 mo in two cases. We could not observe clinical correlates with the immune and biological parameters analyzed. Unexpectedly, no patient developed delayed-type hypersensitivity-like reactions at the sites of repeated intradermal vaccinations. It is possible that the intensity of the elicited immune mechanisms is insufficient to alter the progression of advanced cancer patients. Our inability to observe correlations between clinical outcome and biological parameters might also be related to the small number of patients experiencing stable disease and the heterogeneity in terms of pretreatment tumor burden and tumor types.

Treatment was well tolerated. The most frequently observed toxicities were grades 1 and 2 fever, asthenia, and pain at the injection site. These reactions are probably related to the endogenous and exogenously administered inflammatory mediators. One patient died of a Fournier’s gangrene that developed from a perineal abscess, but relation to treatment is unlikely.

Amazingly high concentrations of IL-12 were observed in the plasma of all patients. The type 1 DC used in the study produced IL-12p70, and thus were very likely to be the main source of IL-12p70 in the patients’ organism. In this regard, the output of IL-12p70 by mature DC in culture and the serum concentrations of the cytokine in the corresponding patients were clearly correlated. In previous clinical studies, we tested DC that had been adenovirally engineered to produce IL-12p70 (37). It is of note that the production of IL-12p70 in DC matured with our mixture is superior to that achieved by adenovirus-mediated transfection of the IL-12 genes (37).

rIL-12 has been used as an efficacious anticancer agent, but systemic administration at high doses for solid malignancies has not progressed because of serious toxicity limiting the therapeutic window (54, 55). The induced levels of circulating IL-12p70 in this trial were so high and unexpected that the question was raised as to whether they could be deleterious. However, renal and liver functions were preserved following treatment in all patients. Evidence for immune inhibition in the presence of excessive and sustained IL-12 has been reported in mice (56), and, therefore, we must interpret these raises of serum IL-12p70 with caution. Nonetheless, high production of IL-12p70 by the DC-injected vaccines correlates with more favorable clinical outcomes at least in glioblastoma patients (7).

IL-12 is a powerful NK-activating factor (22, 24, 28, 57, 58) that in our combined treatment would act in concert with pegylated IFN-α to strongly raise NK activity. Indeed, IL-12 acting as a DC surface molecule is involved in this cross-talk between DC and NK cells along with other receptor-ligand pairs induced by poly(I:C) (23, 28). A clear correlation was found between circulating IL-12 and NK activity. In addition, type 1 DC control NK functions through direct cell-to-cell contact and produce type I IFN, which also stimulates NK activity (23, 35).

As a result of enhanced NK activity and the downstream IFN-α–CXCL10 axis, angiogenesis and vasculogenesis can be inhibited (38). Indeed, we observed a clear decline of CEC that most likely denotes these trains of phenomena (40). The decline in CEC (40) could be related not only to NK activity (25), but also to other activities exerted by type I IFNs (53) and cyclophosphamide (41, 59).

Regarding DC distribution upon injection, our data match those obtained by Verdijk et al. (60). It is of note that ∼1/3 of intranodal injections in inguinal lymph nodes do not distribute to inner lymph node chains probably reflecting unintended perinodal, as opposed to intranodal, delivery (60). It was interesting to observe that those cases in which intranodal injection reached deeper lymph node chains tended to be the patients who showed stronger reactivity to tumor lysate-loaded DC in ELISpot assays. From intradermal injections, only a small fraction of cells reaches the draining lymph nodes. Not withstanding the fact that the actual number of DC required in the lymph nodes for optimal immunization is still unknown, it seems reasonable that wider biodistribution to lymph nodes could maximize the opportunity for T cell stimulation.

As mentioned, we noticed no obvious hardening inflammatory reactions at the vaccination sites in any of the cases, even in the second cycle of treatment. This may reflect the multifactorial immunosuppressive mechanisms that operate in advanced cancer patients (11). Although in some cases PBL-obtained posttreatment showed some degree of reactivity to tumor lysate-loaded DC, the intensity of these adaptive cellular responses is probably too weak to permit meaningful effects on the established tumor masses of advanced cancer patients. It would have been very useful to analyze lymphocyte infiltrates in posttreatment tumor biopsies, but these procedures were not approved in our protocol.

Even though costimulatory molecules and CCR7 are brightly expressed by the injected DC, B7-H1 (PD-L1) expression on their surface might mediate dangerous immunosuppressive mechanisms (61, 62). Hence, combining agents that tamper with this immunosuppressive pathway could be important (62).

In summary, we have developed a DC vaccination strategy that incorporated several novel elements. The treatment was feasible, well tolerated, and induced cellular immunity, as well as several relevant biological effects, including reduction of Tregs, high circulating concentrations of IL-12p70, and decreases in CTC and CEC. The decrease in the number of CEC and CTC supports that this strategy might be especially relevant in the setting of minimal residual disease. Consequently, a randomized clinical trial in colorectal cancer testing this strategy in patients following complete surgical resection of liver metastasis is ongoing at our institution (NCT01348256).

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


