Consolidative Dendritic Cell-based Immunotherapy Elicits Cytotoxicity against Malignant Mesothelioma

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***Rationale*: We previously demonstrated that dendritic cell-based immunotherapy induced protective antitumor immunity with a prolonged survival rate in mice. However, the clinical relevance is still in question. To examine this, we designed a clinical trial using chemotherapy followed by antigen-pulsed dendritic cell vaccination in mesothelioma patients.**

***Objectives*: The aim of this study was to assess the safety and immunological response induced by the administration of tumor lysate-pulsed dendritic cells in patients with mesothelioma.**

***Methods*: Ten patients with malignant pleural mesothelioma received three vaccinations of clinical-grade autologous dendritic cells intradermally and intravenously at 2-week intervals after chemotherapy. Each vaccine was composed of 50×10^6 mature dendritic cells pulsed with autologous tumor lysate and keyhole limpet hemocyanin (KLH) as surrogate marker. Delayed-type hypersensitivity activity to tumor antigens and KLH was assessed, both *in vivo* and *in vitro*. Peripheral blood mononuclear cells during the treatment were analyzed for immunological responses.**

***Measurements and Main Results*: Administration of dendritic cells pulsed with autologous tumor lysate in patients with mesothelioma was safe with moderate fever as the only side effect. There were no grade 3 or 4 toxicities associated with the vaccines or any evidence of autoimmunity. Local accumulations of infiltrating T cells were found at the site of vaccination. The vaccinations induced distinct immunological responses to KLH, both *in vitro* and *in vivo*. Importantly, after three vaccinations, cytotoxic activity against autologous tumor cells was detected in a subgroup of patients.**

***Conclusions*: This study demonstrated that autologous tumor lysate-pulsed dendritic cell–based therapy is feasible, well-tolerated, and capable of inducing immunological response to tumor cells in mesothelioma patients.**

Clinical trial registered with www.clinicaltrials.gov (NCT00280982).

Keywords: antitumor; clinical trial; vaccinations; tumor lysate-pulsed

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

In an earlier study, we described the use of dendritic cell (DC)-based immunotherapy in a mouse model for mesothelioma. The potency of antigen-pulsed DCs was demonstrated by the increase in antitumor immunity leading to a prolonged survival.

What This Study Adds to the Field

We show for the first time the safety and feasibility of tumor lysate–pulsed DCs as therapeutic adjuvants in patients with mesothelioma and found distinct immune responses and antitumor responses in these patients.

Malignant pleural mesothelioma is a fatal disease with median survival time from first signs of illness to death of less than 12 months (1, 2). The incidence of mesothelioma is closely associated with exposure to airborne asbestos fibers (3). Although the application of asbestos is prohibited in most developed countries, incidences of mesothelioma are increasing due to the incubation period of 20 to 50 years from initial exposure to asbestos to the onset of disease. For The Netherlands and most other European countries, deaths related to this disease are expected to continue rising until the year 2020 (4). However, in numerous eastern and developing countries, asbestos is still used, and as consequence, incidences of mesothelioma worldwide will continue to rise (5). This anticipated increase in the incidence of mesothelioma has spurred considerable interest in the development of better treatments for mesothelioma.

Chemotherapy consisting of a combination of pemetrexed and cisplatin is regarded as the standard of care for selected patients with mesothelioma (6). The survival benefit is limited ($\sim$3 mo) and new or additional treatment options like anti-angiogenesis drugs (bevacizumab), photodynamic therapy, gene therapy, and a variety of immunotherapy approaches are currently being tested.

Immunotherapy is a promising approach in the treatment of cancer. It tries to harness the potency and specificity of the immune system to attack cancer cells, aiming for a nontoxic treatment with minor side-effects and a long-lasting immunological memory. One approach of immunotherapy uses dendritic cells (DC) to present tumor-associated antigens (TAA) and thereby generate tumor-specific immunity (7, 8). DC are extremely potent antigen-presenting cells specialized for inducing activation and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTL) and helper $CD4^+$ lymphocytes. We previously investigated the effect of DC-based immunotherapy on the outgrowth of mesothelioma in a murine model (9). Whereas the TAA are not known for mesothelioma, we used tumor cell lysate as antigen source to pulse DC. We established that DC-based immunotherapy induced strong tumor-specific CTL responses leading to prolonged survival in mice (9). The efficacy of immunotherapy was dependent on the tumor load. The most beneficial effects were established at early stages of tumor development. This is in agreement with our current

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knowledge of the effect of immunotherapy in other tumor types (10).

On the basis of these preclinical animal studies, we have now completed a clinical trial in which autologous tumor lysate–pulsed DC were administered intradermally and intravenously in mesothelioma patients after cytoreductive therapy with chemotherapy. Patients received 50×10^6 mature DC pulsed with tumor lysate and keyhole limpet hemocyanin (KLH) every 2 weeks for a total of three injections. The safety, feasibility, and immunological effectiveness of this approach are reported.

METHODS

See the online supplement for more details regarding the preparation of the tumor lysate, flow cytometric analysis of clinical-grade DC, delayed-type hypersensitivity skin testing, immune response assessment against KLH, flow cytometric assays for interferon-γ and granzyme B expression, and the cytotoxicity assays.

Patient Population

The study was approved by the institutional Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands. Procedures followed were in accordance with the ethical standards of this committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. Ten patients, recently diagnosed with malignant pleural mesothelioma of the epithelial subtype, were enrolled after informed consent. Patient characteristics are summarized in Table 1. Patients were eligible for the study when sufficient tumor cells could be obtained from pleural effusion or tumor biopsy material at the time of diagnosis. DC-immunotherapy was planned after completion of the cytoreductive therapy provided that during chemotherapy no major side effects occurred and there was no progressive disease.

A further description is detailed in the online supplement. From all participants, blood and serum samples were taken at regular intervals. Blood was tested for immunological responses and liver and renal functioning. In addition, serum samples were screened for the development of auto-immunity.

Preparation of Tumor Lysate

A detailed description of the preparation of the lysate can be found in the online supplement. Single cell suspensions of tumor tissue (cases 2, 4, 5, and 10) and/or pleural effusions (cases 1, 3, 4, 6, 7, 8 and 9) were counted and resuspended at a concentration of 50×10^6/ml in phosphate buffered saline (PBS). Cells were lysed by six cycles of freezing in liquid nitrogen and thawing at room temperature followed by 100 Gy of irradiation. Large particles were removed by centrifugation ($200 \times g$ for 5 min), and supernatants were passed through a 0.45 μm filter. The resulting tumor lysates were stored in aliquots at −80°C until use.

Dendritic Cell Culture

We used a previously described method to generate clinical-grade mature dendritic cells in conformity with Good Manufacturing Practice guidelines (11). Detailed information is outlined in the online supplement. In brief, concentrated leukocyte fractions were generated through peripheral blood leukapheresis. Peripheral blood mononuclear cells were then enriched using counterflow centrifugal elutriation (Elutra; Gambro BCT, Zaventem, Belgium) as described by Berger and colleagues (12). Fractions were further purified by Ficoll-Paque Premium density gradient centrifugation when percentage of polymorphonuclear cells exceeded 10% (all cases except for patient case 7 and 9; Table 2). Monocytes were resuspended in XVIVO-15 supplemented with 2% pooled human AB serum (DC-culture medium). The next day, half of the medium was replaced by the same volume of DC culture medium supplemented with IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF; CellGenix, Freiburg, Germany). After 6 days of culture, cells were harvested using a pipet and then seeded in fresh DC culture medium supplemented with tumor cell lysate, IL-4, GM-CSF, and KLH (Calbiochem, La Jolla, CA). The next day the maturation cocktail was added (prostaglandin E2 [Pharmacia & Upjohn, Puurs, Belgium], tumor necrosis factor-α [TNF-α], IL-1β, and IL-6 [all CellGenix]). Cells were harvested at Day 9 and 50×10^6 cells were used for immediate vaccination; remaining cells were cryopreserved in dimethyl sulfoxide for later vaccinations and for the delayed type hypersensitivity (DTH) skin testing.

Dendritic Cell Vaccination

Patients received three immunizations with mature DC, loaded with autologous tumor lysate and KLH, in 2-week intervals (Figure 1). Each immunization, consisting of 50×10^6 cells, was administered intradermally and intravenously. Dosage was divided 1/3 intradermally in the forearm and 2/3 though the intravenous route by mixing the components in 100 ml of normal saline drip. Constant monitoring was done for 4-hours after the administration of vaccine therapy. The vaccine was routinely analyzed for DC purity and tested for infectious agents before administration to patients.

RESULTS

Ten patients who met the inclusion/exclusion criteria were enrolled after informed consent; all Caucasian men ranged in age from 56 to 78 years (median 65 yr) and newly diagnosed with malignant pleural mesothelioma of the epithelial subtype. Six patients had more than 150×10^6 cells in their pleural fluid, which was sufficient for pulsing DC; from the others tumor material was obtained by thoracoscopy (>0.2 g wet weight). Nine patients received four cycles of chemotherapy consisting of 500 mg/m^2 of body surface area (BSA) premetrexed (Alimta, Eli Lilly and Co., Fegersheim, France) and 75 mg/m^2 BSA cisplatin every 4 weeks according to the treatment schedule described. One patient received 80 mg/m^2 BSA carboplatin instead of cisplatin because of a hearing implant (case 4). Dietary supplementation with low-dose folic acid and vitamin B_{12} before and during the treatment was given to limit toxicity. During the four cycles of chemotherapy, none of the patients

TABLE 1. PATIENT CHARACTERISTICS AT THE TIME OF DIAGNOSIS

No.	ECOG Performance Status	Chest Pain	Weight Loss (>5%)	PLT (x10^9/L)	LDH (U/L)	HGB (mM)	WBC (x10^9/L)	Age (yr)
1	1	No	Yes	326	298	9.1	9.8	68
2	0	No	Yes	438 H	231	6.5 L	11.0 H	64
3	1	Yes	No	124 L	470 H	7.2	4.0 L	78
4	0	No	No	175	154	10.2	5.6	60
5	0	No	No	481 H	168	7.8 L	7.0	71
6	1	No	Yes	315	368	9.8	7.7	57
7	1	No	Yes	223	354	8.6	7.0	77
8	0	No	Yes	206	268	9.1	5.6	56
9	0	Yes	No	484 H	162	8.8	9.8	66
10	0	Yes	Yes	231	471 H	7.9	4.4	59

Definition of abbreviations: ECOG = Eastern Cooperative Oncology Group; H = above upper limits of reference range of individuals; HGB = hemoglobin level; L = below lower limits of the reference range of individuals; LDH = lactate dehydrogenase enzyme level; PLT = platelet count; WBC = white blood cell count.

H and L values deviate from the normal distribution.

TABLE 2. DENDRITIC CELL CULTURING PROCESS

No.	Material Used for DC Loading	Monoctes at Day 0 (x10^6 Cells)	Additional Purification after Elutra (% Polymorphonuclear Cells Before/After Ficoll)	DC at D10 (x10^6 Cells)*
1	PF	2250	Yes (22/8)	295
2	TT	3000	Yes (75/7)	175
3	PF	2000	Yes (50/5)	160
4	TT/PF	1850	Yes (16/9)	375
5	TT	2400	Yes (47/4)	240
6	PF	2100	Yes (13/6)	230
7	PF	2150	No (7/–)	365
8	PF	3100	Yes (75/7)	276
9	PF	3500	No (9/–)	190
10	TT	1600	Yes (18/8)	255

Definition of abbreviations: DC = dendritic cell; PF = pleural fluid; TT = tumor tissue.
* The initial cell number of the immature DC used for loading was 420 × 10^6 cells.

experienced any serious adverse events. Four patients had a stable disease and six patients showed a partial response after their last chemotherapy and returned at that stage to their usual activities (World Health Organization/Eastern Cooperative Oncology Group performance status of 0 or 1). Patient characteristics are depicted in Table 1.

All ten patients reacted to subcutaneous injected tetanus toxoid in a DTH skin testing 8 to 12 weeks after the last chemotherapy cure indicating that the chemotherapy drugs did not (or no longer) exert their influence on the patients' immune system. Patients underwent a single leukapheresis with a mean total volume processed of 9.0 ± 0.5 L. None of the patients experienced any toxicity during the procedure except for mild citrate reactions that were compensated by calcium administration. The leukapheresis procedure was well tolerated. The products (135 ± 20 ml) were enriched for monocytes using Elutra counterflow elutriation system. Fractions V of most cases (except cases 7 and 9, Table 2) were further purified by Ficoll-Paque Premium density gradient centrifugation because percentages of polymorphonuclear cells exceeded 10% that might otherwise alter DC quality. Blood monocytes were cultured with GM-CSF and IL-4 for 10 days to allow DC differentiation. From all patients, sufficient clinical-grade dendritic cells could be generated for three vaccinations (160 × 10^6 – 375 × 10^6 cells). Although cell numbers showed patient-to-patient variability, the phenotype of the cells harvested at Day 10 of culture were large cells with indistinct and veiled morphology, and more than 95% were negative for CD14 and positive for $CD40^+$, $CD80^{2+}$, $CD83^{2+}$, $CD86^{+++}$, expressing additional high levels of human leucocyte antigen DR (HLA-DR) molecules, which were compatible with the characteristic features of mature DC. Routine sterility testing did not detect microbial contamination in any of the vaccines.

Toxicity

Overall, the vaccination regimen with loaded dendritic cells was well-tolerated in all patients. No dose adjustments or dose discontinuations were necessary. A local skin rash occurred at the site of the intradermal injection after the first vaccination in 8 of the 10 patients. Subsequent vaccinations (second and third) gave a quicker and increased induration and erythema in all patients suggesting that some form of immunity was induced. In one patient (case 1), 3-millimeter skin punch biopsies were taken at Day 2 and Day 14 after the last injection of KLH and lysate-pulsed DC. In addition, adjacent normal, noninjected skin was also biopsied from the same individual. Microscopical examination of the immunohistochemical stainings showed a prominent thickening of the epidermis after 14 days (Figure 2). Compared with normal skin, a mild lymphocytic infiltrate and a more intense interstitial mononuclear cell infiltrate in the mid and deep dermis was demonstrated in the vaccinated site. The infiltrates consisted mainly of HLA-DR, DQ, and DP alleles, macrophages (CD68), and T lymphocytes (CD3, CD4, and CD8) (Figure 2). With regard to the dendritic cells, these were present at increased levels at Day 2 in both epidermis and dermis and probably originated from the vaccine, as demonstrated in other studies (13).

Eight patients developed mild to severe flu-like symptoms after the vaccination, particularly fever, muscle aches, chills, and tiredness. Two patients showed these symptoms after the first vaccination; the others after the second and/or third injection. In seven patients these symptoms normalized after one day. Most patients took paracetamol (acetaminophen) for 1 d as analgesic and antipyretic agent. One patient (case 6), in whom the reaction started after the first injection, the symptoms were more severe and paracetamol treatment was continued for 3 days. This patient did get chills after second and third vaccination. In none of the patients was grade 3 or 4 toxicities observed. No clinical signs and laboratory data of any autoimmune diseases (antinuclear auto-antibodies, extractable nuclear antigens, rheumatoid factor immunoglobulin M, and anticyclic citrullinated peptide [anti-CCP]) were observed in all patients until the final follow-up. There were no substantial changes in the results of routine blood tests.

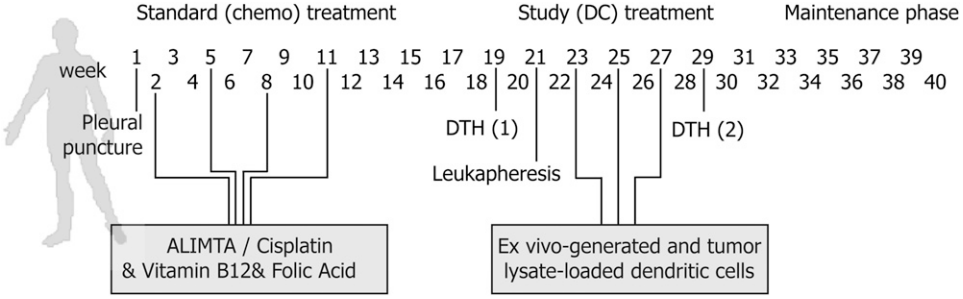

Figure 1. Synopsis of the study consisting of a combined treatment with chemotherapy followed by active immunotherapy using autologous tumor lysate–loaded dendritic cells (DCs). DTH = delayed type hypersensitivity.

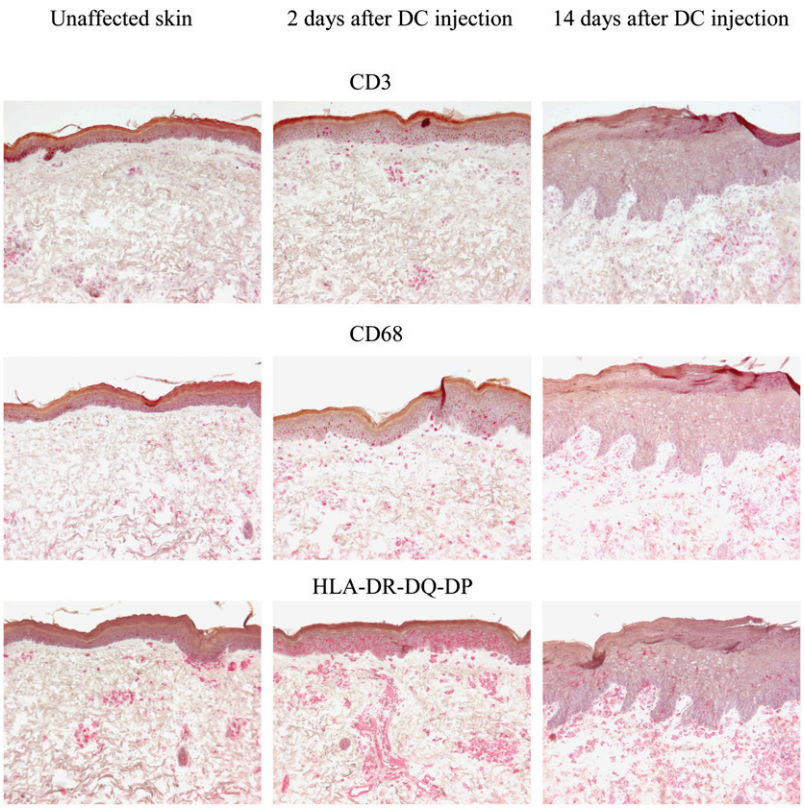

Figure 2. Immunohistochemical stainings for CD3, CD68 and HLA-DR, DQ, and DP alleles of skin biopsies taken from unaffected skin 2 days and 14 days after the second vaccination from the injection site (patient 1; Table 3). DC = dendritic cell.

Efficacy

The clinical responses were evaluated before and after immunotherapy by computed tomography (CT) scans and chest X-rays and analyzed according to the modified Response Evaluation Criteria in Solid Tumors (modified RECIST). Three patients showed partial responses after DC-immunotherapy, one showed stable disease, and six had no response after vaccinations (Table 3 and 4). The CT scans of patient nine, before and after the vaccinations, is shown in Figure 3. Two weeks after the DC vaccinations, a second DTH skin test was performed. Cell lysates of autologous tumors, KLH, DC loaded with tumor lysate and KLH, DC loaded with tumor lysate, and an appropriate positive (tetanus toxoid) and negative (saline) control, were injected intradermally and read 48 hours later. DTH skin testing revealed a response to dendritic cells loaded with tumor lysate and KLH, and to KLH alone in all patients (Table 3). Five patients responded to tumor lysate–loaded dendritic cells without KLH (Table 4). No DTH reaction was observed with tumor lysate in any of the patients. Reactions varied from induration and erythema to only slight erythema.

Serum samples from all patients showed a significant increase of pre-vaccine versus post-vaccine antibodies reactive to KLH, both of the IgG and IgM isotype (Table 4). No or very low amounts of antibodies against KLH were detected in undiluted serum of all patients before vaccination, illustrating the suitability of this antigen to determine the immunocompetence of the vaccine. Responses against KLH gradually increased with the number of vaccinations (Figure 4) suggesting that several vaccinations were necessary to induce a more potent humoral response. Antibodies against KLH in serum, diluted up to 100,000 times, could easily be detectable by ELISA in all patients after three vaccinations (Figure 4). The response remained at the same level for several months after the last DC injection and gradually decreased after 6 to 12 months (data not shown).

Chromium release assays were performed in 6 of 10 patients from whom pleural fluid was obtained (cases 1, 3, 6, 7, 8, and 9, Table 4). Only these samples were suitable because viable single-cell suspensions are needed for labeling with radioactive chromium for the cytotoxicity assay, and therefore, patients from whom tumor tissue was obtained (cases 2, 4, 5, and 10) were excluded for this cytotoxicity assay. Almost no lysis of autologous tumor cells was observed before DC-treatment in these 6 patients. In 4 patients (cases 6, 7, 8, and 9) clear inductions of cytoxicity against autologous tumor cells were measurable. The cytotoxicity levels of patient number 9 increased after every vaccination; for the three other patients (case 6, 7, and 8) three vaccinations were necessary to induce cytotoxicity (Figure 5). Another assay that was used to assess the T cell capacity for cell lysis was the flow cytometric detection of $CD3^+CD8^+$ T cells expressing granzyme B. Nine patients (1 remained at the same level) showed a significantly increased percentage of granzyme B^+ $CD8^+$ T cells after vaccination ($P = 0.023$; paired t test was used for comparing within-subject changes (Figure 6), and the granzyme B expression per CD8 cell increased in most patients (Table 4).

DISCUSSION

To our knowledge, this is the first study assessing autologous monocyte-derived dendritic cells loaded with autologous tumor cell lysate in patients with mesothelioma. The primary objectives of this study were to determine the toxicity and feasibility of clinical grade DC and to investigate if mesothelioma might be susceptible to immunotherapy treatment. Ten patients fulfilled the inclusion and exclusion criteria and were enrolled in the study. Feasibility was defined as producing three doses of 50×10^6 autologous tumor-lysate–loaded DC, each for intravenous (two-thirds) and intradermal (one-third) administration. In all apheresed subjects, sufficient cells were obtained

from a single leukapheresis product, processed *in vitro*, injected (first vaccination), and cryopreserved for later administration (second and third vaccination). These results indicate that our method for producing large amounts of clinical-grade DC is feasible in patients with malignant mesothelioma.

The possibility to harness the potency and specificity of the immune system underlies the growing interest in cancer immunotherapy. One such approach uses the patient's own DC to present tumor-associated antigens and thereby generate tumor-specific immunity (7, 8). We generated DC in large amounts *ex vivo*, in the absence of the suppressing tumor milieu, and subsequently loaded them with a preparation of autologous tumor antigens. To prevent antigens from being presented by immature DCs, which might tolerate tumor antigens and potentially enhance tumor growth (14, 15), cells were matured using a standard cytokine cocktail. Mature DCs are injected both intraveneously (distribution to the liver, spleen and bone marrow) and intradermally where they then migrate to the regional lymphatics. In this way, they can maximally stimulate cytotoxic T cells, B cells, T cells, NK cells and NKT cells that are essential for killing tumors at different immunological organs.

We used a generally known and widely accepted method for the preparation and maturation of clinical grade dendritic cells (11, 12). Autologous tumor lysate was used as the source of tumor antigen to load onto DC because in mesothelioma, specific tumor-associated antigens (TAAs) are undefined to date. Although mesothelin, calretinin, SV-40, Wilms tumor 1 (WT-1), and telomerase have been described as TAA in mesothelioma, these proteins are not expressed on the membranes of all mesothelioma tumors. Besides this, the efficacy of vaccination against a single or a few TAAs is limited by peptide restriction to a given HLA type and the induction of CTL without Th1 response. Therefore, we investigated in an earlier animal study whole tumor lysate as an antigen source with satisfying results (9). We demonstrated that DC immunotherapy was effective in controlling this aggressive cancer in which TAA remain undefined. Other investigators have also shown that human DC pulsed with apoptotic mesothelioma cells were able to induce a CTL response *in vitro* directed against the tumor, illustrating that malignant pleural mesothelioma cells contain unknown TAA that can lead to an antitumoral immune response (16). Autologous tumor lysates might be advantageous in providing the full antigenic repertoire of the tumor and, particularly, unique tumor antigens, which will theoretically decrease the ability of tumors to evade the immune response by down-regulation of a single antigen (17). On the other hand, sufficient amounts of tumor lysate must be available for DC pulsing and this often limits the applicability of tumor-lysate pulsed DC immunotherapy. Forty-three biopsies and pleural fluids were collected from patients with mesothelioma of which 10 patients had sufficient tumor material (pleural fluid $>150 \times 106$ tumor cells or >0.2 g tumor tissue) to pulse the dendritic cells, although most patients had a high tumor burden on the computerized tomography (CT) scan. Tumor material was obtained from patients for dyspnea relief or diagnostic purposes. For ethical reasons, no tumor material was collected from patients without any medical need for removal. In this study, none of the patients developed any clinical or laboratory signs of autoimmune disease, indicating that the fear expressed by some researchers, that such highly stimulatory DC pulsed with undefined tumor lysates might induce autoimmune disease, is unfounded.

From animal studies and reports of other tumor types it is generally accepted that DC-based immunotherapy is most effective in cases of relatively small tumor loads. To decrease

TABLE 3. OVERVIEW OF THE RESPONSES FOUND IN THIS STUDY

Clinical Response	No.	%
Response to prior chemotherapy		
Complete response	0	0
Partial response	6	60
Stable disease	4	40
No response	0	0
Response to immunotherapy		
Complete response	0	0
Partial response	3	30
Stable disease	1	10
No response	6	60
Immunological response		
^{51}Cr release	4 of 6*	66
KLH response in serum	10	100
Skin-test reaction	10	100
KLH	0	0
Tumor lysate	10	100
DC + tumor lysate + KLH DC + tumor lysate – KLH	5	50
Survival in months after diagnosis		
Median (range)	19 (11–34)	90
DOD	9	10
AWD	1	

Definition of abbreviations: DC = dendritic cell; DOD = died of disease; AWD = alive with disease; KLH = keyhole limpet hemocyanin.

* ^{51}Cr release assay was performed in six patients, from remaining patients no viable tumor cells were obtained.

TABLE 4. CLINICAL AND IMMUNE RESPONSE EVALUATION OF PATIENTS WITH MESOTHELIOMA PARTICIPATING IN THE TRIAL

No.	Response on Chemotherapy	IgG anti- KLH*	DTH skin test (loaded DC)	Cyto-toxicity	Difference in Granzyme B Expression(%/MFI)	Radiological Findings Before and After DC Immunotherapy	Interval between Diagnosis and Death (Months)	Follow-up
1	PR	+++	+	—	+2.8/+16.34	PR	23	DOD
2	PR	++	+	ND	0.0/+7.03	PD	34	AWD
3	SD	+	—	—	+4.6/+34.91	PD	15	DOD
4	PR	++	—	ND	+10.0/+6.78	PD	15	DOD
5	SD	++	—	ND	+8.8/+38.82	SD	15	DOD
6	PR	+++	—	+	+21.5/−3.58	PD	13	DOD
7	SD	+	+	+	+1.2/−2.04	PD	11	DOD
8	SD	++	—	+	+4.1/+ 5.86	PR	19	DOD
9	PR	++	+	+	+3.1/+6.78	PR	30	DOD
10	PR	+	+	ND	+35.4/−19.29	PD	15	DOD

Definition of abbreviations: AWD = alive with disease; DC = dendritic cell; DTH = delayed type hypersensitivity; DOD = died of disease; KLH = keyhole limpet hemocyanin; MFI = mean fluorescence intensity; ND = not done; SD = stable disease; PR = partial response; PD = progressive disease.

* IgG antibodies detected against KLH in serum at 1,000 times (+), 10.000 times (++) or at 100.000 times (+++) diluted serum.

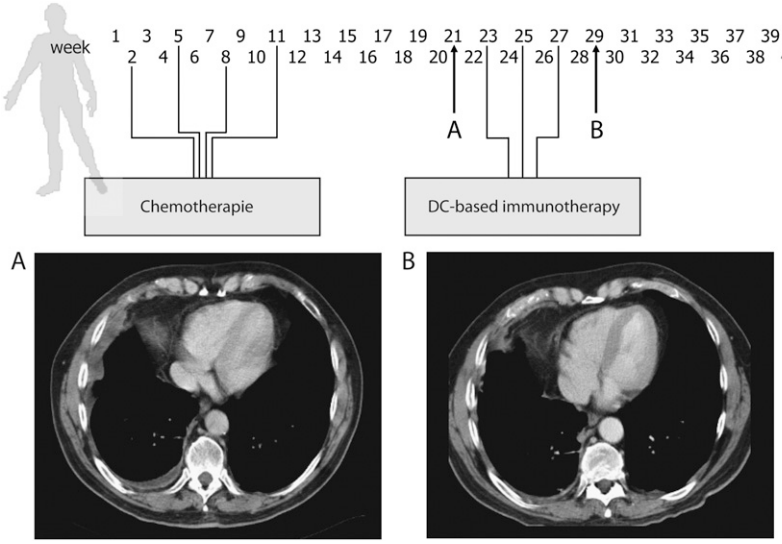

Figure 3. (*A*) CT scans 2 weeks before the first dendritic cell (DC) vaccination and (*B*) two weeks after the third vaccination (indicated by *arrows* on the time scale) revealed a decline in pleural fluid and a regression of the tumor (case 9). Of the 10 participating patients, 6 had progressive disease; 1 had a stable disease, and 3 showed a partial response in this 8-week time period.

a patient's tumor load a combination of pemetrexed and cisplatin (one patient with a hearing implant [cochlear] received pemetrexed and carboplatin as alternative) was given because it is considered the clinical standard of care in mesothelioma. This combination chemotherapy is the only treatment with activity proven in phase III trials and is approved by the U.S. Food and Drug Administration. Patients could only participate in the trial when no progressive disease was present after chemotherapy (exclusion criteria). The downside of these drugs is the high toxicity level (e.g., inducing lymphopaenia), and therefore, a recovery period of 8 to 12 weeks was formulated to achieve immunological recovery from the chemotherapeutic treatment. Preclinical studies have shown certain chemotherapy drugs can synergize with immunotherapy directly (18). Currently several groups are investigating different drugs that can induce optimal immunogenic mesothelioma cell death and can thus be combined with immunotherapy directly leading to an enhanced anticancer immune response (19, 20). A recent report in the form of an abstract has shown that, despite the decrease in lymphocyte numbers, cisplatin/pemetrexed modulates the immune system and provides a rationale for combining cisplatin/pemetrexed with immunotherapy at the same time (21).

Imaging techniques (CT scans) were performed before and after immunotherapy in all participating patients. However, tumor extension in patients with mesothelioma is rather difficult to access due to the widespread tumor over the large surface of the pleura (22). Six patients had progressive disease; one patient had a stable disease, and three showed partial responses after DC-immunotherapy. CT scans 2 weeks before the first DC vaccination and 2 weeks after the third vaccination revealed a regression of the tumor in three patients during this 8-week period. However, these tumor regressions seen on CT scans cannot be attributed solely to the DC treatment but might also be caused by a delayed reaction of the chemotherapy. Imaging scans of a proper control group, patients with high tumor burden in their pleural effusion and not receiving DC vaccinations at corresponding intervals are necessary to draw conclusions on tumor regressions. The evaluation of the overall survival is also difficult to interpret in the absence of a randomized trial and, therefore, these results should be interpreted with caution. As shown in Table 4, median survival was 19 months (range 11–34 mo). Nine patients died of disease; one patient is alive with disease (currently at 48 mo after diagnosis). These survival data are difficult to compare with historical controls as patients are selected. Although the number of patients in this study is limited, these data indicate that a selected group of patients may benefit from DC immunotherapy without major adverse effects.

The immunogenic protein keyhole limpet hemocyanin (KLH) was used in this study as helper antigen and as tracer molecule, allowing *in vivo* and *in vitro* monitoring of immunological responses induced by the vaccinations (23, 24). In the serum samples of all patients, antibodies against KLH induced by DC-therapy were detected, both of the IgG and IgM isotype. Also, all patients revealed strong responses to DCs loaded with tumor lysate and KLH and to KLH alone 48 hours after DTH skin testing. In five patients (50%), DC pulsed with tumor lysate (without KLH) caused induration, which supports the idea that

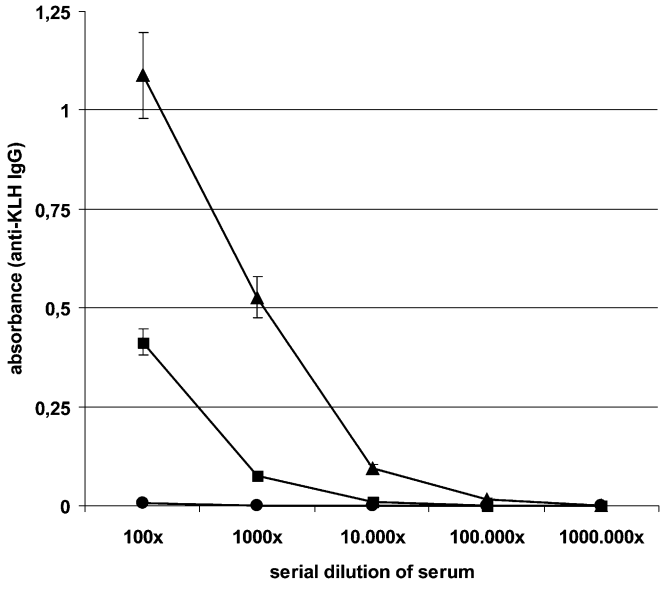

Figure 4. Anti–keyhole limpet hemocyanin (KLH) immunoglobulin responses increase after dendritic cell (DC) injections. Kinetics of antibody responses against KLH was measured in serially diluted serum of representative patient (case 4) at the first (*circles*), second (*squares*), and two weeks after the third (*triangles*) injection of tumor lysate–pulsed DC.

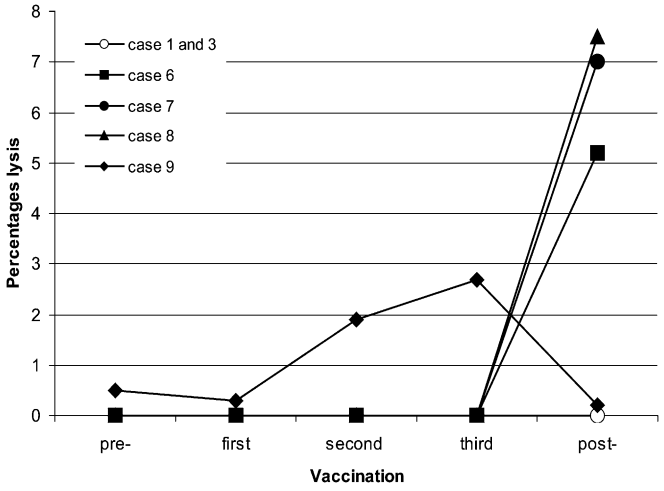

Figure 5. Cytotoxicity for ^{51}chromium-labeled (^{51}Cr) autologous tumor cells by peripheral blood mononuclear cells during dendritic cell (DC) vaccination. Of the six patients that were evaluable, four patients showed increase in ^{51}Cr release caused by lysing of autologous tumor cells. The percentage of lysis was calculated using the formula: corrected % lysis = 100 × (experimental release − spontaneous release[target cells incubated in medium alone])/(maximum release[2% Triton X-100 as lysing agent] − spontaneous release).

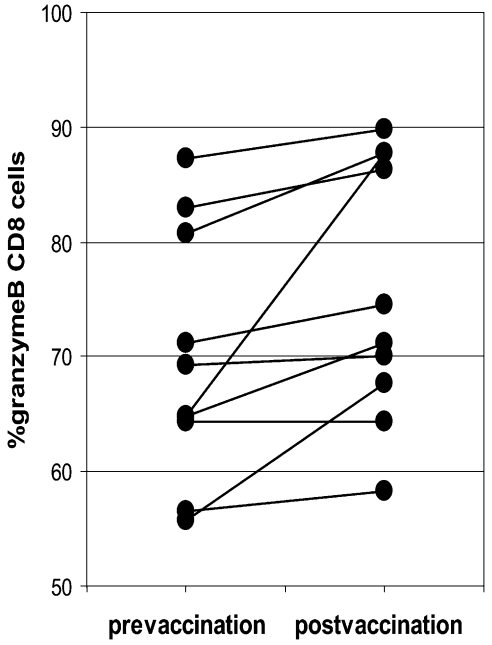

Figure 6. Granzyme B positive CD3$^+$ CD8$^+$ T lymphocytes were measured in blood samples of patients before and after dendritic cell (DC) vaccination using flow cytometry. Significant increases in granzyme B$^+$ T lymphocytes were detected after three vaccinations in all patients (P = 0.023; paired t test).

specific immune responses are induced. This was confirmed by the increase of HLA-DR, DQ and DP$^+$ cells, macrophages and T-lymphocytes in skin biopsies taken from the temporary local skin reactions after DC vaccination. Blood samples revealed an increase in antitumor T cell activity in four of the six patients after DC vaccination. Most studies in other cancer types could not establish these findings directly in peripheral blood and needed more sophisticated techniques to demonstrate T cell activity (25–27). Therefore, our results clearly support the fact that this form of immunotherapy induced specific immune responses in mesothelioma.

Own research and that of others have shown that mesothelioma cells, like other tumor cells, produce many immunosuppressive factors that can affect DC, effector T cells, macrophages, NK and NKT cells. We were the first to demonstrate that human mesothelioma tissue contains significant amounts of Foxp3$^+$ regulatory T cells (28). Depletion of these cells led to increased survival in a transplantable mouse model for mesothelioma. Other studies have revealed that myeloid-derived suppressor cells and M2 macrophages within the tumor promote growth and metastasis by directly acting on tumor cells, endothelial cells, and on antigen-specific T cells (29–32). We cannot exclude that the up-regulation of antitumor activity in our study will be negatively influenced by an immunosuppressive environment. Manipulation of these suppressive factors might therefore be used in combination with DC immunotherapy to improve the outcome of mesothelioma. Recently we started a study with dendritic cell immunotherapy in combination with a low-dose of cyclophosphamide (Endoxan) in mesothelioma patients to inhibit T-regulatory function to increase the success rate of tumor eradication.

The administration of DCs loaded with autologous tumor cell lysate to patients was safe. Some patients developed self-limited fever a few hours after the vaccinations. Local skin reactions were seen at the site of the intradermal injection suggesting that some form of immunity was induced. There was no clinical or radiological evidence of any autoimmunity. Distinct immunological responses to the surrogate marker KLH were induced by the vaccinations, both *in vitro* as *in vivo*. Importantly, antitumor cytotoxicity activity against autologous tumor cells was measured in the blood of patients. An increase in systemic CTL activity was seen in a subset of treated patients (4 out of 6). Multiple vaccinations were necessary as the increase in CTL activity was seen only after 3 vaccinations for most of the patients in this assay. Another immune monitoring assay that was used, the expression of granzyme B in CD8$^+$ T cells, increased significantly in all patients by the vaccination protocol. No correlations between vaccine responders (CTL activity, increase of granzyme B expression, KLH antibodies) and clinical outcomes could be detected.

Although this trial is relatively small with 10 patients, it includes a homogeneous group of patients with regard to histology, prior treatments, performance status, and study design and execution. It is the first human study on DC-based immunotherapy in patients with mesothelioma. In conclusion, DC loaded with autologous tumor cell lysate administrated to patients was safe and feasible and no adverse effects were observed. Antitumor immune responses were detected in a few patients with mesothelioma after DC-immunotherapy. Whether this has a beneficial effect in improving survival will be the subject in successive studies. Influencing the immunosuppressive cells (Tregs, M2 macrophages, and MDSC), cells abundantly present in the tumor environment, and their subsequent effect on DC-mediated anti-tumor responses, seems of critical importance for future clinical trials. Also other sources of antigens to pulse DC must be investigated to make DC immunotherapy more accessible for larger numbers of patients to perform comparative studies.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Hoogsteden HC, Langerak AW, van der Kwast TH, Versnel MA, van Gelder T. Malignant pleural mesothelioma. *Crit Rev Oncol Hematol* 1997;25:97–126.
2. Robinson BW, Musk AW, Lake RA. Malignant mesothelioma. *Lancet* 2005;366:397–408.
3. Stumphius J, Meyer PB. Asbestos bodies and mesothelioma. *Ann Occup Hyg* 1968;11:283–293.
4. Burdorf A, Dahhan M, Swuste P. Occupational characteristics of cases with asbestos-related diseases in the netherlands. *Ann Occup Hyg* 2003;47:485–492.
5. Bianchi C, Bianchi T. Malignant mesothelioma: global incidence and relationship with asbestos. *Ind Health* 2007;45:379–387.
6. Vogelzang NJ. Standard therapy for the treatment of malignant pleural mesothelioma. *Lung Cancer* 2005;50S1:S23–S24.
7. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* 2005;5:296–306.
8. Steinman RM, Dhodapkar M. Active immunization against cancer with dendritic cells: the near future. *Int J Cancer* 2001;94:459–473.
9. Hegmans JP, Hemmes A, Aerts JG, Hoogsteden HC, Lambrecht BN. Immunotherapy of murine malignant mesothelioma using tumor lysate-pulsed dendritic cells. *Am J Respir Crit Care Med* 2005;171: 1168–1177.
10. Cranmer LD, Trevor KT, Hersh EM. Clinical applications of dendritic cell vaccination in the treatment of cancer. *Cancer Immunol Immunother* 2004;53:275–306.
11. de Vries IJ, Adema GJ, Punt CJ, Figdor CG. Phenotypical and functional characterization of clinical-grade dendritic cells. *Methods Mol Med* 2005;109:113–126.
12. Berger TG, Strasser E, Smith R, Carste C, Schuler-Thurner B, Kaempgen E, Schuler G. Efficient elutriation of monocytes within a closed system (elutra) for clinical-scale generation of dendritic cells. *J Immunol Methods* 2005;298:61–72.
13. Verdijk P, Aarntzen EH, Lesterhuis WJ, Boullart AC, Kok E, van Rossum MM, Strijk S, Eijckeler F, Bonenkamp JJ, Jacobs JF, *et al.* Limited amounts of dendritic cells migrate into the T-cell area of lymph nodes but have high immune activating potential in melanoma patients. *Clin Cancer Res* 2009;15:2531–2540.
14. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* 2001;193:233–238.
15. Jonuleit H, Giesecke-Tuettenberg A, Tuting T, Thurner-Schuler B, Stuge TB, Paragnik L, Kandemir A, Lee PP, Schuler G, Knop J, *et al.* A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int J Cancer* 2001;93:243–251.
16. Gregoire M, Ligeza-Poisson C, Juge-Morineau N, Spisek R. Anti-cancer therapy using dendritic cells and apoptotic tumour cells: pre-clinical data in human mesothelioma and acute myeloid leukaemia. *Vaccine* 2003;21:791–794.
17. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998;4:328–332.
18. Gabrilovich DI. Combination of chemotherapy and immunotherapy for cancer: a paradigm revisited. *Lancet Oncol* 2007;8:2–3.
19. McCoy MJ, Nowak AK, Lake RA. Chemoimmunotherapy: an emerging strategy for the treatment of malignant mesothelioma. *Tissue Antigens* 2009;74:1–10.
20. Ampollini L, Soltermann A, Felley-Bosco E, Lardinois D, Arni S, Speck RF, Weder W, Opitz I. Immuno-chemotherapy reduces recurrence of malignant pleural mesothelioma: an experimental setting. *Eur J Cardiothorac Surg* 2009;35:457–462.
21. Nowak A. Chemoimmunotherapy in mesothelioma. 9th International Conference of the International Mesothelioma Interest Group, Amsterdam, The Netherlands, September 25–27, 2008 [abstract 189].
22. van Klaveren RJ, Aerts JG, de Bruin H, Giaccone G, Manegold C, van Meerbeeck JP. Inadequacy of the recist criteria for response evaluation in patients with malignant pleural mesothelioma. *Lung Cancer* 2004;43:63–69.
23. Holtl L, Rieser C, Papesh C, Ramoner R, Herold M, Klocker H, Radmayr C, Stenzl A, Bartsch G, Thurnher M. Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. *J Urol* 1999;161: 777–782.
24. Millard AL, Ittelet D, Schooneman F, Bernard J. Dendritic cell KLH loading requirements for efficient $CD4^+$T-cell priming and help to peptide-specific cytotoxic T-cell response, in view of potential use in cancer vaccines. *Vaccine* 2003;21:869–876.
25. Aarntzen EH, Figdor CG, Adema GJ, Punt CJ, de Vries IJ. Dendritic cell vaccination and immune monitoring. *Cancer Immunol Immunother* 2008;57:1559–1568.
26. De Vries IJ, Bernsen MR, van Geloof WL, Scharenborg NM, Lesterhuis WJ, Rombout PD, Van Muijen GN, Figdor CG, Punt CJ, Ruiter DJ, *et al.* In situ detection of antigen-specific T cells in cryo-sections using MHC class I tetramers after dendritic cell vaccination of melanoma patients. *Cancer Immunol Immunother* 2007;56:1667–1676.
27. de Vries IJ, Bernsen MR, Lesterhuis WJ, Scharenborg NM, Strijk SP, Gerritsen MJ, Ruiter DJ, Figdor CG, Punt CJ, Adema GJ. Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. *J Clin Oncol* 2005;23:5779–5787.
28. Hegmans JP, Hemmes A, Hammad H, Boon L, Hoogsteden HC, Lambrecht BN. Mesothelioma environment comprises cytokines and T-regulatory cells that suppress immune responses. *Eur Respir J* 2006;27:1086–1095.
29. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 2001;166:678–689.
30. Gabrilovich D, Pisarev V. Tumor escape from immune response: mechanisms and targets of activity. *Curr Drug Targets* 2003;4:525–536.
31. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 2007; 25:267–296.
32. Nagaraj S, Gabrilovich DI. Tumor escape mechanism governed by myeloid-derived suppressor cells. *Cancer Res* 2008;68:2561–2563.