

Consolidative dendritic cell-based immunotherapy elicits cytotoxicity against malignant mesothelioma

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At a glance commentary:

Earlier we described in the American Journal of Respiratory and Critical Care Medicine the use of dendritic cell-based immunotherapy in a mouse model for mesothelioma. The potency of antigen-pulsed dendritic cells was demonstrated by the increase in antitumor immunity leading to a prolonged survival. This paved the way to the here described clinical study. Because pre-clinical data in mice had shown that best results were obtained with small tumor loads, ten patients who responded on chemotherapy were selected. We show for the first time the safety and feasibility of tumor lysate-pulsed dendritic cells as therapeutic adjuvants in mesothelioma patients and found distinct immune responses and antitumor responses in these patients.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Footnotes:

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ABSTRACT

Rationale: Earlier we have demonstrated that dendritic cell-based immunotherapy induced protective antitumor immunity with prolonged survival in mice. However, the clinical relevance is still questioned. 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 mesothelioma patients.

Methods: Ten patients with malignant pleural mesothelioma received three vaccinations of clinical-grade autologous dendritic cells intradermally and intravenously at two-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.

Main Results: Administration of dendritic cells pulsed with autologous tumor lysate in mesothelioma patients 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.

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Key words: antitumor, clinical trial, vaccinations, tumor lysate-pulsed

INTRODUCTION

Malignant pleural mesothelioma (MM) is a fatal disease with median survival time from first signs of illness to death of < 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 further rise (5). This anticipated increase in incidence of mesothelioma has spurred considerable interest to develop better treatments for mesothelioma.

Chemotherapy consisting of a combination of pemetrexed and cisplatin is regarded as standard of care for selected patients with mesothelioma (6). The survival benefit is rather limited (~3 months) and new or additional treatment options like anti-angiogenesis drugs (bevacizumab), photodynamic therapy, gene therapy, and a variety of immunotherapy approaches are currently 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 non-toxic 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. Previously we investigated the effect of DC-based immunotherapy on the outgrowth of mesothelioma in a murine model (9). As for mesothelioma the TAA are not known, 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 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 KLH every two 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-gamma 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 (MEC-2005-269). 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. The study is registered with <http://www.clinicaltrials.gov> with identifier NCT00280982. 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, 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 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 (5 min, 200xg), 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 GMP (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 counter-flow centrifugal elutriation (Elutra) as described by Berger et al. (12). Fractions were further purified by Ficoll-Paque PREMIUM density gradient centrifugation when percentage of polymorphonuclear cells (PMN) 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 [DC-CM]). The next day, half of the medium was replaced by the same volume of DC-CM supplemented with interleukin (IL)-4 and granulocyte macrophage-colony stimulating factor (GM-CSF; CellGenix). After 6 days of culture, cells were harvested by pipetting and seeded in fresh DC-CM supplemented with tumor cell lysate, IL-4, GM-CSF, and KLH (Calbiochem, La Jolla, CA, USA). The next day the maturation cocktail was added (prostaglandin E2 [Pharmacia&Upjohn, Puurs, Belgium], tumor necrosis factor-alpha [TNF- α], IL-1 β , and IL-6 [all CellGenix]). Cells were harvested at day 9 and 50×10^6 cells used for immediate vaccination; remaining cells were cryopreserved in DMSO for later vaccinations and for the DTH skin testing.

Dendritic cell vaccination

Patients received three immunizations with mature DC loaded with autologous tumor lysate and KLH with a 2-week interval (Figure 1). Each immunization, consisting of 50×10^6 cells, was intradermally (i.d.) and intravenously (i.v.) administered. Dosage was divided 1/3 i.d. in the forearm and 2/3 though i.v. route by mixing the components in 100 ml of normal saline drip. Constant monitoring was done till 4-h 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 in age ranging from 56 to 78 years (median 65 years) and newly diagnosed with malignant pleural mesothelioma of the epithelial subtype. Six patients had $> 150 \times 10^6$ cells in their pleural fluid, which was sufficient for pulsing DC, from the others tumor material was obtained by thoracoscopy (> 0.2 gram 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 Company, 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 B12 prior to and during the treatment was given to limit toxicity. During the four cycles of chemotherapy, none of the patients experienced any serious adverse events. Four patients had a stable disease and 6 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 (WHO/ECOG) performance status 0 or 1). Patient's characteristics are depicted in table 1.

All ten patients reacted to subcutaneous injected tetanus toxoid in a delayed type hypersensitivity (DTH) skin testing 8 - 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 ($160 \times 10^6 - 375 \times 10^6$ 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 $> 95\%$ were negative for CD14 and positive for CD40^+ , CD80^{++} , CD83^{++} , CD86^{+++} , expressing in addition high levels of

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

The vaccination regimen with loaded dendritic cells was overall 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), three 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, non-injected 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 to 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 consists mainly of HLA-DR,-DQ,-DP+ cells, macrophages (CD68), and T lymphocytes (CD3, CD4, and CD8) (Figure 2). With regards to the dendritic cells, these cells were present at increased levels at day 2 in both epidermis and dermis probably originating from the vaccine as has been demonstrated in other studies (13).

Eight patients developed mild to severe flu-like symptoms after 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 7 patients these symptoms normalized after one day. Most patients took paracetamol (acetaminophen) for 1 day 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 grade 3 or 4 toxicities were observed. No clinical signs and laboratory data of any autoimmune diseases (ANA, ENA, RF IgM and anti-cyclic 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.

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 stable disease and 6 patients had no response after vaccinations (Table 3 and 4). The CT scans of a partial response in patient 9 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 on tumor lysate-loaded dendritic cells without KLH (Table 4). No DTH reaction was observed on tumor lysate in any of the patients. Reactions differed from induration and erythema to only slight erythema.

Serum samples from all patients showed a significant increase of pre- 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 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 – 12 months (data not shown).

Chromium release assays were performed in 6 of the 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 six patients. In four patients (cases 6, 7, 8, and 9) clear inductions of cytotoxicity against autologous tumor cells were measurable. One patient (case 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 also the granzyme B expression per CD8 cell was 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 in- and exclusion criteria and were enrolled in the study. Feasibility was defined as producing 3 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 dendritic cells 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 load them with a preparation of autologous tumor antigens. To prevent that antigens are presented by immature DC's, which might tolerize for tumour antigens and potentially enhance tumour growth (14, 15), cells were matured using a standardized cytokine cocktail. Mature DC are injected both intravenously (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 and NKT cells essential for tumor killing 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 (TAA) are undefined to date. Although mesothelin, calretinin, SV-40, Wilms tumor 1 (WT-1), 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 TAA 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 antigen source with satisfying results (9). We demonstrated that DC immunotherapy was effective in controlling this aggressive cancer in which TAA remain undefined. Also other investigators have shown that human DC pulsed with apoptotic mesothelioma cells were able to induce a CTL response *in vitro* directed against the tumor, illustrating that MM cells contain unknown TAA that can lead to an antitumoral immune response (16, Ebstein, 2004 #219). 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 limited the applicability of tumor-lysate pulsed DC immunotherapy. Forty-three biopsies and pleural fluids were collected from mesothelioma patients; of which 10 patients had sufficient tumor material (pleural fluid > 150x10⁶ tumor cells or > 0.2 gram tumor tissue) to pulse the dendritic cells although most patients had high tumor burden on CT scan. We obtained approval from the Medical Ethics Review Committee to utilize left-over material after dyspnea relief or diagnostic purposes and were not allowed for extra biopsies or fluid collection without any medical need for the patient. In this study, none of the patients developed any clinical or laboratory signs of autoimmune disease, indicating that the fear expressed by of some researchers that such highly stimulatory DC pulsed with undefined tumor lysates might induce autoimmune disease is unfounded.

From animal studies and reports in other tumor types it is generally accepted that DC-based immunotherapy is most effective in case of relatively small tumor loads. To decrease patient's tumor load a combination of pemetrexed and cisplatin (one patient received pemetrexed and carboplatin of hearing problems) 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 US 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 which can induce optimal immunogenic mesothelioma cell death and can thus be combined with immunotherapy directly leading to an

enhanced anti-cancer 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) were performed before and after immunotherapy in all participating patients. However, tumor extension in mesothelioma patients is rather difficult to access due to the widely spread of tumor over the large surface of the pleura (22). Six patients had progressive disease; one patient had a stable disease and 3 showed partial responses after DC-immunotherapy. CT scans two weeks before the first DC vaccination and two weeks after the third vaccination revealed a regression of the tumor in 3 patients during this 8-week period. However, these tumor regressions seen on CT scans can not solely be attributed 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 months). Nine patients died of disease; one patient is alive with disease ($p > 34$ months). These survival data are difficult to compare to 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 dendritic cell 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 dendritic cells loaded with tumor lysate and KLH and to KLH alone 48 h after DTH skin testing. In five patients (50%), DC pulsed with tumor lysate (without KLH) caused induration which supports the idea that 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 temporarily local skin reactions after DC vaccination. Blood samples revealed an increase in anti-tumor T-cell activity in 4 of the 6 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 anti-tumor 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.

Administration of dendritic cells 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, anti-tumor 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 in study design and execution. It is the first human study on dendritic cell-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. Anti-tumor immune

responses were detected in a few mesothelioma patients after DC-immunotherapy. Whether this does have a beneficial effect in improving survival will be subject in successive studies. The elucidation of the DC-mediated immune response in cancer patients, influencing the immunosuppressive cells (Tregs, M2 macrophages and MDSC) in the tumor microenvironment, and the appropriate selection of patients therefore seem to be mandatory 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 in order to perform comparative studies.

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Figure legends

Figure 1: Synopsis of the study consisting of a combined treatment with chemotherapy followed by active immunotherapy using autologous tumor lysate-loaded dendritic cells.

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

Figure 3: CT scans two weeks before the first DC vaccination (A) and two weeks after the third vaccination (B) (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 patients had progressive disease; one patient had a stable disease and 3 patients showed a partial response in this 8 week time period.

Figure 4: Anti-KLH immunoglobulin responses increase after DC injections. Kinetics of antibody responses against KLH was measured in serially diluted serum of representative patient (case 4) at the first (●), second (■), and two weeks after the third (▲) injection of tumor lysate-pulsed DC.

Figure 5: Cytotoxicity for ⁵¹chromium-labeled autologous tumor cells by PBMC during DC vaccination. Of the six patients that were evaluable, four patients showed increase in ⁵¹Cr release caused by lysing of autologous tumor cells. Percentage of lysis was calculated using the formula: corrected % lysis = 100 x (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 DC vaccination using flow cytometry. Significant increases in granzyme B⁺ T lymphocytes were detected after 3 vaccinations in all patients (p=0.023; paired t test).

Table 1: Characteristics at the time of diagnosis per patient

No.	ECOG Performance status	Chest pain	Weight loss (> 5%)	PLT (x10 ⁹ /L)	LDH (U/L)	HGB (mM)	WBC (x10 ⁹ /L)	Age (years)
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

Some values deviate from the normal distribution: **H** indicates above upper limits, **L** below lower limits of the reference range of individuals.

(WBC : white blood cell count; PLT : platelet count; HGB : hemoglobin level; LDH : lactate dehydrogenase enzyme level)

Table 2: Dendritic cell culturing process

No.	Material used for DC loading*	Monocytes 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

* PF = pleural fluid; TT = tumor tissue;

** The initial cell number of the immature DC used for loading was 420x10⁶ cells

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		
KLH	10	100
Tumor lysate	0	0
DC + tumor lysate + KLH	10	100
DC + tumor lysate – KLH	5	50
Survival in months after diagnosis **		
Median (range)	19 (11-34)	
DOD	9	90
AWD	1	10

* ⁵¹Cr release assay was performed in 6 patients, from remaining patients no viable tumor cells were obtained.

** DOD died of disease; AWD alive with disease

Table 4: Clinical and immune response evaluation of the mesothelioma patients 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

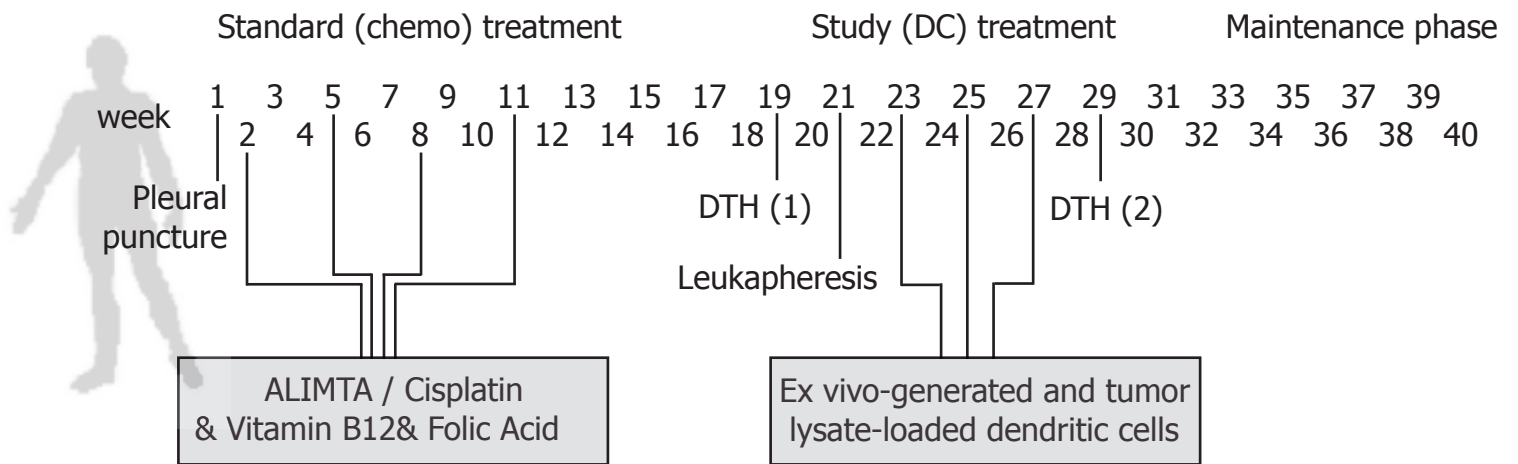
* SD = stable disease; PR = partial response; PD = progressive disease

** immunoglobulin G (IgG) antibodies detected against KLH in serum at 1000 times (+), 10.000 times (++) or at 100.000 times (+++) diluted serum.

*** ND= Not done

**** DOD = died of disease; AWD = Alive with disease

Figure 1



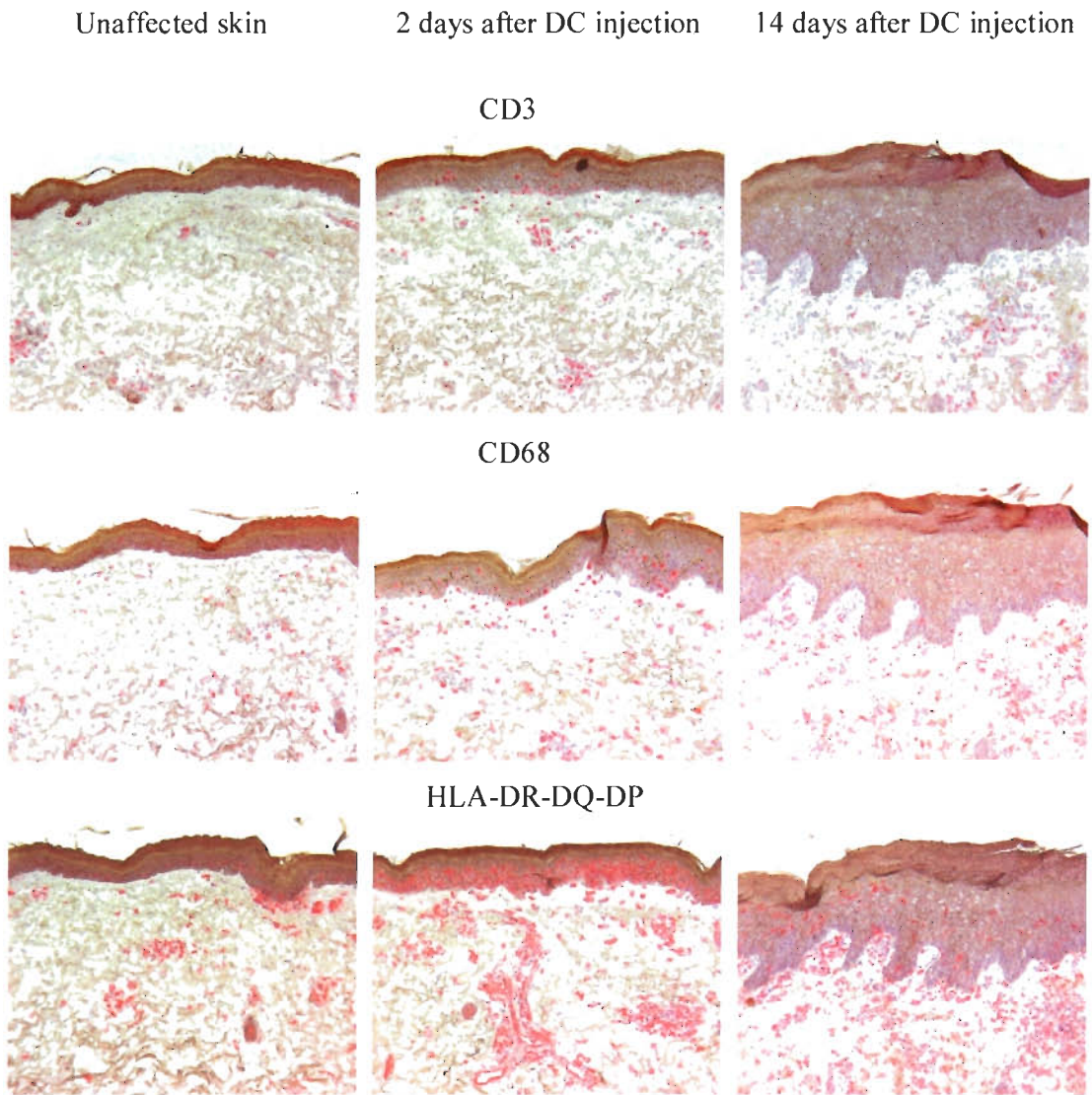
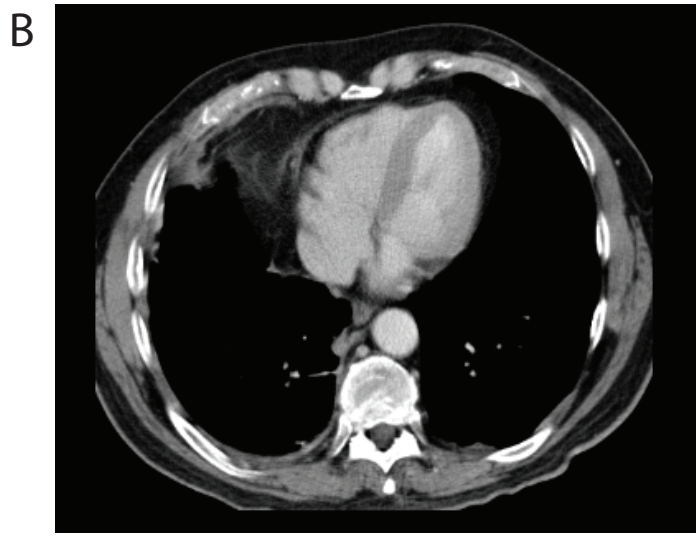
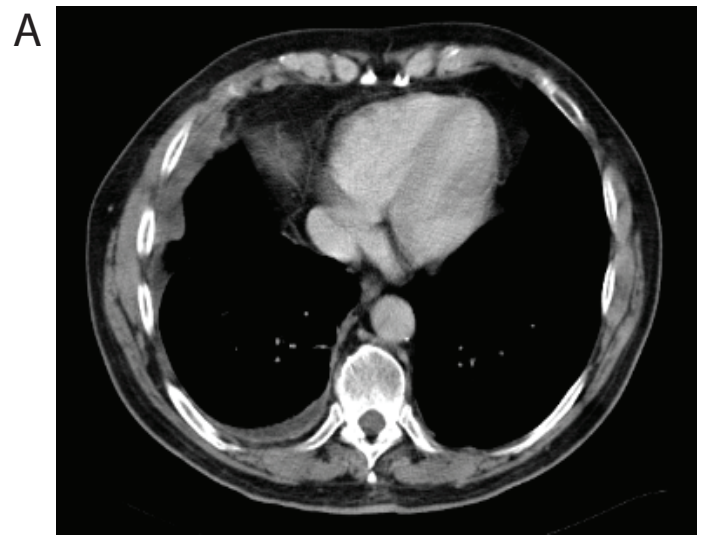
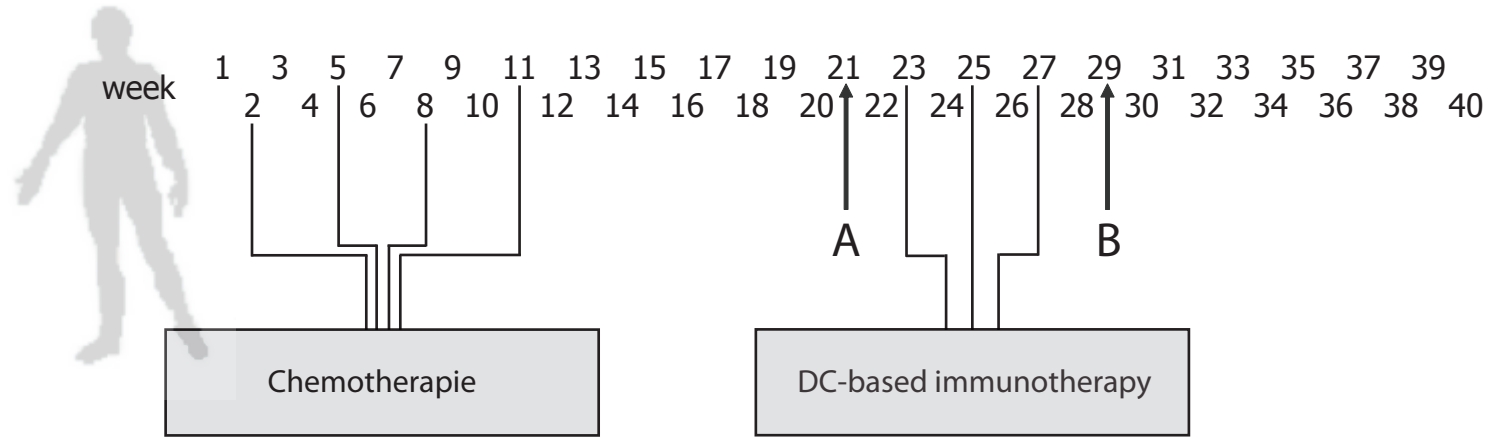


Figure 3



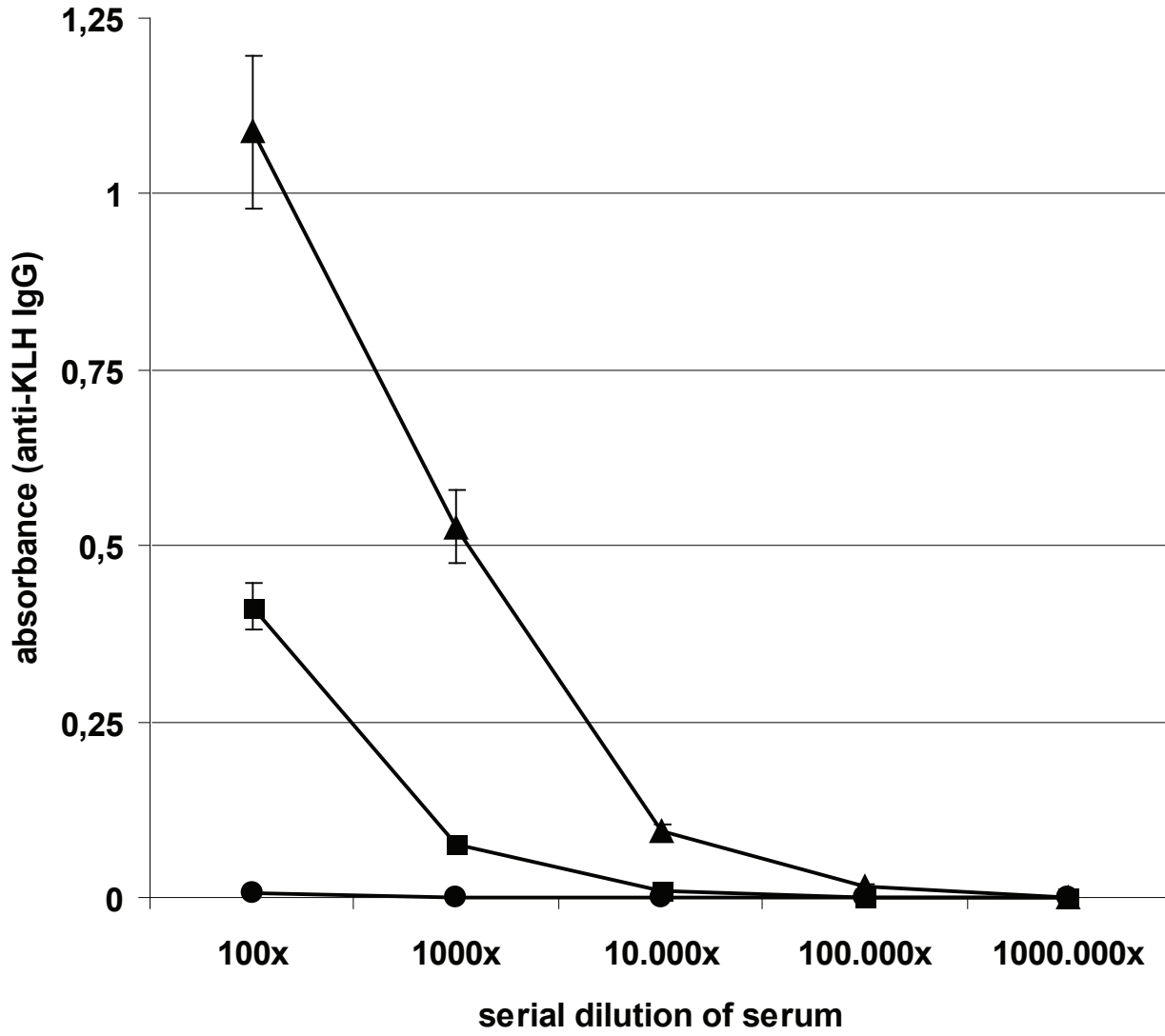
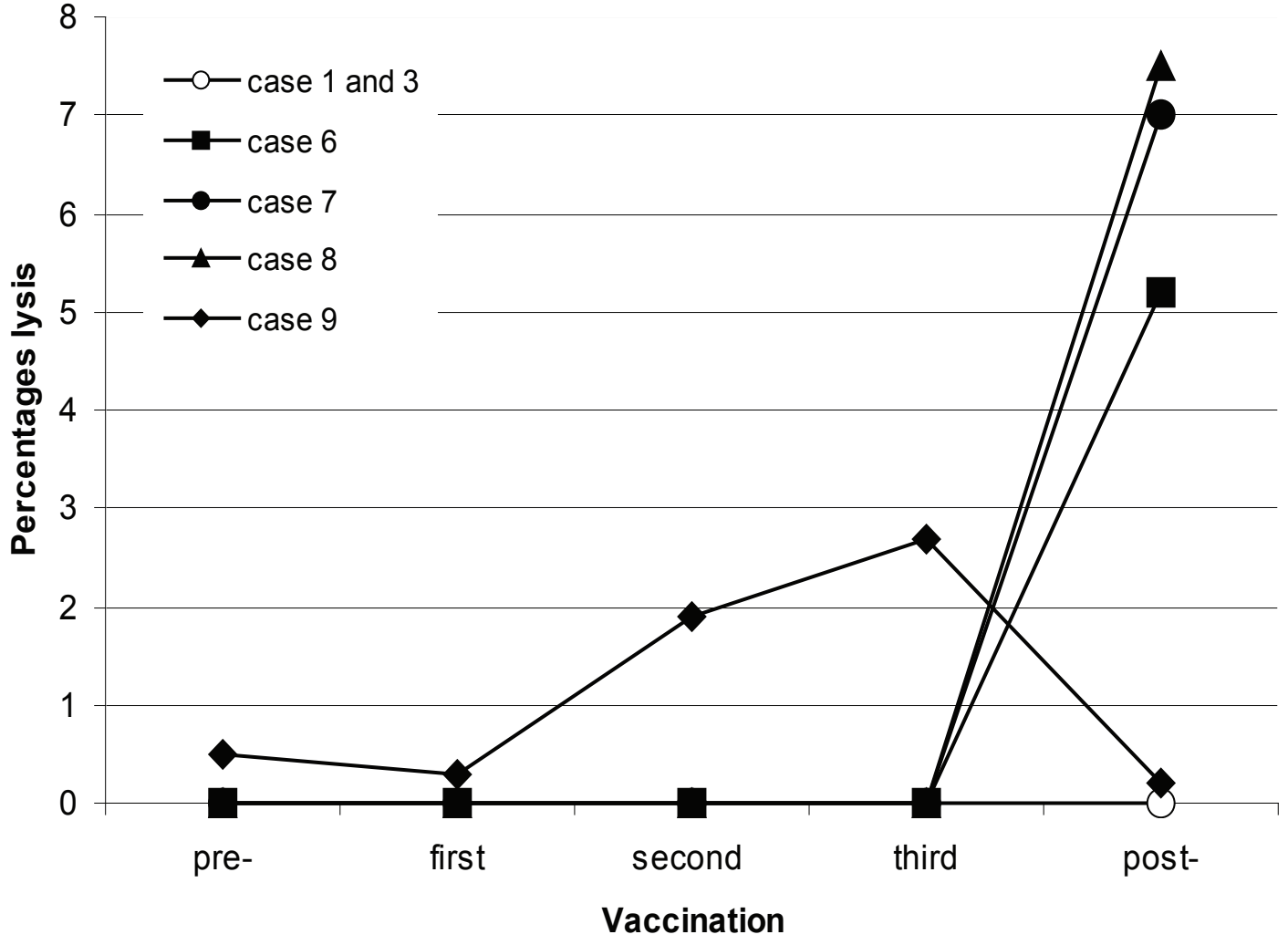
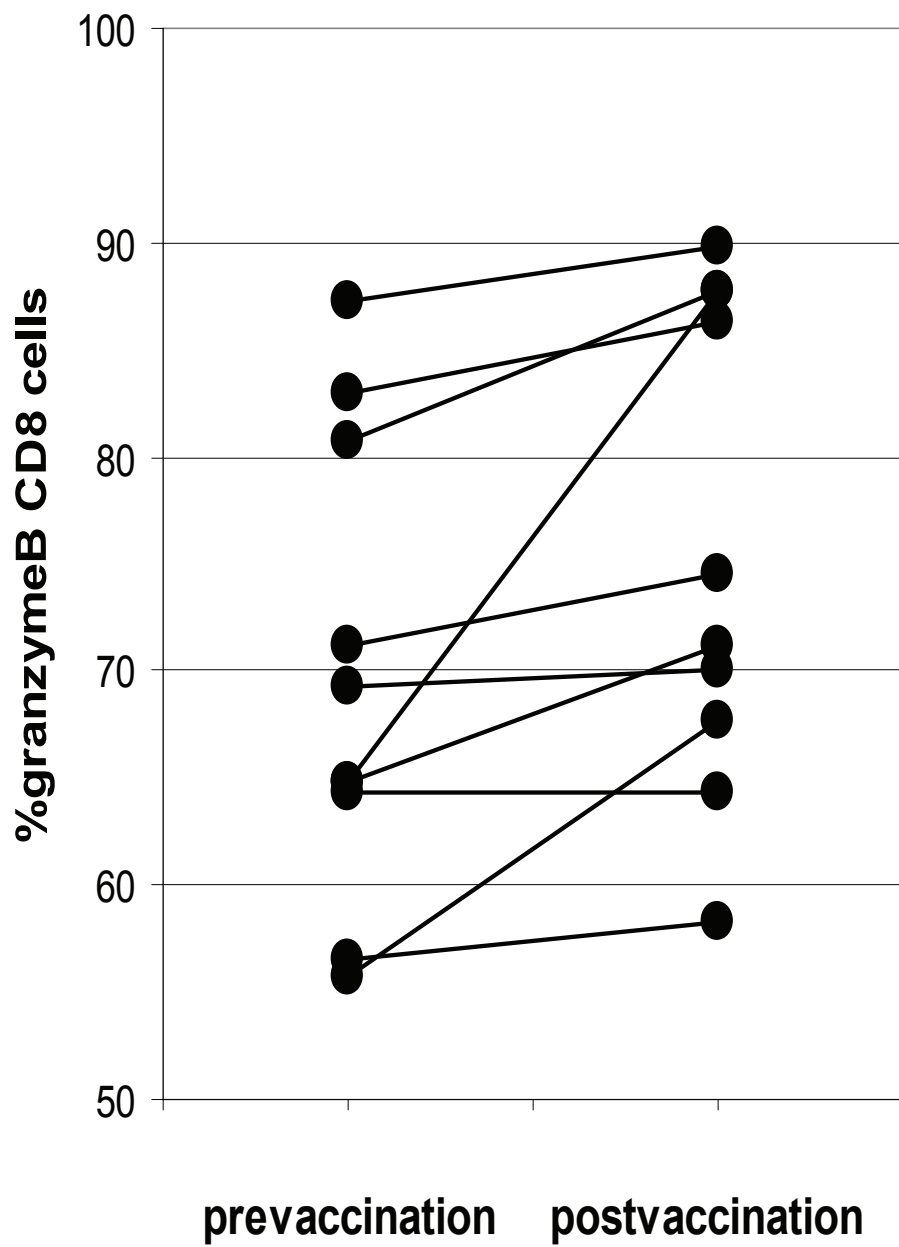


Figure 5





Online Data Supplement

Consolidative dendritic cell-based immunotherapy elicits cytotoxicity against malignant mesothelioma

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Methods

Patient population

At the time of diagnosis, patients with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, with acceptable vital organ functions were included. Serum of all patients was tested negatively for infectious agents (HIV, HTLV I&II, HBV, and HCV). No signs of autoimmune disease were detectable in these patients by testing for antinuclear antibodies (ANA), extractable nuclear antigens (ENA), and rheumatoid factors (RF). An intermission of 8 - 12 weeks after the last chemo cure was determined. A delayed type hypersensitivity (DTH) skin test was performed to investigate if the chemotherapeutic agents still exert their influence on the patients' immune system. A solution of 3.75 Lf of purified tetanus toxoid (NVI, Bilthoven, The Netherlands) was used as positive control and 25 µl 0.9% saline as negative control. Reactivity to tetanus toxoid was measured in all ten patients 48 hours after subcutaneous injection (induration > 5 mm) and was considered evidence for cellular immunocompetence.

Preparation of tumor lysate

Tumor tissue and/or pleural effusions were collected for diagnostic purposes and to relieve symptoms of dyspnea. Tumor tissue (cases 2, 4, 5, and 10) was removed by open pleural biopsy and placed in phosphate-buffered saline (PBS) in sterile containers and transported immediately to the cleanroom facility. Small representative tumor pieces were embedded in Tissue-Tek II optimum cutting temperature ([OCT] Miles, Naperville, IL, USA), snap-frozen

and stored at -80°C . Remaining tumor tissue was dispersed to create a single cell suspension. In case of pleural effusions (cases 1, 3, 4, 6, 7, 8 and 9), fluid was gently aspirated and collected in sterile flasks without anticoagulant or other additives. Effusions were transported immediately to the cleanroom facility and centrifuged at $400 \times g$ for 15 min at room temperature (RT). If necessary small or large amounts of red blood cells in the cell pellet were removed by hypotonic lysis using sterile water or Ficoll-Paque PREMIUM, respectively. Cells were counted and resuspended at a concentration of $50 \times 10^6 / \text{ml}$ in PBS. Cytospin preparations and/or tumor sections were prepared and examined for the presence of tumor cells using the following antibodies: cytokeratin 5/6, cytokeratin 19, thrombomodulin, N-cadherin, vimentin, HBME-1, calretinin, and Wilms' tumor 1 (WT-1) protein (all DAKO, Glostrup, Denmark). Total amounts of malignant cells exceeded 150×10^6 cells and the percentage was at least 30% of total cells (inclusion criteria) or in the case of biopsy material, total wet weight was at least 0.2 gram showing $>30\%$ positivity for tumor markers. Remaining 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 (5 min, $200 \times g$), and supernatants were passed through a $0.45 \mu\text{m}$ 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 GMP (Good Manufacturing Practice) guidelines (1). In brief, concentrated 120 to 150 ml leukocyte fractions were generated through a 4-h restricted peripheral blood leukapheresis, processing on average 9 L of blood (COBE Spectra Apheresis System, Gambro BCT, Zaventem, Belgium). Peripheral blood mononuclear cells were then enriched using counter-flow centrifugal elutriation (Elutra, Gambro BCT, Zaventem, Belgium) as described by Berger et al. (2). Fractions were analyzed by flow cytometry and further purified by Ficoll-Paque PREMIUM (GE Healthcare, Diegem, Belgium) density gradient centrifugation when percentage of polymorphonuclear cells (PMN) exceeded 10 % (all cases except for patient case 7 and 9; Table 2). Monocytes were resuspended at a concentration of 5×10^6 cells/ml in XVIVO-15 (Cambrex Bio Science, Verviers, Belgium) supplemented with 2% pooled human AB serum (DC-culture medium [DC-CM]). The next day, half of the medium was removed and replaced by the same volume of DC-CM supplemented with 1000 IU/ml interleukin (IL)-4 (CellGenix, Freiburg, Germany) and 1600 IU/ml granulocyte macrophage-colony

stimulating factor (GM-CSF; CellGenix). After 6 days of culture, semi-adherent and non-adherent cells were harvested by pipetting. Cells (1×10^6) were seeded per well of a 6-well plate in fresh DC-CM supplemented with tumor cell lysate (1 tumor cell equivalent to 3 DC), 500 IU/ml IL-4, 800 IU/ml GM-CSF, and 10 $\mu\text{g/ml}$ KLH (Calbiochem, La Jolla, CA, USA). The co-loading with the protein KLH, a foreign protein which stimulates T-helper responses, was used to monitor the immune competence. The next day the maturation cocktail was added (prostaglandin E2 [PGE2; 10 $\mu\text{g/ml}$ Pharmacia&Upjohn, Puurs, Belgium], tumor necrosis factor-alpha [TNF- α , 20 ng/ml], IL-1 β [5 ng/ml], and IL-6 [15 ng/ml; all CellGenix]). Cells were harvested at day 9 and 50×10^6 cells used for immediate vaccination; remaining cells were cryopreserved in DMSO for later vaccinations and for the DTH skin testing.

Flow cytometric analysis of clinical-grade DC

An aliquot of the vaccine preparation was retained to examine the expression of extracellular markers. The following monoclonal antibodies were purchased from BD Biosciences / BD Pharmingen (Erembodegem, Belgium): FITC-conjugated CD86 and CD195, PE-conjugated CD83 and CD-95, CD80 - PE-Cy5, CD209 - PerCP-Cy5.5, CD11c - APC, and APC-Cy7 conjugated HLA-DR. The specificity of the antibodies was checked using equivalent concentrations of fluorochrome- and isotype-matched negative control immunoglobulins. Cells were washed with FACS buffer (PBS supplemented with 0.25 % BSA, 0.5 mM EDTA, and 0.05% sodium azide) and counted. At least 0.4×10^6 cells in 100 μl were stained with appropriate dilutions of antibodies. Cells were incubated on ice for 30 min in the dark, washed twice with FACS buffer and analysed by LSR flow cytometry (BD Biosciences). Release criteria for each batch of DC were previously described (8).

Dendritic cell vaccination

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

Delayed-type hypersensitivity skin test

Delayed type hypersensitivity (DTH) skin testing to assess the immunocompetence to tetanus toxoid (positive control), and a physiological salt solution (negative control) was performed on the ventral surface of the forearm two weeks after the third vaccination. Antigen-specific memory cells triggering a local inflammatory response were determined by intradermal application of autologous tumor lysate (10 µg), KLH (5 µg), tumor lysate loaded DC with or without KLH (both 5×10^6 cells). DTH responses were evaluated after 48 h. Three millimeter skin punch biopsies were taken from the intradermal vaccination site at 48 h and day 14 and at a control site, embedded in OCT compound, snap-frozen and stored at -80°C . Tissue sections (6 µm) were cut on a HM-560 cryostat (Microm, Heidelberg, Germany) and immunostaining was carried out using α -HLA-DR,-DQ,-DP, α -CD3, α -CD4, α -CD8 and α -CD68 (all DAKO, Glostrup, Denmark). Binding of antibodies was detected by using the immunoalkaline phosphatase (AP) anti-alkaline phosphatase (APAAP) method (DAKO).

Immune response assessment against KLH

Serum samples were collected into SST serum separation tubes (BD biosciences) before, during and in the lifelong clinical follow-up of the patients. After allowing the serum 30 min to clot, tubes were centrifuged 10 min at 1000 x g. Serum was collected, aliquoted and stored at -80°C until use. Humoral responses to KLH were measured in the serum of patients by ELISA. Microtiter plates (96 wells) were coated overnight at 4°C with 25 µg/ml KLH in PBS per well. After blocking the plates with 1% powdered milk in PBS, different concentrations of patient serum (range, 1 in 100 to 1 in 500,000) were added for 1 h at room temperature. After extensive washing, specific anti-human IgG or anti-human IgM conjugated with horseradish peroxidase were allowed to bind for 1 h at room temperature. Peroxidase activity was revealed with the use of 3,3',5,5'-tetramethyl-benzidine (TMB) as substrate and absorbance was measured in a microtiter plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

Flow cytometric assays for interferon-gamma and granzyme B expression

PBMC from different time points were thawed, seeded at 1×10^5 cells/well into round bottom 96-well plates and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of monensin (GolgiStop, BD Pharmingen) for 4 hours at 37°C . The cells were washed twice and then stained for surface markers with appropriate dilutions of the following monoclonal antibodies: CD3-PE-Cy7, CD4-PE-Cy5, CD8 AF700, and for the intracellular

expressed granzyme-B-PE (Caltag) and IFN- γ -APC (BD Biosciences) using BD Pharmingen's Cytotfix/Cytoperm protocol. Specificity of the antibodies was checked using equivalent concentrations of fluorochrome- and isotype-matched negative control immunoglobulins. Acquisition of the samples was performed on a FACS LSR II cytometer equipped with FACSDiva software (both from BD Biosciences). Final analysis was performed using FlowJo software (Tree Star Inc., Costa Mesa, CA, USA).

Cytotoxicity assays

Blood samples were collected into EDTA vacutainers before, during and in the lifelong clinical follow-up of the patients. Blood peripheral mononuclear cells (PBMC) were purified by Ficoll-Paque density gradient separation. Corresponding patient's pleural effusion cells (p#0 or p#1) from cases 1, 3, 6, 7, 8, and 9 were defrosted, washed extensively and incubated with 100 μ Ci of Na₂⁵¹CrO₄ (MP Biomedicals, Irvine, CA, USA) for 1.5 h at 37°C, washed three times, resuspended in RPMI / 5% FBS to a concentration of 5x10⁴ viable cells/ml. Serial dilutions of PBMC and 5x10³ radiolabeled target cells were added to a 96-well U-bottom microtiter plate (0.2 ml final volume) to achieve the desired effector:target (E:T) ratios. Plates were incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO₂, and cell-free supernatants were collected from each well. The amount of ⁵¹Cr released from lysed target cells was determined by γ scintillation counting. Percent lysis was calculated using the formula: corrected % lysis = 100 x (experimental release - spontaneous release[target cells incubated in medium alone])/(maximum release[2% Triton X-100 as lysing agent]-spontaneous release).

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