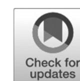
Cancer Therapy: Clinical

Clinical Cancer Research

Autologous Dendritic Cells Pulsed with Allogeneic Tumor Cell Lysate in Mesothelioma: From Mouse to Human



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Abstract

Purpose: Mesothelioma has been regarded as a nonimmunogenic tumor, which is also shown by the low response rates to treatments targeting the PD-1/PD-L1 axis. Previously, we demonstrated that autologous tumor lysate–pulsed dendritic cell (DC) immunotherapy increased T-cell response toward malignant mesothelioma. However, the use of autologous tumor material hampers implementation in large clinical trials, which might be overcome by using allogeneic tumor cell lines as tumor antigen source. The purpose of this study was to investigate whether allogeneic lysate–pulsed DC immunotherapy is effective in mice and safe in humans.

Experimental Design: First, in two murine mesothelioma models, mice were treated with autologous DCs pulsed with either autologous or allogeneic tumor lysate or injected with PBS (negative control). Survival and tumor-directed T-cell responses of these mice were monitored. Results were taken forward in a first-in-human clinical trial, in which 9 patients were treated with 10, 25, or 50 million DCs per vaccination. DC vaccination consisted of autologous monocyte–derived DCs pulsed with tumor lysate from five mesothelioma cell lines.

Results: In mice, allogeneic lysate–pulsed DC immunotherapy induced tumor-specific T cells and led to an increased survival, to a similar extent as DC immunotherapy with autologous tumor lysate. In the first-in-human clinical trial, no dose-limiting toxicities were established and radiographic responses were observed. Median PFS was 8.8 months [95% confidence interval (CI), 4.1–20.3] and median OS not reached (median follow-up = 22.8 months).

Conclusions: DC immunotherapy with allogeneic tumor lysate is effective in mice and safe and feasible in humans. *Clin Cancer Res; 24(4); 766–76. ©2017 AACR.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-17-2522

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Introduction

Malignant pleural mesothelioma (MPM) is a highly lethal neoplasm for which new treatment options are urgently needed (1). Recently reported randomized studies investigating immunotherapy [CTL-associated protein 4 (CTLA-4) inhibitor tremelimumab] and targeted treatment (FAK inhibition, defactinib) were found to be negative for the primary endpoint of an increase in overall survival (2, 3). The phase III MAPS trial targeting angiogenesis (anti-VEGF, bevacizumab) in combination with chemotherapy showed a modest increase in survival (4).

A number of new agents are being tested in early-phase studies in MPM, mostly focusing on immunotherapy with checkpoint inhibitors. Initial results have become available on the blockade of programmed death receptor (PD)-1/programmed death ligand 1 (PD-L1) in MPM showing single-agent response rates ranging between 9% and 25% (5). Response rates to PD-1/PD-L1 inhibitors in these ranges have also been found in other solid malignancies, such as non–small cell lung cancer (NSCLC; ref. 6). As the effect of checkpoint inhibitors is based on the reinvigoration of antitumor T-cell responses, response to therapy is correlated to the presence of tumor-infiltrating T cells' pretreatment (7). Also in mesothelioma, an immune infiltrate including T cells is correlated

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Translational Relevance

Malignant pleural mesothelioma is a highly lethal neoplasm for which new treatment options, like immunotherapy, are urgently needed. Early results from studies investigating checkpoint inhibitors show either no efficacy (anti CTLA-4) or a temporary beneficial effect in a minority of patients (anti PD-l). This is ascribed to the absence of a tumor-directed T-cell response. Previously, we demonstrated that autologous tumor lysate–pulsed dendritic cell immunotherapy increased T-cell response toward malignant mesothelioma. However, the use of autologous tumor material hampers implementation in large clinical trials, which might be overcome by using allogeneic tumor cell lines as tumor antigen source. Here, we present a translational study that provides the basis for allogeneic lysate–loaded dendritic cell (DC) immunotherapy. First, we show efficacy of this strategy in a murine mesothelioma model. Subsequently, we established clinical-grade human mesothelioma cell lines to pulse autologous DC from 9 patients in a first-in-human trial. We show that this treatment is safe and feasible in mesothelioma patients. Our findings pave the way for clinical application of DC immunotherapy in mesothelioma to be evaluated in phase III clinical trials.

to improved survival (8). An immunotherapeutic approach aiming to increase the number of tumor-directed T cells is vaccination. In a recent systematic review on the efficacy of tumor vaccines and cellular immunotherapies, it was established that cell-based vaccination has the highest efficacy in complex tumors like NSCLC (9). Dendritic cell (DC)-based immunotherapy is a potent cell-based immunotherapeutic approach (10, 11), which aims to boost the immune system of cancer patients by enhancing tumor antigen presentation and subsequent activation of tumor-specific (cytotoxic) T cells. DCs are reduced in number in the peripheral blood of mesothelioma patients, and are less functional in terms of activation and antigen presentation compared with healthy controls (12). Therefore, *ex vivo* DC activation and maturation might improve their immune function and direct them to present tumor-associated antigens (TAA). To pulse DCs *ex vivo*, a variety of sources of tumor antigens can be used, such as proteins or peptides, DNA or mRNA in the case of known antigens, or whole tumor cells or tumor lysate, in which undefined antigens are present as well (13).

In previous studies in both murine models and patients with mesothelioma, we have demonstrated that DC vaccination induces tumor-specific CD8 T-cell responses accompanied by promising survival rates (14–16). In these studies, we have used an autologous tumor lysate as a source to pulse DCs, as vaccination with a broad range of antigens can induce a broad repertoire of antitumor T cells and therefore might be more effective (17). Two recent publications showed the potential of a polyvalent vaccination to induce a broad range of T-cell responses in a setting of long synthetic peptide vaccination in melanoma (18, 19). For our DC vaccination strategy in mesothelioma, we generated autologous tumor lysate from patients' own tumor material obtained via pleural drainage, tumor resection, or biopsies, which was used to pulse DCs. This approach was found to be safe and well tolerated by mesothelioma patients (15, 16). However, using an autologous whole tumor cell lysate limited the number of patients that could be included due to logistic limitations, such as the amount and quality of the tumor material. This impeded scaling up the treatment for multicenter clinical trials (20).

An alternative source to pulse the DCs with a broad spectrum of antigens, is the use of an allogeneic tumor lysate generated from cell line cultures, which contain TAA (17). We hypothesized that DC immunotherapy with an allogeneic lysate prepared from tumor cell lines would be able to induce antitumor responses similar to the autologous tumor cell lysate. In this study, we first examined the efficacy of allogeneic tumor lysate DC immunotherapy *in vivo* in two different murine mesothelioma models (CBA/j and BALB/c mice), in which we demonstrated that allogeneic tumor lysate–pulsed DC immunotherapy was similarly effective in improving survival of mesothelioma-bearing mice as autologous tumor lysate DC immunotherapy. To translate these findings to human, we developed a human allogeneic tumor cell lysate, generated from five clinical-grade mesothelioma cell lines. In a first-in-human phase I study, patients with mesothelioma were treated with increasing numbers of autologous DCs pulsed with allogeneic lysate. We found that the treatment is safe, induces an antitumor immune response, and is associated with radiographic responses and promising overall survival.

Materials and Methods

Preclinical

Tumor cell lines culture. The AB1 and AC29 mouse mesothelioma cell lines were kindly provided by Prof. Bruce W.S. Robinson, Queen Elizabeth II Medical Centre (Nedlands, Australia). The AB1 and AC29 cell lines have been derived from malignant mesothelioma after intraperitoneal (i.p.) inoculation of crocidolite asbestos into BALB/c (H-2d) and CBA/j (H-2k) mice, respectively. Both cell lines were obtained in the years 2003–2005, aliquoted, and stored at $-190°C$.

These cell lines were maintained in RPMI1640 GlutaMax supplemented with 50 μg/mL gentamycin (all obtained from Invitrogen) and 5% FBS (HyClone, Thermo Scientific). Tumor cells were cultured from early passages (maximum five passages following cell line acquisition).

Tumor lysate production. AB1 and AC29 tumor cells were harvested from 80% confluent culture flasks and resuspended at a concentration of 5×10^7 cells/mL for AB1 and 1×10^8 cells/mL for AC29 in PBS. Cells were disrupted by five freeze–thaw cycles and subsequent sonication to produce a homogeneous lysate.

Isolation and pulsing of DCs. As in a murine model, it is not possible to obtain sufficient amount of monocyte-derived DCs from peripheral blood, DCs were obtained from bone marrow culture using a modified version of a previously described procedure (21). Instead of flushing the femurs and tibias of naïve BALB/c or CBA/j mice for the bone marrow, major leg bones were crushed using a mortar and pestle with addition of approximately 3 mL of RPMI medium. Cells were passed through a 100-μm cell strainer, depleted of red blood cells, and cultured in DC culture medium, composed of RPMI supplemented with 5% FBS, 50 μg/mL gentamycin, 50 μmol/L betamercaptoethanol (Sigma Aldrich) and 20 ng/mL recombinant murine granulocyte macrophagecolonystimulating factor (GM-CSF) (gift from prof. Bart Lambrecht, VIB-UGent Center for Inflammation Research, Ghent, Belgium). Cells were placed in 90-mm culture dishes (Nunc) with

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a total number of 2×10^6 cells/10 mL at seeding and incubated at 37°C (Innova Co-170, New Brunswick Scientific) in a humidified atmosphere at 5% CO_2 (day 0). On day 3, 10 mL of DC culture medium was added. On day 6, 10 mL of the media was replaced by 10 mL fresh DC medium. After 8 days in culture, DCs were pulsed with AB1 (three tumor cell equivalents per DC) or AC29 (6 AC29 tumor cell equivalents per DC) tumor lysate. After 8 hours, Lipopolysacharide (LPS) was added to allow full maturation. The next day, DCs were harvested by gentle pipetting and purified by Lympholyte-Mammal (Cedarlane) density gradient centrifugation, washed three times with PBS, and resuspended at a concentration of $1–3 \times 10^6$ viable cells in 500 μL PBS.

Verification maturation status of DCs. To verify the purity and maturation of DCs, the cultured cells were assessed with flow cytometry on a FACS LSR II (BD Biosciences). DCs were characterized as $CD11c^+MHC\text{-}II^+$. CD40, CD80, and CD86 were used to verify maturation of DCs. The following antibodies with matched isotype controls were used: anti-CD11c-PE Texas Red, anti-MHC class II-Alexa Fluor 700, anti-CD40-APC (eBioscience), anti-CD80-PerCP-Cy5.5, and anti-CD86-PE-Cy7 (BD Biosciences). DAPI, 4′,6-diamidino-2-phenylindole (Invitrogen) was used as a viability dye.

In vivo survival experiments. Female BALB/c mice and CBA/j mice (Harlan) were housed at the animal care facility of the Erasmus MC (Rotterdam, the Netherlands). The experiment was approved by the local Ethical Committee for Animal Welfare. Experiments were started at 6-8 weeks of age. At day 0, mice were injected intraperitoneally (i.p.) with 0.5×10^6 AB1 tumor cells (BALB/c mice) or 1.5×10^7 AC29 tumor cells (CBA/j mice). The number of tumor cells for these mouse models were titrated to achieve a tumor incidence in untreated mice of at least 80%, with mice generally surviving until at least two weeks after tumor inoculation (14, 22). At day 7, mice received an intraperitoneal injection with PBS or DCs immunotherapy pulsed with either AB1 tumor lysate or AC29 tumor lysate. Six mice were used per group.

Each vaccine contained $1–3 \times 10^6$ DCs, depending on the yield, and equal for all mice within the experiment. Tumor growth, physical wellbeing, body weight, and the survival were monitored every other day for 70 days after tumor injection.

In vitro degranulation assay and flow cytometric analysis. Splenocytes from PBS-treated, DC-treated, or naïve mice were restimulated with tumor cells. A total of 5×10^4 AC29 or AB1 tumor cells were seeded in a flat-bottom 24-well plate (Costar, Corning Inc), to which 1×10^6 splenocytes were added the next day, together with 10 μg/mL CD107a -FITC (BD Biosciences). After one hour, the protein transport inhibitor Golgi stop was added (BD Biosciences). As a positive control, splenocytes were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin (Sigma). Unstimulated splenocytes were used as negative control. After 4 hours of incubation at 37°C, splenocytes were harvested and stained for flow cytometry with anti-CD3-APC-Cy7, anti-CD8-PE-Cy7, anti-CD44-PerCP-Cy5.5, and anti-CD62L-APC (eBioscience). Fixable Viability Dye eFluor 506 (eBioscience) was used to exclude dead cells. For staining of intracellular IFNγ, cells were fixed with 2% paraformaldehyde and permeabilized with 0,5% saponin, before IFNγ was stained intracellularly with anti-IFNγ-Pacific Blue (eBioscience).

Caspase-3/7 assay. $CD8^+$ T cells were isolated from splenocytes from DC-treated mice using the $CD8^+$ Isolation kit (Miltenyi Biotec). Purified $CD8^+$ T cells were then cocultured with AB1 tumor cells or AC29 tumor cells in a 20:1 or 50:1 ratio in a black coated and transparent flat-bottom 96-well microplate (Greiner). Cell PlayerTM Caspase 3/7 reagent (Essen Bioscience) was added at 5 μmol/L final concentration per well to detect apoptosis during the coculture. Cells were cultured in the IncucyteTM FLR (Essen Bioscience) for 4 days at 37°C in a humidified atmosphere at 5% CO_2 and images were captured every 2.5 hours for caspase-3/7 upregulation.

Clinical

Study design. In this first-in-human open-label nonrandomized dose escalation phase I study, we enrolled adult patients with mesothelioma. In total, 9 MPM patients divided over three cohorts (3 patients per cohort) received autologous monocyte–derived DCs (Mo-DC) pulsed with allogeneic tumor cell line lysate. The first cohort received 10 million Mo-DCs per vaccination, the second cohort received 25 million Mo-DCs per vaccination, and the third cohort received 50 million Mo-DCs per vaccination. The autologous Mo-DCs and the allogeneic tumor cell line lysate used to load the autologous Mo-DCs were produced under Good Manufacturing Practice (GMP)-certified conditions. The study was approved by the Central Committee on Research involving Human Subjects (NL4433000014) as defined by the Medical Research Involving Human Subjects Act. Procedures followed were in accordance with the ethical standards of these committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The trial is registered within clinicaltrials.gov, number NCT02395679.

Patient selection. Patients with histologically or cytologically confirmed MPM were eligible to participate in the study. Moreover, patients had to be nonprogressive after at least 4 cycles of pemetrexed and platinum-containing chemotherapy (as determined by CT). Patients that refused chemotherapy treatment as first-line treatment were also eligible. Other inclusion criteria were: measurable disease on CT dimensions according to modified Response Evaluation Criteria In Solid Tumors (RECIST; ref. 23), at least 18 years of age, able to give written informed consent, ambulatory (WHO-ECOG performance status 0 or 1, with an expected survival of at least 3 months), normal organ function and adequate bone marrow reserve (absolute neutrophil count $> 1.0 \times 10^9$/l, platelet count $> 100 \times 10^9$/L, and Hb > 6.0 mmol/L), a positive DTH skin test (induration > 2 mm after 48 hours) against at least one positive control antigen tetanus toxoid, ability to return to the Erasmus MC for adequate follow-up as required by this protocol, written informed consent according to ICH-GCP, and planned start date of vaccination at least 5 weeks after the last dosage of chemotherapy.

A potential subject who met any of the following criteria was excluded from participation in this study: medical or psychologic impediment to probable compliance with the protocol; current use or discontinuation of less than 6 weeks of steroids (or other immunosuppressive agents), with the exception of prophylactic usage of dexamethasone during chemotherapy; prior malignancy except adequately treated basal cell or squamous cell skin cancer, superficial or *in situ* cancer of the bladder or other cancer for which the patient has been disease-free for five years; serious concomitant disease or active infections; history of autoimmune disease

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or organ allografts, or with active acute or chronic infection, including HIV and viral hepatitis; serious intercurrent chronic or acute illness such as pulmonary (asthma or COPD) or cardiac (NYHA class III or IV) or hepatic disease or other illness considered by the study coordinator to constitute an unwarranted high risk for investigational DC treatment; known allergy to shell fish (may contain KLH); pregnant or lactating women; inadequate peripheral vein access to perform leukapheresis; and concomitant participation in another clinical trial.

Procedures. Patients received MesoPher (see below: Production of DC vaccination) via intradermal (i.d.) and intravenous (i.v.) injections with two thirds of the dose intravenous and one third intradermal in three biweekly vaccinations, followed by a booster after 3 and 6 months. The combined intradermal and intravenous administration was chosen to induce a systemic immune response, as proposed by Adema and colleagues (24) and was used in previous phase I trials by our group as well (15, 16). Cohorts of 3 patients received a dose level to evaluate safety. Within each cohort, a patient was required to complete 7 days after the vaccination without toxicities before a new patient was treated. If none of these 3 patients in one cohort had a DLT within 8 weeks of starting the first dose, the next dose level was started.

Outcomes. The primary objective was to determine the safety of the different dose schedules, to establish the recommended dose for further studies. Secondary objectives were antitumor activity according to radiologic examinations, immunologic profiling, and overall survival.

Safety evaluation. Physical examination and hematologic and biochemistry assessments were performed weekly. A 12-lead ECG and laboratory testing was performed before the patient could be included in the trial, and before every vaccination. Toxicity was scored according to the NCI-CTCAE version 4.0.

The following toxicities occurring during 8 weeks after the first vaccination, were defined as dose-limiting (DLT), when considered possibly, probably, or definitively related to study medication. Hematologic toxicity: thrombocytopenia grade 3 during longer than 7 days or grade 4 or a neutropenia grade 3 during longer than 7 days or grade 4. Nonhematologic toxicity: any grade 3/4 toxicity except for diarrhea, nausea, vomiting, hypertension if not adequately treatable, and skin toxicity. Immune-related toxicity: any grade 4 except for rash and (drug related) fever.

Tumor evaluations. Tumor evaluations were performed every 6 weeks by chest CT preferably using contrast and response was determined according to the modified RECIST. Objective responses had to be confirmed 6 weeks after the initial assessment. Duration of response was from first documented response until disease progression.

Immunologic evaluations. Serum samples were collected before start of treatment, on the day of each vaccination, and two weeks after each vaccination. A DTH skin test was performed two weeks after the third vaccination with the lysate, MesoPher, tumor lysate–pulsed DCs without KLH and injection fluid as negative control. DTH reaction was measured 48 hours after intradermal injection. If a positive reaction was observed, a 3- or 6-mm punch biopsy was taken from the skin induration, and was used immediately for isolation of skin-test infiltrating lymphocytes (SKIL).

Rapid expansion of SKILs. Skin biopsies were placed in a 6-well plate with 3-mL medium consisting of RPMI, 7% normal human serum, and low IL2 concentrations (100 IU/mL) for 7 days. Subsequently, T cells were rapidly expanded with a feeder system as described elsewhere (25).

Dextramer staining and analysis. Online prediction algorithms (netCTL: http://www.cbs.dtu.dk/services/NetCTL/ and netMHC-pan: http://www.cbs.dtu.dk/services/NetMHCpan/) were used to select HLA-A2–binding mesothelin peptides with high affinity and predicted MHC presentation. Dextramers were ordered from Immudex. Dextramer A: PE–conjugated HLA-A2 dextramer with peptide SLLFLLFSL (mesothelin$_{20-28}$), Dextramer B: APC-conjugated HLA-A2 dextramer with peptide VLPLTVAEV (mesothelin$_{531-539}$). Dextramer staining was performed according to manufacturer's protocol. Anti-CD3-BV711 and anti-CD8-FITC (clone SK1; BD Biosciences) were used for extracellular staining. DAPI was used as viability dye. Samples were measured on an LSR-II flow cytometer (BD Biosciences). FMO controls were used to determine dextramer-positive populations.

Statistical analysis. All statistical tests on the murine experiments were performed in GraphPad Prism 5. The size of the first-in-human dose escalation study was based on the standard 3+3 dose escalation design, and therefore we did not perform a further sample-size estimation.

Production of DC vaccination (MesoPher). Eligible patients underwent autologous DC pulsed with allogeneic tumor lysate–based immunotherapy (MesoPher). Allogeneic tumor lysate was produced under GMP-compliant conditions and consists of five unique, clinical-grade human multiple myeloma cell lines, which are patented under patent ID P6038325NL. For the production of MesoPher, Mo-DCs were generated from CD14$^+$ monocytes according to a 10-day culture protocol. CD14$^+$ monocytes were enriched from patient-derived apheresis products by CliniMACS procedure (CD14$^+$ procedure, Miltenyi Biotec). Purity of the CD14$^+$ monocytes after CliniMACS procedure was >97% and viability was >95% for all MesoPher preparations. Hereafter, CD14$^+$ monocytes were cultured in T225 flasks (100 million cells per flask) in X-VIVO 15 (Lonza) supplemented with 2% NHS (Sanquin) and adhered overnight at 37°C, 5% CO_2. The following day, half of the medium was refreshed with fresh medium supplemented with 2% NHS, and 1,000 IU/mL rhGM-CSF (Miltenyi Biotec) and 1,600 IU/mL rhIL4 (Miltenyi Biotec) were added. Cells were cultured for three additional days at 37°C, 5% CO_2. On the fifth day of culture, immature unpulsed Mo-DCs were harvested and reseeded in 6-well plates (1 million cells/well) in the presence of tumor lysate (tumor cell equivalent to DC ratio 1:3), and supplemented with 800 IU/mL rhGM-CSF, 500 IU/mL rhIL4, and 10 μg/mL VACMUNE KLH (BioSyn). On day 8 of the culture, immature pulsed Mo-DCs were matured with 5 ng/mL IL1β, 15 ng/mL IL6, 20 ng/mL TNFα (all Miltenyi Biotec), and 10 μg/mL PGE-2 (Pfizer). On day 10 of the culture, cells were harvested and had to comply with the following quality demands to release the batch: sterile, >80% viability, >90% MHC class II$^+$ CD11c$^+$ cells, and >80% CD80$^+$ cells of the MHC class II CD11c$^+$ cells.

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Results

Allogeneic and autologous tumor lysate–loaded DCs mediate comparable survival in mesothelioma-bearing mice

To investigate whether allogeneic tumor lysate–loaded DCs were as effective as autologous tumor lysate–loaded DCs in improving survival in mesothelioma-bearing mice, we set up an *in vivo* experiment using two different mouse models for mesothelioma, as depicted in Fig. 1A. Mesothelioma was induced in these mice as described previously (26). These mouse models are different in both mouse strain and aggressiveness of tumor growth and are chosen to assure robustness of the treatment across different models. In short, BALB/c mice were injected with the syngeneic AB1 cell line and CBA/j mice were injected with the syngeneic AC29 mesothelioma cell line. Seven days following tumor inoculation, BALB/c mice and CBA/j mice received either PBS (vehicle), autologous tumor lysate–pulsed DCs (AB1 for BALB/c and AC29 for CBA/j), or allogeneic tumor lysate–pulsed DC (AC29 for BALB/c and AB1 for CBA/j). The survival of these mice is depicted in Fig. 1B. The results show that DC immunotherapy significantly improved survival in tumor bearing mice of both BALB/c and CBA/j mouse strains compared with PBS-treated control mice ($P < 0.05$), with no significant differences in efficacy between autologous lysate–loaded and allogeneic lysate–loaded DC immunotherapy.

Allogeneic tumor lysate–loaded DCs induce an antitumor T-cell response in mesothelioma-bearing mice

To demonstrate the induction of a tumor-specific T-cell response, splenocytes from surviving CBA/j mice were stimulated with tumor cells. IFNγ production was assessed by intracellular cytokine staining and degranulation was assessed by cell surface expression of CD107. IFNγ^{+} and CD107^{+} splenic CD8 T cells were increased upon stimulation with tumor cells in DC-treated mice, but not untreated mice. No difference was observed between the autologous lysate and allogeneic lysate treatment (Fig. 2A and B). CD8 T cells from all groups were equally capable

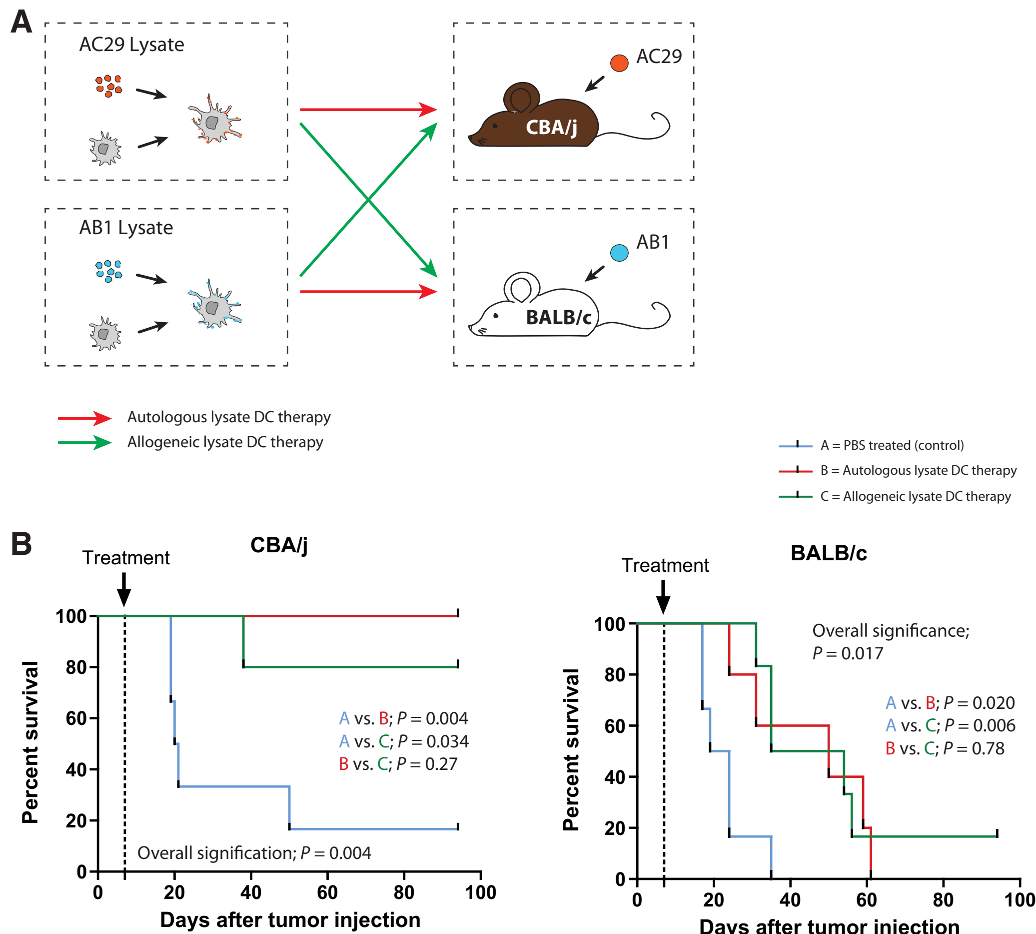

Figure 1.
Efficacy of allogeneic tumor lysate–pulsed dendritic cell immunotherapy in mesothelioma-bearing mice. **A,** Schematic image of the two mesothelioma mouse models and autologous- or allogeneic-pulsed DC immunotherapy. Two different mouse strains were used, CBA/j and BALB/c, inoculated intraperitoneally with their syngeneic mesothelioma cell line (AC29 for CBA/j and AB1 for BALB/c). Syngeneic DCs were cultured from the bone marrow of littermates and pulsed with tumor lysate of either of two tumor cell lines. In the autologous lysate condition, mice were treated with DCs pulsed with the tumor lysate matching their tumor, while in the allogeneic condition, mice were treated with DCs pulsed with the tumor lysate of the allogeneic tumor cell line. **B,** Survival of mesothelioma-bearing mice treated with DC vaccination. Seven days after tumor inoculation, mice were injected intraperitoneally with either PBS (group A, blue), autologous DCs pulsed with autologous tumor cell lysate (group B, red) or autologous DCs pulsed with allogeneic lysate (group C, green). Kaplan–Meier curves are shown for the CBA/j mice (top) and BALB/c mice (bottom). Statistical survival difference between the groups was determined using the log-rank test.

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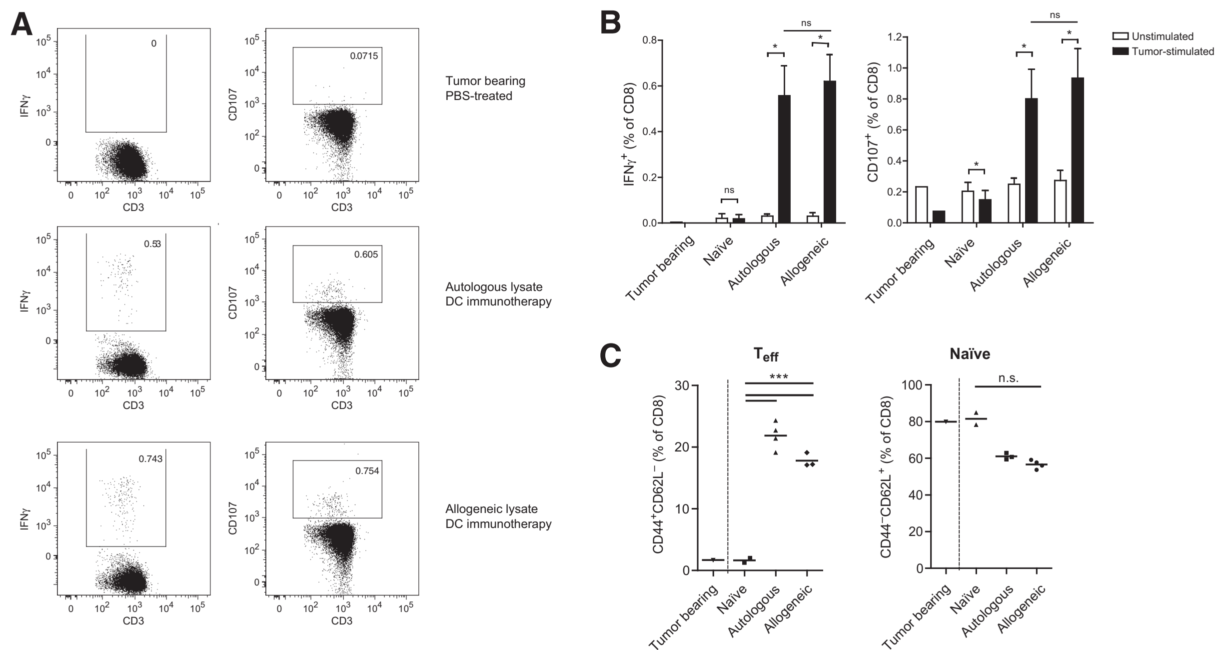

Figure 2.

DC immunotherapy–induced immunologic changes in a murine mesothelioma model. **A,** Representative flow cytometry plots of IFNγ production (left) and CD107 expression (right) in splenic CD8 T cells after coculture with tumor cells. Splenocytes of surviving CBA/j mice (autologous: $n = 4$, allogeneic: $n = 3$), were isolated when the mice were sacrificed and cultured in the presence of AC29 tumor cells. As controls, both tumor-bearing and naïve mice were included (naïve: $n = 2$, tumor-bearing: $n = 1$). **B,** Quantification of IFNγ production (left) and CD107 expression (right) in splenic CD8 T cells, either stimulated with tumor cells or unstimulated. *, $P < 0.05$ (paired Student *t* test for stimulated vs. unstimulated, unpaired Student *t* test for comparison autologous vs. allogeneic treatment). **C,** Proportions of effector phenotype ($CD44^+CD62L^-$) CD8 T cells (T_{eff}, left) and naïve phenotype ($CD44^-CD62L^+$; right) CD8 T cells in splenocytes isolated from treated mice, and tumor-bearing and naïve mice as negative controls. *, $P < 0.05$ (one-way ANOVA).

of IFNγ production and degranulation upon PMA/ionomycin stimulation (not shown). This increased CD8 T-cell functionality after DC treatment coincided with increased proportions of effector memory ($CD44^+CD62L^-$) CD8 T cells in treated versus untreated mice, at the expense of naïve T cells (Fig. 2C). To confirm that the induced T cells in these mice were capable of killing tumor cells, we cocultured splenic CD8 T cells from DC immunotherapy treated and untreated BALB/c mice with AB1 tumor cells. Tumor cell growth was diminished when cocultured with CD8 T cells from DC immunotherapy–treated mice compared with untreated mice, which was due to increased apoptosis as shown by increased caspase expression (Supplementary Fig. S1).

Altogether, these results demonstrate that treatment with DCs pulsed with either autologous or allogeneic tumor lysate induce an effective antitumor immune response and improve survival in mesothelioma-bearing mice.

No dose-limiting toxicity was induced by allogeneic tumor lysate–pulsed DC immunotherapy in a first-in-human trial targeting mesothelioma

The comparable outcome between autologous lysate–pulsed DC immunotherapy and allogeneic lysate–pulsed DC immunotherapy in our mesothelioma mouse models, led us to initiate a first-in-human phase I clinical trial with allogeneic lysate–pulsed DC immunotherapy (MesoPher). To this end, long-term human mesothelioma cell lines were established from pleural effusion cells isolated from patients with pathologically proven mesothelioma. Lysates of five cell lines were combined to pulse DCs with.

In total, 9 patients were treated according to the predefined treatment schedule between January 2015 and October 2015. Data cutoff was in April 2017. Patient characteristics are summarized in Table 1. Five patients were treated as maintenance after chemotherapy while 4 were treatment naïve. Patients received a vaccination regimen of 3 biweekly vaccinations and a booster vaccination after 3 and 6 months. In 2 of 9 patients in the highest dose regimen (patient 7 and 9), no booster vaccination after 6 months could be given due to shortage of DCs to generate 5 vaccinations.

Neither predefined dose-limiting toxicities nor grade 3 or 4 toxicities were established. A summary of all grade toxicity is presented in Table 2, with further specifications of adverse events in Supplementary Table S1. Treatment-related adverse events were mild (grade 1 or 2), although present in all patients. These treatment-related adverse events included an induration of the skin related to the intradermal injections in all patients at vaccination 2 and 3 and a grade 1 or 2 fever in all patients after vaccination. This was a transient fever occurring 4–8 hours after vaccination and lasting for maximally 24 hours.

An immune response was induced in patients after three doses of allogeneic tumor lysate–pulsed DC immunotherapy, in which mesothelin-specific T cells could be detected

As readout for a recall response against the vaccine, a DTH skin test was performed 2 weeks after the third vaccination, using a

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Table 1. Patient characteristics

	Patients ($n = 9$)
Age median (range)	69 (45–79)
Sex	
Male	8 (89%)
Female	1 (11%)
Histologic subtype	
Epithelioid	9 (100%)
Sarcomatoid	0 (0%)
Biphasic	0 (0%)
Stage	
1 (0×)	0 (0%)
2 (1×)	1 (11%)
3 (5×)	5 (56%)
4 (3×)	3 (33%)
ECOG Performance score	
0	7 (78%)
1	2 (22%)
Prior treatment	
Chemotherapy (platinum-based+pemetrexed)	5 (56%)[a]
Radiotherapy	0 (0%)
Surgery	1 (11%)[b]
Other	0 (0%)
Response to chemotherapy (if applicable)	
SD	3 (60%)
PR	2 (40%)

Abbreviations: PR, partial response; SD, stable disease.

[a]Chemotherapy consisted of cisplatin or carboplatin, combined with pemetrexed, with a total of 4 ($n = 4$) or 6 ($n = 1$) cycles.

[b]Surgery in this patient consisted of abdominal debulking surgery (this patient had both pleural and abdominal mesothelioma).

negative control, the lysate only, lysate-pulsed DCs with KLH (MesoPher) and lysate–pulsed DCs without KLH. No skin reactions were seen in the negative control or after injection of the lysate only. All 9 patients responded with redness and thickening of the skin 48 hours after injection of MesoPher with KLH. Injection with lysate-pulsed DCs without the adjuvant KLH resulted in a response in 5 of 9 patients, which all received a dose of at least 25 million DCs.

To assess whether in the positive skin reaction, tumor-specific T cells could be detected, we isolated T cells from a biopsy of the DTH test skin induration. Mesothelin was chosen as a relevant model antigen, as all 9 patients in our study had histologically proven mesothelin expression in diagnostic tumor biopsies. Using dextramers against two different mesothelin epitopes, we assessed reactivity against these epitopes in all HLA-A2–positive patients of which viable T cells were obtained from the skin biopsy. In 4 of 6 evaluable patients, mesothelin-specific T cells could be detected (Fig. 3). In 3 of these 4 patients, T cells against both epitopes were observed, while in one patient (patient 2), T cells against one epitope were detected. These data show that the immune reaction induced by MesoPher contained T cells specific for a relevant tumor-associated antigen, which could be detected in the majority of evaluable patients.

Radiographic responses and promising overall survival after DC immunotherapy

Response and survival data of all patients are summarized in Fig. 4A and Supplementary Fig. S2, with now 8 of 9 patients experiencing progression. In two patients treated both in the 25 million DC cohort, a confirmed partial response was established after the treatment. One patient was pretreated with chemotherapy and one patient treatment naïve. The pretreated patient had a response with a duration of 15 months, while the treatment-naïve patient has an ongoing response with a current duration of 21 months. This patient showed a remarkable decrease of total tumor burden of 70% after the third vaccination (Fig. 4B; Supplementary Fig. S3). In the other 7 patients, a stable disease was established leading to a disease control rate of 100%. Median overall survival has not been reached, since 7 patients are currently alive after a median follow up of 22.8 months (range, 18.5–27.4 months). Median progression-free survival (PFS, defined as time from registration until progression or death, whichever came first) was 8.8 months [95% confidence interval (CI) 4.1–20.3]. PFS at 12 months was 33% (95% CI, 8%–62%). Treatment beyond progression was started in all but one patient after progression and is described in Supplementary Table S2. The one patient that did not start treatment beyond progression has a slow progression of the tumor (and not a 30% enlargement from nadir) and is not experiencing clinical deterioration.

Discussion

We here show that autologous DC therapy with an allogeneic tumor cell lysate is effective in a murine model of mesothelioma and that a comparable treatment is safe and feasible in patients, with promising clinical activity.

The goal of DC-based immunotherapy is to augment the patient's immune system by means of improving tumor cell recognition by T cells. When using autologous tumor lysate to pulse DCs, a personalized set of antigens is presented to the patients' T cells. Practical limitations in obtaining autologous lysate from patients, led to use of an allogeneic lysate derived from tumor cell lines, which relies on the existence of common tumor antigens that are shared between patients.

In two different murine mesothelioma models, a comparable survival was found between mice receiving allogeneic tumor lysate-pulsed DCs and mice receiving autologous tumor lysate-pulsed DCs. Moreover, our *in vitro* experiments show that in this model a similar functional tumor-specific CD8 T-cell response was established, demonstrated by the production of IFNγ and degranulation of the CTLs. This indicates that allogeneic tumor lysate is as immunogenic as autologous tumor lysate when used in a DC immunotherapy setting. We cannot rule out the possibility, however, that allogeneic tumor lysate is slightly less effective, as the groups were too small to definitely compare efficacy between the DC treatments with either allogeneic or autologous lysate. Also, unpulsed DCs, which are known to have some, but a notably inferior, effect on survival (14), were not included as a separate arm in these experiments. But, most importantly, our findings clearly show efficacy of DC immunotherapy using allogeneic tumor lysate.

The results of our murine experiments led us to initiate a first-in-human clinical trial, which showed that treatment was safe and feasible. The trial was designed as a 3+3 design and no

Table 2. Toxicities/adverse events of patients in first-in-human clinical trial

$N = 9$	Any grade, n (%)	Grade 3–4, n (%)
Any AE	9 (100)	0 (0)
Injection site reaction	9 (100)	0 (0)
Fever	9 (100)	0 (0)
Respiratory	6 (67)	0 (0)
Lab abnormalities	8 (89)	0 (0)
Gastrointestinal	1 (11)	0 (0)
Appetite	1 (11)	0 (0)

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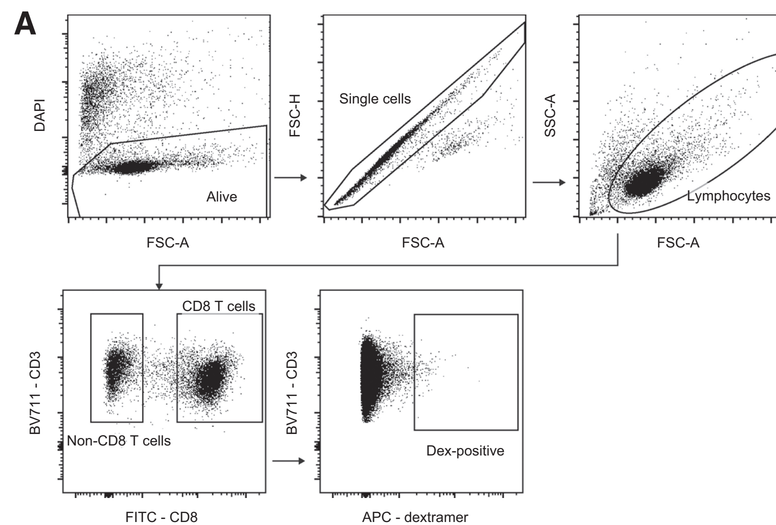

Figure 3.
Mesothelin-specific CD8 T cells in SKILs after injection with allogeneic lysate–pulsed DCs. T cells were isolated from a biopsy of the positive posttreatment DTH test with the vaccine, and expanded *in vitro*. CD8 T cells specific for two mesothelin peptides were detected using FCM with peptide-MHC multimers. Results were obtained of all HLA-A2$^+$ patients of which sufficient T cells could be expanded from the biopsy. **A,** Gating strategy of dextramer binding CD8 and non-CD8 T cells. **B,** Quantification of dextramer-binding CD8 T cells. The bars in these graphs represent peptide-MHC binding T cells, as percentage of either CD8 or non-CD8 T cells, whereby the non-CD8 T cells were used as a negative control.

dose-limiting or major toxicities were established, even in the highest dose. Two partial responses were observed in the 25 million cells dose cohort, which indicates that this dose is high enough to induce radiographic responses. As the yield of monocytes from leukapheresis limited the number of vaccinations that could be given at the 50 million dose cohort, we decided that the dose of 25 million cells was the optimal dose level to be taken forward in further clinical trials. As it was decided to initiate further studies with the 25 million cell dose level, we found it unethical and unnecessary to include further patients in the trial as no additional safety data are to be expected for this dose. In contrast to, for example, targeted therapies where a higher dose is expected to be more effective, as DC immunotherapy relies on the induction of an antitumor immune response and expansion of effector cells within the patient, a larger number of DCs is not necessarily needed to induce a stronger immune response (27). To increase effectivity of DC immunotherapy, it is probably more beneficial to change the microenvironment to facilitate optimal conditions for the expansion and migration of T cells to the tumor. In future studies, it might be of interest to determine effectivity not only by radiographic response, but also by immunologic readout, for example, by monitoring increases in (tumor-specific) CTLs.

Evidence of an immune response induced by vaccination was seen in all patients in the form of a positive response to the DTH skin test after the third vaccination. A DTH response has been shown to correlate with overall survival in a DC vaccination setting in melanoma, although the clinical applicability of this correlation is still largely unclear (28). At the time of the second or subsequent vaccinations, a similar reaction was observed at the site of intradermal injection. This response did not occur after the first vaccination and thus indicates an induction of a tumor lysate–directed response. Interestingly, in the lowest dose cohort and one of the patients of the second cohort, the DTH response was not present if pulsed DCs without KLH were administered. This finding underlines the importance of an adjuvant to enhance immune activation, especially in lower doses of DC vaccination. On the other hand, the positive skin reaction toward pulsed DCs without the adjuvant KLH in the higher doses proves that this DC vaccination induced a response against the lysate, and not against KLH only. This was also confirmed by the presence of mesothelin-specific T cells in the skin induration. Frequencies of mesothelin-specific T cells in the skin biopsy were very low, which was expected, as the full tumor lysate consists of a broad array of tumor antigens, of which mesothelin was chosen as a relevant candidate. Future studies will have to prove whether assessment of particular tumor antigen–specific T-cell responses can be used as a biomarker for clinical responses.

One of the limitations of the study is that in human number of total tumor-specific T cells before and after therapy could not be

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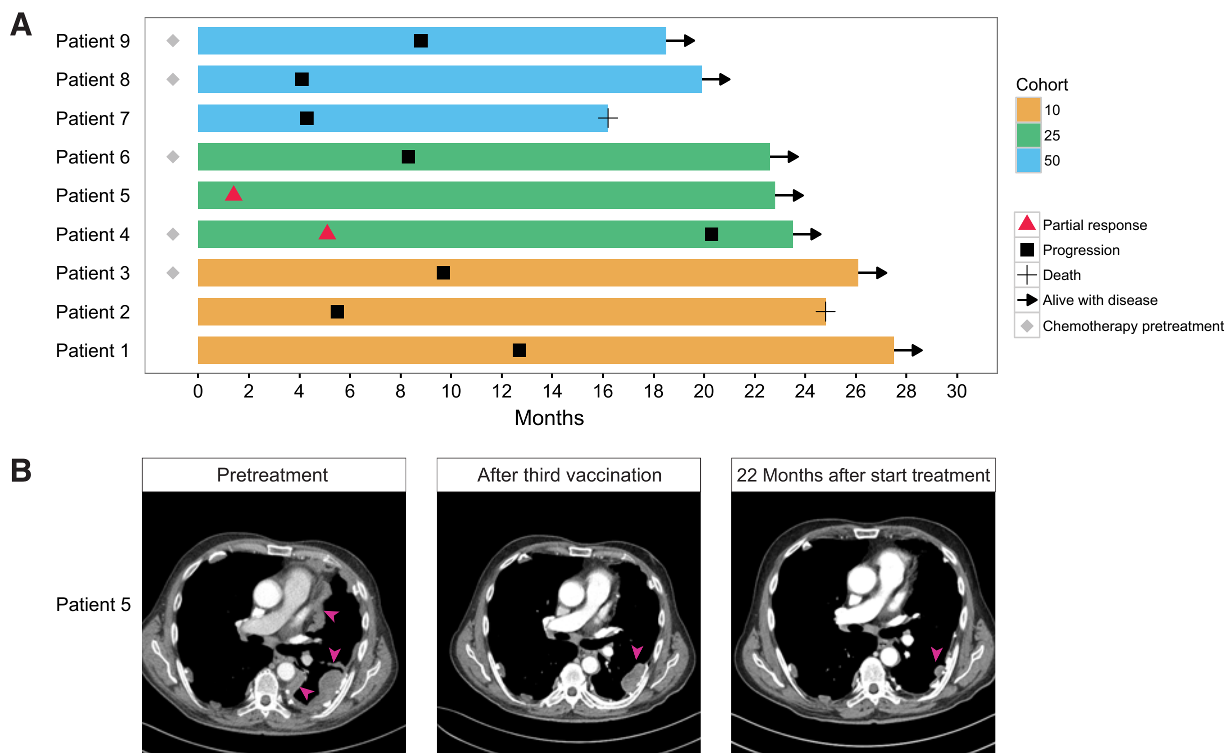

Figure 4.

Overall survival and RECIST responses after first vaccination. **A,** Swimmer plot of patients in phase I clinical trial with MesoPher treatment. Overall survival of patients since date of first vaccination is represented by the filled bars. Start and end of RECIST responses are depicted by the red triangles and black squares, respectively. First evaluation of response was after 6 weeks for all patients. Except patient 5 that experienced a partial response, all other patients had stable disease at first evaluation. Patients treated with chemotherapy prior to inclusion in the study are depicted with a gray diamond. The other patients were treatment naïve. **B,** Pre- and postvaccination CT scans of patient 5. CT scans with contrast of the ongoing response of one treatment-naïve patient receiving allogeneic lysate–pulsed autologous DC vaccination. Tumor mass is indicated with pink arrows. All lesions were solid. This patient was treatment naïve and received 5 doses of 25 million DCs. Pretreatment tumor burden decreased with 70% after the third vaccination (6 weeks after start of treatment) and continued to decrease.

determined in assays similar to the assays performed in mice, as no viable tumor material was available of the patients. Another limitation of the study is that this was a selected patient population with only patients who were not progressing after chemotherapy and 4 treatment-naïve patients, refusing chemotherapy at that moment.

Although conventional radiographic response criteria may be suboptimal in immunotherapy, as they may not reflect tumor growth optimally (20), we determined objective response rate and PFS by modified RECIST criteria (23). The radiographic partial response in 2 of the 9 patients shows the potency of DC immunotherapy to induce tumor reduction. While in the medical literature, safety of allogeneic lysate pulsed DC immunotherapy has been reported for colorectal cancer (29, 30), melanoma (31, 32), urologic cancers (33), and thyroid cancer (34) using doses up to 10 million DCs per vaccination, clinical responses in this study are promising compared with previously reported studies. Although none of these studies had efficacy as primary outcome, the reported radiographic responses were stable disease at best in most studies (29, 31, 33–37), with an exception of one complete response in melanoma (32) and one partial response (out of 20) in a MAGE antigen–expressing colorectal cancer patient (30).

For melanoma, immunotherapy has been successful over the last decades, which has led to FDA approval of three different checkpoint inhibitors (CTLA4 inhibitor ipilimumab and PD1 inhibitors nivolumab and pembrolizumab) and an oncolytic virus therapy (T-VEC). With the high mutational load of melanoma (38), a broad repertoire of tumor-specific T cells can potentially arise (39). MPM, however, is a poorly immunogenic tumor with a lower mutational burden and lower response rates to, for example, checkpoint inhibitors (5). The clinical responses, including a 70% tumor reduction at first radiographic assessment in one patient, indicates that that DC immunotherapy is capable to evoke an effective tumor killing immune response, even in a nonimmunogenic tumor type.

In two recent studies in melanoma, vaccination with long synthetic peptides in most cases led to delayed outgrowth of metastases, while the combination with checkpoint inhibitors was very potent to induce partial or complete responses (18, 19). For mesothelioma, with the potential induction of *de novo* tumor-directed immune responses or enhancement of preexisting responses by DC vaccination, combination therapy of DC vaccination with a checkpoint inhibitor that can reinvigorate this immune response, is anticipated to provide a synergistic effect as well. Indeed, clinical responses toward checkpoint inhibitors were observed in the patients that had received MesoPher previously but ultimately developed disease progression. Out of three patients that received the PD-1 checkpoint inhibitor nivolumab,

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two showed a partial response and one showed stable disease. Moreover, the use of immunologic readouts such as systemic T-cell activation, the presence of tumor antigen–specific T cells or various immunosuppressive cells will also aid in determining the best combination approach.

Other immunotherapy approaches are being explored as well for mesothelioma, most of which exploit the tumor-associated antigen mesothelin. These approaches include adoptive T-cell transfer with mesothelin-CAR T cells, which might also be given regionally and therefore may overcome a potential T-cell homing problem (40). Other approaches include a chimeric anti-mesothelin antibody involving antibody-dependent cellular cytotoxicity (ADCC; ref. 41) and an immunotoxin-coupled anti-mesothelin antibody approach (42).

The overall survival of patients in this study is promising. However, the number of patients in this trial is too limited to draw firm conclusions. Randomized trials with allogeneic-lysate pulsed dendritic cell immunotherapy in malignant pleural mesothelioma are now warranted to establish efficacy of the current approach. In fact, such a trial is planned to open in 2017.

Disclosure of Potential Conflicts of Interest

J.G.J.V. Aerts reports receiving commercial research grants from Ampera and Roche, holds ownership interest (including patents) in Ampera bv, and is a consultant/advisory board member for Amphera, Boehringer Ingelheim, Bristol-Myers Squibb, Eli-Lilly, MSD, and Roche. C. van der Leest is a consultant/advisory board member for Abbvie, AstraZeneca, Boehringer, and Roche. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

Amphera b.v. is a spin-off company from the Erasmus MC University and had no access to patient related data.

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Acknowledgments

The authors would like to thank the department of hematology, the apheresis unit, the pharmacy and the research nurses of the department pulmonary medicine of the Erasmus MC for their support in facilitating the production and delivery of the DC vaccination. The authors also thank Dr. Dave Roelen (Leiden University Medical Center, Leiden, the Netherlands) for performing HLA typing of our patients and Jan von der Thüsen (Department of Pathology, Erasmus MC, Rotterdam, the Netherlands) for the immunostainings of patient biopsies. The human trial was funded by the Dutch Cancer Society (to KWF) and ZonMW (grant number 95104006; granted to J. Hegmans) and Amphera b.v.

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Received August 29, 2017; revised November 1, 2017; accepted December 4, 2017; published OnlineFirst December 12, 2017.

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