A Combination Hybrid-Based Vaccination/Adoptive Cellular Therapy to Prevent Tumor Growth by Involvement of T Cells

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Abstract

Cancer immunotherapy with dendritic cell–tumor cell fusion hybrids induces polyclonal stimulation against a variety of tumor antigens, including unknown antigens. Hybrid cells can prime CTLs, which subsequently develop antitumor responses. The aim of this study was to enhance the known antitumor effect of hybrid vaccination (HC-Vacc) and hybrid-primed adoptive T-cell therapy (HC-ACT) using the poorly immunogenic Lewis lung carcinoma (LLC1) model. The strategy used was a combination of a double HC-Vacc alternating with HC-ACT (HC-Vacc/ACT). Using flat-panel volumetric computer tomography and immunohistochemistry, we showed a significant retardation of tumor growth (85%). In addition, a significant delay in tumor development, a reduction in the number of pulmonary metastases, and increased survival times were observed. Furthermore, the tumors displayed significant morphologic changes and increased apoptosis, as shown by up-regulation of gene expression of the proapoptotic markers Fas, caspase-8, and caspase-3. The residual tumor masses seen in the HC-Vacc/ACT–treated mice were infiltrated with CD4+ and CD8+ lymphocytes and showed elevated IFN expression. Moreover, splenic enlargement observed in HC-Vacc/ACT–treated mice reflected the increased functionality of T cells, as also indicated by increased expression of markers for CTL activation, differentiation, and proliferation (Cd28, Icosl, Tnfisf13, and Tnfisf14). Our findings indicate that the combination therapy of dendritic cell–tumor cell HC-Vacc/ACT is a very effective and a promising immunotherapeutic regimen against poorly immunogenic carcinomas. [Cancer Res 2007;67(11):5443–53]

Introduction

Although cancer cells are altered-self cells, they often do not elicit an appropriate response from the immune system. The failure of the host immune system to eradicate most immunogenic tumors may be due in part to a lack of tumor antigen presentation by dendritic cells (1). Dendritic cells are antigen-presenting cells that induce activation and proliferation of naive CD8+ CTLs and CD4+ T helper cells (T H) in a MHC class I- and class II–restricted manner, respectively (2–4). Various strategies have been developed to introduce tumor-specific antigens into dendritic cells to generate a CTL response against tumor cells. Although the entire repertoire of tumor-associated antigens has yet to be defined, some well-defined tumor antigens have been used for dendritic cell–based vaccination (DC-Vacc) strategies in clinical trials (5, 6). A 3.3% overall response rate was reported in a recent review of 1,306 cancer vaccine trials (7). This result highlights the need to improve cancer vaccines and to develop alternative immunotherapeutic antitumor strategies.

From the numerous approaches used for loading dendritic cells with tumor-specific antigens, the most promising results have been obtained with dendritic cell–tumor cell fusions (hybrids), which theoretically express all tumor-associated antigens but still retain the functions of dendritic cells (6, 8, 9). Moreover, single-modality therapies such as hybrid vaccination (HC-Vacc) or adoptive transfer of hybrid-primed lymphocytes (HC-ACT) has been reported to induce potent antitumor responses against poorly immunogenic tumors in a diverse range of animal tumor models and in selected clinical trials (10–12).

However, despite the promising result of the generation of tumor antigen–reactive CTLs in clinical trials using HC-Vacc, tumor regression has been induced only in rare cases (13–15). This limited success of HC-Vacc can partly be ascribed to insufficient numbers of CTLs and to suboptimal in vivo antitumor function of CTLs induced in response to the HC-Vacc therapy. On the other hand, HC-ACT therapy, which directly provides large numbers of in vitro selected, highly active, tumor-specific T lymphocytes, has produced only a transient clinical response (16, 17). Additionally, our previous animal experiments with HC-ACT therapy also yielded weak antitumor potential, suggesting insufficient T-cell persistence in vivo (11).

On the basis of the aforementioned observations, we hypothesized that a combination therapy of both HC-ACT and HC-Vacc may be an optimal method to induce antitumor immune responses against poorly immunogenic carcinomas by providing sufficient numbers of activated tumor-specific CTLs and potent in vivo stimulation that may lead to consistent tumor and metastasis regression. Several pieces of evidence indicate that T-cell activation is responsible for the apoptosis and subsequent tumor reduction observed in HC-Vacc or HC-ACT therapy, but the mechanisms mediating tumor cell death have not yet been elucidated. Previous studies have established two major pathways for CTL cytotoxicity, which are dependent either on perforin or on the FAS/FAS ligand (FASL; refs. 18, 19). However, the extent to which these cytotoxic mechanisms are active in CTL-mediated tumor destruction in vivo remains to be addressed.

To address the above concerns and to overcome the hurdles of a monospecific therapy, we have used an animal model consisting of C57/BL6 mice harboring large, established s.c. LLC1 tumors to test the effect of a combination therapy of repetitive HC-Vacc and
HC-ACT. For a side-by-side comparison, the tumor-bearing mice were treated with PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT therapeutic regimens, and tumor growth, tumor incidence, survival, and metastasis regression were measured. In addition, the molecular mechanisms responsible for cytotoxicity and potentiation of CTLs in the tumors and spleens of HC-Vacc/ACT–treated mice were assessed in detail.

Materials and Methods

Tumor Cell Lines

The C57/BL6 (H-2b)–derived Lewis lung carcinoma cell line (LLC1) and the B16/F10 melanoma tumor line were obtained from American Type Culture Collection and cultured as described (11,20).

Animals

Female C57/BL6 (H-2b) and BALB/c (H-2b) mice were purchased from Charles Rivers, kept under pathogen-free conditions, and handled in accordance with the European Community’s recommendations for experimentation.

Generation of Bone Marrow–Derived Dendritic Cells

Erythrocyte-depleted C57/BL6 mouse bone marrow cells flushed from marrow cavities of femurs and tibias were cultured in RPMI 1640 that was supplemented with granulocyte macrophage-colony stimulating factor, interleukin 4 (IL-4), and fms-like tyrosine kinase 3. On day 6, the nonadherent cells were plated on Petri dishes with RPMI 1640 supplemented with lipopolysaccharide and on day 10 they were removed and stained with a monoclonal anti-CD11c to confirm the dendritic cell phenotype, as described (11).

Generation of Hybrid Cells

Hybrid cells were generated by fusing bone marrow–derived dendritic cells and tumor cells (LLC1) as previously described (11). Briefly, dendritic cells were stained red using the PKH-67-GL Fluorescent Cell Linker kit, LLC1 cells were stained green using the PKH-67-GL Fluorescent Cell Linker kit (Sigma) according to the manufacturer’s instructions. After removing unbound dye, LLC1 cells were transferred into an electroporation cuvette and pulsed at 200 V/cm for 200 μs using a Gene Pulser (Bio-Rad). Treated LLC1 cells and dendritic cells were then mixed at a ratio of 2:1 and incubated in serum-free RPMI 1640 containing 50% polyethylene glycol. A few minutes later, the cells were resuspended in serum-free RPMI 1640 and incubated for 48 h. After the incubation, fusion efficiency was assessed by confocal microscopy and by counting the proportion of double-stained cells. Later, fused mixtures were sorted using fluorescence-activated cell sorting (FACS; FACSCalibur, Becton Dickinson). The sorted cells displaying both green and red fluorescence were harvested and resuspended in medium for in vitro and in vivo assays.

Therapeutic Regimens

For immunotherapy of LLC1 tumors, C57/BL6 mice were injected s.c. with 1 × 10^6 LLC1 cells/200 μL saline to initiate tumor formation. Four days after tumor initiation, mice were randomly divided into several groups.

HC-Vacc (n = 10). For vaccination therapy, on days 5 and 9 (booster dose), tumor-bearing mice were treated with 2 × 10^6 syngeneic irradiated hybrid cells.

HC-ACT (n = 10). For adoptive cellular therapy, on days 6 and 10 (booster dose), tumor-bearing mice were treated i.v. with 1 × 10^7 spleen cells and lymph node cells that were obtained from irradiated hybrid cell–immunized and reimmunized mice and had been cultured with irradiated LLC1 cells and IL-2.

Combination therapies (PBS, DC-Vacc/ACT, HC-Vacc/ACT; each group n = 22). Four days after tumor initiation, mice received an intrafootpad injection of 2 × 10^6 syngeneic dendritic cells or hybrid cells or PBS. On the following day, mice were treated i.v. with 1 × 10^7 spleen and lymph node cells that had been obtained from mice immunized and reimmunized with dendritic cells, from mice immunized and reimmunized with hybrid cells, or from mice sham-treated with PBS and had been cultured with irradiated LLC1 cells and IL-2. After 3 days, mice from each group received a booster dose of PBS, irradiated dendritic cells, or HC-Vacc followed by a booster dose of spleen and lymph node cells the next day.

To evaluate the therapeutic efficiency of the different treatment regimens, mice were monitored for s.c. tumor growth for an additional 3 weeks after the second combination therapy boost. The size of the tumor was measured as described previously (21). Before being sacrificed, mice were monitored for lung metastasis and spleen size using flat-panel volumetric computed tomography. Twenty-eight days after tumor cell injection, each group of mice was sacrificed. Spleen and lymph node cells were harvested from the mice for proliferation and cytotoxicity assays, and s.c. and lung tumors were harvested for assays of gene expression and histomorphologic changes. A representative experimental scheme is provided in Fig. 1A.

Proliferation and Cytotoxicity Assays for Spleen and Lymph Node Cells

Isolated spleen and lymph node cells (1 × 10^7 per well) were stimulated with irradiated LLC1 cells, syngeneic dendritic cells, syngeneic purified hybrid cells, or allogeneic lymphocytes (BALB/c: 1 × 10^6 per well) and assessed for incorporation of tritiated thymidine ([3H]dTh) on days 3 and 5 of culture as described. For cytotoxicity assays, isolated spleen and lymph node cells were cocultured with irradiated LLC1 cells, syngeneic purified hybrid cells, allogeneic T lymphocytes (BALB/c), or syngeneic B16/F10 tumor cells as targets in various ratios. Cytotoxicity was measured in vitro using the standard cell dilution analysis method (11).

Flat-Panel Volumetric Computed Tomography

The flat-panel volumetric computed tomography is a prototype high-resolution computed tomograph developed by General Electric (GE Global Research). Animals were anesthetized, catheterized, and placed in the center of the volumetric computed tomography gantry. All scans were done with 70 kV/200 mA (scan time: 8 s/rotation; 4.2 cm slab thickness/rotation; reconstructed voxel size 70 × 70 × 70 μm). Data were acquired in an axial mode with two rotation steps, covering 8.4 cm. Reconstruction of the raw data of up to 2,000 frames per rotation was done as described (22).

Immunohistochemical and Immunofluorescence Staining

For histologic analysis, s.c. tumor and lung tissues were fixed in 4% formalin and embedded in paraffin. Three-micrometer-thick paraffin sections were stained with H&E. For immunohistochemical staining, briefly, consecutive 5-μm cryostat sections were fixed for 10 min with ice-cold acetone and methanol, air dried, and then hydrated in PBS. Tissues were blocked in 3% bovine serum albumin/PBS for 30 min at 37°C and incubated with rabbit anti-mouse CD3 (Caltbiochem), rat anti-mouse CD8α (clone 53-6.7), rat anti-mouse CD4/L3T4 (clone H129.19), mouse anti-mouse NK-1.1 (clone PK136; all from PharMingen), or mouse anti-mouse Foxp3 (clone Fio7979; bioscience) for 1 h at 37°C in PBS. For indirect immunofluorescence, slides were incubated with Alexa Fluor 488–labeled goat anti-rabbit IgG and goat anti-rat IgG (Molecular Probes). After incubation, all sections were counterstained with nuclear 4’,6-diamidino-2-phenylindole (DAPI) staining and mounted with Dako fluorescent mounting media (DakoCytomation). Quantification was carried out by computer-aided image analysis using the image processing and analysis system Leica DMLA and QWin 500iW software (Leica Instruments). For each specimen, the immunostained area was analyzed in five randomly selected measurement areas (500 × 370 μm) stained with DAPI, and fluorescence intensity of the markers was expressed as a percentage of the whole measurement area. The final result for each mouse group represents the mean value of all measurements (23).

Apoptosis Detection

Apoptosis in s.c. tumor and lung sections was assessed by cell staining using the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) method (In situ Cell Detection kit; Roche) according to the manufacturer’s recommendations. The stained cells were viewed and quantified under a fluorescence microscope (Leica Instruments).
Quantification was carried out by computer-aided image analysis as mentioned in the immunohistochemical staining section. A positive control for TUNEL labeling was prepared using DNase I (Sigma) treatment. The negative control was obtained by omitting terminal transferase from the labeling procedure.

**In vivo Metastasis Assay**

At the end of the treatment protocol, mice were euthanized and lungs were collected and processed for histopathology. Tissue blocks from all lung lobes were dissected and embedded in paraffin. A total of 50 to 80 3-μm serial sections were made from each lung tissue block. These sections were stained with H&E and were analyzed under a light microscope (Leica Instruments) for the presence of tumor cell clusters. Analysis was done in a blinded fashion. The metastases were classified into three grades based on the number of tumor cells present in the section for each metastasis: grade 1, tumor cells ≤20 to 50; grade 2, tumor cells 50 to 100; and grade 3, tumor cells ≥100, as described (24).

**Reverse Transcription-PCR**

For gene expression analysis, RNA was isolated from tumors and CD3+ cells that were derived from spleen and lymph node cells. Briefly, spleen and lymph node cells were isolated using commercially available murine T-cell isolation columns (R&D Systems) according to the manufacturer’s instructions. The resulting cells were washed, counted, and resuspended...
at the appropriate cell densities for use. Purity was confirmed by flow cytometric analysis using a FITC-conjugated CD3 surface marker (clone 17A2; PharMingen). The columns routinely yielded >90% purity for T cells.

Total RNA was isolated from these samples by the TRIzol (Invitrogen) method according to the manufacturer’s instructions. First-strand cDNA was synthesized with the ImProm-II Reverse Transcription System (Promega Corporation) using oligo(dT)_{12-18} primers according to the

![Graph](image_url)

**Figure 2.** Antitumor effects of HC-Vacc/ACT therapy. A, LLC1 tumor-bearing C57BL/6 mice were treated with PBS (●), DC-Vacc/ACT (○), HC-Vacc (●), HC-ACT (▲), or HC-Vacc/ACT therapy (▼). Tumor volume was measured over time after tumor challenge. Points, average tumor volume; bars, SE. *, P value of treatment groups versus PBS control. B, survival advantage of mice with 4-d initiated LLC1 tumors treated with PBS (●), DC-Vacc/ACT (○), HC-Vacc (●), HC-ACT (▲), or HC-Vacc/ACT therapy (▼). *, P value of treatment groups versus PBS control. C, display of three-dimensional angiography images of datasets acquired with flat-panel volumetric computed tomography. Whole-mouse imaging of PBS-, DC-Vacc/ACT–, HC-Vacc–, HC-ACT–, and HC-Vacc/ACT–treated mice after contrast medium administration. The primary tumors were semiautomatically virtually extracted from the whole-mouse images to facilitate tumor volume measurements. Virtually extracted tumors and spleens from mice treated with PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, and HC-Vacc/ACT (n = 4).
manufacturer’s instructions. For quantitative real-time reverse transcription-PCR (RT-PCR) analysis, 2 µL cDNA was placed into a 25 µL reaction containing SYBR Green PCR mix (Invitrogen) and sequence-specific oligonucleotide primers. The thermal cycle conditions used for all reactions were as follows: denaturation, 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. All real-time reactions were carried out on an MxPro 3000P QPCR System (Stratagene), and analysis was done with the MxPro software. At the end of the PCR cycle, a dissociation curve was generated to ensure the amplification of a single product, and the threshold cycle time (Ct value) for each gene was determined. Relative mRNA levels were calculated based on the Ct values and normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (Hprt1). Specific primers used for amplification were given in the Supplementary Data.

Figure 3. Proliferative activity and cytotoxicity of T lymphocytes isolated from mice treated with DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT. Spleen cells and lymph node cells from LLC1 tumor-bearing mice treated with PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy were either cultured with LLC1 cells, dendritic cells, hybrid cells, or allogeneic lymphocytes [BALB/c; mixed lymphocyte reaction (MLR)]. Proliferative activity was determined from spleen and lymph node cells after (A) the first combination therapy (day 8), (B) the second booster combination therapy (day 11), and (C) at the end of the treatment protocol (day 28). Columns, mean of the proliferation index (cpm of test well / cpm of control; n = 5); bars, SE. *, P value of treatment groups versus PBS control. Cytotoxic capacity of spleen and lymph node cells collected from mice treated as described above were tested against LLC1 cells, syngeneic B16/F10 tumor cells, and allogeneic lymphocytes at an effector/target ratio of 40:1. Mean percentage lysis values are shown for (D) the first combination therapy (day 8), (E) the second booster combination therapy (day 11), and (F) at the end of the treatment protocol (day 28; n = 5). *, P value of treatment groups versus PBS control.
Figure 4. Analysis of lung metastasis by flat-panel volumetric computed tomography and histology. A, lung metastasis detection with flat-panel volumetric computed tomography in C57/BL6 s.c. tumor-bearing mice that were treated with PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy. Flat-panel volumetric computed tomography scanning was done 28 d after LLC1 cell instillation. Representative axial images from healthy mice lung and PBS-, DC-Vacc/ACT–, HC-Vacc–, HC-ACT–, and HC-Vacc/ACT–treated mice lungs (top). Histologic analysis with H&E staining of metastatic lung tumor tissue sections after s.c. injection of LLC1 cells (bottom). The cross-sections show therapy-dependent metastatic tumors in mice receiving PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy. Black and white arrows, lung tumors. B, quantification of metastatic tumors by flat-panel volumetric computed tomography (total number of tumor clusters per lung; n = 5). *, P value of treatment groups versus PBS control. C, quantification of metastatic tumors by histology (number of tumor clusters per lung; grade 1, tumor cells ≤20-50; grade 2, tumor cells 50-100; grade 3, tumor cells >100; n = 5). *, P value of treatment groups versus PBS control. D, histology of s.c. and metastatic lung tumors. Three-micrometer s.c. (top) and lung tumor (bottom) sections from mice treated with PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT were stained with H&E.
Maximal tumor regression and survival. Compared with inhibition of tumor growth (24.7%; Fig. 2), HC-Vacc/ACT treatment resulted in only a modest benefit, and 60% of the mice eventually died by 28 days posttumor inoculation (Fig. 2B). As depicted in Fig. 2A and B, HC-ACT treatment also caused a significant regression of tumor growth (57.5%) along with extended survival of the mice (60%).

The HC-Vacc/ACT therapeutic regimen showed the strongest antitumor effect (85%) among all the treatment groups. This tumor regression was sustained and increased over time, reaching a maximum 28 days after tumor inoculation compared with the other treatment groups (85% versus PBS, 83% versus DC-Vacc/ACT, 60% versus HC-Vacc, and 64% versus HC-ACT). Moreover, the mean survival rate was increased with HC-Vacc/ACT therapy. Importantly, only those mice receiving HC-Vacc/ACT showed 86% survival for the entire observation period extending up to 4 weeks after treatment (Fig. 2B). We also observed a small amount of necrosis in the central part of the s.c. tumors from day 20 to the end of the observation period for the HC-Vacc/ACT therapy group mice (14 of 22 animals).

### Data Analysis

All statistical procedures were done using SPSS statistical software, version 15 (SPSS, Inc.). Data analysis was done using the ANOVA one-way test, with the Scheffe post hoc test for multiple comparisons when appropriate. Data are expressed as mean ± SE. Statistical significance was set at \( P < 0.05 \).

### Results

**Generation of DC/LLC1 fusion hybrids.** Surface markers of LLC1 cells, dendritic cells, and hybrid cells were analyzed by flow cytometry. LLC1 cells were stained with the green membrane marker PKH-67 (95%). The characteristic phenotype and activation of bone marrow–derived dendritic cells was confirmed by expression of CD11c, B7.1, and MHC class II (data not shown) in 60% of analyzed cells. Staining of the DC/LLC1 cell fusion hybrids by both CD11c and the LLC1 cell marker showed that 52% of the cells were double stained (Fig. 1B). The fusion efficiency of DC/LLC1 hybrids was also confirmed by assessing double staining of cells with PKH-67 and the costimulatory molecules B7.1 and MHC class II (data not shown).

To assess the success of the DC/LLC1 cell fusion, confocal microscopy was used. The PKH-67–labeled LLC1 cells and PKH-26–labeled dendritic cells showed green and red staining of the cell membrane, respectively. Hybrids appeared yellow when the two membrane marker stains overlapped (Fig. 1C). The hybrid cells showed a higher percentage of double-positive cells after 48 h of culture, and fused cell nuclei were observed in some cells (Fig. 1C).

**Combination of HC-Vacc and ACT therapy results in maximal tumor regression and survival.** Compared with untreated mice (PBS), vaccination with dendritic cells in combination with ACT (DC-Vacc/ACT) treatment resulted in only a modest inhibition of tumor growth (24.7%; Fig. 2A). In contrast, HC-Vacc treatment resulted in a significant reduction in tumor growth (62.1%) compared with the untreated mice or with the DC-Vacc/ACT treatment regimen. However, this regimen only provided a temporary benefit, and 60% of the mice eventually died by 28 days posttumor inoculation (Fig. 2B). As depicted in Fig. 2A and B, HC-ACT treatment also caused a significant regression of tumor growth (57.5%) along with extended survival of the mice (60%).

Most interestingly, the HC-Vacc/ACT therapeutic regimen showed the strongest antitumor effect (85%) among all the treatment groups. This tumor regression was sustained and increased over time, reaching a maximum 28 days after tumor inoculation compared with the other treatment groups (85% versus PBS, 83% versus DC-Vacc/ACT, 60% versus HC-Vacc, and 64% versus HC-ACT). Moreover, the mean survival rate was increased with HC-Vacc/ACT therapy. Importantly, only those mice receiving HC-Vacc/ACT showed 86% survival for the entire observation period extending up to 4 weeks after treatment (Fig. 2B). We also observed a small amount of necrosis in the central part of the s.c. tumors from day 20 to the end of the observation period for the HC-Vacc/ACT therapy group mice (14 of 22 animals).

**LLC1 tumor imaging in mice after HC-Vacc/ACT therapy.** To obtain detailed information on tumor morphology and metastasis formation of treated LLC1 tumor-bearing mice, noninvasive high-resolution flat-panel volumetric computed tomography was used. Contrast medium application and tomography were well tolerated. Scanning of LLC1 tumor-bearing mice showed that the testicle vein drained into the tumor and that there was severe bone destruction in the femur and knee joint. Similar findings were also observed in mice treated with dendritic cells (i.e., tumor tissue infiltrated the knee joint mainly in the areas of the proximal tibia, fibula, and distal femur). In contrast, surprisingly, HC-Vacc, HC-ACT, and HC-Vacc/ACT therapy mice had normal bone structure (Fig. 2C).

### Table 1. Histopathology of HC-Vacc/ACT therapy

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<tr>
<th>Markers</th>
<th>Cell type</th>
<th>PBS</th>
<th>DC-Vacc/ACT</th>
<th>HC-Vacc</th>
<th>HC-ACT</th>
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<td>CD4</td>
<td>TH</td>
<td>1.51 ± 0.29</td>
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<td>2.39 ± 0.76</td>
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<td>CD8a</td>
<td>CTL</td>
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<td>1.08 ± 0.15</td>
<td>3.95 ± 0.26</td>
<td>6.20 ± 0.51 (P &lt; 0.02)</td>
<td>15.89 ± 1.42 (P &lt; 0.0001)</td>
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<td>CD3</td>
<td>T cell</td>
<td>3.99 ± 0.42</td>
<td>5.95 ± 0.95</td>
<td>7.80 ± 0.82</td>
<td>11.46 ± 1.52 (P &lt; 0.003)</td>
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<tr>
<td>Foxp3</td>
<td>Treg</td>
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<td>3.11 ± 0.58</td>
<td>2.01 ± 0.38</td>
<td>4.85 ± 0.46 (P &lt; 0.04)</td>
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<td>1.93 ± 0.54</td>
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<td>Apoptotic cells</td>
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<td>17.46 ± 1.40 (P &lt; 0.0001)</td>
<td>39.14 ± 3.72 (P &lt; 0.0001)</td>
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**A. Immunohistochemical analysis of s.c. tumors treated with combination therapy**

**B. Immunohistochemical analysis of lung tumors treated with combination therapy**

**NOTE:** (A) Indirect immunofluorescence of s.c. and (B) lung tumors stained with markers for TH-CD4; CTL-CD8a; T cells-CD3; T Reg-Foxp3; and NK-1.1 cells. In addition, the sections were stained with TUNEL to detect in situ cell death. Values for the immunostained areas are given as a percentage of the measurement area (500 × 370 μm; \( n = 3 \)). \( P \) values are of treatment groups versus PBS control.
Figure 5. Quantitative RT-PCR analysis of T-cell activation genes, T-cell proliferation genes, and apoptosis-regulating genes. Expression of the T-cell proliferation genes FASL, Cd28, IL-2b, Tnfsf13b, Tnfrsf13c, Tnfsf14, and Icosl in the spleen (A), the apoptosis genes perforin 1 (Prf1), granzyme B (Gzmb), Fas, Casp8, and Casp3 in the tumor (B) and the T-cell activation genes IL-4, IL-5, IL-10, and IFNγ in the spleen (C) were analyzed by real-time quantitative PCR using the ΔΔCt method for the calculation of the regulation factor (RF). Columns, mean data from triplicate real-time PCR reactions from three different cDNA preparations from each treatment group (PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, and HC-Vacc/ACT) and normalized to Hprt1 (n = 3). *, P value of treatment groups versus PBS control.
Furthermore, extraction of tumor regions from flat-panel volumetric computed tomography images showed that the tumor volume was significantly reduced with HC-Vacc/ACT therapy. Tumor volume of mice treated with HC-Vacc/ACT (1.10 ± 0.26 cm³) was much smaller than that of mice treated with PBS (10.86 ± 0.89 cm³), DC-Vacc/ACT (7.15 ± 0.74 cm³), HC-Vacc (5.12 ± 0.78 cm³), or HC-ACT (5.33 ± 0.64 cm³; Fig. 2C). In addition, we observed an increase in spleen size of mice treated with HC-Vacc/ACT therapy (0.66 ± 0.07 cm³) compared with PBS-treated (0.24 ± 0.04 cm³), DC-Vacc/ACT–treated (0.27 ± 0.05 cm³), HC-Vacc–treated (0.28 ± 0.03 cm³), and HC-ACT–treated (0.48 ± 0.05 cm³) mice (Fig. 2C). Furthermore, relatively homogenous contrast enhancement loss was observed, possibly suggesting an increased local population of T lymphocytes mediating tumor suppression.

**Proliferation and cytotoxic responses of HC-Vacc/ACT therapy.** To assess the lymphocyte proliferation and the induction of LLC1 cell–specific cytotoxicity, spleen and lymph node cells from each group of treated mice were harvested at days 8, 11, and 28. As shown in Fig. 3A to C, T lymphocytes harvested from the HC-Vacc–, HC-ACT–, and HC-Vacc/ACT–treated mice showed a time-dependent improvement in proliferation capacity against LLC1 and hybrid cell stimulator cells, with a marked enhancement against hybrid cell stimulators compared with T lymphocytes that were harvested from PBS- or DC-Vacc/ACT–treated mice.

Induction of LLC1-specific cytotoxicity, as tested against LLC1 tumor cells, B16/F10 (second party tumor) cells, or allogeneic T lymphocytes (Fig. 3D–F), showed a negligible cytotoxic response from T lymphocytes of mice treated with DC-Vacc/ACT against LLC1 cells or B16/F10 tumor cells. In contrast, T lymphocytes obtained from mice treated with HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy showed greatly enhanced cytotoxicity against LLC1 cells at days 11 and 28.

**Effect of HC-Vacc/ACT therapy on pulmonary metastasis.** Because LLC1 tumors metastasize to the lung, it was of interest to also test the effect of HC-Vacc/ACT therapy on the formation of distant metastases. Therefore, we analyzed metastatic growth in mice treated with PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy using flat-panel volumetric computed tomography and H&E staining. In vivo images obtained with flat-panel volumetric computed tomography showed metastases only in the lungs for all groups. Almost every lung of the control mice contained numerous metastatic nodules. In contrast, mice treated with the HC-Vacc/ACT therapy showed a profound reduction in metastasis (Fig. 4A). HC-Vacc/ACT treatment significantly reduced the average number of lung nodules (3.4 ± 0.93) compared with mice treated with PBS (14.8 ± 1.15), DC-Vacc/ACT (10.4 ± 1.36), HC-Vacc (10.0 ± 0.89), or HC-ACT (8.8 ± 1.28; Fig. 4B).

Representative histologic features of metastatic tumor nodules in the lungs of mice that received PBS, DC-Vacc/ACT, HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy are depicted in Fig. 4A. Histologic staining with H&E yielded similar results to the flat-panel volumetric computed tomography, with the lung sections of the HC-Vacc/ACT therapy group showing smaller and fewer tumors in total (9.2 ± 0.80) compared with the PBS (34 ± 3.24), DC-Vacc/ACT (29.2 ± 3.39), HC-Vacc (27.6 ± 2.71), and HC-ACT therapy (18 ± 1.51) groups (Fig. 4C). Interestingly, this reduction was seen in all grades of metastasis (grades 1, 2, and 3).

**Effect of HC-Vacc/ACT therapy on tumor histopathology.** H&E staining of LLC1 tumors showed marked differences between those from animals that received the HC-Vacc, HC-ACT, or HC-Vacc/ACT therapy and the PBS and DC-Vacc/ACT controls (Fig. 4D). The central part of the primary tumors treated with HC-Vacc, HC-ACT, or HC-Vacc/ACT displayed an increased number of tiny cells, which we assumed to be apoptotic cells or infiltrating immune cells. To distinguish between these two possibilities, TUNEL staining was carried out. These stainings showed an increased density of apoptotic cells in the tumors treated with HC-Vacc (20.47 ± 1.87), HC-ACT (17.46 ± 1.40), or HC-Vacc/ACT (39.14 ± 3.72). In contrast, virtually no apoptotic cells were detected in s.c. tumors from mice of the PBS (2.97 ± 0.52) or DC-Vacc/ACT (6.31 ± 0.86) therapeutic regimens. Furthermore, in mice treated with HC-Vacc/ACT, the tumor microenvironment became infiltrated with CD3+ (24.25 ± 1.90), CD4+ (7.60 ± 0.99), and CD8+ T lymphocytes (15.89 ± 1.42), in contrast to any other treatment regimen (Table 1A). However, no significant changes in tumor-infiltrating T regulatory cells (Treg) or natural killer (NK-1.1+) cells were observed in HC-Vacc/ACT–treated primary tumors compared with the other therapeutic groups.

Similarly, an increased number of infiltrating CD3+ (22.13 ± 1.81), CD4+ (13.35 ± 1.02), and CD8+ (18.05 ± 1.47) T lymphocytes, as well as a slight increase in apoptosis, was observed in metastatic lesions of HC-Vacc/ACT–treated mice. In all other treatment groups, no dramatic changes were observed (Table 1B). In addition, the number of infiltrating Treg and NK-1.1+ cells was marginally increased in HC-Vacc/ACT metastatic lesions.

**Influence of HC-Vacc/ACT therapy on T lymphocyte proliferation genes and on tumor apoptotic gene expression.** Because the number of T lymphocytes was increased with the HC-Vacc/ACT treatment, we tested whether regulation of T lymphocyte activation-, differentiation-, and proliferation-associated genes was affected in this treatment group. The expression profiles of T lymphocyte proliferation and activation genes were evaluated by quantitative RT-PCR. The mRNA levels of the CTL activation-, differentiation- and proliferation-associated genes Cd28, Fasl, Icos ligand (Iсол), tumor necrosis factor (TNF) receptor (TNFR) superfamily member 13c (Tnfrsf13c), Tnf superfamily member 13b (Tnfsf13b), and Tnfsf14 were up-regulated in spleens of the HC-Vacc/ACT group compared with the PBS, DC-Vacc/ACT, HC-Vacc, and HC-ACT therapy groups (Fig. 5A). Furthermore, the proapoptotic genes Fas, caspase-8 (Casp8), and caspase-3 (Casp3) were up-regulated in tumors of the HC-Vacc/ACT therapy group compared with tumors from any other group (Fig. 5B). Type 1–related cytokine Ifnγ but not type 1–related cytokines (Il-4, Il-5, and Il-10) was also up-regulated in the HC-Vacc/ACT therapy group (Fig. 5C).

**Discussion**

Our present study shows that the combination of repetitive HC-Vacc and HC-ACT results in highly efficient and persistent tumor growth protection. As compared with control animals, tumor growth retardation of 85% and a roughly 3-fold increase in the survival rate could be shown for mice receiving the combination HC-Vacc/ACT therapy. Furthermore, this combination therapy seemed to significantly prevent the outgrowth of lung metastasis (75%) originating from s.c. LLC1 tumors. The HC-Vacc/ACT therapy might have established a favorable environment for the sustained enrichment of functionally active tumor-specific CTLs that cannot be achieved with single modality therapies.

Efficacy of tumor therapy based on tumor/dendritic cell fusion hybrid vaccination or adoptive transfer of hybrid-primed T lymphocytes has, for the most part, not met expectations (10–12).
Although HC-Vacc approaches have been shown to induce potent protection against solid tumors (25, 26), full mounting of antitumor responses is apparently restricted by an inability to maintain long-term, tumor-specific effector T-cell activity. In contrast, the effect of HC-ACT immunotherapy using ex vivo selected and expanded antigen-specific T-cell clones is restricted by limited expansion in vivo, shortened life span, and frequently by inefficient target invasion (27, 28). Additionally, CTL depletion caused by programmed cell death of the majority of the expanded T lymphocytes may also limit the tumor-reducing potential of ACT (29).

We assumed that hybrid vaccination followed by hybrid ACT may boost the in vivo expansion, polyclonality, and antitumor activity of adoptively transferred tumor-specific T cells, thereby reconstituting effective long-lasting immunity in vivo. In accordance with this hypothesis, we found an increase in CD4+ and CD8+ T cells at primary tumor sites and in metastatic lesions of HC-Vacc/ACT–treated mice with LLC1 tumors. Consistent with this, we also observed dramatic splenic hypertrophy in the HC-Vacc/ACT–treated group, as has also been described in other fusion vaccination tumor models (11, 30). These expanded T cells are functional and tumor directed, as impressively shown by the proliferation and cytotoxicity data presented here, where distinct activities selective for the tumor cells were detected. Given the suggestions from both mouse experiments and human clinical trials that the most effective T-cell populations are those that comprise both CTL and T_{H} subsets, it was no surprise that the strongest antitumor responses were obtained with the HC-Vacc/ACT therapy.

Most importantly, the HC-Vacc/ACT therapy benefits probably achieved by increased functionality of T cells can be partly explained by the up-regulation in circulating effector cells of several costimulatory molecules, including members of the B7 family (Icosl, Cd28) and the TNF/TNFFR families (Tnfsf13b, Tnfsf14, and EASL). Thus, a short priming period and adequate T-cell activation by these molecules may have contributed to avoiding late-stage differentiation and activation-induced cell death of effector T cells. Moreover, considering the role of highly expressed Cd28 in amplifying T-cell receptor (TCR)–mediated T-cell activation and proliferation, and Icos in fine-tuning effector T-cell differentiation and function, it is reasonable to assume that up-regulation of these molecules might boost preestablished antitumor responses (31, 32).

Interestingly, the engagement of both Icos and Cd28 along with their ligands and concomitant increase in IFNγ but not IL-10 indicate that both pathways can contribute to Th1 activation in HC-Vacc/ACT mice. Conversely, increased levels of TNF superfamily members can enhance T-cell activation, homing signals, the initiation of cytolytic responses, and suppression of T_{Reg} cells (33–35).

Most interestingly, a 3-fold rise of apoptosis was achieved in primary tumors in the HC-Vacc/ACT therapy group, confirming the in vivo cytolytic efficiency of CTLs generated by this treatment regimen. In contrast, despite a significant reduction of lung metastasis in HC-Vacc/ACT–treated animals, only negligible changes in apoptosis in the metastatic tumors could be observed. This phenomenon might be explained by an altered proportion of T_{Reg} cells and CTLs (CD4+ and CD8+ cells) in the metastatic lesions compared with the primary tumor sites. These data are consistent with the observation that T_{Reg} cells are overrepresented in lesions from cancer patients (36). Proliferation of T_{Reg} cells may be influenced by tumor cells themselves. This assumption is based on the observation that tumors are able to convert dendritic cells into transforming growth factor–β–expressing immature dendritic cells that can promote CD8+ CCR7+ IL-10+ T_{Reg} cell proliferation (37).

Another possibility is that vaccination might promote T_{Reg} function and tumor trafficking instead of selectively improving the function of antitumor effector cells (38).

Our study showed that CTLs were consistently implicated as critical components for tumor regression in vivo. However, the molecular mechanisms by which hybrid-primed CTLs effectively cause tumor cell death in vivo have not been identified. Previous studies have established two major pathways for CTL cytotoxicity, which are dependent on perforin or Fas (18, 19). Up-regulation of IFNγ levels in HC-Vacc/ACT–treated animals emphasizes the fact that a response was mediated by type 1 CD8+ T cells, which predominantly secrete IFNγ and kill either through a perforin- or Fas-mediated pathway, whereas type 2 CD8+ T cells preferentially secrete IL-4, IL-5, and IL-10 and kill through a perforin-mediated pathway (39, 40). The assumption that the FASL-dependent pathway was used is strongly supported by our finding of elevated levels of Fas, caspase-3, and caspase-8 in primary tumor sites in HC-Vacc/ACT–treated mice.

In conclusion, we have shown that a combination therapy with repetitive hybrid cell vaccination and adoptive T-cell therapy has the strongest efficacy to date in suppressing primary and metastatic tumor growth. The therapeutic effect was accompanied by a clear enhancement of distinct T lymphocyte subsets in the primary and metastatic tumor lesions, as well as by FAS-mediated apoptosis. Future studies in preclinical and clinical settings will evaluate the efficacy of this combined method in particular as a consolidation strategy after surgical or chemotherapeutic reduction of the primary tumor mass.

Acknowledgments


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