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Telomerase mRNA-Transfected Dendritic Cells Stimulate Antigen-Specific CD8$^+$ and CD4$^+$ T Cell Responses in Patients with Metastatic Prostate Cancer

Zhen Su,2* Jens Dannull,2* Benjamin K. Yang,* Philipp Dahm,* Doris Coleman,* Donna Yancey,* Sylvia Sichi,* Donna Niedzwiecki,‡ David Boczkowski,† Eli Gilboa,† and Johannes Vieweg3*

Telomerase reverse transcriptase (hTERT) represents an attractive target for cancer immunotherapy because hTERT is reactivated in most human tumors. A clinical trial was initiated in which hTERT mRNA-transfected dendritic cells (DC) were administered to 20 patients with metastatic prostate cancer. Nine of these subjects received DC transfected with mRNA encoding a chimeric lysosome-associated membrane protein-1 (LAMP) hTERT protein, allowing for concomitant induction of hTERT-specific CD8$^+$ and CD4$^+$ T cell responses. Treatment was well tolerated. Intense infiltrates of hTERT-specific T cells were noted at intradermal injection sites after repeated vaccination. In 19 of 20 subjects, expansion of hTERT-specific CD8$^+$ T cells was measured in the peripheral blood of study subjects, with 0.9–1.8% of CD8$^+$ T cells exhibiting Ag specificity. Patients immunized with the chimeric LAMP hTERT vaccine developed significantly higher frequencies of hTERT-specific CD4$^+$ T cells than subjects receiving DC transfected with the unmodified hTERT template. Moreover, CTL-mediated killing of hTERT targets was enhanced in the LAMP hTERT group, suggesting that an improved CD4$^+$ response could augment a CTL response. Vaccination was further associated with a reduction of prostate-specific Ag velocity and molecular clearance of circulating micrometastases. Our findings provide a rationale for further development of hTERT-transfected DC vaccines in the treatment of prostate and other cancers. *The Journal of Immunology, 2005, 174: 3798–3807.

Human telomerase reverse transcriptase (hTERT)$^4$ presents an attractive target against a broad range of tumors, because it is silent in normal tissues, but reactivated and overexpressed in >85% of human solid tumors, including prostate cancer (1). Because hTERT activity is essential for maintaining the proliferative capacity of tumor cells, the risk of Ag escape by genetically unstable tumors may be reduced when targeting hTERT Ags in a vaccination setting. Therefore, hTERT may represent a more suitable target for cancer immunotherapy than other self-Ags expressed by tumors such as MART-1, gp100, NY-ESO-1, and proteins of the MAGE family (2, 3).

Several studies have suggested that hTERT may represent a broadly applicable Ag for use in active immunotherapy protocols. Dendritic cells (DC) pulsed with the HLA-A0201-restricted hTERT peptide 540–548 stimulated CTL responses in vitro (4, 5) and in advanced cancer patients (6). In another study, hTERT peptide-specific CTL clones failed to recognize hTERT- and HLA-A2-expressing tumor cells due to lack of Ag processing by the proteasome (7). Furthermore, immunizing with class I-restricted hTERT peptides does not stimulate CD4$^+$ T cell help, and peptides corresponding to defined hTERT-specific class II-restricted epitopes have just recently been identified (8, 9).

We have previously shown that immunization of mice with hTERT mRNA-transfected DC stimulated tumor-specific CTL responses in vitro and inhibited the growth of unrelated tumors in tumor-bearing mice (10). Similarly, hTERT-transfected DC stimulated hTERT-specific CTL that were capable of recognizing and lysing autologous tumor cells (10), allogeneic HLA-A0201-matched tumor cell lines (11), and hTERT mRNA-transfected DC with similar efficacy. Thus, the use of hTERT mRNA-transfected DC to stimulate a CTL response in vitro and their utility to serve as surrogate targets in immunological assays provide a broadly applicable strategy for the functional validation of candidate Ags in immunotherapy settings. Another advantage of using RNA over peptides as Ag is that RNA encodes multiple epitopes for many HLA alleles, and, therefore, extends the scope of vaccination to cancers in which potent T cell epitopes have not yet been identified. Finally, methods were recently described to introduce RNA into DC with high efficiency by electroporation, allowing easy access of RNA-encoded Ags into the cytoplasmic translation machinery upon entry into the cells (12, 13). Cytoplasmic Ags are channeled preferentially through the class I presentation pathway, thereby activating primarily Ag-specific CD8$^+$ T cells (14). Yet, recent studies have underscored the critical role of the CD4$^+$ T cell arm of the immune system in facilitating the initiation, potentiation, and maintenance of an effective antitumor immune response (15–17).

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4 Abbreviations used in this paper: hTERT, human telomerase reverse transcriptase; DC, dendritic cell; LAMP, lysosome-associated membrane protein-1; PSA, prostate-specific Ag; DTH, delayed-type hypersensitivity; PSA/dt, PSA doubling time.

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As a potential approach to enhance an Ag-specific CD4+ T cell response, we have recently shown that chimeric hTERT transcripts containing the lysosomal targeting signal of lysosome-associated membrane protein-1 (LAMP) (18, 19) can direct hTERT Ag processing into the class II pathway (11, 20). DC transfected with mRNA encoding a chimeric LAMP hTERT protein exhibited enhancement in their ability to stimulate CD4+ T cell responses in vitro, while allowing for concomitant induction of hTERT-specific CD8+ T cell responses (11). Therefore, a clinical study was initiated to test the utility of hTERT mRNA-transfected DC as a general strategy for inducing potentially therapeutic T cell responses in patients with metastatic prostate cancer. In this study, we immunized 20 eligible subjects with hTERT- or LAMP hTERT mRNA-transfected DC and analyzed vaccine safety, immunologic efficacy, and biochemical (prostate-specific Ag (PSA)) response to therapy.

Materials and Methods

Study design

Treatment of patients was performed following written informed consent on an institutional review board-approved protocol. Patients with histologically confirmed metastatic adenocarcinoma of the prostate were eligible for this study. Patients were required to have adequate hepatic, renal, and neurological function, a life expectancy of >6 mo, and a Karnofsky performance status of >70%. Patients must have had recovered from all toxicities related to any prior therapy and not have received any chemotherapy, radiation therapy, or immunotherapy for at least 6 wk before study entry. In patients treated with medical hormonal therapy, evidence of appropriate testosterone suppression was obtained before study entry. Continuation of gonadal androgen suppression was conducted on all patients on luteinizing hormone-releasing hormone analogs. Excluded from study were patients with CNS metastases, patients with a history of autoimmune disease, with serious intercurrent chronic or acute illnesses, or with concurrent second malignancy other than nonmelanoma skin cancer, or controlled superficial bladder cancer. Patients on steroid therapy or other immunosuppressive agents were also excluded.

Vaccine preparation

Cell production was performed in a dedicated cell processing facility at Duke University using standardized, Food and Drug Administration-approved protocols. For DC culture, a concentrated leukocyte fraction was harvested by leukapheresis. PBMC were isolated from the leukapheresis product by density gradient centrifugation over polysucrose/sodium diatrizoate (Histopaque; Sigma-Aldrich), and cells were resuspended in serum-free AIM-V medium (Invitrogen Life Technologies). PBMC were incubated in a humidified incubator for 1 h at 37°C to allow plastic adherence. The semiadherent cell fraction was used for DC culture by incubation in serum-free X-VIVO 15 medium (Cambrex Bio Science) supplemented with recombinant human IL-4 (500 U/ml) and recombinant human IL-10 (100 U/ml; Genzyme). Before administration, cells were phenotypically characterized to ensure that they met the typical phenotype of fully mature DC. Lin-105, HLA class I and II, CD86high, CD83high.

Dose and immunization schedule

Subjects were randomized to receive three or six intradermal injections of hTERT- or LAMP hTERT mRNA-transfected DC. The injections were given intradermally at weekly intervals and consisted of 1 × 107 cells suspended in 200 μl of 0.9% sodium chloride (Abbott Laboratories) at each vaccination cycle. All subjects were evaluated for toxicity, immunological and biochemical (PSA) response to therapy. Follow-up visits occurred bi-weekly for three visits, monthly for one visit, then every 4 mo or until the subject was removed from the study.

Evaluation of immune status

IFN-γ ELISPOT assays were performed using PBMC obtained before, during, and after vaccination. PBMC were cultured overnight in RPMI 1640 medium supplemented with 10% FCS. CD4+ and CD8+ T cells were isolated from PBMC by negative depletion. After blocking wells with complete medium, 1 × 105 T cells and 1 × 104 RNA-transfected DC in 100 μl of complete medium were added to each well of flat-bottom 96-well multiwell plates (Multiscreen-IP; Millipore) precoated with 2 μl of IFN-γ capture Ab. Plates were incubated for 20 h at 37°C, and biotinylated IFN-γ detection Ab (Endogen) was added to each well. Cells were then incubated for an additional 2 h at room temperature, and then streptavidin-alkaline phosphatase (1 μg/ml; Sigma-Aldrich) and substrate (Kirkegaard & Perry Laboratories) was added. After washing, spots were counted using an automated Zeiss KS Elispot Compact reader (Carl Zeiss).

CTL assays were performed by coculturing the RNA-transfected DC with autologous PBMC at a DC:PBMC ratio of 1:10. Cells were restimulated once, and IL-2 (10 U/ml) was added after 5 days and every other day thereafter. After 20 days of culture, effector cells were harvested for cytolytic assays. Target cells were labeled with 100 μCi of Na2[35Cl] (NEN) in 200 μl of complete RPMI 1640 for 1 h at 37°C in 5% CO2, and 35Cl-labeled target cells were incubated in complete RPMI 1640 medium with effectors cells for 4 h at 37°C. Then, 50 μl of supernatant was harvested, and release of 35Cl was measured with a gamma counter. Results from triplicate wells were averaged, and the percentage of specific lysis was calculated.

For Ag-specific proliferation assays, purified CD4+ T cells were seeded into 96-well flat-bottom microplates in the presence of LAMP hTERT- or control mRNA-transfected DC. Cultures were incubated in flat-bottom 96-well plates precoated with the background control. After 5 days of culture, 1 μCi of [methyl-3H] thymidine (NEN) was added to each well, and incubation was continued for an additional 16 h. Cells were collected onto glass fiber filters (Wallac) with a cell harvester, and uptake of thymidine was determined using a liquid scintillation counter.

Analysis of cutaneous injection sites

Skin punch biopsies were obtained under local anesthesia at the periphery of erythematous delayed-type hypersensitivity (DTH) sites that developed after the third injection. The diameter of the DTH reaction was measured 48 h after vaccination, with induration/erythema >4 mm being considered positive. To determine T cell infiltration, biopsy specimens were analyzed by immunohistochemistry. After freezing, tissues were cut at 10 μm, and sections were placed on slides. Acetone-fixed cryosections were incubated with the primary Ab. Binding of the primary Ab was detected using biotinylated secondary Abs followed by avidin-conjugated horseradish peroxidase or alkaline phosphatase. In one patient treated with hTERT mRNA-transfected DC, biopsy tissue was minced and cultured in complete medium supplemented with IL-2- (50 U/ml) and 10 μg/ml PHA-P. Irradiated, allogeneic PBMC were used as feeder cells. Every 2 days, half of the medium was replaced by fresh IL-2-containing complete medium. After 2 wk of culture, T cells were tested for Ag-specific recognition and lysis in 3Cr cytolytic assays.

FACS analysis

Four-color FACS analyses were performed using a BD Biosciences FACSCalibur. Fluorochrome-conjugated Abs as well as isotypic control Abs (CalTAG) were used for T cell staining. A total of 1 × 105 cells was suspended in staining buffer (PBS with 1% FCS, 2 mM EDTA, and 0.1% sodium azide) and incubated for 20 min at 4°C with the Ab. Data were analyzed and presented using CellQuest software. For detection of IFN-γ, isolated CD4+ and CD8+ T cells were activated for 6 h in the presence of autologous mRNA-transfected DC, and cytokine secretion was measured using the IFN-γ secretion assay detection kit (Miltenyi Biotec). Cytometric bead arrays were performed using the human Th1/Th2 cytokine cytometric bead array kit according to the manufacturer’s protocol (BD Pharmingen).

Biostatistical assessment

Pre- and posttreatment serum PSA levels were collected on all patients who completed immunotherapy. PSA doubling time (PSAa) was calculated as described previously (21) using a linear regression model to obtain estimates of the change of serum PSA over time. Two curves were fit for each patient based on all available data points from the PSA nadir to the most recent pretreatment value (pretreatment PSAa), and using all posttherapy data obtained at 2 wk up to 10 wk after completion of the trial (posttherapy week).
PSAdt). For negative values (PSA reductions) or for PSAdt approaching infinite (stable disease), PSAdt was arbitrarily set at 100.0 mo. Differences between the pre- and posttreatment PSAdt (see Fig. 6A) as well as increases in Ag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells after vaccination (Table I) were compared using the Wilcoxon matched-pairs signed rank test, analyzing the null hypothesis that the rates of change in PSA or T cell response were equivalent before and after immunotherapy. A two-sided p value of <0.05 was considered statistically significant.

**Enumeration of circulating tumor cells**

Total RNA was extracted from PBMC using the RNeasy Maxi kit (Qiagen) according to the protocol provided by the manufacturer. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies). cDNA standards were generated by reverse transcription. Primer-specific amplification of mRNA encoding the relevant genes was performed by a technique identical with that validated the Ag-presenting function of the matured, hTERT mRNA-transfected DC by demonstrating Ag-specific T cell proliferation and the stimulation of hTERT-specific T cell responses in vitro (10, 11).

**Results**

**Patient and vaccine characteristics**

Twenty subjects with metastatic prostate cancer were enrolled and randomized to receive either hTERT- or LAMP hTERT mRNA-transfected DC. In each treatment arm, dose escalation was performed using a starting dose of 1 × 10<sup>7</sup> DC. Table I displays the patient characteristics, dose, and treatment assignments are provided in Table I. The vaccines were given strictly intradermally, and the vaccination site was changed at each injection cycle according to a standardized protocol. To generate DC, all patients underwent a single, 2-h restricted leukapheresis processing 10–12 liters of peripheral blood. PBMC were isolated from the leukapheresis product by Ficoll gradient centrifugation, yielding an average number of 8 × 10<sup>6</sup> ± 4 × 10<sup>6</sup> PBMC. DC were generated by culturing plastic semiallogeneic mononuclear precursors in serum-free medium supplemented with the cytokines IL-4 and GM-CSF. After days, immature DC were transfected with hTERT or LAMP hTERT mRNA by electroporation and then matured with the proinflammatory cytokines IL-1β, IL-6, TNF-α, and PGE<sub>2</sub>, as described previously (23). DC yield after culture and mRNA transfection was 8.0 ± 3.5% of the starting PBMC population (6 × 10<sup>8</sup> ± 1 × 10<sup>8</sup> DC). The mRNA-transfected and cytokine-matured DC consistently exhibited the phenotype HLA-Class I<sup>high</sup>, HLA-DR<sup>high</sup>, CD3<sup>neg</sup>, CD14<sup>low</sup>, CD83<sup>high</sup>, and CD86<sup>high</sup>, consistent with mature, monocye-derived DC.

This clinical trial was preceded by extensive preclinical studies that validated the Ag-presenting function of the matured, hTERT mRNA-transfected DC by demonstrating Ag-specific T cell proliferation and the stimulation of hTERT-specific T cell responses in vitro (10, 11).

**Vaccine safety**

Vaccination was well tolerated and no major (more than grade I) treatment-related toxicities were encountered. Four patients demonstrated grade I constitutional symptoms in the form of fatigue or...
flu-like symptoms of short (<48 h) duration. All but two subjects exhibited grade I injection site reactions consisting of inflammatory erythema/induration lasting 48–72 h. To assess autoimmune toxicities, serum levels of anti-nuclear Ab, rheumatoid factor, anti-thyroglobulin Ab, and thyroid-stimulating hormone were measured. In two subjects (13-LMP; 17-TRT), a transient increase in anti-nuclear Ab was observed that subsided within a 2-wk period. No patient developed clinical signs of autoimmune pathology. In one subject, grade III anemia and thrombocytopenia were reported to regulatory agencies, but were considered to be unrelated to vaccine treatment.

**Analysis of intradermal injection sites**

All but two patients enrolled in this trial (05-TRT; 11-TRT), developed pronounced (grade I) inflammatory responses at the site of injection, lasting ~48–72 h. As shown in Fig. 1A, these inflammatory responses increased in size over the duration of five vaccination cycles. There was a trend toward more pronounced DTH reactions in subjects enrolled in the LAMP hTERT group. In six patients who developed such skin reactions, biopsies were obtained 48 h after the third dose (Fig. 1B). Histological analysis of the vaccination site consistently demonstrated profound infiltration with CD4+ and, to a lesser extent, CD8+ T cells in subjects enrolled in both treatment arms, consistent with DTH reactions. The numbers of CD4+ T cells in these specimens were consistently higher than cells expressing the T cell marker CD3, suggesting that other CD4-expressing cells, such as macrophages or neutrophils were involved in this inflammatory response. Interestingly, CD4+ and CD8+ T cells were preferentially clustered in close proximity to hair follicle epidermal cells (arrows) that, according to prior reports, express hTERT (24), as well as HLA class I and class II Ags (25). To determine the Ag specificity of the infiltrating T cells, skin tissue from a patient who was vaccinated with hTERT mRNA-transfected DC was enzymatically digested and T cells were expanded for 2 wk in IL-2 (50 U) and PHA-P in the presence of (irradiated) allogeneic feeder cells. T cells were then analyzed in standard cytotoxicity assays for their capacity to recognize and lyse hTERT-, GFP (control) mRNA-transfected DC targets, or K562 target cells. As shown in Fig. 1C, the ex vivo-expanded T cells were capable of lysing hTERT-, but not GFP-expressing or K562 target cells.

In summary, we show that intradermal injections with ex vivo-matured hTERT- and LAMP hTERT mRNA-transfected DC elicited pronounced inflammatory reactions at the site of vaccination. T cells expanded from DTH sites were hTERT specific and lysed hTERT-expressing target cells. In contrast, DC with an intermediate degree of maturation, applied in prior trials, did not evoke any detectable inflammatory responses or T cell infiltrates at cutaneous injection sites (22, 26).

**FIGURE 1.** Analysis of cutaneous T cell infiltrates. A. Study patients were immunized with weekly doses of hTERT- ([]) or LAMP hTERT (■) mRNA-transfected DC. The diameter of induration was measured 48 h after every injection, each time by the same investigator. Erythema or induration >4 mm was considered significant. B. Skin biopsies were obtained 48 h following the third vaccination, and immunohistochemistry was performed to characterize T cell infiltrates. T cell infiltrates predominantly consisted of CD4+ and, to a lesser extent, CD8+ T cells that were preferentially clustered in close proximity to hair follicle cells (arrows). C. To determine Ag specificity of the infiltrating T cells, skin biopsies were obtained 48 h following the third vaccination and cultured in complete medium supplemented with IL-2 (50 U/ml) and 10 μg/ml PHA-P. Irradiated, allogeneic PBMC were used as feeder cells. After 2 wk of culture, T cells were tested for Ag specificity in standard 3HCr cytolytic assay at the indicated E:T ratios. hTERT mRNA-loaded DC were used as targets (■). As controls, GFP mRNA-loaded DC (○) and K562 cells (△) were used to control for unspecific-, or NK cell-mediated killing.
T cell monitoring in the peripheral blood of study subjects

Because the site of injection was constantly changed in all patients with each treatment cycle, the emergence of a cutaneous T cell infiltrate suggests that vaccine-induced, hTERT-specific T cells residing in the peripheral blood were recruited into the skin compartment following repeated antigenic challenge with the vaccine. To provide direct evidence for the presence of vaccine-induced hTERT-specific T cells in the peripheral blood of the study subjects, we performed IFN-γ ELISPOT analyses and determined the frequencies of hTERT-specific CD4+ and CD8+ T cells from PBMC samples of 12 subjects treated with three weekly vaccinations using hTERT- or LAMP hTERT mRNA-transfected DC. PBMC were collected at baseline and 2 wk after the third vaccination. CD4+ and CD8+ T cells were then isolated using magnetic bead separation techniques. Each T cell subset was cultured overnight with hTERT mRNA-transfected DC targets, and visible spots were enumerated using an automated ELISPOT reader. As controls, DC transfected with mRNA encoding GFP or a chimeric LAMP β-actin protein were used. As demonstrated in Fig. 2, all 12 subjects had undetectable levels of hTERT-specific CD8+ or CD4+ T cells in preimmunization samples. In contrast, a significant increase of hTERT-specific CD8+ T cells after vaccination was observed in virtually all patients (except for patient 08-TRT), and 9 of 12 patients developed a significant hTERT-specific CD4+ T cell response. In contrast, no measurable T cell reactivities against GFP-, or LAMP β-actin (data not shown) mRNA-transfected DC targets were noted, suggesting that the targeting sequence of LAMP-1 does not carry any intrinsic immunogenicity and that cross-reactivities against other LAMP-expressing cells may not be an issue in a vaccination setting. Consistent with our preclinical studies (11), we show (Table I) that a higher proportion of patients vaccinated with LAMP hTERT mRNA-transfected DC exhibited hTERT-specific CD4+ T cell responses and that these CD4+ T cell responses were of a significantly higher magnitude than in subjects vaccinated with the unmodified hTERT vaccine (p = 0.004).

To determine the cytolytic function of the vaccine-induced T cells, hTERT-specific CTL lines were generated from the PBMC of four subjects (two patients treated with hTERT-, and two with LAMP hTERT mRNA-transfected DC) following one restimulation cycle with the respective mRNA-transfected DC. CTL were then analyzed in standard cytolytic assays as described previously (22). As shown in Fig. 3, CTL generated from the two subjects treated with LAMP hTERT mRNA-transfected DC exhibited a higher lytic activity against hTERT-presenting targets than CTL stimulated from the PBMC of subjects enrolled in the hTERT group, suggesting that an enhanced hTERT-specific CD4+ response is capable of augmenting CTL-mediated tumor cell killing and, therefore, vaccine efficacy.

In summary, we show that three weekly doses of hTERT- or LAMP hTERT mRNA-transfected DC are capable of stimulating Ag-specific T cell responses in advanced-stage prostate cancer patients. As suggested by our preclinical studies, it appears that vaccination with LAMP hTERT mRNA-transfected DC resulted in superior stimulation of an hTERT-specific CD4+ response in vivo. Moreover, vaccine-induced CTL exhibited lytic activity against
were eliminated. The remaining hTERT-specific CD8+ T cells were remarkably high, with 0.9–1.8% of CD8+ T cells in the LAMP hTERT group exhibited higher CD4+ T cell numbers in the peripheral blood of prostate cancer patients, suggesting that tolerizing mechanisms did not negatively impact vaccination efficacy (30). Our data further suggest that the LAMP-driven enhancement of CD4+ T cell immunity may facilitate the development of central memory T cells that secreted high levels of IL-2 upon antigenic challenge (Fig. 4B).

Next, the Ag-specific proliferative capacity of CD4+ T cells isolated from pre- and postvaccination PBMC of two subjects treated with hTERT-15 (15-TRT) and LAMP hTERT (19-LMP) mRNA-transfected DC (18-LMP; 19-LMP) resulted in the stimulation of a robust CD8+ T cell response that continuously surged over the entire treatment course and peaked ~2–4 wk after the sixth and final dose. The peak levels of hTERT-specific effector CD8+ T cells were remarkably high, with 0.9–1.8% of CD8+ T cells demonstrating Ag specificity. T cell expansion was followed by rapid contraction, during which ~80–90% of the CD8+ T cell effector cells were eliminated. The remaining hTERT-specific CD8+ T cells were maintained for a minimum of 16 wk. As shown in Table 1, subjects in the LAMP hTERT group exhibited higher CD4+ T cell frequencies after vaccination than patients vaccinated with the unmodified hTERT RNA, who developed only low or insignificant numbers of hTERT-specific CD4+ T cells over the entire treatment course. As observed in other reports (27), the emergence of a T cell response in the peripheral blood correlated with the development of a cutaneous DTH reaction in most subjects analyzed (Fig. 4A, right panels).

To characterize the phenotype of the vaccine-induced, IFN-γ-secreting CD8+ T cells, PBMC were collected from the four study subjects 2 mo after the sixth vaccination and stained with a panel of T cell markers (Fig. 4B). In addition, the ability of the vaccine-induced CD8+ T cells to produce the cytokines IFN-γ, TNF-α, and IL-2 was analyzed after in vitro restimulation (Fig. 4C). Following vaccination with hTERT mRNA-transfected DC, hTERT-specific CD8+ IFN-γ+ T cells (obtained during study week 16), displayed a phenotype of clonally expanded effector CTL at an advanced stage of differentiation and exhibited high expression of CD45RA, CD57, but low expression of CD28 and CD62L (not shown). They also lacked cell surface markers typically found on memory T cells during early stages of differentiation, namely CD27, CD45RO, and CCR7 (Fig. 4B). Upon restimulation, hTERT-specific CD8+ T cells produced the cytokines IFN-γ and TNF-α, but not IL-2, consistent with clonally expanded cytotoxic effector memory T cells (Fig. 4C). In contrast, CD8+ T cells isolated from subjects vaccinated with LAMP hTERT mRNA-transfected DC exhibited higher levels of CD62L and CCR7 (data not shown) and gained the ability to secrete IL-2 (Fig. 4C), suggesting the development of central T cell memory (28, 29).

In summary, the experiments shown in Fig. 4A demonstrate the temporal evolution of an hTERT-specific T cell response in the peripheral blood of study subjects vaccinated with hTERT mRNA-transfected mature DC. Six weekly injections were associated with expanding numbers of hTERT-specific T cells in the peripheral blood of prostate cancer patients, suggesting that tolerizing mechanisms did not negatively impact vaccination efficacy (30). Our data further suggest that the LAMP-driven enhancement of CD4+ T cell immunity may facilitate the development of central memory T cells that secreted high levels of IL-2 upon antigenic challenge (Fig. 4B).

Analysis of vaccine-induced CD4+ T cells

To characterize the LAMP hTERT vaccine-induced CD4+ T cell responses, we performed phenotypic and functional analyses (Fig. 5). CD4+ IFN-γ+ T cells revealed high expression of CD45RA, CD95, CD57 (not shown), and CD44 (not shown), intermediate expression of CD45RO, CD62L, but no expression of CD27 (not shown), and CCR-7, consistent with an effector memory T cell phenotype (Fig. 5A). In addition, high expression of CCR-5 was noted in these T cell subsets that may contribute to the recruitment of CD4+ T cells into peripheral tissues and tumor lesions (31). Next, the Ag-specific proliferative capacity of CD4+ T cells isolated from pre- and postvaccination PBMC of two subjects treated with hTERT-15 (15-TRT) and LAMP hTERT (19-LMP) mRNA-transfected DC was analyzed. CD4+ T cells were isolated from pre- and posttherapy PBMC samples (2 wk after the sixth injection) and restimulated once with LAMP hTERT- or with GFP mRNA-transfected DC (control). Ag-specific CD4+ T cell proliferation was determined in standard proliferation assays.

As shown in Fig. 5B, only background levels of hTERT-specific CD4+ T cell proliferation was detected in prevaccination PBMC samples, whereas CD4+ T cells from patients treated with LAMP hTERT-19 (19-LMP), but not with hTERT mRNA-transfected DC (15-TRT) proliferated after antigenic exposure. LAMP hTERT vaccine-induced CD4+ T cells exhibited lytic function against hTERT-expressing targets (Fig. 5C) and produced the cytokines IL-2, IL-5, and IFN-γ, but little IL-4 (D), suggesting a mixed Th1/Th2-type response.

These results provide further evidence that vaccination with LAMP hTERT mRNA-transfected DC is capable of stimulating an improved hTERT-specific effector/memory CD4+ T cell response in advanced prostate cancer patients. The LAMP hTERT vaccine-induced CD4+ T cells proliferated vigorously in the presence of Ag and, in two subjects analyzed, recognized and lysed hTERT- or not GFP-expressing targets.
Clinical response to vaccination

Serum PSA was measured before and after therapy and PSAAdt was calculated. A total of 12 patients (7 treated with 3 cycles and 5 receiving 6 cycles) with a follow-up of at least 2 mo was available for analysis. Five patients that elected to undergo other forms of systemic or experimental therapy after completion of the trial (03-LMP; 04-TRT; 08-TRT; 10-LMP; 14-LMP) and one patient (09-TRT) who was lost to follow up were excluded. The median pretreatment PSAAdt for patients receiving 3 and 6 vaccination cycles was 4.6 and 2.9 mo, respectively (Fig. 6A). Although no objective clinical responses were noted after vaccination, PSAdt calculations revealed an improvement of PSAdt in the 6-cycle group to 100.0 mo, whereas median posttreatment PSAdt in the 3-vaccine dose group was 3.8 mo (p = 0.61). The change in pre- and posttreatment PSAdt was statistically significant for the 6-cycle group (p = 0.04).

Whenever excess cells not used for immune monitoring were available, we analyzed the kinetics of circulating tumor cells in the treated subjects, as described previously (22). Eighteen patients were evaluable for analysis. Total RNA was extracted from 1 × 10⁷ PBMC collected at several time points before, during, and after the vaccination phase. PSA mRNA was amplified by real-time PCR and PSA mRNA copy numbers were normalized to those specific for the reference gene β-actin amplified from the same PBMC sample. To determine background levels, we determined the average mRNA copy number of PSA from 1 × 10⁷ PBMC of 10 age-matched healthy male volunteers (Fig. 6B, dotted lines). As exemplified in Fig. 6B, among a total of nine patients analyzed in the LAMP hTERT group, four subjects had measurable elevations (above background) in pretreatment samples, all of which were transiently reduced when compared with pretreatment levels during and after vaccination. Among nine patients analyzed in the hTERT group, in six patients detectable (above background) pretreatment PSA mRNA copy numbers could be amplified. Again, a transient reduction or clearance of PSA-expressing circulating tumor cells was observed in five of six subjects.
FIGURE 5. Analysis of vaccine-induced CD4⁺ T cells. A, The cell surface expression of CD4⁺ memory T cell markers was analyzed on hTERT-specific CD4⁺ IFN-γ⁺ T cells using flow cytometry (gray histograms, isotypic controls). B, Ag-specific proliferation of hTERT-specific CD4⁺ T cells before (pre) and after vaccination (post). Negative selection of CD4⁺ T lymphocytes was performed using magnetic bead separation. The isolated T cells were cocultured in triplicate wells with or without (data not shown) LAMP hTERT mRNA-transfected DC. After 5 days of culture, thymidine was added to each well, and incorporation was determined using liquid scintillation counting. C, Cytolytic assays for isolated CD4⁺ T cells were performed using LAMP hTERT or GFP (control) mRNA-transfected DC as targets. D, For detection of intracellular cytokines, CD4⁺ T cells from a patient vaccinated with LAMP hTERT mRNA-loaded DC were stimulated with LAMP hTERT mRNA-transfected DC for 8 h with addition of brefeldin A during the last 4 h. Cells were then fixed, permeabilized, and stained using Abs against IL-2, IL-4, IL-5, and IFN-γ (gray histograms, isotypic controls for anti-cytokine Abs). Cytokine expression by CD4⁺CD69⁺ cells was analyzed by flow cytometry.

FIGURE 6. Impact of vaccination on PSAdt and circulating tumor cells. A, Serum PSA was measured from 12 evaluable study subjects, and pre- and posttherapy PSAdt time was calculated. Linear regression was used to obtain estimates of the pre- and posttherapy PSAdt of individual study subjects. Comparisons between pre- and postvaccination PSAdt were performed using the Wilcoxon matched-pairs signed rank test. Results from seven eligible patients immunized with three weekly cell doses, and from five subjects vaccinated with six doses of hTERT- (▪) and LAMP hTERT (×) mRNA-transfected DC are depicted. B, Real-time PCR was used to quantitatively assess the kinetics of circulating tumor cells at baseline (wk −4 and 0), during (wk 2, 4, 6) and after vaccination (wk 26) in study patients using primers and probes specific for PSA. PSA mRNA was amplified from total RNA extracted from 1 × 10⁷ PBMC, and the corresponding copy numbers were quantitated and subsequently normalized to those specific for the reference gene β-actin amplified from the same PBMC sample. To further improve the sensitivity of this assay, the average mRNA copy number of each marker amplified from PBMC of 12 age-matched healthy male volunteers (dotted lines) was determined. As exemplified in four subjects (01-LMP; 06-LMP; 05-TRT; 08-TRT), vaccination with hTERT mRNA-transfected DC led to a transient clearance of circulating tumor cells in 9 of 10 subjects who had elevated numbers of PSA-expressing circulating tumor cells before therapy.
In summary, these data suggest that vaccination with hTERT mRNA-transfected DC was associated with a short-term impact on PSA-AH and with transient elimination of circulating tumor cells in selected patients. As shown in Figs. 4 and 6, it appears that the impact on these surrogate markers was closely associated with the presence of a measurable, hTERT-specific T cell response and disappeared as soon as the vaccine-induced T cell response subsided. Therefore, continued vaccination or further boosting appears necessary to maintain the hTERT-specific T cell response and enhance a potential clinical impact.

Discussion

We have previously shown that high-level hTERT activity can be induced on DC after transfection with full-length hTERT mRNA, and that these DC can stimulate hTERT-specific CTL in vitro. CTL generated in this manner recognized and lysed autologous prostate cancer after intradermal immunization with hTERT- or LAMP hTERT mRNA-transfected DC. Following six vaccination cycles, expansion of hTERT-specific CD8+ T cells was stimulated at frequencies comparable to those seen after vaccination for infectious diseases that result in clearance of the infection (32) (Fig. 4A). No vaccine-related major toxicities or autoimmune manifestations were seen in any subject. Nevertheless, the potential for subclinical autoimmune reactivities against hTERT-expressing normal tissues must be considered, because hTERT is reactivated in many proliferating tissues such as activated T and B cells, bone marrow, hair follicles (see Fig. 1), germ cells, and intestinal tissues.

A major objective of this study was to enhance an hTERT-specific CD4+ T cell response by immunization with a chimeric LAMP hTERT vaccine. The data presented in Figs. 1–5 cumulatively suggest that vaccination with LAMP hTERT mRNA-transfected DC resulted in 1) more pronounced DTH reactions (Fig. 1), 2) enhanced CD4+ T cell responses (Figs. 3 and 4), 3) increased Ag-specific proliferative responses (Fig. 5), and 4) improved CTL-mediated lytic activity (Fig. 2), when compared with immunization with the unmodified hTERT template. Although there was a strong trend toward an improved CD4+ T cell response in the three subjects treated with six weekly vaccine doses of LAMP TERT mRNA-transfected DC, no formal statistical analysis could be performed due to limited subject numbers. However, a significant enhancement of CD4+ T cell immunity was noted in subjects enrolled at dose level 1 (three weekly cell doses), demonstrating that the attachment of the LAMP sequence to the hTERT template can result in a significantly enhanced CD4+ T cell memory response (Table I). Improvement of T cell memory was also independently suggested by the ability of LAMP hTERT-stimulated CD8+ T cells to secrete the cytokine IL-2 (Fig. 4C). Recent studies performed in murine models demonstrated that only central memory, but not effector memory T cell subsets have the ability to 1) rapidly proliferate after re-exposure to Ag, 2) to produce IL-2, and 3) to persist long-term in vivo by undergoing homeostatic proliferation in response to IL-15 and IL-7 (29, 33). These findings may have important implications for determining the optimal time for boosting, because sufficient numbers of central memory T cells should be present before a booster immunization is given. Unexpectedly, vaccination with the unmodified hTERT template alone was sufficient to stimulate a modest, but detectable CD4+ T cell response (Figs. 2 and 4) in vivo. It is possible that cytoplasmic Ags like hTERT may gain access to the class II presentation pathway via autophagy, whereby cytoplasmic material is sequestered into endoplasmic reticulum-derived vesicles, called autophagosomes, which then fuse with endocytic or lysosomal vesicles (34).

Recent studies have not only underscored the importance of DC maturation to enhance the immunologic potency of the administered DC, but also raised the concern that vaccination with immature DC could induce immune suppression (30). Furthermore, highly efficient transfection protocols have been recently developed that allow mRNA transfection of DC by electroporation (12, 35). Therefore, in this trial, ex vivo-matured and electroporated DC were clinically applied for the first time in human subjects with metastatic prostate cancers. Despite these modifications, the number of vaccine-induced T cells detected in the peripheral blood after three vaccination cycles does not appear to be strikingly different from what was seen in our prior clinical trials, in which immature monocyte-derived DC were passively transfected with PSA mRNA (22), or total tumor RNA (26), and injected into patients who then consistently exhibited a T cell response after vaccination. It is possible that hTERT as an Ag with low immunogenicity has counterbalanced the benefits of DC maturation and RNA electroporation. Alternatively, effects of the electroporation technology or the DC maturation protocol must be also considered as potential factors that may negatively impact DC function.

An important feature of this early clinical experience is the consistency of detecting hTERT-specific T cell responses in the immunized subjects, thus providing a baseline of biological activity that we seek to improve in future studies. We expect that this trial will aid in the decision-making process to systematically develop increasingly effective DC vaccination protocols targeting hTERT expressed by prostate and other cancers.

Disclosures

J. Vieweg and E. Gilboa are consultants and shareholders (options) for MERIX Biosciences.

References


