Vaccination of Cancer Patients Against Telomerase Induces Functional Antitumor CD8+ T Lymphocytes

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Abstract

Purpose: High-level expression of the telomerase reverse transcriptase (hTERT) in >85% of human cancers, in contrast with its restricted expression in normal adult tissues, points to hTERT as a broadly applicable molecular target for anticancer immunotherapy. CTLs recognize peptides derived from hTERT and kill hTERT+ tumor cells of multiple histologies in vitro. Moreover, because survival of hTERT+ tumor cells requires functionally active telomerase, hTERT mutation or loss as a means of escape may be incompatible with sustained tumor growth.

Experimental Design: A Phase I clinical trial was performed to evaluate the clinical and immunological impact of vaccinating advanced cancer patients with the HLA-A2-restricted hTERT I540 peptide presented with keyhole limpet hemocyanin by ex vivo generated autologous dendritic cells.

Results: As measured by peptide/MHC tetramer, enzyme-linked immunospot, and cytotoxicity assays, hTERT-specific T lymphocytes were induced in 4 of 7 patients with advanced breast or prostate carcinoma after vaccination with dendritic cells pulsed with hTERT peptide. Tetramer-guided high-speed sorting and polyclonal expansion achieved highly enriched populations of hTERT-specific cells that killed tumor cells in an MHC-restricted fashion. Despite concerns of telomerase activity in rare normal cells, no significant toxicity was observed. Partial tumor regression in 1 patient was associated with the induction of CD8+ tumor infiltrating lymphocytes.

Conclusions: These results demonstrate the immunological feasibility of vaccinating patients against telomerase and provide rationale for targeting self-antigens with critical roles in oncopogenesis.

Introduction

As a potential molecular therapeutic target for cancer, the telomerase reverse transcriptase (hTERT) has been intensively scrutinized because of its near universal expression in human cancer cells, and its critical functional role in tumor growth and development (1). One proposed clinical strategy is hTERT-directed immunotherapy, supported by the identification of immunogenic hTERT epitopes that trigger tumor-lytic T cells in preclinical in vitro human studies (2). Telomerase maintains chromosomal integrity by protecting telomeric DNA that would otherwise be lost during successive rounds of cell division in rapidly dividing cells such as tumor cells (3, 4). Because >85% of all human cancers express telomerase activity (5), there is potential to extend strategies of hTERT-specific immunotherapy to the majority of patients with common cancers. Targeting hTERT immunologically may also minimize immune escape due to antigen loss, providing a test of the hypothesis that molecules essential to the neoplastic process and presented by HMC antigens can function as effective tumor antigens for which mutation or deletion is incompatible with sustained tumor growth (6). Tumor antigen down-regulation in the face of specific, high-avidity T cells has been a well-documented mechanism of immunoresistance in trials targeting antigens associated with but not required for the growth of particular cancers (7–9). In contrast, pharmacological or genetic inhibition of hTERT activity in human tumors that express telomerase activity leads to growth arrest in vitro without the outgrowth or development of hTERT-negative escape mutants (10–12).

In our previous experiments, hTERT-specific CTLs generated in vitro from healthy individuals or cancer patients using the hTERT peptide I540 (ILAKFLHWWL) label brightly with peptide/MHC tetramers and kill a range of hTERT+ tumor cell lines and primary tumors in a peptide-specific, MHC-restricted fashion (13, 14). A second group independently generated I540-specific CTLs and confirmed lysis of telomerase-positive, HLA-A*0201 (HLA-A2)+ tumors (15). The I540 peptide binds strongly to HLA-A2, the most frequently expressed HLA allele among nearly 50% of Caucasians, Asians, and Hispanics, and 33% of African-Americans. Two other lines of evidence demonstrate that hTERT I540 peptide is naturally processed and presented by tumors. First, I540-specific CTLs lyse HLA-A2+, telomerase-negative sarcoma tumor cells only after retroviral infection with full-length
hTERT but not vector alone (13). Second, Lev et al. (16) described recently the isolation of human antibodies from a large nonimmune repertoire of human Fab fragments displayed on phage that bind with high affinity to the IS40 peptide/HLA-A2 complex. These antibodies were used to directly visualize the specific HLA-A2/hTERT epitope on antigen-presenting cells as well as on the surface of tumor cells. In contrast, Ayyoub et al. (17) reported that hTERT IS40-specific clones failed to recognize telomerase-positive HLAA2+ tumor cells and that in vitro proteasome digestion studies showed inadequate hTERT processing.

Multiple rounds of peptide stimulation are required to generate hTERT-specific CTLs in vitro, suggesting that the circulating precursor frequency of this repertoire even in patients with advanced cancer is quite low (14). Thus, successful strategies for hTERT vaccination will likely need to prime or expand a low-frequency CD8+ T-cell repertoire that may be limited by mechanisms of tolerance or immunological ignorance to this self-antigen.

Safety presents another important issue for telomerase-directed therapeutics, including immunotherapy. Certain normal cells express telomerase activity and may present a risk for deleterious clinical side effects. Although in vitro studies in human systems suggest that hTERT is a poor autoantigen for telomerase-positive hematopoietic progenitor cells or activated T lymphocytes (13–15, 18), the sensitivity of the assays used may be too low. Murine studies in vivo demonstrate the generation of TERT-specific protective immunity without the development of autoimmunity against TERT-expressing cells (19), but mouse models may not adequately predict effects of targeting telomerase in humans. Both neoplastic and benign murine tissue have a far greater telomere length reserve than human cells, with a pattern of mouse TERT expression in normal cells far more extensive than hTERT expression in normal human tissue (20). A number of other candidate hTERT-directed therapies, including oligonucleotide hTERT template inhibitors (21) and hTERT-promoter directed oncolytic gene therapies (22) have shown encouraging efficacy in preclinical xenograft models but potential human toxicity of these approaches also remains uncharacterized.

We now report the safe induction of hTERT-specific CD8+ T cells after repeated vaccination of advanced cancer patients against the HLA-A2-restricted hTERT IS40 peptide presented with keyhole limpet hemocyanin (KLH) by ex vivo generated autologous dendritic cells (DCs). Immune responses and clinical evidence of antitumor activity were observed in the absence of toxicity. These results support ongoing efforts to develop immunotherapy directed against hTERT and other widely expressed self-antigens linked to oncogenesis.

Materials and Methods

Study Design and Patients. The clinical protocol was an open-label prospective single-institution study of HLA-A2-positive patients with progressive metastatic breast cancer resistant to conventional cytotoxic therapy or progressive hormone-independent prostate cancer. The protocol was approved by the Dana-Farber/Partners Cancer Care Institutional Review Board and conducted with Food and Drug Administration approval of an investigator-sponsored investigational new drug application. Signed, written informed consent was obtained, as required, from each patient. To be eligible, patients had to be ≥18 years of age with a baseline Eastern Cooperative Oncology Group Clinical performance status ≤1. At baseline, they had to have adequate hematological function (hemoglobin >10 g/dl, white blood count >3,000 cells/mm³, absolute lymphocyte count >1,000 cells/mm³, and platelet count >75,000 cells/mm³), adequate renal function (serum creatinine <1.5 times upper limit of normal), adequate hepatic function (total bilirubin <1.5 upper limit of normal and aspartate aminotransferase and alanine aminotransferase <2.5 times the upper limit of normal), and a contrast computed tomography or magnetic resonance imaging scan of the brain negative for metastatic disease. For female patients of childbearing potential, a negative pregnancy test prior to leuphapheresis was required. Patients were excluded for a history of brain metastases; positivity for HIV, hepatitis B virus, or hepatitis C virus; active infection; use of chemotherapy, radiation therapy, immunotherapy, immunosuppressive drugs, glucocorticoids, hematopoietic growth factors, or other investigational drugs within 30 days of leukapheresis; use of anticoagulants or nonsteroidal anti-inflammatory drugs within 7 days of leukapheresis; history of bone marrow or stem cell transplantation; history of autoimmune disease; significant comorbid disease; and history of alcohol abuse or illicit drug use within 12 months of enrollment. Concomitant use of chemotherapy, radiation therapy, immunotherapy, steroids, immunosuppressive agents, nonsteroidal anti-inflammatory drugs, anticoagulants, or other investigational drugs was not allowed.

Vaccine Production. Before vaccination, patients underwent one or two leukaphereses, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation. DCs were generated from plastic adherent blood mononuclear precursors by plating PBMC at the equivalent of 25 × 10⁶ monocytes in triple flask tissue culture vessels (500 cm² total surface area; Nunc, Rochester, NY) in 25 ml of Iscove’s modified Dulbecco’s medium with 5 mm glutamine and 25 mM HEPES (Life Technologies, Inc., Rockville, MD) and 1% autologous plasma (DC medium) for 2 h at 37°C in a 5% CO₂ incubator. Nonadherent cells were subsequently removed, and the adherent cells were cultured in 25 ml of DC medium containing 50 ng/ml clinical grade granulocyte macrophage colony-stimulating factor (Immunix, Seattle, WA) and 20 ng/ml of sterile, good manufacturing practice-grade recombinant human interleukin 4 (R&D Systems, Minneapolis, MN). Cytokines were replenished on day 3 or 4. After 7 days, nonadherent cells were collected, washed in Iscove’s modified Dulbecco’s medium, and pulsed for 2 h at 37°C in a 5% CO₂ incubator at 2 × 10⁶ cells/ml with KLH (10 μg/ml; sterile, endotoxin-free good manufacturing practice grade; Intracel Corporation, Rockville, MD), and one of three peptides synthesized by standard Fmoc chemistry (>94% pure, good manufacturing practice grade; Multiple Peptide Systems, San Diego, CA). Peptides used were hTERT IS40 (ILAKFLHVL) at 60 μg/ml, HIV RT-pol476 (ILKEPVHGV; Ref. 23) at 40 μg/ml, and influenza MP58 (GILGFVFTL; Ref. 24) at 10 μg/ml. After pulsing, DCs were harvested, counted, and an appropriate number of DC were resuspended in 1.0 ml of PBS (Life Technologies, Inc.) and transferred to a syringe for sterile
administration. Release criteria included >70% viability by trypan-blue exclusion, negative final product gram stains, and negative bacterial cultures initiated on days 0 and 4. All of the cultures were endotoxin-free and Mycoplasma negative.

**Study Treatments.** Eligible patients were s.c. administered a total of $15 \times 10^6$ autologous DCs every other week for up to six vaccinations. For each vaccination, three injections were given: $5 \times 10^6$ DC pulsed with I540 hTERT peptide and KLH were injected in a right-sided extremity, $5 \times 10^6$ DC pulsed with MP58 influenza peptide and KLH were injected in a left-sided extremity, and $5 \times 10^6$ DC pulsed with RT-pol476 HIV peptide and KLH were injected in the same left-sided extremity. Injections alternated, when possible, between upper and lower extremities. Injections were given either on the inner aspect of the arm within 10 cm of the axilla or on the anterior aspect of the thigh within 10 cm of the inguinal region. Physical exam and laboratory assessment were performed at the time of each vaccination and within 30 days of the final vaccination.

Bone marrow aspirates were obtained at the time of the first and third vaccination. In 1 patient, biopsy of skin tumor nodules was performed per protocol at baseline and repeated after the third vaccination. Aspirates and biopsy tissue were evaluated at the clinical pathology laboratory of the Brigham and Women's Hospital. Toxicities were evaluated and graded according to the National Cancer Institute's Common Toxicity Criteria. Tumor staging studies were performed at baseline and repeated within 30 days of the final vaccination, and clinical response was determined using standard criteria.

**Samples for Immunoassessment.** Phlebotomy was performed at the time of each vaccination and within 30 days of the final vaccination, together with a simultaneous measurement of the complete blood count and differential. PBMCs were isolated by Ficoll centrifugation and frozen at $-150^\circ$C before the performance of immunoassessment assays. Phenotypic analysis of lymphocyte subsets was performed using monoclonal antibody (mAb) and isotype controls by flow cytometry as described previously (13). Immunoperoxidase studies of tumor biopsy samples were done using routine clinical techniques and diamobenzidine as a chromogen in the Department of Pathology, Brigham and Women's Hospital. In situ hybridization for hTERT mRNA was performed on prevaccination paraffin-embedded archival tumor tissue using probes as described previously (18).

**MHC Class I Tetramer Analysis.** Soluble HLA-A2 tetramers were prepared with immunizing peptides and β2-microglobulin as described (25), conjugated to phycoerythrin, and validated using peptide-specific CTLs as described (14). Control tetramer was made with the HLA-A2-binding peptide L11 (LGFGYPYVY; Ref. 26) from HTLV-1 tax and validated using L11-specific clones (14). Cells were incubated with tetramers and with mAbs CD8-FITC (ImmuneTech, Marseilles, France), CD4-PerCP (Becton-Dickinson, San Jose, CA), and CD14-PerCP (Becton-Dickinson) for 30 min at room temperature. For phenotypic analyses, mAbs used were CD45RA-allophycocyanin (APC), CD45RO-APC, CD28-APC, CD27-FITC (PharMingen, San Diego, CA), anti-CCR7-FITC (R&D Systems), and CD8-APC or CD8-FITC (ImmuneTech).

**In Vitro Peptide Stimulation.** Thawed PBMCs (10^6/well) were incubated with autologous irradiated (32Gy) PBMCs (10^6/well) in the presence of peptide (10 μg/ml: New England Peptide, Fitchburg, MA) or β2-microglobulin (2.5 μg/ml; Sigma, St. Louis, MO) in complete medium (RPMI 1640 with 10% human AB serum, 2 mM glutamine, 20 mM HEPES, and 15 μg/ml gentamicin) and 10 ng/ml of interleukin 7 (Endogen, Woburn, MA) in 24-well tissue culture plates. After 24 h and again on day 5, interleukin 2 (20 IU/ml; Chiron Corp., Emeryville, CA) was added, and cells were analyzed on day 8.

**Enzyme-Linked Immunospot (ELISPOT) Analysis.** For IFN-γ analysis, uncultured or in vitro-stimulated PBMCs at $2.5 \times 10^6$ cells/well were added to ImmunoSpot plates (Cellular Technology, Cleveland, OH) precoated with 10 μg/ml of anti-IFN-γ mAb (Mabtech, Nacka, Sweden) in the presence or absence of 5 μg/ml of peptide overnight at 37°C. After washing, wells were incubated with 1 μg/ml biotin-conjugated anti-IFN-γ mAb (Mabtech) followed by streptavidin-alkaline phosphatase (Mabtech). Purified anti-CD3 mAb was used as a positive control. Experiments were performed in triplicate. Spots were developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium color development substrate (Promega, Madison, WI). Spots were counted using a Prior ProScan analyzer and Image Pro Plus software (Hitech Instruments, Edge- mont, PA), and results shown as mean ±1 SD.

**Polyclonal Expansion and Tetramer-Guided High Speed Sorting.** PBMCs (1 × 10^6 cells/ml) were stimulated with irradiated K562 cells (0.5 × 10^6 cells/ml) expressing 4-1BB-ligand and CD32, and loaded with anti-CD3 (OKT3) and anti-CD28 (9.3) mAb as described (27). Cultured T cells were restimulated with K562 transfectants every 8–10 days. For cell sorting, cells were labeled with CD8 mAb and hTERT I540 tetramer before isolation using a MoFlo sorter (DakoCytomation, Fort Collins, CO). Cells obtained after sorting were stimulated using irradiated K562 transfectants, and ELISPOT analyses were performed after the first expansion/sort cycle. Four-h chromium release assays were performed after the first or second expansion/sort cycle for which specific lysis of target cells was calculated from triplicate determinations using cpm of (experimental result – spontaneous release)/maximum release – spontaneous release). SD was <5%. SW-480, SK-OV-3, and HBL-100 cells (American Type Culture Collection, Manassas, VA) were evaluated for HLA-A2 expression and for telomerase activity as described previously (13). CD40-activated B cells were generated as described previously (13).

**Lymphocyte Proliferation Assay.** Cryopreserved PBMCs obtained before and after vaccination were thawed and assayed simultaneously by incubating with 1 μg/ml of KLH (Intracel Corp.) or medium alone at 100,000 PBMCs/well in a 96-well round-bottomed tissue culture plate. On day 6, 1 μCi of [3H]thymidine was added and plates were harvested 18 h later. Experiments were performed in six replicates. Stimulation index was defined as the ratio of cpm with and without KLH, and shown as mean ±1 SD.

**Results**

**Toxicities and Clinical Response.** A total of 34 vaccinations were given to 7 HLA-A2+ adult patients with hormone-refractory metastatic prostate cancer or chemotherapy-resistant metastatic breast cancer (Table 1). The immunogen used was...
HLA-A2-restricted hTERT peptide I540 loaded with KLH onto ex vivo generated DCs. Manufactured DCs expressed high levels of CD11c, CD86, and HLA-DR but low levels of CD83, CD80, and CD14 (Fig. 1), consistent with an intermediate differentiation of precursors between fully immature and fully mature monocyte-derived DCs (28). Inoculation with 10^6 peptide-pulsed DCs per vaccination was well-tolerated, and no grade 3 or 4 adverse events or laboratory abnormalities were observed, except for tumor pain in UPIN 901 related to spinal cord tumor progression 1 week after the first vaccine. Escalation to the second (30^6 DCs per vaccination) and third (90^6 DCs/vaccination) dose level was not made because the three or more leukaphereses found to be required was deemed infeasible for patients. Because hTERT is expressed in normal bone marrow, bone marrow aspirates were evaluated pre- and postvaccination, but no histological changes were observed (data not shown). Similarly, given previous observations that hTERT-specific CTLs can lyse activated B cells in vitro (13), serum immunoglobulin levels and absolute peripheral B lymphocyte counts were monitored, but no significant reductions were observed after vaccination.

For 4 patients (UPIN 901, UPIN 904, UPIN 905, and UPIN 907), baseline archival tumor tissue was available for the evaluation of hTERT expression by in situ hybridization; in each case, extensive hTERT expression was observed in tumor cells (Fig. 2, A–D).

Among 6 evaluable patients, 1 mixed clinical response was observed. This patient (UPIN 907) had metastatic breast cancer confined to multiple skin nodules on her chest wall that before vaccination had progressed despite chemotherapy, radiation therapy, and hormonal therapy. After vaccination, partial tumor nodule regression was observed without the appearance of new nodules or new sites of disease. Bidimensional measurement of the largest lesion demonstrated a 60% reduction, but overall objective criteria for partial response were not met. Sequential biopsies of one lesion performed before and after vaccination demonstrated the induction postvaccination of a predominant CD8+ lymphoid infiltrate into the tumor (Fig. 2, E–H), whereas only poorly differentiated carcinoma with no infiltrating lymphocytes was observed in the baseline biopsy. Because the biopsy specimen was placed directly into formalin, isolation of tumor-infiltrating lymphocytes in a cell suspension was not possible.

For 4 patients (UPIN 901, UPIN 902, UPIN 903, and UPIN 904), baseline archival tumor tissue was available for the evaluation of hTERT expression by in situ hybridization; in each case, extensive hTERT expression was observed in tumor cells (Fig. 2, A–D).

Table 1  Patient characteristics and responses

<table>
<thead>
<tr>
<th>Patient</th>
<th>Carcinoma</th>
<th>Age (yrs)</th>
<th>Prior therapy for advanced disease</th>
<th>No. of vaccinations given</th>
<th>Telomerase reverse transcriptase CTL immune response</th>
<th>Clinical response</th>
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<tbody>
<tr>
<td>UPIN 901</td>
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<td>60</td>
<td>H</td>
<td>1</td>
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<td>NE</td>
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<td>UPIN 902</td>
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<tr>
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</tr>
<tr>
<td>UPIN 907</td>
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<td>52</td>
<td>C, H, R</td>
<td>3</td>
<td>Yes</td>
<td>MR</td>
</tr>
</tbody>
</table>

a HI Prostate, hormone-independent prostate cancer; Met Breas, metastatic breast cancer.

b H, Hormonal therapy; C, chemotherapy; I, immunotherapy; R, radiation therapy; T, trastuzumab.

c NE, not evaluable; SD, stable disease; PD, progressive disease; MR, mixed response.
possible. Biopsy staining with hTERT I540 tetramer was also not possible, as the current state-of-the-art of this technique requires imbedded fresh tissue. Formalin fixation also made evaluation of HLA-A2 expression by the tumor impossible.

Four other evaluable patients (each with prostate cancer) had stable disease by standard radiographic assessment postvaccination, and 1 patient (with breast cancer) had progressive disease. For each prostate cancer patient, prostate specific antigen

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**Fig. 2** Evaluation of tumor biopsies from patient UPIN 907 before (A–E) and after vaccination (F–H). Telomerase reverse transcriptase expression was revealed by specific hybridization with the antisense probe (A and B) but not the control sense probe (C and D). Biopsy of tissue before vaccination is shown in both bright field (A and C) and dark field (B and D). Similar results were obtained for each of four patients evaluated by in situ hybridization. H&E evaluation of the baseline biopsy for UPIN 907 (E) demonstrated poorly differentiated breast carcinoma. After vaccination (F), biopsy showed infiltration by lymphocytes, most of which were shown to express CD45 (G) and CD8 (H) by immunohistochemistry of serial sections. Original magnification A–D, ×400; E–H, ×200.
levels increased modestly postvaccination (1.88 ± 0.44 average fold increase over 3 months), although in 2 cases, PSA levels fell slightly below baseline at the midpoint of the vaccine schedule before rising again. Hormonal therapy for each patient with prostate cancer was uninterrupted before and during the trial; 2 patients had undergone bilateral orchiectomy previously (15 months and 3 years before vaccination), and 3 patients remained on standard-dose leuprolide (initiated 7 months, 2.5 years, and 6 years before vaccination).

**Induction of CD8+ T-Cell Responses to hTERT Peptide.** We used peptide/MHC tetramers to track the induction of hTERT I540-specific CD8+ cells after vaccination, examining both uncultured PBMCs and PBMCs sensitized to peptide for 1 week in vitro. At baseline in each patient, no hTERT I540-specific CD8+ cells were identified down to the limit of detection (<0.03% of CD8+ cells) in uncultured PBMCs. However, in 3 patients after vaccination, tetramer+ CD8+ cells were observed in uncultured PBMCs, ranging from 0.26% in patient UPIN 906 to 0.58% in patient UPIN 904 (Fig. 3A).

Tetramer analyses of PBMCs after in vitro sensitization (IVS) with I540 hTERT peptide corroborated these results. Again, at baseline in all of the patients, no I540 hTERT tetramer+ CD8+ cells were detectable in PBMCs stimulated in vitro for 1 week with hTERT I540 peptide (Fig. 3A) or the negative control HTLV-1 L11 peptide (data not shown). However, in the same 3 patients with detectable hTERT-specific CD8+ T cells in uncultured PBMCs, a 0.9%–6.3% population of hTERT I540 tetramer+ CD8+ cells was identified after IVS (Fig. 3A). These cultures did not stain with the negative control HTLV-1 L11 tetramer. Baseline or postvaccine PBMCs stimu-

![Fig. 3](attachment:fig3.png)
induced in vitro with HTLV-1 L11 peptide failed to stain with either hTERT I540 tetramer or HTLV-1 L11 tetramer (data not shown). In a fourth patient, for whom no hTERT I540 tetramer+ CD8+ cells were detectable in uncultured PBMCs, such cells were evident after IVS of PBMCs with hTERT I540, but not HTLV-1 L11, peptide (Fig. 3A). Interestingly, this was the patient with a mixed clinical response and evidence on biopsy for the induction of CD8+ tumor infiltrating lymphocytes postvaccination (Fig. 2).

Multiple DC vaccinations were required for the induction of hTERT I540-specific CD8+ cells, as no tetramer+ cells were identified before the third or fourth vaccination in either uncultured PBMCs or in vitro peptide-sensitized PBMCs. Of the 3 nonresponding patients, 1 received only one vaccination, although the other 2 received a full course.

Functional Activity of hTERT-Specific CD8+ T Cells. Uncultured PBMCs and PBMCs sensitized to peptide for 1 week in vitro were also evaluated by ELISPOT analysis to determine the cytokine-secreting capacity of hTERT-specific CD8+ cells induced after vaccination. For uncultured PBMCs, no hTERT-specific responses were detected pre- or postvaccination. However, for each of the 4 patients with tetramer responses, hTERT I540-stimulated PBMCs secreted IFN-γ when rechallenged during ELISPOT analysis with hTERT I540 peptide but not HTLV-1 L11 negative control peptide (Fig. 3B). No IFN-γ secreting hTERT-specific cells were identified in any patient before vaccination (Fig. 3B).

To analyze tetramer-binding cells for antitumor cytotoxicity, tetramer+ CD8+ cells from PBMCs were enriched in vitro using a combination of polyclonal expansion and tetramer-guided, high-speed cell sorting. To do this, postvaccination PBMCs from 3 responding patients (UPIN 903, UPIN 904, and UPIN 906) were expanded polyclonally for 7 days using K562 transfectants expressing ligands for CD3, CD28, and 4-1BB (27), and then sorted to enrich hTERT I540 tetramer+ CD8+ cells and hTERT I540 tetramer− CD8+ cells. Sorted cells were then restimulated with K562 transfectants and resorted. This approach was chosen to obtain highly purified populations of effector and control CD8+ T cells, which is generally not achievable by conventional methods of peptide-loaded autologous APCs for in vitro stimulation of CTLs. For each donor, highly enriched populations of tetramer+ and tetramer− CD8+ cells were achieved (range, 62.2% to 99.4%; Fig. 4). By ELISPOT analysis, these hTERT I540 tetramer+ cells specifically secreted IFN-γ in response to hTERT I540 peptide, whereas hTERT I540 tetramer− CD8+ cells did not (Fig. 4). CD8+ tetramer+ cells were tested for lytic function and found to lyse HLA-A2+, telomerase+ carcinoma cells but not HLA-A2−, telomerase+ carcinoma tumor cells (Fig. 4). T2 cells pulsed with hTERT I540 peptide were also lysed but not T2 cells pulsed with HIV RT-pol I476 peptide. Tetramer−CD8+ purified cells did not exhibit any lytic activity (Fig. 4).

Finally, autologus CD40-activated B cells (telomerase+) were also examined as targets and were lysed by tetramer+ CD8+ T cells (14.9%, 14.2%, and 5.7% at E:T ratios of 30:1, 10:1, and 3:1, respectively) but not by tetramer− CD8+ T cells (specific lysis <0.2% at each E:T ratio).

Induction of T Cell Responses to KLH. Four of 7 patients demonstrated priming to KLH after vaccination (Fig. 5A). For those patients who completed six vaccinations, KLH responses were highest when measured at the end of study; however, evidence for KLH priming was observed in some patients after one or two vaccinations.

Immune Responses to Control Peptides. Patients in this trial were also simultaneously injected with DCs loaded with influenza MP58 and HIV RT-pol peptides as internal immunological controls. Three patients (UPIN 901, UPIN 903, and UPIN 906) responded to influenza MP58 peptide after vaccination, based on an increase in the percentage of MP58 tetramer+ CD8+ cells observed in uncultured PBMCs (Fig. 5B). These MP58-specific cells proliferated specifically to MP58 peptide during IVS (Fig. 5B) and secreted IFN-γ by ELISPOT in response to rechallenge with MP58 but not HTLV-1 L11 peptide (data not shown). Two other patients (UPIN 902 and UPIN 907), both with detectable influenza MP58 CD8+ T cells at baseline, maintained nearly the same percentage of MP58 tetramer+ CD8+ cells in uncultured PBMCs after vaccination. For both pre- and postvaccination samples, MP58-specific cells from these 2 patients also proliferated and secreted IFN-γ in response to MP58 peptide (data not shown). The 2 other patients (UPIN 904 and UPIN 905), each with undetectable MP58-specific CD8+ cells at baseline, failed to show evidence for specific MP58 responses after vaccination.

Finally, 1 patient (UPIN 906) demonstrated evidence for priming to HIV RT-pol peptide after vaccination. RT-pol tetramer+ CD8+ cells were detectable after four vaccinations in uncultured or in vitro sensitized PBMCs (Fig. 5B), but were not detectable at baseline with or without IVS. These results demonstrate the ability of this vaccine formulation to induce CD8+ T-cell responses in patients to both recall and neo epitopes.

Surface Phenotypic Analysis of Uncultured Tetramer+ CD8+ Cells. Uncultured tetramer+ CD8+ cells induced by vaccination were examined without in vitro manipulation for expression of CD45RA, CD45RO, CCR7, CD27, and CD28 by multiple parameter flow cytometry. For each of the 3 patients with detectable hTERT-specific CD8+ T cells in uncultured PBMCs after vaccination, hTERT I540 tetramer+ CD8+ cells predominantly expressed CD45RA+ but lacked CCR7 (Fig. 5C). The expression of other markers was mixed (Fig. 5C). After tetramer isolation and polyclonal in vitro expansion of postvaccination samples, hTERT I540-specific T cells expressed CD45RO but not CD45RA, CCR7, or CD27 (data not shown). CD45RA and CCR7 expression patterns on antigen-specific human CD8+ T cells correlate with memory cell function (29, 30). CD8+ CD45RA+ CCR7− cells in peripheral blood, for example, are thought to delineate lytic effector memory cells (29–31). Two of these patients (UPIN 903 and UPIN 906) also had detectable influenza MP58-specific CD8+ T cells after vaccination, and similarly, uncultured influenza MP58 tetramer+ CD8+ cells were predominantly CD45RA+ CCR7− (Fig. 5C). The expression of other markers was again mixed, with a pattern similar to hTERT I540 tetramer+ cells.
Fig. 4 Polyclonal expansion and cell sorting of telomerase reverse transcriptase (hTERT) IS40 tetramer+ CD8+ cells from vaccinated patients. Polyclonal expansion and tetramer-guided high speed sorted achieved highly enriched populations of tetramer+ CD8+ cells or tetramer–CD8+ cells for (A) UPIN 903, (B) UPIN 904, and (C) UPIN 906. Enzyme-linked immunospot activity was evaluated after one sort/expansion cycle and for each patient, P < 0.01 (Student’s t test) comparing HTLV-1 responses to hTERT responses. Cytolysis of SW-480 (HLA-A2+, telomerase+), HBL-100 (HLA-A2+, telomerase+), and SK-OV-3 (HLA-A2−, telomerase+) carcinoma cells or peptide-loaded T2 cells was analyzed after the first or second cycle.
Discussion

The purpose of this clinical investigation was to determine whether vaccination of cancer patients with hTERT peptide can induce hTERT-specific CD8+ T-cell responses as a first clinical test of telomerase as a broadly applicable tumor-associated target for immunotherapy. Here, we demonstrate the successful generation of functional hTERT-specific CD8+ T cells in patients with advanced prostate or breast cancer after repeated vaccination with autologous DCs loaded with hTERT peptide and KLH. Although undetectable before vaccination, hTERT-specific CD8+ cells after vaccination were identified by peptide/MHC tetramers, proliferated, and secreted IFN-γ after in vitro peptide sensitization, killed tumors, and demonstrated phenotypic characteristics of tumor-lytic CD8+ T cells.

Importantly, the induction of hTERT-specific CD8+ T cells was observed in the absence of significant toxicity. Unlike other treatment modalities such as chemotherapy, where toxicity is usually measured as a function of circulating drug levels, cellular immunotherapy approaches targeting self-antigens carry the risk of autoimmunity as the primary toxicity. Previous trials have demonstrated that significant toxicities related to delivery mechanisms of peptide-based cancer vaccines are rare (32).

Although a repertoire of T cells with receptors reactive to hTERT I540 peptide (and other hTERT peptides) clearly exists
in human peripheral blood (13–15, 18, 19, 33–35), the circulating precursor frequency of this repertoire, even in patients with advanced cancer, is low (14). Consequently, the measurable induction of hTERT I540-specific CD8+ T cells after vaccination in this study likely reflects the expansion of a low-frequency population of precursor CD8+ cells, rather than the boosting of a recall response. This expansion may reflect one or a combination of immunological effects, including: (a) priming of naïve CD8+ T cells specific for I540; (b) activation of immunologically ignorant CD8+ T cells (36, 37); or (c) reversal of peripherally tolerated specific CD8+ T cells. In vivo T-cell priming has been observed previously as a consequence of DC vaccination (38, 39), and here, HIV peptide responses in 1 patient (who by the required prestudy evaluation was HIV seronegative) and KLH responses in 4 patients argue that the vaccine formulation used in this study was biologically able to prime specific T cells. In those patients with a statistically significant KLH response postvaccine compared with baseline, the mean stimulation index was 31 (range, 5–80); in previous studies of vaccination with KLH-loaded DCs, mean stimulation indices range from roughly 10 to 50, depending on the formulation (mature versus semimature), type of subject (healthy versus cancer patient), and route of administration (38–40). Fully immature DCs used in two studies failed to prime (41, 42). Vaccination of patients in this study may have activated immunologically ignorant T cells for which previous physiological presentation of hTERT peptide failed to trigger clonal expansion. hTERT peptide vaccination may also draw upon a T-cell repertoire affected at some level by central or peripheral tolerance mechanisms. Immunological studies (13, 15) and studies using human antibodies (16) have provided evidence that hTERT I540 peptide is naturally processed and presented by human tumor cells in the groove of MHC class I. Lev et al. (16) described recently the isolation of human antibodies from a large nonimmune repertoire of human Fab fragments displayed on phage that bind with high affinity to the I540 peptide/HLA-A2 complex. The extent to which hTERT I540 is presented or cross-presented in thymus or other normal tissues remains poorly understood. In mouse model systems, vaccination with DCs transfected with TERT mRNA induces protective immunity without toxicity (19), important because mTERT is a much more broadly expressed self-antigen in mice. It is possible, therefore, that the induction of hTERT I540-specific CD8+ T cells after vaccination in this study reflects, in part, a modulation of CD8+ T-cell tolerance to hTERT in patients.

Partial tumor regression was observed in 1 patient after vaccination and was associated on biopsy with the induction of CD8+ infiltrating lymphocytes at the site of tumor. hTERT-specific immune responses in this study were demonstrated in peripheral blood. It would be important in future studies to know explicitly whether vaccination induces tumor-homing hTERT-specific T cells. Furthermore, subsequent studies of hTERT vaccination, building on the immunological and safety data reported here, may be able to increase the amplitude of anti-hTERT responses by using more potent immunotherapeutic modalities. One attractive possibility is the use of ex vivo fully matured DCs for peptide presentation (28). Another possibility is adoptive T-cell therapy (43, 44), possibly in combination with depletion of T-regulatory cells. Our observation that artificial APCs can ex vivo expand hTERT-specific CD8+ T cells from postvaccine, but not prevaccine samples, suggests that hTERT-specific adoptive immunotherapy in combination with vaccination may be possible. The breadth of anti-hTERT responses could also be improved by incorporating additional hTERT T-cell epitopes, including those restricted to MHC class II (45). Attempts to disrupt negative regulatory elements of both host and tumor origin will also be critical in future studies.

In considering the use of autologous DCs as a vaccine modality, our study adds to many others suggesting the clinical utility of this approach. Specific antitumor responses and some clinical responses have been observed in pilot trials; however, differences in study design, source of DC precursors, maturation stage, dose, and route of administration have complicated the generation of a consensus regarding DC manufacturing (28). Two small trials in metastatic melanoma suggest that mature monocyte-derived DCs are more effective at inducing immune responses than immature DCs (42, 46). Similar observations have been made in two normal donors injected with immature DCs loaded with influenza peptide (41). Nevertheless, immune responses have been observed previously with immature or semimature DCs, particularly if injected intranodally (40, 47). One explanation for these findings has been that mature, but not immature, DCs are migratory and capable of reaching draining lymph nodes (42).

Finally, given our observations that hTERT-specific CD8+ T cells can be induced in vivo in patients, it will become important to evaluate whether hTERT vaccination results in the development or outgrowth of telomerase-negative tumor cells. Given the requisite role of hTERT in telomerase-positive tumors, modulation of hTERT as a means of immune escape might be incompatible with sustained tumor growth.

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