Telomerase-Specific T-Cell Immunity in Breast Cancer: Effect of Vaccination on Tumor Imunosurveillance


Abstract

The human telomerase reverse transcriptase (hTERT) is nearly universally overexpressed in human cancer, contributes critically to oncogenesis, and is recognized by cytotoxic T cells that lyse tumors. CD8+ T cells specific for hTERT naturally occur in certain populations of cancer patients in remission, but it remains poorly understood whether such T cells could contribute to tumor immunosurveillance. To address this issue, we induced hTERT-specific T cells in vivo via peptide vaccination in 19 patients with metastatic breast cancer who otherwise had no measurable T-cell responses to hTERT at baseline. Tumor-infiltrating lymphocytes (TIL) were evident after, but not before vaccination, with 4% to 13% of post-vaccine CD8+ TIL specific for the immunizing hTERT peptide. Induction of TIL manifested clinically with tumor site pain and pruritus and pathologically with alterations in the tumor microenvironment, featuring histiocytic accumulation and widespread tumor necrosis. hTERT-specific CD8+ T cells were also evident after vaccination in the peripheral blood of patients and exhibited effector functions in vitro including proliferation, IFN-γ production, and tumor lysis. An exploratory landmark analysis revealed that median overall survival was significantly longer in those patients who achieved an immune response to hTERT peptide compared with patients who did not. Immune response to a control cytomegalovirus peptide in the vaccine did not correlate with survival. These results suggest that hTERT-specific T cells could contribute to the immunosurveillance of breast cancer and suggest novel opportunities for both therapeutic and prophylactic vaccine strategies for cancer.

Introduction

The re-emerging concept of tumor immunosurveillance builds on findings that T lymphocytes can recognize peptide antigens expressed on the surface of tumor cells in the context of MHC molecules (1, 2). Among the several dozen such antigens that have been previously described, most are neither broadly expressed in cancer nor critical to the oncogenic process, and the appearance of antigen-loss mutations in tumor cells in the face of immune pressure is well-described (3–5). We therefore sought to understand natural and induced immune responses to a class of antigens termed "universal tumor antigens" that not only trigger T cell reactivity against a broad range of tumor types but also play critical functional roles in tumor growth and development (6). We hypothesized that such molecules, if essential to the neoplastic process and presented by MHC molecules, might function as effective mediators of cellular immunosurveillance for which mutation as a means of immune escape would be incompatible with sustained tumor growth.

A prototype antigen for this hypothesis is the human telomerase reverse transcriptase (hTERT; ref. 7). 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 (8–10). Although telomerase activity and hTERT expression is absent in most normal human cells, telomerase activity is found in >85% of all human cancers (11), including most types of cancer stem cells described thus far (12). hTERT expression plays a critical functional role in oncogenesis (13), and inhibition of hTERT in cancers that express telomerase activity leads to growth arrest in vitro without the development of escape mutants (14, 15).

Naturally occurring CD8+ T cells specific for the hTERT peptide I540 (ILAKFLHWL) have been observed in high numbers in blood from certain populations of cancer patients in remission following standard therapies (16, 17), but whether such T cells represent an active component of cancer immunosurveillance—and one that might be therapeutically exploited—remains unknown. We and others have previously observed that the I540 peptide binds with high affinity to HLA-A2 and can be used to generate specific CTLs in vitro that lyse a wide range of hTERT+ tumor cell lines and primary tumors (18–21). In healthy individuals or patients with a heavy burden of metastatic cancer, the precursor frequency of I540-specific CD8+ T cells in peripheral blood is very low (20). In contrast, >80% of HLA-A2+ patients with chronic myelogenous leukemia in durable remission following treatment with imatinib, IFN-α, or stem cell transplantation harbor large numbers of circulating, tetramer-positive I540-specific CTL in peripheral blood, ranging from 0.1% to 13.2% of freshly isolated CD8+ T cells (16). In our own study of patients with acute myelogenous leukemia or chronic myelogenous leukemia in remission following allogeneic stem cell transplantation or imatinib, we also found that 80% of patients had I540 tetramer–reactive CD8+ T cells in the blood (range, 0.1–1.2%; data not shown). Similarly, >90% of HLA-A2+ patients with prostate cancer in remission following prostatectomy...
showed I540-specific CD8+ T cells (range, 0.1–1.4%) that recognize HLA-matched or autologous tumor and specifically secrete IFN-γ in vitro (17).

In this study, the goal was to determine the effect of I540-specific CD8+ T cells on cancer immunosurveillance by inducing such cells in patients who at baseline exhibit no measurable T-cell responses to hTERT I540. To do this, we vaccinated patients with metastatic breast cancer with hTERT I540 peptide emulsified in adjuvant and delivered s.c. with granulocyte macrophage colony-stimulating factor (GM-CSF). We found that hTERT-specific CD8+ T cells, absent at baseline in peripheral blood or tumors, were readily and safely induced in patients with metastatic breast cancer, infiltrated tumors, and were associated with tumor necrosis. An exploratory landmark analysis revealed an association between an hTERT-specific CD8+ T cell immune response and overall survival.

Materials and Methods

Patients and vaccination protocol. The clinical protocol was an open-label prospective study of HLA-A2+ patients with metastatic breast cancer refractory to at least one conventional therapy for metastatic disease. The protocol was approved by the University of Pennsylvania 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 from each patient. To be eligible, patients had to be >18 years of age and HLA-A2+ with a baseline Eastern Cooperative Group Clinical performance status ≤2. At baseline, patients had to have adequate hematologic function (WBC count >3,000 cells/mm³, hemoglobin >10, and platelet count >75,000 cells/mm³), adequate renal function (serum creatinine ≤1.5 times the upper limit of normal), adequate hepatic function (total bilirubin ≤1.5 times the upper limit of normal and aspartate aminotransferase and alanine aminotransferase ≤2.5 times the upper limit of normal), and imaging studies of the brain that were negative for metastatic disease. Patients were excluded if pregnant or lactating or for a history of brain metastases; positivity for HIV, HTLV-1, hepatitis B or C viruses; active infection; use of chemotherapy, radiation therapy, immunotherapy, immunosuppressive drugs, glucocorticoids, hematopoietic growth factors, or other investigational drug within 30 days of treatment; history of stem cell transplantation; or alcohol abuse or illicit drug use. Concomitant use of chemotherapy, radiation therapy, steroids, immunosuppressive drugs, antiangiulants, or other investigational drugs was not allowed.

Eligible patients were vaccinated four times every other week and then monthly until toxicity or clinically significant disease progression. Vaccinations consisted of aqueous solutions of hTERT peptide I540 (ILAKFLHLWLV) or cytomegalovirus (CMV) peptide N945 (NLVPMVATV; each peptide was ≥92% pure and of good manufacturing practice grade; Merck Biosciences AG) emulsified in the adjuvant Montanide ISA 51 (Seppic, Inc.) and delivered s.c. in the thigh (right thigh, hTERT I540 emulsion; left thigh, CMV N945 emulsion). Sargamostim (clinical grade GM-CSF; Berlex Laboratories, Inc.) was also given s.c. at each of the two peptide-injection sites (70 μg) per vaccination.

Dose escalation and evaluation of toxicity and tumor response. Three cohorts of patients (5–8 patients/cohort) were enrolled at three dose levels: 10 μg per peptide per injection, 100 μg per peptide per injection, or 1,000 μg per peptide per injection. Toxicity was graded according to National Cancer Institute Common Toxicity Criteria (version 2.0). Dose-limiting toxicity (DLT) was defined as treatment-related (a) grade 3 or higher hematologic or nonhematologic toxicity; (b) grade 2 or higher autoimmune reaction; or (c) grade 2 or higher allergic reaction, as determined by a protocol-specific Data Safety Monitoring Board. Patients who did not receive at least three vaccinations were deemed un evaluable for immune responses and were replaced in the dose cohort. A dose level would have been considered too toxic, and enrollment at that or higher dose levels halted, if two or more patients at that level experienced DLT. Five patients were initially treated at the 10 μg dose level but one received only two vaccines and was replaced (for a total of six patients at this dose level, none with DLT). Five patients were then treated at the 100 μg dose level and another five patients at the 1,000 μg dose, all without DLT. Permission was then received to treat three additional patients at the 100 μg dose level. Tumor response was assessed every 2 to 3 months according to Response Evaluation Criteria in Solid Tumors standards.

Patient and normal volunteer samples. Tumor biopsy samples were obtained from accessible tumor lesions of enrolled patients and processed in formalin for histopathologic analysis or placed in saline and mechanically dispersed as a single-cell suspension for flow cytometric analysis. Peripheral blood mononuclear cells (PBMC) were obtained from patient phlebotomy samples by Ficoll centrifugation and frozen at −150°C prior to the performance of immunosassessments assays. PBMC were also obtained from 19 healthy volunteers following written, informed consent using an Institutional Review Board–approved clinical protocol.

Immunohistochemistry. Tissues were processed, sectioned, and stained in the Department of Pathology and Laboratory Medicine at the Hospital of the University of Pennsylvania. Immunoperoxidase studies were done using paraffin sections and Dako Envision technology (Dako) involving biotinylated secondary antibodies and a highly sensitive streptavidin-enzyme-chromogen detection system. The primary antibodies used were CD45 (1:300, M0701; Dako), CD4 (1:200, 4B12; Novocarsta), CD8 (1:100, C8/144B; Dako), GMIP-17 (1:900, TLA-1; Immunotech), CD68 (1:500; Dako), CD79a (1:500, JCB-117; Dako), CD20 (1:150, L26; Dako), and CD56 (1:100; NCAM, Monosan). Positive control staining was done using sections from a standard lymph node sample (Burkitt lymphoma).

Flow cytometric and MHC class I tetramer analysis. Phenotypic analysis of lymphocyte subsets was done using monoclonal antibody and isotype controls by flow cytometry as previously described (20) using a FACScanto cytomter and FACSDiva software (BD Biosciences Immunocytochemistry Systems). Soluble HLA-A2 tetramers were purchased from Beckman Coulter Immunomics or manufactured at the Abramson Cancer Center Human Immunology Core. The cutoff for a measurable response of tetramer+ CD8+ T cells in peripheral blood was defined as the mean + 3 SD for the percentage of tetramer+ CD8+ cells among peripheral CD8+ T cells from 9 HLA-A2–negative healthy volunteers (i.e., 0.1%) or from 10 HLA-A2+ negative healthy volunteers (also 0.1%).

Intracellular cytokine analysis. Lymphocytes (5 × 10⁶/mL) were incubated in complete media (RPMI with 10% human AB serum, 2 mmol/L glutamine, 20 mmol/L HEPES, and 15 μg/mL gentamicin) with 1 μg/mL of peptide (New England Peptide) or phorbol 12-myristate 13-acetate/ionomycin for 5 h with brefeldin A added for the last 4 h. Cells were then labeled with fluorochrome-conjugated monoclonal antibody against cell surface molecules at 4°C, and then fixed and permeabilized (Cytofix/ Cytopermit kit, BD Bioscienes) before staining with anti–IFN-γ monoclonal antibody or isotype control and analysis by flow cytometry. For intracellular cytokine staining of lymphocytes stimulated in vitro with peptide for 8 days, T2 cells (2.5 × 10⁶/mL; American Type Culture Collection) loaded with peptide (1 μg/mL) and [β2-microglobulin (2.5 μg/mL; Sigma) were used as stimuli.

In vitro peptide stimulation. Thawed PBMC (10⁶/well) were incubated with autologous irradiated (32 Gy) PBMC (10⁶/well) in the presence of peptide (1 μg/mL; New England Peptide) and [β2-microglobulin (2.5 μg/mL) in complete media and 10 ng/mL of interleukin 7 (Sigma) in 24-well tissue culture plates. After 24 h, and again on day 5, interleukin 2 (20 IU/mL; Chiron Corp.) was added, and cells were analyzed on day 8.

Cytotoxicity assays. Standard chromium release assays were done as previously described (20). Specific lysis of target cells calculated from triplicate determinations using counts per minute of (experimental result – spontaneous release) / (maximum release – spontaneous release). SD was ≤5%. Tumor cell lines HBL-100, SW-480, and SK-OV-3 (American Type Culture Collection) were evaluated for HLA-A2 expression and for telomerase activity as previously described (20).

Statistical analysis. Landmark analyses were employed to assess the association between T cell immune responses and overall survival. In analyses of survival by treatment response, the landmark method avoids the statistical drawback of the follow-up time and is useful for long-term follow-up.

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This method provides unbiased estimates of survival, conditional on the inclusion of patients continuing on-study at the landmark. Although subjective, the selection of the landmark in this study was driven by time course of immunologic response (time to maximal tetramer response) and end of biweekly injections but not by clinical outcome. Moreover, to avoid biases of outcome-driven post hoc analysis, patients were categorized as immunologic non- or low-responders versus immunologic high-responders, by the median percentage of hTERT I540 tetramer+ CD8+ T cells or CMV N495 tetramer+ CD8+ T cells at the landmark. Overall survival was defined as the interval from the landmark date to date of death due to any cause or last patient contact, if alive, and was estimated by the method of Kaplan and Meier. The 95% confidence interval (95% CI) was calculated. Overall survival was compared between immunologic non/low-responders and high-responders at the landmark interval (95% CI) was calculated. Overall survival was compared between immunologic non/low-responders and high-responders at the landmark date of death due to any cause or last patient contact, if alive, and was estimated by the method of Kaplan and Meier. The 95% confidence interval (95% CI) was calculated. Overall survival was compared between immunologic non/low-responders and high-responders at the landmark.

Results

**hTERT I540–specific CD8+ T cells in breast cancer.** Nineteen HLA-A2+ patients with metastatic breast cancer were studied (Table 1). At baseline, in all patients, hTERT I540 tetramer+ CD8+ T cells were not measurable among freshly isolated PBMC or among PBMC stimulated for 8 days *in vitro* with I540 peptide. Similarly, there were no CD8+ T cells that secreted IFN-γ in response to direct challenge with hTERT I540 peptide in intracellular cytokine analyses. To further evaluate for natural T cell responses to the I540 epitope, six patients with accessible tumor lesions in the skin, breast, or lymph nodes underwent biopsy at baseline. By flow cytometry of biopsy cell suspensions, few CD45+ tumor-infiltrating leukocytes (TIL) were observed, including no CD45+ CD8+ T cells that were labeled with hTERT I540 tetramer (Fig. 1; Supplemental Table S1). Importantly, in all patients examined, tumor CD8+ T cells labeled poorly with the hTERT I540–specific CD8+ T cells were rare in these patients.

**CMV peptide was included in the vaccine.**

To determine the effect of vaccination on tumor infiltration, postvaccination tumor biopsies were obtained in the same patients who had undergone biopsy at baseline. In three of these six patients (50%), hTERT I540–specific CD8+ T cells were observed in the tumor after—but not before—vaccination, with 4.4% to 12.7% of CD8+ T cells in the tumor binding to the hTERT I540 tetramer compared with <0.6% of cells binding to a negative control tetramer (Fig. 1; Supplemental Table S1). Importantly, in all patients examined, tumor CD8+ T cells labeled poorly with the CMV N495 tetramer before or after vaccination, even though this CMV peptide was included in the vaccine.

### Table 1. Patient characteristics

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<th>Patient no.</th>
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<th>No. of vaccines received</th>
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Abbreviations: C, chemotherapy; E, experimental therapy; H, hormonal therapy; R, radiation; S, surgery; T, trastuzumab; NE, not evaluated (lost to follow-up); SD, stable disease; PD, progressive disease.

*Response included regression of chest wall lesions not meeting criteria for partial response.
In two patients, hTERT-specific CD8+ T cells in the biopsy samples were accompanied by the development of widespread tumor necrosis involving 50% to 80% of the specimens after vaccination. For UPIN 004 (10 µg dose level), few TILs were noted at baseline (Fig. 2A); however, after four vaccinations, biopsy revealed the induction of TILs, primarily CD4+ and CD8+ T cells in the absence of tumor necrosis (Fig. 2B, top row). One week after the eighth vaccination, the patient acutely developed moderately severe pain at known tumor sites, and a repeat biopsy revealed large areas of necrosis within the tumor (Fig. 2B, middle row), a likely pathologic explanation for the clinical syndrome. Immunohistochemistry of this biopsy revealed a marked infiltration of CD68+ histiocytes, but few T cells, within the necrotic regions (data not shown) and a persistent infiltration of CD4+ and CD8+ T cells surrounding small foci of residual viable carcinoma cells (Fig. 2B, bottom row). At each time after vaccination, immunohistochemistry revealed a notable fraction of TILs positive for GMP-17 (Fig. 2B, bottom row), which marks cytotoxic granules in CTL. Intrapumoral CD79a+ B cells (Fig. 2B, bottom row) and peritumoral B cell follicles (data not shown), but not CD56+ natural killer cells, were also observed after vaccination.

Results were similar for UPIN 010 (100 µg dose level), with the development of tumor necrosis observed in biopsy samples after the fifth vaccination but not at baseline (Fig. 2C and D). This was the same time course as the appearance of intratumoral hTERT I540 tetramer+ CD8+ T cells as measured by flow cytometry (Supplemental Table S1). Tumor necrosis seen on histopathology was accompanied clinically by the development of moderately severe pruritus and pain at tumor sites in the week after the fifth vaccine. Immunohistochemistry revealed the infiltration of both CD8+ and CD4+ T cells (Fig. 2C and D), including GMP-17+ TILs and histiocytes in the absence of CD56+ natural killer cells (data not shown).

hTERT-specific CD8+ T cells after vaccination in unstimulated peripheral blood. To further assess the induction of I540-specific T cells following vaccination, tetramer and intracellular cytokine analyses were done on peripheral blood T cells obtained after vaccination. For six patients (32%) after (but not before) vaccination, hTERT I540 tetramer+ CD8+ T cells in unstimulated PBMC were identified (median maximal response, 0.42%; range, 0.15–2.01%; patient 008 in Fig. 3). Median time to maximal tetramer response was 11 weeks (range, 10 weeks to 29 months). One of these patients was at the 10 µg dose level, 4 were at the 100 µg dose level, and 1 was at the 1,000 µg dose level. Intracellular cytokine analysis corroborated these findings: patients in whom a tetramer immune response was noted after vaccination also exhibited IFN-γ secreting CD8+ T cells after challenge of post-vaccine PBMC with hTERT I540 peptide (Fig. 3).
hTERT-specific CD8+ T cells after *in vitro* stimulation of peripheral blood T cells. As a further test for the induction of circulating hTERT I540–specific T cells after vaccination, PBMC obtained after vaccination were incubated *in vitro* with I540 peptide (or control peptide) and analyzed on day 8 with peptide/MHC tetramers. For 13 of 19 patients (68%), measurable populations of hTERT tetramer+ CD8+ T cells were clearly identified after (but not before) vaccination among *in vitro* stimulated lymphocytes (patient 009 in Fig. 4A). Three such patients were at the 10 µg dose level, six patients were at the 100 µg dose level, and four patients were at the 1,000 µg dose level. The median maximal percentage of tetramer+ CD8+ T cells was 1.58% (range, 0.20–24.18%), compared with <0.10% of such cells at baseline in these patients or in the tetramer-negative patients at any time point (Fig. 4B). Six patients who were positive for hTERT I540 CD8+ T cells in this *in vitro* stimulation assay also showed hTERT I540 tetramer+ CD8+ T cells in unstimulated blood. Seven patients were positive only after *in vitro* stimulation (none positive at baseline for either assay).

hTERT I540–specific T cells from *in vitro* peptide-stimulated cultures were then examined in functional assays for reactivity against peptide-expressing targets and telomerase-positive tumor cells. In seven out of seven patients tested, hTERT I540–specific CD8+ T cells secreted IFN-γ in response to HLA-A2+ T2 cells.

**Figure 2.** Histopathologic analysis of tumor biopsy samples obtained from patient 004 (A and B) and patient 010 (C and D). For patient 004, biopsies were obtained before vaccination (A), after the fourth vaccination (B, top and middle rows); and after the eighth vaccination (B, bottom row) and analyzed by H&E staining (A, left; B, top left and middle rows) or immunohistochemistry for CD3 (A, second panel; B, second panel top row), CD8 (A, third panel; B, third panel top row and left panel bottom row), CD4 (A, right; B, top row right, second panel bottom row), GMP-17 (B, third panel bottom row), and CD79a (B, right panel bottom row). For patient 010, biopsies were obtained before vaccination (C) or after the fifth vaccination (D) and analyzed by H&E staining (C and D, left) or immunohistochemistry for CD8 (C and D, middle) or CD4 (C and D, right).
loaded with I540 peptide, but not control peptide (patient 004 in Fig. 4C). In standard chromium-release cytotoxicity assays, hTERT I540–specific CD8+ T cells from seven of nine patients tested lysed HLA-A2+ carcinoma cells expressing telomerase but not HLA-A2–negative carcinoma cells (Fig. 4D). No IFN-γ or cytotoxicity responses were observed for in vitro–stimulated CD8+ T cells in baseline PBMC samples.

**CD8+ T-cell responses to CMV peptide.** Peripheral blood T cells obtained before and after vaccination were also characterized for reactivity against CMV N495 peptide using a similar set of tetramer and functional assays. Fourteen patients (74%) were CMV seropositive at baseline, and CMV N495 tetramer+ CD8+ T cells were measurable at baseline in 11 patients from either unstimulated blood (n = 9; median, 0.72%; range, 0.13–10.7%) or following in vitro stimulation with N495 peptide (n = 11; median, 6.62%; range, 0.13–59.5%), or both (n = 9). In the three other seropositive patients, CMV N495–specific T cells were not measurable even with in vitro stimulation at baseline. In five CMV-seronegative patients, CMV N495–specific T cells were not measurable at baseline from either unstimulated or stimulated blood, as expected.

After vaccination, in all but two seropositive patients, CMV N495 tetramer+ T cells were measurable among CD8+ T cells analyzed from unstimulated blood (median maximal response, 0.51%; range, 0.16–6.47%) and after in vitro stimulation (median maximal response, 2.51%; range, 0.57–55.4%; data not shown). Moreover, evidence for T cell priming was observed in three CMV-seronegative patients in whom CD8+ tetramer+ cells were evident after in vitro stimulation following the fourth (1.94% CMV tetramer+ CD8+ T cells), seventh (0.31%), or eighth (0.17%) vaccination without CMV antibody seroconversion during the treatment period. The latter finding suggests that the peptide/Montanide/GM-CSF vaccine formulation used in this study was able to prime human CD8+ T cells in vivo.

**Tumor response and overall survival.** No objective clinical responses as measured by Response Evaluation Criteria in Solid Tumors standards were observed in 18 evaluable patients. Best response was stable disease in nine patients (50%). One patient with stable disease at dose level 2 (UPIN 011) had regression of multiple chest wall lesions after vaccination, but overall, tumor regression in this patient did not meet the criteria for a partial response.

We then did an exploratory landmark survival analysis to assess the association between the induction of hTERT I540–specific CD8+ T cells in peripheral blood and overall survival. The landmark method avoids bias in the length of on-study observation time favoring responders, in analyses of survival by treatment response. Time zero (the landmark) was chosen as the date of the immune response evaluation after the fourth vaccination (median on-study day 70, range 55–78). This landmark was chosen because the median time to maximal tetramer response was 11 weeks after vaccination (i.e., ∼1 month after the fourth vaccination) and coincided with the end of biweekly vaccinations. Sixteen patients who received at least four vaccines were included in the landmark analysis. Patients were categorized as immunologic non- or low-responders or immunologic high-responders, based on the median percentage (40%) of in vitro–stimulated I540 hTERT tetramer+ CD8+ T cells at the landmark. To be sure, the selection of the landmark was subjective, although it was driven by time course of immunologic response and not by clinical outcome, and this method also did not account for change in immune response status after the landmark.

The median overall survival from the landmark was longer for the nine hTERT immunologic high-responders as compared with that for the seven non/low-responders (32.2 months; 95% CI, 5.3–59.1 months versus 17.5 months; 95% CI, 7.5–27.6 months, respectively; P = 0.03; Fig. 5A). Among the hTERT immunologic high-responders were three patients with prolonged survival (33+, 34+, and 40+ months). Because hTERT immunologic response might simply reflect healthier patients with better prognosis regardless of treatment, we did an additional landmark analysis to test the association between immune response to CMV peptide and overall survival. Patients were categorized as CMV non- or low-responders versus high-responders based on the median percentage (∼10%) of CMV+ CD8+ T cells at the landmark. The
Figure 4. Functional analysis of hTERT I540–specific CD8+ T cells before and after vaccination. A, PBMC for patient 009 at baseline and after the fourth vaccination were stimulated with I540 peptide in vitro for 8 d then analyzed with peptide/MHC tetramers reactive for a negative control peptide or hTERT I540 peptide. Percentages in the boxes are for CD8+ T cells. B, the maximum in vitro stimulation response observed for each patient. C, intracellular IFN-γ production at baseline and after the fourth vaccination for I540-stimulated CD8+ T cells from patient 004 in response to T2 cells loaded with L11 tax peptide (negative control), T2 cells loaded with I540 hTERT peptide, or phorbol 12-myristate 13-acetate (PMA) and ionomycin (positive control). D, tumor lysis at baseline and after the fourth vaccination for I540-stimulated CD8+ T cells for patient 004 in response to HBL-100 carcinoma cells (●; telomerase-positive, HLA-A2+), SW-480 carcinoma cells (●; telomerase-positive, HLA-A2+), and SK-OV-3 carcinoma cells (●; telomerase-positive, HLA-A2–negative).
median overall survival from the landmark was similar for the six CMV high-responders as compared with that for the eight CMV non/low-responders (18.6 months; 95% CI, 0.0–41.9 months versus 21.3 months; 95% CI, 4.6–37.9 months, respectively, \( P = 0.83 \); Fig. 5B).

Discussion

In this study, we mobilized a T-cell immune response specific for the I540 hTERT epitope \textit{in vivo} in metastatic breast cancer patients and showed the induction of hTERT-specific, tumor-infiltrating CD8+ T cells with major clinicopathologic consequences including tumor necrosis. None of these effects on the tumor microenvironment have been previously described for telomerase-based immunotherapy of cancer. hTERT-specific CD8+ T cells, absent at baseline in these patients, also appeared in peripheral blood after vaccination and exhibited multiple effector functions \textit{in vitro} including proliferation, IFN-\( \gamma \) production, and tumor lysis. We observed longer overall survival in patients who achieved an immune response to I540 hTERT peptide compared with patients who did not; on the other hand, immune response to the control N495 CMV peptide included in the vaccine as a control for this purpose was not associated with overall survival. Overall, our data suggest that functional hTERT I540–specific CD8+ T cells induced \textit{in vitro} by vaccination could alter the histopathologic and immunologic variables of tumor immunosurveillance in patients with breast cancer.

These findings have important implications in the context of a re-emerging view of cancer immunosurveillance (2). Although tumorigenesis is initiated by genetic events, additional pressures within the tumor microenvironment, particularly cellular immune responses, further influence tumor growth and development. Tumor cells express antigens as a result of genomic instability and aberrant gene expression, making them immunologically distinct from normal cells and potential targets of the host immune system (1). Cytotoxic CD8+ T cells are considered chief mediators of tumor rejection based on the molecular recognition of peptide antigens expressed on the surface of tumor cells in the context of MHC molecules (1, 2). For humans with cancer, the presence and type of tumor-infiltrating T cells independently predicts clinical outcome across a broad range of histologies (23–26). Nevertheless, the epitope specificities of such prognostically important T cells remain largely unknown, particularly in carcinomas.

The data reported here suggest that hTERT-specific T cells may influence tumor immunosurveillance in patients with breast cancer via infiltration and recognition of tumor lesions. Our study focused on the immunodominant hTERT I540 epitope in HLA-A2+ patients, but numerous other hTERT-derived T cell epitopes, including those restricted to MHC class I and class II, have been identified that induce \textit{in vitro} T cell responses against telomerase-positive human tumors (27). Several approaches for hTERT immunotherapy have been reported (reviewed in ref. 27). In breast cancer, hTERT is part of a growing list of tumor antigens that are considered relevant immunotherapeutic targets, including HER-2/neu, MUC-1, and cyclins (28–30). For example, vaccination with her2/neu peptides induces specific cellular immunity in patients with breast cancer (31–33), and HER-2/neu vaccination may offer clinical benefit particularly in the setting of minimal residual disease (32, 33). These findings underscore an increasing appreciation that, beyond melanoma and renal cell carcinoma, carcinomas of the breast can be targeted by cellular immunity (24, 28–30, 34).

The therapeutic induction of CD8+ T cell responses to the hTERT I540 epitope has been explored in other clinical settings. Parkhurst and colleagues administered I540 peptide in Montanide (but without GM-CSF) to 14 patients with various cancers (35). I540-specific CD8+ T cells were identified after (but not before) vaccination in seven patients, and several highly avid T cell clones were generated after vaccination that recognized T2 cells pulsed with I540 peptide. None of these clones, however, recognized HLA-A2+ hTERT+ tumor cells, similar to \textit{in vitro} data reported by another group (36). The investigators’ interpretation of
this data that hTERT I540 peptide is not presented on the surfaces of tumor cells in the context of HLA-A2 is discordant with previously published in vitro and in vivo findings from our group and three other groups (17–19, 21, 37). In a recent publication, Dupont and colleagues (21) make note of these discrepancies and address it using a novel antigen-presenting cell (APC) system developed independently in that laboratory. These investigators found that (a) the I540 epitope is naturally processed on the surface of both tumor cells and APCs, and (b) I540-specific CD8+ T cells efficiently lyse tumors expressing endogenous hTERT in an HLA-restricted manner. They suggested that a more potent stimulation signal to I540-specific T cells than peptide-pulsed PBMC used in other studies may be required for optimal CTL generation in vitro. In addition, the direct isolation of the I540 peptide from the groove of HLA-A2 on primary tumor cells (16), as well as K562 cells expressing both HLA-A2 and hTERT (38), have been shown by mass spectroscopy.

Naturally occurring I540-specific T cells have also been observed in patients, described best for patients with leukemia or prostate cancer in durable remission after standard therapy (refs. 16, 17; data not shown). In acute viral infections, such expansion of antigen-specific CD8+ T cells in peripheral blood is typically associated with immune control of disease. In cancer, however, even extensive expansion of tumor antigen–specific CD8+ T cells in vivo is not thought to guarantee a therapeutic effect (39). In many cases, tumor-specific T cells have been shown to be impaired, and cytokine production is absent (40). Naturally occurring I540-specific CD8+ T cells isolated from patients with prostate cancer seem to be functionally competent and even able to lyse autologous tumor cells in vitro (17); however, in leukemia, the functional status of naturally occurring I540-specific CD8+ T cells is less certain (16). In this study, we found that the induction of I540-specific CD8+ T cells in patients who otherwise had no measurable T-cell responses to the epitope at baseline resulted in changes in important variables of immunosurveillance, in particular, T cell infiltration of tumors. One implication of these data is that naturally occurring hTERT-specific T cell responses observed in certain patients in remission may also actively contribute to tumor immunosurveillance; however, our data do not rule out another possibility that the existence of naturally occurring hTERT-specific T cell responses may only represent a (late) consequence of T cell priming by APC that have cross-presented antigen from tumor cells killed by other processes.

Neither natural T cell responses to the I540 hTERT epitope nor responses induced by vaccination have been associated with autoimmune consequences despite the expression of telomerase in normal tissues of patients such as hematopoietic progenitor cells, activated lymphocytes, and certain epithelial cells (41). In vitro, hTERT-specific CTL do not lyse telomerase-positive CD34+ hematopoietic progenitor cells or activated T lymphocytes (18, 19). We have also been unable to reveal hTERT CTL-mediated toxicity against normal bone marrow cells using immunodeficient mice reconstituted with patient hematopoietic progenitors after exposure to hTERT-specific CTL (42). Using multiple cancer models in immunocompetent mice, Nair and colleagues found that TERT-specific vaccination generates robust protective immunity without the development of autoimmunity against TERT-expressing cells (43). There are as yet no long-term safety data after hTERT immunotherapy, and it remains conceivable that prolonged or more robust hTERT immune responses specific to I540 or other epitopes may result in toxicity on normal hTERT-expressing cells. The clinical consequences of long-term telomerase insufficiency in patients with inherited mutations in hTERT or associated genes are well-described, serious, and important to bear in mind (44–47).

In summary, our data suggest that despite being a self-antigen, hTERT can function as a physiologic target of cellular immunosurveillance in breast cancer. If efficient hTERT-specific immunity can be successfully elicited in cancer patients without the induction of severe autoimmunity, hTERT would be a candidate for a widely applicable cancer vaccine. As is true for other strategies of cancer immunotherapy, understanding and disrupting negative regulatory elements of both host and tumor origin will be critical in such future studies, as reviewed extensively elsewhere (48). The critical functional role of hTERT in human cancer may limit its deletion or mutation, and moreover, because hTERT can be predicted to be associated with >85% of human cancers, the opportunity for vaccinating individuals as an immunoprevention strategy can be envisioned for hTERT-based therapies. This not only includes the testing of hTERT vaccination in the adjuvant (minimal residual disease) clinical setting, for example, but also ultimately in healthy individuals considered at high risk for cancer based on genetic factors and medical history. Any preventative cancer vaccine would require a very narrow toxicity profile, and whether this is achievable for hTERT or any other universal tumor antigen is an important question for future studies.

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