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SPECIFIC AIMS OF THE PROJECT

- 1. To evaluate the sensitivity and specificity of hTERT and CYP1B1 expression in ductal lavage samples for the detection of breast cancer.**
- 2. Determine the safety and feasibility of vaccinating advanced breast cancer patients with hTERT-expressing mature autologous dendritic cells, assessing the generation of hTERT-specific immunity and clinical response as a result of vaccination.**

A. INTRODUCTION

Molecular targets to facilitate early detection and preventative therapy for women at high risk for breast cancer have not been characterized. Two recently characterized intracellular enzymes -- human telomerase reverse transcriptase (hTERT) and the cytochrome P450 isoform 1B1 (CYP1B1), each overexpressed in >90% of invasive breast cancers but rarely found in normal tissue -- may fill this gap. Such targets, if found at the earliest time of malignant transformation, may be ideally suited not only for early detection but also cancer prevention by vaccination. A growing clinical experience in advanced cancer patients has underscored the safety and feasibility of vaccination strategies. The universal expression of hTERT and CYP1B1 provide an opportunity for both early detection and cancer vaccination. We hypothesize that the candidate universal tumor antigens hTERT and CYP1B1 can be detected in ductal lavage specimens, specific for the presence of carcinoma. We further hypothesize that immunologic responses can be elicited in advanced breast cancer patients using vaccines incorporating hTERT, providing a safety and feasibility platform for ultimately vaccinating women at high risk for breast cancer.

The ability to detect a target antigen in breast ductal lavage fluid has the potential for early detection and also the potential to create more focused treatment plans targeting such an antigen. Evidence of immunologic response to hTERT based vaccines in advanced cancer patients, as well as demonstration of safety and feasibility, would justify the pursuit of preventative vaccination in high-risk individuals, particularly those in whom such antigens are expressed.

B. BODY

Aim 1: To evaluate the sensitivity and specificity of hTERT and CYP1B1 expression in ductal lavage samples for the detection of breast cancer.

Goals at one year according to statement of work: IRB approval and patient accrual. UPCC 02102, "Sensitivity of ductal lavage for the detection of breast cancer," received IRB approval and patient recruitment began. Patients were eligible if a) they had a BIRADS 4 category

mammogram for which biopsy was to be performed, and b) they were having an MRI performed prior to biopsy either on one of two radiology studies investigating this issue or performed clinically. Seven patients were approached for recruitment and 4 patients were enrolled. Two of four patients did not have nipple aspirate fluid (NAF) and therefore were not candidates for ductal lavage. In one patient cannulation of the duct was stopped due to pain, despite the use of local and intraductal anesthetic. Previous reports have suggested an 80% rate of collection of NAF. However, rates of NAF appear to vary with breast cancer risk with highest rates of NAF in women with the greatest risk. Although our patients had category 4 mammograms, none had family history of breast cancer or has had a subsequent cancer diagnosis; therefore, these were not high-risk patients.

Moreover, since the time our protocol was designed and initiated, studies by other groups have shown that the sensitivity for the detection of ductal lavage for the detection of breast cancer is low. Brogi et al performed ductal lavage in 26 women undergoing mastectomy for carcinoma, as well as four control women. No lavage specimen was clearly malignant and only 4 samples contained marked atypical, despite the fact that on pathology 24 of the mastectomy specimens contained in-situ carcinoma and 20 had invasive carcinoma.¹ Thus, the use of cytology in ductal lavage for detection appears quite limited. Despite its limitations in breast cancer detection, ductal lavage remains interesting as a tool for risk stratification, particularly if molecular markers can be used to assess risk. We are now collaborating with Dr. Gillian Mitchell's group at the Family Cancer Centre at the Peter MacCollum Cancer Centre in Victoria, Australia. In this project, women with BRCA1 and BRCA2 mutations are undergoing serial ductal lavages to examine molecular markers present in ductal lavage specimens. These women encompass a very high-risk group who are much more likely to have NAF and also more likely to have a ductal lavage with sufficient cells for analysis. In addition, at the University of Pennsylvania in our Cancer Risk Evaluation Program, we follow over 2500 families, with more than 320 families and more than 700 individuals having BRCA1 or BRCA2 mutations. Our collaboration with this group will allow access to a large collection of ductal lavage samples from BRCA1 and BRCA2 mutation carriers. A revised statement of work will be submitted to the grants officer for approval.

Aim 2: Determine the safety and feasibility of vaccinating advanced breast cancer patients with hTERT-expressing autologous dendritic cells, assessing the generation of hTERT-specific immunity and clinical response as a result of vaccination.

Goals at one year according to statement of work: Obtain IND and IRB approval for dendritic cell trial and begin enrollment. It has been previously described that the I540 telomerase peptide on tumors is recognized by HLA-A2-restricted cytotoxic T lymphocytes and leads to tumor lysis in vitro. This year we have published our initial phase I trial examining hTERT pulsed dendritic cell vaccination in 7 individuals with advanced cancer (5 prostate, 2 breast)². Immunologic studies and extensive bone marrow assays have been completed demonstrating an immune response to vaccine in 4 of 6 evaluable patients with no dose limiting toxicities and no significant bone marrow toxicity. In the statement of work, we proposed the use of mature dendritic cells for vaccination, as opposed to the immature dendritic cells used in our first study. A Standard Operating Procedure for the derivation of mature DC for clinical use was developed at the University of Pennsylvania and an IND application was prepared for FDA review.

Earlier this year, a important study was reported comparing the use of adoptively transferred mature dendritic cell vaccines to chemotherapy in patients with metastatic melanoma,

and showed no benefit of dendritic cell vaccination vs. chemotherapy, with an overall low response rate of <6% in both arms³. In contrast, the clinical use of peptide with GM-CSF to target dendritic cells in cancer patients in vivo has resulted in promising immunological and clinical responses, including durable molecular remissions in patients with acute leukemia⁴. In light of these findings, we have therefore begun to explore the use of telomerase peptide delivered with GM-CSF as a method of targeting dendritic cells in vivo, rather than ex vivo, in patients with breast cancer. In a phase I clinical trial, we have vaccinated 14 HLA-A2+ women with metastatic breast cancer subcutaneously with 10 ug (n=6), 100 ug (n=5), or 1000 ug (n=3) of hTERT I540 peptide emulsified in Montanide adjuvant and administered with GM-CSF per UPCC Protocol 11102 (Domchek PI) and under the auspices of BB-IND 10675 (Vonderheide, sponsor). No dose limiting toxicities have been observed, including no bone marrow toxicity. Grade 1 and 2 injection site reactions have been observed in most patients. One of 5 evaluable patients at dose level one completed all 8 vaccinations and has stable disease (14+ months) and four patients had progressive disease after completing 3, 6, 4, and 4 vaccinations. At the second dose level, 5 patients are evaluable, and one has had a minor response (chest wall tumor regression, s/p 7 vaccinations and 5+ months), 2 patients have stable disease (s/p all 8 vaccinations and stable 8+ months; s/p 8 vaccinations and stable 8+ months), and two patients had progressive disease after completing 6 vaccinations each. Restaging studies for patients at the third dose level are pending or planned.

Preliminary immunoassessment assays of patients treated at the first two dose levels have been performed. In one patient at the 10 ug dose level and in 4 of 5 patients at the 100 ug level, hTERT-specific CD8+ T cells were observed after vaccination (>0.10% hTERT tetramer+ CD8+ T cells) compared to ≤0.05% before). These cells could be specifically expanded by in vitro stimulation. Five patients have undergone tumor biopsy on study, and flow cytometric and histopathologic analyses have been performed. In 3 patients, hTERT-specific CD8+ tumor infiltrating lymphocytes were observed by tetramer analysis (between 5%-12% tetramer+ cells among CD8+ T cells compared to a negative tetramer control stain of ≤0.06% CD8+ T cells). In two patients, TILs were associated with marked tumor necrosis involving 50%-80% of the biopsy specimens.

Immunohistochemistry has been completed in two patients. TILs consisted of both CD8+ and CD4+ T cells (but not CD56+ NK cells). Significant numbers of TILs in each patient were positive for TIA-1, a marker of cytotoxic granules in bona fide CTLs. This data suggest that I540 telomerase peptide vaccination leads to in vivo immune recognition of carcinoma by effector lymphocytes and tumor necrosis.

Plans for Year Two

Plans for the next year of support include work on SPECIFIC AIMS ONE and TWO.

For AIM ONE, we will examine molecular markers in ductal lavage fluid of BRCA1 and BRCA2 mutation carriers.

For AIM TWO: We will complete our second-generation hTERT vaccine in breast cancer patients. Upon its completion later this year, we intend to pursue a phase I/II study in which metastatic breast cancer patients will be treated with immune-modulatory doses of cyclophosphamide followed by telomerase peptide vaccination and GM-CSF. The use of

cyclophosphamide is intended to deplete in vivo negative regulatory CD25+ CD4+ lymphocyte known to markedly infiltrate breast tumors.

KEY RESEARCH ACCOMPLISHMENTS

1. Approval and initiation of UPCC Protocol
2. Established collaboration with Dr. Gillian Mitchell assessing molecular markers in BRCA1 and BRCA2 mutation carriers.
3. Completion of data analysis and publication of a manuscript detailing our feasibility trial of telomerase dendritic cell vaccination.²
4. Completion of SOP for mature dendritic cells.
5. IND and IRB approval for the use of hTERT peptide vaccination in women with advanced breast cancer with 14 patients treated to date at three dose levels.

REPORTABLE OUTCOMES:

A. Publications During This Funding Period (2003-2004)

1. Domchek SM, Eisen A, Calzone K, Stopfer J, Blackwood A, Weber BL. Application of breast cancer risk prediction models in clinical practice. *Journal of Clinical Oncology*. 2003; 21:593-601.
2. Bendell JC, Domchek SM, Burstein HJ, Harris LN, Younger J, Kuter I, Bunnell C, Rue M, Gelman R, Winer EP. Central nervous system metastases in women who receive trastuzumab-based therapy for metastatic breast cancer. *Cancer*. 2003; 97:2972-7.
3. Domchek SM. Chemoprevention in BRCA1 and BRCA2 mutation carriers. *Seminars in Breast Disease*, 2003; 6:30-33
4. Stein S, DeMichele A, Domchek S, Fox K. Gemcitabine and trastuzumab combinations for patients with metastatic breast cancer overexpressing HER2/neu. *Clinical Breast Cancer*. 2004; 4:S117-120.
5. Vonderheide RH, Domchek SM, Schultze JL, George DJ, Hoar KM, Chen DY, Stephans KF, Masutomi K, Loda M, Xia Z, Anderson KS, Hahn WC, Nadler LM. Vaccination of cancer patients against telomerase induces functional anti-tumor CD8+ T lymphocytes, *Clin Can Res*, 2004, 10: 828-839.
6. Domchek SM and Weber BL. Inherited Genetic Factors in Breast Cancer. In: *Diseases of the Breast*. Third Edition. Philadelphia, Lippincott Williams & Wilkins. 2004.
7. Huang J, Domchek SM, Brose MS, Rebbeck TR, Nathanson KL, Weber BL. Germline *CHEK2**1100delC mutations in breast cancer patients with multiple primary cancers. *Am J Hum Gen*. 2004; *in press*.

B. Abstracts

1. Domchek SM, Tweed AJ, Friebel T, Rebbeck TR, Weber BL. Estrogen-receptor status in breast cancers of BRCA1-mutation carriers following oophorectomy: implications for tamoxifen chemoprevention. American Society of Clinical Oncology. Chicago, Illinois 2003.
2. Stein SH, Davidson R, Tweed AJ, DeMichele A, Domchek S, Fox K, Cohen D. Renal dysfunction with IV bisphosphonates in patients with metastatic breast cancer. American Society of Clinical Oncology. Chicago, Illinois 2003.
3. Domchek SM, Stein SH, DeMichele A, Schucter L, Fox KR, Vonderheide RH. Vaccination of metastatic breast cancer patients against the universal tumor antigen telomerase. San Antonio Breast Symposium. San Antonio, TX 2003.

4. Domchek SM, Tweed AJ, Stopfer J, Weiner M, Weber BL. Physician and patient-related barriers to participation in genetic testing for breast cancer susceptibility. American Society of Clinical Oncology, New Orleans, Louisiana 2004.
5. Isaacs C, Skates SJ, Lehman C, Marcom K, Bowen DJ, Domchek S, Tomlinson G, Armstrong DK, Schnall M. Mammographic, MRI and ultrasound characteristics of BRCA1, BRCA2, and other genetically high-risk women enrolled in a prospective multi-institutional breast cancer screening trial. American Society of Clinical Oncology, New Orleans, Louisiana 2004.

C. Funding

None

CONCLUSIONS

Our initial feasibility trial of ductal lavage raised important issues regarding recruitment and feasibility in performing ductal lavage in a group of women who were not high risk for breast cancer. Therefore, given our large cohort of BRCA1 and BRCA2 mutation carriers who are at very high risk for breast cancer and are excellent candidates for chemoprevention, we are involved in a collaborative effort examining ductal lavage in this group. With regard to telomerase peptide vaccination, we have published the first DC paper and are in the middle of a second trial targeting DCs in vivo, rather than ex vivo. Preliminary data suggest immune responses and some anti-tumor biological activity. These projects have the potential for a major impact in terms of detection, prevention and treatment of breast cancer.

REFERENCES (Also see "Publications" in "Reportable Outcomes")

1. Brogi E, Robson M, Panageas KS, et al: Ductal lavage in patients undergoing mastectomy for mammary carcinoma: a correlative study. *Cancer* 98:2170-6., 2003
2. Vonderheide RH, Domchek SM, Schultze JL, et al: Vaccination of cancer patients against telomerase induces functional antitumor CD8+ T lymphocytes. *Clin Cancer Res* 10:828-39., 2004
3. Schadendorf D NF, Broecker EB, Enk A, Grabbe S, Ugurel S, Edler L, Schuler G: Dacarbazine versus vaccination with autologous peptide-pulsed dendritic cells as first-line treatment of patients with metastatic melanoma: Results of a prospective-randomized phase III study. American Society of Clinical Oncology New Orleans, LO, 2004
4. Mollrem J KS, Lu s, Rios R, Stricher H, Wand C, Giralt S, O'Brien S, Cortes J, Champlin R, Martin T, Wieder E: Peptide Vaccination with PR1 Elicits Active T Cell Immunity That Induces Cytogenetic Remission in Acute Myelogenous Leukemia. American Society of Hematology Philadelphia, PA, 2002

APPENDICES

1. *Curriculum Vitae*, Domchek
2. Vonderheide RH, et al. Vaccination of cancer patients against telomerase induces functional anti-tumor CD8+ T lymphocytes, *Clin Can Res*, 2004, 10: 828-839.

UNIVERSITY OF PENNSYLVANIA - SCHOOL OF MEDICINE
Curriculum Vitae

September, 2004

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Education

9/86-6/90	B.A.	Dartmouth College, Hanover, NH (Engineering Sciences)
9/93-6/94		Oxford University, England (English Literature)
8/90-6/95	M.D.	Harvard Medical School

Postgraduate Training and Fellowship Appointments

7/95-6/96	Intern, Internal Medicine, Massachusetts General Hospital
7/96-6/98	Resident, Internal Medicine, Massachusetts General Hospital
7/98-7/01	Clinical Fellow in Hematology and Oncology, Dana-Farber Cancer Institute
1/00-12/00	Chief Medical Resident, Massachusetts General Hospital

Faculty Appointments

1/00-10/01	Instructor in Medicine, Harvard University
11/01-	Assistant Professor in Medicine, University of Pennsylvania

Hospital and Administrative Appointments

1/00-6/01	Assistant in Medicine, Massachusetts General Hospital
7/01-10/01	Assistant in Medicine, Dana-Farber Cancer Institute

Specialty Certification

1998	American Board of Internal Medicine
2001	American Board of Internal Medicine: Medical Oncology

Licensure

1998	Massachusetts
2001	Pennsylvania

Awards, Honors and Membership in Honorary Societies

1989	Choate Scholar, Dartmouth College
1989	Phi Beta Kappa, Dartmouth College
1990	<i>Summa cum laude</i> , Dartmouth College
1993	Marshall Scholar, Oxford University
1995	<i>Magna cum laude</i> , Harvard Medical School
2000	Chief Medical Resident, Massachusetts General Hospital
2001	Landenberger Scholar, University of Pennsylvania
2002	Ann B. Young Assistant Professor in Cancer Research, University of Pennsylvania
2003	Department of Defense, Physician-Scientist Award

Memberships in Professional and Scientific Societies

1999-	American Society of Clinical Oncology
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Editorial Positions

2000-	Ad Hoc reviewer, <i>Cancer</i>
2001-	Ad Hoc reviewer, <i>Journal of Clinical Oncology</i>
2002-	Ad Hoc reviewer, <i>New England Journal of Medicine</i>
2002-	Ad Hoc reviewer, <i>Journal of Human Genetics</i>
2002-	Ad Hoc reviewer, <i>Clinical Cancer Research</i>
2003-	Ad Hoc reviewer, <i>Journal of General Internal Medicine</i>

Academic Committees at Massachusetts General Hospital

1999-2000	Internship Selection Committee, Internal Medicine Residency
2000	Internal Review of Pediatrics Program
2000	Teaching and Training Council, Internal Medicine Resident
2000	Curriculum Committee, Internal Medicine Residency
2000	Training Program Council, Internal Medicine Residency

Academic Committees at University of Pennsylvania

2003	Educational Taskforce for Department of Medicine Strategic Planning Initiative
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Major Teaching Responsibilities at Harvard University

2000	Chief Resident in Medicine, Massachusetts General Hospital
	<ul style="list-style-type: none">• Planned and led daily residents' reports• Provided case discussion at multiple residents' reports• Planned and supervised daily lecture series for the medical housestaff• Planned and ran weekly Morbidity & Mortality conferences• Provided case discussion at multiple Morbidity & Mortality conferences• Served as ward attending for two months and supervised team of residents, interns and medical students• Taught at case conferences for Harvard Medical students• Served as medical consult attending for one month and supervised team of residents• Directed and organized the entire medical residency program which included 130 interns and residents

Major Teaching Responsibilities at University of Pennsylvania

11/01-	Assistant Professor of Medicine, University of Pennsylvania
	<ul style="list-style-type: none">• Serve as inpatient attending for four weeks a year, supervising team of fellows, residents, interns and medical students• Serve as inpatient oncology consult four weeks a year, supervising oncology fellows• Preceptor to medical students and residents in outpatient clinic• Preceptor to residents in the Women's Health Elective
7/02	"Cancer screening trials", Educational series for medical oncology fellows
5/03	"Breast cancer genetics", Medical student, endocrinology course
5/03	"Tamoxifen decision-making", Medical student decision making course
5/04	"Breast cancer genetics", Medical student women's health course
6/04	"Adjuvant therapy in breast cancer", University of Pennsylvania radiation oncology residents, June 1 2004
7/04	"Cancer screening trials", Educational series for medical oncology fellows

Lectures by Invitation

8/98	Fellow Conference on Breast Cancer, M.D. Anderson Cancer Center "Predictors of skeletal complications in metastatic breast cancer", Houston, TX
11/98	New England Cancer Society Annual Meeting, "Predictors of skeletal complications in metastatic breast cancer", Worcester, MA.

- 8/00 "Chemotherapy concepts for house officers",
Massachusetts General Hospital Medical Housestaff lecture series, Boston, MA
- 2/01 "Breast cancer", Massachusetts General Hospital Medical Housestaff lecture series, Boston, MA
- 3/01 Harvard Medical Student Subinternship teaching series, monthly presentation,
Boston, MA
- 5/01 "Breast cancer, risk and prevention", Newton-Wellesley Hospital, Newton,
MA
- 6/01 "Hormonal replacement therapy and the risk of breast cancer", Living Well
series, Dana-Farber Cancer Institute, Boston, MA
- 7/01 "Hormonal therapies in breast cancer", Educational series for radiation oncology
residents, Dana-Farber Cancer Institute, Boston, MA
- 9/01 "Breast cancer", Massachusetts General Hospital Medical Housestaff lecture
series. Boston, MA
- 9/02 "Ductal lavage for the detection of breast cancer: how it works and what's the
evidence?", Changing Concepts in Breast Cancer 2002 Conference, University
of Pennsylvania, Philadelphia, PA
- 10/02 "Breast cancer: risk, screening, prevention and management", Moravian
College, Bethlehem, PA
- 10/02 "Breast cancer genetics: who to test and how to manage", Moravian College,
Bethlehem, PA
- 10/02 "What is a clinical trial?", Pennsylvania Breast Cancer Coalition, Harrisburg, PA
- 10/02 "Ductal lavage", Life After Breast Cancer, University of Pennsylvania,
Philadelphia, PA
- 11/02 "Hormone replacement therapy and breast cancer risk", FOCUS panel discussion, University of
Pennsylvania, Philadelphia, PA
- 1/03 "Management of BRCA1 and BRCA2 mutation carriers", San Antonio Update, Baylor College of
Medicine, Washington D.C.
- 6/03 "Update on breast cancer susceptibility genes". Medical Grand Rounds, Chester County Hospital,
West Chester, PA.
- 8/03 "Breast, ovarian and colon cancer genetics." Medical Grand Rounds. Pocono Medical Center, East
Stroudsburg, PA
- 9/03 "Breast cancer genetics", Life After Breast Cancer, University of Pennsylvania, Philadelphia, PA
- 9/03 "Breast cancer genetics: How to test and how to manage". Medical Grand Rounds. Lancaster
General Hospital, Lancaster, PA.
- 1/04 "Breast cancer genetics". Medical Grand Rounds, St. Joseph's Hospital, Reading, PA
- 5/04 "Update in adjuvant therapy for breast cancer", Teich Lecture, Beth-Israel Medical Center, New
York
- 5/04 "Risk models in clinical practice", National Cancer Institute Risk Modeling Meeting, Washington,
DC
- 6/04 "ASCO update: Breast cancer prevention, detection and genetics", University of Pennsylvania
CME course, Philadelphia PA

Organizing Roles in Scientific Meetings

2004- American Society of Clinical Oncology, Education Committee, Tumor Biology and Genetics

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Research publications, peer reviewed

1. Gurmankin AD, Domchek SM, Stopfer J, Fels C, Armstrong K. Patients' resistance to risk information in genetic counseling for BRCA1/2. *Archives of Int Med*. 2004, *in press*.
2. Huang J, Domchek SM, Brose MS, Rebbeck TR, Nathanson KL, Weber BL. Germline CHEK2*1100delC mutations in breast cancer patients with multiple primary cancers. *Am J Hum Gen*. 2004; *in press*.
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- telomerase induces functional anti-tumor CD8+ T lymphocytes. *Clinical Cancer Research*. 2004; 10:828-39.
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 - Piccione E, Case RD, Domchek SM, Hu P, Chaudhuri M, Backer JM, Schlessinger J, Shoelson SE. Phosphatidylinositol 3-kinase p85 SH2 domain specificity defined by direct phosphopeptide/SH2 domain binding. *Biochemistry*. 1993; 32:3197-202.
 - Domchek SM, Auger KR, Chatterjee S, Burke TR Jr, Shoelson SE. Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphonopeptide. *Biochemistry*. 1992; 31:9865-70.

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- Domchek SM, Vonderheide RH, Younger J, Iglehart JD, Winer EP. Assessment of long-term survivors of metastatic breast cancer. American Society of Clinical Oncology. Orlando, Florida 2002.
- Domchek SM, Tweed AJ, Friebel T, Rebbeck TR, Weber BL. Estrogen-receptor status in breast cancers of BRCA1-mutation carriers following oophorectomy: implications for tamoxifen chemoprevention. American Society of Clinical Oncology. Chicago, Illinois 2003.
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Editorials, Reviews, Chapters

- Domchek SM and Garber JE. Genetic Testing for Breast Cancer. *Principles & Practice of Oncology: Updates*. 2001; 15: 1-15.
- Domchek SM and Weber BL. Inherited Genetic Factors in Breast Cancer. Chapter in *Diseases of the Breast*. 2004.

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

Robert H. Vonderheide,³ Susan M. Domchek,^{1,2}
 Joachim L. Schultze,^{1,2} Daniel J. George,^{1,2}
 Kara M. Hoar,¹ Dih-Yih Chen,³
 Katherine F. Stephans,¹ Kenkichi Masutomi,¹
 Massimo Loda,¹ Zhinan Xia,¹
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 Lee M. Nadler^{1,2}

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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 oncogenesis.

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 MHC antigen 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 (ILAKFLHWL) 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 found 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

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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 I540 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 I540-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 I540 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 Administra-

tion 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 leukapheresis 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 monocyte precursors by plating PBMC at the equivalent of 25×10^6 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 (Immunex, 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 2×10^6 cells/ml with KLH (10 μ g/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 I540 (ILAKFLHWL) at 60 μ g/ml, HIV RT-pol476 (ILKEPVHGV; Ref. 23) at 40 μ g/ml, and influenza MP58 (GILGFVFTL; Ref. 24) at 10 μ g/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 15×10^6 autologous DCs every other week for up to six vaccinations. For each vaccination, three injections were given: 5×10^6 DC pulsed with I540 hTERT peptide and KLH were injected in a right-sided extremity, 5×10^6 DC pulsed with MP58 influenza peptide and KLH were injected in a left-sided extremity, and 5×10^6 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°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 diaminobenzidine 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 $\beta 2$ -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 (LL-FGYPVYV; Ref. 26) from HTLV-1 tax and validated using L11-specific clones (14). Cells were incubated with tetramers and with mAbs CD8-FITC (Immunotech, 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 (Immunotech).

In Vitro Peptide Stimulation. Thawed PBMCs (10^6 /well) were incubated with autologous irradiated (32Gy) PBMCs

(10^6 /well) in the presence of peptide ($10 \mu\text{g/ml}$; New England Peptide, Fitchburg, MA) and $\beta 2$ -microglobulin ($2.5 \mu\text{g/ml}$; Sigma, St. Louis, MO) in complete medium (RPMI 1640 with 10% human AB serum, 2 mM glutamine, 20 mM HEPES, and 15 $\mu\text{g/ml}$ 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 2.5×10^4 cells/well were added to ImmunoSpot plates (Cellular Technology, Cleveland, OH) precoated with 10 $\mu\text{g/ml}$ of anti-IFN- γ mAb (Mabtech, Nacka, Sweden) in the presence or absence of 5 $\mu\text{g/ml}$ of peptide overnight at 37°C . After washing, wells were incubated with 1 $\mu\text{g/ml}$ 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, Edgemont, 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 $\text{cpm of (experimental result - spontaneous release) / (maximum release - spontaneous release)}$. 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 $\mu\text{g/ml}$ 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

Table 1 Patient characteristics and responses

Patient	Carcinoma ^a	Age (yrs)	Prior therapy for advanced disease ^b	No. of vaccinations given	Telomerase reverse transcriptase CTL immune response	Clinical response ^c
UPIN 901	HI Prostate	60	H	1	No	NE
UPIN 902	HI Prostate	58	H, C	6	No	SD
UPIN 903	HI Prostate	72	H, I	6	Yes	SD
UPIN 904	HI Prostate	52	H, C	6	Yes	SD
UPIN 905	HI Prostate	57	H, C	6	No	SD
UPIN 906	Met Breast	40	C, H, T	6	Yes	PD
UPIN 907	Met Breast	52	C, H, R	3	Yes	MR

^a HI Prostate, hormone-independent prostate cancer; Met Breast, 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.

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 15×10^6 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 (30×10^6 DCs per vaccination) and third (90×10^6 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

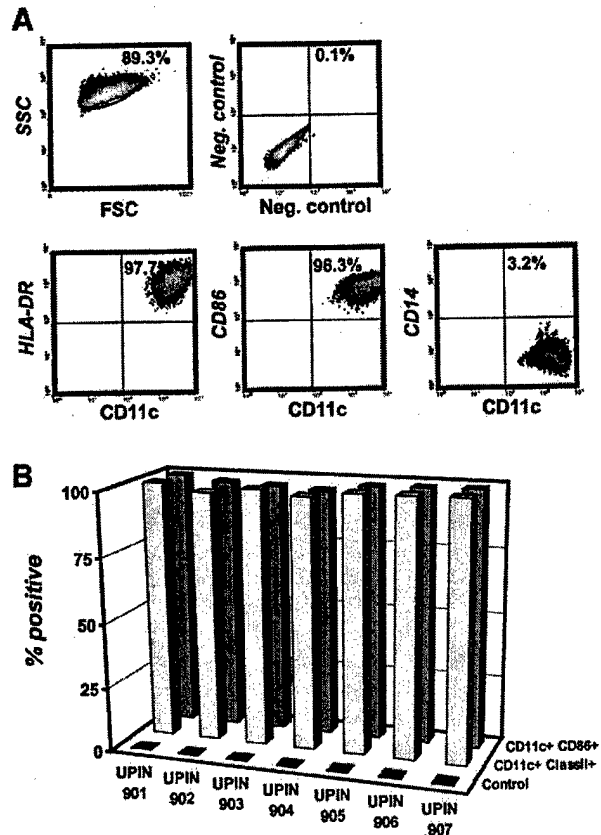


Fig. 1 Flow cytometric analysis of dendritic cell (DC) preparations used for vaccination. A, after 7 days in culture, an aliquot of cells for each DC preparation was stained with monoclonal antibody (mAb) specific for CD11c, HLA-DR MHC class II, CD86, and CD14 and isotype control mAb (shown for one vaccine for UPIN 904). After gating for forward and side scatter profiles, cell surface expression of these markers was determined in two-color analysis. The percentage of double-positive cells is shown in each panel. B, The percentage of CD11c+ CD86+ cells and CD11c+ MHC class II+ cells for all vaccine preparations is shown as the mean percentage per patient. SD of percentage of double-positive cells for each patient was $<0.8\%$ (range, 0.4–7.5%).

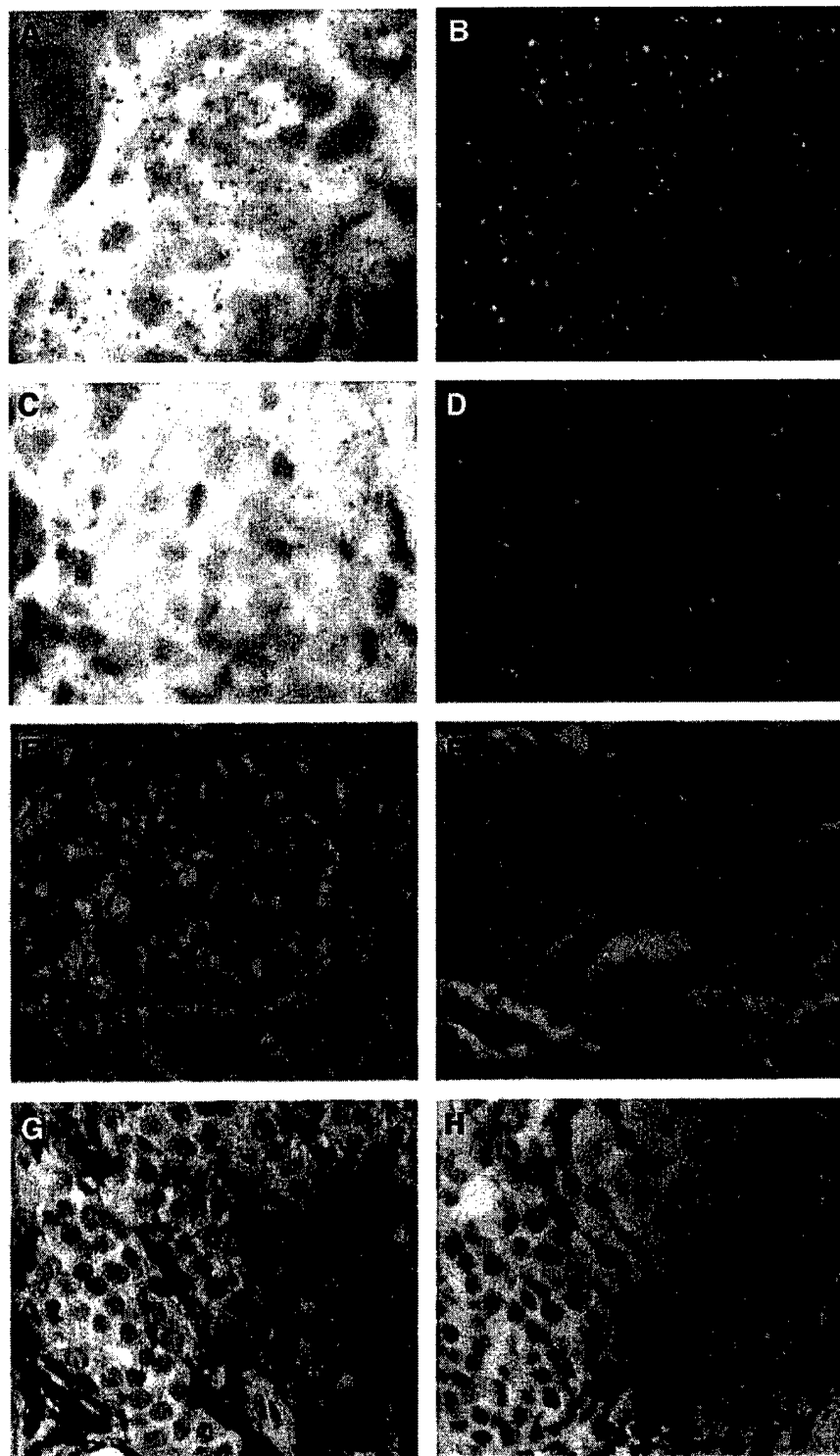


Fig. 2 Evaluation of tumor biopsies from patient UPIN 907 before (A-E) and after vaccination (F-H). Telomerase reverse transcriptase expression was revealed by specific hybridization with the antisense probe (A and B) but not the control sense probe (C and D). Biopsy of tissue before vaccination is shown 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, $\times 400$; E-H, $\times 200$.

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

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 detec-

tion ($<0.03\%$ 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-

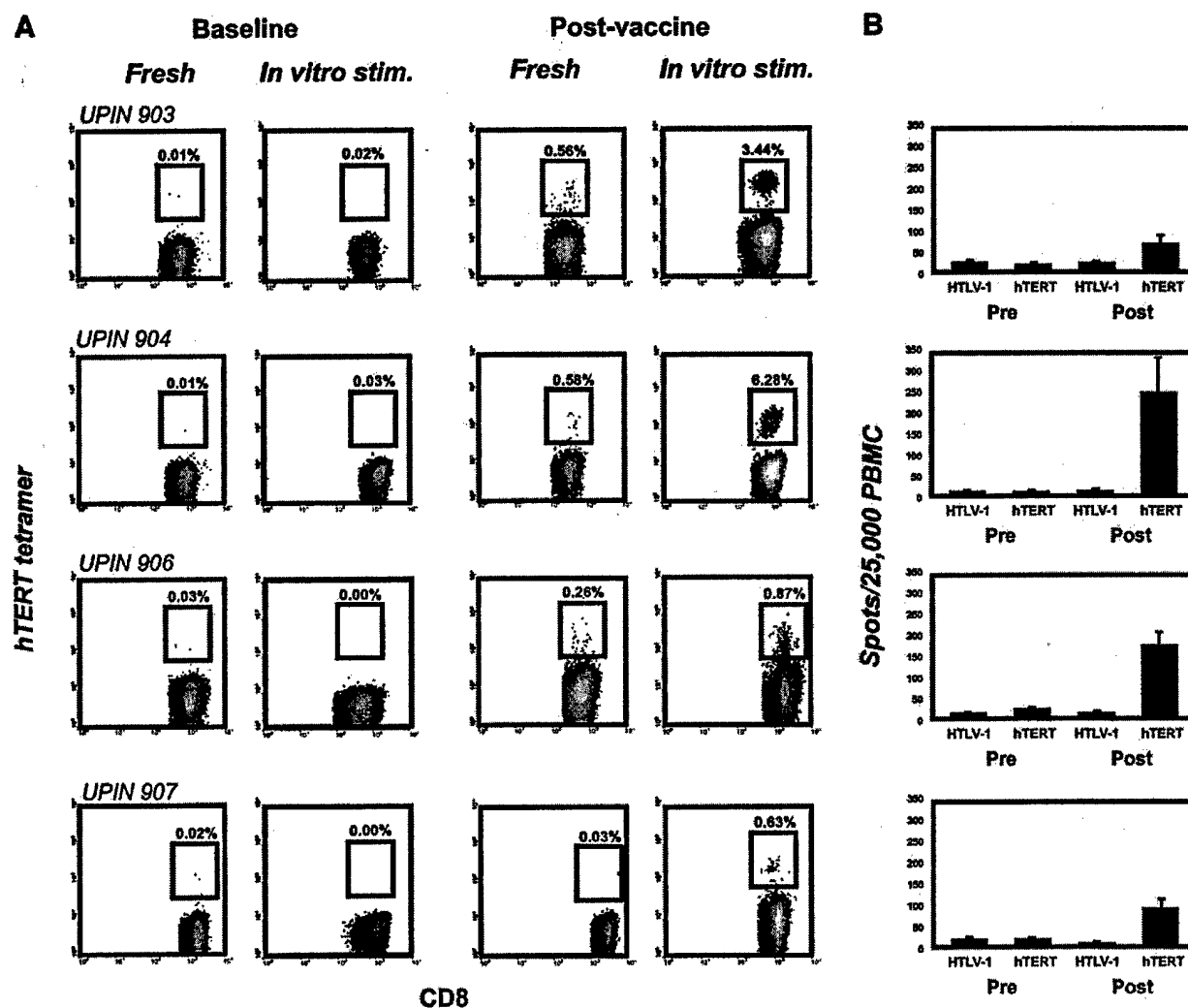


Fig. 3 Telomerase reverse transcriptase (hTERT)-specific CD8+ T-cell responses detectable in 4 patients by tetramer and functional analyses. **A**, CD8+ CD4- CD14- cells were analyzed from uncultured (fresh) peripheral blood mononuclear cell (PBMC) for binding to hTERT I540 tetramer either at baseline or postvaccine. Baseline or postvaccine PBMCs were also subjected to *in vitro* sensitization (IVS) with hTERT I540 peptide and CD8+ CD4- CD14- cells were analyzed for binding to tetramer. **B**, PBMCs from either pre- or postvaccine samples were subjected to IVS with hTERT I540 and then analyzed by ELISPOT for IFN- γ secretion to either hTERT I540 peptide or HTLV-1 peptide. For each patient shown, $P < 0.01$ (Student's *t* test) for pre- versus postvaccine hTERT responses; bars, \pm SD.

lated *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, autologous 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.

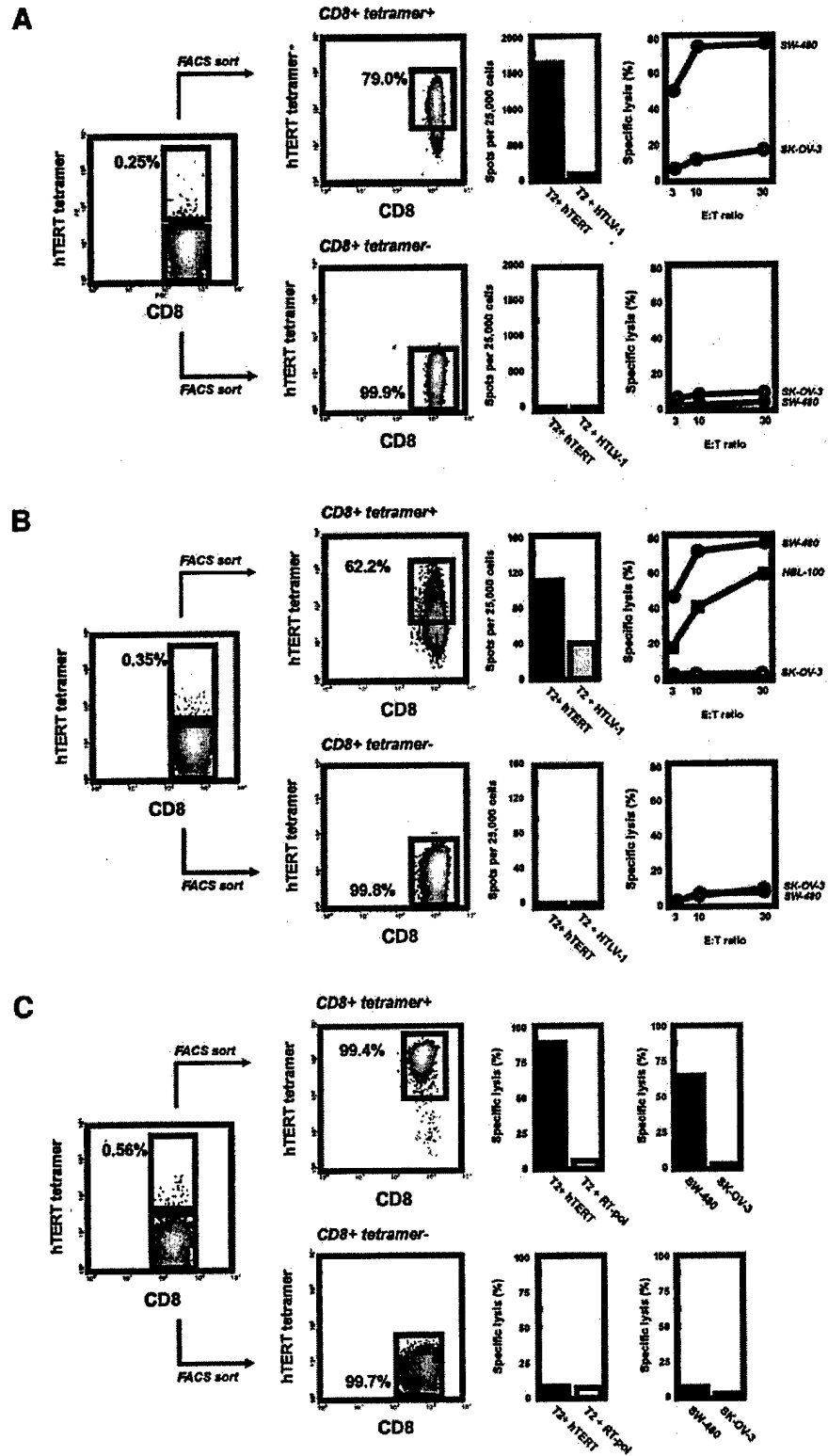


Fig. 4 Polyclonal expansion and cell sorting of telomerase reverse transcriptase (*hTERT*) I540 tetramer+ CD8+ cells from vaccinated patients. Polyclonal expansion and tetramer-guided high speed sorted achieved highly enriched populations of tetramer+ CD8+ cells or tetramer- CD8+ cells for (A) UPIN 903, (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.

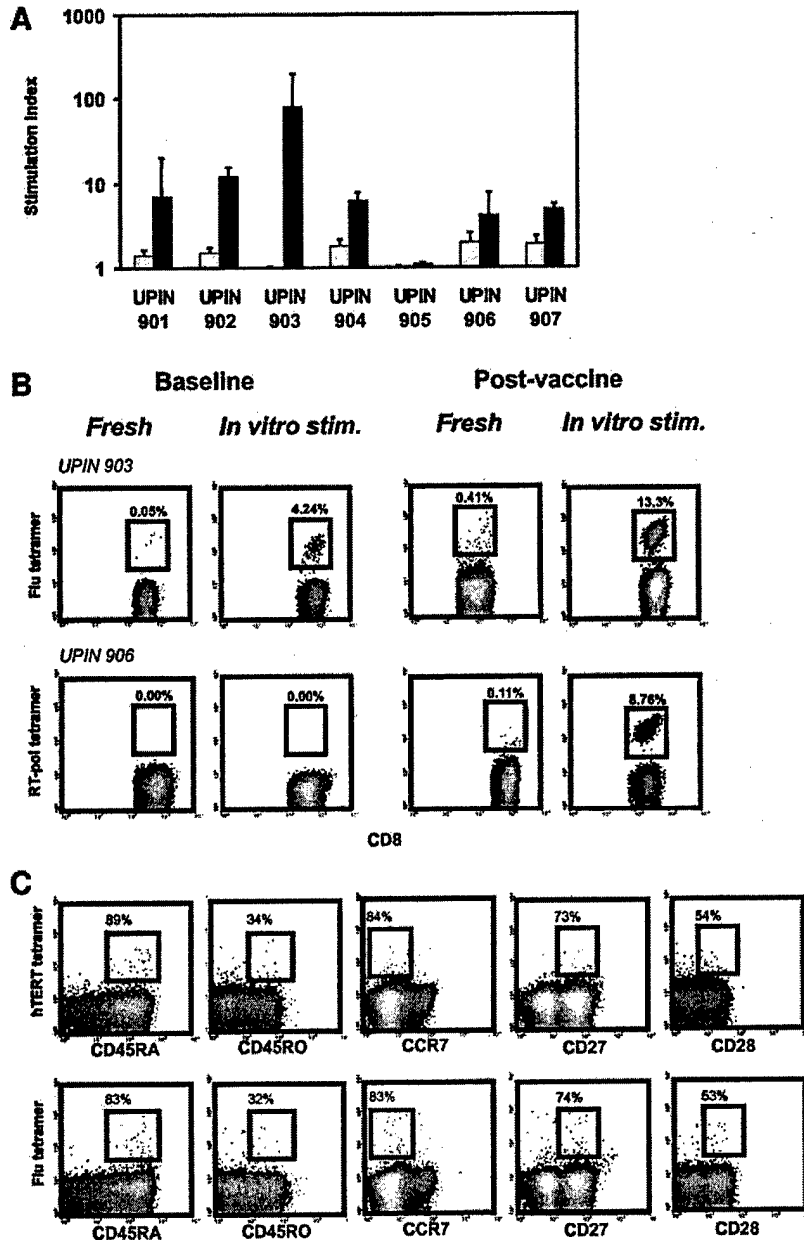


Fig. 5 Immune responses to control antigens and surface phenotype of patient CD8⁺ T cells. **A**, *in vitro* lymphocyte proliferative responses to keyhole limpet hemocyanin before (□) and after (■) vaccination. For UPIN 902, UPIN 903, UPIN 904, and UPIN 907, $P < 0.03$ (Student's *t* test). **B**, CD8⁺ CD4⁻ CD14⁻ cells were analyzed from uncultured (fresh) peripheral blood mononuclear cells (PBMCs) for binding to Flu MP58 tetramer (shown for UPIN 903) or HIV RT-pol476 tetramer (shown for UPIN 906) either at baseline or postvaccine. Baseline or postvaccine PBMCs were also subjected to *in vitro* sensitization with peptide and CD8⁺ CD4⁻ CD14⁻ cells were analyzed for binding to tetramer. In each case, staining with HTLV-1 tetramer was below the limit of detection (<0.03%; data not shown). **C**, cell surface phenotype of telomerase reverse transcriptase I540-specific CD8⁺ T cells (*top row*) or influenza MP58-specific CD8⁺ T cells (*bottom row*) among uncultured, postvaccine CD8⁺ CD4⁻ CD14⁻ PBMCs from patient UPIN 903. Results for hTERT-specific CD8⁺ T cells from UPIN 904 and UPIN 906 and influenza MP58-specific CD8⁺ T cells from UPIN 906 were similar (data not shown); bars, \pm SD.

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 tolerized 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 individual *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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