

Research Article

Highly Optimized DNA Vaccine Targeting Human Telomerase Reverse Transcriptase Stimulates Potent Antitumor Immunity

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Abstract

High levels of human telomerase reverse transcriptase (hTERT) are detected in more than 85% of human cancers. Immunologic analysis supports that hTERT is a widely applicable target recognized by T cells and can be potentially studied as a broad cancer immunotherapeutic, or a unique line of defense against tumor recurrence. There remains an urgent need to develop more potent hTERT vaccines. Here, a synthetic highly optimized full-length hTERT DNA vaccine (phTERT) was designed and the induced immunity was examined in mice and non-human primates (NHP). When delivered by electroporation, phTERT elicited strong, broad hTERT-specific CD8 T-cell responses including induction of T cells expressing CD107a, IFN- γ , and TNF- α in mice. The ability of phTERT to overcome tolerance was evaluated in an NHP model, whose TERT is 96% homologous to that of hTERT. Immunized monkeys exhibited robust [average 1,834 spot forming unit (SFU)/ 10^6 peripheral blood mononuclear cells (PBMC)], diverse (multiple immunodominant epitopes) IFN- γ responses and antigen-specific perforin release (average 332 SFU/ 10^6 PBMCs), suggesting that phTERT breaks tolerance and induces potent cytotoxic responses in this human-relevant model. Moreover, in an HPV16-associated tumor model, vaccination of phTERT slows tumor growth and improves survival rate in both prophylactic and therapeutic studies. Finally, *in vivo* cytotoxicity assay confirmed that phTERT-induced CD8 T cells exhibited specific cytotoxic T lymphocyte (CTL) activity, capable of eliminating hTERT-pulsed target cells. These findings support that this synthetic electroporation-delivered DNA phTERT may have a role as a broad therapeutic cancer vaccine candidate. *Cancer Immunol Res*; 1(3); 179–89. ©2013 AACR.

Introduction

Immunotherapy for cancer through induction of antitumor cellular immunity has recently reemerged as an important experimental therapy for the treatment of nonresponsive cancers. However, most tumor-associated antigens (TAA) are expressed in one or a few tumor types as tumors generally exhibit tissue-specific features (1). In contrast, human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is highly expressed in more than 85% of human tumors from diverse cancer phenotypes, with little or no expression in normal somatic cells (2–6). Expression of hTERT

correlates with telomerase activity, which may be a requirement for tumor survival (7). Telomerase activation/hTERT expression is associated with little loss of telomere length and accounts for the unlimited proliferative capacity of cancer cells. As expression of hTERT is directly linked to tumor cell growth and contributes crucially to the long-term survival of tumor cells, loss of telomerase activity will lead to hTERT-positive tumor cell death by apoptosis (8). In addition, targeting hTERT may have the potential to eliminate cancer stem cells as recent studies have suggested that cancer stem or stem-like cells express hTERT (9–11). These findings collectively point to hTERT as an attractive TAA and provide the basis of developing hTERT-based universal vaccine for cancer immunotherapy (12–14).

Therapeutic hTERT vaccines have been widely studied because of their potential to stimulate the killing of tumor cells by enhancing the activity of telomerase-specific cytotoxic CD8 T cells (12). Many studies have been conducted to develop hTERT peptide vaccines containing motifs that either bind to MHC class I (I540 and 572Y) or MHC class II molecules (GV1001; refs. 15–18). Moreover, multiple strategies are being explored to use full-length hTERT recombinant constructs targeting multiple CD8 and CD4 epitopes simultaneously. As a result, the potency of autologous dendritic cells transduced with hTERT mRNA (19, 20) and viral vector-based vaccines

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(21, 22) has been reported with partial success in animal models. However, findings from initial clinical trials of hTERT vaccines in patients with cancer have shown that these approaches suffer from limited induction of CD8⁺ T cells and had limited impact on overall survival (13). Therefore, there remains an urgent need to develop more potent hTERT therapeutic vaccines with a broader T-cell footprint.

Several clinical trials have evaluated the efficacy of DNA vaccination against a variety of cancers (23). Although there are some indications of limited immune responses in vaccinated patients with melanoma or prostate cancer (24, 25), previous DNA vaccines generally seem to induce weak cellular immunity in humans. Recent synthetic DNA design strategies, such as codon/RNA optimization, the addition of highly efficient immunoglobulin leader sequences (26–28), use of more efficient DNA delivery methods including *in vivo* electroporation (29), have been applied to improve the immune responses induced by DNA vaccines in humans, with recent significant success (30). However, these new approaches have not been combined to test a new hTERT DNA vaccine.

In this report, we attempt to extend this improved immune potency to construct a synthetic DNA vaccine expressing a full-length *hTERT* with modifications using a combination of approaches in gene optimization. The hTERT DNA was delivered by electroporation and its immunogenicity and antitumor effect were evaluated in non-human primates (NHP) and mice. These data strongly support further study of the hTERT DNA vaccine in combination with electroporation delivery as a potential immunotherapy platform against an array of human and animal malignancies.

Materials and Methods

Immunogen design and expression

A synthetic hTERT DNA vaccine was generated using the *hTERT* sequence retrieved from GenBank (accession number: AF018196) with several modifications (Fig. 1A). The full-length *hTERT* gene was 3,512 bp and subcloned into an expression vector pGX0001.

In vitro hTERT expression was detected using TNT Quick Coupled Transcription/Translation System (Promega). The gene product was immunoprecipitated using an anti-HA (hemagglutinin) tag monoclonal antibody (Sigma-Aldrich) and analyzed by SDS-PAGE. The synthesized protein was detected by autoradiography.

An indirect immunofluorescent assay was conducted to confirm hTERT expression as previously described (31). Briefly, human rhabdomyosarcoma cells were transfected with pH_{hTERT} and pGX0001 (1 µg/well) using TurboFectin8.0 Transfection Reagent (OriGene). Forty-eight hours later, the cells were fixed and incubated with anti-hTERT (C-term) monoclonal antibody (Millipore) overnight at 4°C. The slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Cell Signaling Technology), and analyzed by fluorescent microscopy (Leica Microsystems, Inc.) using the SPOT Advanced software (SPOT Diagnostic Instruments, Inc.).

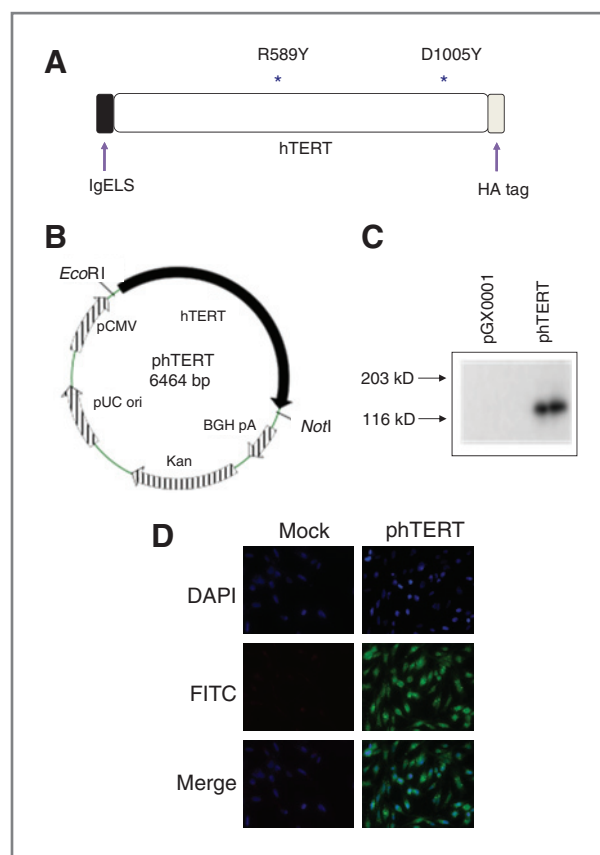


Figure 1. Design and expression of hTERT DNA vaccine. A, schematic of hTERT antigen. The * denotes the incorporated point mutation. B, map of pH_{hTERT}. C, detection of pH_{hTERT} expression by *in vitro* translation. The gene product was immunoprecipitated using an anti-HA (hemagglutinin) tag monoclonal antibody, visualized by SDS-PAGE and autoradiography. D, immunofluorescence assay of pH_{hTERT}. Transfected rhabdomyosarcoma cells expressing hTERT protein showed typical FITC-fluorescence using a commercial hTERT (C-term) monoclonal antibody. DAPI, 4',6'-diamidino-2-phenylindole.

Mice studies

Mice and immunization. Female 8-week-old C57BL/6 mice were purchased from The Jackson Laboratory. Their care was in accordance with the guidelines of NIH and University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Mice were divided into two groups and immunized with 50 µg of hTERT DNA by intramuscular injection into the quadriceps followed by electroporation using the CELLECTRA adaptive constant current device (Inovio Pharmaceuticals, Inc.; ref. 32). The mice received four immunizations, two weeks apart. One week after the last immunization, the mice were sacrificed and splenocytes were isolated for immunology studies.

ELISpot assay. Mouse IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay was conducted as previously described (31). Peptides spanning the entire hTERT protein, each containing 15 amino acids overlapping by eight amino acids, were synthesized by GenScript. The entire set of peptides was pooled at a concentration of 2 µg/mL/peptide into four pools for stimulation of the IFN-γ release.

CD8⁺ T-cell depletion. CD8⁺ lymphocytes were depleted from splenocytes using Dynabeads mouse CD8 (Lyt2; Life Technologies). After CD8⁺ T cells depletion, IFN- γ ELISpot was conducted as described earlier.

Intracellular cytokine staining. Intracellular cytokine staining (ICS) was conducted as described previously (32). Briefly, splenocytes from vaccinated and naïve mice were stimulated with hTERT peptides, stained with FITC anti-mouse CD107a, and followed by ViViD Dye (Invitrogen). Cells were then stained with the following extracellular antibodies: APC-Cy7 anti-mouse CD3e, PerCP-Cy5.5 anti-mouse CD4, and APC anti-mouse CD8a (BD Biosciences). Intracellular cytokines were subsequently stained with the following antibodies: Alexa Fluor 700 anti-mouse IFN- γ and PE-Cy7 anti-mouse TNF (BD Biosciences).

Cell line. TC-1 cell line is a well-characterized lung epithelial cell line immortalized with HPV16 E6/E7, and transformed with the *c-Ha-ras* oncogene (33). TC-1 cells were purchased from American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C.

In vivo tumor challenge study. Ten female 8-week-old C57BL/6 mice were immunized with 50 μ g of pH₁TERT four times biweekly. One week after the last immunization, each mouse was challenged with 5×10^4 TC-1 cells injected subcutaneously including 10 naïve mice that served as a control. Tumors were measured twice weekly with digital calipers spanning the shortest (width) and longest surface diameters (length; ref. 34). Tumor volumes were calculated according to the formula: $V = \text{length} \times \text{width}^2 \times \pi/6$ (35). Mice were sacrificed when tumor diameter reached 20 mm in compliance with our IACUC protocol.

In vivo tumor treatment study. Female C57BL/6 mice were separated into two groups of 10 mice: naïve and hTERT group. On day 0, all mice were injected subcutaneously with 5×10^4 TC-1 cells in the right flank. All mice in the hTERT group were immunized with 50 μ g of pH₁TERT on days 3, 10, 17, and 24. Tumors measurement was conducted as described earlier.

In vivo cytotoxicity study. An *in vivo* cytotoxicity assay was conducted as previously described (36, 37). Splenocytes from naïve mice were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) at a concentration of 1 μ mol/L or 1 nmol/L. CFSE^{hi} (1 μ mol/L)-labeled cells were pulsed with the relevant peptides (hTERT peptides), whereas CFSE^{lo} (1 nmol/L)-labeled cells were pulsed with the irrelevant peptides (HPV6 E6/E7 peptides). Equal frequency of CFSE^{hi} and CFSE^{lo} cells was combined and 10^7 cells were intravenously injected into naïve or pH₁TERT-immunized mice. Forty-eight hours later, splenocytes were isolated and analyzed by flow cytometry. The percentage killing was calculated as follows: $100 - [(\% \text{ relevant peptide pulsed in immunized} / \% \text{ irrelevant peptide pulsed in immunized}) / (\% \text{ relevant peptide pulsed in naïve} / \% \text{ irrelevant peptide pulsed in naïve})] \times 100$.

Rhesus monkey studies

Immunization and PBMC isolation. Four rhesus macaques were vaccinated with pH₁TERT, four times intramuscularly

followed by electroporation using CELLECTRA adaptive constant current electroporation device, 6 weeks apart, at 2 mg DNA/each immunization. Blood was collected 2 weeks after each immunization and peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Hypaque density gradient centrifugation.

IFN- γ and perforin ELISpot assay. Monkey IFN- γ and perforin ELISpot were conducted as previously described (38, 39). Antigen-specific responses were determined by subtracting the number of spots in the negative control wells from the wells containing peptides. After subtracting the negative control, the mean value in the wells with the PBMCs collected postvaccination had to exceed 50 SFU/ 10^6 PBMCs and be at least four times higher than prevaccination reactivity to be considered as a positive response.

Statistical analysis. Standard and paired Student *t* tests were applied to analyze statistical significance of all quantitative data produced in this study, and $P < 0.05$ was considered statistically significant.

Results

Design and construction of the full-length hTERT DNA vaccine

As indicated in Fig. 1A, the hTERT immunogen was developed with several modifications, including codon/RNA optimization and the addition of a highly efficient leader sequence, to enhance the expression and immunogenicity of pH₁TERT. Two mutations (R589Y and D1005Y) were incorporated into the hTERT sequence to assist in breaking tolerance (40). The modified gene was subcloned into pGX0001 and named as pH₁TERT for further study (Fig. 1B)

Expression of hTERT

In vitro expression of hTERT was verified by T7 coupled transcription and translation reaction. After immunoprecipitation with the anti-HA tag monoclonal antibody, hTERT expression was analyzed by 12% SDS-PAGE. The hTERT protein migrated to the corresponding molecular weight at approximately 130 kDa (Fig. 1C). No protein band was detected in the pGX0001 vector lane. An indirect immunofluorescence assay was conducted to further confirm hTERT expression. As shown in Fig. 1D, the cells expressing hTERT protein showed typical FITC-fluorescence, supporting the expression of hTERT in a relatively native conformation. As a control, expression was not detected in pGX0001-transfected cells.

Vaccination with pH₁TERT induces strong CD8-mediated hTERT-specific responses in mice

IFN- γ ELISpot was conducted to assess antigen-specific cellular immune responses induced by pH₁TERT after four immunizations (Fig. 2A). The total response against four pools of hTERT peptides in pH₁TERT-immunized mice was $1,817 \pm 211$ SFU/ 10^6 splenocytes, which was significantly greater than the immune responses in the naïve group (20 ± 6 SFU/ 10^6 splenocytes; $P = 0.01$; Fig. 2B). The C-terminus of the hTERT protein (peptide pool 4) was the most immunogenic region, accounting for about half the response elicited by pH₁TERT (970 ± 113 SFU/ 10^6 splenocytes).

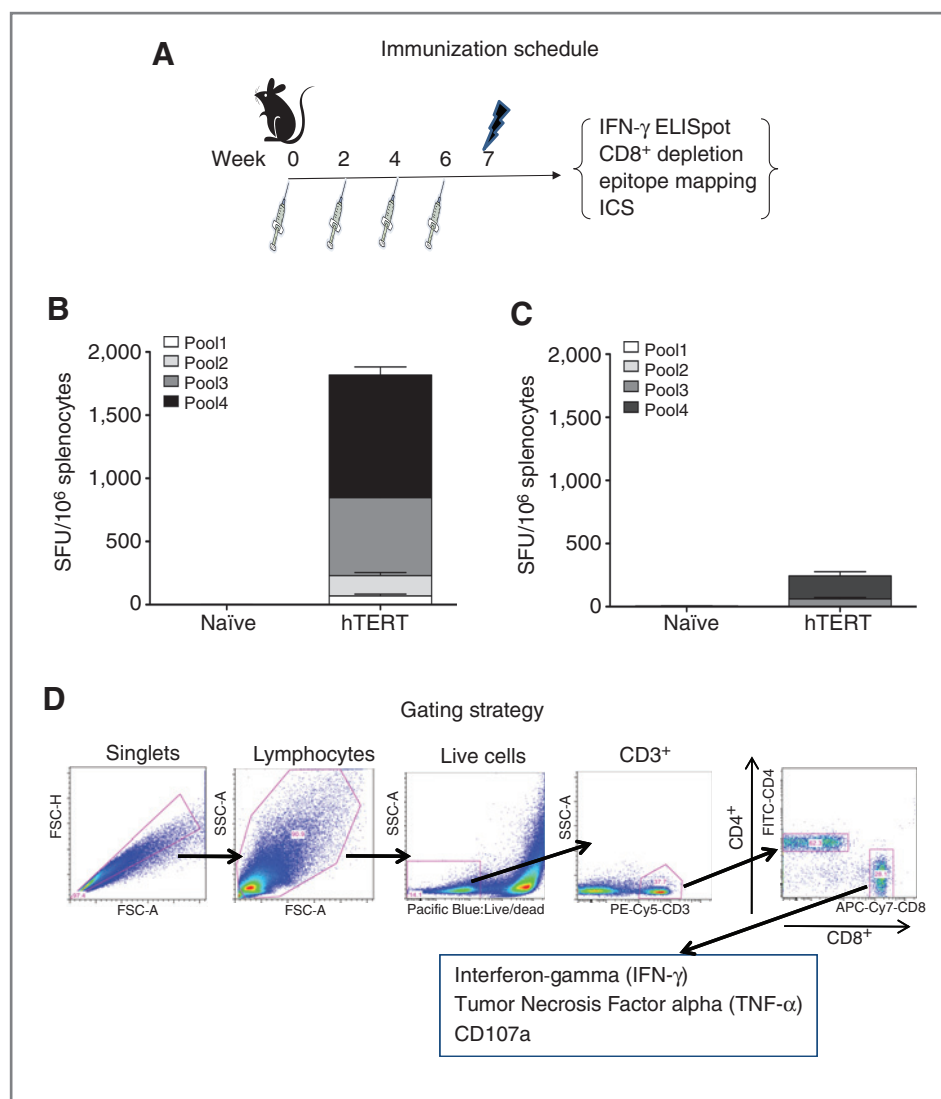


Figure 2. phTERT elicited robust CD8⁺ immune response in C57BL/6 mice. **A**, immunization schedule. **B**, total IFN- γ responses induced by phTERT. Frequencies of IFN- γ -secreting cells/10⁶ splenocytes after four immunizations with phTERT were determined by IFN- γ ELISpot assay. Splenocytes from each mouse (10 mice/group) were stimulated with hTERT peptides. Results were presented as mean \pm SEM. **C**, total IFN- γ responses induced by phTERT after CD8 depletion. CD8 T cells were depleted from splenocytes and IFN- γ ELISpot assay was conducted. **D**, schematic diagram of gating strategy of ICS.

Another ELISpot assay was conducted after CD8⁺ T-cell depletion to determine whether CD8⁺ cells were responsible for the detected robust IFN- γ responses. The results indicated that the spot number was reduced to 244 ± 78 in the phTERT-vaccinated mice (a 85% decrease in the frequency of IFN- γ -producing cells) after CD8⁺ T-cell depletion (Fig. 2C), supporting that the vaccine-induced IFN- γ production is mediated mainly by CD8⁺ T cells.

The breadth of T-cell response was suggested to be important for antitumor effect. Accordingly, an additional ELISpot assay was conducted against 26 hTERT matrix peptide pools. There were nine matrix pools showing more than 50 spots, indicating that phTERT could elicit a broad range of T-cell immune responses (Supplementary Fig. S1A). There were eight epitope-comprising peptides in the region from amino acid 288 to 869 (Table 1). The C-terminus (amino acid 862-1158) had the most epitope-comprising peptides (8 peptides), which was consistent with the result in Fig. 2B. Individual peptides in pool 4 were used to confirm

the matrix mapping result against the C-terminus of hTERT, and two immunodominant epitope-containing peptides (RKTVVNFPVEDEALG and KNPTFFLRVISDTAS) were identified (Supplementary Fig. S1B). As shown in Supplementary Fig. S1C, some of the identified peptides were conserved between mTERT and hTERT. All peptides listed in Table 1 were confirmed to contain one H2-D^b-restricted epitope by using Immune Epitope Database (IEDB) analysis resource Consensus tool (<http://tools.immuneepitope.org>), suggesting effective processing of this antigen.

Vaccination with phTERT in mice enhances magnitude of IFN- γ , CD107a, and TNF- α production in CD8 T cells

ICS assays were conducted to further characterize the responses induced by phTERT. The secretion of IFN- γ , CD107a, and TNF- α in both CD4⁺ and CD8⁺ T cells were determined (Fig. 2D). As shown in Fig. 2E, the average frequency of CD8⁺IFN- γ ⁺ cells in immunized mice (0.8%) was significantly higher than that of the naïve group (0.1%; $P < 0.05$). The

Figure 2. (Continued) E, secretion of CD107a, IFN- γ , and TNF- α postvaccination in both CD4⁺ and CD8⁺ cells. Splenocytes were stimulated with hTERT peptides for 5 hours before surface and intracellular staining. Background-subtracted percentages of hTERT-specific CD4⁺ or CD8⁺ T cells producing CD107a, IFN- γ , and TNF- α were calculated. The experiment shown is representative of three different experiments using 5 mice per group.

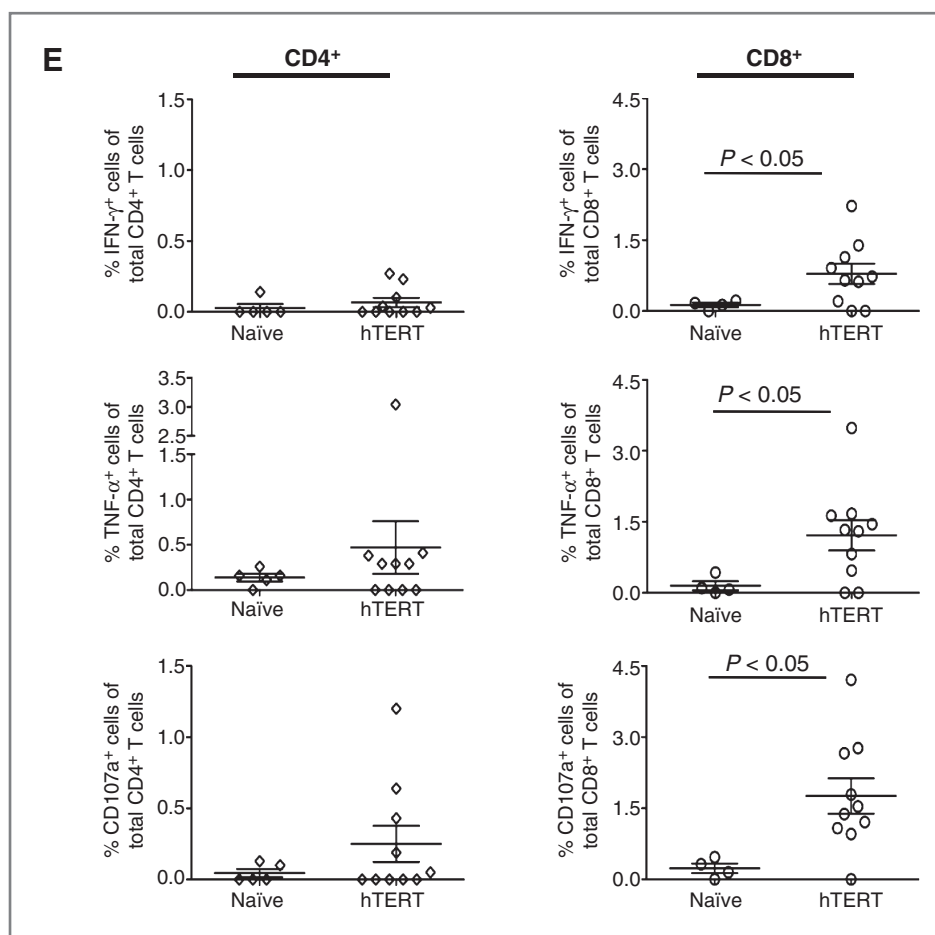


Table 1. Identified epitope-comprising peptides in mice immunized with pHTERT

	Number of epitope-comprising peptides	Sequence of epitope-comprising peptides
aa 1–296 (pool 1)	None	N/A
aa 288–582 (pool 2)	4	TGARRLVETIFLGSR ETIFLGSRPWMPGTP RPLFLELLGNHAQCP LGNHAQCPYGVLKKT
aa 575–869 (pool 3)	4	DGLRPIVNMDYVVG NMDYVVGARTFRREK SVLNAYERARRPGLLG ARRPGLLGASVLGLD
aa 862–1158 (pool 4)	8	RKTWNFPVEDEALG PVEDEALGGTAFVQM DTRTLEVQSDYSSYA QSDYSSYARTSIRAS NSLQTVCTNIYKILL TNIYKILLQAYRFH KNPTFFLRVISDTAS RVISDTASLCYSILK

NOTE: Two identified immuno-dominant epitope-comprising peptides are highlighted in bold.
Abbreviation: aa, amino acid.

percentage of TNF- α -secreting cells from the total CD8⁺ T-cell population in pHtERT-immunized mice was approximately 1.2% on average, whereas the naïve group produced only 0.1% ($P < 0.05$). After showing that antigen-specific CD8⁺ T cells had the ability to secrete IFN- γ and TNF- α , we investigated whether these CD8⁺ T cells exhibited a phenotype of putative CTLs. CD107a, a marker of cytolytic degranulation on lymphocytes, such as CD8⁺ T cells, was used to evaluate the CTL potential of vaccine-induced T cells. Following stimulation with hTERT peptides, the percentage of CD107a-positive CD8 cells was 1.8%, which was significantly higher than the percentage in the naïve group. There was a trend showing the increased production of IFN- γ , TNF- α , and CD107a in CD4⁺ T cells in the immunized mice. However, the differences in average frequencies of CD4⁺IFN- γ ⁺, CD4⁺ TNF- α ⁺, and CD4⁺ CD107a⁺ cells were not statistically significant compared with what were observed in the naïve mice. The immune responses elicited by pHtERT are heavily skewed toward driving CD8⁺ lymphocytes with the potential to lyse hTERT-expressing tumor cells.

Vaccination with pHtERT is capable of breaking tolerance and generating robust hTERT-specific CTLs in rhesus macaques

The induction of T-cell immunity against the tumor antigen hTERT could be controlled by mechanisms of central and peripheral tolerance. Sequence homology analysis indicated that hTERT shares 64% identity with mouse TERT, and 96% identity with rhesus macaque TERT. Consequently, immune tolerance is expected to play a major role in testing the efficacy of an hTERT vaccine in NHPs. Furthermore, rhesus T-cell immunity is much closer to human T-cell immunity serving as a highly relevant model for immunotherapeutic vaccine development. Therefore, we moved forward to determine whether pHtERT is able to break tolerance and induce cellular responses in rhesus macaques. The prebleed blood samples were studied to establish the background level of immune response of each individual animal in the study.

The results showed that the hTERT-immunized monkeys exhibited very low background level of immune response (5 SFU/10⁶ PBMCs) with a dramatic increase in vaccine-induced responses following each immunization. The average number of IFN- γ -producing cells after the first, second, third, and fourth immunization was 11, 177, 834, and 1,834 SFU/10⁶ PBMCs (Fig. 3A), respectively. These results showed that immunization with pHtERT could elicit strong boostable hTERT-specific cellular responses. Epitope mapping was also conducted to investigate the diversity of the observed immune responses in rhesus macaques. Except for animal 5015 with only two matrix pools showing 50 or more SFU/10⁶ PBMCs, the rest of immunized animals exhibited responses to 9 (M4628), 13 (M5012), and 10 (M5021) matrix pools with 50 or more spots out of 26 pools, suggesting that there were multiple dominant and subdominant epitopes in response to the vaccine (Supplementary Fig. S2A). Most identified epitope-comprising peptides are either 100% conserved between NHP and humans or exhibit only a single amino acid difference (Supplementary Fig. S2B and Table 2), supporting that this approach can break

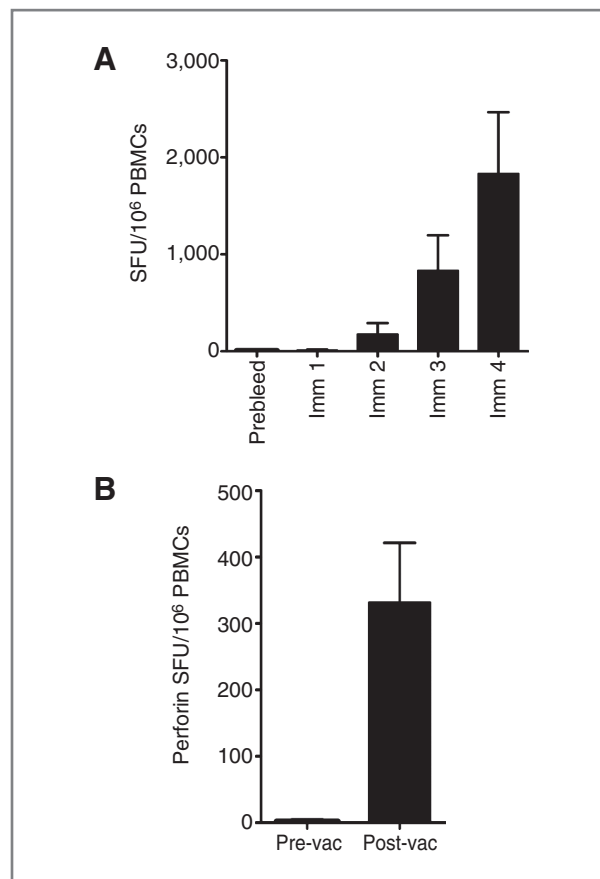


Figure 3. pHtERT elicited robust CTLs in rhesus macaques. **A**, total IFN- γ responses induced by pHtERT following each immunization. Four rhesus macaques were vaccinated with pHtERT, four times intramuscularly followed by electroporation, 6 weeks apart, at 2 mg DNA/each immunization. PBMCs were isolated and stimulated with hTERT peptides for 24 hours. Frequencies of hTERT-specific IFN- γ -secreting cells/10⁶ PBMCs were determined by IFN- γ ELISpot assay. **B**, enhanced perforin release in immunized monkeys. Prebleed and PBMCs after last immunization were stimulated with hTERT peptides for 24 hours. Frequencies of hTERT-specific perforin-secreting cells/10⁶ PBMCs were determined by perforin ELISpot assay. Results are presented as mean \pm SEM.

tolerance in relevant species to epitopes with importance to human immunotherapy.

As perforin is known to be a major cytolytic protein and key effector molecule for T cell-mediated cytotoxicity, perforin ELISpot was used to determine whether hTERT-specific T cells were capable of releasing perforin from cytotoxic granules. Results showed that the average numbers of perforin-producing cells were 332 SFU/10⁶ PBMCs (Fig. 3B), indicating that vaccination elicited CTLs that could destroy hTERT-expressing target cells.

Assessment of physiologic parameters in NHP

To examine whether there is any CTL-mediated host toxicity, we assessed a number of physiologic parameters in NHP (Supplementary Table S1). No significant weight loss was observed and white blood cell (WBC) counts remained within

Table 2. Summary of epitope mapping results in rhesus macaques immunized with pHtTERT

Monkey ID	No. of epitope-comprising peptides	No. of epitope-comprising peptides with 100% identity between hTERT and RhTERT	No. of epitope-comprising peptides with single aa difference between hTERT and RhTERT
M4628	17	5	9
M5012	41	19	14
M5015	1	1	0
M5021	24	12	7

Abbreviations: aa, amino acid; RhTERT, rhesus macaque TERT.

normal range. No elevation of alkaline phosphatase (ALK P), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) indicated that induction of hTERT-specific immune responses did not cause significant damage to the liver. No evidence of impaired kidney function was seen, as creatinine and blood urea nitrogen (BUN) remained within normal limits. Creatine phosphokinase (CPK) was evaluated to determine whether electroporation or induction of immune responses negatively influenced skeletal or cardiac muscle. Elevation of CPK was not detected. Overall, we did not observe any vaccine-induced adverse effects in NHP despite evidence of strong hTERT-specific CTLs *in vivo*.

Vaccination with pHtTERT elicits antitumor immunity and delays the E6/E7-expressing tumor growth

It is important to determine whether the robust pHtTERT-induced T cells could actually exhibit antitumor immunity in an *in vivo* challenge model. As hTERT may have value as a broad immunogen to prevent tumor recurrence posttreatment or in identified high-risk individuals, we first studied immunization of mice followed by tumor challenge. High level of mTERT expression is detected in TC-1 tumor cells (41), therefore, an *in vivo* TC-1 tumor challenge study was conducted to assess whether vaccination with pHtTERT could mediate antitumor immunity (Fig. 4A). The data showed that immunized mice exhibited significantly smaller tumors compared with those in the naïve group (Fig. 4B and D) at all days postchallenge till day 35 ($P < 0.05$). Thirty-seven days posttumor challenge, the mice in naïve group either died or were euthanized because the diameters of tumors reached 20 mm. In contrast, about 70% of pHtTERT-immunized mice still survived 37 days posttumor implantation (Fig. 4C). These data indicated that pHtTERT induced potent antitumor immunity, and immunized animals exhibited delayed tumor growth and improved survival.

hTERT-specific CD8 T cells induced by vaccination eliminated target cells *in vivo*

Although we showed the upregulation of CD107a and increased release of perforin in hTERT-specific CD8 T cells, we thought it would be important to confirm CTL activities *in vivo*. Therefore, an *in vivo* cytotoxicity assay was conducted to evaluate the ability of vaccine-induced CD8 T cells to eliminate

target cells. CFSE-labeled splenocytes were pulsed with either hTERT or HPV6 E6/E7 peptides and adoptively transferred into either naïve or pHtTERT-immunized mice. The killing activity was evaluated by gating on CFSE-labeled splenocytes (Fig. 4E). As shown in Fig. 4F, the average percentage killing observed in 5 immunized mice was about 73%, indicating a strong antigen-specific killing of target cells. No killing of irrelevant T cells was observed. The result confirmed that the vaccine-induced CD8 T cells had killing capacity to initiate target cell death *in vivo*.

Vaccination with pHtTERT slows the tumor growth in tumor-bearing mice

Given the results obtained from the prophylactic tumor study, an *in vivo* tumor therapy study was conducted to analyze in an initial fashion the therapeutic effect of vaccination with pHtTERT. We initiated the study by challenging mice with 5×10^4 TC-1 cells on day 0. Three days after TC-1 cells implantation, 10 mice in the hTERT group were immunized with pHtTERT and boosted on day 10, 17, and 24 (Fig. 5A). All mice exhibited tumor growth, however, the tumors in pHtTERT-immunized mice were significantly smaller than those in the naïve group at day 39 ($P < 0.05$; Fig. 5B). Six of 10 pHtTERT-immunized mice still survived 39 days posttumor implantation, whereas all mice in naïve group were either dead or euthanized (Fig. 5C). Therefore, vaccination with pHtTERT slows tumor growth and improve survival rate of tumor-bearing C57BL/6 mice.

Discussion

Overexpression of hTERT has been linked to development and progression of more than 85% of cancer types in a variety of species. Peptides derived from hTERT can be processed by tumors and presented in the context of MHC class I molecules, thus triggering hTERT-specific T cells (42–44). Although there has been a great deal of important activity, there remains a need for improved hTERT immunogens.

DNA vaccines have emerged as an attractive approach for antigen-specific immunotherapy. This technology has significant potential, compared with traditional protein and peptide vaccines, in terms of generating CTL responses (45). However, few studies have been conducted to develop hTERT DNA

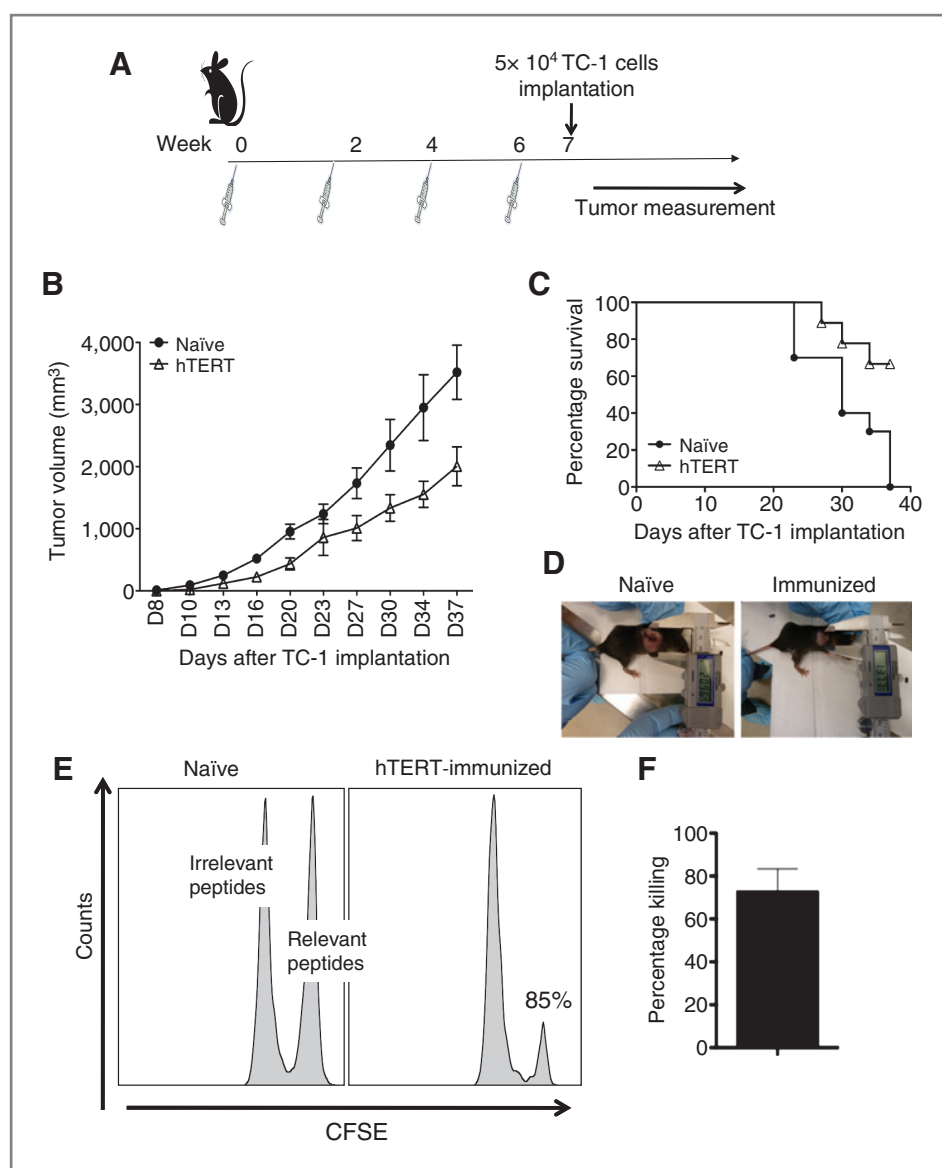


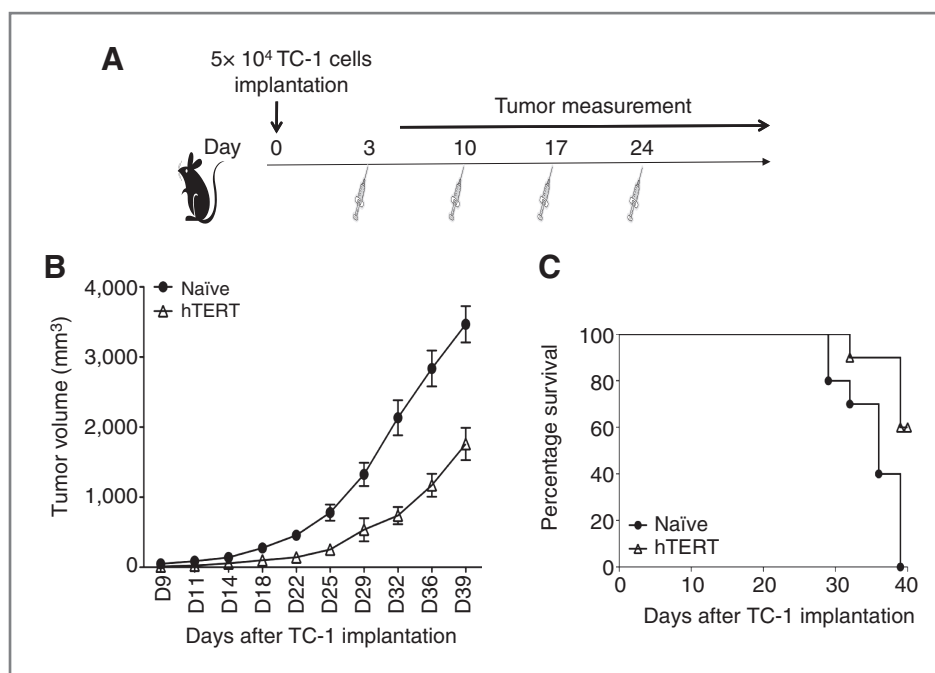
Figure 4. Preventive antitumor immunity induced by pHtERT. **A**, experimental design. Ten mice were immunized with 50 μ g of pHtERT four times biweekly. Each mouse was challenged with 5×10^4 TC-1 cells injected subcutaneously including 10 naïve mice, which served as a control. Tumors were measured twice weekly. **B**, delayed tumor growth in immunized mice. Tumor measurements for each time point are shown only for surviving mice. **C**, vaccination with pHtERT extended survival. **D**, representative image of tumor size in naïve or pHtERT-vaccinated group at day 34 after TC-1 implantation. **E**, percentage killing of a representative pHtERT-immunized mouse. CFSE-labeled splenocytes were pulsed with either hTERT or HPV6 E6/E7 peptides and adoptively transferred into naïve or pHtERT immunized mice through the tail vein. Forty-eight hours later, CFSE-labeled cells were recovered and analyzed by fluorescence-activated cell sorting (FACS) to quantify percentage killing. **F**, average percentage killing of 5 pHtERT-immunized mice. Results were presented as mean \pm SEM. The experiment shown is representative of two repeated experiments.

vaccines. Previously, the DNA platform was studied in prime-boost strategies due to the low immunogenicity of hTERT DNA vaccines (46–48). Here, we developed a novel hTERT DNA vaccine using gene optimization strategies and showed that the vaccine delivered by electroporation induced strong cellular immune responses in both mice and NHP. The immune responses observed in monkeys were much higher than what has been previously described for other hTERT DNA vaccines. The T-cell immune responses after just three immunizations with 2 mg of pHtERT by electroporation in monkeys (834 SFU/ 10^6 PBMCs) were already comparable to these induced by a DNA/electroporation prime Ad6 boost strategy (which included 5 mg dose DNA delivered with electroporation five times followed by 10^{11} VP of Ad6 boost two times; ref. 48). It is likely that the combination of better construct optimization strategies and a more potent CELLECTRA electroporation delivery system accounts for the increased vaccine-induced responses.

Recently, by using a similar combination of the approaches in gene optimization and gene delivery, we have successfully shown that a novel human papillomavirus (HPV) therapeutic DNA vaccine could induce robust cellular immune responses to E6/E7 of HPV16 and 18 with cytolytic functionality in women previously treated for high-grade cervical dysplasia (30). The immune responses boosted significantly further when the fourth immunization of pHtERT was conducted ($1,834$ SFU/ 10^6 PBMCs), suggesting enhanced antigen-specific immune responses may be obtained by multiple vaccinations. This ability to boost T-cell responses without antivector responses or other limitations could be an important advantage in clinical studies.

Cytotoxic CD8 T cells are considered crucial components of antitumor immunity that attack tumor cells presenting TAA peptide with MHC class I on their surface (49, 50). As a result, one major focus in the field of cancer immunotherapy has been

Figure 5. Therapeutic effect induced by pH₂TERT in tumor-bearing C57BL/6 mice. **A**, experimental design. Mice were separated into two groups of 10 mice, naïve and hTERT group. On day 0, all mice were injected subcutaneously with 5×10^4 TC-1 cells. Starting on day 3, all mice in the hTERT group were immunized with 50 μ g of pH₂TERT at weekly intervals four times. **B**, vaccination with pH₂TERT delayed tumor growth in tumor-bearing mice. Tumor measurements for each time point are shown only for surviving mice. **C**, vaccination with pH₂TERT extended survival in tumor-bearing mice. The experiment shown is representative of two repeated experiments.



on the stimulation of antigen-specific CD8⁺ CTL responses. Research has indicated that patients with breast cancer who mounted an hTERT-specific CTL exhibit significantly longer median overall survival (51). Here, we first showed vaccination of mice with the hTERT DNA vaccine significantly enhanced the numbers of CD107a-, IFN- γ -, and TNF- α -producing cells, indicating the generation of putative antigen-specific CTLs. Second, the vaccine-induced T cells exhibited ability to release perforin in the monkeys immunized with pH₂TERT. Moreover, instead of conducting the ⁵¹Cr-release assay, we applied an *in vivo* cytotoxicity assay to confirm the CTL function *in vivo* by flow cytometry. This assay allowed us to measure cytotoxicity quantitatively by counting the loss of antigen-specific target cells. Our data showed vaccination-elicited CTL that could destroy target cells (the average percentage killing observed in the immunized mice was about 73%). The clinical benefit of the vaccine-induced CTL responses needs to be further investigated.

Effective cancer therapeutic vaccines that mediate clinical responses in patients with cancer may require generation of broadly targeting CTLs against multiple epitopes to limit tumor immune escape. Recently, a therapeutic vaccine for renal cell cancer consisting of multiple tumor-associated peptides was able to induce T cells associated with longer patient survival (52). Expanded diversity of the T-cell responses in these trials was associated with clinical benefit, indicating that targeting multiple epitopes in immunotherapy improved clinical efficacy. Liao and colleagues showed that a peptide vaccine including four hTERT HLA-A0201-restricted CTL epitopes could elicit stronger antitumor immunity than their corresponding linear peptides (16). Therefore, multi-epitope-containing DNA vaccines may represent promising tools for inducing antitumor responses in patients with cancer. By delivering large numbers of CTL epitopes, DNA vaccines may

avoid T-cell epitope restriction to a particular MHC haplotype and possible immunoselection of epitope loss variants (53). Here, we confirmed that vaccination with pH₂TERT could not only induce multiple H2-D^b-restricted epitopes in mice, but also could elicit multiple dominant and subdominant epitopes in monkeys. A broad spectrum of T-cell responses was induced by vaccination, implying a possible clinical benefit and suggesting potential improved performance in the clinic.

It has been reported that hTERT antigen-specific CTLs are effective in targeting human cancers *in vivo* (8, 22). As hTERT T cells could be considered effective immune surveillance to prevent recurrence posttreatment or to limit tumor development in identified high-risk individuals, we studied immunization of mice followed by tumor challenge for their ability against tumor challenge. Previous studies have shown that xenogeneic melanoma-associated DNA vaccines can elicit effective antitumor immunity against murine melanoma (54, 55). Yamano and colleagues found that a human C-terminal TERT DNA vaccine induced effective antitumor immunity against TS/A murine breast cancer (46). Because the amino acid homology of NTE, RT, and CTE regions between hTERT and murine TERT are 61.8%, 65.5%, and 69.9%, respectively, the hTERT has regions of homology, which may induce testable antitumor immunity against murine TERT-expressing tumor cells. Hence, we used a TC-1 tumor challenge model to evaluate the antitumor effect of hTERT-specific CTL. The results show that vaccination with pH₂TERT conferred delayed tumor growth and longer overall survival. As this antitumor effect was potentially associated with T-cell tumor infiltration, it will be interesting to conduct more studies to investigate the phenotype of these tumor-infiltrating CD8 CTLs. On the basis of the data we observed in the NHP study, we would expect that closer matching of a mouse TERT vaccine to

the native mouse sequence would further improve the effectiveness.

A major challenge with regard to hTERT and other TAA immunotherapy vaccines is to develop therapies capable of generating a robust CTL against this self-antigen in a safe manner. Although hTERT is overexpressed in most tumor cells, its expression can also be detected in rare normal cells such as hematopoietic progenitor cells, spermatogonia in the testis, activated lymphocytes, and certain epithelial cells (56). Consequently, the question of whether vaccine-induced hTERT-specific CTLs carries the risk of inducing autoimmune responses with pathologic consequence was raised. Many studies have shown the hTERT-specific CTLs have no detectable effect on hTERT-positive CD34⁺ hematopoietic progenitor cells or activated T cells (43, 44) and do not result in autoimmune responses that target normal hTERT-expressing cells (22, 57). In addition, clinical studies in patients with cancer using hTERT-based vaccines have not shown toxicity (8, 19, 58). These findings may reflect relatively low levels of hTERT expression or ineffective processing of hTERT peptides in normal cells. In the present study, several important physiologic parameters were evaluated and no vaccine-induced adverse effects were detected in phTERT-immunized monkeys despite evidence of strong hTERT-specific CTLs.

Taken together, we report that administration of a synthetic highly optimized hTERT DNA vaccine in combination with adaptive constant current electroporation delivery platform was capable of breaking immune tolerance and eliciting robust and diverse CTLs in mouse and NHP models. These vaccine-induced CTLs seemed not to be associated with any major toxicities or organ damage, and were effective in

mounting a potent antitumor response. These data support further study of phTERT in the setting of cancer immunotherapy, to improve tumor immune surveillance in high-risk individuals, and prevention of disease recurrence.

Disclosure of Potential Conflicts of Interest

N.Y. Sardesai has ownership interest (including patents) in Inovio Pharmaceuticals, Inc. D.B. Weiner has commercial research grant from Inovio Pharmaceuticals, Inc. and MVI, ownership interest (including patents) in Inovio Pharmaceuticals, Inc., and is a consultant/advisory board member of Inovio Pharmaceuticals, Inc., Novartis, VGXi, Pfizer, Merck, and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Yan, P. Pankhong
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