Preventive Vaccination with Telomerase Controls Tumor Growth in Genetically Engineered and Carcinogen-Induced Mouse Models of Cancer

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Abstract
The telomerase reverse transcriptase, TERT, is an attractive target for human cancer vaccination because its expression is reactivated in a conspicuous fraction of human tumors. Genetic vaccination with murine telomerase (mTERT) could break immune tolerance in different mouse strains and resulted in the induction of both CD4+ and CD8+ telomerase-specific T cells. The mTERT-derived immunodominant epitopes recognized by CD8+ T cells were further defined in these mouse strains and used to track immune responses. Antitumor efficacy of telomerase-based vaccination was investigated in two cancer models closely resembling human diseases: the TRAMP transgenic mice for prostate cancer and a carcinogen-induced model for colon cancer. TERT overexpression in tumor lesions was shown in both models by immunohistochemistry, thus reinforcing the similarity of these tumors to their human counterparts. Repeated immunizations with mTERT-encoding DNA resulted in a significant delay of tumor formation and progression in both the prostate cancer and the colon cancer models. Moreover, evaluation of the intratumoral infiltrate revealed the presence of telomerase-specific T cells in vaccinated mice. The safety of vaccination was confirmed by the absence of histomorphologic changes on postnecropsy analysis of several organs and lack of adverse effects on blood cell counts. These results indicate that TERT vaccination can elicit antigen-specific immunosurveillance and imply this antigen as a potential candidate for preventive cancer vaccines. [Cancer Res 2008; 68(23):9865–74]

Introduction
TERT is considered a very attractive antigen for cancer therapy (1). Telomerase is a ribonucleoprotein in which the protein component, TERT, uses its RNA as template for adding telomeric repeat sequences to the ends of chromosomes. TERT is shut down in most human somatic tissues but reactivated in 85% of tumors, conferring unlimited replicative potential to neoplastic cells. When telomerase is reactivated in tumor cells, TERT is processed and presented together with class I MHC molecules and tumors are recognized by T lymphocytes specific against telomerase (2, 3). These findings have justified cancer vaccination clinical trials based either on autologous dendritic cells transfected with human TERT–derived peptides (4, 5).

To exploit the immunogenicity versus tolerance issue, we vaccinated mice with mouse TERT (mTERT)–based DNA vaccines. Gene-based vaccination strategies are probably the most promising approaches to induce effective cell-mediated immunity (CMI) against cancer (6). Delivery of antigens by DNA injection allows access to multiple antigen-presenting pathways. Moreover, increased expression of the antigen and enhanced immunogenicity can be achieved by physical methods, including in vivo electroporation (7, 8), and molecular modifications, such as the use of codon-optimized sequences and the fusion of the target antigen to microbial-derived proteins (9, 10). DNA vaccines are safe and easy to produce; moreover, they can be used repeatedly for long-term maintenance of antitumor CMI.

There are differences in telomerase activity between humans and rodents; in particular, telomerase has higher basal levels of activity in mouse tissues compared with humans. Nevertheless, reactivation of telomerase is commonly occurring in mouse tumors as well and abrogation of telomerase activity in mouse cancerous cells impairs their metastatic potential (11, 12). Many of the data about the efficacy of genetic vaccination with mTERT were obtained with transplantable tumor models but the correlation of responses to immunization with overexpression of telomerase was not investigated (13). Transplantable tumor cell lines are useful for immunologic studies but genetically engineered and chemically induced tumors in rodents are considered better models for evaluating results transferable to the clinical setting (14). The TRAMP mouse model is genetically engineered to expresses SV40 large T antigen preferentially in the prostate epithelium to develop prostate tumors (15). The carcinogen 1,2-dimethylhydrazine (DMH) is widely used for the induction of colon cancers in mouse (16, 17). Because our immunohistochemistry data showed mTERT overexpression in both prostate and colon tumors, we used these experimental models to monitor the effect of mTERT-based vaccination. Here, we show that TERT-based genetic vaccination induces telomerase-specific CD8+ T cells able to infiltrate tumor lesions and affects various stages of tumor progression in both tumor models without causing any detectable adverse effect.

Materials and Methods

Mice and cell lines. TRAMP mice and TRAMP-C2 cell line were a gift from N. M. Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA). The presence of PB-Tag gene in TRAMP mice was tested by PCR as...
described (15) and heterozygous TRAMP mice were used in the experiments. C57Bl/6 (H-2b) and BALB/c (H-2b) mice were from Charles River. Mice were treated in accordance with national legislation and European guidelines by means of an internal review process. MBL-2 (H-2d) is a C57Bl/6 leukemia line, B16 (H-2b) is a C57Bl/6 melanoma line, and CT26 (H-2b) is a BALB/c colon carcinoma.

DNA vectors and immunization procedures. The synthetic codon optimized sequence coding for TPA-mTERT-LTB (mTERT-LTB) was obtained by GENART and cloned into vector pV1JnsA (18). cDNA encoding mTERT wild-type sequence was obtained by Geron Corp. and obtained by GENEART and cloned into vector pV1JnsA (18). cDNA optimized sequence coding for TPA-mTERT-LTB (mTERT-LTB) was performed according to commonly used protocols available at the DNA vaccine Web site (8).

Leukocyte cultures. Spleens were removed from the animals and cell suspension was prepared. Mouse peripheral blood mononuclear cells (PBMC) were obtained by retroocular bleeding. RBCs were removed by osmotic lyses. Mixed leukocyte cultures (MLC) were set up with TRAMP splenocytes (3 × 10^7) coincubated with γ-irradiated allogeneic BALB/c splenocytes (3 × 10^5). Mixed leukocyte peptide cultures (MLPC) were set up with either naive or vaccinated TRAMP splenocytes (5 × 10^5) restimulated in vitro with 1 μmol/L of mTERT_198-205 peptide (VGRNFTNL) or β-galactosidase (β-gal) were used. DNA immunization was performed according to commonly used protocols available at the DNA vaccine Web site (8).

Enzyme-linked immunosorbent spot assays. Ninety-six-well MAIP plates (Millipore) were coated overnight with a 2.5 μg/mL solution of rat anti-mouse IFN-γ (BD Pharmingen). Cells were plated at 1 × 10^5 per well in triplicate and incubated for 20 h at 37°C with 2 μg/mL peptides. After incubation, plates were washed with PBS and 0.05% Tween 20 and incubated overnight at 4°C with anti-mouse biotin-conjugated anti-IFN-γ antibody. The day after, streptavidin-alkaline phosphate conjugate was added for 2 h. Plates were developed by adding nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Pierce) and spots were then counted using an automated enzyme-linked immunosorbent spot (ELISPOT) reader.

ELISA. Splenocytes (10^5 cells) from MLPCs were restimulated for 24 h in triplicate with an equal amount of target cells, the supernatants were harvested, and released IFN-γ was measured by ELISA (Endogen). Cytotoxicity assay. Cytotoxicity was measured by 51Cr release assay. MLPCs were incubated either with MBL-2 cells pulsed with the peptides or with B16 target cells pretreated when indicated for 24 h with IFN-γ (25 ng/mL). Target cells were labeled with 51Cr (100 μCi; Amersham) and then mixed with effector cells. Assays were performed in triplicate and supernatants were harvested to measure 51Cr released. The percent of specific lysis was calculated as previously described (20).

Histologic evaluation of prostate lesions in TRAMP. The urogenital tract was removed at necropsy and prepared for pathologic evaluation: tissues were fixed in paraformaldehyde/lysine/periodate for 3 h at 4°C, infiltrated with 30% sucrose in PBS overnight at 4°C, and frozen in OCT. Histologic sections were analyzed with a standard H&E stain. The sections were evaluated by a pathologist blinded to the treatment groups according to Kaplan-Lefko guidelines (21). The presence of normal tissue, prostate intraepithelial neoplasia (PIN; epithelial stratification with occasional mitotic figures or cribriform pattern), adenocarcinoma (ADC; abnormal glands or multiple papillae with vessel-richstromal axes lined by epithelial cells with numerous mitoses), poorly differentiated carcinoma (PDC; sheets of small cells with a low nucleus-cytoplasm ratio with no glandular formation, numerous mitotic, and apoptotic figures) was scored.

The Lasso tool (Adobe Photoshop software) was used to perform quantitative image analysis.

Immunohistochemistry. For the detection of telomerase, cryostat sections (4–10 μm) of colon and prostate were incubated, respectively, with the anti-mouse telomerase ab177 and with ab23099 (Abcam run out of the ab177 during our study; Abcam) for overnight at 4°C. Briefly, sections were rinsed in PBS-0.1% Triton and incubated with the secondary anti-rabbit antibody. To reveal the reaction, we used an ABC Complex (Vector) for 30 min at room temperature. Only in colon tumors, staining was enhanced with nickel-3,3'-diamobenzidin.

For the detection of tumor-infiltrating lymphocytes (TIL), prostate tissue slides were incubated with rat anti-mouse CD8 (AbD Serotec) for 30 min followed by 30-min incubation with goat anti-rat IgG (Jackson Immunoresearch). To reveal the reaction, slides were incubated for 30 min with alkaline phosphatase–conjugated streptavidin and stained with fuxin chromogen substrate for 15 min followed by a hematoxylin counterstain.

Cytofluorimetric evaluation of TERT-specific TILs. TILs were isolated by means of a mechanical separation and by enzymatic digestion with 300 units/mL DNase, 0.1% hyaluronidase, and 1% collagenase buffer. After 30-min incubation at 37°C, the cells were filtered through a 70-μm sieve and resuspended in fluorescence-activated cell sorting (FACS) buffer with anti-mouse Fc-γR for 10 min at room temperature. After washing, cells were labeled with mTERT_198-205 (H-2b) tetramer-PE (5 μg/mL; Beckman Coulter) for 20 min at room temperature. Samples were then stained at 4°C with rat anti-CD8-PerCP(Cy5.2) or FITC. Cells were analyzed with FACS Canto II and data were elaborated using DIVA software (BD Biosciences).

Induction of colon tumors by DMH and evaluation of tumor lesions. Treatment of BALB/c mice with DMH [20 mg/kg in Tris-HCl (pH 6.5)] was started at week 6 of age and was performed once a week for 6 wk. Animals were sacrificed to evaluate tumor lesions and colons were dissected, split open on filter paper, and fixed in 4% formalin-1× PBS. Lesions were stained in 1% methylene blue observed microscopically and scored by two independent operators. Colons were discolored by 70% ethanol and paraffin embedded, and 5-μm-thick sections were H&E stained for tumor grading. Tumor grading has been assessed and G1-G2 stages were assigned based on tumor differentiation grade and mitotic event number following AJCC Cancer Staging Manual, Sixth Edition.

Safety assessment. Twenty BALB/c female mice, 6 wk old, were immunized as described in the previous section. Immunized mice and age-matched control mice were sacrificed at 38 wk of age. Hematologic counts were performed (ADIVA 120) and several organs were collected (liver, lungs, spleen, kidneys, heath, injection sites in femoral quadriceps, lymph nodes, bone marrow, and Peyers patches). Tissues were immediately fixed in 10% buffered formalin. Histopathologic evaluation was performed by the Safety Assessment and Pathology (MSD-Chibret, Paris, France).

Statistical analysis. The Wilcoxon-Mann-Whitney U test was used to examine the null hypothesis of rank identity between two sets of nonparametric data. Student's t test was used to compare parametric groups. Kaplan-Maier plots and the Mantel-Haenszel test were used to compare survival of mice belonging to different treatment groups. All P values presented are two sided.

Results

DNA vaccination with mTERT breaks tolerance in healthy mice. The mTERT gene sequence was modified by fusing Escherichia coli LTB sequence to its COOH terminal part (22). LTB is a secreted protein; therefore, to fully incorporate its adjuvant function in the vaccine, we introduced a secretion signal before the telomerase sequence. Moreover, for safety purposes, the mTERT sequence was modified in two amino acid positions to be catalytically inactive (23). This fusion construct was used to exploit the induction of anti-TERT CMI in healthy mice of both C57Bl/6 and BALB/c strains. Mice were therefore subjected to five weekly injections before measuring CMI. The immune response was evaluated among splenocytes by ICS for IFN-γ–producing cells. TERT peptides were divided into
three pools (mTERT pool 1 containing the sequence from amino acids 1–388, pool 2 containing the sequence from amino acids 377–811, and pool 3 containing the sequence from amino acids 801–1122). This approach allows detection of both CD4+ and CD8+ antigen-activated T cells (24). ICS indicated that the CD8+ T-cell epitope was contained within the mTERT pool 1, whereas CD4+ T-cell reactivity was stimulated by pools 2 and 3 in BALB/c mice (Fig. 1A). C57Bl/6 mice showed CD8+ T-cell reactivity against both mTERT pools 1 and 2, whereas CD4+ T-cell response in this strain was detected only toward the NH2-terminal region of the mTERT protein (mTERT-1 peptide pool; Fig. 1B). Intervals of 1 or 2 weeks between immunizations were comparable in terms of CMI induction and two DNA injections were sufficient to induce an immune response against mTERT (data not shown). Finally, we compared our optimized mTERT fusion construct to mTERT plain construct in BALB/c mice. The mTERT plain construct was also immunogenic, inducing a CD4+ T-cell response comparable with that obtained with the LTB fusion construct, but a weaker CD8+ T-cell response (Fig. 1C). CD4+ T-cell reactivity was also detected toward LTB sequence but only when present in the construct (Fig. 1C). Overall, these results indicate that DNA vaccination with mTERT is immunogenic in a “self” context.

**Immune response induced by vaccination against telomerase is long lived and safe.** Twenty BALB/c mice were immunized by five weekly injections with mTERT-LTB. Immune response was analyzed from each individual mouse by ICS on PBMC. PBMCs were collected before starting the immunization (week 6 of age), shortly after the end of the immunization protocol (week 13), and finally at 38 weeks of age. Blood samples were evaluated for their reactivity against mTERT peptide pool 1, previously shown to contain the class I MHC-restricted epitope for BALB/c mice. The anti-TERT CD8+ T-cell response was not changed even a long time after the immunization (Fig. 1D). In spite of the presence of circulating antitelomerase CD8+ T cells, no alteration of blood cell

**Figure 1.** Evaluation of immune reactivity in mice immunized with mTERT. A, BALB/c mice immunized with mTERT-LTB. B, C57Bl/6 mice immunized with mTERT-LTB. For all four panels, cutoff values were set as the average reactivity of six mice immunized with mock plasmid plus 2 SD (0.05% for CD4+ and 0.09% for CD8+ T cells, respectively). In A and B, splenocytes were exposed to the three TERT peptides pools. Columns, average frequencies from 10 individual mice; bars, SD. *, responses significantly different from mice immunized with mock plasmid (P < 0.05, Student’s t test). C, comparison of CMI evaluated by ICS in BALB/c mice immunized either with mTERT construct (black columns) or with mTERT-LTB fusion (gray columns). Columns, average of eight mice per group; bars, SD. *, responses significantly different in the two groups (P < 0.05, Student’s t test). D, longevity of CD8+ T cells induced by vaccination in BALB/c mice immunized with mTERT-LTB. PBMCs were exposed to mTERT-1 peptide pool and analyzed by ICS for IFN-γ production before (week 6) and twice after the immunization (weeks 13 and 38). Columns, average values of 10 mice; bars, SD.
counts was observed between vaccinated (n = 10) and age-matched control (n = 4; average WBC of 7.1 × 10^3/mm^3 and RBC of 11.1 × 10^6/mm^3 in vaccinated mice versus WBC of 7.6 × 10^3/mm^3 and RBC of 11.5 × 10^6/mm^3 in untreated mice). Moreover, no significant histomorphologic changes were noted in mice from vaccinated group compared with untreated mice. Two mice among those receiving the vaccine exhibited focal skeletal muscle fiber atrophy with focal or multifocal mineralization, which suggested marginal postinjury scar, possibly related to the immunization procedure.

**Identification of mTERT immunogenic epitopes in BALB/c and C57Bl/6 mice strains.** To characterize better mTERT epitopes recognized by CD8+ T cells in different mouse strains, midi-pools consisting of twelve 15-mers were prepared so that each individual peptide was present in two different midi-pools. Each pair of peptide pools, including the same 15-mer, was plated on 96-well plates using a matrix scheme. Reactivity was found against the midi-pools D, Q, and R as shown in Fig. 2A for BALB/c mice. Once the reactive overlapping 15-mers were identified, the putative 9-mer epitopes were synthesized and tested individually. Only one epitope corresponding to the mTERT167-175, AYQVCGSPL, was recognized in all BALB/c mice (Fig. 2B). Two different epitopes recognized by CD8+ T cells were instead identified in C57Bl/6 mice: mTERT198-205 VGRNFTNL and mTERT505-512 SLGKYGKL. However, although the peptide mTERT198-205 was recognized by freshly isolated splenocytes of immunized mice (Fig. 2C) and could also expand mTERT-specific CD8+ T cells following in vitro stimulation (shown in next figures), mTERT505-512 failed to expand primed T cells (data not shown). We thus decided to use mTERT198-205 VGRNFTNL epitope for subsequent monitoring of the immune response in C57Bl/6 mice.

**DNA vaccination with mTERT induces CD8+ T-cell response in TRAMP mice.** The TRAMP model of prostate cancer was genetically engineered to target SV40 T antigen expression almost exclusively in the prostate tissue, leading to progressive prostate cancer development in all the mice (15). Despite tumor growth, TRAMP mice remained immunocompetent at different stages.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Identification of the epitopes recognized by CD8+ T cells in BALB/c and C57Bl/6 mice. A, ELISPOT analysis for the identification of reactive 15-mer peptide contained in mTERT pool 1 was performed by determining the reactivity against a series of midi-pools indicated with letters from A to W. B, ELISPOT analysis of the reactivity of five single BALB/c mice (1–5) toward the H-2d–restricted CD8+ T-cell epitope mTERT167-175 (AYQVCGSPL). C, ELISPOT analysis of the reactivity of five individual C57Bl/6 mice (1–5) toward the H-2b–restricted epitope mTERT198-205 (VGRNFTNL). In B and C, IFN-γ–producing cells are enumerated as SFC/10^6 splenocytes from mice immunized with mTERT-LTB plasmid. Cutoff values were set as the average reactivity of six mice immunized with mock plasmid plus 2 SD (12 SFC/10^6 splenocytes).
including the late stages of tumor development (week 48). In fact, splenocytes of TRAMP mice of 8, 24, 36, and 48 weeks of age recognized in vitro allogeneic target cells (Fig. 3A). Surprisingly, in 48-week-old TRAMP mice, there was a weak but reproducible spontaneous immune response against the immunodominant mTERT198-205 epitope, which was absent in younger TRAMP mice \( (P = 0.021; \text{Fig. 3A}) \). However, it was clear that this spontaneous response was unable to control tumor progression, similarly to what was observed in humans (25).

Starting from week 6, TRAMP mice were vaccinated twice, at 2-week intervals, with either \( \beta \)-gal–encoding or mTERT-encoding plasmids. Two weeks after the second vaccine dose, IFN-\( \gamma \) ELISPOT revealed that mTERT immunization was able to prime and expand antigen-specific CD8\(^+\) T cells recognizing only the mTERT198-205 epitope and not the control \( \beta \)-gal\(_{96-103}\) peptide \( (P = 0.035; \text{Fig. 3B}) \).

The numbers of spot-forming colonies (SFC)/10\(^6\) cell obtained in this assay were comparable with those reported in Fig. 2C for wild-type C57Bl/6 mice. Mice vaccinated with \( \beta \)-gal–coding plasmid recognized only \( \beta \)-gal\(_{96-103}\) peptide and not mTERT198-205 peptide \( (P = 0.0045; \text{Fig. 3B}) \). The numbers of IFN-\( \gamma \)–producing splenocytes in TRAMP mice vaccinated with either mTERT or mTERT-LTB plasmid DNA were similar, suggesting the induction of comparable immune responses in C57Bl/6 mice (Fig. 3B).

The antigen-specific T-cell population was expanded in vitro by stimulating effector lymphocytes with mTERT198-205 peptide in MLPC. As expected, these peptide-stimulated cultures were able to release IFN-\( \gamma \) when coincubated with mTERT198-205-pulsed cell targets (Fig. 3C). Moreover, the TERT-specific T cells also recognized to the same extent unpulsed, syngeneic B16 melanoma and TRAMP-C2 prostate cancer cells. This recognition of naturally processed TERT peptides was increased on pretreatment of B16 cells with IFN-\( \gamma \), known to up-regulate surface class I MHC expression (Fig. 3C). Finally, we also evaluated the cytotoxicity of TERT-specific MLPC. Lytic activity was shown both against target cells pulsed with mTERT198-205 epitope and, to a lower extent, against B16 and TRAMP-C2 tumor cells naturally processing the endogenous TERT antigen (Fig. 3D).

Vaccination of TRAMP mice reduces the prostate areas affected by the tumor and increases long-term survival. As described in human prostatic carcinoma, overexpression of telomerase was appreciable from the early stages of prostate epithelial transformation in TRAMP mice (26). A large proportion of the nuclei of the hyperplastic epithelium, in fact, showed a moderate to strong staining for mTERT (Fig. 4A, i–iv). The positivity was conserved along the neoplastic progression to PIN and ADC. Noteworthy, the staining was stronger at the edges of positivity was conserved along the neoplastic progression to PIN and ADC. Noteworthy, the staining was stronger at the edges of positivity was conserved along the neoplastic progression to PIN and ADC. Noteworthy, the staining was stronger at the edges of

To evaluate the in vitro vaccination efficacy, TRAMP mice were immunized with both TERT plasmids from the 6th week of age. DNA was administered at biweekly intervals and the immunization schedule was repeated every 10 weeks. A first group of vaccinated TRAMP mice was euthanized at week 24 for histologic examination. Pathologic evaluation indicated that prostates of control plasmid–treated animals had extensive areas of PIN and ADC with unpolarized proliferating cells (Fig. 4B, i), whereas prostates of mTERT-vaccinated TRAMP mice showed areas of PIN constituted by more differentiated and polarized cells (Fig. 4B, ii). Quantitative image analysis of prostates confirmed that only 42.4% of their area was occupied by ADC in the mouse group vaccinated with mTERT compared with 62.2% in the controls \( (P < 0.05) \). Conversely, normal prostatic tissue was more represented (30.64% versus 7.37%; \( P < 0.05 \)) in mTERT-vaccinated mice (Fig. 4B, i–iii). A second group of TRAMP mice was monitored until either spontaneous death or the appearance of either painful or life-threatening complications. A clear effect of vaccination on overall survival was observed with both plasmid constructs expressing TERT \( (P = 0.004, \text{cumulative data are shown in Fig. 4C}) \).

Telomerase-specific T cells infiltrate prostate tumors. TRAMP mice immunized at the 6th week of age were euthanized at week 24 and tumor sections were evaluated by immunohistochemistry to detect tumor-infiltrating CD8\(^+\) T cells. CD8\(^+\) T cells were present in both mock- and mTERT-vaccinated mice, indicating that this tumor is permissive for T-cell infiltration (Fig. 5A, i and ii). However, many CD8\(^+\) T cells were in direct contact with tumor epithelial cells in mTERT-vaccinated mice (Fig. 5A, ii). To identify mTERT-specific CD8\(^+\) T cells, we used mTERT198-205-K\(^b\) fluorescent tetramers (tet). Firstly, we showed the specificity of this reagent by staining, both \( \text{ex vivo} \) and after \( \text{in vitro} \) stimulation with mTERT198-205 peptide, telomerase-specific splenocytes induced by vaccination (Fig. 5B). Once this reagent was validated, 24-week-old TRAMP mice were sacrificed and single-cell suspension derived from prostate tissue was stained with mTERT198-205-K\(^b\) fluorescent tetramers and anti-CD8 monoclonal antibodies (mAb) and analyzed by FACS (Fig. 5C, i and ii). CD8\(^+\)/tet\(^+\) cells were detected among tumor-infiltrating CD8\(^+\) T cells in three of three mTERT-vaccinated mice, with an average of 33.4 ± 6 CD8\(^+\)/tet\(^+\)/5 × 10\(^5\) total events analyzed by FACS (Fig. 5D). CD8\(^+\) infiltrating T cells were also detected in three of three mice vaccinated with the control plasmid but the tet\(^+\) cells were not present in the infiltrate (average, 1.8 ± 1 CD8\(^+\)/tet\(^+\)/5 × 10\(^5\) total events; Fig. 5D, mTERT versus mock vaccinated, \( P = 0.02 \)). Confirming histologic data, the percentage of tumor-infiltrating CD8\(^+\) T cells was comparable in the two groups (3,220 ± 1,200 CD8\(^+\)/tet\(^+\)/5 × 10\(^5\) total events in the control and 2,868 ± 712 CD8\(^+\)/tet\(^+\)/5 × 10\(^5\) total events in mTERT-vaccinated mice).

Vaccination of DMH-treated BALB/c controls tumor growth. A second cancer model was used to evaluate efficacy of DNA vaccination approach. Mouse exposure to DMH carcinogen led to progressive development of multiple tumors in the colon in all exposed mice (16). Tumor progression resembles human colorectal cancer, both macroscopically and microscopically, being characterized by the formation of aberrant crypt proceeding to adenoma and finally carcinoma (17). We firstly showed mTERT overexpression in samples obtained from DMH-treated animals. In control animals (vehicle treated), mTERT staining was mostly restricted to the lower segment of the colon crypts, where proliferative cells are residing (stem cells and committed stem cells; Fig. 6A, i). On the contrary, in DMH-induced animals, the immunostaining showed an upper shift of mTERT pattern of expression and positive cells were detected in all three segments of the crypt (bottom, mid, and upper segments; Fig. 6A, ii) already 6 weeks after DMH induction of tumors. This observation agrees with the proliferative shift induced by the carcinogen, which has been extensively described in this model of colon rectal cancer (27). It is noteworthy that mTERT not only is expressed in the mid-third of the crypt, where transit cells migrate and are still differentiating and partly proliferating, but also in the upper third of the crypt, where terminally differentiated cells and apoptotic cells are residing in normal situation. At very late stages after DMH induction (35 weeks), several ADCs were detectable, and mTERT signal was
clearly appreciated in the mucosa adjacent to large tumors and in several areas of the tumoral mass (Fig. 6A, iii and iv).

We performed two different vaccination experiments in this model. In the "prophylactic setting," we immunized mice 4 weeks after DMH induction, when macroscopic tumor lesions were not yet detectable in the large bowel. In the "early therapeutic setting," the effect of the mTERT vaccination on later tumor stage development was investigated by starting the immunization schedule 15 weeks after DMH induction. Mice received five weekly DNA injections of the TERT-LTB fusion construct. DMH treatment did not compromise the immune responsiveness in mice (28), and in agreement with previous findings, we found that the immune responsiveness against mTERT198-205 was indistinguishable from that measured in control BALB/c mice (average, 155 ± 94 SFC/10^6)

Figure 3. Evaluation of TERT-specific immune response in vaccinated TRAMP mice. A, TRAMP mice of different ages (8, 24, 36, and 48 wk) were euthanized and their splenocytes were used to set up allogeneic and peptide-stimulated cultures (MLC and MLPC, respectively). MLCs (black columns) and MLPCs (gray columns) were tested in IFN-γ ELISA. MLPC values of IFN-γ released in the presence of cells pulsed with control β-gal96-103 peptide were subtracted from the values obtained with the mTERT198-205 peptide-pulsed cells. For allogeneic MLCs, IFN-γ release is reported as the difference between the values obtained in the presence of the allogeneic targets (CT26 cells) and syngeneic targets (MBL-2 cells). Columns, mean; bars, SD. Student's t test; mTERT198-205 responsiveness of 48-wk TRAMP mice versus 8, 24, and 36 wk, P = 0.02. Where no SD is present, data are greater than the assay limit. B, splenocytes of TRAMP mice, vaccinated at 6th week of age, with either TERT-LTB fusion (TERT-LTB), full-length TERT (TERT), β-gal plasmid (β-gal), or control empty plasmid (control) were tested ex vivo in an IFN-γ ELISPOT assay with either mTERT198-205 (right) or β-gal96-103 (left) peptides. Columns, mean of triplicate wells of one representative experiment; bars, SD. Student's t test statistics: mTERT198-205 mice vaccinated with mTERT plasmid DNA versus mice vaccinated with β-gal–encoding plasmid DNA, P = 0.005; mTERT198-205 mice vaccinated with mTERT-LTB fusion plasmid DNA versus mice vaccinated with β-gal–encoding plasmid DNA, P = 0.014; mTERT198-205 mice vaccinated with mTERT plasmid DNA versus mice vaccinated with mTERT-LTB fusion plasmid DNA, P = 0.6 (not significant). C, MLPCs from mice immunized with either mTERT (black columns) or β-gal (gray columns) were tested in IFN-γ ELISA against mTERT198-205 peptide-pulsed or β-gal96-103 peptide-pulsed MBL-2 cells, TRAMP-C2 cells, and B16 melanoma cell line either untreated (B16) or pretreated with IFN-γ (B16 IFN-γ) for 24 h. As additional control, B16 melanoma cells pulsed with mTERT198-205 peptide was included (B16 mTERT). Columns, mean of triplicate wells; bars, SD. Where no SD is present, data are greater than the assay limit. D, MLPCs were tested in a cytotoxicity test by 51Cr release assay against mTERT198-205 peptide-pulsed or β-gal96-103 peptide-pulsed MBL-2, TRAMP-C2 prostate cancer cells, and B16 melanoma cells, either treated or untreated with IFN-γ.
Figure 4. Efficacy of TERT vaccination controlling prostate cancer progression in TRAMP mice.

A, immunohistochemistry of TERT expression during prostate carcinogenesis. i, mTERT, scarcely expressed in the normal portion of the prostatic duct (black arrow), is hyperexpressed in numerous cells of the early hyperplastic lesions (gray arrow). ii, in ducts affected by PIN (irregularly shaped glands with cribriform architecture), TERT remains widely hyperexpressed, particularly in zones with evident architectural disorder (circled). iii, TERT positivity seems diffuse in the ADC cells and particularly marked along the invasion front (circled). iv, in PDC tumors, TERT is irregularly expressed in most cells.

B, histology of prostate tissues in vaccinated TRAMP mice sacrificed at the 24th week of age. i, prostates of control plasmid–treated animals showed extensive areas of PIN and ADC with unpolarized proliferating cells. ii, prostates of TERT-vaccinated TRAMP mice showed areas of PIN constituted by more differentiated and polarized cells.

iii, table summarizing the percentage of prostate areas occupied by normal tissue, PIN, and ADC in control and mTERT-vaccinated mice. Values are expressed as mean ± SD.*: statistically significant difference from the control (P < 0.05, n = 23 mice in each experimental group).

C, survival curves. The cycle of vaccination, composed of two DNA injections at biweekly interval, was repeated every 10 wk, starting from week 6 (bottom arrowheads). Survival curves are from TRAMP mice vaccinated with either mTERT (empty circle; n = 15) or control plasmids (black circle; n = 15). Mantel-Haenszel test: mTERT plasmid DNA versus empty plasmid DNA, P = 0.004.
in vehicle treated versus $118 \pm 83$ SFC/$10^6$ in DMH treated; $n = 6$ mice/group).

Vaccination efficacy was evaluated by euthanizing mice 12 weeks after DMH induction for the prophylactic setting. At this time, only benign tumors are detectable; the most frequent lesion is multiple aberrant foci crypts and few early adenomas are also present. A significant reduction in the number of multiple aberrant foci crypts and of early adenomas was observed ($P = 1 \times 10^{-8}$, Fig. 6B, and $P = 9 \times 10^{-9}$, data not shown, respectively). In the second experiment (vaccination in early therapeutic setting), mice were sacrificed at week 28 from DMH induction. Analysis of tumor was performed by counting the number of late adenoma lesions and measuring their size. Results showed that the vaccination significantly reduced the number of late adenomas, as well as the adenoma size, compared with untreated mice ($P = 0.026$, data not shown; $P = 0.0001$, Fig. 6C). In addition, histologic evaluation was performed on 12 late adenomas isolated from mice in each experimental group. Notably, 70% of the adenomas isolated from mice vaccinated with mTERT-LTB were in the G1-G2 differentiation state, whereas 85% of those isolated from control mice were already in a more advanced G3 histology grade (Fig. 6D, $P = 0.01$). Overall, these results indicate a powerful effect of vaccination on prevention of early benign lesions (aberrant crypt foci and early adenomas), whereas the effect on more advanced stages is mainly on progression of existing lesions toward larger size and less differentiated tumors.

**Discussion**

To the best of our knowledge, this article shows, for the first time, that mTERT-based vaccination can induce mTERT epitope-specific CD8+ T cells and alter the complex chain of cancerogenesis events in experimental models other than transplantable tumors, leading to a significant prolongation of survival. TERT antigen thus joins, to all intents and purposes, HER2/NEU oncogene and prostate stem cell antigen (PSCA) in the list of potential candidates to design new vaccines for tumor prevention (29–31). Moreover, given the broader expression of telomerase in cancer, compared with HER2/NEU oncogene and PSCA, TERT-based immunotherapy widens preventive vaccination application to a larger fraction of human cancers.

Although telomerase overexpression was described in both models of colon and prostate cancers, once neoplastic lesions were established, the initial phase of cancer progression seemed to be

![Figure 5](attachment:image-url)

**Figure 5.** Analysis of TILs in prostates from TRAMP mice. A, immunohistochemistry evaluation of CD8+ T cells. Sections from 24-wk-old TRAMP mouse vaccinated either with the control plasmid (i) or with mTERT plasmid (ii). Arrows, lymphocytes in close contact with epithelial cells. B, FACS staining of splenocytes from TRAMP mice with anti-CD8 mAb and TERT-tet. Ex vivo analysis from a pool of five mice immunized with either the control plasmid (i) or the mTERT plasmid (ii). Cytofluorimetric analysis of splenocytes week after in vitro stimulation with mTERT peptide: iii, mice immunized with control plasmid; iv, mice immunized with mTERT plasmid. C, FACS analysis of TILs in mouse prostates of 24-wk-old TRAMP mice. The frequencies of double-positive CD8+tet+ population are shown in a representative control (i) and mTERT-vaccinated mouse (ii). D, average numbers of double-positive CD8+tet+ cells on the total events acquired by FACS ($5 \times 10^5$) in the two groups ($P = 0.02$, Student’s t test; $n = 3$ mice/group).
more affected from active vaccination. This was also true when comparing the higher significance of the mTERT vaccination on either “prophylactic” or “early therapeutic” settings of the DMH-induced colon cancer model (Fig. 6). This effect might depend either on progressive weakening of the vaccination efficacy or establishment of evasive maneuvers from neoplastic cells. Indeed, the data about the functional activity of mTERT-specific CD8+ T cells suggest that the overall avidity of the effectors generated by vaccination is low (Fig. 3C and D). These CD8+ T cells, in fact, recognize very efficiently mTERT198-205 peptide-pulsed target cells but much less wild-type tumors (Fig. 3C and D). This low functional avidity is the consequence of the low frequency of mTERT-specific CD8+ T cells and this population can be enriched by in vitro repeated stimulations with the antigen followed by cloning of CD8+ T cells possessing a T-cell receptor with the highest avidity for mTERT198-205 peptide-Kb complexes. TERT-based immunotherapy might thus benefit from the adoptive transfer of TERT-specific CD8+ T cells and our initial studies indicate that this approach might affect more advanced forms of prostate cancer in TRAMP mice.

There are no data supporting TERT antigen loss in vaccinated versus untreated mice at late disease stages (data not shown), and thus, progressive weakening of the immune response due to active escape from tumor seems a more likely possibility. In TRAMP mice, we found that the combination of arginase (ARG) and nitric oxide synthase (NOS) inhibitors was essential to restore full tumor recognition by TILs infiltrating advanced prostate tumors, and

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similar data were obtained with human prostate organ cultures (32). These results allowed us to identify a dominant mechanism based on L-arginine metabolism by which mouse and human prostate cancers restrain tumor-specific T lymphocytes and offered novel perspectives for the immunotherapy of cancer. We are, in fact, developing ARG and NOS dual inhibitors to be administered to tumor-bearing mice in combination with either vaccination or the adoptive transfer of mTERT-specific CD8+ T cells.

The results of our studies have also other important implications for the development of clinical cancer vaccines. The TERT-LTB fusion construct seems to prime a better CD8+ T-cell response in some mouse strains (Fig. 1C) and this might even be more relevant when the genetic heterogeneity of human beings is considered. Moreover, we showed that repeated immunization with mTERT resulted in a sustained immune response for prolonged periods without the appearance of overt autoimmune manifestations and hematopoiesis impairment. These findings suggest that normal tissues and hematopoietic stem cells do not become target of antitelomerase T cells. Telomerase, under normal circumstances, is likely segregated in molecular complexes and not efficiently processed and presented in the context of MHC class I molecules and therefore ignored by the immune system. This hypothesis is further strengthened by the observation that a spontaneous immune response against telomerase is observed in TRAMP mice (Fig. 3A) and in some patients with cancer (33). In line with our observations, a naturally arising response against the ubiquitous histone H4 protein was recently described among CD8+ T cells infiltrating prostate cancer in TRAMP mice, indicating that ubiquitous proteins may become tumor antigens only in the context of the tumor (34). Telomerase and histone H4 thus belong to a category of antigens that are normally ignored by the immune system, for which a T-cell repertoire with antitumor activity exists and can be rescued by active immunization without apparent side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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