

Identification of HLA DR7-restricted Epitopes from Human Telomerase Reverse Transcriptase Recognized by CD4+ T-Helper Cells¹

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ABSTRACT

CD4+ T cells play critical roles in initiating, regulating, and maintaining antitumor immune responses. One way to improve current tumor vaccines that mainly induce CTLs would be to activate antigen-specific CD4+ T cells that recognize MHC class II restricted tumor associated antigens. Human telomerase reverse transcriptase (hTERT) is preferentially expressed by various tumors and, therefore, could be a universal tumor antigen. In this study, we used a combined approach of using the prediction software TEPITOPE to select class II epitope candidates and *in vitro* T-cell biological analysis to identify class II-restricted epitope(s) in hTERT. We first identified several HLA-DR7-restricted class-II epitope candidates in hTERT by examining human T-cell responses to synthetic peptides. We then characterized these HLA-DR7-restricted hTERT epitope candidates by establishing and analyzing peptide-specific T-cell clones. It was demonstrated that CD4+ T cells specific for the HLA-DR7-restricted hTERT₆₇₂ epitope (RPGLLGASVGLDDI) can respond to naturally processed hTERT proteins. Furthermore, the hTERT₆₇₂-specific T cells recognized hTERT antigen from various tumors, including prostate cancer, breast cancer, melanoma, and leukemia. Thus, the identification of the naturally processed HLA-DR7-restricted hTERT epitope, together with the previous finding of class I-restricted hTERT epitopes, provide a basis for the combined application of class I- and II-restricted hTERT epitopes to induce potent, long-term CD4+ and CD8+ T-cell responses against a broad spectrum of tumors.

INTRODUCTION

Human telomerase is a ribonucleoprotein that mediates RNA-dependent synthesis of telomeric DNA, the distal ends of eukaryotic chromosomes that stabilize the chromosomes during replication. Once activated, the telomerase synthesizes telomeric DNA and compensates for its loss with each cell division. Maintenance of a constant telomere length ensures chromosomal stability, prevents cells from aging, and confers immortality (1, 2). High hTERT³ activity was found in >80% of tumors of different histological origins and types (1, 3), whereas normal tissues show little or no telomerase activity (1, 4–6). Because of its preferential expression by tumor, several groups have postulated that hTERT may be a TAA and subsequently demonstrated that CTL responses against hTERT can be induced (7–9). Thus, hTERT could serve as a universal antigen for tumor immunotherapy.

Accumulating evidence indicates that CD4+ Th cells play critical roles in initiating, regulating, and maintaining antitumor immune responses (10, 11). Th cells provide helper activity for the induction

and maintenance of CD8+ CTLs (10, 12–14). They also have effector functions against tumors via macrophage activation, cytokine production, or direct killing of MHC class II-positive tumors (10, 13, 15). Dissection of cellular interactions reveals that Th cells must recognize antigens on the same APC that cross-presents the CTL epitopes in a cognate manner (16, 17). One way to improve tumor vaccines that induce CTLs would be to include T-helper epitopes for the TAA.

In this study, we used a combined approach of analyzing the hTERT protein sequence by the MHC class II epitope prediction program TEPITOPE (18–20) and *in vitro* T-cell biological analysis to identify class II-restricted epitope(s) in hTERT. We identified several DR7-restricted class-II epitope candidates in hTERT by examining human T-cell responses to synthetic peptides derived from predicted sequences. We additionally characterized the hTERT epitopes by establishing and examining peptide-specific T-cell clones. The results of this study demonstrate that the hTERT₆₇₂-specific T cells can recognize hTERT antigen from tumors of different histological origins and types.

MATERIALS AND METHODS

Cell Lines, Blood Donors, Monoclonal Antibodies, and Cell Culture Medium. Prostate cancer cell line (LNCaP-FGC), breast cancer cell lines (BT-474 and MDA-MB231), melanoma cell lines (SK-MEL37 and NA-6-MEL), human leukemia cell lines (HL-60 and Jurkat), and the hTERT-negative cell line GM847 were from American Type Culture Collection and Dr. O. M. Pereira-Smith (Baylor College of Medicine). Peripheral bloods were obtained from adult healthy donors with their consent (donor B15: DRB1*07, 07; B24: DRB1*07*04^a; B22: DRB1*07, 03; B14: DRB1*07, 11; B03: DRB1*07, 15; and B05: DRB1*07*04^b). HLA typing of peripheral blood donors was performed by PCR-SSP DNA-based procedures in the HLA, Flow and Diagnostic Immunology Laboratory of the Methodist Hospital (Houston, TX). The study was approved by Baylor's Institutional Review Board on Human Subjects Committee.

The monoclonal antibodies G46.6 (HLA-DR), G46-2.6 (HLA-ABC), SPVL3 (HLA-DQ), RPA-T4 (CD4), HIT8a (CD8), and SJ25C1 (CD19) were purchased from BD PharMingen (San Diego, CA) and Immunotech (Miami, FL). Medium used for cell culture were AIM-V serum-free medium (Life Technologies, Inc., Grand Island, NY), RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and L-glutamine/penicillin/streptomycin, and CellGenix DC serum-free medium (CellGenix, Freiburg im Breisgau, Germany). Human recombinant IL-2 was purchased from Boehringer Roche (Indianapolis, IN).

Peptide Synthesis and Recombinant Protein Production. TEPITOPE is a Windows application that enables the identification of HLA class II ligand binding epitopes (18–20). Peptides corresponding to the predicted HLA-DR7 binding sequences (1% threshold) were synthesized and purified in the MD Anderson Cancer Center Peptide Core (Houston, TX). The purity of the 15-mer peptides was >90% by high-performance liquid chromatography. Synthetic peptides were reconstituted in distilled water or DMSO at a concentration of 5 mg/ml. Peptides used in the study were hTERT₅₄₅ (LHWLMSVYVVELLS; aa545-aa559), hTERT₅₇₃ (LFFYRKSVWSKLQSI; aa573-aa587), hTERT₆₇₂ (RPGLLGASVGLDDI; aa672-aa686), hTERT₈₈₀ (AKTFLRTLVRGVPEY; aa880-aa894), and hTERT₉₁₆ (GTAFFVQMPAHGLFPW; aa916-aa930) from hTERT (GenBank accession no. NM003219), and Her-2_{aa304-318} (LSTDVGSCTLVLCPLH) from human Her-2. Recombinant hTERT_{aa540-aa1003}-Fc and Neu_(extracellular domain)-Fc fusion proteins were produced in SF9 insect cells by use of a baculovirus expression system (Life Technologies, Inc.), purified by affinity binding to Protein A (Sigma

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³ The abbreviations used are: hTERT, human telomerase reverse transcriptase; APC, antigen-presenting cell; rIL, recombinant human interleukin; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; SAC, *Staphylococcus aureus* Cowan I; TAA, tumor-associated antigen; Th, T-helper lymphocyte; TRAP, telomeric repeat amplification protocol; GM-CSF, granulocyte macrophage colony-stimulating factor.

Chemical Co.), and tested by Western blot analysis with anti-hTRT (Santa Cruz Biotechnology) or anti-Neu (Oncogene) antibodies, respectively.

Assay for Telomerase Activity by TRAP. A sensitive TRAP PCR ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) was used to analyze the functional telomerase activity in tumor cell lines and B lymphocytes. In brief, cells were counted, washed with PBS, and lysed (200 μ l for 2×10^5 cells) on ice for 30 min. After pelleting of cellular debris by centrifugation, the supernatant was recovered and stored at -80°C until further use. For the TRAP reaction, 25 μ l of reaction mixture were transferred into a PCR tube containing 2 μ l of cell lysate in 23 μ l dH₂O. The mixture was incubated for 30 min at 25°C to allow primer elongation, and then heated to 94°C for 5 min for telomerase inactivation and subjected to 30 PCR thermocycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. The amplification product was denatured, hybridized to a digoxigenin-labeled probe, and detected by an ELISA, as described in detail by the manufacturer.

Generation of T-Cell Lines and Clones with hTRT-derived Peptides. The PBMCs of the donor were plated in round-bottomed 96-well plates (Costar) at 200,000 cells/well in AIM-V medium. Peptides were added into each well at the concentration of 20 μ g/ml. The total number of wells set up for each peptide was 48. After a week of incubation, the culture medium was removed, and cells were resuspended in AIM-V medium and tested for specific proliferative responses with corresponding peptides (20 μ g/ml) in the presence of 10^5 autologous irradiated (6,000 rad) PBMCs as a source of APCs. Cell proliferation assays were incubated at 37°C in a 5% CO₂ incubator for 72 h, and during the last 16 h, the cultures were pulsed with 1 μ Ci [³H]thymidine/well. The incorporation of radioactivity into DNA, which correlates with cell proliferation, was measured in a β scintillation counter (TopCount NXT^R; Packard) after automated cell harvesting (Packard).

A T-cell line was considered to be reactive to hTRT-derived peptide if the cpm was >1000 and exceeded the reference cpm (in the absence of peptides) by at least three times. The frequency of peptide-specific T cells was determined by dividing the number of positive wells divided by the total number of PBMCs seeded in the initial culture (21, 22). hTRT-specific T-cell lines were cloned by limiting dilution at 5 cells/well in the presence of 10^5 irradiated allogeneic PBMCs as accessory cells and 5 μ g/ml of phytohemagglutinin protein (Sigma Chemical Co.). Cultures were refed with fresh RPMI 1640 containing 10 IU/ml of rhIL-2 every 3–4 days. After approximately 12–14 days, growth-positive wells became visible and were tested for specific responses to hTRT peptides in a proliferation assay as described above.

PBMC-derived DC Culture. Human DCs were prepared as described recently (23). Briefly, PBMCs were isolated by Ficoll-Hypaque gradient centrifugation (Pharmacia), washed in PBS, and resuspended in serum-free DC medium (CellGenix). After adherence to plastic for 2 h, the adherent cell fraction was cultured in serum-free DC medium with 1000 IU/ml recombinant

human GM-CSF (R&D Systems) and 1000 IU/ml rhIL-4 (R&D Systems). On day 5, DCs were matured by stimulating with a cytokine mixture consisting of recombinant human tumor necrosis factor α (10 ng/ml, R&D Systems), rhIL-1 β (1000 ng/ml; R&D Systems), rhIL-6 (10 ng/ml; R&D Systems), and prostaglandin E₂ (1 μ g/ml; Sigma Chemical Co.) as described previously (24).

Antigen-specific Responses of T-Cell Clones. T cells ($2\text{--}3 \times 10^4$ cells/well) were cocultured with irradiated (4,000 rad) DCs ($1\text{--}1.5 \times 10^3$ cells/well) in complete RPMI 1640 in the presence of various concentrations of antigen (peptides and recombinant protein) in round-bottomed 96-well plates. In some cases, recombinant protein (10 μ g/ml) or tumor cell lysates (see below) were pulsed on DCs at day 4 during DC culture 24 h before the addition of the DC maturation mixture.

To identify the MHC molecules involved in antigen presentation, inhibition of antigen-induced T-cell proliferation was analyzed by the addition of various antibodies against MHC class I and MHC class II molecules at a final concentration of 20 μ g/ml. Antigen-specific T-cell responses were measured by [³H]thymidine incorporation during the last 16 h of 72 h culture. In some instances, culture supernatants were collected before the addition of [³H]thymidine for the determination of cytokine production using ELISA kits (PharMingen, San Diego, CA).

Tumor cell lysates were prepared by five freeze-thaw cycles of 5×10^7 tumor cells resuspended in 2 ml of serum-free DC medium. The cells were sonicated for 10 min and then centrifuged at $15,000 \times g$ for 30 min (4°C). Supernatant was recovered, aliquoted, and stored at -80°C until later use. The supernatant (20 μ l) was added to a total of 5×10^5 DC in 500 μ l of DC culture medium.

Analysis of T-Cell Responses to *in Vitro*-activated Normal B Lymphocytes. B lymphocytes were purified from PBMCs by CD19+ selection with a MACS B-cell isolation kit (Milteyi Biotec, Auburn, CA). Purified B cells (5×10^4 /well, round-bottomed 96-well plates) were *in vitro*-stimulated by culture in complete RPMI 1640 supplemented with SAC (1:10,000 dilution; Roche Molecular Biochemicals, Indianapolis, IN) for 3 days (25). Telomerase activity was determined in nonactivated and SAC activated purified B lymphocytes by TRAP, as described above. Before cocultivation with autologous T cells, the surface expression of CD19 and HLA-DR on activated B cells was analyzed by fluorescence-activated cell sorter. T cells (3×10^4 cells/well) were cocultured with irradiated (4,000 rad) B lymphocytes (3×10^4 cells/well) in complete RPMI 1640 in round-bottomed 96-well plates. Cell proliferation was determined by [³H]thymidine incorporation assay 72 h later.

RESULTS

T-Cell Responses to Predicted Peptides Derived from hTRT.

To evaluate hTRT as a potential MHC class II-restricted TAA, we first used TEPITOPE, a T-cell epitope prediction program (20), to analyze the

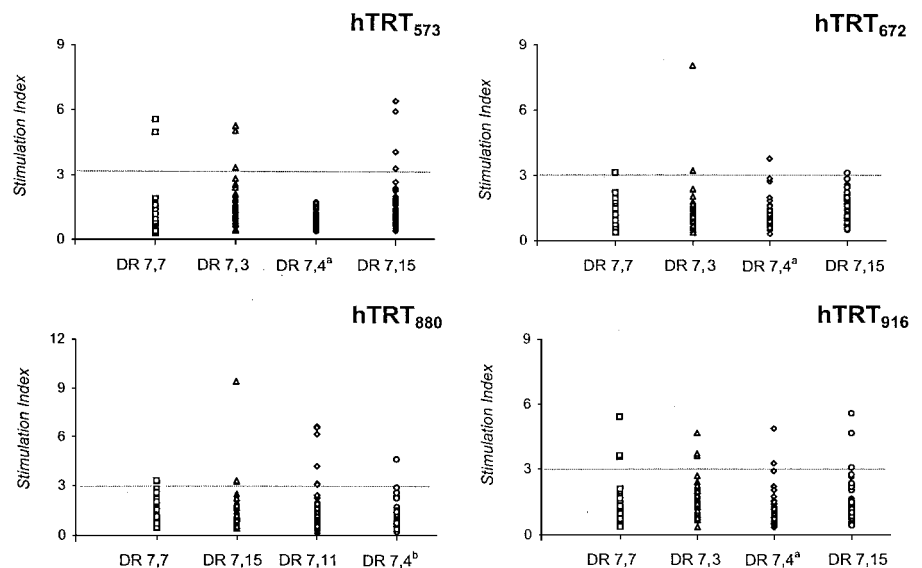


Fig. 1. Proliferative T-cell responses to hTRT-derived peptides. PBMCs (2×10^5 /well) from HLA-DR7+ healthy adult donors (B03: DRB1*07*15; B05: DRB1*07*04^b; B14: DRB1*07*11; B15: DRB1*07*07; B22: DRB1*07*03; and B24: DRB1*07*04^a) were cultured with one of five different 15-mer peptides (hTRT₅₄₅, hTRT₅₇₃, hTRT₆₇₂, hTRT₈₈₀, and hTRT₉₁₆) in 96-well plates for 7 days. A total of 48 wells were seeded per donor and per peptide. [³H]thymidine incorporations of the primed T cells were measured after restimulation with autologous PBMCs (1×10^5) and corresponding peptides. Wells were scored positive if the mean cpm of T cells stimulated with peptides exceeded cpm without peptide stimulation by at least 3 times. The results are reported as stimulation indexes (SI) of each tested well of different donors. None of the T-cell wells from the tested donors ($n = 3$) for hTRT₅₄₅ was positive for hTRT₅₄₅ (data not shown).

hTERT protein sequence. At a prediction threshold of 1% (the highest stringency), five sequence motifs in hTERT were predicted to bind to HLA-DR7. Accordingly, five 15-mer peptides corresponding to the predicted sequences (hTERT₅₄₅: LHWLMSVYVVVELLRS; hTERT₅₇₃: LFFYRKSVWSKLQSI; hTERT₆₇₂: RPGLLGASVLGLDDI; hTERT₈₈₀: AKTFLRLTLVRGVPEY; and hTERT₉₁₆: GTAFVQMPAHGLFPW) were synthesized and purified. To primarily test if the peptides are recognized by human CD4⁺ T cells, PBMCs from several HLA-DR7⁺ healthy donors were stimulated with each peptide in 96-well plates for 7 days. T-cell responses were assessed by measuring [³H]thymidine incorporation after restimulation with the corresponding peptides and autologous PBMCs as APCs. As shown Fig. 1, four peptides (hTERT₅₇₃, hTERT₆₇₂, hTERT₈₈₀, and hTERT₉₁₆) elicited proliferative T-cell responses from the donor T cells and, therefore, were considered as MHC class II-restricted epitope candidates. None of the T cells from the tested donors responded to hTERT₅₄₅ (data not shown).

Specificity and MHC Restriction of hTERT Peptide-reactive T-Cell Clones. To additionally characterize these epitopes, we set out to generate peptide-specific T-cell clones from each of these epitopes. Individual peptide-reactive T-cell clones were generated from hTERT₉₁₆-reactive T-cell lines and from hTERT₆₇₂-reactive T-cell lines by limiting dilution (22). However, T cells specific for the peptides hTERT₅₇₃ and hTERT₈₈₀ failed to grow up for additional analysis despite repeated attempts in different donors. Most established T-cell clones strongly responded to autologous PBMCs in the presence of hTERT₉₁₆ or hTERT₆₇₂ with stimulation indexes ranging from 9 to 120 (data not shown).

As depicted in Fig. 2, A and B, the specificity and MHC restriction of hTERT₆₇₂ and hTERT₉₁₆ T-cell clones was additionally assessed. The responses of hTERT₆₇₂ T cells to their corresponding peptide were inhibited by an anti-HLA-DR antibody but not by anti-HLA-ABC and anti-HLA-DQ antibodies indicating that the observed response was HLA-DR-restricted (Fig. 2A). The hTERT₆₇₂ T-cell response was specific, because the T cells did not respond to stimulation with irrelevant 15-mer peptides derived from HER-2 or with other hTERT peptides. As shown in Fig. 2B, the specificity and MHC class II restriction of hTERT₉₁₆ T-cell clones were also demonstrated.

Avidity of CD4⁺ T-Cell Clones. We additionally examined if the peptide-reactive cells were CD4⁺ T cells. The T-cell responses to hTERT₆₇₂ or hTERT₉₁₆ were inhibited by anti-CD4 antibodies, but not by anti-CD8 antibodies, indicating that the observed responses were CD4-restricted (data not shown). Moreover, we used flow cytometric analysis to verify the phenotype of the T-cell clones. As shown in Fig. 3, the T-cell clones were exclusively CD4-positive but CD8-negative. To evaluate the avidity of hTERT₉₁₆ and hTERT₆₇₂ T-cell clones for their ligands, peptide titration curves were generated with autologous DCs. The half maximal cell proliferation of the hTERT₉₁₆ T-cell clone from donor B24 was obtained between hTERT₉₁₆ peptide concentrations of 6.67 and 0.67 μ M, compared with peptide concentrations of 0.67 and 0.067 μ M for the hTERT₆₇₂ T-cell clone from the same donor B24 (Fig. 4). T-cell clones specific for the peptides hTERT₉₁₆ and hTERT₆₇₂ were also generated from donor B15. The T-cell clones from donor B15 showed similar responses to peptides. Thus, it is evident that the T-cell receptor of the hTERT₆₇₂ T-cells exhibited higher avidity than the hTERT₉₁₆ T cells.

Recognition of Natively Processed Epitopes by CD4⁺ T-Cell Clones. One essential feature of functional CD4⁺ T cells is their ability to recognize naturally processed antigens. Thus, we tested whether the T cells from different HLA-DR⁺ donors could recognize and respond to naturally processed and presented epitopes of hTERT. A recombinant hTERT protein (aa₅₄₀₋₁₀₀₃) that contains all sequences of the predicted peptides tested and an irrelevant Neu-Fc protein were produced and used to pulse PBMC-derived DCs. As shown in Fig. 5,

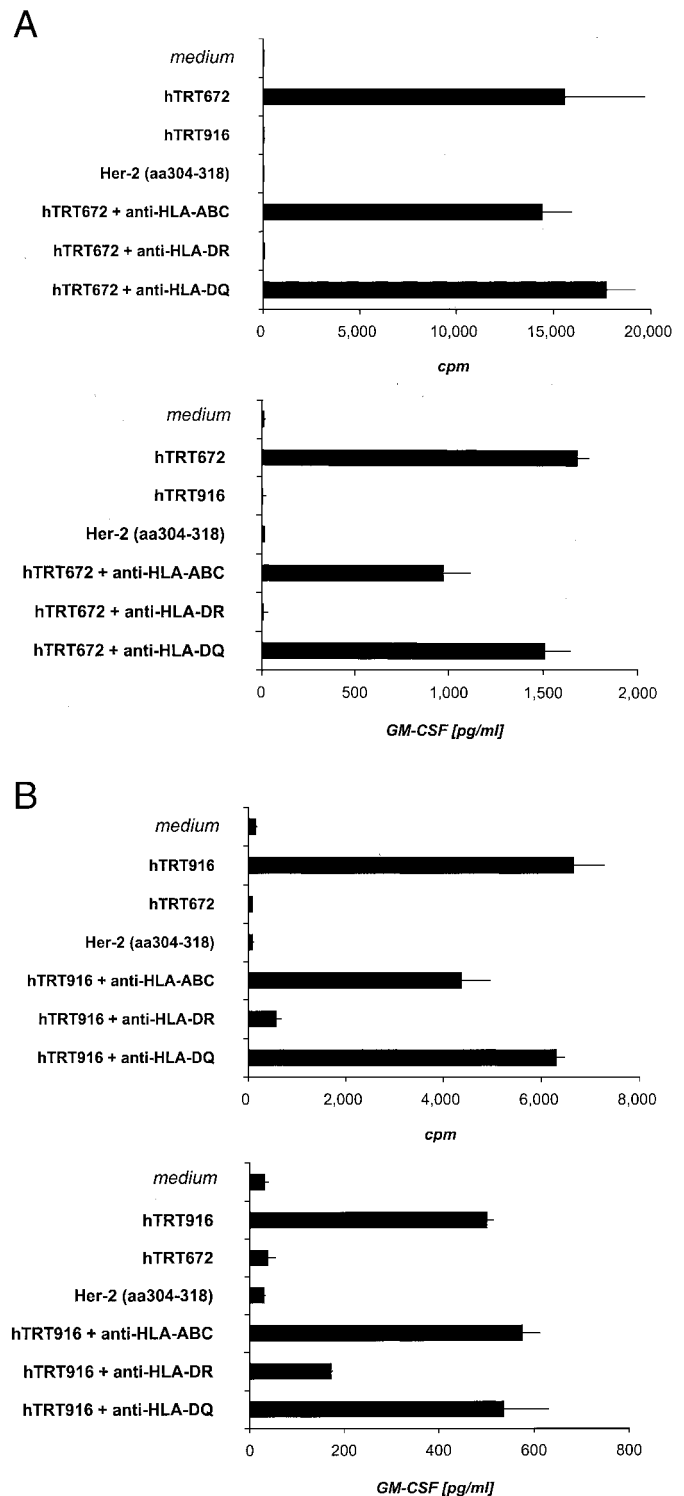


Fig. 2. Specificity of T-cell responses. Individual T-cell clones were established from hTERT₆₇₂- and hTERT₉₁₆-reactive T-cell lines from DR7⁺ donor B24 (DRB1*07:04) by limiting dilution culture. The hTERT₆₇₂ T-cell clone B24-43.2 and the hTERT₉₁₆ T-cell clone B24-48.5 (3×10^4 cells/well) were restimulated with autologous PBMC-derived DCs (1.5×10^3 /well) pulsed with the same concentration (10 μ g/ml) of hTERT₆₇₂ and hTERT₉₁₆, respectively, or an irrelevant peptide derived from HER-2 in the presence of anti-HLA-DR, anti-HLA-ABC, or anti-HLA-DQ monoclonal antibodies (20 μ g/ml). GM-CSF release and [³H]thymidine incorporation of the hTERT₆₇₂ T cells (A) and hTERT₉₁₆ T cells (B) were measured 48–72 h later. Values represent the means of duplicate wells; bars, \pm SD. The representative result of one of three repeated experiments is shown.

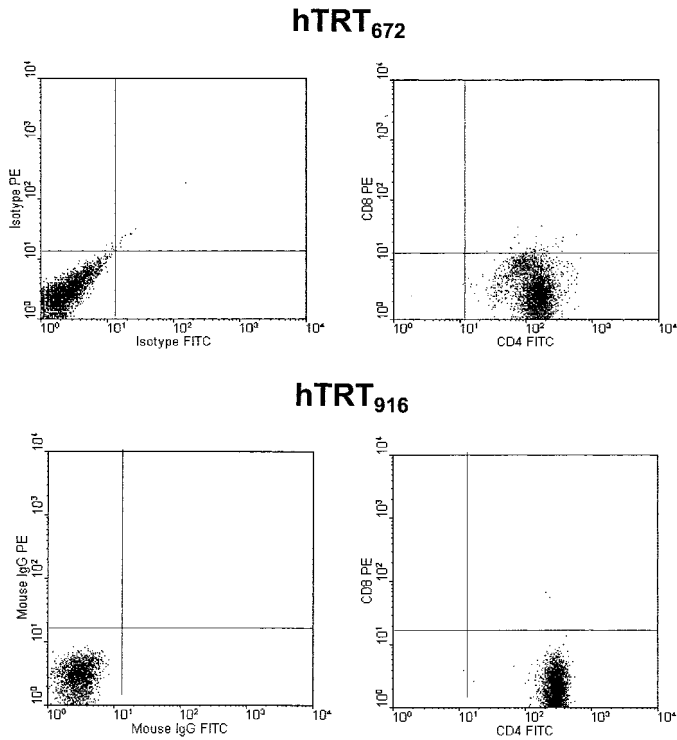
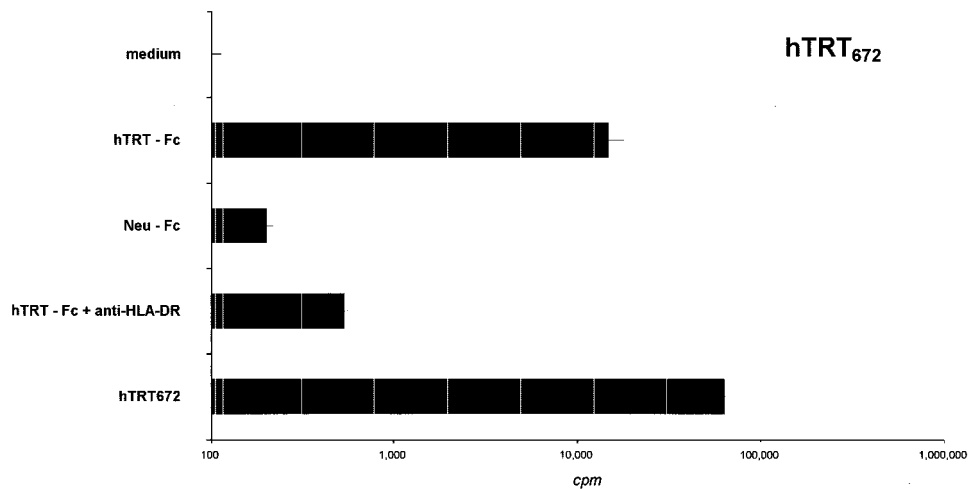


Fig. 3. Flow cytometric assay of T-cell clones. The hTERT₆₇₂ T-cell clone B24-43.2 and the hTERT₉₁₆ T-cell clone B24-48.5 were double-stained with antihuman CD4-FITC and CD8-PE antibodies or isotype controls (mouse IgG-FITC and IgG-PE). The cells were then examined by flow cytometric analysis. More than 95% of the T-cell populations were CD4-positive and CD8-negative.

the hTERT₆₇₂ T-cell clone B24-43.2 recognized the hTERT protein after processing and presentation by autologous DCs, as demonstrated by active T-cell proliferation. By contrast, the T-cell clone did not respond to the irrelevant Neu-Fc proteins presented by autologous DCs. The T-cell response was inhibited by the anti-HLA-DR antibody. However, the hTERT₉₁₆-specific T cells were found to only marginally recognize the hTERT proteins pulsed on DCs (data not shown). The T-cell clones specific for the peptides hTERT₉₁₆ and hTERT₆₇₂ generated from a different donor (B15) showed similar responses to proteins. These results indicate that the synthetic peptide sequence hTERT₆₇₂ recognized by the CD4+ T cells is a naturally processed MHC class II epitope.

Fig. 5. hTERT₆₇₂-CD4+ T-cell responses to natively processed hTERT. The hTERT₆₇₂-specific T-cells (B24-43.2; 5×10^4 /well) were stimulated with irradiated autologous PBMC-derived DCs (2.5×10^3 /well) pulsed with recombinant hTERT-Fc protein (10 μ g/ml) in the presence or absence of anti-HLA-DR antibody (20 μ g/ml). The hTERT₆₇₂ T-cell clone was also stimulated with DCs pulsed with irrelevant recombinant Neu-Fc proteins (10 μ g/ml) or the positive control hTERT₆₇₂ peptides (10 μ g/ml). T-cell proliferations were determined by [³H]thymidine incorporation assays during the last 16 h of 72 h of culture. Values shown are the means of duplicate determinations; bars, \pm SD. The representative result of one of three repeated experiments is shown. Two clones from different donors were tested.



CD4+ T-Cell Response against Various Tumors. CD4+ T cells react with APCs that take up and process the tumor antigen protein from apoptotic and dead tumor cells. Thus, we tested the capacity of the hTERT₆₇₂-reactive CD4+ T-cell clones to become activated when cocultured with APCs pulsed with lysates from different tumor types. The functional telomerase activity in eight tumor lines from different tissues and organs, including prostate cancer, breast cancer, melanoma, leukemia, and lymphoma, was determined by TRAP, a two-step PCR-based primer extension assay (1). All of these tumor lines, except the GM847 cell line, were tested positive for hTERT. We then examined if the hTERT₆₇₂ T-cell clone responded to autologous DCs pulsed with these tumor lysates. As shown in Fig. 6, the T cells proliferated and secreted GM-CSF after stimulation with each of

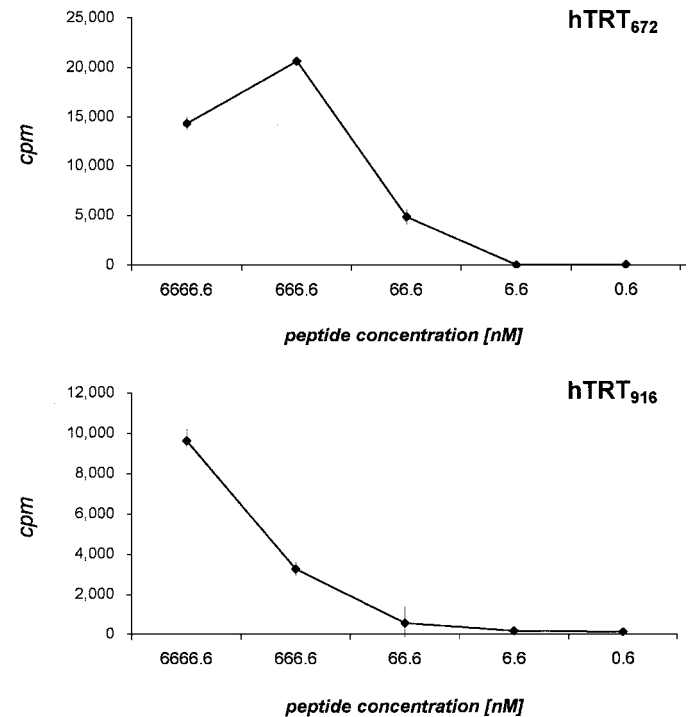


Fig. 4. Peptide titration of T-cell clones. The hTERT₆₇₂- and hTERT₉₁₆-specific CD4+ T-cell clones were cultured with irradiated autologous DCs in the presence of various concentrations of peptides hTERT₆₇₂ and hTERT₉₁₆. Each data point represents the mean of duplicate samples; bars, \pm SD. The representative result of one of three repeated experiments is shown. Two clones from different donors were tested for each peptide.

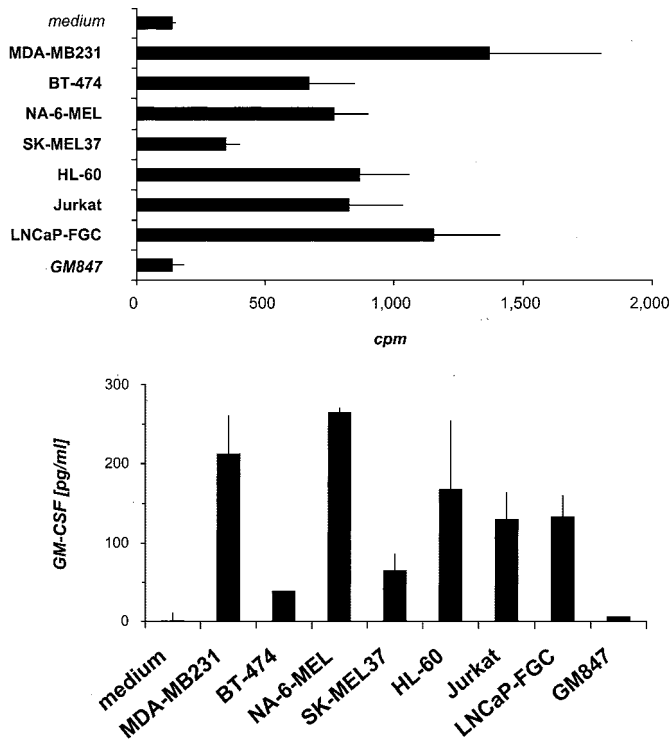


Fig. 6. hTERT₆₇₂ CD4⁺ T-cell responses to hTERT-positive tumor cell lysates. The hTERT₆₇₂ T-cell clones (2.5×10^4 /well) were stimulated with autologous DCs (2.5×10^3 /well) pulsed with hTERT-positive tumor lysates, including the prostate cancer line LNCaP-FGC, breast cancer lines (BT-474 and MDA-MB231), melanoma lines (SK-MEL37 and NA-6-MEL), and leukemia lines (HL-60 and Jurkat), or hTERT-negative cell lysate (GM847). GM-CSF release and [³H]thymidine incorporation by the T cells were measured. Values shown are the means of duplicate determinations; bars, \pm SD. T-cell clones were considered to be reactive to tumor lysate-pulsed DCs if the GM-CSF release and [³H]thymidine incorporation exceeded the reference values (medium only and GM847-pulsed DCs) by at least three times.

hTERT⁺ tumors of different tissues and organs, including prostate cancer (LNCaP-FGC), breast cancer (BT-474 and MDA-MB231), and melanoma cell lines (SK-MEL37 and NA-6-MEL), but not to the stimulation with the hTERT-negative cells (GM847). This result indicates that the hTERT₆₇₂ T cells broadly recognize the hTERT epitope derived from tumors of different tissues and organs.

Because hTERT is also expressed in certain types of normal cells, such as activated human peripheral blood B-lymphocytes that are also MHC class II-positive (7–9), we tested whether hTERT₆₇₂-reactive CD4⁺ T cells can recognize their antigen on autologous telomerase-positive B cells. B lymphocytes were purified from PBMCs by selection of CD19⁺ cells and were activated by SAC (1:10,000). Surface expression of CD19 and HLA-DR on the isolated and activated B cells was confirmed by flow cytometric analysis (data not shown). As published recently (25), the enzymatic activity of telomerase, which correlates with hTERT gene expression, was significantly up-regulated in PBMC-derived B lymphocytes after SAC treatment, as examined by TRAP (data not shown). However, the functional telomerase activity in activated B cells was significantly lower than that in tumor cells. After autologous irradiated B cells (nonactivated or activated) were cocultured with the hTERT₆₇₂ T cells, the hTERT₆₇₂ T cells failed to proliferate (Fig. 7), indicating that the hTERT₆₇₂ T cells do not respond to normal hTERT-expressing B lymphocytes. This result is in accordance with previous observations that hTERT-specific CTLs did not lyse hTERT-positive CD34⁺ cells, probably because of the low quantity of hTERT peptides presented with MHC molecules under physiological conditions (7–9).

DISCUSSION

CD4⁺ T cells play an important role in antitumor responses through several different mechanisms. They provide crucial help in the induction and maintenance of CTL responses. They produce lymphokines that may have a direct effect on tumor cells. Experiments in animal models have demonstrated the importance of antigen-specific CD4⁺ T cells in the elimination of tumors (10, 13, 14). Peptide vaccination to elicit antitumor immunity remains an attractive means to treat cancer patients. Because the main focus of current tumor vaccination effort has been to induce CTL responses (10, 11), a logical way to enhance the potency of these tumor vaccines would be to include CD4⁺ T-cell epitopes for the same tumor antigen.

Because telomerase is preferentially expressed by tumors of different histological origins and types (1, 3), it may serve as a universal tumor antigen. CD4⁺ T cells do not become activated *in vivo*, and subsequently provide “help” to CTLs and perform other functions unless their epitopes are expressed on MHC class II-positive tumor cells or on APCs that have captured and processed the tumor antigen. Thus, it is important to establish that class II epitopes represented by the corresponding peptides are naturally processed through the MHC class II pathway. Our results show that the class II epitope hTERT₆₇₂ is presented on APCs, in the context of the HLA-DR7 allele, that have processed hTERT protein or dead tumor cells/lysates. The capacity of the peptide-reactive CD4⁺ T cells to recognize APCs that have processed antigens derived from tumor cells demonstrates that the epitope represented by the corresponding peptide is naturally processed through the MHC class II pathway. Thus, these results suggest that the identification of MHC class II-restricted hTERT epitopes may

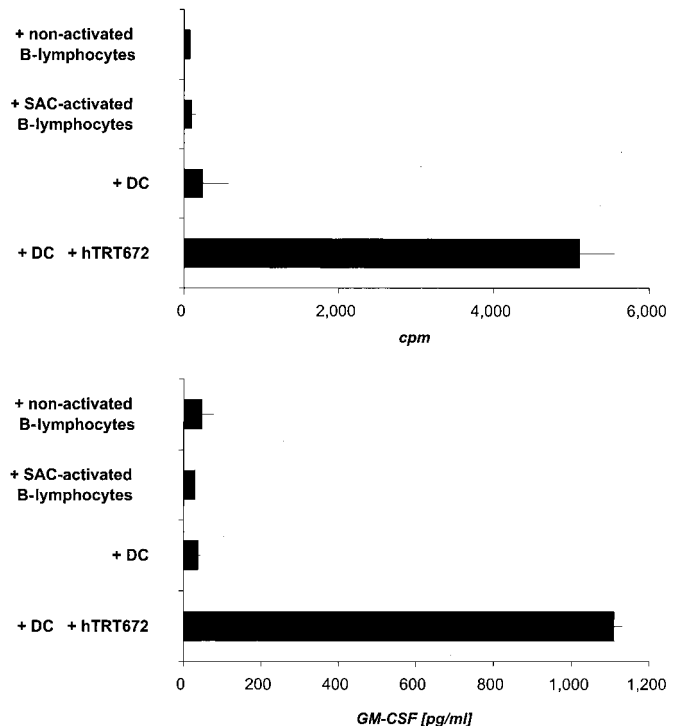


Fig. 7. hTERT₆₇₂ T-cell response to autologous activated B lymphocytes. Normal B lymphocytes were isolated from PBMCs of donor B24 by anti-CD19 magnetic beads and then activated with SAC (1:10,000) for 3 days. Purified B cells (3×10^4 /well) with or without SAC activation were irradiated (4,000 rad) and then cocultured with autologous hTERT₆₇₂-specific T cells (3×10^4 /well). Autologous PBMC-derived DCs with or without hTERT₆₇₂ pulsing were also cocultured with hTERT₆₇₂-specific T cells as positive and negative control. GM-CSF release and T-cell proliferation were determined 48–72 h later by ELISA and [³H]thymidine incorporation assays, respectively. Values shown are the means of triplicate determinations; bars, \pm SD. The representative result of one of two repeated experiments is shown. Two clones from different donors were tested.

lead to the development of effective subunit therapeutic vaccines that induce both hTERT-specific CTL (7–9) and T-helper responses against a broad spectrum of tumors.

Because hTERT is a self-antigen, and expressed in stem cells and mature hematopoietic cells (26, 27), hTERT vaccination could result in autoimmunity and destruction of normal cells. However, it was reported that the hTERT-specific CTLs did not lyse CD34⁺ cells (7, 9). The result of this study showed that hTERT₆₇₂-specific CD4⁺ T cells did not respond to autologous hTERT-positive B cells, probably because the quantity of hTERT peptides generated under physiological condition is insufficient to stimulate CD4⁺ T cells. From numerous tumor vaccination trials with tumor antigens shared by normal tissues, no apparent autoimmunity was observed (28–30). These data suggest that hTERT-based vaccination might be safe in cancer patients.

hTERT contributes to cell transformation and is preferentially expressed in ~85% of tumors of different tissues and organs (1, 4–6, 31, 32). Several groups postulated that hTERT may be a TAA because of its preferential expression by tumors and subsequently showed that CTL responses could be induced against hTERT (7–9), although it has been questioned whether the identified CTL epitope hTERT540 is a naturally processed epitope (33). Here, we identified several class II-restricted epitopes in hTERT, including a naturally processed epitope. This study also demonstrated that hTERT-specific CD4⁺ T cells can be readily activated with proper stimulation. The identification of class II-restricted epitopes in hTERT and the ability to induce hTERT-specific CD4⁺ Th responses, together with previous reports of hTERT-specific CTLs (7–9, 34), provide a rationale for combined application of class I- and II-restricted hTERT epitopes to stimulate potent and long-term CD4⁺ Th and CD8⁺ CTL responses against various tumors. Moreover, the broad expression of hTERT in most tumors (1, 5, 6) raises the possibility of using class II-restricted hTERT epitopes as adjuvants to provide cognate T-cell help to CTLs that recognize other TAAs.

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REFERENCES

- Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. *Science (Wash. DC)*, *266*: 2011–2015, 1994.
- Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A., and Weinberg, R. A. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell*, *90*: 785–795, 1997.
- Shay, J. W., and Bacchetti, S. A survey of telomerase activity in human cancer. *Eur. J. Cancer*, *33*: 787–791, 1997.
- Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., and Cech, T. R. Telomerase catalytic subunit homologs from fission yeast and human. *Science (Wash. DC)*, *277*: 955–959, 1997.
- Sommerfeld, H. J., Meeker, A. K., Piatyszek, M. A., Bova, G. S., Shay, J. W., and Coffey, D. S. Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res.*, *56*: 218–222, 1996.
- Ramakrishnan, S., Eppenberger, U., Mueller, H., Shinkai, Y., and Narayanan, R. Expression profile of the putative catalytic subunit of the telomerase gene. *Cancer Res.*, *58*: 622–625, 1998.
- Vonderheide, R. H., Hahn, W. C., Schultze, J. L., and Nadler, L. M. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity*, *10*: 673–679, 1999.
- Nair, S. K., Heiser, A., Boczkowski, D., Majumdar, A., Naoy, M., Lebikowski, J. S., Vieweg, J., and Gilboa, E. Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. *Nat. Med.*, *6*: 1011–1017, 2000.
- Minev, B., Hipp, J., Firat, H., Schmidt, J. D., Langlade-Demoyen, P., and Zanetti, M. Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc. Natl. Acad. Sci. USA*, *97*: 4796–801, 2000.
- Pardoll, D. M., and Topalian, S. L. The role of CD4⁺ T cell responses in antitumor immunity. *Curr. Opin. Immunol.*, *10*: 588–594, 1998.
- Rosenberg, S. A. Progress in human tumour immunology and immunotherapy. *Nature (Lond.)*, *411*: 380–384, 2001.
- Mumberg, D., Monach, P. A., Wanderling, S., Philip, M., Toledano, A. Y., Schreiber, R. D., and Schreiber, H. CD4(+) T cells eliminate MHC class II-negative cancer cells *in vivo* by indirect effects of IFN- γ . *Proc. Natl. Acad. Sci. USA*, *96*: 8633–8638, 1999.
- Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D., and Levitsky, H. The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.*, *188*: 2357–2368, 1998.
- Ossendorp, F., Mengede, E., Camps, M., Filius, R., and Melief, C. J. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.*, *187*: 693–702, 1998.
- Abbas, A. K., Murphy, K. M., and Sher, A. Functional diversity of helper T lymphocytes. *Nature (Lond.)*, *383*: 787–793, 1996.
- Bennett, S. R., Carbone, F. R., Karamalis, F., Miller, J. F., and Heath, W. R. Induction of a CD8⁺ cytotoxic T lymphocyte response by cross-priming requires cognate CD4⁺ T cell help. *J. Exp. Med.*, *186*: 65–70, 1997.
- Ridge, J. P., Di Rosa, F., and Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature (Lond.)*, *393*: 474–478, 1998.
- Engelhard, V. H. Structure of peptides associated with class I and class II MHC molecules. *Annu. Rev. Immunol.*, *12*: 181–207, 1994.
- Hammer, J., Sturniolo, T., and Sinigaglia, F. HLA class II peptide binding specificity and autoimmunity. *Adv. Immunol.*, *66*: 1–100, 1997.
- Manici, S., Sturniolo, T., Imro, M. A., Hammer, J., Sinigaglia, F., Noppen, C., Spagnoli, G., Mazzi, B., Bellone, M., Dellabona, P., and Protti, M. P. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. *J. Exp. Med.*, *189*: 871–876, 1999.
- Zhang, J., Medaer, R., Stinissen, P., Hafler, D., and Raus, J. MHC-restricted depletion of human myelin basic protein-reactive T cells by T cell vaccination. *Science (Wash. DC)*, *261*: 1451–1454, 1993.
- Zhang, J., Markovic-Plese, S., Lacet, B., Raus, J., Weiner, H. L., and Hafler, D. A. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.*, *179*: 973–984, 1994.
- Schroers, R., Sinha, H., Segal, I., Schmidt-Wolf, C., Rooney, C., Brenner, M., Sutton, R., and Chen, S.-Y. Transduction of human PBMC-derived dendritic cells and macrophages by HIV-1-based lentiviral vectors. *Molecular Therapy*, *1*: 171–179, 2000.
- Jonleitet, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J., and Enk, A. H. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.*, *27*: 3135–3142, 1997.
- Hu, B. T., and Insel, R. A. Up-regulation of telomerase in human B lymphocytes occurs independently of cellular proliferation and with expression of the telomerase catalytic subunit. *Eur. J. Immunol.*, *29*: 3745–3753, 1999.
- Weng, N. P., Levine, B. L., June, C. H., and Hodes, R. J. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J. Exp. Med.*, *183*: 2471–2479, 1996.
- Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S., and Yamakido, M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.*, *155*: 3711–3715, 1995.
- Morgan, D. J., Kruwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M., and Sherman, L. A. Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol.*, *160*: 643–651, 1998.
- Overwijk, W. W., Lee, D. S., Surman, D. R., Irvine, K. R., Touloukian, C. E., Chan, C. C., Carroll, M. W., Moss, B., Rosenberg, S. A., and Restifo, N. P. Vaccination with a recombinant vaccinia virus encoding a “self” antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. *Proc. Natl. Acad. Sci. USA*, *96*: 2982–2987, 1999.
- Hu, J., Kindsvogel, W., Busby, S., Bailey, M. C., Shi, Y. Y., and Greenberg, P. D. An evaluation of the potential to use tumor-associated antigens as targets for antitumor T cell therapy using transgenic mice expressing a retroviral tumor antigen in normal lymphoid tissues. *J. Exp. Med.*, *177*: 1681–1690, 1993.
- Blackburn, E. H. Telomerases. *Annu. Rev. Biochem.*, *61*: 113–129, 1992.
- Greider, C. W. Mammalian telomere dynamics: healing, fragmentation shortening and stabilization. *Curr. Opin. Genet. Dev.*, *4*: 203–211, 1994.
- Ayyoub, M., Migliaccio, M., Guillaume, P., Lienard, D., Cerottini, J. C., Romero, P., Levy, F., Speiser, D. E., and Valmori, D. Lack of tumor recognition by hTERT peptide 540–548-specific CD8(+) T cells from melanoma patients reveals inefficient antigen processing. *Eur. J. Immunol.*, *31*: 2642–2651, 2001.
- Gilboa, E. The makings of a tumor rejection antigen. *Immunity*, *11*: 263–270, 1999.

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Identification of HLA DR7-restricted Epitopes from Human Telomerase Reverse Transcriptase Recognized by CD4+ T-Helper Cells

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