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Identification of HLA DR7-restricted Epitopes from Human Telomerase Reverse Transcriptase Recognized by CD4+ T-Helper Cells[1]

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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 (hTRT) 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 hTRT. We first identified several HLA-DR7-restricted class-II epitope candidates in hTRT by examining human T-cell responses to synthetic peptides. We then characterized these HLA-DR7-restricted hTRT epitope candidates by establishing and analyzing peptide-specific T-cell clones. It was demonstrated that CD4+ T cells specific for the HLA-DR7-restricted $hTRT_{672}$ epitope (RPGLLGASVLGLDDI) can respond to naturally processed hTRT proteins. Furthermore, the $hTRT_{672}$-specific T cells recognized hTRT antigen from various tumors, including prostate cancer, breast cancer, melanoma, and leukemia. Thus, the identification of the naturally processed HLA-DR7-restricted hTRT epitope, together with the previous finding of class I-restricted hTRT epitopes, provide a basis for the combined application of class I- and II-restricted hTRT 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 hTRT[3] 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 hTRT may be a TAA and subsequently demonstrated that CTL responses against hTRT can be induced (7–9). Thus, hTRT 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 hTRT 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 hTRT. We identified several DR7-restricted class-II epitope candidates in hTRT by examining human T-cell responses to synthetic peptides derived from predicted sequences. We additionally characterized the hTRT epitopes by establishing and examining peptide-specific T-cell clones. The results of this study demonstrate that the $hTRT_{672}$-specific T cells can recognize hTRT 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 hTRT-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 $hTRT_{545}$ (LHWLMSVYVVELLRS; aa545-aa559), $hTRT_{573}$ (LFFYRKSVWSKLQSI; aa573-aa587), $hTRT_{672}$ (RPGLLGASVLGLDDI; aa672-aa686), $hTRT_{880}$ (AKTFLRTLVRGVPEY; aa880-aa894), and $hTRT_{916}$ (GTAFVQMPAHGLFPW; aa916-aa930) from hTRT (GenBank accession no. NM003219), and $Her\text{-}2_{aa304-318}$ (LSTDVGSCTLVCPLH) from human Her-2. Recombinant $hTRT_{aa540-aa1003}$-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: hTRT, human telomerase reverse transcriptase; APC, antigen-presenting cell; rhIL, 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 μl for 2 $\times$ 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 futher use. For the TRAP reaction, 25 μl of reaction mixture were transferred into a PCR tube containing 2 μl of cell lysate in 23 μl dH_20. 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 μg/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 μg/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% CO_2 incubator for 72 h, and during the last 16 h, the cultures were pulsed with 1 μCi [^{3}H]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 μg/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-1β (1000 ng/ml; R&D Systems), rhIL-6 (10 ng/ml; R&D Systems), and prostaglandin E_2 (1 μg/ml; Sigma Chemical Co.) as described previously (24).

Antigen-specific Responses of T-Cell Clones. T cells (2–3 $\times$ 10^4 cells/well) were cocultured with irradiated (4,000 rad) DCs (1–1.5 $\times$ 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 μg/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 μg/ml. Antigen-specific T-cell responses were measured by [^{3}H]thymidine incorporation during the last 16 h of 72 h culture. In some instances, culture supernatants were collected before the addition of [^{3}H]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 $\times$ 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 μl) was added to a total of 5 $\times$ 10^5 DC in 500 μl 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 $\times$ 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 $\times$ 10^4 cells/well) were cocultured with irradiated (4,000 rad) B lymphocytes (3 $\times$ 10^4 cells/well) in complete RPMI 1640 in round-bottomed 96-well plates. Cell proliferation was determined by [^{3}H]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

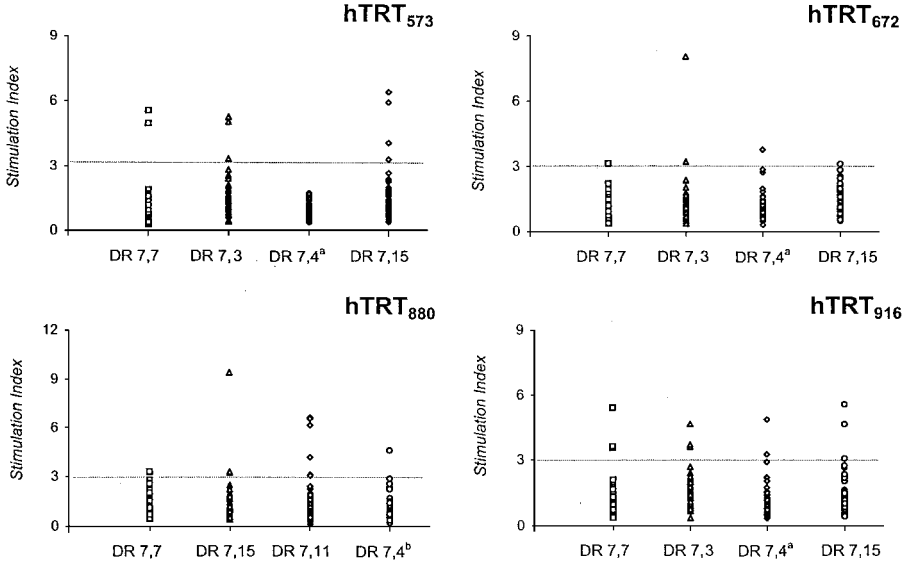


Fig. 1. Proliferative T-cell responses to hTRT-derived peptides. PBMCs (2 $\times$ 10^5/well) from HLA-DR7+ healthy adult donors (B03: DRB1*07*15; B05: DRB1*07*04^b; B14: DRB1*07*11; B15: DRB1*07*07; B22: DRB1*07*03; and B24: DRB1*07*04^a) were cultured with one of five different 15-mer peptides ($hTRT_{545}$, $hTRT_{573}$, $hTRT_{672}$, $hTRT_{880}$, and $hTRT_{916}$) in 96-well plates for 7 days. A total of 48 wells were seeded per donor and per peptide. [^{3}H]thymidine incorporations of the primed T cells were measured after restimulation with autologous PBMCs (1 $\times$ 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 $hTRT_{545}$ was positive for $hTRT_{545}$ (data not shown).

hTRT protein sequence. At a prediction threshold of 1% (the highest stringency), five sequence motifs in hTRT were predicted to bind to HLA-DR7. Accordingly, five 15-mer peptides corresponding to the predicted sequences ($hTRT_{545}$: LHWLMSVYVVELLRS; $hTRT_{573}$: LFFYRKSVWSKLQSI; $hTRT_{672}$: RPGLLGASVLGLDDI; $hTRT_{880}$: AKTFLRTLVRGVPEY; and $hTRT_{916}$: 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 [^{3}H]thymidine incorporation after restimulation with the corresponding peptides and autologous PBMCs as APCs. As shown Fig. 1, four peptides ($hTRT_{573}$, $hTRT_{672}$, $hTRT_{880}$, and $hTRT_{916}$) 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 $hTRT_{545}$ (data not shown).

Specificity and MHC Restriction of hTRT 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 $hTRT_{916}$-reactive T-cell lines and from $hTRT_{672}$-reactive T-cell lines by limiting dilution (22). However, T cells specific for the peptides $hTRT_{573}$ and $hTRT_{880}$ 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 $hTRT_{916}$ or $hTRT_{672}$ 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 $hTRT_{672}$ and $hTRT_{916}$ T-cell clones was additionally assessed. The responses of $hTRT_{672}$ 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. 2*A*). The $hTRT_{672}$ 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 hTRT peptides. As shown in Fig. 2*B*, the specificity and MHC class II restriction of $hTRT_{916}$ 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 $hTRT_{672}$ or $hTRT_{916}$ 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 $hTRT_{916}$ and $hTRT_{672}$ T-cell clones for their ligands, peptide titration curves were generated with autologous DCs. The half maximal cell proliferation of the $hTRT_{916}$ T-cell clone from donor B24 was obtained between $hTRT_{916}$ peptide concentrations of 6.67 and 0.67 μM, compared with peptide concentrations of 0.67 and 0.067 μM for the $hTRT_{672}$ T-cell clone from the same donor B24 (Fig. 4). T-cell clones specific for the peptides $hTRT_{916}$ and $hTRT_{672}$ 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 $hTRT_{672}$ T-cells exhibited higher avidity than the $hTRT_{916}$ 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 hTRT. A recombinant hTRT protein ($aa_{540-1003}$) 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,

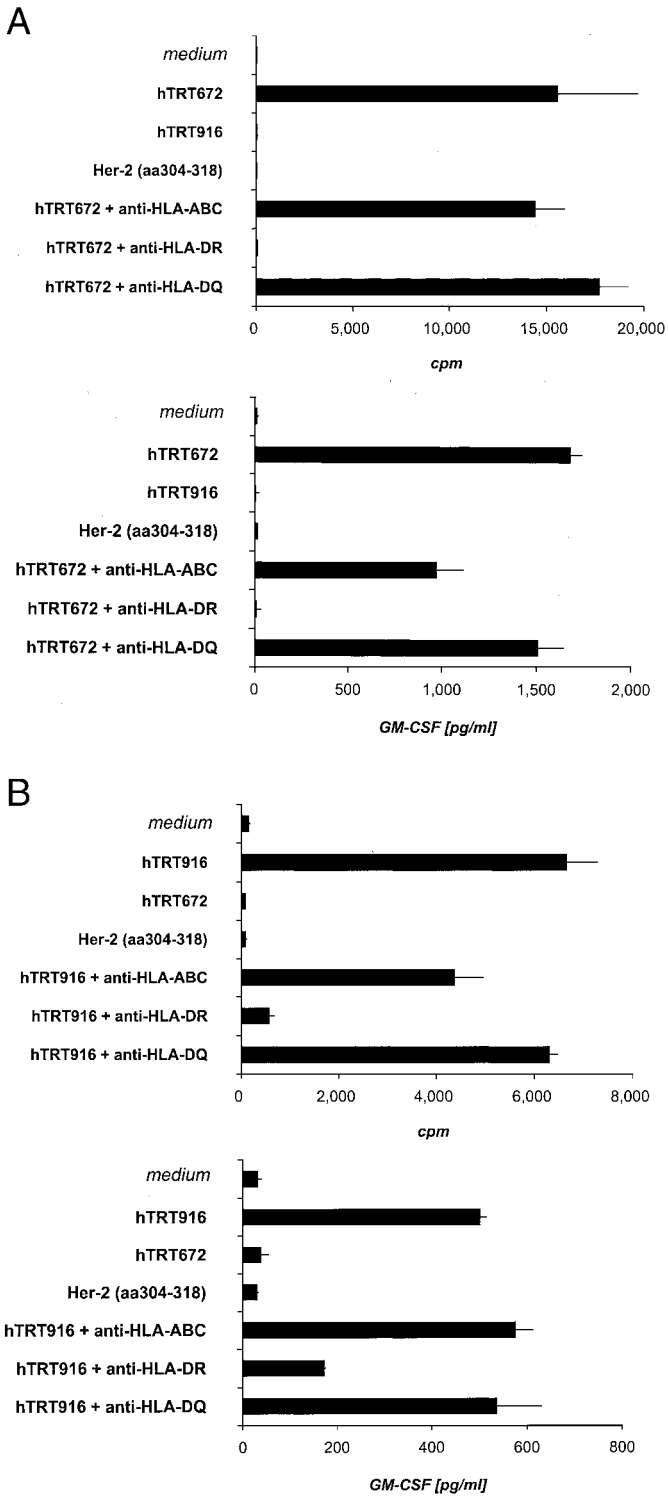


Fig. 2. Specificity of T-cell responses. Individual T-cell clones were established from $hTRT_{672}$- and $hTRT_{916}$-reactive T-cell lines from DR7+ donor B24 (DRB1*07*04) by limiting dilution culture. The $hTRT_{672}$ T-cell clone B24–43.2 and the $hTRT_{916}$ T-cell clone B24–48.5 (3×10^4 cells/well) were restimulated with autologous PBMC-derived DCs (1.5×10^3/well) pulsed with the same concentration (10 μg/ml) of $hTRT_{672}$ and $hTRT_{916}$, 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 μg/ml). GM-CSF release and [^{3}H]thymidine incorporation of the $hTRT_{672}$ T cells (*A*) and $hTRT_{916}$ T cells (*B*) were measured 48–72 h later. Values represent the means of duplicate wells; *bars*, ±SD. The representative result of one of three repeated experiments is shown.

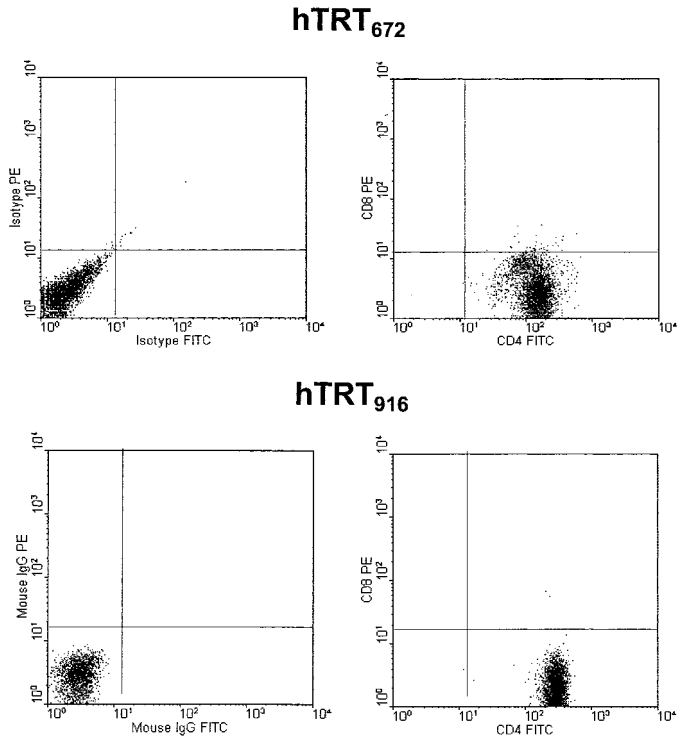


Fig. 3. Flow cytometric assay of T-cell clones. The $hTRT_{672}$ T-cell clone B24−43.2 and the $hTRT_{916}$ T-cell clone B24−48.5 were double-stained with antihuman CD4-FITC and CD8-PE antibodies or isotype controls (mouse IgG-FITC and IgG-PE). The cells were then examined by flow cytometric analysis. More than 95% of the T-cell populations were CD4-positive and CD8-negative.

the $hTRT_{672}$ T-cell clone B24−43.2 recognized the hTRT 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 $hTRT_{916}$-specific T cells were found to only marginally recognize the hTRT proteins pulsed on DCs (data not shown). The T-cell clones specific for the peptides $hTRT_{916}$ and $hTRT_{672}$ generated from a different donor (B15) showed similar responses to proteins. These results indicate that the synthetic peptide sequence $hTRT_{672}$ recognized by the CD4+ T cells is a naturally processed MHC class II epitope.

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 $hTRT_{672}$-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 hTRT. We then examined if the $hTRT_{672}$ 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

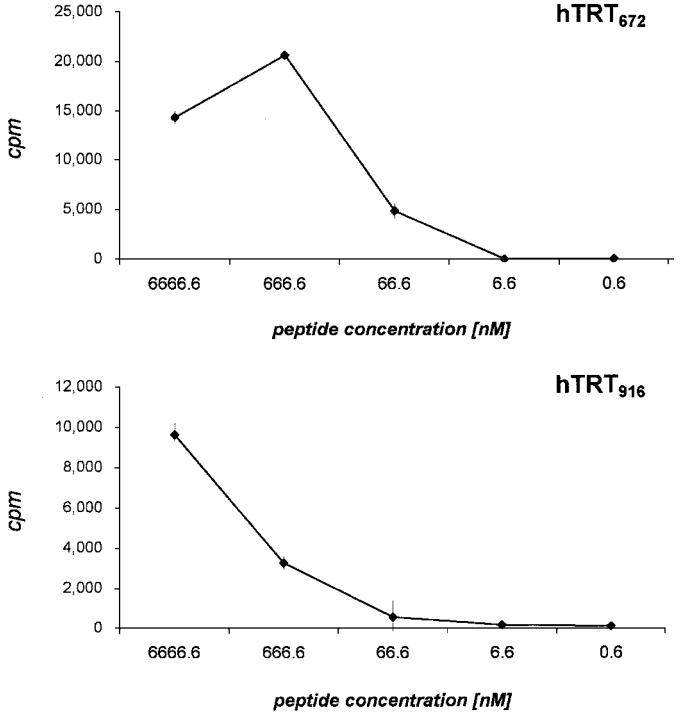
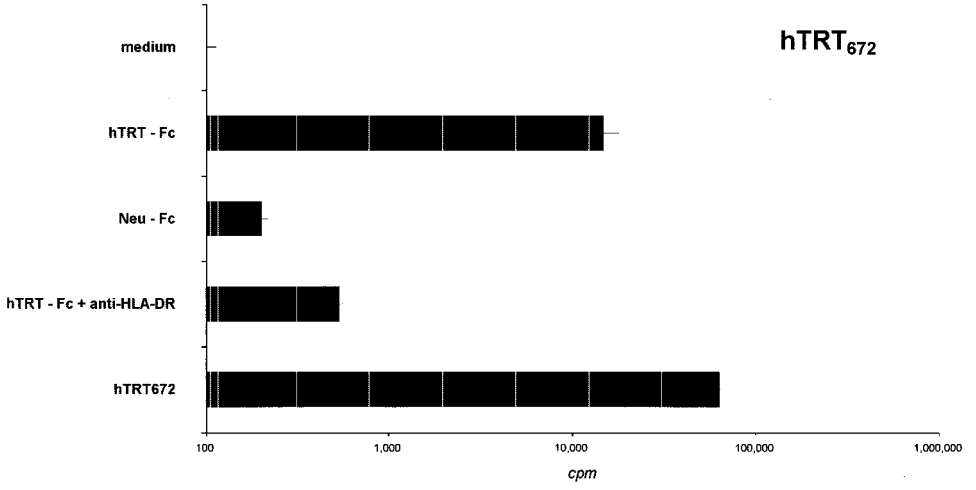


Fig. 4. Peptide titration of T-cell clones. The $hTRT_{672}$- and $hTRT_{916}$-specific CD4+ T-cell clones were cultured with irradiated autologous DCs in the presence of various concentrations of peptides $hTRT_{672}$ and $hTRT_{916}$. Each data point represents the mean of duplicate samples; *bars*, ±SD. The representative result of one of three repeated experiments is shown. Two clones from different donors were tested for each peptide.

Fig. 5. $hTRT_{672}$ -CD4+ T-cell responses to natively processed hTRT. The $hTRT_{672}$-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 hTRT-Fc protein (10 μg/ml) in the presence or absence of anti-HLA-DR antibody (20 μg/ml). The $hTRT_{672}$ T-cell clone was also stimulated with DCs pulsed with irrelevant recombinant Neu-Fc proteins (10 μg/ml) or the positive control $hTRT_{672}$ peptides (10 μg/ml). T-cell proliferations were determined by [^{3}H]thymidine incorporation assays during the last 16 h of 72 h of culture. Values shown are the means of duplicate determinations; *bars*, ±SD. The representative result of one of three repeated experiments is shown. Two clones from different donors were tested.



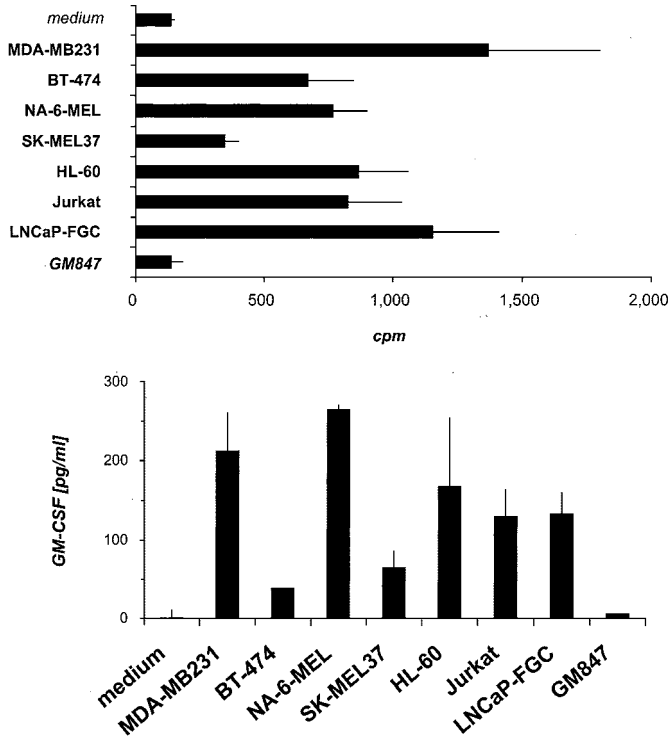


Fig. 6. $hTRT_{672}$ CD4+ T-cell responses to hTRT-positive tumor cell lysates. The $hTRT_{672}$ T-cell clones (2.5×10^4/well) were stimulated with autologous DCs (2.5×10^3/well) pulsed with hTRT-positive tumor lysates, including the prostate cancer line LNCaP-FGC, breast cancer lines (BT-474 and MDA-MB231), melanoma lines (SK-MEL37 and NA-6-MEL), and leukemia lines (HL-60 and Jurkat), or hTRT-negative cell lysate (GM847). GM-CSF release and [^{3}H]thymidine incorporation by the T cells were measured. Values shown are the means of duplicate determinations; *bars*, ±SD. T-cell clones were considered to be reactive to tumor lysate-pulsed DCs if the GM-CSF release and [^{3}H]thymidine incorporation exceeded the reference values (medium only and GM847-pulsed DCs) by at least three times.

hTRT+ 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 hTRT-negative cells (GM847). This result indicates that the $hTRT_{672}$ T cells broadly recognize the hTRT epitope derived from tumors of different tissues and organs.

Because hTRT 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 $hTRT_{672}$-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 hTRT 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 $hTRT_{672}$ T cells, the $hTRT_{672}$ T cells failed to proliferate (Fig. 7), indicating that the $hTRT_{672}$ T cells do not respond to normal hTRT-expressing B lymphocytes. This result is in accordance with previous observations that hTRT-specific CTLs did not lyse hTRT-positive CD34+ cells, probably because of the low quantity of hTRT 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 $hTRT_{672}$ is presented on APCs, in the context of the HLA-DR7 allele, that have processed hTRT 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 hTRT epitopes may

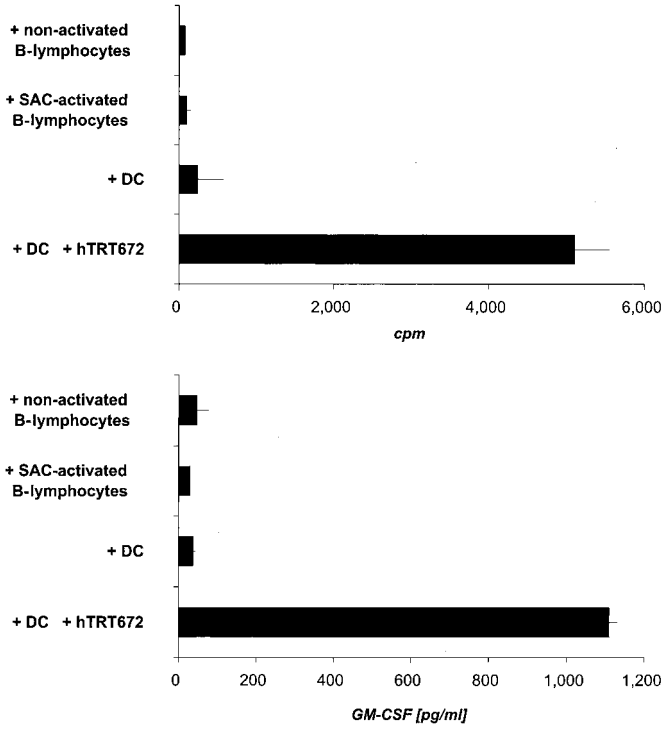


Fig. 7. $hTRT_{672}$ T-cell response to autologous activated B lymphocytes. Normal B lymphocytes were isolated from PBMCs of donor B24 by anti-CD19 magnetic beads and then activated with SAC (1:10,000) for 3 days. Purified B cells (3×10^4/well) with or without SAC activation were irradiated (4,000 rad) and then cocultured with autologous $hTRT_{672}$-specific T cells (3×10^4/well). Autologous PBMC-derived DCs with or without hTRT672 pulsing were also cocultured with $hTRT_{672}$-specific T cells as positive and negative control. GM-CSF release and T-cell proliferation were determined 48–72 h later by ELISA and [^{3}H]thymidine incorporation assays, respectively. Values shown are the means of triplicate determinations; *bars*, ±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 hTRT-specific CTL (7–9) and T-helper responses against a broad spectrum of tumors.

Because hTRT is a self-antigen, and expressed in stem cells and mature hematopoietic cells (26, 27), hTRT vaccination could result in autoimmunity and destruction of normal cells. However, it was reported that the hTRT-specific CTLs did not lyse $CD34^+$ cells (7, 9). The result of this study showed that $hTRT_{672}$-specific CD4+ T cells did not respond to autologous hTRT-positive B cells, probably because the quantity of hTRT 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 hTRT-based vaccination might be safe in cancer patients.

hTRT 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 hTRT may be a TAA because of its preferential expression by tumors and subsequently showed that CTL responses could be induced against hTRT (7–9), although it has been questioned whether the identified CTL epitope hTRT540 is a naturally processed epitope (33). Here, we identified several class II-restricted epitopes in hTRT, including a naturally processed epitope. This study also demonstrated that hTRT-specific CD4+ T cells can be readily activated with proper stimulation. The identification of class II-restricted epitopes in hTRT and the ability to induce hTRT-specific CD4+ Th responses, together with previous reports of hTRT-specific CTLs (7–9, 34), provide a rationale for combined application of class I- and II-restricted hTRT epitopes to stimulate potent and long-term CD4+ Th and CD8+ CTL responses against various tumors. Moreover, the broad expression of hTRT in most tumors (1, 5, 6) raises the possibility of using class II-restricted hTRT epitopes as adjuvants to provide cognate T-cell help to CTLs that recognize other TAAs.

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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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