Human Telomerase Reverse Transcriptase-Specific T-Helper Responses Induced by Promiscuous Major Histocompatibility Complex Class II-Restricted Epitopes

Roland Schroers,[1] Lei Shen,[1] Lisa Rollins, Cliona M. Rooney, Kevin Slawin, Grete Sonderstrup, Xue F. Huang, and Si-Yi Chen[2]

Center for Cell and Gene Therapy [R. S., L. S., L. R., C. M. R., X. F. H., S-Y. C.] and Departments of Molecular and Human Genetics [R. S., L. S., L. R., S-Y. C.], Pediatrics [C. M. R., X. F. H.], and Urology [K. S.], Baylor College of Medicine, Houston, Texas 77030, and Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305 [G. S.]

ABSTRACT

An effective tumor vaccine may require the induction of both CTL and T-helper (Th) cell responses against tumor-associated antigens. Human telomerase reverse transcriptase (hTERT) is highly expressed in >85% of cancer cells and thus is a potential target for tumor vaccines. We therefore sought to identify promiscuous Th epitopes in hTERT, which can be presented by more than one MHC class II allele. Each of 10 peptides derived from hTERT that were predicted to bind to MHC class II molecules was found to be able to induce primary human T-cell responses *in vitro*. We then established $CD4^+$ T-cell clones specific for these peptides and found that only $hTERT_{766}$ (LTDLQPYMRQFVAHL)-specific $CD4^+$ Th cells were effective in recognizing naturally processed hTERT antigen. We further found that the naturally processed epitopes $hTERT_{766}$ and $hTERT_{672}$ (which was identified previously) were promiscuous and capable of inducing $CD4^+$ T-cell responses in the context of several commonly found HLA-DR alleles, including DR1, DR7, and DR15 for $hTERT_{672}$, and DR4, DR11, and DR15 for $hTERT_{766}$. We further demonstrated that immunization of humanized HLA-DR4 transgenic mice with $hTERT_{766}$ peptide elicited antigen-specific Th responses that can recognize the antigenic peptides derived from hTERT protein and various hTERT-positive tumors, such as breast cancer, melanoma, and leukemia. It was also shown that T-cell precursors specific for the naturally processed epitopes are part of the T-cell repertoires in healthy donors and prostate cancer patients. Thus, these promiscuous, naturally processed Th epitopes in hTERT could be used to develop improved cancer vaccines through the simultaneous stimulation of CTL and Th cells against a broad spectrum of hTERT-positive tumors.

INTRODUCTION

Human telomerase, a ribonucleoprotein enzyme consisting of a telomerase RNA component, telomerase protein 1, and hTERT,[3] plays a key role in determining telomere length and cellular replicative life span (1, 2). hTERT, the catalytic subunit of human telomerase, is highly expressed in >85% of cancer cells, but not in most normal cells (3–6), suggesting the potential value of telomerase as a universal target for antitumor therapy.

The design and implementation of effective T-cell-based cancer treatments including vaccines against hTERT will likely require the identification of MHC class I- and II-restricted epitopes in this ribonucleoprotein. Identification of a MHC class I CTL epitope in hTERT ($hTERT_{540}$) has been reported by several groups (7–9), but whether it represents a naturally processed epitope remains in question (10). In the absence of antigen-specific Th responses, however, the hTERT-reactive CTLs may not survive or expand sufficiently to be effective in generating clinically significant antitumor responses. To improve antitumor vaccines, therefore it may be necessary to augment stimulated CTLs with helper epitopes from the target antigen.

In a recent study, we found that one of five hTERT peptides ($hTERT_{672}$) predicted to bind to HLA-DR7 is a naturally processed epitope presented by this MHC class II allele (11). In this study, we aimed to identify "promiscuous" Th epitopes in hTERT, which are recognized by Th cells in the context of more than one MHC class II allele. As we reported here, 10 hTERT peptides that were predicted to bind to MHC class II molecules were tested. It is found that only the Th cells generated with a $hTERT_{766}$ peptide effectively recognized naturally processed antigen, indicating that this peptide is a naturally processed epitope. We found that the naturally processed epitope

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[1] These authors contribute equally to this work.
[2] To whom requests for reprints should be addressed, at Center for Cell and Gene Therapy, Alkek Building N1004, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-1236; Fax: (713) 798-1083; E-mail: sychen@bcm.tmc.edu.

[3] The abbreviations used are: hTERT, human telomerase reverse transcriptase; DC, dendritic cell; APC, antigen-presenting cell; Th, T-helper; PBMC, peripheral blood mononuclear cell; ATCC, American Type Culture Collection; PSMA, prostate-specific membrane antigen; mAb, monoclonal antibody; AA, amino acid; rhIL, recombinant human interleukin; PE, phycoerythrin; SI, stimulation indices; CFA, complete Freund's adjuvant; LCL, lymphoblastoid cell line; TCR, T-cell receptor.

Table 1 Synthetic peptide sequences derived from hTERT

Peptide	AA position	AA sequence
$hTERT_{631}$	631–650	RPIVNMDYVVGARTFRREKR
$hTERT_{706}$	706–720	LYFVKVDVTGAYDTI
$hTERT_{766}$	766–780	LTDLQPYMRQFVAHL
$hTERT_{787}$	787–801	RDAVVIEQSSSLNEA
$hTERT_{805}$	805–823	LFDVFLRFMCHHAVRIRGK
$hTERT_{854}$	854–868	FAGIRRDGLLLRLVD
$hTERT_{894}$	894–908	YGCVVNLRKTVVNFP
$hTERT_{930}$	930–944	WCGLLLDTRTLEVQS
$hTERT_{951}$	951–965	RTSIRASLTFNRGFK
$hTERT_{971}$	971–989	RRKLFGVLRLKCHSLFLDL

$hTERT_{766}$ and the previously identified $hTERT_{672}$ are promiscuous and capable of inducing $CD4^+$ T-cell responses in the context of several commonly found HLA-DR alleles, including DR1, DR7, and DR15 for $hTERT_{672}$ and DR4, DR11, and DR15 for $hTERT_{766}$. We further demonstrated that immunization of humanized HLA-DR4 transgenic mice with $hTERT_{766}$ peptide elicited strong antigen-specific Th responses that can recognize the antigenic peptides derived from hTERT protein and hTERT-positive tumors, such as breast cancer, melanoma, and leukemia.

MATERIALS AND METHODS

Epitope Prediction and Peptide Synthesis. TEPITOPE software from Dr. Juergen Hammer[4] was used to predict potential HLA-DR-binding peptides with promiscuous binding characteristics (12, 13). The prediction threshold was set at 1%, and peptides were selected on the basis of their ability to bind to at least three of the following eight HLA-DR molecules: DRB1*0101; DRB1*0301; DRB1*0401; DRB1*0701; DRB1*0801; DRB1*1101; DRB1*1501; and DRB5*0101. Based on the prediction, 10 peptides from hTERT (Table 1) were synthesized. An irrelevant peptide $EBNA_{482}$ (AEGLRALLARSHVER) from the EBV protein EBNA-1 was also synthesized. Peptides at a purity of >90% were produced by Genemed Synthesis (San Francisco, CA) and dissolved in 100% DMSO at a concentration of 10 mg/ml.

Recombinant Protein, mAbs, Tissue Culture Reagents, and Cell Lines. 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., Grand Island, NY), purified by affinity binding to protein A (Sigma, St. Louis, MO), and tested by Western blot analysis with anti-hTERT (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Neu (Oncogene, La Jolla, CA) antibodies, respectively. The following hybridomas were used to produce mAbs: HB55 (L243, antihuman HLA-DR; ATCC); HB95 (W6/32, antihuman MHC class I; ATCC); HB103 (Genox3.53, antihuman HLA-DQ; ATCC); HB180 (9.3F10, antihuman MHC class II; ATCC); and 2D6 (antihuman HLA-DR and HLA-DQ monomorphic). Antihuman CD4 (RPA-T4, FITC labeled), antihuman CD8 (HIT8a, PE labeled), antihuman CD4 (PE labeled), antihuman HLA-DR (FITC labeled), and antimouse CD4 (FITC labeled) were all purchased from BD PharMingen (San Diego, CA). Media used for cell culture were AIM-V serum-free medium (Life Technologies, Inc.), RPMI 1640 supplemented with 10% FBS (Life Technologies, Inc.) and L-glutamine/penicillin/streptomycin, and CellGenix DC serum-free medium (CellGenix). rhIL-2 was purchased from Boehringer Roche (Indianapolis, IN). A panel of homozygous EBV-LCLs, including DR1(HH), DR4(P678), DR7(T1), DR11(P125), DR15(P612), and DR17(LA), were established. Breast cancer cell line MDA-MB231, melanoma cell line NA-6-MEL, human leukemia cell lines HL-60 and Jurkat were from ATCC, and the hTERT-negative cell line GM847 was from Dr. O. M. Pereira-Smith (Baylor College of Medicine).

Blood Samples and HLA Typing. Heparinized blood was collected from healthy volunteers and prostate cancer patients (Methodist Hospital, Houston, TX). PBMCs were isolated by Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient separation. HLA typing was performed by PCR-sequence-specific primer DNA-based procedures (The Methodist Hospital, Houston, TX). The Institutional Review Board on Human Subjects (Baylor College of Medicine) approved this research.

T-Cell Proliferation Assay and Generation of T-Cell Clones. The donor's PBMCs were plated in U-bottomed 96-well plates (Costar) at 200,000 cells/well in AIM-V media. Peptides were added to each well at a concentration of 20 μg/ml; a total of 48 wells were prepared for analysis of each peptide. After a week of incubation, the culture medium was removed, and cells were resuspended in AIM-V media and tested for specific proliferative responses to corresponding peptides (20 μg/ml) in the presence of 10^5 autologous irradiated (6000 rads) PBMCs as a source of APCs. In cell proliferation assays, cells were incubated at 37°C in a 5% CO_2 incubator for 72 h, and cultures were pulsed with 1 μCi [^{3}H]thymidine/well during the last 16 h. The incorporation of radioactivity into DNA was measured in a beta scintillation counter (TopCount NXT; Packard) after automated cell harvesting (Packard). The results were presented either as SI (mean cpm of peptide-pulsed PBMCs/mean cpm of PBMC not exposed to peptides) or cpm (14, 15). A T-cell line/well was considered to be positively reactive to peptide if the cpm were >1000 and SI was >3 (11, 16). The frequency of peptide-specific T cells was determined by dividing the number of positive wells by the total number of PBMCs seeded into the initial culture (16). Overall positive events were defined if there was significant difference at a 95% confidence level between the numbers of positive wells of the peptide-containing wells compared with that of the non-peptide-containing wells. Because these low frequency events were in accordance with Poisson's distribution, ≥3 of 48 peptide-containing wells were defined as a positive event ($P < 0.05$).

hTERT-specific T-cell lines were cloned by limiting dilution at 0.3 cell/well in the presence of 10^5 irradiated allogeneic PBMCs as accessory cells and 5 μg/ml phytohemagglutinin protein (Sigma). Cultures were refed with fresh RPMI 1640 medium containing 10 IU/ml interleukin 2 every 3–4 days. After approximately 12–14 days, growth-positive wells became visible and were tested for specific responses to hTERT peptides in a proliferation assay, as described above.

[4] www.vaccinome.com.

Preparation of PBMC-Derived Human DCs. Human DCs were prepared as described recently (17). Briefly, PBMCs were resuspended in serum-free DC medium (CellGenix) and incubated at 37°C in humidified 5% CO_2. The cell fraction adherent to plastic was cultured in serum-free DC medium with 1000 IU/ml recombinant human granulocyte macrophage colony-stimulating factor (R&D Systems) and 1000 IU/ml rhIL-4 (R&D Systems). On day 5, DCs were stimulated to maturity 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), as published previously (18).

Analysis of Antigen-Specific Proliferative Responses of T-Cell Clones. $CD4^+$ T cells ($2–3 \times 10^4$ cells/well) were mixed with irradiated APCs in the presence of various concentrations of antigen (peptides and recombinant protein) in U-bottomed 96-well culture plates. APCs consisted of either DCs (1.5×10^3 cells/well) or EBV-LCLs (3×10^4 cells/well). In some cases, recombinant protein (10 μg/ml) was pulsed on DCs at day 4 during DC culture before the addition of the DC maturation mixture. To identify the MHC restriction molecules involved in antigen presentation, we analyzed the inhibition of antigen-induced T-cell proliferation by adding 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 a 72-h culture.

Peptide Immunization of HLA-DR4 Transgenic Mice. Humanized HLA-DR4 transgenic mice (HLA-DRB1*0401), which are murine class II deficient and transduced with human CD4 molecule, were generated by Dr. Grete Sonderstrup in the Department of Microbiology and Immunology of Stanford University (19–21). The transgenic mice have been successfully used to identify human class II-restricted epitopes and to study immune responses (19–23). HLA-DR4 expression on the transgenic mice was analyzed by flow cytometry. DR4 transgenic 6–10-week-old mice were used for experiment. The transgenic mice were immunized twice at a 1-week interval with 100 μg of $hTERT_{766}$ peptide emulsified in CFA (final volume, 100 μl), administered s.c. into the rear back. Control group mice were injected with PBS emulsified in CFA.

Evaluation of T-Cell Responses by IFN-γ ELISPOT Assay. IFN-γ ELISPOT assay has been used to analyze peptide-specific T-cell responses by determining the frequency of Th precursors specific for the peptide. Mice were sacrificed 14 days after the last immunization, and splenocytes were obtained for assessing IFN-γ production. Briefly, 96-well MultiScreen-IP plates (Millipore Corp., Bedford, MA) were coated with 100 μl/well capture mAb against mouse IFN-γ (AN-18; Mabtech Inc., Cincinnati, OH) at a concentration of 10 μg/ml and incubated overnight at 4°C. The plates were washed four times with PBS and then blocked with RPMI 1640 plus 10% FBS for 2 h at 37°C. After washing, freshly isolated splenocytes were plated at 4×10^5 cells/well in RPMI 1640 with 10% FBS, in the presence or absence of peptide $hTERT_{766}$ (20 μg/ml), recombinant hTERT proteins (20 μg/ml), or hTERT-positive tumor lysates (50 μl/well). Tumor cell lysates were prepared by three freeze-thaw cycles of 5×10^7 tumor cells resuspended in 5 ml of RPMI 1640 with 10% FBS. Then the cells were centrifuged at $15,000 \times g$ for 30 min at 4°C. Supernatant was recovered, aliquoted, and stored at −80°C for later use, as described previously (11). After 20 h of cell culture in the incubator, the cells were removed by washing three times with PBS and four times with PBS/Tween 20 (0.05%). Biotinylated antimouse IFN-γ antibody (R4-6A2; Mabtech Inc.), diluted to 1 μg/ml in PBS/Tween 20 containing 0.5% BSA, was added and incubated for 2 h at 37°C. The plates were then washed six times with PBS/Tween 20 (0.05%), and subsequently, avidin-peroxidase-complex (Vector Laboratories, Burlingame, CA) was added, incubated for 1 h at room temperature, and removed by washing three times with PBS and PBS/Tween 20 (0.05%). The color of the plates was developed by adding horseradish peroxidase substrate 3-amino-9-ethylcarbozole (Sigma). The plates were then washed with tap water and air dried in dark. The plates were evaluated using an automated ELISPOT reader (Zellnet Consulting Inc., New York, NY).

Statistical Analysis. Two-sided Student's *t* test was used to evaluate the differences in the T-cell proliferation and ELISPOT assay among different groups. The overall significance level was set at 5%.

RESULTS

Initial Identification of Promiscuous MHC Class II-Restricted Epitopes in hTERT. To identify promiscuous MHC class II Th epitopes in hTERT, we first examined the AA sequence of this protein for the presence of peptide sequences containing binding motifs for multiple HLA-DR alleles, using the algorithm program TEPITOPE (12, 13). Because Th cells generally prefer to recognize peptides of about 15 residues in length, 10 predicted peptides corresponding to promiscuous binding motifs of 15-mer or longer were synthesized and purified (Table 1). Human T-cell responses to these peptides were assessed by isolating PBMCs from HLA-typed healthy donors with HLA-DR1, HLA-DR3, HLA-DR15, or other alleles and seeding them into 96-well plates that were subsequently stimulated with each peptide. After a week of stimulation, the cultures were tested for their capacity to respond to the peptides presented by autologous PBMCs. Cultures exhibiting at least a 3-fold increase in their proliferative response to peptides were considered positive. SI representing PBMC responses to each of the 10 peptides are shown in Fig. 1, *A* and *B*. Almost all donors tested responded to $hTERT_{631}$, $hTERT_{706}$, $hTERT_{854}$, $hTERT_{894}$, $hTERT_{930}$, $hTERT_{951}$, $hTERT_{766}$, $hTERT_{787}$, $hTERT_{805}$, and $hTERT_{971}$, indicating that the 10 peptides are viable Th epitope candidates. Importantly, several peptides ($hTERT_{631}$, $hTERT_{894}$, $hTERT_{766}$, $hTERT_{787}$, and $hTERT_{805}$) were capable of inducing T-cell responses to more than one MHC class II allele, indicating some degree of promiscuity.

Specificity and MHC Restriction Analysis of $CD4^+$ T-Cell Clones. To further characterize the peptides that induced positive T-cell responses, we selected and expanded cultures that exhibited at least a 3-fold increase in their proliferative response to peptides and cloned them by limiting dilution. T-cell clones that proliferated in response to six peptides ($hTERT_{631}$, $hTERT_{706}$, $hTERT_{766}$, $hTERT_{787}$, $hTERT_{805}$, and $hTERT_{894}$) were successfully generated. Those specific for the remaining

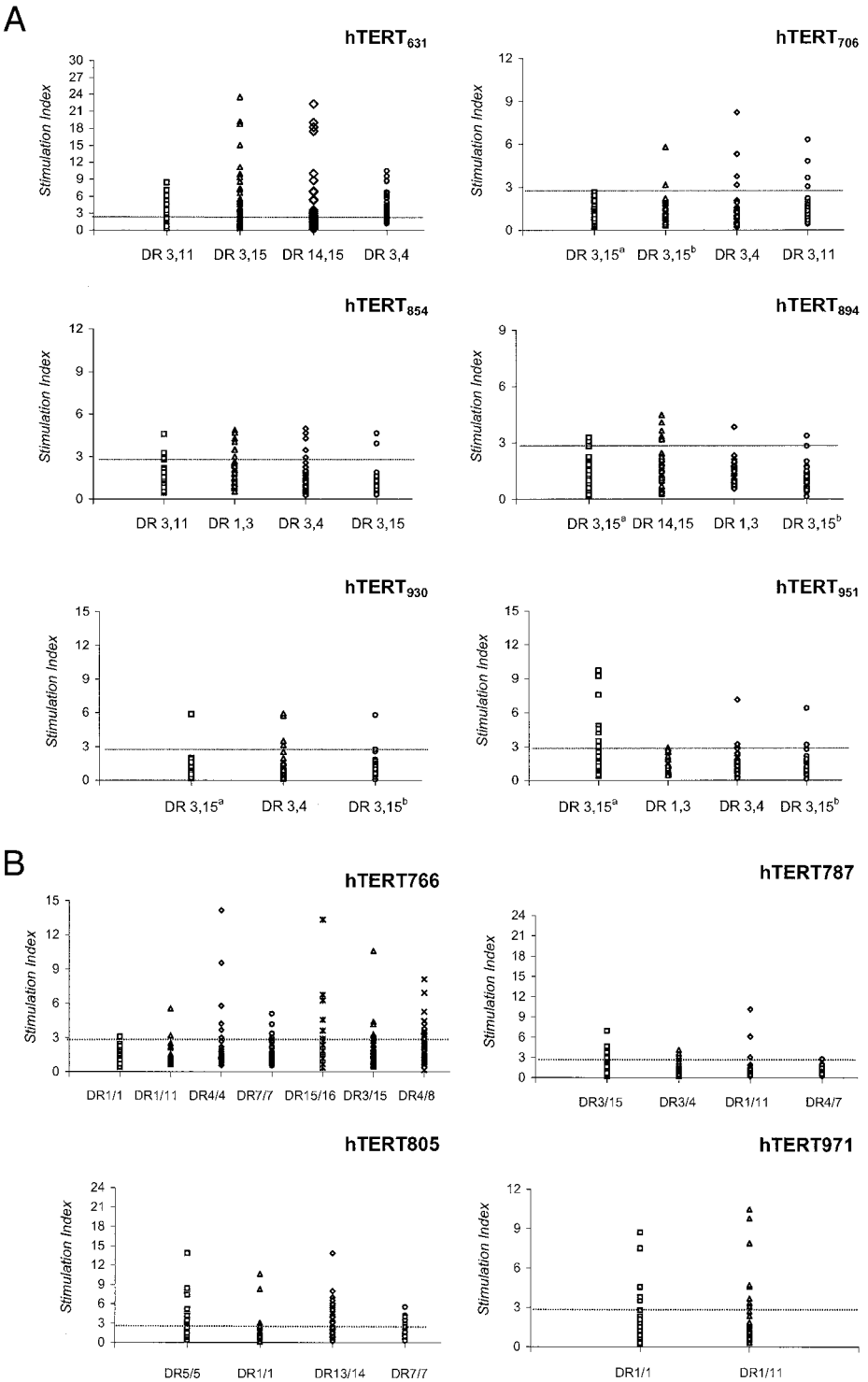

Fig. 1 Proliferative T-cell responses to hTERT-derived peptides. PBMCs from healthy adult donors were cultured with 1 of 10 different hTERT peptides (*A*, $hTERT_{631}$, $hTERT_{706}$, $hTERT_{854}$, $hTERT_{894}$, $hTERT_{930}$, and $hTERT_{951}$; *B*, $hTERT_{766}$, $hTERT_{787}$, $hTERT_{805}$, and $hTERT_{971}$; 20 μg/ml) for 7 days in U-bottomed 96-well plates (2×10^5 cells/well). [^{3}H]Thymidine incorporation of the primed T cells was measured after restimulation with autologous PBMCs (1×10^5) as APCs with or without corresponding peptides. SI (the mean cpm of peptide-pulsed PBMCs/the mean cpm of PBMCs not exposed to peptides) for each tested well are shown. A T-cell line/well was considered specific for a peptide when the cpm exceeded 1000 and SI was >3.

peptides ($hTERT_{854}$, $hTERT_{930}$, $hTERT_{951}$, and $hTERT_{971}$) failed to expand to sufficient numbers for further analysis, despite repeated attempts with different donors' blood. Fig. 2 shows the specificity of the T-cell clone responses to the various peptides. All T-cell clones responded vigorously to the stimulation with the corresponding peptides but did not respond to stimulation with irrelevant 15-mer peptides derived from EBNA1 ($EBNA_{482}$) or with irrelevant, noncorresponding peptides.

Furthermore, we used antibody blocking assays to test

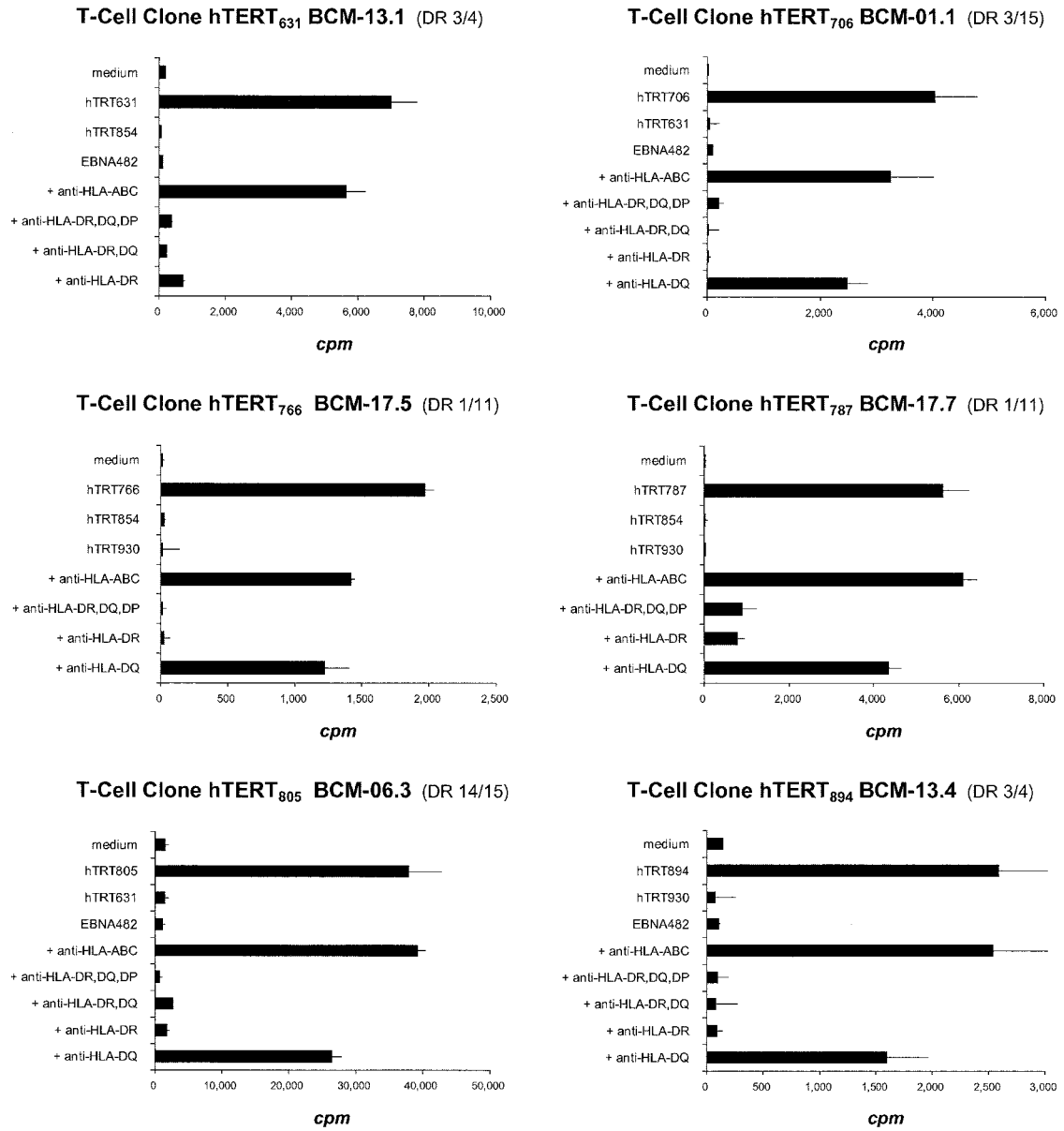

Fig. 2 Specificity and MHC restriction of T-cell responses. T-cell clones for each hTERT-derived peptide were established by limiting dilution culture. Subsequently, the T cells (3×10^4 T cells/well) were restimulated with autologous PBMC-derived DCs (1.5×10^3 DCs/well) in the presence of hTERT-derived peptides or an irrelevant 15-mer peptide from EBV ($EBNA_{482}$) at the same concentration of 20 μg/ml. Antibodies against MHC class I and MHC class II molecules were added together with specific hTERT peptides to analyze the MHC restriction. Cellular proliferation was measured by [^{3}H]thymidine incorporation assays. The proliferation of T cell clones cocultured with corresponding peptides was significantly higher than the proliferation with medium control and with the addition of HLA-DR antibody ($P < 0.05$). Mean and SD are shown for triplicate wells. The data shown are representative of three repeated experiments.

whether the T-cell responses to peptides were MHC class II restricted. As shown in Fig. 2, responses of the $hTERT_{631}$-, TRT_{706}-, $hTERT_{766}$-, $hTERT_{787}$-, $hTERT_{805}$-, and $hTERT_{894}$-specific T-cell clones were all inhibited by anti-HLA-DR and anti-HLA-DR/DQ/DP antibodies, but not by anti-HLA-ABC (class I) and anti-HLA-DQ antibodies. Flow cytometric analysis of T-cell clones (Fig. 3) confirmed that these clones were $CD4^+$ and $CD8^-$.

Identification of Natively Processed Epitopes. The effectiveness of antitumor immunotherapy based on $CD8^+$ and $CD4^+$ T cells depends on the ability of the latter to recognize naturally processed antigen presented by APCs. This property depends in turn on correct processing of the epitope in the MHC class II pathway and the avidity of the epitope for its MHC/T-CR complex (24). To determine whether the newly identified peptides were naturally processed antigens, we first evaluated

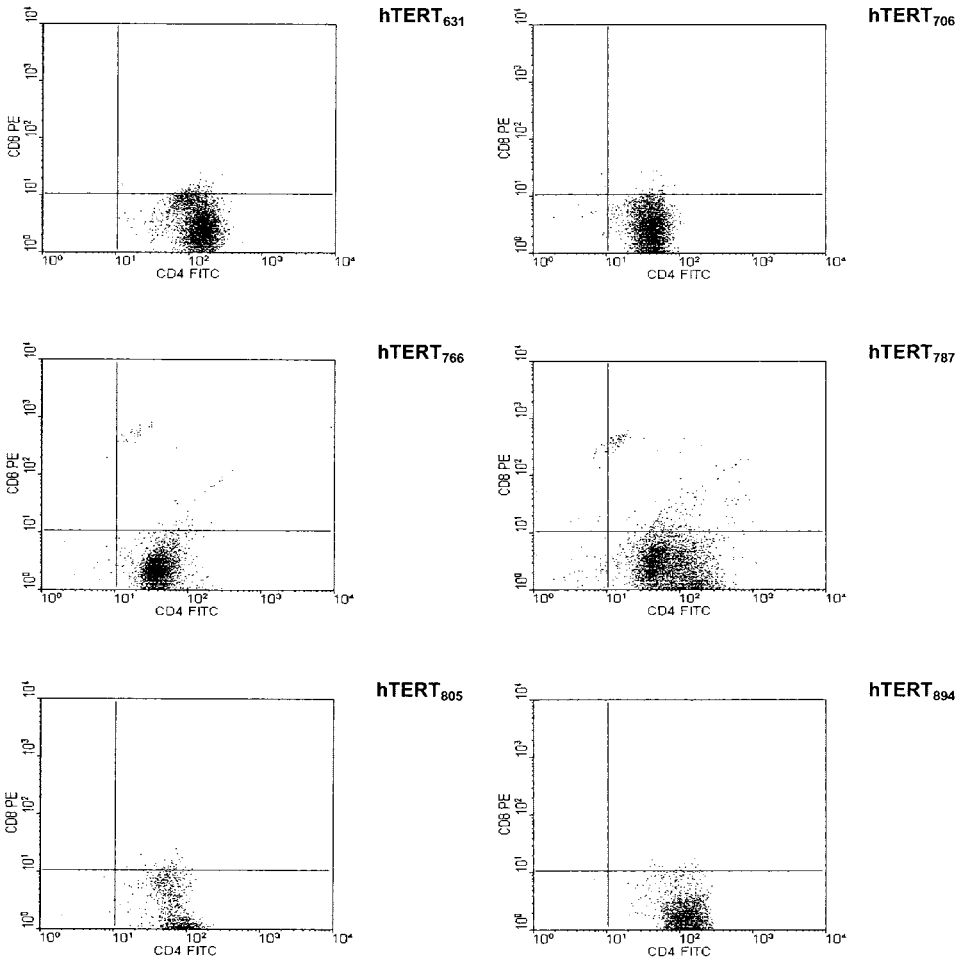

Fig. 3 Fluorescence-activated cell-sorting analysis of T-cell clones. The hTERT-reactive T-cell clones (Fig. 2) were double-stained with antihuman CD4-FITC and CD8-PE antibodies or isotype controls (mouse IgG-FITC and IgG-PE). The cells were then examined by flow cytometric analyses.

the avidity of the specific T-cell clones for their ligands. Peptide titration curves were generated with autologous DCs as APCs. For peptides $hTERT_{631}$, $hTERT_{766}$, and $hTERT_{787}$, the peptide concentrations required to obtain half of the maximal proliferation exceeded 1.0 μM (Fig. 4). For $hTERT_{706}$, $hTERT_{805}$, and $hTERT_{894}$, half-maximal proliferation was observed at higher concentrations (>5.0 μM).

The ability of these T-cell clones to recognize naturally processed antigen in the form of recombinant hTERT protein was tested in experiments in which autologous PBMCs or DCs were used as APCs, and recombinant hTERT proteins were used as a source of antigen (Fig. 5*A*). As shown in Fig. 5*B*, the $hTERT_{766}$-specific T-cell clone responded to hTERT protein-pulsed DCs, and this activity was inhibited by anti-HLA-DR antibody. The response of the $hTERT_{766}$-specific T-cell clone to the hTERT protein was specific because the T cells did not react to stimulation with autologous DCs pulsed with irrelevant recombinant Neu-Fc protein. Subsequent testing of T-cell clones specific for $hTERT_{787}$ or $hTERT_{631}$, which showed avidities similar to that of $hTERT_{766}$-specific clones, failed to detect significant proliferative responses when either PBMCs or DCs were used as APCs (data not shown). Thus $hTERT_{631}$ and $hTERT_{787}$ appear to be cryptic epitopes, not produced by APCs that normally process protein antigen. T-cell clones specific for $hTERT_{706}$, $hTERT_{805}$, or $hTERT_{894}$ had lower avidities for their ligands and were unable to proliferate when stimulated with PBMCs or DCs pulsed with the corresponding hTERT protein (data not shown). This result suggests either of two possibilities: either these epitopes may be cryptic; or the affinity of the T cells for the epitopes was low, requiring a higher number of peptide/MHC complexes than normally expressed on the APCs to trigger proliferative T-cell responses. Taken together, the data indicate that of the 10 peptides tested, $hTERT_{766}$ represents a naturally processed Th epitope in hTERT.

Promiscuity of Naturally Processed hTERT Epitopes. Ideally, a peptide-based vaccine would be effective in patients representing several different HLA-DR genotypes. We then tested the promiscuity of the naturally processed epitope $hTERT_{766}$ as well as the previously identified eptiope $hTERT_{672}$ (17). $CD4^+$ T-cell clones were stimulated with corresponding peptide using different types of homozygous LCLs as APCs. Homozygous LCLs used were DR1(HH), DR4(P678), DR7(T1), DR11(P125), DR15(P612), and DR17(LA). As shown in Fig. 6*A*, the $hTERT_{672}$-specific T-cell clone responded to the peptide $hTERT_{672}$ presented by HLA-DR1, HLA-DR7, and HLA-DR15. The $hTERT_{766}$-specific T-cell clone responded

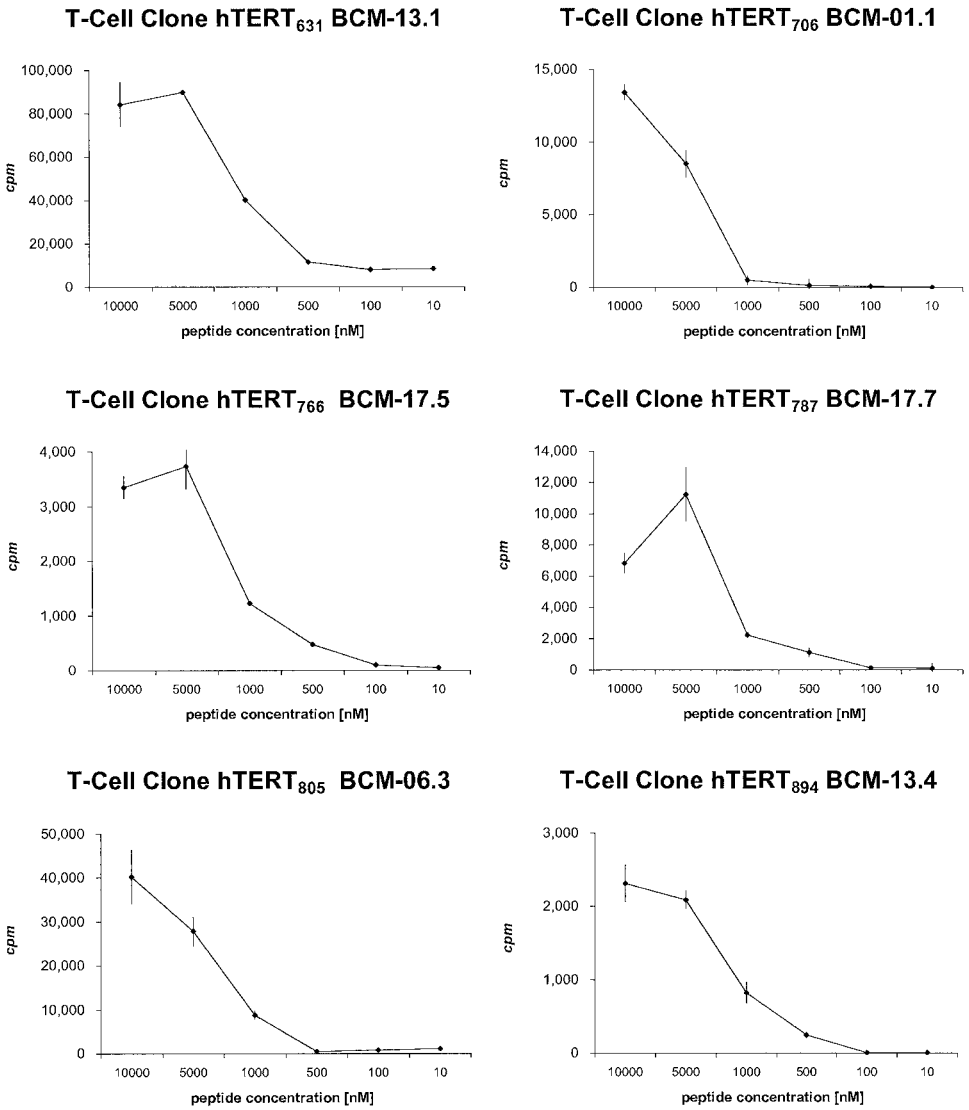

Fig. 4 Peptide titration experiments. Dose-response curves were created for each peptide-specific T-cell clone to estimate the epitope avidity of the MHC/TCR complex. T-cell clones (3×10^4 cells/well) were cocultured with autologous PBMC-derived DCs (1.5×10^3 DCs/well) and with hTERT-specific peptides at various concentrations. Each data point represents the mean and SD of triplicate samples of [^{3}H]thymidine incorporation of the T cells. The data are from one experiment that is representative of three repeated experiments.

to peptide $hTERT_{766}$ presented by HLA-DR4, HLA-DR11, and HLA-DR15 (Fig. 6*B*). These results indicate that both naturally processed epitopes are promiscuous and capable of inducing $CD4^+$ T-cell responses in the context of several commonly found HLA-DR alleles.

We then asked whether T-cell responses against these naturally processed epitopes could be induced using PBMCs from healthy donors with different genetic backgrounds. We also primarily assessed the frequency of hTERT-specific $CD4^+$ T cells in humans (which was calculated as the number of positive wells/total number of T cells in all wells tested) because others have demonstrated that an antigen-specific T-cell line derived from a 96-plate well (200,000 cells/well) most likely originates from a single T-cell precursor (16). As shown in Fig. 1*B*, $hTERT_{766}$ broadly induced T-cell responses from healthy donors with different genetic backgrounds, further demonstrating the promiscuity of the epitope. The frequencies of T-cell precursors specific for the naturally processed epitopes $hTERT_{766}$ and $hTERT_{672}$ in different healthy donors were $0.1–1.14 \times 10^{-6}$ and $0–0.83 \times 10^{-6}$, respectively. We also tested whether T-cell responses against the peptides could be induced using PBMCs from cancer patients. Testing was restricted to one naturally processed epitope, $hTERT_{672}$, due to the limited amount availability of cancer patient blood. Of seven prostate cancer patients tested, T cells from three patients with different HLA-DR alleles responded to the $hTERT_{672}$ stimulation (data not shown). The estimated precursor frequencies of T cells specific for the epitope $hTERT_{672}$ in the positive donors with different DR types are about $0–0.41 \times 10^{-6}$.

Antigen-Specific T-Cell Response Induced by $hTERT_{766}$ Immunization of HLA-DR4 Transgenic Mice. To further assess the therapeutic potential of these epitopes, we used humanized HLA-DR4 transgenic mice (19–21) to determine whether immunization with the peptide can induce a $CD4^+$ Th response specific not only for the peptide but also for the hTERT protein. The HLA-DR molecule expression on trans-

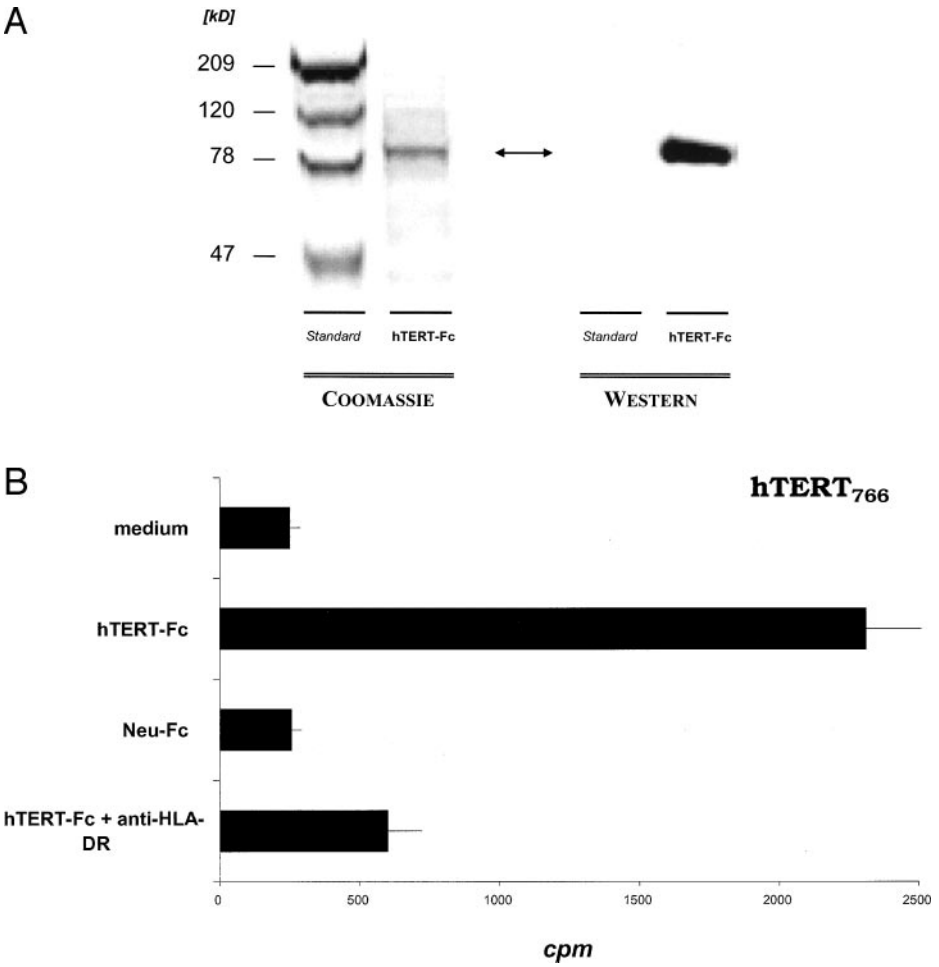

Fig. 5 *A*, analysis of recombinant hTERT protein. SF9 insect cells cells were infected with hTERT-Fc baculovirus. After lysis of whole cells, the Fc-tagged hTERT protein was purified by protein A affinity chromatography. The purified protein was detecetd on a 10% SDS-PAGE gel by Coomassie Blue staining. A Western blotting using an antibody against hTERT showed a single band of approximately 79 kDa. *B*, responses of T-cell clone $hTERT_{766}$ to natively processed hTERT protein. The $hTERT_{766}$-specific T cells (clone $hTERT_{766}$ BCM-17.5; 2×10^4 cells/well) were stimulated with irradiated autologous PBMC-derived DCs (1×10^3 DCs/well) pulsed with recombinant hTERT-Fc protein or irrelevant Neu-Fc protein control (10 μg/ml). After 3 days of culture, [^{3}H]thymidine incorporation was determined. The proliferation of T cells cocultured with hTERT-Fc-pulsed DCs was significantly higher than that of T cells with control Neu-Fc protein-pulsed DCs ($P < 0.05$) and with addition of anti-HLA DR antibody ($P < 0.05$). Values shown are the mean of triplicate determinations (*bars*, SD). The representative result of one of three repeated experiments is shown.

genic mice was detected by flow cytometric assay. The spleen cells were isolated and stained with FITC-conjugated mouse antihuman HLA-DR, PE-conjugated mouse antihuman CD4, or FITC-conjugated rat antimouse CD4 (BD PharMingen). These transgenic mice are human HLA-DR, $CD4^+$, and mouse $CD4^-$ by flow cytometric assay (data not shown). Two weeks after the last immunization, the transgenic mice were sacrificed, and the responses of their splenocytes to peptides, recombinant hTERT protein, and hTERT-positive tumor cells, including breast cancer, melanoma, and leukemia, were examined by using IFN-γ ELISPOT assays. The splenocytes of $hTERT_{766}$-immunized mice responded strongly to the $hTERT_{766}$ stimulation, producing IFN-γ at a frequency of 147 spots/million splenocytes. In contrast, the splenocytes of PBS-immunized control mice produced IFN-γ at a background frequency of 4 spots/million splenocytes to the peptide $hTERT_{766}$ (Fig. 7*A*). Furthermore, $hTERT_{766}$-specific responses could be inhibited by antihuman CD4 antibody. The splenocytes of $hTERT_{766}$-immunized mice did not respond to irrelevant peptide $EBNA_{482}$ stimulation. To exclude the possible allogeneic and xenogeneic effects of T-cell responses from the immunized transgenic mice, we immunized the DR4 transgenic mice with an irrelevant DR4-binding peptide, $PSMA_{459}$, a newly identified Th epitope in PSMA (25). As shown in Fig. 7*B*, the splenocytes of $PSMA_{459}$-immunized mice strongly responded to stimulation with the peptide $PSMA_{459}$ but not to the $hTERT_{766}$ peptide, indicating that the T-cell response from the immunized transgenic mice to the $hTERT_{766}$ peptide is specific.

Because most of tumor cells are MHC class II negative, the tumor-specific MHC class II-restricted $CD4^+$ T cells are not able to recognize these tumor cells directly. $CD4^+$ T cells induced by peptide immunization can react with APCs that take up and process the tumor antigen protein. Thus, we tested whether transgenic mouse T cells become activated when cocultured with splenocytes containing T cells and APCs pulsed with the recombinant hTERT proteins. As shown in Fig. 8*A*, when stimulated with the recombinant hTERT protein, the splenocytes of $hTERT_{766}$-immunized mice produced IFN-γ at a frequency of 41 spots/million cells, significantly higher than the splenocytes of the PBS-immunized mice. Furthermore, the splenocytes of $hTERT_{766}$-immunized mice produced IFN-γ at a background frequency when stimulated with irrelevant Neu-Fc proteins (4 spots/million cells). In addition, Fig. 8*C* showed that the splenocytes of $PSMA_{459}$-immunized mice strongly responded to stimulation with the PSMA protein (25), but not to the hTERT protein. These results indicate that $hTERT_{766}$ immunization

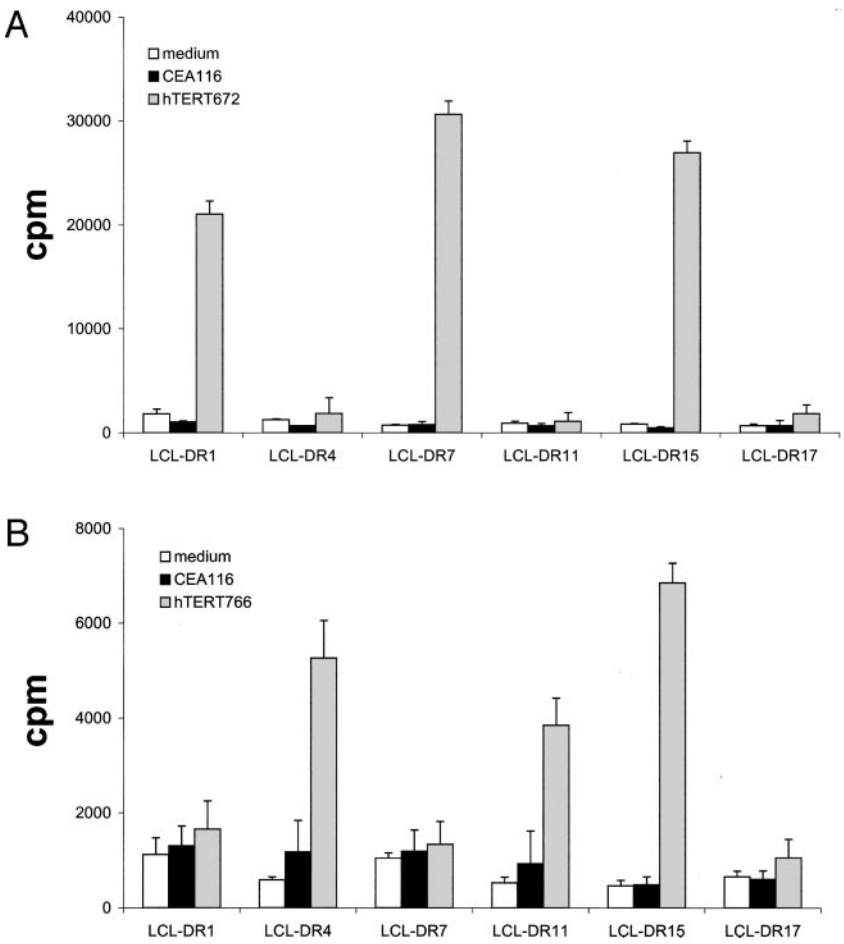

Fig. 6 *A* and *B*, presentation of $hTERT_{672}$ and $hTERT_{766}$ by different HLA-DR alleles. $hTERT_{672}$-specific T cells (*A*) or $hTERT_{766}$-specific T cells (*B*; 3×10^4 cells/well) were cocultured with one of irradiated homozygous EBV-LCLs of different HLA-DR genotypes (3×10^4/well) pulsed with peptide $hTERT_{672}$, $hTERT_{766}$, or an irrelevant peptide, CEA_{116} (20 μg/ml). The results represent the peptide-induced proliferative responses in the presence of different HLA-DR alleles. Mean and SD are shown for triplicate wells. If invisible, the SD fell within the size of the symbol. The data indicate that $hTERT_{672}$ and $hTERT_{766}$ epitopes can be promiscuously presented by several commonly found HLA-DR alleles.

activates T cells that specifically recognize antigenic peptides processed from hTERT proteins.

Finally, we tested whether activated $CD4^+$ T cells can recognize APCs that directly take up and process the tumor antigen from tumor cells. The functional telomerase activity in tumor lines was determined by TRAP, a two-step PCR-based primer extension assay (4). Human leukemia cell lines (HL-60 and Jurkat), melanoma cell line NA-6-MEL, and breast cancer cell line MDA-MB231 were tested positive for hTERT, and GM847 was negative (11). As shown in Fig. 8*B*, when stimulated with hTERT-positive tumor cell lysates, the splenocytes of $hTERT_{766}$-immunized mice produced IFN-γ at a frequency of 157 spots/million cells to MDA-MB231, 38 spots/million cells to NA-6-MEL, and 21–25 spots/million cells to HL-60 and Jurkat, significantly higher than the splenocytes stimulated with hTERT-negative GM847 tumor lysates (3 spots/million cells). These results indicate that T cells activated by $hTERT_{766}$ immunization specifically respond to antigenic peptides derived from various hTERT-positive tumors.

DISCUSSION

Peptide and peptide/DC vaccination to elicit antitumor immunity is an attractive strategy for cancer therapy. To date, tumor vaccination efforts have been mainly directed to the induction of CTL responses (26, 27) with numerous MHC class I CTL epitopes from a large variety of tumor-associated antigens (28, 29). Although impressive antitumor responses have been elicited in some patients, the results have been disappointing overall (30–32). One explanation appears to lie in failure to exploit $CD4^+$ Th cells, which play critical roles in initiating, regulating, and maintaining antitumor immune responses (26, 28). They exert helper activity for the induction and maintenance of $CD8^+$ CTLs (26, 33), as well as other antitumor activities (26, 34–38). Thus, effective tumor vaccines may require not only CTLs but also Th epitopes from tumor-associated antigens.

Attempts to identify MHC class II-restricted Th epitopes that could be used to enhance antitumor responses have yielded only a few promising candidates (29, 39). Recently, several groups have used computer-based algorithms to identify tumor-associated Th epitopes, including tyrosinase, MART-1, NY-ESO, and MAGE-3 (24, 40–45). In the present study, we combined a computer-based analytical program with *in vitro* T-cell biological analysis to identify Th epitopes in hTERT. Of 10 hTERT peptides that were predicted to bind to MHC class II molecules, all were capable of inducing primary T-cell response *in vitro*. By establishing $CD4^+$ T-cell clones for these peptides,

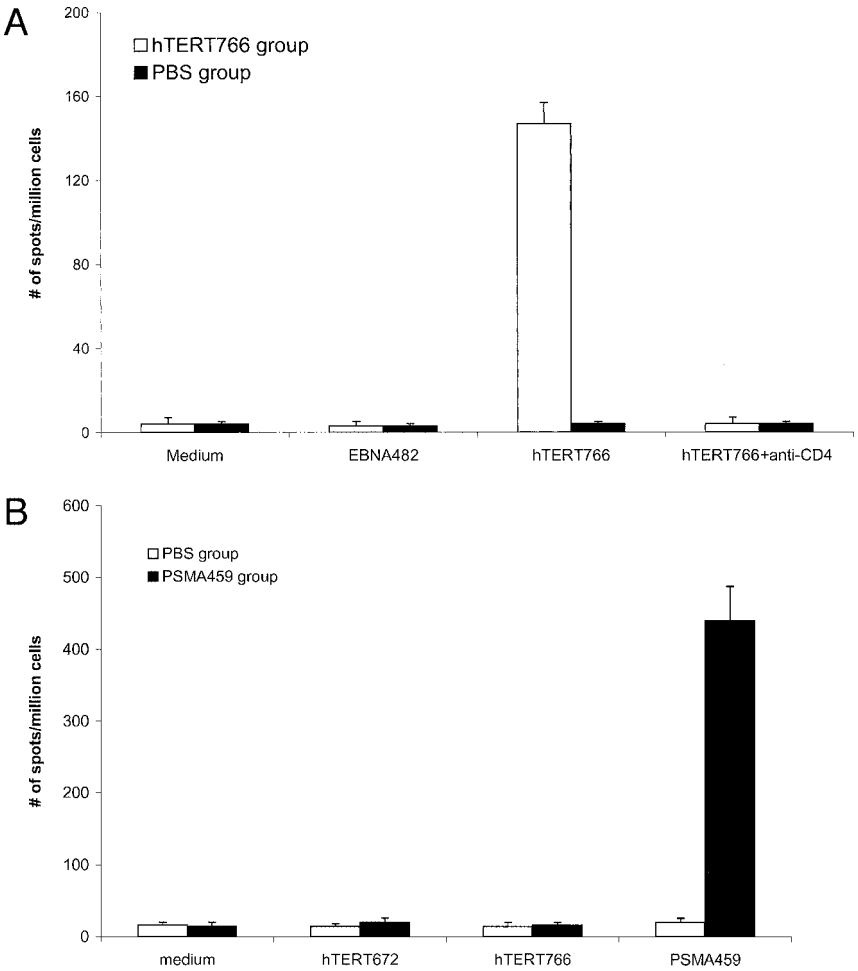

Fig. 7 *A* and *B*, peptide-specific Th response induced by immunization with $hTERT_{766}$. HLA-DR4 transgenic mice (4 mice/group) were twice immunized with 100 μg of $hTERT_{766}$ or an irrelevant DR4-binding peptide $PSMA_{459}$ or PBS (100 μl) emulsified with CFA. Fourteen days later, after the second immunization, the splenocytes of each mouse group were pooled and tested for peptide $hTERT_{766}$-induced IFN-γ production by ELISPOT. *A*, $hTERT_{766}$-immunized transgenic mice. *B*, $PSMA_{459}$-immunized transgenic mice. Data show the mean and SD of spots from triplicate wells from one experiment that is representative of two experiments performed. The frequency of peptide-specific T cells of $hTERT_{766}$-immunized mice was significantly higher than that of PBS control group ($P < 0.01$).

we were able to demonstrate that only the clone specific for $hTERT_{766}$ can recognize naturally processed and expressed hTERT epitopes, indicating that the epitope corresponding to this synthetic peptide is naturally processed through the MHC class II pathway.

A major aim of this study was to identify T-cell epitopes presented by multiple common HLA alleles, such as HLA-DR1, HLA-DR4, and HLA-DR7, so that any resultant vaccines could be used in patients with a spectrum of genotypes (46–50). Thus, by exploiting the predictive algorithm of Hammer *et al.* (12), which focuses on promiscuous HLA-DR binding peptides, we identified two naturally processed epitopes, $hTERT_{766}$ and $hTERT_{672}$, that may constitute cross-reactive MHC class II molecules. Indeed, subsequent testing demonstrated that $hTERT_{766}$ can be presented by multiple DR alleles tested, including DR4, DR11 and DR15, and $hTERT_{672}$ can be presented by multiple DR alleles tested, including DR1, DR7, and DR15. Thus, these two naturally processed Th epitopes are promiscuous, enhancing their potency as tumor vaccine candidates.

We further evaluated the therapeutic potential of these naturally processed, promiscuous hTERT Th epitopes by using humanized HLA-DR4 transgenic mice (19–21) to determine whether a $CD4^+$ Th response specific not only for the peptide but also for the hTERT protein and hTERT-positive tumor cells can be induced. It was found that immunization of humanized HLA-DR4 transgenic mice with $hTERT_{766}$ peptide elicited strong antigen-specific Th responses that can recognize the antigenic peptides derived from hTERT protein and various hTERT-positive tumors such as breast cancer, melanoma, and leukemia. hTERT is expressed in stem cells and mature hematopoietic cells (51, 52), and therefore, hTERT vaccination could result in autoimmunity and destruction of normal cells. However, the result of this study showed that hTERT-specific $CD4^+$ T cells did not respond to autologous hTERT-positive LCLs without peptide pulsing (Fig. 6). In addition, no abnormality of the peptide-immunized HLA-DR4 transgenic mice was observed (data not shown). This result is in agreement with our previous observation (11), and other reports that the hTERT-specific CTLs did not lyse $CD34^+$ cells (7, 9), probably because the quantity of hTERT peptides generated under physiological condition is insufficient to stimulate $CD4^+$ T cells.

hTERT is a potential universal target for tumor vaccine because it is highly expressed in ~85% of cancer cells (1–5, 10, 53, 54). Several studies identified CTL epitopes in hTERT and demonstrate that CTL responses were induced against hTERT-

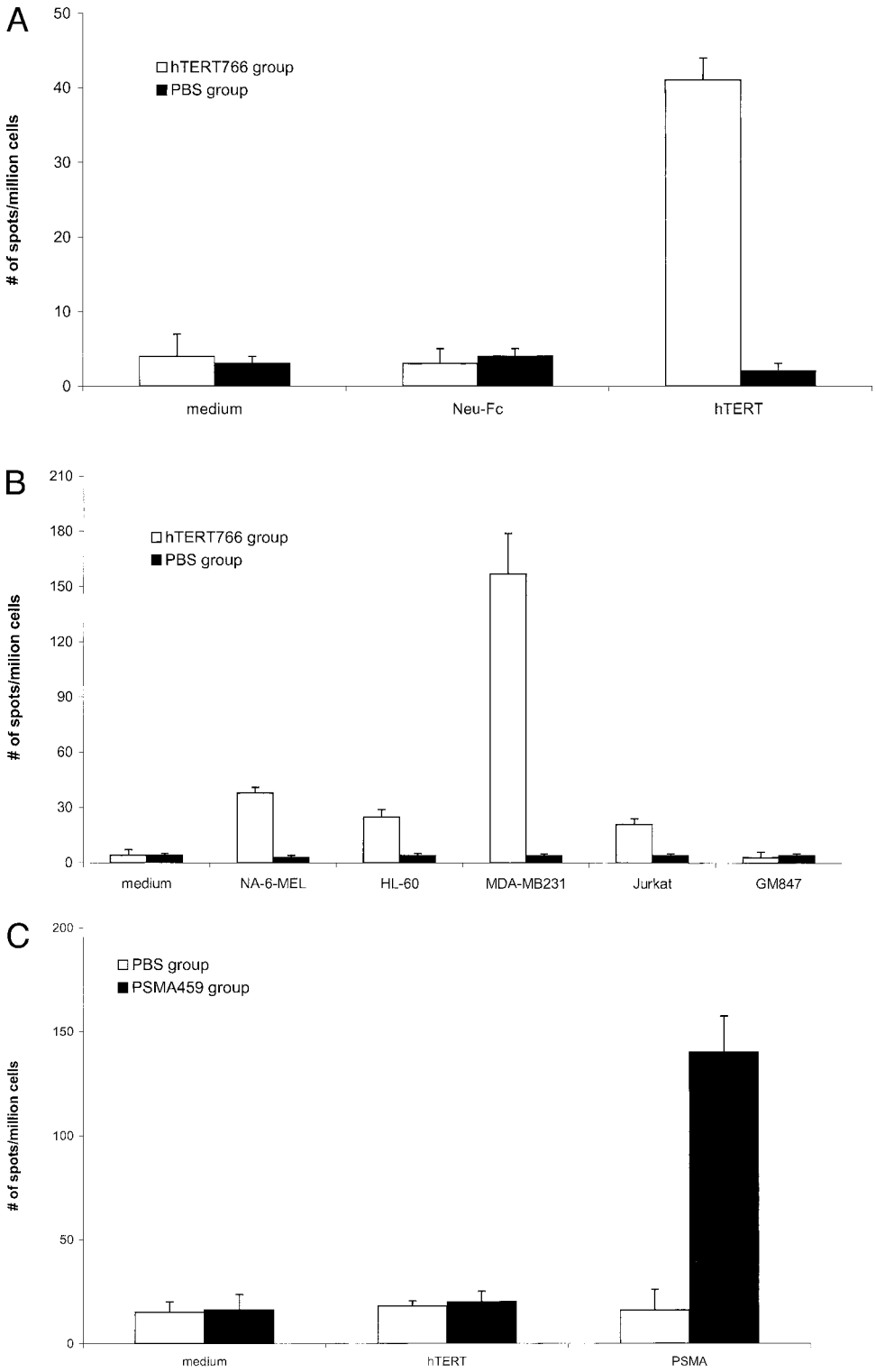

Fig. 8 *A–C,* Th responses to antigenic peptides derived from hTERT proteins and hTERT-positive tumors. Splenocytes from HLA-DR4 transgenic mice (4 mice/group) immunized with 100 μg of $hTERT_{766}$ or the irrelevant peptide $PSMA_{459}$ (25) or PBS (100 μl) emulsified with CFA were assessed for the production of IFN-γ by ELISPOT. *A* and *B,* recombinant hTERT proteins or irrelevant neu-Fc proteins (20 μg/ml; *A*) and hTERT-positive tumor lysates or hTERT-negative GM847 cell lysates (50 μl; *B*) were used to pulse the splenocytes of $hTERT_{766}$- or PBS-immunized mice. Data show the mean and SD of spots from triplicate wells from one experiment that is representative of two experiments performed. The frequency of IFN-γ-secreting cells of $hTERT_{766}$-immunized group when stimulated with hTERT proteins or tumor lysates was significantly higher than that of Neu-Fc proteins and hTERT-negative GM847 controls ($P < 0.01$). *C,* splenocytes from $PSMA_{459}$-immunized transgenic mice responded to stimulation with PSMA proteins, but not to hTERT proteins (20 μg/ml). The data indicate that T-cell responses induced by the $hTERT_{766}$ epitope specifically respond to antigenic peptides derived from hTERT proteins and hTERT-positive tumors.

positive tumor cells *in vitro* (7–9, 55). Although controversy remains as to whether the hTERT540–549 epitope is a naturally processed one (7, 56–59), several studies demonstrated that human DCs transfected with an adenoviral vector expressing hTERT or with hTERT mRNA induced hTERT-specific CTLs that killed hTERT-positive tumors (8, 56). Furthermore, human DCs transfected with mRNA encoding a chimeric hTERT/lysosome-associated membrane protein (LAMP-1) protein were

capable of stimulating concomitant hTERT-specific $CD8^+$ and $CD4^+$ T-cell responses *in vitro* (60). Taken together, our identification of promiscuous, naturally processed hTERT epitopes and the evidence of strong hTERT-specific Th responses in humanized HLA-DR4 transgenic mice induced by the peptide immunization should provide a basis for the development of improved vaccines through simultaneous stimulation of CTLs (7–9) and Th against a broad spectrum of hTERT-positive tumors.

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Correction: Article on Human Telomerase Reverse Transcriptase-Specific T-Helper Responses

In the article on hTERT-Specific T-Helper Responses in the October 15, 2003, issue of *Clinical Cancer Research*, Figure 5A was incorrect. The corrected figure appears below.

Schroers R, Shen L, Rollins L, et al. Human Telomerase Reverse Transcriptase-Specific T-Helper Responses Induced by Promiscuous Major Histocompatability Complex Class II-Restricted Epitopes. Clin Cancer Res 2003;9:4743–55.

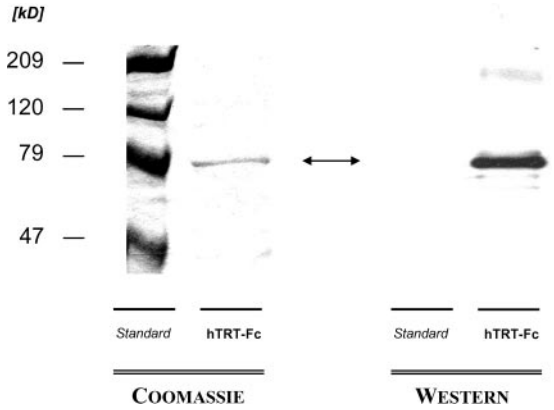

Fig. 5 A, analysis of recombinant hTERT protein. SF9 insect cells were infected with hTERT-Fc baculovirus. After lysis of whole cells, the Fc-tagged hTERT protein was purified by protein A affinity chromatography. The purified protein was detected on a 10% SDS-PAGE gel by Coomassie Blue staining. A Western blotting using an antibody againist hTERT showed a single band of approximately 79 kDa.

Clinical Cancer Research

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Clin Cancer Res 2003;9:4743-4755.