Immunogenic HLA-B*0702-Restricted Epitopes Derived from Human Telomerase Reverse Transcriptase That Elicit Antitumor Cytotoxic T-Cell Responses

Olivier Adotévi,[1,4] Karine Mollier,[2] Christine Neuveut,[3] Sylvain Cardinaud,[4] Emmanuelle Boulanger,[4] Blandine Mignen,[5] Wolf-Hervé Fridman,[1] Maurizio Zanetti,[6] Pierre Charneau,[2] Eric Tartour,[1] François Lemonnier,[4] and Pierre Langlade-Demoyen[4]

Abstract

Purpose: The human telomerase reverse transcriptase (hTERT) is considered as a potential target for cancer immunotherapy because it is preferentially expressed in tumor cells. To increase the applicability of hTERT-based immunotherapy, we set out to identify CTL epitopes in hTERT restricted by HLA-B*0702 molecule, a common MHC class I allele.

Experimental Design: HLA-B*0702-restricted peptides from hTERT were selected by using a method of epitope prediction and tested for their immunogenicity in human (*in vitro*) and HLA-B*0702 transgenic mice (*in vivo*).

Results: All the six hTERT peptides that were predicted to bind to HLA-B*0702 molecule were found to induce primary human CTL responses *in vitro*. The peptide-specific $CD8^+$ CTL lines were tested against various $hTERT^+$ tumor cells. Although differences were observed according to the tumor origin, only three CTL lines specific for p277, p342, and p351 peptides exhibited cytotoxicity against tumor cells in a HLA-B*0702-restricted manner. In addition, this cytotoxicity was inhibited by the addition of peptide-loaded cold target cells and indicated that these epitopes are naturally processed and presented on the tumor cells. Further, *in vivo* studies using humanized HLA-B*0702 transgenic mice showed that all the candidate peptides were able to induce CTL responses after peptide immunization. Furthermore, vaccination with a plasmid DNA encoding full-length hTERT elicited peptide-specific CTL responses, indicating that these epitopes are efficiently processed *in vivo*.

Conclusions: Together with previously reported hTERT epitopes, the identification of new CTL epitopes presented by HLA-B*0702 increases the applicability of hTERT-based immunotherapy to treating cancer.

Human telomerase is a ribonucleoprotein enzyme considered as a widely expressed tumor-associated antigen (1, 2). This enzyme, which consists of a RNA subunit and a catalytic subunit [human telomerase reverse transcriptase (hTERT)], mediates the RNA-dependent synthesis of telomeric DNA (3, 4). Telomere attrition limits the replication potential of most somatic cells, whereas tumor cells acquire immortality by continuous telomere maintenance, which is predominantly due to the reactivation and overexpression of hTERT (5, 6). High hTERT expression (>85%) has been frequently shown in telomerase-positive primary human tumors and cancer cell lines but is low or undetectable in normal tissues (7–9). Because hTERT activity is essential for maintaining the proliferative capacity of tumor cells, the risk of antigen escape by genetically unstable tumors may be reduced when targeting hTERT for vaccination. Thus, hTERT may represent an attractive target for cancer immunotherapy than other self-antigen expressed by tumor cells, such as gp100, MART-1, and cancer testis antigens (e.g., the MAGE family of protein; refs. 10, 11).

There is increasing evidence that peptides derived from hTERT are specifically recognized by $CD8^+$ CTLs (12–14). Furthermore, hTERT-specific CTLs have been found in patients suffering from various malignancies, such as leukemia, colorectal, prostate, and breast cancer, and indicated that natural immune response against hTERT is induced in cancer patients (15–18). Recently, clinical studies have shown that hTERT-specific T-cell responses can be induced *in vivo* in $HLA\text{-}A^*0201^+$

Authors' Affiliations: [1]Institut National de la Sante et de la Recherche Medicale U255, Université René Descartes, Unité d'Immunologie Biologique, Hôpital Européen Georges Pompidou, Assistance-Public Hôpitaux de Paris; [2]Unité de Virologie Moléculaire and [3]Unité d' Oncogenèse et Virologie Moléculaire, Institut National de la Sante et de la Recherche Medicale U579 and [4]Immunité Cellulaire Antivirale, Institut Pasteur, Paris, France; [5]Etablissement Français du Sang, site Hôpital Henri Mondor, Assistance-Public Hôpitaux de Paris, Créteil, France; and [6]Department of Medicine and Cancer Center, University of California at San Diego, La Jolla, California

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Requests for reprints: Olivier Adotévi, Hôpital Européen Georges Pompidou, Assistance-Public Hôpitaux de Paris, 20 rue Leblanc, 75908 Paris Cedex 15, France. Phone: 33-56-09-39-80; Fax: 33-56-09-20-80; E-mail: Olivier.adotevi@hop.egp.ap-hop-paris.fr.

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cancer patients, opening the use of hTERT peptides for anticancer therapeutic vaccination (19, 20). Although HLA-A*0201 is the most frequent in the Caucasian population expressed in ~50% (21), an effective T-cell-based cancer treatment, including vaccines, against hTERT will likely require the identification of other MHC class I–restricted but also MHC class II–restricted epitopes in this antigen. Thus, the search of CTL epitopes has been extended to other MHC class I alleles, such as HLA-A3 (22), HLA-A24 (23), and, more recently, HLA-A1 types (24). T helper epitopes in hTERT have also been reported for several HLA-DR molecules to improve antitumor vaccines (25, 26).

The goal of this study was to identify immunogenic peptides restricted by HLA-B*0702 molecule, which accounts for ~15% to 20% of the Caucasian population (27). As we reported here, the immunogenicity of six hTERT peptides that were predicted to bind to HLA-B*07027 molecules was analyzed. It was found that all the peptides were able to induce HLA-B*0702-restricted CTL responses in healthy donors. Three of the six peptide-specific CTL lines effectively recognized HLA-matched, telomerase-positive tumor cell lines, indicating that they are naturally processed and expressed epitopes. Subsequently, hTERT peptide and DNA immunization of humanized HLA-B*0702 transgenic elicited strong and antigen-specific $CD8^+$ T-cell responses *in vivo*, indicating that these epitopes could serve as potential immunogen for cancer vaccines.

Materials and Methods

Mice

Humanized HLA-B*0702 transgenic mice, which expressed a HLA-B*0702 α1α2, H2-K^d α3 chimeric construct in combination with constitutive murine β2-m molecule, had their H2-D^b and H2-k^b genes deleted, as described previously (28). These mice were on a C57BL/6 background and used at 8 to 10 weeks of age. They were bred and maintained under specific pathogen-free conditions in our animal facility (Pasteur Institute, Paris, France).

Human blood cells

Buffy coats from HLA-$B7^+$ healthy platelet donors were purchased from Henri Mondor Hospital Blood Bank, Assistance-Public Hôpitaux de Paris (Créteil, France). Experiments were done in accordance to an approved French Blood Bank Institute protocol.

Cell lines

The following human cells lines from American type Culture Collection (Manassas, VA) were used: renal cancer cell line (U293T), EBV-transformed B lymphoblastoid cell line (JY), Burkitt's lymphoma cell line (Raji), T lymphoma cell line (Jurkat), and T/B-LCL hybrid lymphoblastoid cell line (T1). Transporter associated with antigen-processing–deficient T2 cells transfected with the HLA-B*0702 gene (referred to as T2-B7) were from P. Creswell laboratory (Yale University Medical Center, New Haven, CT; ref. 29). The BBG.1 EBV^+ primary effusion lymphoma B-cell line was kindly provided by H. Collandre (Laboratoire de Virologie Medicale Moleculaire, Grenoble, France; ref. 30). The melanoma cell lines (LB34, LB373, and KUL68) were kindly provided by P. Coulie (Institute of Cellular Pathology, Université Catholique de Louvain, Brussels, Belgium). Human T1 cell line and murine lymphoma RMA cell line were stably transfected with HLA-B*0702 monochain gene (referred to as T1-B7 and RMA-B7; refs. 28, 31).

Epitope selection

We used the predictive algorithm SYFPEITHI to analyze amino acid sequences of hTERT protein (access via http://www.uni-tuebingen.de/uni/kxi/; ref. 32). Six peptides were retained based to their highest predictive score to bind to HLA-B*0702 molecule (Table 1). HLA-B*0702-restricted CTL epitopes derived from human cytomegalovirus pp65 265-274 RPHERNGFTV (R10V) and HIV-1 GP41 ($p_{843-851}$) IPRRIRQGL (31) were used as control peptides. All synthetic peptides were synthesized by PRIMM (Milan, Italy) to a minimum of 85% purity.

Table 1. Binding capacity and *in vivo* immunogenicity of HLA-B*0702-restricted hTERT epitopes

hTERT peptides	Sequence	Amino acid position	Score	Relative avidity*	Peptide immunization†		DNA immunization‡	
					R/T	Specific lysis (%)	R/T	Specific lysis (%)
p1	M*P*RAPRCR*A*	1-9	23	0.4	4/6	40; 39; 35; 38	3/5	26; 45; 24
p4	A*P*RCRAVRS*L*	4-13	25	0.3	6/6	52; 53; 48; 51; 56; 61	5/5	42; 37; 34; 49; 22
p68	A*P*SFRQVSC*L*	68-77	25	0.4	6/6	63; 78; 75; 69; 70; 59	5/5	46; 24; 32; 39; 27
p277	R*P*AEEATS*L*	277-285	23	2.5	4/6	44; 33; 45; 39	3/5	31; 22; 33
p342	R*P*SFLLSS*L*	342-350	23	4.7	2/6	36; 29	2/5	28; 31
p351	R*P*SLTGARR*L*	351-360	23	1.5	5/6	47; 51; 37; 36; 54	3/5	37; 30; 28

NOTE: The designation, the position of the peptides, and the score numbers based on HLA-binding prediction algorithm (32) are indicated. Letters in italics correspond to canonic major anchor residues to HLA-B*0702 molecules.

*The binding and stabilizing capacity to HLA-B*0702 molecule was measured *in vitro* by MHC-binding stabilization assay on T2-B7 (28) and the relative avidity showed was the ratio of the concentration of tested versus reference peptides need to reach 20% of the maximal amount of stabilized molecules as defined with high concentration of reference peptide.

†HLA-B*0702 transgenic mice were injected (six mice per peptide group) s.c. with 50 μg of each hTERT peptide with hepatitis B virus core T13L helper peptide coemulsified in incomplete Freund's adjuvant (28). Ten days later, spleen cells of individual mouse were stimulated *in vitro* with corresponding hTERT peptide. Cytolytic activities of effector cells were assayed by ^{51}Cr release assay against peptide-loaded RMA-B7 target cells. The indicated specific lysis (for a 60:1 E/T cell ratio) was calculated by subtracting nonspecific lysis observed with RMA-B7 pulsed with R10V control peptide that already <5%. The number of responders (when specific lysis is >10%) versus tested mice (R/T) is indicated.

‡Three weeks after pCMV-hTERT DNA injection, splenocytes of five transgenic mice were harvested and restimulated independently with lipopolysaccharide blasts loaded with each hTERT-HLA-B*0702 peptide. The cytotoxic activity of effector cells was measured after one round *in vitro* restimulation against RMA-B7 cells pulsed with either hTERT peptide or R10V control peptide. Specific lysis for an E/T cell ratio of 60:1 was calculated as described. The number of responder versus five mice tested (R/T) is shown.

HLA-B*0702-binding assay

The relative avidity of hTERT-derived peptides for HLA-B*0702 was measured using a MHC stabilization assay on T2-B7 cells and compared with the avidity of a reference peptide as described previously (14, 28). T2-B7 cells were incubated overnight at 37°C with various concentrations of hTERT peptides or reference peptide R10V. HLA-B7 expression was measured by flow cytometry using an anti-HLA-B7 monoclonal antibody (mAb) ME1 following by FITC-conjugated $F(ab')_2$ goat anti-mouse IgG antibody (BD PharMingen, Franklin Lakes, NJ). Results are expressed as values of relative avidity, which is the ratio of the concentration of test peptide necessary to reach 20% of the maximal binding by the reference peptide, so that the lower the value the stronger the binding.

Evaluation of hTERT expression

hTERT expression was measured by intracellular staining using two antibodies: an anti-hTERT mAb (TEL, clone 2C4, Abcam, Paris, France) and anti-hTERT polyclonal antibody (TERT, clone H-231, Santa Cruz Biotechnology, Santa Cruz, CA; refs. 33, 34). Briefly, 10^6 cells were washed thrice in PBS-bovine serum albumin 0.5% (staining buffer), fixed in PBS plus 4% paraformaldehyde (5 minutes at 37°C), and washed. Anti-hTERT antibody was added in staining buffer plus 0.1% saponin and incubated for 30 minutes at room temperature. Cells were washed in permeabilization buffer, and detection antibody FITC labeled was added (30 minutes at room temperature). Cells were then washed once in permeabilization buffer and twice in staining buffer before being analyzed by flow cytometry.

Generation of hTERT-specific CTL lines in human

For CTL line generation, peripheral blood mononuclear cells (PBMC) from HLA-B7$^+$ normal healthy donors were stimulated with hTERT-derived peptides *in vitro* in 24-well plates with autologous, irradiated adherents cells in the presence of interleukin-2 and interleukin-7 as described previously (13, 14). After three to four rounds of culture, CTLs were screened in a standard ^{51}Cr release assay.

Measurement of human CTL activity

Human CTL activity was measured by standard ^{51}Cr release assay as described previously (13, 14). The specific lysis was calculated by subtracting nonspecific lysis observed with the HLA-B*0702-restricted HIV-1-derived control peptide. Donors were considered as responders when specific lysis of >10% was observed.

Human IFN-γ ELISPOT assay

CD8$^+$ lymphocytes were selected within peptide-specific CTL lines using magnetic beads system (CD8 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany). More than 90% purity was reached in the CD8$^+$ fraction, and CD16/CD56 natural killer cell fraction was <5%. Peptide-specific CD8$^+$ CTL lines were tested against tumor cells and their ability to produce IFN-γ was measured by ELISPOT assays. Presensitized CD8$^+$ T cells (5×10^4 and 1×10^4) and 2×10^4 target cells [T2-B7 or peptide-pulsed T2-B7 (1 μg/mL)] or γ-irradiated various tumor cells were added to each well. After incubation for 16 hours at 37°C, IFN-γ spot-forming cells were detected according to the manufacturer's instructions (Diaclone, Besançon, France). The spots were counted on a Bioreader 2000 system (Biosys, Karben, Germany).

MHC restriction and cold target inhibition assays

The hTERT-specific CD8$^+$ T cells used in these assays were purified from peptide-specific CTL line cultures as mentioned above. Studies on MHC restriction were done by using blocking antibodies (13). Briefly, ^{51}Cr-labeled target cells were incubated with an anti-HLA class I framework mAb, W6/32 (BD PharMingen), or ME1 (anti-HLA-B7 mAb), or G46.6 (anti-HLA-DR mAb; BD PharMingen) at an optimal concentration (10 μg/mL) for 30 minutes at 4°C before adding effector cells to determine HLA restriction. Cold target inhibition assay was done as described previously (13, 23). Briefly, T2-B7 cells were incubated with 1 μg/mL of each hTERT-derived peptide for 2 hours at 37°C. After washing thrice, T2-B7 or peptide-pulsed T2-B7 cells were used as cold target cells. Various numbers of cold target cells were incubated with effector cells for 1 hour and 5×10^3 ^{51}Cr-labeled tumor cells were added to the wells. The cytotoxicity assay was done as described previously.

In vivo immunogenicity studies

Peptide immunization. HLA-B*0702 transgenic mice were injected s.c. at the base of the tail with 50 μg hTERT peptide or PBS as control along with 140 μg I-A^b MHC class II helper peptide TPPAYRPPNAIL (T13L), which correspond to amino acids 128 to 140 of the hepatitis B virus core protein in incomplete Freund's adjuvant (28, 35). Ten days later, IFN-γ ELISPOT assay has been used to analyze *ex vivo* peptide-specific T-cell responses. Spleen cells of immunized mice were harvested and CD8$^+$ T lymphocytes were isolated using mouse CD8 T-cell isolation kit (Miltenyi Biotec). Serial dilutions of CD8$^+$ T lymphocytes were cultured with 2×10^4 irradiated human tumor cells (5,000 rads) or RMA-B7 cells pulsed previously (or not) with hTERT peptide (5 μg/mL), fixed with 1% paraformaldehyde, and washed thrice before use. After incubation for 24 hours at 37°C, IFN-γ spot-forming cells were detected according to the manufacturer's instructions.

DNA immunization. A plasmid DNA vector coding for the hTERT expressed under the control of cytomegalovirus promoter (pCMV-hTERT; ref. 36) was purified on plasmid Giga kit columns under endotoxin-free conditions (Qiagen, Hilden, Germany). Anesthetized HLA-B*0702 transgenic mice were injected with 50 μL cardiotoxin into each regenerating tibialis anterior muscles 5 to 6 days before DNA injection. For DNA-based vaccination, 50 μL pCMV-hTERT (1 μg/μL in PBS) or 50 μL PBS as control was injected directly into each pretreated muscle at days 0 and 14 (37). Ten days after the last immunization, Spleen cells of immunized mice were harvested and Ficoll-purified lymphocytes were isolated, and hTERT-specific T cells were detected *ex vivo* using ELISPOT assay as describe above. For mouse CTL induction, splenocytes of peptide- or DNA-immunized mice were separately stimulated for 5 to 6 days with peptide-pulsed (10 μg/mL) γ-irradiated (5,000 rads) syngeneic splenocytes or lipopolysaccharide blast cells (35, 37). The specific cytotoxic activity of effector CTL was measured by standard ^{51}Cr release assay (35).

Results

Selection and binding capacity of HLA-B*0702-restricted epitopes. Using a CD8$^+$ T-cell epitope prediction algorithm (32), we first analyzed the 8– to 10–amino acid sequence of hTERT protein for the presence of peptide sequences containing binding motifs for HLA-B*0702 molecules. We retained six hTERT peptides based on their highest predictive score (Table 1). Because immunogenicity of MHC class I–restricted peptides reflects to some degree their binding capacity for MHC class I molecules (38), we sought direct proof of the strength of interaction between the six hTERT peptides and the HLA-B*0702 molecule in a binding/stabilization assay that used transporter associated with antigen-processing–deficient T2-B7 cells. The peptide relative avidity shown in Table 1 indicated that the six peptides were excellent binders to HLA-B*0702 molecules. Three peptides (p1, p4, and p68) displayed a relative avidity of ≤1 and the three others (p277, p342, and p351) exhibited a relative avidity of ≥1.5. Thus, exception for one peptide (p342, relative avidity, 4.7), there was a good correlation between the binding assay and the prediction scores of the candidate peptides.

Generation of primary CTL responses against hTERT in healthy subjects. To check out the presence of precursor T cells for the

six hTERT peptides in the human repertoire and their possible expansion on antigen stimulation, we used PBMC of eight HLA-B7$^+$ healthy donors in an *in vitro* immunization protocol (13). After four rounds of *in vitro* immunization, effector CTLs were tested for their lytic activity against peptide-pulsed T2-B7 cells. As shown in Fig. 1, hTERT-specific CTLs were generated in all normal donors tested. The frequency of responders against each hTERT peptide varied among donors. Overall, four peptides (p1, p68, p277, and p351) immunization yielded specific CTL responses in the majority of the subjects (>60% of responders). In contrast, the immunization with p4 and p342 peptides generated specific CTL responses but in fewer instances (37% and 25% of responders, respectively). The weaker response observed with p342 *in vitro* immunization could be related to its lower relative affinity for HLA-B*0702 molecule compared with other peptides (Table 1). No significant cytotoxicity was observed against nonpulsed T2-B7 cells (data not shown) or pulsed with irrelevant control peptide (Fig. 1). These results clearly indicated that these six hTERT peptides were able to generate specific CTL responses, implying that precursor CTL for these epitopes were present in the peripheral repertoire of HLA-B7$^+$ healthy subjects. Therefore, we asked whether the peptide-specific CTL lines generated from healthy individuals would be able to recognize HLA-matched hTERT$^+$ tumor cells.

hTERT peptide-specific CTLs kill various human tumor cells. To assess if hTERT-derived peptides are naturally processed in tumor cells, the cytotoxic activity of the hTERT peptide-specific CTL lines was tested against a panel of tumor cell lines of various tissue origin. hTERT expression was first verified in the tumor cells by flow cytometry using an intracellular staining with anti-hTERT mAb. All the tumor cells selected express high levels of hTERT protein (Fig. 2A; data not shown). The human tumor cells lysis was detected by ^{51}Cr release assay using peptide-specific CTL lines obtained from the PBMC of four healthy donors after subsequent *in vitro* immunization with candidate peptide as described above.

The results presented in Table 2 show the three of the six peptide-specific CTL lines that exhibited cytotoxicity against various HLA-B7$^+$ hTERT$^+$ tumor cells but not against HLA-B7$^-$ hTERT$^+$ tumor cells. The p351-specific CTL exhibited cytotoxic activity against all the hTERT$^+$ tumor cells tested. In contrast, the p277-specific and p342-specific CTLs exerted cytotoxicity against tumor cells of lymphoid origin as leukemia or lymphoma cells but were not reactive against nonlymphoid tumor cells (melanoma or renal cancer). These results were significant as the tumor lysis was similarly observed with peptide-specific CTL lines from multiple healthy donors (Table 2). However, the CTL lines from the same healthy donors, specific for the three other peptides (p1, p4, and p68), failed to kill all the tumor cell lines, although they were reactive against T2-B7 cells pulsed with corresponding peptide (data not shown). Thus, p277, p342, and p351 peptide-specific CTLs were able to kill hTERT$^+$ tumor cells, although differences were observed depending on tumor origin.

The ability of the CTL to recognize human tumor cells was also assessed using IFN-γ ELISPOT assay. As shown in Fig. 2B, the p351-specific CD8$^+$ T-cell line was reactive against all tumor cell lines in a HLA-B*0702-restricted fashion. In these experiments, IFN-γ untreated nonlymphoid cells were also used as target cells and were recognized by p351-specific CTL although at the lower levels (Fig. 2B). In contrast, the two other CD8$^+$ T-cell lines specific for p277 and p342, respectively, failed to recognize the nonlymphoid tumor cells, whereas they were reactive against tumor cells line from lymphoid (Fig. 2C and D). Similar responses were observed with peptide-specific CD8$^+$ T cells from three additional healthy donors (data not shown) and support the tumor cells lysis observed with ^{51}Cr release assay.

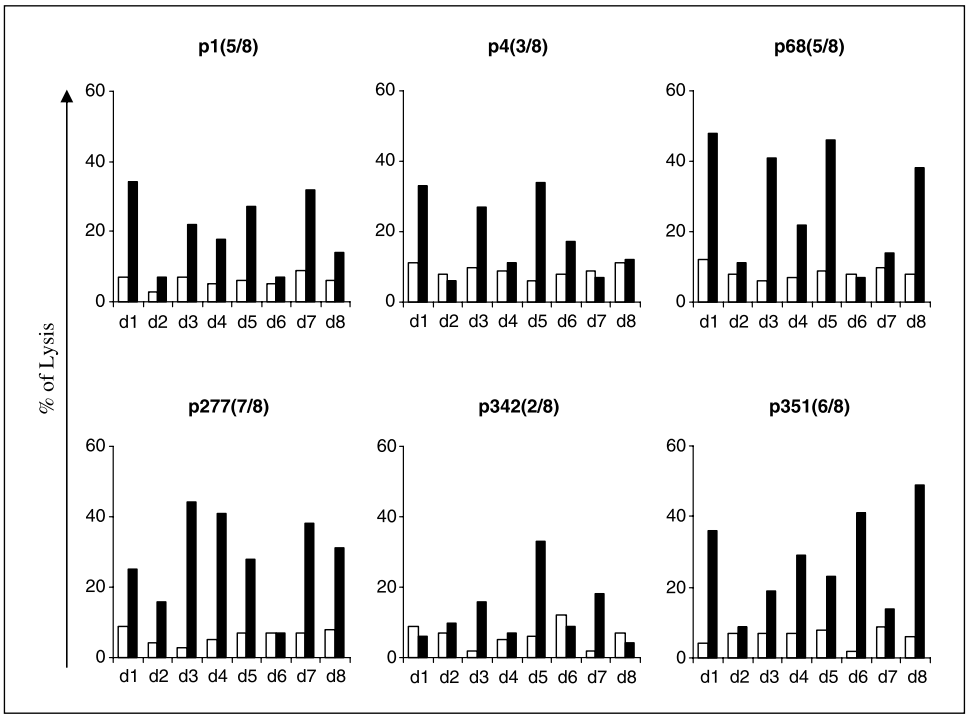

Fig. 1. Generation of hTERT-specific CTLs in HLA-B7$^+$ healthy donors. PBMC from eight HLA-B7$^+$ healthy donors (d1-d8) were stimulated *in vitro* with each hTERT peptide-pulsed autologous antigen-presenting cells (15). After three or four rounds of weekly stimulation, effector cells were assayed for cytolytic activity against ^{51}Cr-labeled T2-B7 cells pulsed with the indicated hTERT peptide (black columns) or control HIV-1-derived peptide (white columns). Percentage of lysis at E/T ratio of 30:1 is shown for each peptide, and the number of responders (when specific lysis is >10%) versus tested donors is indicated (R/T).

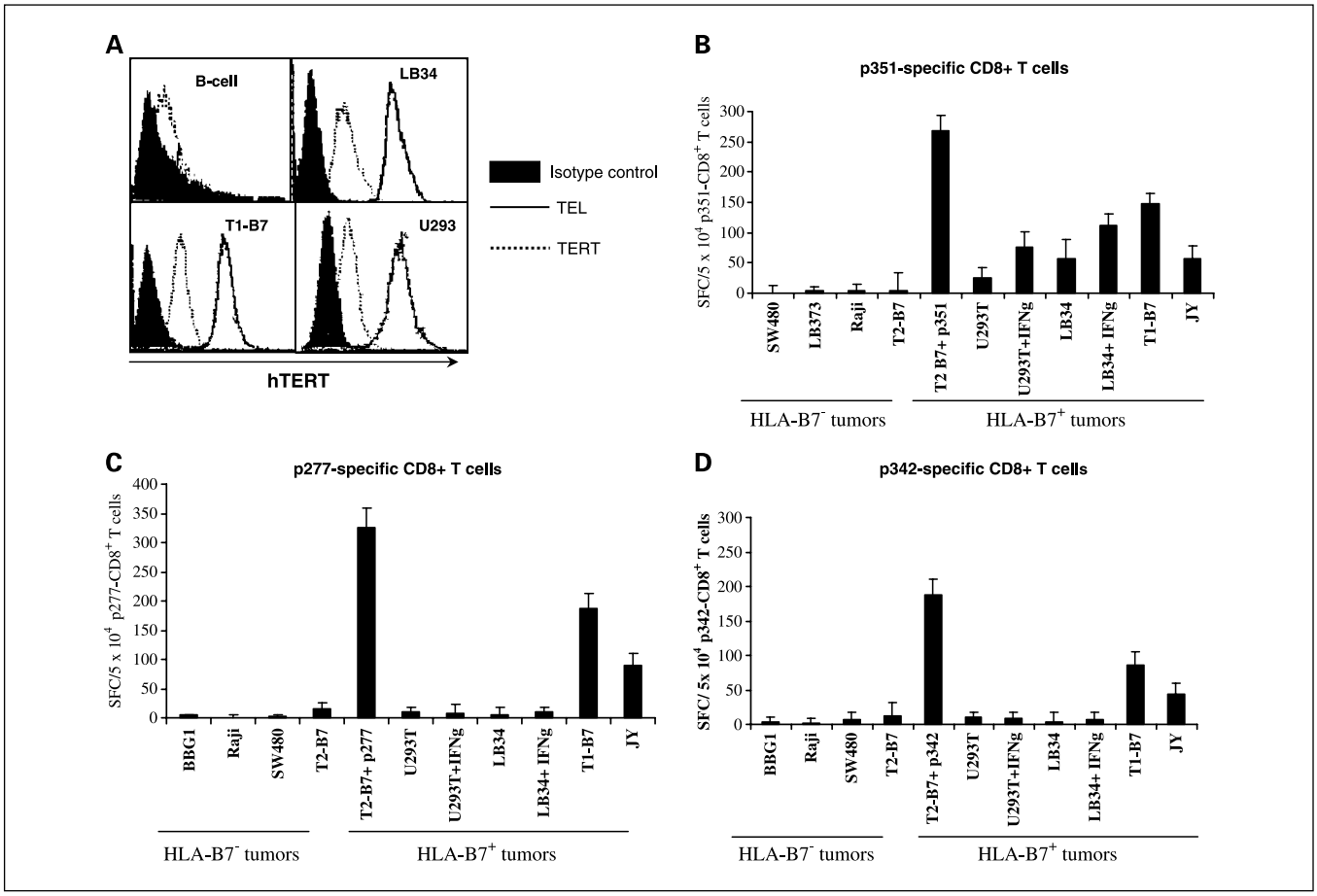

Fig. 2. Tumor cell recognition by HLA-B*702-restricted $CD8^+$ T lymphocytes. *A,* hTERT expression on human tumor cell lines and normal cells. hTERT expression was measured by flow cytometry using intracellular staining with two anti-hTERT antibodies TEL (clone 2C4) and TERT (clone H-231; see Materials and Methods). CD40L-activated B lymphocytes were used as control normal cells. *B* to *D,* assays for IFN-γ-producing hTERT peptide-specific $CD8^+$ T cells. $CD8^+$ cells were selected within peptide-specific CTL obtained from one healthy donor (d5) using magnetic beads system. Each peptide-specific $CD8^+$ T cells was cultured with γ-irradiated HLA-$B7^+$ or HLA-$B7^-$ tumor cells. The ability of peptide-specific $CD8^+$ CTL to recognize tumor cells were assayed by IFN-γ ELISPOT assay. The nonlymphoid tumor cell lines (LB34, melanoma; U293T, renal cancer) were pretreated or not with IFN-γ (100 units/mL) 48 hours and washed several times before ELISPOT assay. T2-B7 or T2-B7 pulsed with corresponding peptide were used as positive control. Columns, mean of spots from triplicate wells from one experiment; bars, SD. The number of specific IFN-γ-producing cells (spot-forming cells) per 5×10^4 peptide-specific $CD8^+$ T cells.

Further, to address whether hTERT-specific CTLs could kill autologous normal cells, we did cytotoxicity assay using normal PBMCs and activated B lymphocytes as target cells. Activated lymphocytes belong to the rare normal cells that are known to express telomerase (9, 13). To this end, autologous B lymphocytes from healthy donor were isolated and activated *in vitro* with CD40L. Although freshly isolated PBMC cells are telomerase negative (data not shown), the CD40-activated B lymphocytes were found to express low levels of hTERT as measured by flow cytometry compared with tumor cell lines (Fig. 2A). As expected, autologous PBMCs were not lysed by the peptide-specific CTL lines. They also failed to recognize CD40-activated B cells, whereas these cells were susceptible to CTL lysis after pulsing with peptide (Table 2). These findings indicate that activated nontransformed B lymphocytes are unlikely a target of the candidate peptide-specific CTLs.

MHC restriction and cold target inhibition assays. Studies on MHC class I restriction were done by using blocking antibodies during the cytotoxicity assay. As shown in Fig. 3A, the lysis of HLA-B*0702$^+$ melanoma (LB34) and T1-B7 cell lines by the p351-specific $CD8^+$ CTL was inhibited by a HLA-B7 mAb or by a pan-anti-MHC class I mAb. In contrast, an anti-HLA-DR mAb has no effect on the cytotoxic activity (Fig. 3A). Similar inhibition of cytotoxic activity was also observed with the CTL lines specific for p277 and p342 in the presence of blocking antibodies (data not shown). These data indicated that the CTL lines exerted the cytotoxicity against tumor cells in a HLA-B7-restricted manner.

To further confirm that the tumor cell lysis by peptide-specific $CD8^+$ CTL lines was mediated by specific recognition of naturally processed hTERT peptides, we did cold target competition assay. As shown in Fig. 3B, the addition of p351-pulsed T2-B7 cells resulted in decreased cytotoxicity of p351-specifc $CD8^+$ CTL against tumor cells, whereas the addition of nonpulsed T2-B7 showed no effect on cytotoxic activity. The same experiment also revealed that the lysis mediated by p277-specific $CD8^+$ CTL and p342-specific $CD8^+$ CTL was inhibited by adding p277 and p342 peptide-loaded T2-B7 as cold target cells, respectively (Fig. 3C and D). Together, these data strongly suggest that the hTERT-derived epitopes p277, p342, and p351 were naturally processed and presented on tumor cell lines from lymphoid origin, whereas only p351 hTERT epitope

seemed to be naturally processed on the nonlymphoid tumor cell lines, such as melanoma and renal cancer cells.

Immunogenicity of hTERT peptides in HLA-B*0702 transgenic mice. To further assess the therapeutic potential of these naturally processed epitopes, we used humanized HLA-B*0702 monochain transgenic mice (28, 31). Mice were twice injected with hTERT peptide together with a T helper peptide in incomplete Freund's adjuvant. Ten days later, the splenocytes were harvested and antigen-specific $CD8^+$ T-cell responses were monitored *ex vivo* by IFN-γ ELISPOT assay. As shown in Fig. 4A, the $CD8^+$ T lymphocytes of p351-immunized mice responded strongly to the p351 loaded on RMA-B7 cells, producing IFN-γ at a frequency of 134 spots/10^5 $CD8^+$ T cells. In contrast, the $CD8^+$ T lymphocytes of PBS-injected control mice produced IFN-γ at a background frequency of 6 spots/10^5 $CD8^+$ T cells to the p351 peptide stimulation (Fig. 4A). Similar strong peptide-specific $CD8^+$ T lymphocytes were induced in p277-immunized transgenic mice compared with PBS-injected mice (168 versus 5 spots/10^5 $CD8^+$ T cells; Fig. 4B). In contrast, immunization with peptide p342 induced a weaker response at a frequency of 38 spots/10^5 $CD8^+$ T cells (Fig. 4C). We further check if the $CD8^+$ T cells educated in HLA-B*0702 transgenic mice could recognize natural epitopes presented on human cell lines. For this purpose, the freshly isolated $CD8^+$ T lymphocytes from splenocytes of peptide-immunized mice were assayed against $hTERT^+$ human tumor cells. As shown in Fig. 4D, the induced peptide-specific $CD8^+$ T cells from transgenic mice were reactive against human HLA-matched tumor cell lines. The p351-specific $CD8^+$ T lymphocytes produced IFN-γ in the presence of all cell lines, whereas p277- and p342-specific $CD8^+$ T cells were only reactive against leukemia cell line T1-B7 (Fig. 4D), in agreement with data observed in human. These data indicated that hTERT-specific $CD8^+$ T cells induced in transgenic mice can react to naturally expressed antigens on human cell lines.

In this study, we were able to generate CTLs with all hTERT peptides *in vitro* in normal human individuals. Therefore, a reasonable expectation would be that they may also be immunogenic *in vivo*. To this end, the HLA-B*0702 transgenic mice were immunized with each candidate peptide and the ability of peptide to induce CTL responses was determined by ^{51}Cr release assay. As shown in Table 1, most candidate peptides (p1, p4, p68, p277, and p351) were able to induce CTL responses in after immunization. As indicated, two peptides (p4 and p68) elicited strong CTL responses in all immunized mice, and three peptides (p1, p277, and p351) induced intermediate CTL responses in at least half of

Table 2. CTL of healthy donors kill various tumor cell lines

Cell target	Cell origin	hTERT*	HLA-B7†	Peptide-specific CTL cytotoxicity‡							
				p277			p342		p351		
				Donor 1	Donor 3	Donor 5	Donor 5	Donor 7	Donor 1	Donor 3	Donor 5
T2-B7	T-B hybrid		+	6	3	7	1	3	5	8	2
T2-B7 + peptide			+	55	47	41	37	48	48	53	56
T1-B7	T-B hybrid	+	+	41	51	28	27	31	39	44	48
JY	B lymphoblast	+	+	13	26	31	21	22	32	29	16
Jurkat	T lymphoma	+	+	7	29	18	8	17	ND	21	26
LB34	Melanoma	+	+	0	0	2	7	3	42	25	51
KUL68	Melanoma	+	+	7	4	1	ND	6	14	21	ND
U293T	Renal cell	+	+	4	8	2	1	9	19	22	25
LB337	Melanoma	+	–	3	3	1	5	1	6	1	2
SW480	Colon cancer	+	–	0	1	ND	3	0	5	4	3
BBG1	Primary effusion lymphoma	+	–	6	0	5	1	0	0	0	2
T1	T-B hybrid	+	–	3	0	1	10	6	3	8	1
Raji	B lymphoma	+	–	6	3	1	4	0	1	3	ND
PBMC	Autologous	–	+	0	2	1	2	0	0	0	1
CD40-B cell	Autologous	low	+	7	0	6	2	3	9	0	4
CD40-B cell + peptide	Autologous	low	+	35	46	34	36	29	22	39	51

Abbreviation: ND; not determined.

*hTERT expression on target cells was determined by flow cytometry using anti-hTERT mAb (see Materials and Methods).

†HLA-B7 expression on tumor cell lines was measured by flow cytometry.

‡The p277- and p351-specific CTLs were obtained from three healthy donors (donors 1, 3, and 5) and the p342-specific CTL from two healthy donors (donors 5 and 7) after subsequent rounds of weekly restimulation with indicated hTERT peptide (13). The CTL lines were assayed for their lytic activity against T2-B7 cells or T2-B7 pulsed with indicated peptide and tested against HLA-$B7^+$ $hTERT^+$ tumor cells and HLA-$B7^-$ $hTERT^+$ tumor cells. The nonlymphoid tumor cell lines (melanoma and renal cancer cells) were incubated with 100 units/mL recombinant IFN-γ for 48 hours before cytotoxicity assay. Autologous B lymphocytes were isolated and activated for 48 hours with trimeric CD40L (40 μg/mL) and used as normal target cells. Cellular cytotoxicity was measured in a standard ^{51}Cr release assay, and specific lysis for a 30:1 E/T ratio is shown.

transgenic mice. In contrast, p342 peptide induced a weaker response (30% of injected mice), in agreement of CTL responses, induced *in vitro* in healthy donors. The CTL responses were peptide specific, as no significant cytotoxicity was observed against nonpulsed RMA-B7 target cells, excluding cross-reactivity with murine telomerase (data not shown).

hTERT-specific CD8⁺ T-cell responses after DNA-based immunization. To fully characterize the immunogenic potential of these epitopes, it is necessary to establish which among the various candidate peptides was processed and presented from hTERT protein. To this end, HLA-B*0702 transgenic mice were immunized with a plasmid DNA encoding hTERT. The frequencies of antigen-specific splenocytes were quantified *ex vivo* by IFN-γ ELISPOT assay. As shown in Fig. 4E, significant frequencies of peptide-specific, IFN-γ-producing cells were detected in DNA-immunized mice. These responses were observed in the presence of all the six candidate hTERT peptides, although differences were observed. Four epitopes p4, p68, p277, and p351 yielded greater peptide-specific T-cell responses than the two other p1 and p342 (Fig. 4E).

Next, peptide-specific CTL induction was also evaluated after DNA immunization. To this end, spleen cells of individual DNA-immunized transgenic mice were restimulated *in vitro* with each peptide for 5 to 6 days before ^{51}Cr release assay. As shown in Table 1, DNA injection elicited efficient peptide-specific CTL responses in individual mice, although different response profiles were observed. Specific CTL responses were induced in all DNA-immunized mice responses against two candidate peptides p4 and p68 and indicated that they are efficiently processes *in vivo*. Three peptides, p1, p277 and p351, have an intermediate pattern of recognition, as half of vaccinated animals developed antigen-specific CTL responses. In contrast, p342-specific CTL response was induced in only 25% of DNA-immunized mice. Interestingly, this peptide was also poorly immunogenic after peptide immunization but seemed to be naturally processed and presented on the tested tumor cells. Thus, following DNA-based vaccination, processing of hTERT protein leads to multiepitopic CD8⁺ T-cell responses in humanized HLA-B*0702 transgenic mice.

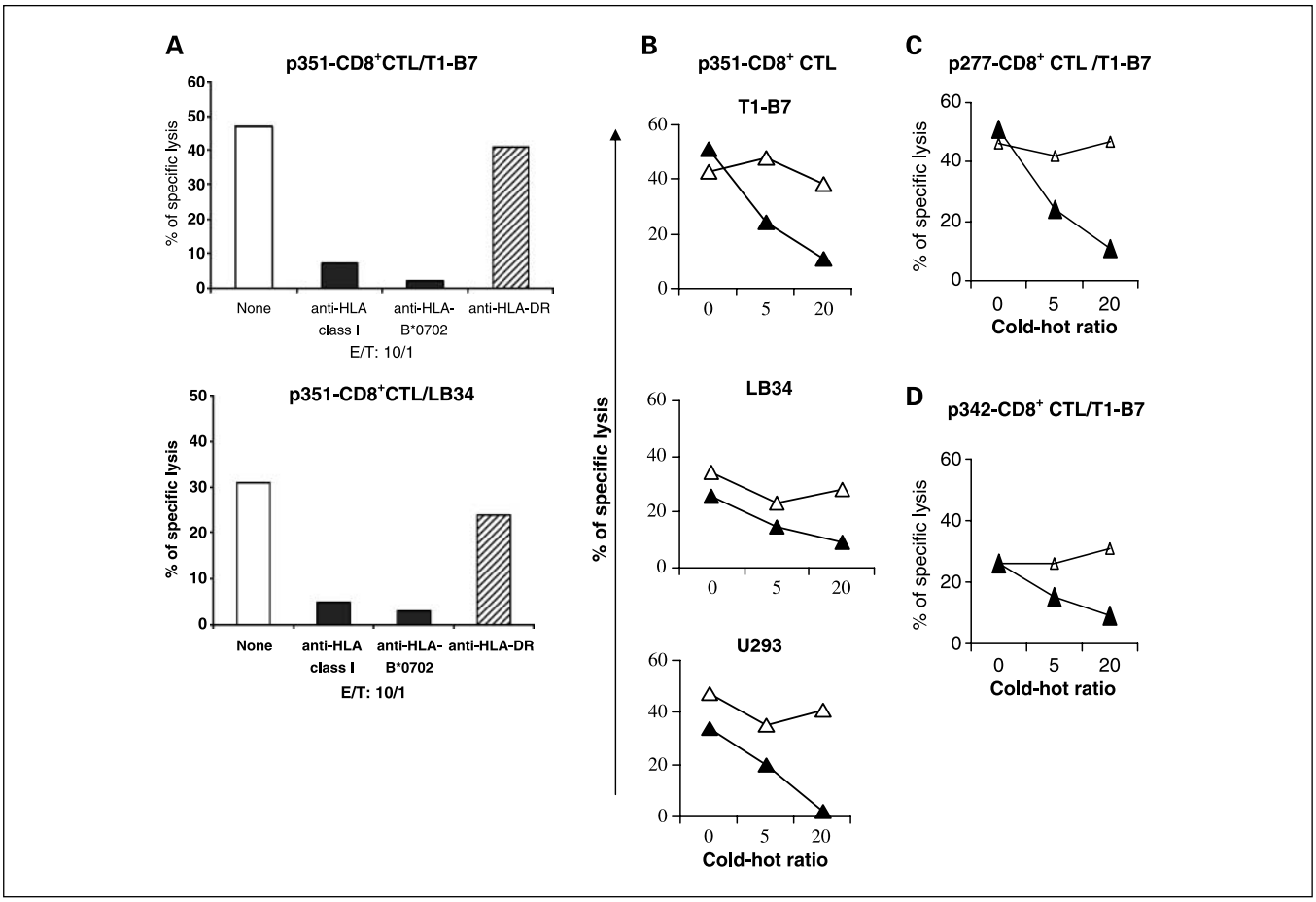

Fig. 3. MHC restriction and cold target inhibition assays. *A,* anti-HLA class I mAbs inhibited cytolytic activity of p351-specific CTL. The cytotoxicity of p351-specific CD8⁺ CTL against HLA-B7⁺ tumor cells, LB34 melanoma cell line and T1-B7 leukemia cell line pretreated with or without two anti-HLA class I mAbs (anti-HLA class I framework, W6/32; anti-HLA-B7, ME1), or anti-HLA-DR mAb (G46.6), was determined by standard ^{51}Cr release assay. Specific lysis at 30:1 ratio. *B* to *D,* cold target cell inhibition of peptide-specific CD8⁺ CTL cytotoxicity. The cytotoxic activities of p277, p342, and p351 peptide-specific CD8⁺ CTL lines against the mixture of ^{51}Cr-labeled tumor cells (hot) and unlabeled T2-B7 cells (cold) were determined by standard ^{51}Cr release assay at an effector to ^{51}Cr-labeled target ratio of 20:1. *B,* cytolytic activity of p351-specific CD8⁺ CTL against ^{51}Cr-labeled T1-B7, LB34, and U293T cell lines mixed with various numbers of nonpulsed T2-B7 (△) or p351-pulsed T2-B7 cells (▲). *C* and *D,* cytotoxicity of p277- and p342-specific CD8⁺ CTLs against ^{51}Cr-labeled T1-B7 cells mixed with nonpulsed T2-B7 cells (△) or p277- and p342-pulsed T2-B7 cells (▲). (▲). Percentage of specific lysis at various cold-hot ratio. The melanoma (LB34) and renal cancer cells (U293T) were incubated previously with IFN-γ before use as describe above.

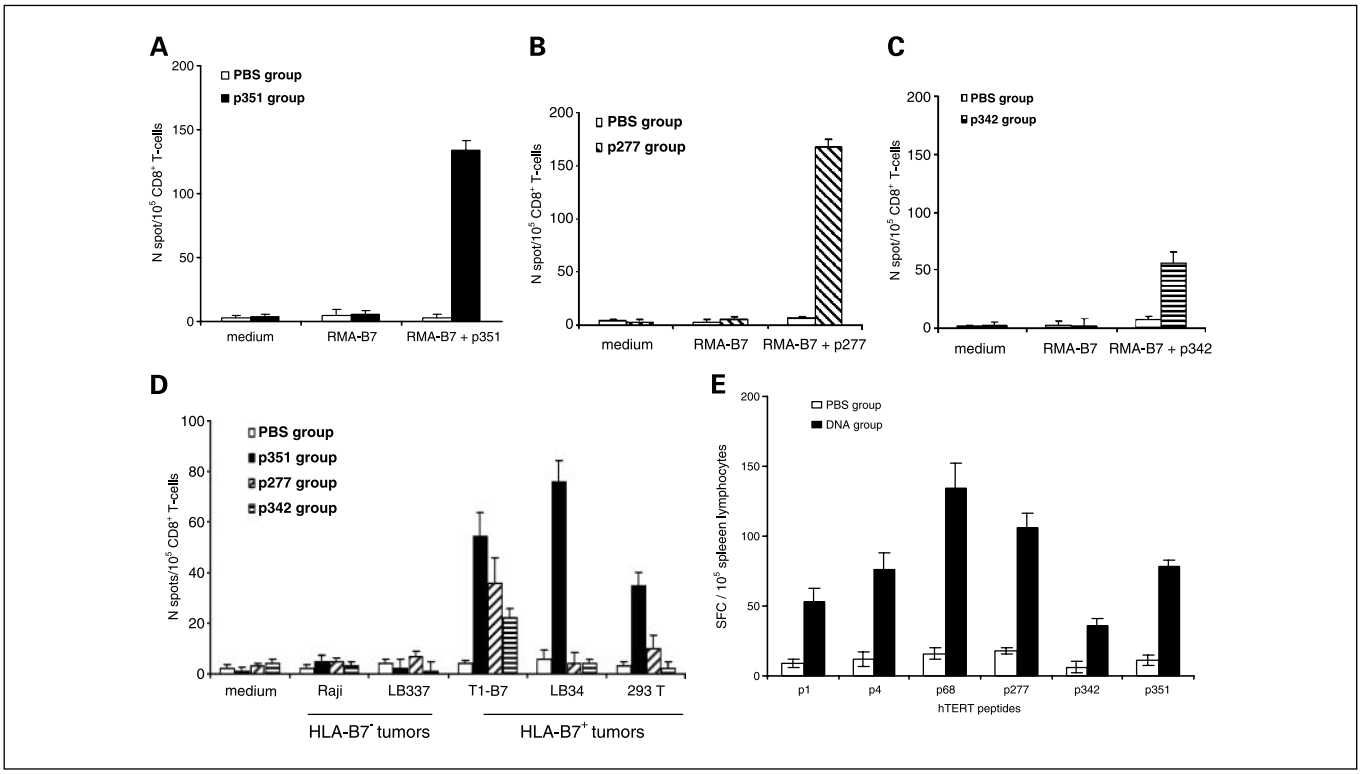

Fig. 4. Immunogenicity of hTERT peptides in HLA-B*0702 transgenic mice. *A* to *C,* frequencies of peptide-specific IFN-γ-secreting $CD8^+$ T cells after immunization with naturally processed hTERT peptides (p277, p342, and p351). HLA-B*0702 transgenic mice (three mice per group) were immunized at J0 and J14 with 100 μg of each peptide together with T13L helper peptide or injected with PBS (100 μL) in incomplete Freund's adjuvant. Ten days after the last immunization, splenocytes from immunized mice were pooled and $CD8^+$ T lymphocytes were isolated by negative selection and next tested for peptide-induced IFN-γ by ELISPOT assay. The $CD8^+$ T cells were cultured in the presence of RMA-B7 cells or corresponding peptide-loaded RMA-B7 cells (5 μg/mL) for 18 to 20 hours. *D,* recognition of $hTERT^+$ human tumor cells by mouse peptide-specific $CD8^+$ T cells. The induced peptide-specific $CD8^+$ T cells were cultured with 2×10^4 irradiated $HLA\text{-}B7^+$ or $HLA\text{-}B7^-$ human tumor cells. Specific IFN-γ-producing cells were measured 24 hours later by ELISPOT assay. *A* to *D,* representative of two experiments. Columns, mean of spots from triplicate wells from one experiment; bars, SD. *E,* multi-hTERT epitopes CTL cell responses after DNA immunization. Three weeks after plasmid hTERT-DNA or PBS injection, Ficoll-purified lymphocytes were isolated from pooled splenocytes (three mice per group). Serial dilutions of spleen lymphocytes were cultured in the absence or presence of each indicated peptide (5 μg/mL) for 24 hours. The peptide-specific IFN-γ-producing cells were detected using ELISPOT assay. The number of specific IFN-γ spots was calculated after subtracting nonspecific values observed in the absence of peptide stimulation (<8 spots/10^5 $CD8^+$ T cells). Representative of three experiments. Columns, mean of spots from triplicate wells from one representative experiment; bars, SD.

Discussion

The search for immunogenic hTERT-derived peptides for each HLA type is a formidable task but a necessary step to develop immunotherapy that targets hTERT on tumor cells in the widest assortment of the human population. We and others previously identified immunogenic CTL epitopes restricted by common HLA types, such as HLA-A2.1 (13–15), HLA-A3 (22), HLA-A24 (23), and HLA-A1 (24). Here, we extend the identification of hTERT-derived immunogenic peptides to HLA-B*0702, which is the most prevalent (75%) allele within the HLA-B7 supertype that accounts for ~25% of the Caucasian population (27). The HLA-B7 supertype includes several other haplotypes: B*3501-03, B*51, B*5301, B*5401, B*0703-05, B*1508, B*5501-02, B*5601-02, B*6701, and B*7801 alleles. These HLA molecules share a peptide-binding specificity for P in position 2 and a hydrophobic aliphatic (A, L, I, M, or V) or aromatic (F, W, or Y) residue in the COOH-terminal position (39, 40). In this study, we reported six new hTERT-derived epitopes that elicit HLA-B*0702-restricted CTL responses. The binding affinity of these peptides to HLA-B*0702 molecule seemed to be high and correlated with their highest score predicted by the peptide-motif prediction algorithm used (32).

A potential disadvantage in targeting hTERT-derived antigens for immunotherapy is that they may be subject to self-tolerance. Nevertheless, we were able to generate *in vitro* hTERT peptide-specific CTL responses in $HLA\text{-}B7^+$ normal individuals, proving that the T repertoire for hTERT was available in normal individuals as reported previously (13, 14). By establishing $CD8^+$ CTL lines specific for these peptides, we showed that three of them (p277, p342, and p351) can recognize naturally processed and expressed epitopes on various tumor cells. The tumor recognition analysis showed that the lymphoid tumor cell lines tested (leukemia and lymphoma) naturally expressed three of the six candidate epitopes (p277, p342, and p351), whereas the nonlymphoid tumor cell lines (melanoma and renal cancer cells) express only peptide p351. These results were reproducibly in multiple healthy donors. Heterogeneity expression of hTERT protein on tumor cells could not explain the difference observed in tumor recognition, because all the tumor cell lines used highly expressed this antigen. In addition, the cytotoxic activity exerted by the peptide-specific CTL lines was equally inhibited by corresponding peptide loaded on cold target cells. However, the fact that lymphoid tumor cell lines expressed more hTERT epitopes compared with nonlymphoid ones could find partial explanation on the processing

mechanisms used by these cell lines. It has been reported that different processing by proteasome and immunoproteasome could modulate epitope generation in cancer cells (41, 42). Several antigenic peptides are not processed with the same efficiency by these two proteasome types (43, 44). Most nonlymphoid cells (normal or tumoral) constitutively express standard proteasomes and switch to immunoproteasomes when exposed to IFN-γ (45). Thus, we speculate that only constitutive proteasome type could be used in nonlymphoid tumor cells tested, whereas tumor cell lines from lymphoid lineage could preferentially used immunoproteasome to trimmed hTERT. Recent data have also shown that IFN-γ treatment can modulate hTERT epitope expression and presentation in lung cancer cells (46). In this study, the nonlymphoid tumor cell lines used for cytotoxicity assay were previously treated with IFN-γ to increase MHC class I expression. However, in the experiments using ELISPOT assay, IFN-γ untreated nonlymphoid cells were also able to stimulate peptide-specific CTL although at the lower level. This could be due to the low levels of HLA-B7 molecules on these cells observed in absence of IFN-γ pretreatment (data not shown). Further experiments should be carried out to determine the expression profile of these epitopes on many other tumor cells, including primary tumor cells from cancer patients.

Although hTERT-specific CTL lines used for tumor recognition were generated from healthy donors, these data could be extended to cancer patients, as we and other showed previously that hTERT-specific CTLs from both cancer patients and healthy individuals equivalently killed tumor cell lines (14, 47).

To assess for the therapeutic potential of these hTERT epitopes, we used the humanized H-2D/$Kb^{-/-}$, HLA-B*0702 transgenic mice (28, 31). All the six peptides were able to induce specific CTL responses *in vivo*, indicating that the HLA-B*0702 transgenic mice possessed the adequate T-cell repertoire allowing recognition of the hTERT-derived epitopes. We found an overall correlation of CTL responses with peptide affinity for HLA-B*0702 molecule supporting previous works that showed direct correlation between avidity for the MHC molecule and immunogenicity (38, 48). Interestingly, mouse peptide-specific $CD8^+$ T cells can recognize endogenous peptides on human tumor cells, indicating that T cells educated in these mice are able to recognize peptide bound to the native HLA-B*0702 molecule. By using a plasmidic DNA coding hTERT immunization of transgenic mice, we showed that all the candidate peptides are processed *in vivo*.

Although telomerase expression is mainly restricted to tumor cells, rare normal cells, including germ cells and hematopoietic progenitor and activated lymphocytes, are known to express telomerase (7, 8). Consequently, any hTERT-based vaccine therapy may result in autoimmunity and destruction of normal cells that express this antigen. In the present study, the hTERT-specific $CD8^+$ CTL lines generated from healthy donors were not reactive against autologous CD40-activated B cells without peptide pulsing. These findings may reflect the relatively low levels of hTERT found in these cells after a short *in vitro* stimulation (48 hours) with CD40L and support previous data that showed that hTERT-specific CTLs failed to lyse CD40-activated B lymphocytes (14). Nevertheless, it has been shown that CD40-activated B cells could be susceptible to hTERT-specific lysis in HLA-A2 and HLA-A3 antigen system. However, in these studies, the B cells used become $hTERT^+$ after several weeks of *in vitro* stimulation with CD40L (12, 22). Further experiments with other hTERT-expressing normal cells are needed to fully evaluate autoreactivity of hTERT-specific T cells in HLA-B*0702 antigen system. Next, we also address whether autoimmunity could be observed in vaccinated HLA-B*0702 transgenic mice. No deleterious effects were observed in the peptide- or DNA-immunized mice even several weeks after vaccination (data not shown). Autoimmunity development seems to be unlikely in peptide immunization, because the comparison of telomerase amino acid sequences between human and mouse revealed that, for all the candidate peptides, there was at least one amino acid difference with mouse sequences. However, it cannot be excluded that many other epitopes from hTERT could cross-react with mouse telomerase-derived peptides, including MHC class II–restricted epitopes, especially when mice are immunized with plasmidic DNA encoding the full-length hTERT. Therefore, the fact that autoimmunity does not develop after hTERT-DNA immunization may support the observations of clinical studies in cancer patients using hTERT-based vaccines that did not mention autoimmunity (19, 20, 49).

In conclusion, we presented the successful identification of several immunogenic hTERT peptides restricted to HLA-B*0702. We show that this identification requires a multistep approach involved *in vitro* and *in vivo* steps using both mice and human PBMC. To date, such systematic approach has enabled the identification of hTERT-derived epitopes, such as HLA-A2 (14, 15), HLA-DR (26), and, now, HLA-B*0702 peptides, with immunogenic characteristics that could justify their use in immunotherapy of cancer patients.

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Olivier Adotévi, Karine Mollier, Christine Neuveut, et al.

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