HER-2/*neu* and hTERT Cryptic Epitopes as Novel Targets for Broad Spectrum Tumor Immunotherapy[1]

Antonio Scardino,[2]* David-Alexandre Gross,[2]* Pedro Alves,* Joachim L. Schultze,† Stéphanie Graff-Dubois,* Olivier Faure,* Sophie Tourdot,* Salem Chouaib,* Lee M. Nadler,† François A. Lemonnier,‡ Robert H. Vonderheide,[3]† Angelo A. Cardoso,[3]† and Kostas Kosmatopoulos[4]*

Tolerance to tumor-nonmutated self proteins represents a major obstacle for successful cancer immunotherapy. Since this tolerance primarily concerns dominant epitopes, we hypothesized that targeting cryptic epitopes that have a low affinity for HLA could be an efficient strategy to breach the tolerance to tumor Ags. Using the P1Y heteroclitic peptide approach, we identified low affinity cryptic HLA-A*0201-restricted epitopes derived from two widely expressed tumor Ags, HER-2/*neu* and hTERT. The P1Y variants of four HER-2/*neu* (neu_{391}, neu_{402}, neu_{466}, neu_{650})- and two hTERT ($hTERT_{572}$ and $hTERT_{988}$)-derived low affinity peptides exhibited strong affinity for HLA-A*0201 and stimulated specific CTL from healthy donor PBMCs. These CTL specifically recognized HER-2/*neu*- and hTERT-expressing tumor cells of various histological origins. In vivo studies showed that HLA-A*0201 transgenic HHD mice vaccinated with the P1Y variant peptides generated CTL that specifically lysed Ag-expressing tumor cells, thus recognizing the cognate endogenous Ags. These results suggest that heteroclitic variants of low affinity, cryptic epitopes of widely expressed tumor Ags may serve as valid tools for tumor immunotherapy. ***The Journal of Immunology*****, 2002, 168: 5900–5906.**

Tumor-specific CTL recognize tumor Ag (TuAg)[5]-derived peptides complexed with MHC class I molecules. The large majority of the known human TuAgs are nonmutated self proteins that either are overexpressed by tumors or are developmentally regulated (1, 2). HER-2/*neu* and hTERT are two Ags that are particularly appealing for a broad spectrum immunotherapy because of their overexpression in a variety of tumors of different origins (3, 4). HER-2/*neu* is a 185-kDa transmembrane glycoprotein with tyrosine kinase activity and homology to the epidermal growth factor receptor (5). The HER-2/*neu* gene is present in normal cells as a single copy. In contrast, the HER-2/*neu* gene is amplified and the associated protein is overexpressed in breast, ovary, uterus, lung, renal, stomach, and pancreas cancers (6–8). TERT is a reverse transcriptase that maintains telomere length in rapidly dividing cells, a crucial mechanism for the unrestricted survival of cancer cells (9). Recently, we and others identified the hTERT as a widely expressed TuAg found in greater than 85% of all human cancers (10, 11). To date, multiple high affinity, dominant epitopes derived from these two Ags have been identified and used as targets of specific CTL responses (10–19).

However, like the majority of TuAgs, HER-2/*neu* and hTERT are also expressed on normal tissues, including the thymus (20). This raises the problem of the tolerance of their specific CTL repertoire, and consequently their inability to trigger a strong and efficient antitumor response, as it has been demonstrated for other TuAgs (21–25). Breaking tolerance to TuAgs is, indeed, one of the major goals of tumor vaccination (26). Tolerance to self Ags involves dominant epitopes and much lesser cryptic epitopes (26–28). Hence, we can hypothesize that the recruitment of the CTL repertoire specific for cryptic determinants should circumvent immunologic tolerance. Results obtained in the murine p53 and more recently in the transgenic adenocarcinoma mouse prostate mouse model fit in favor of this hypothesis (21, 24, 25). Cryptic tumor epitopes might, therefore, be considered good candidates for tumor immunotherapy provided that they are efficiently presented by tumor cells.

In this study, we used the heteroclitic peptide approach (29) to identify six low affinity cryptic HER-2/*neu* and hTERT epitopes presented by HLA-A*0201. These epitopes, in their P1Y heteroclitic form, stimulated CTL that specifically lysed HER-2/*neu*- or hTERT-expressing tumor cells of various histological origins.

Materials and Methods

Animals

HHD mice have been previously described (30). They are β_2-microglobulin $(\beta_2m)^{-/-}$, $D^{b-/-}$ and express an HLA-A*0201 monochain composed of a chimeric heavy chain (α1 and α2 domains of HLA-A*0201 and the α3 and intracellular domains of D^b) linked by its N terminus to the C terminus of the human β_2m by a 15-aa peptidic arm.

*Institut National de la Santé et de la Recherche Médicale Unité 487, Institut Gustave Roussy, Villejuif, France; †Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115; and ‡Unité d'Immunité Cellulaire Antivirale, Departement Sida Retrovirus, Institut Pasteur, Paris, France

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[2] A.S. and D.-A.G. equally contributed to this work.

[3] R.H.V. and A.A.C. equally contributed to this work.

[4] Address correspondence and reprint requests to Dr. Kostas Kosmatopoulos, Institut National de la Santé et de la Recherche Médicale Unité 487, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94800, Villejuif, France. E-mail address: kostas@igr.fr

[5] Abbreviations used in this paper: TuAg, tumor Ag; β_2m, β_2-microglobulin; DC, dissociation complex; MAGE, melanoma Ag; RA, relative affinity; wt, wild type; hTERT, human telomerase reverse transcriptase; mTERT, murine telomerase reverse transcriptase.

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Cell lines

Murine RMA/HHD and RMAS/HHD cells were obtained by transfection of RMA and RMAS cells with the HHD construct (30). Human tumor cells used as targets for HER-2/*neu*-specific CTL were the HLA-A*0201$^+$ HER-2/*neu*$^+$ MCF-7 (breast cancer), HCT-116 (colon cancer), PUB/N (lung cancer) kindly provided by M. Chouaib (Institut Gustave Roussy, Villejuif, France), LAW (renal cell carcinoma) kindly provided by F. Triebel (Institut Gustave Roussy), and the HLA-A*0201$^+$ HER-2/*neu*$^-$ ZR75.1 (breast cancer) and SUP/M2 (anaplastic lymphoma) kindly provided by C. Gambacorti (Instituto di Tumori, Milan, Italy). The level of HER-2/*neu* expression by these lines has been reported previously (18). Human tumor cells used as targets for hTERT-specific CTL were the HLA-A*0201$^+$ hTERT$^+$ U266 (myeloma), 36 M (ovarian carcinoma), KO29 (melanoma) and SKW6.4 (EBV-transformed lymphoblasts), and the HLA-A*0201$^+$ hTERT$^-$ U20S (osteosarcoma) and the HLA-A*0201$^-$ hTERT$^+$ HSS (myeloma) and Calu-1 (lung cancer). The hTERT expression by these lines has been described previously (10). Unless indicated otherwise, the human cell lines were obtained from American Type Culture Collection (Manassas, VA) or as previously reported (10). The TAP1/2 mutant T2 cells and the NK-sensitive K562 cells were also used. All cells were grown in RPMI 1640 medium supplemented with 10% FCS.

Peptides

Peptides were synthesized by Synt:em S.A. (Nimes, France).

Viruses

T39 (vac-*neu*) HER-2/*neu*-expressing recombinant and TBC-Wy wild-type (vac-wt) vaccinia viruses were kindly provided by G. Mazzara (Therion Biologics, Cambridge, MA).

Generation of CTL in HHD mice

HHD mice were injected s.c. at the base of the tail with 100 μg peptide emulsified in IFA in the presence of 140 μg of the I-A^b-restricted hepatitis B virus core-derived Th epitope (128–140; sequence TPPAYRPPNAPIL). After 11 days, spleen cells (5×10^7 cells in 10 ml) were stimulated in vitro with 10 μM peptide. On day 6 of culture, the bulk responder populations were tested for specific cytotoxicity.

Generation of CTL from human PBMC

Dendritic cells were prepared by cultivating plastic adherent PBMC in the presence of 700 U/ml GM-CSF (R&D Systems, Minneapolis, MN) and 100 U/ml IL-4 (R&D Systems) for 7 days. Immunofluorescence staining of this dendritic cell-enriched population showed that >80% were CD80/B7-1$^+$, CD86/B7-2$^+$, and HLA-DR$^+$ cells. Activated B cells were prepared by culture of Percoll-isolated B cells over a monolayer of CD40 ligand-expressing fibroblasts in B cell medium supplemented with 2.8 μg/ml cyclosporin A (31). After 14 days, greater than 85% of the cells were CD19$^+$, CD80$^+$, CD86$^+$, HLA-DR$^+$.

Dendritic cells were harvested after 7 days, pulsed overnight with peptide (10 μM) in the presence of soluble CD40 ligand to induce their maturation, irradiated at 36 Gy, and added to autologous T cells at a T:dendritic cell ratio of 20:1 in culture medium (RPMI 1640 supplemented with 10% human AB serum, 10 nM L-glutamine, and gentamicin). Three days later, primed T cells were harvested, isolated by Ficoll centrifugation, and replated in culture medium supplemented with 20 IU/ml IL-2. On day 7, and weekly thereafter, T cells were restimulated with peptide-pulsed irradiated dendritic cells (first restimulation) or activated B cells (second, third, and fourth restimulations). Cytotoxicity was tested 5–7 days after the fourth restimulation.

Cytotoxic assay

Murine RMA, RMAS/HHD, and RMA/HHD cells, and human tumor cells were used as targets. For virus infection, RMA and RMA/HHD cells were incubated with vac-wt or vac-*neu* recombinant viruses (10 PFU/cell) for 16 h. Targets were labeled with 100 μCi ^{51}Cr for 90 min, washed four times, and plated in 96-well round-bottom plates (10^4 cells/well in 100 μl RPMI 1640 + 3% FCS). RMAS/HHD and T2 cells were pulsed with 1 μM peptides at 37°C for 90 min. Variable numbers of effector cells (in 100 μl) were then added and incubated at 37°C for 4 h. After incubation, 100 μl supernatant was collected, and radioactivity was measured in a gamma counter. Percentage of specific lysis was determined as: percentage of specific lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100. Spontaneous release was always <20% of maximal release induced by 3 N HCl.

*Measurement of peptide relative affinity to HLA-A*0201*

T2 cells (3×10^5 cells/ml) were incubated with various concentrations of peptides ranging from 100 to 0.1 μM in serum-free RPMI 1640 medium supplemented with 100 ng/ml human β_2m at 37°C for 16 h. Cells were then washed twice and stained with the BB7.2 mAb, followed by FITC-conjugated goat anti-mouse Ig mAb to quantify the expression of HLA-A*0201. For each peptide concentration, the HLA-A*0201-specific staining was calculated as the percentage of the staining obtained with 100 μM of the reference peptide HIVpol$_{589}$ (IVGAETFYV). The relative affinity (RA) is determined as: RA = concentration of each peptide that induces 20% of HLA-A*0201-expression/concentration of the reference peptide that induces 20% of HLA-A*0201 expression. The lower the RA value, the stronger is the peptide binding to HLA-A*0201. The definitive RA value for each peptide was determined from at least three independent experiments. In all experiments, 20% of HLA-A*0201 expression using the reference peptide was obtained at 1–3 μM.

*Assessment of peptide/HLA-A*0201 complex stability*

T2 cells (10^6/ml) were incubated overnight with 100 μM of each peptide in serum-free RPMI 1640 medium supplemented with 100 ng/ml β_2m at 37°C. Cells were then washed four times to remove free peptides, incubated with brefeldin A (10 μg/ml) for 1 h to block cell surface expression of newly synthesized HLA-A*0201 molecules, washed, and incubated at 37°C for 0, 2, 4, 6, or 8 h. Subsequently, cells were stained with the BB7.2 mAb, followed by FITC-conjugated goat anti-mouse Ig mAb. For each time point, peptide-induced HLA-A*0201 expression was calculated as: mean fluorescence of peptide-preincubated T2 cells − mean fluorescence of T2 cells treated in similar conditions in the absence of peptide. DC_{50} (dissociation complex, DC) was defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at $t = 0$. The definitive DC_{50} value for each peptide was determined from at least three independent experiments.

Results

*Low affinity HER-2/*neu *and hTERT peptides*

Four previously described HER-2/*neu* peptides with low affinity for HLA-A*0201 (*neu*$_{391}$, *neu*$_{402}$, *neu*$_{466}$, and *neu*$_{650}$) (18, 29) were used in this study. Two hTERT peptides predicted by the Bioinformatics and Molecular Analysis Section algorithm (32) to have low HLA-binding affinity were also included. Their capacity to bind and stabilize HLA-A*0201 molecules was assessed using the T2-binding assay. As shown in Table I, all these peptides form unstable complexes with HLA*0201 ($DC_{50} < 2$ h). Analysis of the relative binding affinity showed that *neu*$_{391}$, *neu*$_{402}$, hTERT$_{572}$, and hTERT$_{988}$ were weak binders to HLA-A*0201 (RA > 5),

Table I. *HLA A2.1 affinity of HER-2/*neu *and hTERT peptides*

Peptides	Sequence	RA[a]	DC_{50} (h)[b]
Native			
neu$_{369}$[c]	KIFGSLAFL	2.3	>6
neu$_{402}$[c]	TLEEITGYL	19.0	<2
neu$_{391}$[c]	PLQPEQLQV	>70	2
neu$_{650}$[c]	PLTSIISAV	1.4	2
neu$_{466}$[c]	ALIHHNTHL	4.8	2
hTERT$_{540}$	ILAKFLHWL	0.5	6
hTERT$_{572}$	RLFFYRKSV	25.3	<2
hTERT$_{988}$	DLQVNSLQTV	28.6	<2
Heteroclitic			
neu$_{402Y}$[c]	YLEEITGYL	3.6	4
neu$_{391Y}$[c]	YLQPEQLQV	1.3	>6
neu$_{650Y}$[c]	YLTSIISAV	0.2	4–6
neu$_{466Y}$[c]	YLIHHNTHL	1.4	>6
hTERT$_{572Y}$	YLFFYRKSV	2.2	5
hTERT$_{988Y}$	YLQVNSLQTV	2.1	>6

[a] The RA is determined as described in *Materials and Methods*.

[b] DC_{50} was defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at $t = 0$.

[c] Results of RA and DC_{50} have been published previously (see Refs. 18 and 29).

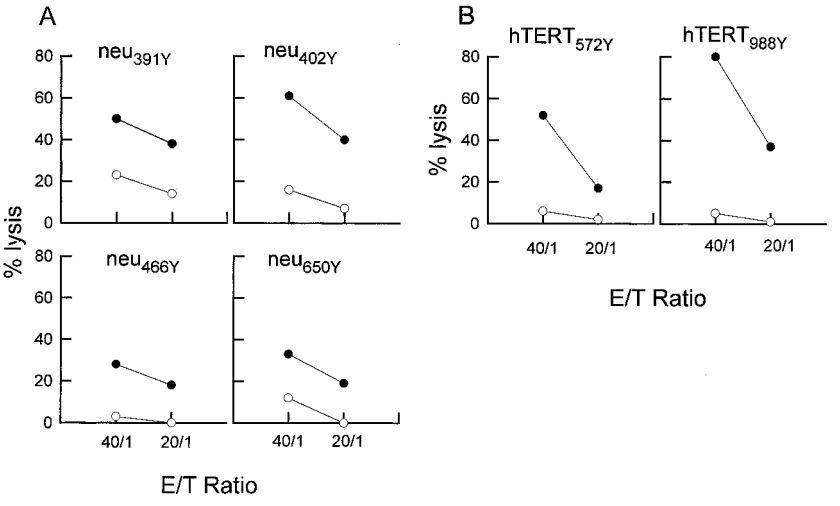

FIGURE 1. Generation of peptide-specific CTL from healthy donor PBMC. CTL were generated by in vitro stimulation of $CD8^+$ PBMC from healthy donors with P1Y variants of HER-2/*neu* (*A*) and hTERT (*B*) low affinity peptides, as described in *Materials and Methods*. CTL were tested for cytotoxicity against T2 cells loaded with an irrelevant ($HIVgag_{76}$, *A*; $MAGE3_{271}$, *B*) (○), the native cognate (●), and the P1Y variant (■) peptides.

whereas the peptides neu_{466} and neu_{650} exhibited a strong binding affinity (RA < 5).

Generation of CTL against low affinity peptides

The generation of specific CTL is a prerequisite for the identification of CTL epitopes. However, low affinity peptides are generally nonimmunogenic in either humans or HLA-A*0201 transgenic mouse models (33).

To generate CTL against low affinity peptides, we used the P1Y heteroclitic peptide approach we described previously (29). It consists of substituting the amino acid at position 1 with a tyrosine. Indeed, P1Y variants of neu_{391}, neu_{402}, neu_{466}, neu_{650}, $hTERT_{572}$, and $hTERT_{988}$ exhibited a high affinity (RA < 5 and $DC_{50} \geq 4$ h), fulfilling the criteria of peptides predicted to be immunogenic (Table I). We then used the heteroclitic P1Y variants of the low affinity HER-2/*neu* and hTERT peptides to induce CTL from the PBMC of healthy donors. Four to five donors were tested for each peptide. The generation of CTL was evaluated on cognate peptide-pulsed T2 cell targets after the fourth in vitro restimulation. Peptide-specific CTL were generated from PBMC stimulated with all these heteroclitic peptides. CTL killed T2 cells pulsed with the cognate peptide, but not T2 cells pulsed with an irrelevant peptide ($HIVgag_{76}$ or $MAGE3_{271}$) (Fig. 1).

*Low affinity HER-2/*neu *and hTERT peptides are naturally processed by tumor cells expressing the relevant TuAg*

To evaluate whether these low affinity HER-2/*neu* and hTERT peptides are naturally processed and presented by tumor cells, peptide-specific CTL were tested for their ability to lyse HER-2/*neu*- or hTERT-expressing HLA-A*0201$^+$ tumor cells. For the HER-2/*neu*-derived peptides, CTL generated were tested as effector cells in cytotoxic assays using MCF-7 (HLA-A*0201$^+$, HER-2/*neu*$^+$), ZR75.1 (HLA-A*0201$^+$, HER-2/*neu*$^-$), and K562 (NK-sensitive) cells as targets. As shown in Fig. 2*A* for a representative case, CTL specific for neu_{391Y}, neu_{402Y}, neu_{466Y}, and neu_{650Y} lysed the MCF-7 cells, but not the ZR75.1 nor the K562 cells. Similarly, for the hTERT-derived peptides, CTL generated by stimulation with $hTERT_{572Y}$ and $hTERT_{988Y}$ lysed the SKW6.4 cells (HLA-A*0201$^+$, hTERT$^+$), but failed to lyse the U20S (HLA-A*0201$^+$ hTERT$^-$) and the K562 cells (Fig. 2*B*). We further demonstrated that the MCF-7 lysis by HER-2/*neu*-reactive CTL involves the specific recognition of the HLA-A*0201/*neu* peptide complexes in cold target inhibition experiments. MCF-7 killing was inhibited by T2 cells loaded with the corresponding native HER-2/*neu* peptide, but not by T2 cells loaded with the irrelevant $HIVgag_{76}$ peptide (Fig. 3). Inhibition was dependent on the cold-hot target ratio.

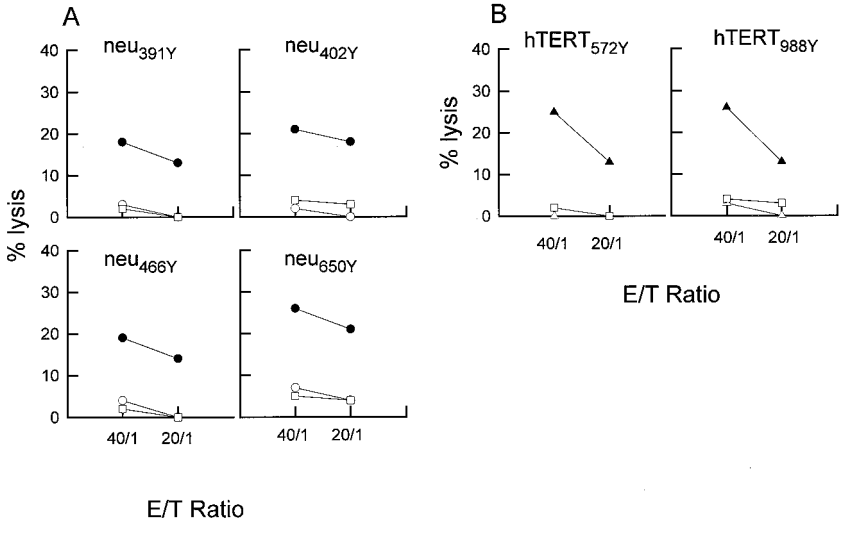

FIGURE 2. Recognition of HER-2/*neu*- and hTERT-expressing tumor cells by human CTL. CTL were generated by in vitro stimulation of $CD8^+$ PBMC from healthy donors with P1Y variants of HER-2/*neu* (*A*) and hTERT (*B*) low affinity peptides and tested against: *A*, ZR7.5 (HLA-A*0201$^+$ HER-2/*neu*$^-$) (○), MCF-7 (HLA-A*0201$^+$ HER-2/*neu*$^+$) (●), and K562 (□); and *B*, U20S (HLA-A*0201$^+$ hTERT$^-$) (△), SKW6.4 (HLA-A*0201$^+$ hTERT$^+$) (▲), and K562 (□) targets.

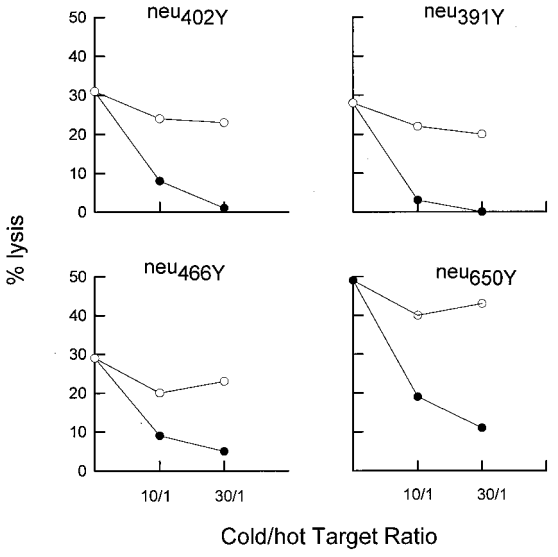

FIGURE 3. Tumor cell killing by HER-2/*neu*-specific CTL involves specific HLA-A*0201/*neu* peptide recognition. CTL were generated by in vitro stimulation of $CD8^+$ PBMC with P1Y heteroclitic low affinity HER-2/*neu* peptides. They were tested for cytotoxicity against MCF-7 cells in the presence of cold T2 cells loaded with the corresponding native HER-2/*neu* peptide (●) or with the irrelevant $HIVgag_{76}$ peptide (○). Effector-hot target ratio: 40:1.

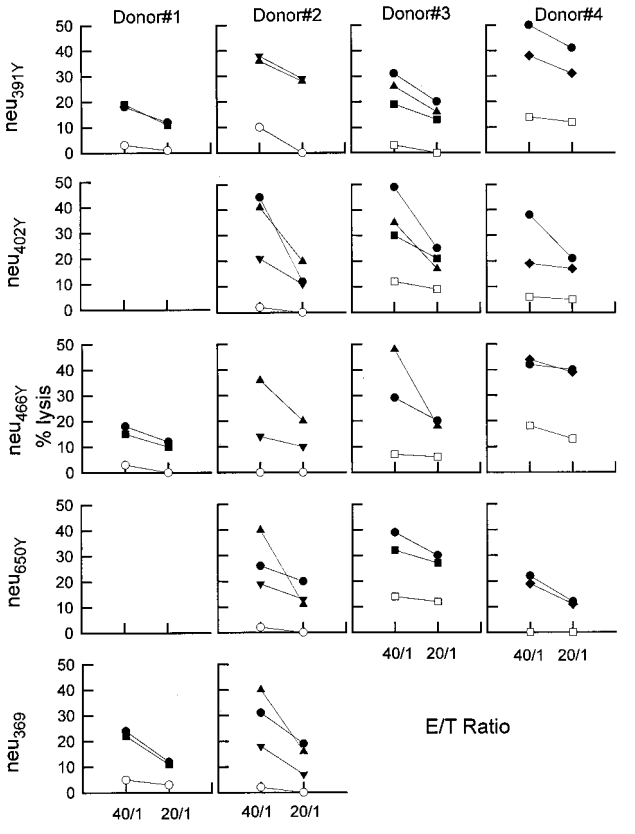

FIGURE 4. Recognition by HER-2/*neu* peptide-specific CTL of tumor cells of various origins. CTL were generated by in vitro stimulation of $CD8^+$ PBMC from four healthy donors with P1Y variants of HER-2/*neu* peptides and tested for cytotoxicity against the HLA-A*0201$^+$ HER-2/*neu*$^-$ ZR75.1 (○), SUP/M2 (□), and the HLA-A*0201$^+$ HER-2/*neu*$^+$ MCF-7 (●), HCT-116 (■), LAW (▲), and PUB/N (▼) targets.

These studies demonstrate that the low affinity, cryptic epitopes neu_{391}, neu_{402}, neu_{466}, neu_{650}, $hTERT_{572}$, and $hTERT_{988}$ are naturally processed and presented by human tumor cells expressing the relevant TuAg.

*Low affinity HER-2/*neu *and hTERT epitopes are presented by tumor cells of various histological origins*

HER-2/*neu* and hTERT are appealing TuAgs because of their wide expression on multiple cancers. To determine the potential of low affinity, cryptic HER-2/*neu* and hTERT epitopes for broad spectrum immunotherapy, we tested the capacity of CTL induced by the PY1 variants of these epitopes to lyse malignant cells derived from tumors of different histological origins.

For the HER-2/*neu* epitopes, CTL specific for the four cryptic epitopes were used as effectors, as well as CTL generated using the dominant peptide neu_{369} (12). The CTL were tested for their capacity to lyse the HLA-A*0201$^+$ HER-2/*neu*$^+$ MCF-7, HCT116, LAW, and PUB/N cells. These cell lines express variable amounts of HER-2/*neu* (18). The HLA-A*0201$^+$ HER-2/*neu*$^-$ cell lines ZR75.1 and SUP/M2 were used as controls. As shown in Fig. 4, CTL generated using the PY1 variants of the cryptic epitopes lysed all HER-2/*neu*$^+$ tumor cells (black symbols) irrespective of their tissue of origin, including those with low expression of HER-2/*neu* (LAW) (18). Importantly, these CTL showed a cytotoxic efficacy equivalent to that mediated by neu_{369}-specific CTL (Fig. 4, donors 1 and 2), thus demonstrating that presentation of low affinity epitopes does not require high expression of the endogenous Ag.

A similar analysis was performed for the hTERT-derived epitopes (Fig. 5). Four HLA-A*0201$^+$ hTERT$^+$ tumor cells (U266, 36 M, KO29, and SKW6.4) were used as targets for CTL specific for the two hTERT cryptic epitopes or the dominant peptide $hTERT_{540}$ (10, 11). The HLA-A*0201$^+$ hTERT$^-$ cell line U20S and two HLA-A*0201$^-$ hTERT$^+$ cells (HSS and Calu-1) were used as negative controls. CTL lysed all the HLA-A*0201$^+$ hTERT$^+$ cells (filled symbols) irrespective of their histological origin. In contrast, they did not lyse targets that exclusively express hTERT or HLA-A*0201 (open symbols). CTL specific for the cryptic epitopes and those specific for the dominant epitope $hTERT_{540}$ recognized hTERT$^+$ tumor cells with equivalent efficacy (Fig. 5, donors 1 and 2).

These studies indicate that low affinity, cryptic HER-2/*neu* and hTERT epitopes are presented by tumor cells of different types and may serve as targets for broad spectrum antitumor immunotherapeutic strategies.

*P1Y heteroclitic low affinity HER-2/*neu *and hTERT peptides induce in vivo tumor-specific CTL*

The capacity of the cryptic HER-2/*neu* and hTERT epitopes to induce in vivo tumor-specific CTL was assessed using the HLA-A*0201-transgenic HHD mouse model. Mice were vaccinated with the native peptides and their P1Y heteroclitic variants. After 11 days, their spleens were removed and the splenic cells were restimulated in vitro with the corresponding native peptide. Results in Fig. 6 show that native peptide-vaccinated mice did not develop CTL, confirming previous results showing that low affinity peptides are not immunogenic in HHD mice (29). In contrast, CTL were generated in mice vaccinated with HER-2/*neu* (Fig. 6*A*) and hTERT (Fig. 6*B*) heteroclitic peptides. These CTL lysed RMAS/HHD targets pulsed with the cognate and the P1Y heteroclitic, but not an irrelevant peptide.

CTL specific for HER-2/*neu* peptides recognized endogenous HER-2/*neu* since they lysed RMA/HHD cells infected with HER-2/*neu* recombinant (vac-*neu*), but not with vac-wt vaccinia virus. They did not kill RMA cells infected with the vac-*neu* virus either (Fig. 7*A*), thus demonstrating the HLA-A*0201 restriction of the

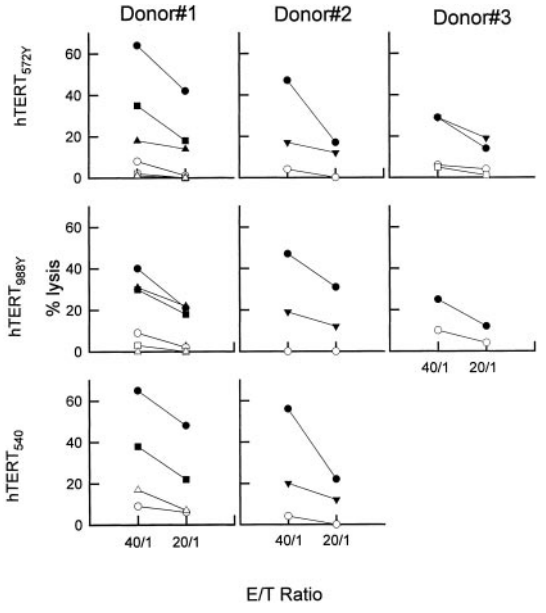

FIGURE 5. Recognition by hTERT peptide-specific CTL of tumor cells of various origins. CTL were generated by in vitro stimulation of $CD8^+$ PBMC from three healthy donors with P1Y variants of hTERT peptides and tested for cytotoxicity against the HLA-A*0201$^+$ hTERT$^-$ U20S (□), the HLA-A*0201$^-$ hTERT$^+$ HSS (○), Calu-1 (△), and the HLA-A*0201$^+$ hTERT$^+$ U266 (●), 36 M (■), SKM6.4 (▲), and KO29 (▼) targets.

endogenous HER-2/*neu* recognition. To evaluate the endogenous TERT recognition by TERT-specific CTL, we used the HLA-A*0201$^+$ mTERT$^+$ RMA/HHD cells as targets, since the $hTERT_{572}$ and $hTERT_{988}$ epitopes are identical in mouse and human TERT sequences. CTL induced in mice vaccinated with $hTERT_{572Y}$ and $hTERT_{988Y}$ lysed RMA/HHD cells, but failed to lyse TERT$^+$ HLA-A*0201-negative RMA cells (Fig. 7*B*).

These studies show that the P1Y heteroclitic variants of low affinity HER-2/*neu* and TERT epitopes are immunogenic, and induce, in vivo, CTL capable of specifically lysing tumor cells expressing the relevant TuAg.

Discussion

This work aimed at determining whether cryptic epitopes derived from widely expressed TuAgs can serve as targets of antitumor CTL and whether they have potential for use in tumor immunotherapy. We identified six low affinity HLA-A*0201-restricted epitopes of the ubiquitous HER-2/*neu* and hTERT TuAgs and showed that they are targets of antitumor CTL. These epitopes were presented by various tumor cells irrespective of their histological origin and the level of Ag expression. Moreover, we showed that these epitopes used as heteroclitic P1Y variants induce a tumor-specific CTL response in vivo in HLA-A*0201 transgenic HHD mice.

This observation, if extended to low affinity epitopes of other widely expressed TuAgs, is of great importance for a broad spectrum tumor immunotherapy. In fact, the majority of TuAgs, including HER-2/*neu* and hTERT, corresponds to nonmutated self proteins overexpressed in tumors, and the CTL repertoire against their high affinity immunodominant epitopes may be tolerized. Such tolerance mainly concerns high avidity CTL. This has been demonstrated in HLA-A*0201/K^b mice for HLA-A*0201-restricted p53-specific and tyrosinase-specific CTL (21–23), and more recently in HHD mice for the HLA-A*0201-restricted CTL specific for the mTERT (D.-A. Gross, unpublished observations). Low affinity cryptic epitopes are weakly or not at all involved in tolerance induction. For instance, B6 mice possess a fully functional CTL repertoire against low affinity D^b-restricted mgp100 and K^b-restricted gp75 epitopes (25, 34). Moreover, in the transgenic adenocarcinoma mouse prostate model, specific CTL can be generated against a subdominant/cryptic SV40T epitope, but not against a dominant SV40T epitope (24). Given that the efficacy of tumor immunotherapy depends on the avidity of recruited CTL (35, 36), these results point out the potential of using low affinity tumor epitopes, provided that they are able to mobilize their specific CTL repertoire and that they are presented by tumor cells efficiently enough to be recognized by CTL.

An effective strategy to mobilize CTL targeting low affinity epitopes is the use of heteroclitic variants that have a higher affinity and more stable binding to HLA molecules (25, 34, 37). We have

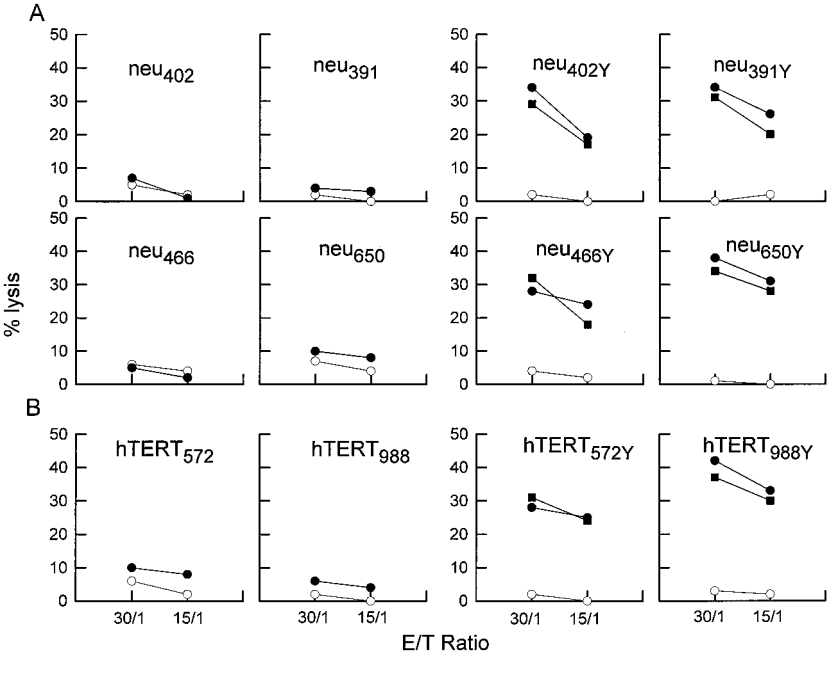

FIGURE 6. In vivo generation of peptide-specific CTL in HHD mice. HHD mice were vaccinated with the native and the P1Y heteroclitic low affinity HER-2/*neu* (*A*) and hTERT (*B*) peptides, and their spleen cells were in vitro stimulated with the cognate native peptides, as described in *Materials and Methods*. CTL were tested for cytotoxicity against RMAS/HHD targets coated with an irrelevant (HIV-gag_{76}) (○), the cognate native (●), and the P1Y heteroclitic (■) peptide.

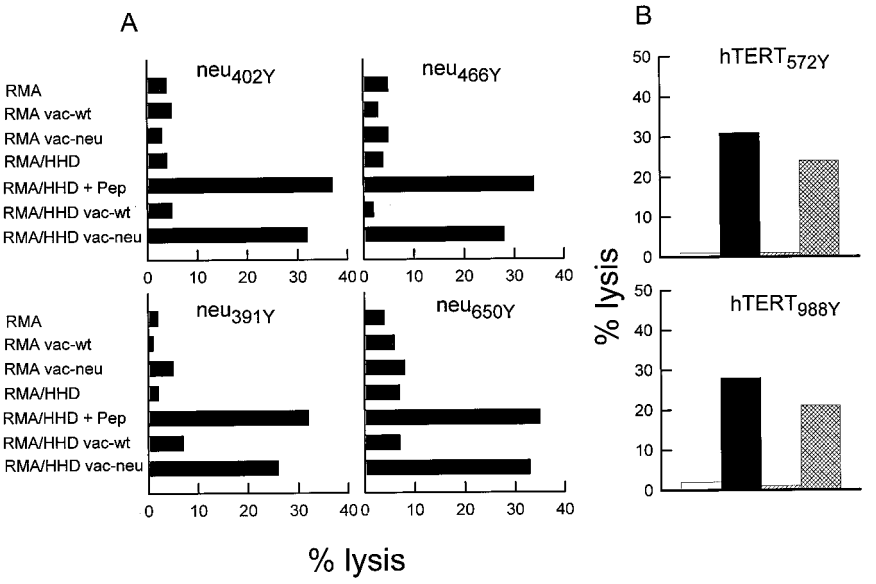

FIGURE 7. Tumor cell recognition by CTL induced in HHD mice. HHD mice were vaccinated with the P1Y heteroclitic low affinity HER-2/*neu* and hTERT peptides, and their spleen cells were in vitro stimulated with the cognate native peptides. *A*, HER-2/*neu*-specific CTL were tested for cytotoxicity against RMA and RMA/HHD targets infected with the vac-*neu* or the vac-wt viruses, as indicated, at a 30:1 E:T ratio. *B*, hTERT-specific CTL were tested against RMAS/HHD cells coated with the irrelevant HIVgag$_{76}$ (□) or with the native cognate peptide (■), RMA (▨), and RMA/HHD (▩) targets at a 30:1 E:T ratio.

previously described two heteroclitic variant approaches that enabled the generation of CTL directed to low affinity D^b-restricted and HLA-A*0201-restricted epitopes (29, 37). Heteroclitic variants of low affinity D^b epitopes from flu nucleoprotein induced a potent protective immunity against lethal challenge of mice with flu virus (37). In this study, we demonstrate in HHD mice that this strategy can be used to mobilize antitumor CTL specific for cryptic HER-2/*neu* and hTERT epitopes. We are presently evaluating the potential of P1Y heteroclitic peptides as tumor vaccines in HHD mouse tumor models.

The low affinity peptides we described are presented by tumor cells at a level sufficient to be targets of antitumor CTL, an observation that raises several issues. First, low affinity HER-2/*neu* and hTERT epitopes are presented by tumor cells of different origins; they can, therefore, be used alone or in association with other previously described HER-2/*neu* and hTERT epitopes for a broad spectrum immunotherapy (38–40). The vaccine potential of a polyepitopic vector containing the four HER-2/*neu* P1Y variants along with intermediate or high affinity HER-2/*neu* epitopes described by us and by others is being currently studied in HHD mice. Second, low affinity epitopes, even those with the lowest affinity, such as *neu*$_{391}$ and *neu*$_{402}$, are efficiently presented by tumor cells expressing low amounts of HER-2/*neu* such as LAW. The level of HER-2/*neu* expression is not, therefore, a barrier to the use of low affinity epitopes for tumor immunotherapy. This is most likely explained by the fact that CTL effectors require a small number of peptide/HLA complexes on the target surface to be activated (41). Third, six of six low affinity peptides we have studied in this work are naturally processed by tumor cells. Although the small number of peptides does not permit any conclusion about the diversity of the epitopic repertoire of an Ag, these results suggest that the epitopic repertoire contains a very large number of cryptic epitopes. This is strengthened by our previous observation that three of five flu nucleoprotein-derived peptides with low or intermediate D^b affinity tested are processed by flu-infected murine cells (42) and by recent results showing that low HLA-A*0201 affinity peptides from MAGE are presented by tumor cells.[6] It is noteworthy that all these peptides had the appropriate HLA primary anchor motifs (P5N and P9 M for D^b molecule; P2L and P9/10V/L for HLA-A*0201 molecule). This raises the question of whether the presence of anchor motifs is by itself sufficient to ensure a natural presentation independently of the overall HLA affinity. Investigation of a large panel of randomly selected peptides having the anchor motifs or not and exhibiting variable affinities is necessary before drawing any definitive conclusion.

In summary, we provide results demonstrating that low affinity tumor epitopes for HER-2/*neu* and hTERT are efficiently presented by tumor cells and can, therefore, be used for tumor immunotherapy.

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References

1. Van Pel, A., P. van der Bruggen, P. G. Coulie, V. G. Brichard, B. Lethe, B. van den Eynde, C. Uyttenhove, J. C. Renauld, and T. Boon. 1995. Genes coding for tumor antigens recognized by cytolytic T lymphocytes. *Immunol. Rev. 145:229.*
2. Robbins, P. F., and Y. Kawakami. 1996. Human tumor antigens recognized by T cells. *Curr. Opin. Immunol. 8:628.*
3. Disis, M. L., and M. A. Cheever. 1997. HER-2/*neu* protein: a target for antigen specific immunotherapy of human cancer. *Adv. Cancer Res. 71:343.*
4. Meyerson, M., C. M. Counter, E. N. Eaton, L. W. Ellisen, P. Steiner, S. D. Caddle, L. Ziaugra, R. L. Beijersbergen, M. J. Davidoff, Q. Liu, et al. 1997. hEst2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell 90:785.*
5. Coussens, L., T. L. Yang-Feng, Y.-C. L. E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science 230:1132.*
6. Reese, D. M., and D. J. Slamon. 1997. HER-2/*neu* signal transduction in human breast and ovarian cancer. *Stem Cells 15:1.*
7. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science 235:177.*
8. Berchuck, A., A. Kamel, R. Whitaker, B. Kerns, G. Olt, R. Kinney, J. T. Soper, R. Dodge, D. L. Clarke-Pearson, P. Marks, et al. 1990. Overexpression of HER-2/*neu* is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res. 50:4087.*
9. Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science 266:2011.*
10. Vonderheide, R. H., W. C. Hahn, J. L. Schultze, and L. M. Nadler. 1999. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity 10:673.*
11. Minev, B., J. Hipp, H. Firat, J. D. Schmidt, P. Langlade-Demoyen, and M. Zanetti. 2000. Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc. Natl. Acad. Sci. USA 97:4796.*

[6] S. Graff-Dubois et al. Generation of CTL recognizing an HLA-A*0201 restricted epitope shared by MAGE-A$_1$, -A$_2$, -A$_3$, -A$_4$, -A$_6$, -A$_{10}$, and -A$_{12}$ tumor antigens: implication in a broad-spectrum tumor immunotherapy. *Submitted for publication.*

12. Fisk, B., T. L. Blevins, J. T. Wharton, and C. G. Ioannidis. 1995. Identification of an immunodominant peptide of HER-2/*neu* protooncogene recognized by ovarian tumor specific cytotoxic T lymphocytes lines. *J. Exp. Med. 181:2109.*
13. Peiper, M., P. S. Goedegebuure, D. C. Linehan, E. C. Ganguly, C. Douville, and T. J. Eberlein. 1997. The HER-2/*neu* derived peptide p654–662 is a tumor associated antigen in human pancreatic cancer recognized by cytotoxic T lymphocytes. *Eur. J. Immunol. 27:1115.*
14. Linehman, D. C., P. S. Goedegebuure, G. E. Peoples, S. O. Rogers, and T. J. Eberlein. 1995. Tumor specific and HLA A2 restricted cytolysis by tumor associated lympocytes in human metastatic breast cancer. *J. Immunol. 155:4486.*
15. Brossart, P., G. Stuhler, T. Flad, S. Stevanovic, H. G. Rammensee, L. Kanz, and W. Brugger. 1998. HER-2/*neu* derived peptides are tumor associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by in vitro induced specific cytotoxic T lymphocytes. *Cancer Res. 58:732.*
16. Yoshiro, I., P. S. Goedegebuure, G. E. Peoples, A. S. Parikh, J. M. DiMaio, H. K. Lyerly, A. F. Gazdar, and T. J. Eberlein. 1994. HER-2/*neu* derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. *Cancer Res. 54:3387.*
17. Rongcun, Y., F. Salazar-Onfray, J. Charo, K. J. Malmberg, K. Ervin, H. Maes, K. Kono, C. Hising, M. Petersson, O. Larsson, et al. 1999. Identification of new HER-2/*neu* derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. *J. Immunol. 163:1037.*
18. Scardino, A., P. Alves, D. A. Gross, S. Tourdot, S. Graff-Dubois, S. Chouaib, F. A. Lemonnier, L. M. Nadler, A. A. Cardoso and K. Kosmatopoulos. 2001. Identification of novel HER-2/*neu* immunogenic epitopes presented by renal cell carcinoma and other epithelial tumors. *Eur. J. Immunol. 31:3261.*
19. Keogh, E., J. Fikes, S. Southwood, E. Celis, R. Dhesnut, and A. Sette. 2001. Identification of new epitopes from four different tumor associated antigens: recognition of naturally processed epitopes correlates with HLA-A*0201 binding affinity. *J. Immunol. 167:787.*
20. Liu, K., M. M. Schoonmaker, B. L. Levine, C. H. June, R. J. Hodes, and N. P. Weng. 1999. Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. *Proc. Natl. Acad. Sci. USA 96: 5147.*
21. Theobald, M., J. Biggs, J. Hernandez, J. Lustgarten, C. Labadie, and L. A. Sherman. 1997. Tolerance to p53 by A2.1 restricted cytotoxic T lymphocytes. *J. Exp. Med. 185:833.*
22. Hernandez, J., P. P. Lee, M. M. Davis, and L. A. Sherman. 2000. The use of HLA A2.1/p52 peptide tetramers to visualize the impact of self-tolerance on the TCR repertoire. *J. Immunol. 164:596.*
23. Colella, T. A., T. N. J. Bullock, L. B. Russel, D. W. Mullins, W. W. Overwijk, C. J. Luckey, R. A. Pierce, N. P. Restifo, and V. H. Engelhard. 2000. Self-tolerance to the murine homologue of a tyrosinase-derived melanoma antigen: implications for tumor immunotherapy. *J. Exp. Med. 191:1221.*
24. Grossmann, M. E., E. Davila, and E. Celis. 2001. Avoiding tolerance against prostatic antigens with subdominant peptide epitopes. *J. Immunother. 24:237.*
25. Overwijk, W. W., A. Tsung, K. R. Irvine, M. R. Parkhurst, T. J. Goletz, K. Tsung, M. W. Carroll, C. Liu, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1998. Gp100/pmel 17 is a murine tumor rejection antigen: induction of self-reactive tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med. 188: 277.*
26. Restifo, N. P. 2001. Hierarchy, tolerance, and dominance in the antitumor T-cell response. *J. Immunother. 24:193.*
27. Cibotti, R., J. M. Kanellopoulos, J. P. Cabaniols, O. Halle-Pannenko, K. Kosmatopoulos, E. Sercarz, and P. Kourilsky. 1992. Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants. *Proc. Natl. Acad. Sci. USA 89:416.*
28. Nanda, N. K., and E. E. Sercarz. 1995. Induction of anti-self-immunity to cure cancer. *Cell 82:13.*
29. Tourdot, S., A. Scardino, E. Saloustrou, D. A. Gross, S. Pascolo, P. Cordopatis, F. A. Lemonnier, and K. Kosmatopoulos. 2000. A general strategy to enhance immunogenicity of low-affinity HLA-A2.1-associated peptides: implication in the identification of cryptic tumor epitopes. *Eur. J. Immunol. 30:3411.*
30. Pascolo, S., N. Bervas, J. M. Ure, A. G. Smith, F. A. Lemonnier, and B. Perarnau. 1997. HLA-A2.1-restricted education and cytolytic activity of $CD8^+$ T lymphocytes from β2-HLA-A2.1 monochain transgenic H-$2D^b$ β_2m double knockout mice. *J. Exp. Med. 185:2043.*
31. Schultze, J. L., S. Michalaki, M. J. Seamon, G. Dranoff, K. Jung, J. Daley, J. C. Delgado, J. G. Gribben, and L. M. Nadler. 1997. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J. Clin. Invest. 100:2757.*
32. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA A2 binding peptides based on independent binding of individual peptide side chains. *J. Immunol. 152:163.*
33. Sette, A., A. Vitielo, B. Reherman, P. Fowler, R. Nayersina, M. W. Kast, C. J. M. Melief, C. Oserdorf, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of cytotoxic T cell epitopes. *J. Immunol. 153:5586.*
34. Dyal, R., W. B. Bowne, L. W. Weber, J. LeMaoult, P. Szabo, Y. Moroi, G. Piskun, J. J. Lewis, A. N. Houghton, and J. Nicolic-Zugic. 1998. Heteroclitic immunization induces tumor immunity. *J. Exp. Med. 188:1553.*
35. Zeh, H. J., III, D. Perry-Lalley, M. E. Dudley, S. A. Rosenberg, and J. C. Yang. 1999. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J. Immunol. 162:989.*
36. Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc. Natl. Acad. Sci. USA 93:4102.*
37. Tourdot, S., M. Oukka, J. C. Manuguerra, N. Malgafa, I. Vergnon, N. Riché, M. Brulley-Rosset, P. Cordopatis, and K. Kosmatopoulos. 1997. Chimeric peptides: a new approach to enhance immunogenicity of peptides with low MHC binding affinity: application in antiviral protection. *J. Immunol. 159:2391.*
38. Thomson, S. A., M. A. Sherritt, J. Medveczky, S. L. Elliott, D. J. Moss, G. J. Fernando, L. E. Brown, and A. Surhbier. 1998. Delivery of multiple CD8 cytotoxic T cell epitopes by DNA vaccination. *J. Immunol. 160:1717.*
39. Ishioka, G. Y., J. Fikes, G. Hermanson, B. Livingston, C. Crimi, M. Qin, M. F. del Guercio, C. Oserrof, C. Dahlberg, J. Alexander, et al. 1999. Utilization of MHC class I transgenic mice for development of minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes. *J. Immunol. 162:3915.*
40. Firat, H., F. Garcia-Pons, S. Tourdot, S. Pascolo, A. Scardino, Z. Garcia, M. L. Michel, R. W. Jack, G. Jung, K. Kosmatopoulos, et al. 1999. H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. *Eur. J. Immunol. 29:3112.*
41. Sykulef, Y., M. Joo, I. Viturina, T. J. Tsomidis, and H. N. Eissen. 1996. Evidence that a single peptide MHC complex on a target cell can elicit a cytotoxic T cell response. *Immunity 4:565.*
42. Oukka, M., J. C. Manuguerra, N. Livaditis, S. Tourdot, N. Riché, I. Vergnon, P. Cordopatis, and K. Kosmatopoulos. 1996. Protection against lethal viral infection by vaccination with non-immunodominant peptides. *J. Immunol. 157:3039.*