Identification of HER-2/neu immunogenic epitopes presented by renal cell carcinoma and other human epithelial tumors

Antonio Scardino[1], Pedro Alves[1,5], David A. Gross[1], Sophie Tourdot[1], Stephanie Graff-Dubois[1], Eric Angevin[2], Hüseyin Firat[3], Salem Chouaib[1], François Lemonnier[4], Lee M. Nadler[5], Angelo A. Cardoso[5] and Kostas Kosmatopoulos[1]

[1] INSERM 487, Institut Gustave Roussy, Villejuif, France
[2] Laboratoire d'Immunologie Clinique, Institut Gustave Roussy, Villejuif, France
[3] Genethon III, CNRS, URA 1923, Evry, France
[4] Unité d'Immunité Cellulaire Antivirale, Departement Sida Retrovirus, Institut Pasteur, Paris, France
[5] Department of Adult Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, USA

HER-2/neu is a tumor-associated antigen overexpressed in a large variety of human tumors. Eight HER-2/neu peptides displaying HLA-A*0201 anchoring motifs were selected and tested for their binding affinity to HLA-A*0201 and their capacity to elicit cytotoxic T lymphocyte (CTL) responses in both HLA-A*0201 transgenic mice and in HLA-A*0201$^+$ healthy donors. Two high-affinity (p5 and p48) and one intermediate-affinity (p1023) peptides triggered CTL responses in both transgenic mice and humans, comparable to those observed for the well-known HER2/neu dominant peptide p369. CTL induced in transgenic mice lysed HLA-A*0201$^+$ RMA cells infected with recombinant HER-2/neu but not cells infected with wild-type vaccinia virus. Human CTL lysed HLA-A*0201$^+$ HER-2/neu$^+$ tumor cells of different origins (breast, colon, lung and renal cancer) irrespective of the expression levels of HER-2/neu. Importantly, primed CTL specific for these epitopes were detected in freshly isolated tumor-infiltrating lymphocytes from three renal cell carcinoma patients. Therefore, the HER-2/neu peptides p5, p48 and p1023 may be good candidates for immunotherapy of a broad spectrum of tumors, including renal cell carcinoma.

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1 Introduction

Tumor-specific CTL have been detected in several cancers patients and could serve as a therapeutic arm in the form of adoptive immunotherapy or via their mobilization by tumor cell vaccines [1, 2]. However, manipulation of CTL for clinical use requires the identification and characterization of their associated tumor antigens and their cognate epitopes. The recent demonstration in humans that vaccination of naive hosts with peptide-pulsed dendritic cells elicits peptide-specific T cell-mediated immunity [3] provides a strong argument for the clinical use of tumor antigen-derived peptides. To date, several tumor antigen epitopes have been identified, most of them in malignant melanoma [4]. The characterization of antigenic epitopes shared and efficiently presented by multiple tumors, such as the recently identified dominant epitope of hTERT [5], will hopefully result in the design of immunotherapeutic strategies of broad clinical impact.

HER-2/neu is a 185-kDa transmembrane glycoprotein with tyrosine kinase activity, the coding gene of which is present in normal cells as a single copy [6, 7]. In contrast, the HER-2/neu gene is amplified and its respective protein overexpressed in multiple human cancers, including carcinoma of breast, ovary, uterus, lung, kidney, stomach and pancreas [7, 8]. Moreover, HER-2/neu overexpression has been associated with poor prognosis in both intraductal carcinomas of the breast (20–40% of cases) and ovarian cancer (30% of the patients) [9, 10]. Analysis of the fine specificity of HLA-A*0201-restricted HER-2/neu-specific TIL revealed a number of dominant epitopes [11–16], and their use in immunotherapy protocols has been proposed. Strikingly, preliminary clinical

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Abbreviations: RCC: Renal cell carcinoma **TIL:** tumor-infiltrating lymphocytes **RA:** Relative affinity **FI:** Fluorescence index

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observations in HER-2/neu$^+$ breast, colorectal and ovarian cancer patients showed that vaccination with the dominant peptide p369 induced specific CTL that failed to lyse the primary tumor cells [17]. These findings may be due to the recruitment of low-avidity CTL and the elimination or inactivation of the high-avidity specific CTL repertoire, thus emphasizing the need for the identification of novel HER-2/neu immunogenic epitopes with a higher vaccination potential, which could be employed on multiepitopic vaccine strategies. The efficacy of such vaccination approach has been recently demonstrated in animal models [18–20].

In this manuscript, we describe three novel HLA-A*0201-restricted HER-2/neu epitopes (p5, p48 and p1023) immunogenic in both HLA-A*0201 transgenic mice and human healthy donors. These epitopes are naturally processed and presented by HER-2/neu$^+$ lung, breast, colon and renal tumor cells. More importantly, renal cell carcinoma (RCC) patients developed p5-, p48- and p1023-specific anti-tumor CTL responses, and epitope-specific CTL were detected in their freshly isolated tumor-infiltrating lymphocytes (TIL). Therefore, these epitopes may be considered in the design of broad-spectrum anti-tumor polyepitopic immunotherapeutic strategies for the treatment of human epithelial tumors.

2 Results

2.1 Selection of HLA-A*0201-binding HER-2/neu peptides

To identify novel HLA-A*0201-restricted HER-2/neu epitopes that could trigger anti-tumor T cell responses, we searched for peptides with optimal HLA-A*0201 anchor motifs (L/M at position 2 and V/L at the C-terminal position) [21]. Seven nonameric peptides – p5, p48, p391, p402, p466, p650 and p661 – have been selected (Table 1). Two additional peptides were included: p369, which is a well-known dominant HER-2/neu peptide [11]; and the decamer p1023, predicted by the BIMAS software (http://www-bimas.dcrt.nih.gov/molbio/hla_bind/index.html) to have a high half-time of dissociation from HLA-A*0201 molecule.

2.2 Affinity of HER-2/neu peptides for the HLA-A*0201 molecule

The capacity of the selected HER-2/neu peptides to bind to HLA-A*0201 and form stable peptide/HLA-A*0201 complexes was assessed (Table 1). The HER-2/neu peptides could be separated into three groups: (I) high-affinity peptides (p5, p48, and p369) that strongly bind to HLA-A*0201 [relative affinity (RA) <5] and form stable complexes [dissociation complex $(DC)_{50}$ >2 h]; (II) intermediate-affinity peptides (p661 and p1023) that are poor HLA-A*0201 binders (RA >5) but can efficiently stabilize HLA-A*0201 (DC_{50} >2 h), and (III) low-affinity peptides (p391, p402, p466 and p650) that do not form stable peptide/HLA-A*0201 complexes (DC_{50} <2 h), while varying in their ability to bind to HLA-A*0201 (p466 and p650 are high affinity; and p391 and p402 are low affinity).

Table 1. HLA-A*0201 affinity of HER-2/neu peptides

Peptides	Sequence	RA	DC_{50}
p5	ALCRWGLLL	2.3	>8
p48	HLYQGCQVV	1.7	>8
p369	KIFGSLAFL	2.3	6–8
p661	ILLVVVLGV	>60	2–4
p1023	YLVPQQGFFC	19.7	4
p391	PLQPEQLQV	>70	2
p402	TLEEITGYL	19.0	<2
p466	ALIHHNTHL	4.8	2
p650	PLTSIISAV	1.4	2

2.3 Induction of CTL responses specific for the HER-2/neu peptides

The capacity of HER-2/neu peptides to prime *in vivo* specific CTL responses was evaluated using the HLA-A*0201 transgenic HHD mice. Mice immunized with p5, p48 and p1023 developed CTL that lysed peptide-pulsed, but not unpulsed, RMAS-HHD targets (Fig. 1). As expected, vaccination with the control p369 peptide also resulted in the triggering of significant peptide-specific CTL responses. In contrast, mice immunized with p391, p402, p466, p650 and p661 failed to generate significant peptide-specific CTL. These findings support our previous observation that peptides that do not form stable complexes with HLA-A*0201 (DC_{50} <2 h) are generally not immunogenic, irrespective of their binding affinity [22].

The HER-2/neu peptides immunogenic in the HHD mice (p5, p48, p1023 and the control p369) were then evaluated for their capacity to trigger the generation of CTL from the peripheral blood of HLA-A*0201$^+$ healthy donors. CTL were generated from four different donors and their cytolytic activity evaluated after three or four

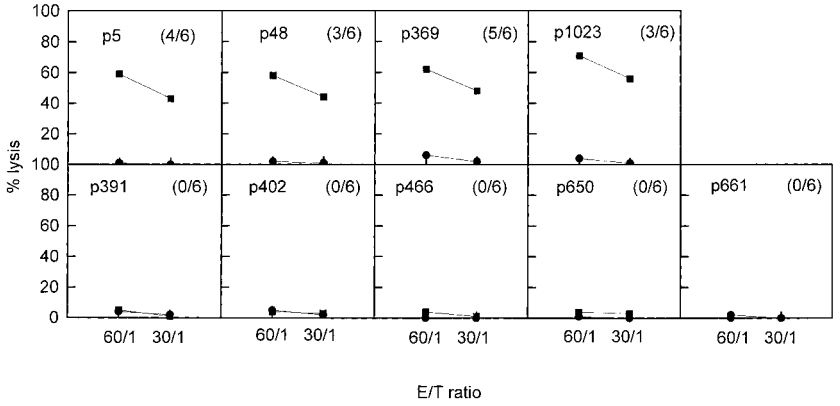

Fig. 1. Generation of HLA-A*0201-restricted HER-2/neu peptide-specific CTL in HHD mice. HHD mice were immunized with peptide and 11 days later their spleen cells were stimulated *in vitro* with 10 μM of the corresponding peptide. Cytotoxicity was tested against peptide pulsed (■) or unpulsed (●) RMAS HHD targets. The number of responding versus tested mice is indicated in parentheses.

restimulations, using the TAP-deficient T2 cells as targets. As shown in Fig. 2, for one representative donor, CTL lysed T2 cells pulsed with their cognate peptide but not unpulsed T2 cells. Similar results were obtained for the four donors tested.

2.4 Natural processing and presentation of p48 and p1023 by HER-2/neu-expressing tumor cells

While processing and presentation of p369 and, more recently, p5 epitopes [23] has been documented on human tumor cells, is unknown whether this is also the case for p48 and p1023. To evaluate the natural processing of these HER-2/neu peptides two different strategies were employed.

First, CTL lines were established from HHD mice primed with p5, p48, p369, and p1023, and their cytotoxic activity was tested using RMA-HHD cells infected with a vaccinia virus expressing HER-2/neu (vac-neu) or the appropriate control (vac-wt). p369-specific CTL were used as a positive control of HER-2/neu peptide presentation by vac-neu-infected cells. Flow cytometric analysis showed that RMA-HHD cells infected with vac-neu, but not vac-wt, expressed surface HER-2/neu (data not shown). As displayed in Fig. 3, CTL specific for p5, p48, p369 and p1023 lysed vac-neu-infected, but not vac-wt-infected, RMA-HHD targets, thus suggesting that p48 and p1023, likewise p5 and p369, are naturally processed and presented by the target cells.

Secondly, CTL lines primed with these peptides were generated from healthy donors and their cytolytic activity evaluated using the HLA-A*0201$^+$ HER-2/neu$^+$ (MCF-7) and HLA-A*0201$^+$ HER-2/neu$^-$ (ZR75.1) breast cancer cells, and the NK-sensitive K562 cells, as targets. As shown in Fig. 4A, CTL lines specific for p5, p48, p369, or p1023 lysed MCF-7 but not ZR75.1 cells nor NK-sensitive K562 cells. CTL lysed the HLA-A*0201$^+$ HER-2/neu$^-$ ZR75.1 cells only when these cells were pulsed with the respective HER-2/neu cognate peptide. These findings demonstrate that p48 and p1023 are naturally processed by tumor cells and efficiently presented by HLA-A*0201 molecules.

Epitope specificity and HLA-A*0201-restriction of HER-2/neu$^+$ tumor cell lysis were confirmed in blocking and cold-target inhibition assays. Lysis of MCF-7 cells by the peptide-specific CTL was significantly inhibited by an anti-HLA-A*0201-specific mAb but not by an anti-HLA-DR specific mAb (Fig. 4B). Moreover, cold T2 cells pulsed with the cognate but not with the irrelevant pfluM peptide significantly inhibited MCF-7 cell lysis by CTL

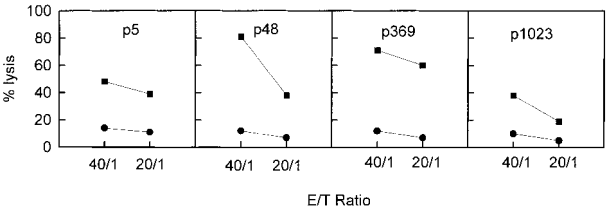

Fig. 2. Generation of HLA-A*0201-restricted HER-2/neu peptide-specific CTL from healthy donor PBMC. CTL were induced from purified T cells stimulated *in vitro* with autologous dendritic or CD40-activated B cells. CTL activity was tested against peptide pulsed (■) or unpulsed (●) T2 cells, 5–7 days after the 3rd fourth stimulation.

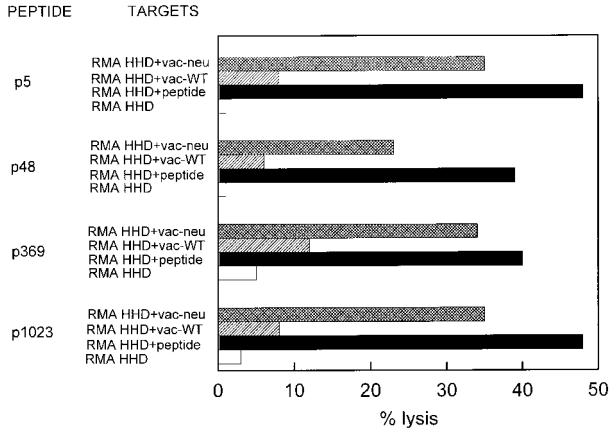

Fig. 3. Recognition of HER-2/neu-expressing murine tumor cells by murine CTL. Peptide-specific CTL induced in vaccinated HHD mice were tested against peptide-pulsed, vac-wt-infected and vac-neu-infected RMA HHD targets, as indicated. E/T ratio: 60/1.

(Fig. 5). This inhibition was dose dependent, as it was more effective at a 50:1 cold-to-hot target ratio.

2.5 Presentation of p5, p48, and p1023 HER-2/neu peptides by breast, lung, colon and renal human carcinomas

As our aim was to identify tumor epitopes expressed by multiple human cancers, we evaluate whether these peptides were efficiently presented by cell lines derived from different HER-2/neu-expressing epithelial tumors (see Table 2 for intensity of HER-2/neu expression). CTL specific for p5, p48, p369 and p1023 were generated from healthy donors (n=4) and tested for their capacity to lyse HLA-A*0201$^+$ HER-2/neu$^+$ breast (MCF-7), lung (1355 and PUB/N), colon (HCT-116) and renal (A-498, DOB and LAW) cancer cell lines. Two HLA-A*0201$^+$ HER-2/neu$^-$ cell targets (ZR75.1 and SUPM2) were employed as negative controls, and K562 cells were used as NK-sensitive targets. As shown in Fig. 6, CTL specific for all these peptides lysed HLA-A*0201$^+$ HER-2/neu$^+$ tumor cell targets, irrespective of their cellular origin. In contrast, these CTL did not lyse the two HER-2/neu$^-$ targets or the K562 cells. These results clearly demonstrate that the newly identified HER-2/neu epitopes p48 and p1023, alike p5 and p369, are presented by HER-2/neu-expressing tumors of different tissue origins.

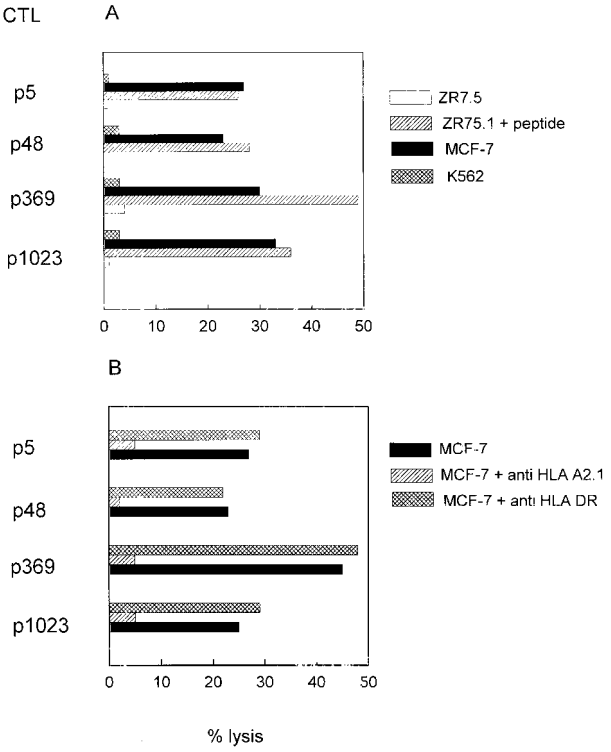

Fig. 4. Recognition of HER-2/neu-expressing human tumor cells by human CTL. (A) Peptide-specific CTL generated from HLA-A*0201 healthy donor PBMC were tested against ZR7.5 (HLA-A*0201$^+$, HER-2/neu$^-$), MCF-7 (HLA-A*0201$^+$, HER-2/neu$^+$) and K562 targets at E/T ratio: 40/1. (B) Peptide-specific CTL were tested against MCF-7 targets in the presence of 1/100 diluted anti-HLA-A*0201 and anti-HLA-DR mAb, as indicated. E/T ratio: 40/1.

2.6 Natural occurrence of p5-, p48-, p369-, and p1023-specific CTL in RCC patients

As these four HER-2/neu peptides were processed and presented by three RCC cell lines (DOB, LAW and A-498), we sought to determine whether epitope-specific CTL responses naturally occur in HER-2/neu$^+$ RCC patients. The presence of CTL reactive to HER-2/neu peptides was assessed in freshly isolated TIL obtained from three HLA-A*0201$^+$ HER-2/neu$^+$ (Dob, Law and Gue) and one HLA-A*0201$^+$HER-2/neu$^-$ (Deb) RCC patients using an ELISPOT assay (Table 3). Dob and Law were the patients from whom the cell lines DOB and LAW were derived, respectively. CTL specific for p5, p48, p369, and p1023 epitopes were detected in the TIL from the three HER-2/neu$^+$ patients (35–50 IFN-γ-secreting cells per 10^5 TIL for Dob and Gue and 10–15 IFN-γ-secreting cells per 10^5 TIL for Law). In contrast, peptide-specific CTL were not detected in the TIL from the HER-2/neu$^-$ patient.

Table 2. HER-2/neu expression by tumor cells

Cell line	FI
ZR75.1	0.12
SUP/M2	0.11
MCF-7	1.94
HCT116	1.23
PUB/N	1.33
1355	0.95
A-498	0.45
DOB	0.95
LAW	0.35

These results demonstrate, for the first time, that RCC patients develop an endogenous anti-HER-2/neu immune response directed against the different HER-2/neu peptide determinants, including the novel p48 and p1023 epitopes.

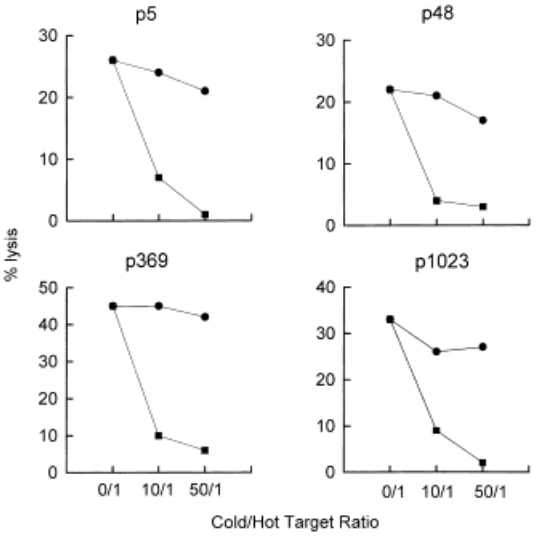

Fig. 5. HER-2/neu specificity of peptide induced human CTL. Human CTL were tested against ^{51}Cr-labeled MCF-7 cells in the presence of cold T2 cells pulsed with the cognate (p5, p48, p369 and p1023) (■) or the irrelevant pfluM (●) peptide. E/T ratio: 40/1.

3 Discussion

The identification of the epitopes that may serve as targets for the patient's anti-tumor T cell-mediated immunity constitutes a major challenge in the development of effective immunotherapeutic strategies. Here we describe two novel HER-2/neu-derived HLA-A*0201-binding immunogenic epitopes (p1023 and p48) and confirm the natural processing of the p5 epitope. These epitopes are presented by human epithelial tumors of multiple origins, including breast, colon, lung and renal cancer. We provide evidence that RCC patients possess TIL that specifically recognize these HER-2/neu epitopes, thus demonstrating endogenous priming of

Table 3. Peptide-specific IFN-γ-secreting cells in freshly isolated TIL from RCC patients[a)]

	IFN-γ-secreting cells (10^5 TIL)			
	HER-2/neu$^+$ RCC patients			HER-2/neu$^-$ RCC patient
Peptides	Dob	Gue	Law	Deb
No peptide	8 ± 3	6 ± 6.5	3 ± 1	2 ± 1.7
pHIVgag76	10 ± 6	7 ± 7.3	2 ± 2	6 ± 4.2
p369	47 ± 8.7*	49 ± 14.7*	10 ± 0.6*	3 ± 1.9
p5	48 ± 8.7*	35 ± 1.5*	NT	3 ± 2.8
p48	50 ± 4.2*	40 ± 9*	15 ± 6.4*	5 ± 3.8
p1023	36 ± 5.7*	38 ± 8.2*	13 ± 7.2*	7 ± 1.5
pfluM58	44 ± 5.1*	139 ± 35.5*	130 ± 37*	252 ± 21
PHA	1,011 ± 131	1,162 ± 356	1,073 ± 29	968 ± 45

a) Peptide-specific CTL in freshly isolated TIL was measured by ELISPOT assay. Results represent the mean ± SD of quadruplicates; NT: not tested.

* $p<0.03$ compared to HIVgag76.

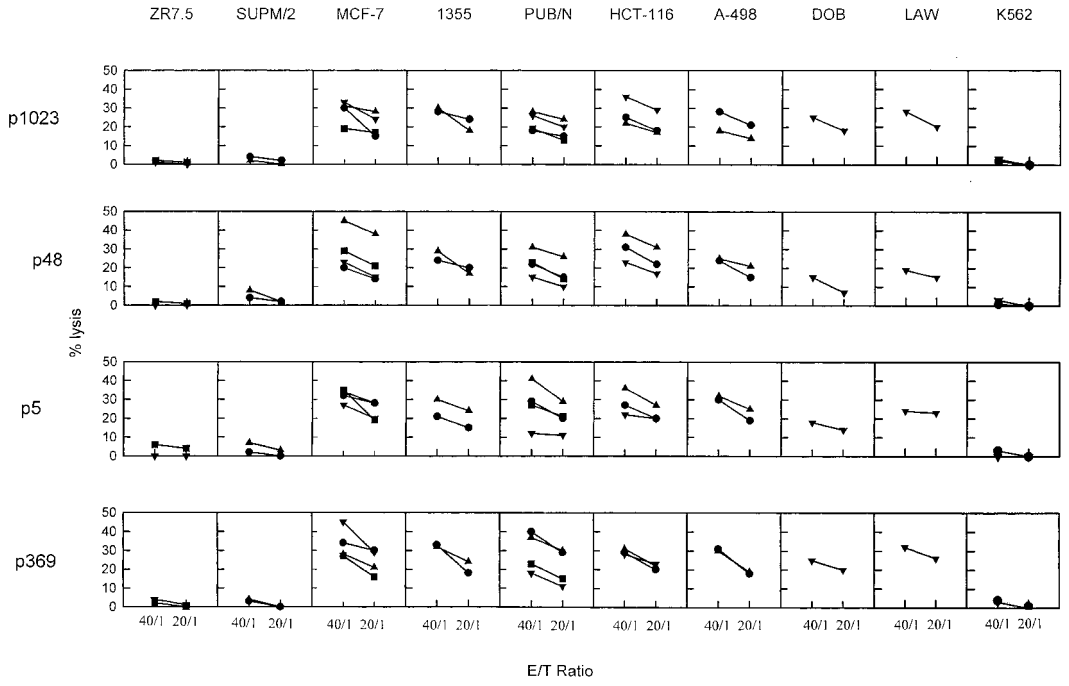

Fig. 6. Recognition by human CTL of HER-2/neu-expressing cells from breast, lung, colon and renal tumors. Peptide-specific CTL generated from PBMC of four different healthy donors (●, ■, ▲, ▼) were tested against HLA-A*0201$^+$, HER-2/neu$^-$ ZR7.5 (breast cancer), SUPM2 (anaplastic lymphoma) and HLA-A*0201$^+$, HER-2/neu$^+$ MCF-7 (breast cancer), 1355 (lung cancer), PUB/N (lung cancer), HCT-116 (colon cancer), A-498 (RCC), DOB (RCC) and LAW (RCC) targets at E/T ratios of 40/1 and 20/1.

tumor-reactive T cells. This constitutes the first demonstration that HER-2/neu is a tumor-associated antigen in RCC and that p5, p48 and p1023 epitopes, in addition to p369, participate in the anti-tumor CTL response naturally developed in these patients.

The identification of multiple HLA-A*0201-restricted HER-2/neu epitopes has an evident interest for tumor immunotherapy. First, it allows for the selection of the epitopes with the highest potential for vaccination. These epitopes must, optimally, be immunogenic and recruit a wide, avid and functional CTL repertoire, which quality is strongly influenced by negative selection. HER-2/neu is expressed at low levels in normal tissues and might be involved in negative selection, as suggested by the existence of tolerance to HER-2/neu peptides in a murine model [24]. Since negative selection mostly concerns the immunodominant epitopes with high HLA affinity, such as p369, it is possible that the p369-specific CTL repertoire may be partially tolerized, particularly the high-avidity CTL. This may explain the inability of CTL from breast, ovarian and colorectal cancer patients vaccinated with the p369 to recognize the HER-2/neu expressing tumor cells [17]. In this regard, the p1023 epitope, which displays an intermediate affinity to HLA-A*0201, may be a good candidate for immunotherapy as its CTL repertoire should, theoretically, be less affected by negative selection than the CTL repertoire directed to dominant high-affinity epitopes. Second, HER-2/neu epitopes with the highest potential for vaccination could be included in immunotherapy protocols using polyepitopic vectors that allow for the generation of a polyspecific, robust and efficient CTL response [18–20]. We have recently evaluated the immunogenic potential of a vector containing multiple HER-2/neu HLA-A*0201 epitopes and observed the generation of CTL specific for all the epitopes and, more importantly, the induction of *in vivo* anti-tumor protective immunity (Scardino et al., manuscript in preparation).

One important observation is that the p5, p48, and p1023 epitopes are naturally processed and efficiently presented by HER-2/neu-expressing tumors of different tissue origins. This is relevant since the expression of an antigen by tumor cells does not necessarily result in the presentation of all its putative immunogenic epitopes. It has been suggested that the presentation of HER-2/neu peptides to CTL may vary in lung and ovarian cancer, likely reflecting differences of HLA-A*0201 and HER-2/neu expression levels or their presentation of other tumor-associated antigens (TAA) [15]. In the present study, we show that tumor cells that express equivalent

levels of HLA-A*0201, but not of HER-2/neu, are efficiently lysed by epitope-specific CTL, thus suggesting that the efficient presentation of these epitopes is not conditioned by the level of HER-2/neu expression. This is particularly evident for the RCC lines A-498 and LAW, which express lower levels of HER-2/neu than the tested lung, colon and breast tumor cell lines.

RCC is known to be immunogenic since tumor-reactive effector T cells can be isolated and expanded from these patients' peripheral blood and tumor sites [25, 26]. Accordingly, immunotherapy has been pursued as a valuable modality in the search for novel, more efficient and specific strategies for the treatment of this cancer. Increasing efforts are being placed on the identification of the antigenic peptides targeted by CTL responses to RCC. However, with the exception of the recently described g250 [27], the TAA identified in RCC are either mutated proteins or molecules rarely expressed in primary renal tumor cells [28–31], thus limiting their clinical use. In contrast, HER-2/neu is a nonmutated protein overexpressed in a high percentage of RCC [32]. Here, we show the presence of anti-HER-2/neu CTL in freshly isolated TIL from RCC patients. This is the first demonstration that a naturally occurring T cell-mediated immune response targeting a widely expressed antigen such as HER-2/neu exist in RCC patients. These findings underscore the rationale for the use of these HER-2/neu peptides in the design of vaccination strategies for RCC.

In conclusion, we identified novel HER-2/neu immunogenic peptides that are involved in the endogenous T cell response to RCC. These peptides are naturally processed and presented by different epithelial tumors and serve as targets for epitope-specific CTL. These observations suggest that these peptides may be used in clinical approaches to amplify anti-tumor specific immunity by recruiting T cells targeting multiple immunogenic epitopes.

4 Materials and methods

4.1 Animals

The HHD mice were previously described [33]. They are $\beta 2m^{-/-}$, $D^{b-/-}$ and express an HLA-A*0201 monochain composed of a chimeric heavy chain ($\alpha 1$ and $\alpha 2$ domains of HLA-A*0201 and the $\alpha 3$ and intracellular domains of D^b) linked by its N terminus to the C terminus of the human $\beta 2m$ by a 15-amino acid peptide arm.

4.2 Cells

Murine RMA-HHD and RMAS-HHD cells were obtained by transfection of murine RMA cells and their TAP-deficient variant RMAS cells with the HHD construct [33]. The HLA-A*0201 expressing human tumor cells were: T2 (deficient in TAP1 and TAP2 transporters); 1355 (lung cancer), HCT-116 (colon cancer) and A-498 (RCC), kindly provided by Dr. W. Brugger (Tübingen, Germany); DOB and LAW (RCC) established by Dr. F. Triebel (Villejuif, France); PUB/N (lung cancer) established by Dr. F. Mami-Chouaib (Villejuif, France); MCF-7 and ZR75.1 (breast cancers); and SUPM2 (anaplastic lymphoma), kindly provided by Dr. Gambacorti, (Milan, Italy). NK-sensitive K562 cells were also used. Cells were grown in RPMI 1640 medium supplemented with 10% FCS. Freshly isolated TIL from RCC patients were provided by Drs. A. Caignard and F. Triebel (Villejuif, France).

4.3 Peptides and viruses

Peptides were synthesized by Synt:em (Nîmes, France). T39 (vac-neu) HER-2/neu-expressing recombinant and TBC-Wy (vac-wt) wild-type vaccinia viruses were kindly provided by Dr. G. Mazzara (Therion Biologics, Cambridge, MA).

4.4 Generation of CTL in HHD mice

HHD mice were injected subcutaneously at the base of the tail with 100 μg of peptide emulsified in incomplete Freund's adjuvant (IFA) in the presence of 140 μg of the IA^b restricted HBVcore-derived T-helper epitope (128–140; sequence TPPAYRPPNAPIL. Spleen cells (5×10^7 cells in 10 ml) were harvested on day 11 and *in vitro* stimulated with peptide (10 μM). After 6 days in culture, the bulk responder populations were tested for specific cytotoxicity.

4.5 Generation of CTL from human PBMC

Dendritic cells (DC) were prepared by cultivating plastic-adherent PBMC in the presence of 700 U/ml GM-CSF (R&D Systems Inc. MN) and 100 U/ml IL-4 (R&D Systems Inc. MN) for 7 days. Immunofluorescence staining of this DC-enriched population showed that >80% of the cells 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 CD40L-expressing fibroblasts in B cell medium supplemented with 2.8 μg/ml cyclosporin A [34]. After 14 days, greater than 85% of the cells were $CD19^+$, $CD80^+$, $CD86^+$, HLA-DR^+.

DC were harvested, pulsed overnight with peptide (10 μM) in the presence of sCD40L to induce their maturation, irradiated at 36 Gy, and added to autologous T cells at a T:DC ratio of 20:1 in culture medium (CM; RPMI 1640 supplemented with 10% human AB serum, 10 nM L-glutamine and gentamycin). After 3 days, primed T cells were harvested, isolated by Ficoll centrifugation and replated in CM supplemented with 20 IU/ml IL-2. On day 7, and weekly thereafter, T cells were restimulated with peptide-pulsed irradiated DC

(1st restimulation) or activated B cells (2nd, 3rd, and 4th restimulations). Cytotoxicity was tested 5–7 days after the 4th restimulation.

4.6 Cytotoxic assay

Murine RMAS-HHD and RMA-HHD cells, and human MCF-7, 1355, PUB/N, A-498, DOB, LAW, HCT-116, ZR75.1, SUPM2 and K562 cells were used as targets for cytotoxicity. For virus infection, 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 of ^{51}Cr for 90 min, washed four times and plated in 96-well round-bottom plates (10^4 cells/well in 100 μl of RPMI 1640 + 3% FCS). Uninfected RMA-HHD and RMAS-HHD murine, T2, SUPM2 and ZR75.1 human cells were pulsed with 1 μM of peptides at 37°C for 90 min. Varying numbers of effectors (100 μl) were then added to the wells and incubated at 37°C for 4 h. After incubation, 100 μl of supernatant was collected and radioactivity was measured in a γ-counter. Percentage of specific lysis was determined as: lysis = [(experimental release – spontaneous release)/(maximal release – spontaneous release)×100]. Spontaneous release was always <20% of maximal release induced by 3 N HCl.

4.7 Quantification of peptide-specific T cells

The presence of peptide-specific T cells in freshly isolated TIL from RCC patients was determined by an ELISPOT assay designed to detect IFN-γ-producing cells; 10^5 TIL were stimulated with 10^5 T2 cells unpulsed or pulsed with HER-2/neu peptides, pHIVgag76 (negative control) or fluM58 (positive control) (10 μM for 16 h at 37°C). PHA-stimulated TIL were used as internal control in each experiment. After 24-h incubation, IFN-γ-producing cells were measured as described [35]. IFN-γ-secreting cells were counted using the automated image analysis system ELISPOT Reader (AID Strassberg, Germany). For each peptide, cultures were performed in quadruplicates. The Wilcoxon two tail-rank test was performed to determine statistical significant differences between the number of IFN-γ-secreting cells in the wells stimulated with the HER-2/neu and the HIV-gag76 peptides.

4.8 Flow cytometric analysis of HER-2/neu expression by tumor cells

Expression of HER-2/neu by human tumor cells and vac-neu infected RMA-HHD cells was evaluated by staining of cells with the C-18 mAb (Santa Cruz Biotechnology) followed by FITC-conjugated goat-anti-rabbit Ig Ab. Fluorescence index (FI) was determined as: FI = (mean fluorescence with C-18 mAb – mean fluorescence with control Ab)/mean fluorescence with control.

4.9 Measurement of peptide RA to HLA-A*0201

T2 cells (3×10^5 cells/ml) were incubated with concentrations of peptides ranging from 100 μM to 0.1 μM in serum-free RPMI medium supplemented with 100 ng/ml of human β2m 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 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 mean 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.

4.10 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 medium supplemented with 100 ng/ml β2m 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} was defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at t=0.

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Correspondence: Kostas Kosmatopoulos, INSERM 487, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif, France
Fax: +33-1-42115288
e-mail: kostas@igr.fr