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Identification of a Novel Immunogenic HLA-A*0201-Binding Epitope of HER-2/neu with Potent Antitumor Properties

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HER-2/neu oncoprotein is overexpressed in a variety of human tumors and is associated with aggressive disease. Immunogenic HER-2/neu CTL epitopes have been used as vaccines for the treatment of HER-2/neu positive malignancies with limited success. By applying prediction algorithms for MHC class I ligands and proteosomal cleavages, in this study, we describe the identification of HER-2/neu decamer LIAHNQVRQV spanning residues 85–94 (HER-2(1085)). HER-2(1085) proved to bind with high affinity to HLA-A2.1 and was stable for 4 h in an off-kinetics assay. This peptide was immunogenic in HLA-A2.1 transgenic (HHD) mice inducing peptide-specific CTL, which responded to tumor cell lines of various origin coexpressing human HER-2/neu and HLA-A2.1. This demonstrates that HER-2(1085) is naturally processed from endogenous HER-2/neu. Five of sixteen HER-2/neu+ HLA-A2.1+ breast cancer patients analyzed had HER-2(1085)-reactive T cells ranging from 0.35–70% of CD8+ T cells. Depletion of T regulatory cells from PBMC enabled the rapid expansion of HER-2-A2/I HER-2(1085)pentamer+/CD8+ cells (PENT+), whereas significantly lower numbers of CTL could be generated from unfractionated PBMC. HER-2(1085)-specific human CTL recognized the HER-2/neu+ HLA-A2.1+ tumor cell line SKBR3.A2, as determined by IFN-γ intracellular staining and in the high sensitivity CD107α degranulation assay. Finally, HER-2(1085) significantly prolonged the survival of HHD mice inoculated with the transplantable ALC.A2.1.HER tumor both in prophylactic and therapeutic settings. These data demonstrate that HER-2(1085) is an immunogenic peptide, capable of eliciting CD8-mediated responses in vitro and in vivo, providing the platform for further exploitation of HER-2(1085) as a possible target for anticancer immunotherapy.


The HER-2/neu gene product has attracted much interest as a target for cancer immunotherapy. HER-2/neu is a transmembrane glycoprotein and member of the epidermal growth factor receptor family (1). HER-2/neu amplification and/or overexpression have been reported in primary breast cancer and also in ovarian, colorectal, and pancreatic adenocarcinomas (2–6). HER-2/neu has become an important target for cancer immunotherapy for several reasons. In particular, HER-2/neu is present in high proportions of tumor cells (7) and single tumor cells frequently show an intense expression of this molecule (8), suggesting that anti-HER-2/neu therapy would specifically target most tumor cells. Furthermore, CTL and IgG specific for HER-2/neu have been detected in patients with HER-2/neu+ breast cancer (9), demonstrating that this molecule is capable of inducing both cellular and humoral immune responses in vivo. Finally, tumor-reactive CTL responses have been induced in vitro using various MHC class I-binding synthetic peptides derived from the HER-2/neu sequence (9). Vaccination trials with HER-2/neu CTL peptides have in their majority included epitope p369–377 (10–12). In many of these studies, vaccine-specific CTL could be generated, which recognized HER-2/neu (369–377) on indicator tumors. In other series of studies, patients were immunized with mixtures of potential T peptides, which encompassed CTL epitopes (13–15). Most of the vaccinated patients developed long-lasting T cell immunity to HER-2/neu CTL epitopes and exhibited increased frequencies of T lymphocytes specifically recognizing the vaccine.

HER-2/neu peptide-based vaccinations have, so far, produced modest clinical responses. Because HER-2/neu is a self protein, immune tolerance mechanisms may hamper the development of an antitumor immunologic response in vivo (16–18). To this end, several approaches are now being invented and applied in preclinical models to circumvent tolerance against HER-2/neu resulting in persistent and efficient antitumor responses. Such methodological approaches are mainly based on the selective depletion of T regulatory (Treg) cells either without any further immunomodulation (18, 19) or combined with peptide vaccination (20). Plasmid DNA vaccination and regimens involving active immunization combined with administration of TLR agonists efficiently prevented and/or delayed the onset of mammary carcinomas (21–23). In our recent study (24), we have shown that immunization with a xenogenic HER-2/neu CTL peptide induced enhanced rejection of a transplantable HER-2/neu+ tumor expressing the self-CTL epitope. Translation of these modalities in phase I/II trials may

4Cancer Immunology and Immunotherapy Center and 5Breast Cancer Clinic, St. Savvas Cancer Hospital, Athens, Greece; 7Pathophysiology Department, Laikon General Hospital and Medical School, National and Kapodistrian University of Athens, Athens, Greece; and 9Interdisciplinary Research Institute, Universite Libre de Bruxelles, Brussels, Belgium

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2 Address correspondence and reprint requests to Dr. Angelos D. Gritzapis, Cancer Immunology and Immunotherapy Center, St. Savvas Cancer Hospital, 171 Alexandras Avenue, 11522 Athens, Greece. E-mail address: adgritzapis@ciic.gr

3 Abbreviations used in this paper: Treg, T regulatory; MFI, mean fluorescence intensity; PENT, pentamer.

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provide the basis for improved clinical outcome. However, in parallel with studies aiming at improving the effectiveness of active immunizations with known HER-2/neu CTL epitopes, it is mandatory to identify novel immunogenic HER-2/neu CTL epitopes for further development of HER-2/neu-based treatment strategies.

In this study, we used SYPFEITHI, which is a database for the prediction of MHC class I ligands and PAProC, a prediction algorithm for proteasomal cleavages, for the selection of potential HER-2/neu CTL epitopes. In this way, we could identify a HER-2/neu decamer spanning residues 85–94 (HER-2(1085)) which was used to detect HLA-A2 on individuals’ PBMC along with an anti-CD25-PE mAb (clone 2A3) (BD Biosciences) and goat-anti-mouse IgG microbeads. Phenotype studies showed that more than 90% of effector CD8+ cells was performed (see above). In those wells where PENT had been added, PENT was tested for dose-dependent binding to T cells in a HLA-A*0201 stabilization assay, as described previously (28). In brief, T cells were incubated overnight in serum-free RPMI 1640 (Biochrom) in the presence of 10 ng/ml β2-microglobulin (Sigma-Aldrich) without (background immunofluorescence) or with peptide over a range of concentrations from 1 nmol/L to 10 μmol/L. Stability of HLA-A*0201 was assessed by flow cytometry after staining the cells with the BB7.2 mAb and FITC-conjugated anti-mouse IgG (DakoCytomation). Results are expressed as fold of increase of mean fluorescence intensities (MFI) in the presence of peptide relative to MFI without peptide.

Measurement of peptide/HLA-A*0201 complex stability

The method for this has been reported previously (29). In brief, T cells were incubated overnight at 37°C without peptide or with 1 μmol/L of peptide in serum-free RPMI 1640 (Biochrom) supplemented with β2-microglobulin at 100 ng/ml. Next, cells were incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 1 h, washed, and further incubated for 0, 2, 4, and 6 h. Subsequently, cells were stained with BB7.2 mAb followed by staining with a FITC-conjugated anti-mouse IgG (DakoCytomation). MFI was measured at 0 h was considered as 100%. MFI measured at all other time-points are expressed relative to MFI at 0 h and calculated as follows: [MFI (0 h) – MFI (2, 4, or 6 h)/MFI (0 h)] × 100.

Ex vivo analysis of HER-2(1085)-specific T cells

Heparinized peripheral blood samples were obtained from each patient (or healthy donor) and PBMC were isolated by density gradient centrifugation. PBMC were cultured, in parallel with autologous irradiated (3000 rads) PBMC, using goat-anti-mouse IgG (DakoCytomation) as a second Ab, in a standard indirect immunofluorescence assay.

Materials and Methods

Patients and healthy donors

PBMC from HLA-A*0201+ (n = 16) and HLA-A*0201− (n = 10) breast cancer patients and HLA-A*0201+ (n = 16) healthy subjects were collected. HLA-2/neu status for breast cancer patients was +3, as determined by immunohistochemical staining. The investigation was approved by the Institutional Ethics Committee and written informed consent was received from all individuals. The BB7.2 mAb (a gift from Prof. G. Rammensee, Department of Immunology, University of Tuebingen, Tuebingen, Germany) was used to detect HLA-A*2.1+ HER-2/neu tumor cell line. HER-2(1085) also induced CTL in vivo and protected HHD mice against the growth of transplantable tumors.

Cell lines

The HER-2/neu overexpressing ovarian cancer cell line SKOV3 (donated by C. G. Ioannides, Department of Gynecologic Oncology and Immunology, University of Texas, Austin, TX and M.D. Anderson Cancer Center, Houston, TX) was maintained in culture in αMEM medium (Biochrom) supplemented with 10% FCS (Biochrom). The HER-2/neu overexpressing breast cancer cell line SKBR3 was purchased from the American Type Culture Collection and was also grown in αMEM medium. The murine ALC lymphoma cell line was provided by Prof. R. Kiessling (Unite d’Immunité Cellulaire Antivirale, Institut Pasteur, Paris, France).

All mice were maintained in pathogen-free conditions in the animal facilities of our center, all protocols were reviewed by the St. Savvas Cancer Hospital competent authority for compliance with the Greek and European regulations on Animal Welfare and with Public Health Service recommendations.

Epitope prediction

Epitope prediction was done as described previously (26). In brief, potential HLA-A*0201 ligands from the sequence of HER-2/neu were isolated using a matrix pattern suitable for calculations of peptides fitting to the HLA-A*0201 motif. Such motif predictions are available at www.syfeithi.de. Epitope prediction was done according to the algorithm for cleavages by human proteasomes.

Peptide synthesis

The decamer HER-2/neu (85–94) LIAHNQQVRQV [HER-2(1085)] and control gp100-derived peptide gp (154–162) [gp9(162)] were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer using Fmoc-chemistry and analyzed by mass spectrometry. Peptides were >95% pure as analyzed by reverse-phase HPLC. The influenza flu matrix peptide (aa 58–66; flu (58–66)) was purchased from EZBiolab.

T2 binding assay

HER-2(1085) was tested for dose-dependent binding to T cells in a HLA-A*0201 stabilization assay, as described previously (28). In brief, T2 cells were incubated overnight in serum-free RPMI 1640 (Biochrom) in the presence of 100 ng/ml β2-microglobulin (Sigma-Aldrich) without (background immunofluorescence) or with peptide over a range of concentrations from 1 nmol/L to 10 μmol/L. Stability of HLA-A*0201 was assessed by flow cytometry after staining the cells with the BB7.2 mAb and FITC-conjugated anti-mouse IgG (DakoCytomation). Results are expressed as fold of increase of mean fluorescence intensities (MFI) in the presence of peptide relative to MFI without peptide.

In vitro generation of HER-2(1085)-specific CTL

Unfractionated or CD4+ CD25+ Treg cell-depleted PBMC from HER-2/neu−, HLA-A*0201+ breast cancer patients were used for the generation of HER-2(1085)-specific CTL. CD4+ CD25+ Treg cells were depleted from PBMC before cell culture in a two-step procedure using anti-CD25-PE mAb (clone 2A3) (BD Biosciences) and goat-anti-mouse IgG microbeads (Miltenyi Biotec) according to the manufacturer’s procedures. In brief, in a first step, PBMC were labeled with anti-CD25-PE mAb and, in a second step, CD25+ cells among PBMC were depleted using goat-anti-mouse IgG microbeads. Phenotype studies showed that PBMC were totally devoid of CD4+ CD25+hi cells (data not shown). The Treg cell-depleted PBMC were cultured, in parallel with autologous total PBMC, in the presence of 10 μg/ml of HER-2(1085) or control flu (58–66) peptide in RPMI 1640 culture medium supplemented with 10% FCS, antibiotics, 10% II/2, 2% IL-2 (Chiron) and 5 ng/ml of IL-7 (Immunotools). Cells were plated at 2 × 106 cells/ml in 96-well U-bottom plates (Costar/Corning) and placed in an incubator with 5% CO2 and humidified atmosphere. Weekly restimations were performed with irradiated autologous PBMC pulsed with HER-2(1085) at a ratio of 5:1 CTL to autologous irradiated (3000 rads) PBMC. Every other day, fresh IL-2 was added at 10 IU/ml. At 5–7 days after the last restimulation, cells were harvested, washed, and resuspended in fresh RPMI 1640 culture medium, supplemented with 10% FCS, and plated in 96-U-bottom plates at 1 × 106 cells/well together with 0.5 × 106 targets (either SKBR3.A2 or mock transfectants of SKBR3). One hour after the start of stimulation, 10 μg/ml of brefeldin A were added to each well. An FITC-labeled anti-CD107a mAb (BD Biosciences) was added toward the half of the wells. After 2 h of stimulation in a CO2 incubator, flow cytometric PE-labeled HER-2(1085) pentamer analysis of effector CD8+ cells was performed (see above). In those wells where anti-CD107a had been added, PENT+/CD8+ cells were also analyzed.
for CD107 expression. In the rest of the wells, intracellular IFN-γ staining was performed using a Fix and Perm kit (Caltag) and FITC-conjugated anti-IFN-γ mAb (BD Bioscience). Stained cells were analyzed on a FACSCalibur.

In vivo generation of HER-2(1085)-specific CTL

In vivo generation of HER-2(1085)-specific CTL was tested in HHD mice. Mice were immunized according to our protocol described recently (24). This included three s.c. injections every 5 days (i.e., days 0, 5, and 10) at the base of the tail, each one consisting of 100 μg HER-2(1085) emulsified in 200 μl IFA, and 5 i.p. injections (i.e., days 2, 4, 6, 8, and 10) with 100 ng of recombinant murine GM-CSF (Endogen) in 0.3 ml of PBS per injection. Splenic CD8+ T cells from the immunized mice were then used as effectors in cytotoxicity assays.

Cytotoxicity assay

HHD mice were immunized with HER-2(1085) in IFA and GM-CSF as above. One day following the last injections (i.e., day 11), CD8+ T cells were isolated from total immune splenocytes by negative selection using the CD8+ T cell isolation kit (Miltenyi Biotec). Cytotoxic activity of splenic CD8+ CTL from immunized HHD mice was determined by standard chromium release assay, as described (30). In brief, effector CTL in 100 μl medium were placed in 96-well-U-bottom plates (Costar). Tumor cell targets were labeled with sodium chromate (Radiochemical Centre) and added (5 × 10^4 cells in 100 μl) to effectors at the indicated ratios. For peptide recognition, T2 cells were incubated overnight together with 20 μg/ml peptide, washed, and then labeled. After 4 h of incubation in a CO2 incubator, percent cytotoxicity was determined according to the formula: percent lysis = 100 × (test 51Cr release – spontaneous 51Cr release)/(maximum 51Cr release – spontaneous 51Cr).

Protective tumor model

This was performed as recently described (24). In brief, HHD mice were immunized with 100 μg of either HER-2(1085) or control gp(9154) peptide in IFA plus GM-CSF, as above. One day after the last injection, mice were inoculated s.c. with 2 × 10^4 transplantable ALC.A2.1.HER tumor cells. Mice were observed for survival for >150 days. Tumor size was expressed in IFA plus GM-CSF, as above.
as the product of the perpendicular diameters of individual tumors (in mm²). The observation was terminated with euthanasia when tumor mass grew up to 200–250 mm² in size.

Therapeutic tumor model

The procedures for this model were actually the same as those described recently (24). In brief, HHD mice were inoculated s.c. with 2 × 10⁴ \( \text{H11003}^{\text{ALC.A2.1.HER}} \) tumor cells. Once palpable tumors were established (i.e., 22–26 days after tumor inoculation), mice were immunized with HER-2(1085), or gp(9154) in IFA plus GM-CSF, as above. In the experiments shown in Fig. 7, we compared the efficacy of IFA or GM-CSF, administered separately, as immunological adjuvants for vaccination against HER-2(1085). For this, HHD mice received HER-2(1085) in 1) IFA plus normal saline, 2) IFA plus GM-CSF, and 3) GM-CSF plus normal saline.

Statistics

Tumor sizes among the respective groups were compared with Wilcoxon’s rank-sum test. Kaplan-Meier curves were plotted for survival analyses. All \( p \) values were two tailed and considered significant at \( p < 0.05 \).

Results

Binding of HER-2(1085) to HLA-A*0201 molecules

Prediction of the candidate 10-mer epitope LIAHNQVRQV spanning residues 85–94 from HER-2/neu oncoprotein, [HER-2(1085)] was done according to the HLA-A*0201 motif using the SYFPEITHI algorithm. Based on the presence of isoleucin (I) at position 2 and glutamic acid (Q) at positions 6 and 9, HER-2(1085) has a binding score of 21 for HLA-A*0201. In addition, according to the PAProC algorithm, this peptide has sites for proteosomal cleavages that allow its natural processing (proteasome type I cleaves HER-2/neu at positions 85 and 94). The capacity of HER-2(1085) to bind to HLA*0201 and to form stable peptide/HLA-A*0201 complexes was tested in standard functional T2 binding and MHC peptide stability assays, respectively. As shown in Fig. 1, A and B, this peptide exhibited a comparable strong binding and stabilization capacity compared with peptides having high binding activity for HLA-A*0201 molecules, such as HER-2(9369), which represents an immunogenic epitope of HER-2/neu (9, 12, 14) and flu (58–66) (24).

Generation of HER-2(1085)-specific CTL in vivo

We next sought to investigate whether active immunization with HER-2(1085) could generate CTL, specifically recognizing this peptide either pulsed onto T2 cells or naturally expressed on human tumor cell lines. For this purpose, we immunized HHD mice with HER-2(1085) in IFA plus GM-CSF, according to the protocol described in Materials and Methods. CD8⁺ T cells isolated from total immune splenocytes were subsequently used as effectors in cytotoxicity assays against various targets. As shown in Fig. 2, such effectors efficiently lysed T2 cells pulsed with HER-2(1085);
Ex vivo detection of HER-2(1085)-reactive T cells in patients with breast cancer

To verify recognition of the HER-2(1085) epitope by human specific CD8+ T cells, PBMC from HLA-A*0201+ breast cancer patients whose tumors overexpressed HER-2/neu were tested ex vivo for recognition of HER-2(1085) synthetic peptide, using flow cytometric HLA-A2*0201+ pentamer analysis. Among the HER-2/neu+ patients examined, positive were defined as those with ex vivo frequencies higher than 0.30% which is calculated from the highest frequency detected in healthy donors’ CD8+ T cells (i.e., 0.18%) plus 2 SD of the mean value for this group (i.e., 2 × 0.06%) (Fig. 3A). Of 16 patients tested, 11 were negative having fewer than 0.30% PENT+/CD8+ cells while the percentage of HER-2(1085) PENT+/CD8+ in the positive ones (n = 5) ranged from 0.35–0.70 (Fig. 3A). In contrast, the frequencies of HER-2(1085) PENT+/CD8+ cells among all HER-2/neu+, HLA-A*0201+ breast cancer patients (n = 10) and healthy donors (n = 16) tested, were equal or below 0.13 and 0.18%, respectively; Fig. 3A).

Fig. 3B, shows two representative dot plots from ex vivo analysis of HER-2/neu+ HLA-A*0201+ patients’ PENT+/CD8+ cells.

Generation of functionally active HER-2(1085)-specific CTL from Treg-depleted PBMC

The potency of HER-2(1085) to induce specific CTL responses upon vaccination in HHD mice (Fig. 2) may not necessarily reflect an analogous situation in humans. To assess whether this peptide is also capable of generating human CTL responses, unfractionated PBMC from patients who have been scored as positive in the previous figure (Fig. 3A) were stimulated and restimulated over a period of a total of 15–21 days, with irradiated peptide-pulsed autologous PBMC in the presence of IL-7 and/or IL-2. As it has been reported by other groups that Treg cells prevent in vitro expansion of tumor-reactive T cells (31, 32), we also did HER-2(1085)-specific CTL generation following the depletion of CD4+CD25+ Treg cells. Our data depicted in Fig. 4 show that stimulation of Treg cell-depleted PBMC from an HLA-A*0201+ patient with HER-2/neu+ breast cancer (patient no. 3) with HER-2(1085), in the presence of IL-2 and IL-7, resulted in the induction of 19.19% of HER-2(1085) PENT+/CD8+ T cells. Functionality of these cells was determined by intracellular IFN-γ staining and in the high sensitivity CD107a degranulation assay (33) upon recognition of the SKBR3-A2 breast cancer cell line. As also shown in Fig. 4, a high percentage (76.44%) of HER-2(1085) PENT+/CD8+ cells stained positive for intracellular IFN-γ. In addition, the vast majority of PENT+/CD8+ cells (90.45%) were also positive for CD107a membrane expression (Fig. 4), demonstrating the potential for granule-dependent perforin/granzyme-mediated cytotoxicity. Without Treg cell depletion, only 3.46% of HER-2(1085) PENT+/CD8+ cells could be generated from the same patient (Fig. 5). In another HER-2/neu+, HLA-A*0201+ patient (patient no. 5), 8.33% of HER-2(1085) PENT+/CD8+ T cells were generated by the stimulation of Treg cell-depleted PBMC (Fig. 4), which were overpopulated by IFN-γ− (71.11%) and CD107a− (91.12%) cells. Without CD4+CD25+ Treg cell depletion, only 2.95% of PENT+/CD8+ cells could be generated from patient no. 5 (Fig. 5). Similar results were also obtained with HLA-A*0201−, HER-2/neu− patients no. 8 and no. 9, where a 4- and 7.5-fold enhancement respectively in the percentage of functionally active HER-2(1085) PENT+/CD8+ cells generated in Treg cell-depleted PBMC cultures was noticed (Fig. 4) compared with unfractionated PBMC cultures (Fig. 5). Functional specificity of PENT+/CD8+ cells was assessed after incubation with mock transfectants of SKBR3 cells (not expressing HLA-A*0201), where only negligible percentages of both PENT+/CD8+IFN-γ+ and PENT+/CD8+/CD107− (below 3%) could be detected in all cases (data not shown). In addition, no staining with HER-2(1085) PENT+ and anti-CD8 could be observed, in control cultures with regulatory T cell-depleted PBMC stimulated under the same conditions but with the flu (58–66) peptide (0–0.08%; data not shown).

Inhibition of ALC.A2.1.HER tumor growth after HER(1085) vaccination of syngeneic HHD mice

We next tested the efficacy of HER-2(1085) to induce antitumor protective immunity in HHD mice, inoculated with transplantable ALC tumors coexpressing both human HER-2/neu and HLA-A*0201 (ALC.A2.1.HER). In our recent report (24), we have demonstrated that protective vaccination with a HER-2/neu peptide emulsified in IFA elicits the most effective antitumor immunity when GM-CSF is coadministered. By applying the same protocol, a significant protection against the growth of ALC.A2.1.HER in HHD mice was observed. From this group of mice, two of eight became long-term survivors, whereas the rest (six) survived up to day 158 post tumor cell inoculation (Fig. 6A). In contrast, mice
receiving the control gp(9154) peptide, GM-CSF, or no treatment, lived significantly less (range of survival in these groups: 68–87 days post tumor inoculation) (Fig. 6; \(p < 0.01\)).

We also explored the therapeutic efficacy of HER-2 (1085) in the potential treatment of established tumors. According to this, HHD mice were inoculated with ALC.A2.1.HER tumor cells and, once tumors were palpable, mice were treated by multiple injections of HER-2 (1085) and GM-CSF (24). As shown in Fig. 6B, a highly pronounced delay of tumor growth was observed when mice were treated in this way. Three of eight mice were cured whereas in the remainder (\(n = 5\)) tumor growth was significantly delayed compared with untreated mice or mice treated with control peptide gp(9154) (Fig. 6B; \(p < 0.005\)). Mice which survived resisted a second challenge with ALC.A2.1.HER but not with the wild-type ALC or the single transfectants ALC.HER or ALC.A2.1 (data not shown).

Next, mice were vaccinated with HER-2 (1085) using either IFA or GM-CSF, and the therapeutic efficacy of these vaccines was compared with our standard protocol, which includes both adjuvants. As expected (24), vaccination with peptide in IFA slightly,
through significantly, increased the period of time required to develop large tumors (i.e., > 200 mm² size). These were observed by days 70–90 post tumor inoculation (p < 0.05 compared with untreated HHD mice; Fig. 7). In contrast, administration of vaccinations with peptide plus GM-CSF induced a strong antitumor effect (two of seven mice rejected their tumors and the rest developed large tumors between days 100–140; Fig. 7). Although there was a trend for increased survival in the peptide/IFA/GM-CSF group (in three of seven mice the tumor regressed and in the remainder, large tumors were developed by days 130–150; Fig. 7) compared with the peptide/GM-CSF group, the differences were not statistically significant.

**Discussion**

In this study, we describe a novel HER-2/neu-derived HLA-A*0201-binding immunogenic epitope [HER-2(1085)] and confirm natural processing and expression. HER-2(1085) also proved to be efficient in vivo both as preventive and therapeutic vaccine and in vitro by generating HER-2(1085)-specific CD8 T cells from the peripheral blood of HER-2/neu+ HLA-A*0201+ breast cancer patients. In accordance with recent studies of NY-ESO-specific CD4+ T cell responses (31) and WT1-specific CD8+ T cell responses (32) in cancer patients, increased percentages of HER-2(1085)-specific CD8+ T cells among our breast cancer patients’ PBMC could only be generated following the depletion of Treg cells. This suggests that the HER-2/neu expressing patients’ tumor might induce a HER-2/neu-specific effector CD8+ T cell response, which was inhibited by Treg cells.

When tested in functional assays, the affinity of HER-2(1085) for binding to HLA-A2 molecules was comparable to that of HER-2(9369), which has already been classified as a good binder because it binds to HLA molecules with high affinity (34) and is strongly immunogenic (35). Moreover, we could demonstrate similar slow off-kinetic profiles for HER-2(1085) and HER-2(9369). Thus, the strong immunogenicity of HER-2(1085) might be explained by its enhanced ability to remain bound to MHC class I molecules on APC for a longer period, allowing better presentation to CTLs. It is also possible that the increased immunogenicity of HER-2(1085) might be related to increased functional binding activity for T cells (36). This might be the indirect result of the increased stability of the complexes (i.e., TCR-MHC-peptide), allowing more avid TCR contact.

We provide evidence that breast cancer patients posses in their peripheral blood CD8+ T cells specifically recognizing HER-2(1085). Thus, 5 of 16 patients examined (31.25%) were scored as positive having in their peripheral blood 0.35–0.70% CD8+ T cells which stained intensively with the HLA-A2/HER-2(1085) pentamer. This observation represents, to our knowledge, the first demonstration of HER-2/neu-specific T cells detected ex vivo. The relatively high percentages of peptide-specific CD8+ T cells detected ex vivo can be attributed to autovaccination, induced by the release of tumor Ag from dying apoptotic/necrotic cells as the result of standard chemotherapy applied to the patients before this study. At the present time, we do not have any understanding as to the reasons why some patients have increased percentages of circulating HER-2(1085)PENT+/CD8+ T cells while others do not. If we consider that the population of CD8+ T cells that recognizes HER-2(1085) is monocular, it is possible to attribute the difference to a stochastic limiting event, which may be difficult to identify, such as the encounter between an initial CTL precursor and the peptide presented in the context of HLA-A2 on APCs (e.g., the autologous tumor or dendritic cells in case of cross-processing). In contrast if HER-2(1085) is recognized by CD8+ T cells expressing a limited set of TCR Vα and Vβ genes (oligoclonal recognition) different explanations must be found. One possible explanation is that the prevaccination state of the immune system of the patients is different. For instance, some patients might have acquired immune tolerance for their tumor Ag (which may be associated with increased percentages of Treg cells). Another possibility is that the sensitivity of the tumors to immune attack varies widely among patients and that detectable CTL expansions can only be recorded following a successful interaction of responder CTL with the tumor.

The HHD model is mostly relevant at identifying HLA-A*0201-restricted peptides as vaccine candidates. To the best of our knowledge, ALC.A2.1.HER, along with the recently described D122 clone from Lewis lung carcinoma cotransfected to express HLA-A2.1 and prostatic acid phosphatase (37), are the only carcinoma models in HHD mice and therefore represent a powerful tool for assessing the efficiency of peptide-based vaccines. According to our active immunization results, HHD mice prophylactically vaccinated with HER-2(1085) emulsified in IFA plus GM-CSF showed a significantly delayed ALC.A2.1 HER tumor growth and some of them became even long-term survivors. Mice that rejected their tumors became resistant to a second challenge with the same tumor but not the wild-type (ALC) or its single transfectants (ALC.A2.1 or ALC.HER) (data not shown). These type of experiments demonstrated the specificity of the in vivo antitumor response as well as the capacity of our vaccine to induce immunological memory. Importantly, vaccinations with HER-2(1085) plus GM-CSF proved also to be successful in therapeutic settings. There was a trend for a more efficient antitumor activity in vivo induced by peptide/IFA/GM-CSF vs peptide/GM-CSF vaccination, but to a modest level. Both IFA (Montamide ISA-51) and GM-CSF have been used as vaccine adjuvants in clinical trials without causing any significant toxicities (12, 38–41). The design of phase I clinical trials with patients assigned randomly to receive peptide in GM-CSF alone or in IFA plus GM-CSF will be definitely more informative about the effectiveness of these protocols in humans.

In clinical trials, there have been attempts to eliminate the negative immunoregulatory effects of Treg cells in vivo and potentiate tumor-specific immune responses (42, 43). In two studies (42, 44), reduction of circulating Treg cells before vaccination improved stimulation of antitumor effector T cells compared with vaccination alone. These results are encouraging and suggest that selective depletion of Treg cells in vivo before administration of cancer vaccination may elicit objective clinical responses. The dramatic increase in the frequencies of antitumor CTL among T reg cell-depleted patients’ PBMC during in vivo priming with HER-2(1085), strongly supports the concept of in vivo T reg cell depletion before vaccination. Whether Tregs suppress HER-2(1085)-specific CTL directly or through APCs remains to be determined (45, 46). In our culture condition, Tregs may directly suppress CTL induction or may inhibit APC, such as DC maturation and differentiation to DC to elicit CTL. These possibilities are not necessarily mutually exclusive. Additional experiments are surely needed to elucidate the mode of action of Tregs in our model.

Trastuzumab induced HER-2/neu receptor internalization has been shown to involve enhanced tumor lysis by HER-2/neu specific CTLs (10, 47, 48). Importantly, PBMC from breast cancer patients receiving a HER-2/neu vaccine showed increased recognition and lysis of HER-2/neu+ breast and ovarian tumor cell lines treated with trastuzumab (10). It has been postulated that treatment of patients with trastuzumab may increase the amount of peptide complexed and presented on MHC class I molecules that can be recognized by CTLs (47). Moreover uptake of HER-2/neu-trastuzumab immune complexes by dendritic cells in vivo may result in
more efficient CD8 HER-2/neu-specific responses (49). Combining HER-2/neu peptide vaccination with trastuzumab treatment may, therefore, result in improved clinical results. This could be accomplished through a more efficient killing of autologous tumor cells by both vaccine primed and expanded CTLs and trastuzumab-induced increases of HER-2/neu peptide expression on MHC class I molecules of tumor cells. The finding from our recent report (50) demonstrating decreased percentages of Treg cells, in breast cancer patients under treatment with trastuzumab, support further investigation into the use of HER-2(103) in vaccination trials alone or in combination with trastuzumab. The inclusion of HER-2(103) in multipeptide vaccines (e.g., together with HER-2(966) and HER-2(964), both of which are already in clinical trials (9, 51, 52)) offers another possibility for its potential use in HER-2/neu peptide-based vaccines.

In summary, by combining prediction algorithms for HLA-
A*0201 ligands and proteosomal cleavages, we have identified an immunogenic T cell epitope from the tumor Ag HER-2/neu. This epitope is of great interest for the analysis of T cell responses in tumor patients. Importantly, this epitope broadens the applicability of HER-2/neu-based treatment strategies.

Disclosures
The authors have no financial conflict of interest.

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