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ORIGINAL ARTICLE

Identification and characterization of a HER-2/*neu* epitope as a potential target for cancer immunotherapy

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Abstract Our aim is to develop peptide vaccines that stimulate tumor antigen-specific T-lymphocyte responses against frequently detected cancers. We describe herein a novel HLA-A*0201-restricted epitope, encompassing amino acids 828–836 (residues QIAKGMSYL), which is naturally presented by various HER-2/*neu*$^+$ tumor cell lines. HER-2/*neu*(828-836), [HER-2(9_{828})], possesses two anchor residues and stabilized HLA-A*0201 on T2 cells in a concentration-dependent Class I binding assay. This peptide was stable for 3.5 h in an off-kinetic assay. HER-2(9_{828}) was found to be immunogenic in HLA-A*0201 transgenic (HHD) mice inducing peptide-specific and functionally potent CTL and long-lasting anti-tumor immunity. Most important, using HLA-A*0201 pentamer analysis we could detect increased ex vivo frequencies of CD8$^+$ T-lymphocytes specifically recognizing HER-2(9_{828}) in 8 out of 20 HLA-A*0201$^+$ HER-2/*neu*$^+$ breast cancer patients. Moreover, HER-2(9_{828})-specific human CTL recognized the tumor cell line SKOV3.A2 as well as the primary RS.A2.1.DR1 tumor cell line both expressing HER-2/*neu* and HLA-A*0201. Finally, therapeutic vaccination with HER-2(9_{828}) in HHD mice was proven effective against established transplantable ALC.A2.1.HER tumors, inducing complete tumor regression in 50% of mice. Our data encourage further exploitation of HER-2(9_{828}) as a promising candidate for peptide-based cancer vaccines.

Keywords HER-2/*neu* · Peptide vaccines · HHD mice · CTL · Breast cancer

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Introduction

It has long been established that small peptide products derived from the proteolytic processing of proteins synthesized by tumor cells, when bound to MHC Class I molecules, can be recognized by specific cytotoxic T-lymphocytes (CTL) [1, 2]. Tumor peptide-specific CTL, adoptively transferred or in vivo activated by active immunization, exert anti-tumor therapeutic activity which usually results, at least as encountered in preclinical tumor models, in regression of established tumors [3]. Thus, the development of therapies aiming at cancer immunotherapy is critically dependent on the identification of tumor-associated antigens. The use of genetic approaches based on genomic or cDNA expression libraries [4, 5] along with the utilization of biochemical methods based on acid elution of MHC Class I-bound peptides [6, 7], have enabled the identification of several immunodominant tumor protein-derived epitopes recognized by CTL [4–8]. However, an alternative method for identifying tumor epitopes recognized by CTL

is based mainly on algorithms predicting the binding of the corresponding peptides on MHC Class I alleles [9, 10]. Nonetheless, in the search for identifying suitable tumor peptide epitopes and notwithstanding the nature of the methodology employed, it is the generation of peptide-specific CTL that has been proven to be critical in confirming the immunogenicity of the peptides in question [11, 12].

Overexpression of the HER-2/*neu* oncogene by a significant number of human carcinomas has been associated with more aggressive disease [13, 14]. Despite its high oncogenic potential, HER-2/*neu* oncoprotein has been considered as a promising target for cancer therapeutics as it has been proven to be immunogenic in vivo inducing specific CTL and IgG responses in a considerable subset of patients with HER-2/*neu* positive breast cancer [13]. Moreover, various MHC Class I- and Class II-binding peptides from the HER-2/*neu* sequence were also able to elicit in vitro tumor-reactive CTL and T helper (Th) responses, respectively [14–18]. However, synthetic peptides corresponding to HER-2/*neu* immunogenic epitopes have greatly failed to function as therapeutic vaccines in phase I/II trials [13, 19–22]. Due to the fact that HER-2/*neu* is a self protein, tolerance mechanisms may provide a serious obstacle toward the development of efficient anti-tumor responses in vivo [23–25]. Therefore, we [26] and others [25, 27–30] have invented and applied several approaches in preclinical models to circumvent tolerance against the HER-2/*neu* protein, resulting in more persistent and efficient anti-tumor responses [16, 25–29]. However, in parallel with the studies aiming at improving the effectiveness of a vaccine, it has been considered essential to identify novel immunogenic CTL epitopes, aiming at providing insight into the design of more efficacious peptide-based treatment modalities.

In this study, by applying the data obtained by prediction algorithms for MHC Class I ligands and proteasomal cleavage databases, we describe the identification of an immunogenic nonamer from the human HER-2/*neu* sequence, encompassing amino acids 828 to 836 (residues QIAKGMSYL), and reveal several features of the immune response to this epitope as well as of its anti-tumor properties both in vitro and in vivo. Our data clearly point to the fact that HER-2(9_{828}) could act as a potent cancer vaccine.

Materials and methods

Patients and healthy donors

Venous blood was drawn from HLA-A*0201$^+$ ($n = 20$) and HLA-A*0201$^-$ ($n = 10$) HER-2/*neu*$^+$ breast cancer patients and HLA-A*0201$^+$ ($n = 10$) healthy volunteers. Using a standard indirect immunofluorescence assay, detection of HLA-A*0201 on individuals' PBMC was enabled when staining with the BB7.2 mAb (a gift from Prof. H. G. Rammensee, Department of Immunology, University of Tuebingen, Tuebingen, Germany) along with a PE-conjugated anti-mouse IgG (DakoCytomation, Glostrup, Denmark), which was used as a second antibody. This investigation received approval from the Institutional Ethics Committee and a written informed consent from all individuals.

Mice

HHD mice are $\beta 2m^{-/-}$, $Db^{-/-}$, and express a HLA-A*0201 monochain composed of a chimeric H chain ($\alpha 1$ and $\alpha 2$ domains of HLA-A*0201 and the $\alpha 3$ intracellular domain of D^b) linked by its NH_2 terminus to the COOH terminus of the human β_2m [31]. HHD mice were provided by Prof. Francois Lemonnier (Unite d'Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France). Mice were maintained in pathogen-free conditions in the animal facilities of our center. All protocols were reviewed by the St. Savas Cancer Hospital competent authority in compliance with the Greek and European regulations on Animal Welfare and with Public Health Service recommendations.

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 Alpha MEM medium (Biochrom AG, Germany) supplemented with 10% FCS (Biochrom AG, Germany), with the addition of 2 mM *L*-Glutamine (Biochrom AG, Germany), 100 μg/ml gentamicin and 2.5 mM 2-mercapthoethanol (Promega, Madison, WI, USA). The murine ALC lymphoma cell line was provided by Prof. R. Kiessling (Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden). This was grown in vivo as ascites by serial passages in C57BL/6 syngeneic mice. HLA-A*0201 transfectants of SKOV3 (SKOV3.A2), and HLA-A*0201 and human HER-2/*neu* transfectants of ALC (ALC.A2.1.HER) were produced as recently described [26]. RS.A2.1.DR1 is a breast cancer cell line developed in our laboratory from primary tumor cells of a surgically excised HLA-A*0201$^+$ HLA-DR1$^+$ HER-2/*neu*$^+$ tumor. This was grown in vitro in Dubellco's MEM (Gibco, Carlsbad, CA, USA) medium supplemented with 10% FCS, 10% NCTC 109 medium (Gibco, Carlsbad, CA, USA), 2 mM *L*-Glutamine (Biochrom AG, Germany), 100 μg/ml gentamicin, 0.1 mM MEM non-essential amino acids (Gibco, Carlsbad, CA, USA), 0.5 mM sodium bicarbonate (Gibco, Carlsbad, CA, USA), 2.5 mM 2-mercapthoethanol (Promega, Madison, WI, USA), and 100 IU/ml human insulin. In all transfected cell lines, expression of

the transgene was maintained with the addition of 500 μg/ml Geneticin (Biochrom AG, Germany) in the culture medium.

Epitope prediction

Epitope prediction was done as described by isolating potential HLA-A*0201 ligands from the sequence of HER-2/*neu* using a matrix pattern suitable for calculation of peptides fitting to the HLA-A*0201 motif [10]. Such motif predictions are available at http://www.syfpeithi.de. Epitope prediction was combined with PAProC (http://www.paproc.de/) [32], which is a prediction tool for cleavages by human proteasomes, and peptide sequence alignment was performed using Blastp database (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Peptide synthesis

Peptides HER-2(9_{828}), HER-2(9_{657}), HER-2(9_{369}), and HER-2(9_{767}), derived from the human HER-2/*neu* sequence, as well as the gp100-derived peptide gp(154-162) [gp(9_{154})] and the influenza matrix peptide flu(58-66) were purchased from EZBiolab (EZ Biolab, Carmel, USA). Peptides were synthesized by standard solid-phase chemistry and analyzed by mass spectrometry. Peptide purity was >95% as analyzed by reverse-phase HPLC.

T2 binding assay

HER-2(9_{828}) was tested for dose-dependent binding to T2 cells in a HLA-A*0201 stabilization assay, as described recently [18, 26]. In brief, T2 cells were incubated overnight in serum-free RPMI 1640 (Biochrom AG, Germany) in the presence of 100 ng/ml β2-microglobulin (Sigma–Aldrich, Saint Louis, MO, USA) without peptides (background immunofluorescence) or with peptides [HER-2(9_{828}), HER-2(9_{369}), HER-2(9_{767}), flu(58-66)] over a range of concentrations from 1 nmol/L to 10 μmol/L. Stability of HLA-A*0201 was assayed by flow cytometry after staining the cells with the BB7.2 mAb and PE-conjugated anti-mouse IgG (DakoCytomation, Glostrup, Denmark). 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 off-kinetics assay has been reported recently [18]. In brief, T2 cells were incubated overnight at 37°C without peptide or with 10 μmol/L of peptides [HER-2(9_{828}), HER-2(9_{369}), HER-2(9_{767}), flu(58-66)] in serum-free RPMI 1640 (Biochrom AG, Germany) supplemented with β2-microglobulin at 100 ng/ml. Next, cells were incubated with Brefeldin A (10 μg/ml; Applichem GmbH, Germany) 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 PE-conjugated anti-mouse IgG (DakoCytomation, Glostrup, Denmark). MFI 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 h, 4 h, or 6 h)/MFI (0 h)] × 100.

In vivo generation of HER-2(9_{828})-specific CTL

In vivo generation of HER-2(9_{828})-specific CTL was tested in HHD mice by applying a protocol similar to that recently published by us [18]. Briefly, 6 to 8-week-old mice were injected thrice with HER-2(9_{828}) peptide (100 μg per injection emulsified in 100 μl Incomplete Freund's adjuvant subcutaneously at the base of the tail) every 5 days (i.e., days 0, 5, and 10). On the same days, GM-CSF was administered intraperitoneally at 100 ng per mouse in 100 μl PBS. Pooled splenic $CD8^+$ T cells from the immunized mice were used as effectors/responders in the following assays.

ELIspot assay

$CD8^+$ T cells were isolated from total immune HHD splenocytes (collected from killed animals one day or 30 days after the last injection) by negative selection using the $CD8^+$ T-cell isolation kit (Miltenyi Biotec, Auburn, CA, USA) [18]. Cells were pooled and plated in ordinary 96-U bottom plates at 0.5×10^5 cells/well in quadruplicates for 24 h at 37°C. Irradiated (3,000 rad) stimulatory-target cells were added to the $CD8^+$ T-cell responders at a cell ratio of 1:1 in a total volume of 200 μl/well RPMI 1640 culture medium, supplemented with 10% FCS. The next day, well contents were transferred into the 96-well flat-bottomed PVDF ELIspot plate and incubated for another 24 h. Washing steps as well as the visualization of spots were performed according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). IFNγ production was estimated by counting spots in the automated 4-plate ELIspot reader (Eli.Expert; A.EL.VIS GmbH, Germany) using the Eli.Analyse Software (Version V5.0) of the same company. Specific spots were calculated by subtracting the mean number of spots obtained in the control cultures (i.e., those set up in the absence of stimulatory cells) from the mean number of spots in each experimental group.

Flow cytometric degranulation assay

$CD8^+$ T cells were isolated from total immune HHD splenocytes (collected from killed animals one day after the last injection) by negative selection using the $CD8^+$ T-cell isolation

kit [18]. Cells were pooled, resuspended in fresh RPMI 1640 culture medium (supplemented with 10% FCS) and plated in 96-U-bottom plates at 0.5×10^5 cells/well together with an equal amount of the cancer cell line targets (E:T = 1:1). A 10 µl mix of FITC-labeled anti-mouse CD107a (clone 1D4B; BD Biosciences, San Jose, CA, USA) and anti-mouse CD107b (clone ABL-93; BD Biosciences, San Jose, CA, USA) mAb was also added to half of the wells. One hour after the start of stimulation, 10 µg/ml of Brefeldin A (Applichem GmbH, Germany) and 2 µM of Monensin (eBioscience, San Diego, CA, USA) were added to each well. Following a 6-h stimulation in a CO_2 incubator, cells were washed in 2 ml of wash buffer (2% FBS, 0.1% sodium azide in PBS), resuspended in residual liquid and stained with an APC-labeled anti-mouse CD8a mAb (clone 53-6.7; eBioscience, San Diego, CA, USA) for 20 min at 4°C. Cells were then fixed and permeabilized using the BD Cytofix/Cytopem™ Fixation/Permeabilization Kit (BD Biosciences, San Jose, CA, USA), according to the protocol provided by the manufacturer and intracellularly stained with a PE-conjugated anti-mouse IFNγ mAb (Clone XMG1.2; eBioscience, San Diego, USA). Flow cytometric analysis of effector IFNγ^+/CD107$^+$/CD8$^+$ cells was performed on a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software.

CFSE-based in vivo cytotoxicity assay

In vivo cytotoxicity assay was done as described recently with a few modifications [25]. Briefly, a single-cell suspension of 20×10^6 naive HHD splenocytes per 600 µl was labeled with two concentrations, 0.5 (CFSE^{low}) or 5.0 µmol/L (CFSE^{high}), of the fluorescent dye CFSE (Molecular Probes, Leiden, the Netherlands) for 10 min at 37°C. The reaction was stopped by the addition of equal volume of FBS followed by a 30 min incubation at room temperature. Splenocytes labeled with the highest concentration of 5.0 µmol/L (CFSE^{high}) were pulsed either with the HER-2(9_{828}) or with the HER-2(9_{657}) peptide for 1 h at room temperature. The two splenocyte populations (CFSE^{high} and CFSE^{low} cells) were mixed together in equal amounts and a total of 15×10^6 splenocytes were injected intravenously into each vaccinated HHD mouse. Mice were killed 48 h later, and single-cell suspensions from spleens were processed individually to evaluate the presence of CFSE^{high} and CFSE^{low} cells. The specific cytolytic activity was calculated as 100 × (percentage CFSE^{low} cells − percentage CFSE^{high} cells)/percentage CFSE^{low} cells.

In vivo therapeutic mouse model

Following a similar vaccination protocol as previously described [18], 6–8 week-old sex-matched HHD mice were inoculated subcutaneously with 5×10^4 ALC.A2.1.HER cells. As much as 10–12 days later, once palpable tumors were established, mice were vaccinated thrice with a 5-day interval subcutaneously at the base of the tail with either 100 µg of the HER-2(9_{828}) peptide [or peptide gp(9_{154})] emulsified in IFA or IFA alone. On the same days as above, GM-CSF was also administered intraperitoneally at 100 ng per mouse. Mice were observed for survival for >60 days. Tumor size was monitored every 4 days and was expressed as the product of the perpendicular diameters of individual tumors (in mm^2). The observation was terminated with euthanasia when tumor mass grew more than 250 mm^2 in size.

Ex vivo analysis of HER-2(9_{828})-specific T cells

Heparinized peripheral blood samples were collected from each patient or healthy donor and PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Biochrom AG, Germany). PE-labeled HLA-A*0201 pentamer (PENT) presenting the HER-2(9_{828}) epitope was provided by Proimmune (The Magdalen Centre, Oxford Science, Oxford, U.K.). The cellular staining protocol followed was provided by the manufacturer. Briefly, $1–2 \times 10^6$ PBMC were washed in 2 ml of wash buffer (0.1% sodium azide, 0.1% BSA in PBS), spun down (500*g* for 5 min), and resuspended in residual liquid (α50 µl). One test (10 µl) of PENT peptide was added to the cells and was mixed by pipetting. After incubation at room temperature for 10 min, cells were washed in 2 ml wash buffer, resuspended in residual liquid (50 µl), and further incubated for 20 min on ice with FITC-labeled CD45 (clone 2D1; BD Biosciences, San Jose, CA, USA) and APC-labeled anti-CD8 mAb (clone SK1; BD Biosciences, San Jose, CA, USA). Dead cell exclusion was performed by staining 10 min before analysis with the fluorescent dye 7-*Amino-actinomycin D* (7-AAD) (BD Pharmigen, San Jose, CA, USA). PENT$^+$/CD8$^+$ cells were analyzed on a FACSCalibur Flow Cytometer with the use of the CellQuest software.

In vitro generation of HER-2(9_{828})-specific CTL

CD4$^+$CD25$^+$ Treg cell-depleted PBMC from two HER-2/*neu*$^+$, HLA-A*0201$^+$ breast cancer patients (Patient 1 and 2) exhibiting the highest ex vivo HER-2(9_{828})-PENT$^+$/CD8$^+$ frequencies (0.030 and 0.045%, respectively) were used for the generation of HER-2(9_{828})-specific CTL. CD4$^+$CD25$^+$ Treg cells were depleted from PBMC prior to cell culture in a two-step procedure as described recently [18]. Briefly, PBMC were initially labeled with anti-CD25-PE mAb (clone 2A3) (BD Biosciences) following the depletion of CD25$^+$ cells among PBMC using goat-anti-mouse IgG microbeads (Miltenyi Biotec, Bergish-Gladbach,

Germany). Phenotype studies showed that PBMC were totally devoid of $CD4^{+}CD25^{high}$ cells (data not shown). In vitro stimulation of Treg cell-depleted PBMC with HER-$2(9_{828})$ was performed as recently described [18]. As much as 5–7 days after the last re-stimulation, cells were assessed for cytotoxic activity by standard chromium release assay, as described recently [18]. Briefly, in vitro-generated CTL were resuspended in 100 µl of fresh RPMI medium supplemented with 10% FCS and plated on 96-well-U-bottomed plates (Costar, Cambridge, MA). Tumor cell targets were labeled with sodium chromate (Radiochemical Centre, Amersham, The Netherlands), resuspended at a concentration of 0.3×10^5 cells/ml and added to effectors at the indicated ratios (E:T = 10:1, 20:1, 40:1). For peptide recognition, T2 cells were pulsed with either 20 µg/ml of the HER-$2(9_{828})$ or the HER-$2(9_{657})$ control peptide, washed and then labeled. After a 6 h incubation in a CO_2 incubator, % cytotoxicity was determined according to the following formula: % lysis = 100 × (test ^{51}Cr release − spontaneous ^{51}Cr)/(maximum ^{51}Cr release − spontaneous ^{51}Cr).

Statistics

All experimental data were evaluated using Student's *t* test at 95% confidence interval to determine the statistical significance of differences between groups, with $P < 0.05$ being considered significant.

Results

Binding of HER-$2(9_{828})$ to HLA-A*0201 molecules

Binding prediction of the candidate 9-mer epitope [HER-$2(9_{828})$] (QIAKGMSYL, spanning residues 828–836 from the HER-2/*neu* oncoprotein) to HLA-A*0201 allele, was performed using the SYFPEITHI algorithm. Based on the presence of the amino acids isoleucin (I) at position 2 and leucin (L) at position 9, HER-$2(9_{828})$ has a binding score of 22 for HLA-A*0201. In addition, according to the PAProC algorithm, this peptide has sites for proteasomal cleavages that allow its natural processing (proteasome type III cleaves HER-2/*neu* at positions 828 and 836). The capacity of HER-$2(9_{828})$ to bind to HLA*0201 and form stable HLA-A*0201/Peptide complexes was tested in standard functional T2 binding and MHC/peptide stability assays, respectively. As shown in Fig. 1a and b, this peptide exhibited a rather intermediate binding affinity and MHC/peptide complex stability, as compared with peptides displaying high binding affinity for HLA-A*0201 molecules, such as HER-$2(9_{369})$ and influenza (58-66) [26, 33, 34] and peptides defined as intermediate binders to the HLA-A*0201 molecule [i.e., HER-$2(9_{767})$] [12].

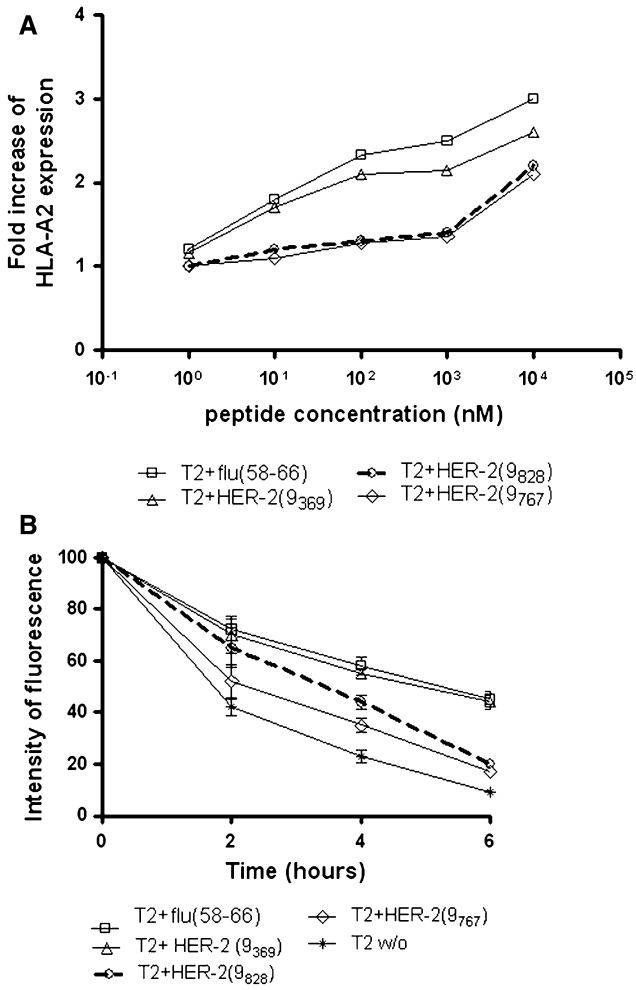

Fig. 1 **a** Binding of HER-$2(9_{828})$ to HLA-A*0201 molecules on T2 cells. Fold increase of MFI for HLA-A2.1 expression in the presence of peptides at the indicated concentrations over MFI observed after overnight incubation of T2 cells in plain medium. Control peptides flu(58-66) and HER-$2(9_{369})$ bind with high-affinity to HLA-A2.1 and HER-$2(9_{767})$ is an intermediate binder. One representative experiment of three performed is shown. **b** Stabilization of HLA-A*0201/peptide complexes on T2 cells. T2 cells were incubated with peptides (10 µmol/L) overnight, then washed, treated over 1 h with brefeldin A washed and subsequently incubated for the indicated times at 37°C. MFI are expressed relative to those observed in the initial labeling experiment, which was done after the treatment with brefeldin A (0 h) (considered as 100% of MFI). Mean values ± SD from three experiments are shown

Generation of HER-$2(9_{828})$-specific CTL in HHD mice

We next sought to investigate whether active immunization with HER-$2(9_{828})$ could generate $CD8^{+}$ T cells specifically recognizing this peptide as naturally processed and expressed on tumor cell lines. For this purpose, we immunized HHD mice with HER-$2(9_{828})$ in IFA plus GM-CSF, according to the protocol described in Sect. "Materials and methods". $CD8^{+}$ T cells isolated from total immune splenocytes were subsequently used as responders in IFNγ-based

ELIspot assays in vitro against various targets. As shown in Fig. 2a, such responders efficiently recognized the HER-2/*neu* overexpressing human tumor cell line SKOV3, transfected to express HLA-A*0201 (i.e., SKOV3.A2), as well as the mouse lymphoma cell line ALC.A2.1.HER, a double transfectant of HER-2/*neu* and HLA-A*0201 [18]. Significantly lower IFNγ responses were observed when the non-transfectants of the above cell lines (i.e., SKOV3 and ALC) were used as stimulators ($P < 0.001$ in both cases; Fig. 2a), which were comparable to those mediated by CD8$^+$ T-cell responders from control mice immunized with adjuvants (i.e., IFA plus GM-CSF) in the absence of peptide. Statistically significant differences between the peptide-immunized and non-peptide immunized HHD mice were also obtained against the human HER-2/*neu*$^+$, HLA-A*0201$^+$, HLA-DR1$^+$ RS.A2.1.DR1 cancer cell line ($P < 0.001$; Fig. 2a). Overall, these results suggest that HER-2(9_{828}) peptide is immunogenic in vivo and that this epitope is naturally expressed by HER-2/*neu*$^+$, HLA-A*0201$^+$ tumor cell lines.

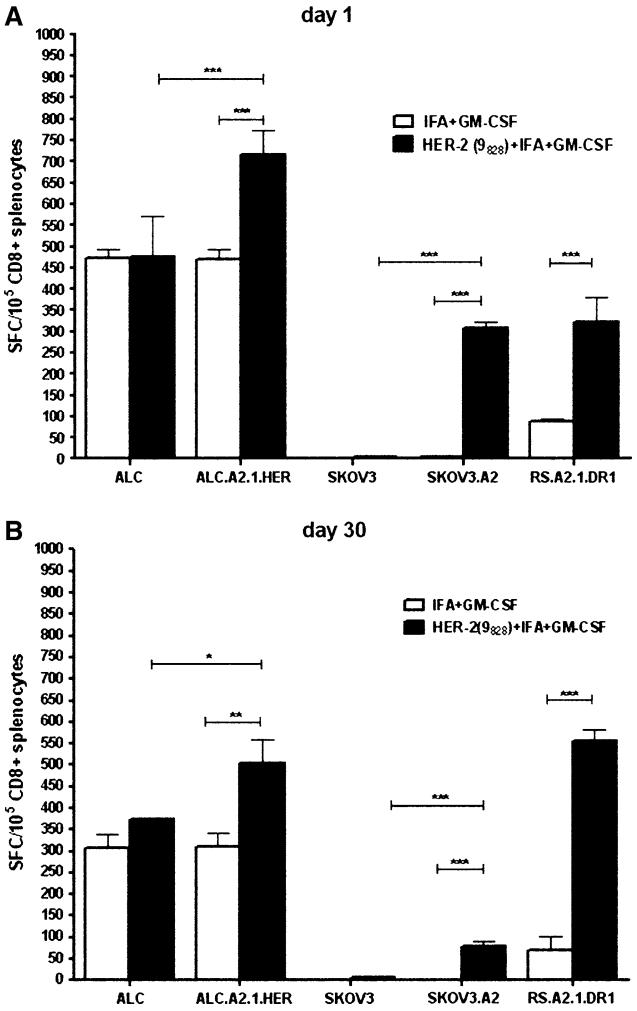

Fig. 2 Vaccination with HER-2(9_{828}) in IFA plus GM-CSF generates CTL in HHD mice recognizing HER-2/*neu*$^+$ HLA-A*0201$^+$ tumor cell lines. **a** One day after the last vaccination, CD8$^+$ splenic T cells from immunized HHD mice were tested as responders against stimulatory cell lines transfected to express both HER-2/*neu* and/or HLA-A*0201 (i.e., ALC.A2.1.HER and SKOV3.A2,) or non-transfected [i.e., ALC (HER-2/*neu*$^-$, HLA-A*0201$^-$) and SKOV3 (HER-2/*neu*$^+$, HLA-A*0201$^-$)]. The HER-2/*neu*$^+$, HLA-A*0201$^+$, RS.A2.1.DR1 cell line was also used as a stimulator. CD8$^+$ splenic T cells were plated in ELIspot plates with the indicated stimulatory cells at an E:T = 1:1 ratio. Following a 48 h incubation, spots indicative of IFNγ-producing CD8$^+$ T cells were counted. Five mice were vaccinated for each group and the mean ± SD values from the pooled data are shown. **b** Vaccination with HER-2(9_{828}) in IFA plus GM-CSF generates long-lasting immunity in HHD mice. As much as 30 days after the last vaccination, CD8$^+$ splenic T cells were plated in ELIspot plates with the same stimulatory cell lines as in (**a**), at a 1:1 ratio. After a 48 h incubation, spots indicative of IFNγ-producing CD8$^+$ T cells were counted. Five animals per group were vaccinated and the mean values ± SD from the pooled data are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

To evaluate the capacity of peptide vaccination to induce a long-lasting tumor-specific immune response, CD8$^+$ effector T cells were isolated from the spleens of HHD mice 30 days after immunization with HER-2(9_{828}) peptide and tested as in Fig. 2a. A similar pattern of IFNγ responses were observed in the ELIspot assay (Fig. 2b) as with CTL effectors collected just one day after immunization with HER-2(9_{828}) demonstrating that this peptide is capable of inducing memory anti-tumor CTL immunity lasting at least 30 days.

Similar results regarding the capacity of HER-2(9_{828}) to generate functionally active CD8$^+$ CTL can be deduced from the flow cytometric degranulation assay. In this assay, the potential for granule-dependent perforin/granzyme-mediated target cell killing was determined. Using flow cytometry, we were able to measure the cumulative exposure of granular membrane proteins CD107a/b on the cell surface of CTL, characterized as positive markers of degranulation, with the simultaneous staining of intracellular IFNγ secreted by CTLs [18, 35]. CD8$^+$ T cells from HHD immunized mice exhibited high levels of intracellular IFNγ and a high potential of granule-dependent perforin/granzyme-mediated target cell killing as detected by the increased surface expression of the granular membrane protein CD107 upon recognition of ALC.A2.1.HER, SKOV3.A2, and RS.A2.1.DR1 cancer cell lines (Fig. 3). Vaccination of HHD mice with IFA and GM-CSF in the absence of the peptide elicited a significantly weaker response as characterized by a low percentage of IFNγ^+/CD107$^+$ cells among the isolated splenic CD8$^+$ T cells upon encountering the ALC.A2.1.HER and SKOV3.A2 targets ($P < 0.05$ and $P < 0.001$, respectively; Fig. 3). Statistically significant differences among the two vaccinated groups of mice were also observed against the RS.A2.1.DR1 cell line, although at a lesser degree ($P < 0.05$; Fig. 3).

The relatively high background responses observed against certain stimulatory cell lines (e.g., ALC, RS.A2.1.DR1) in the above assays (Figs. 2, 3) may be attributed to an IFA plus GM-CSF induced non-specific activation of

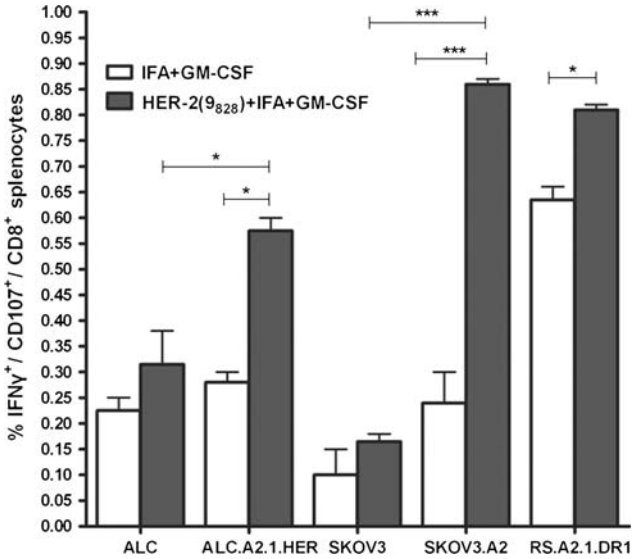

Fig. 3 Functional characterization of $CD8^+$ effectors from vaccinated HHD mice. $CD8^+$ splenic T cells freshly isolated from HHD animals vaccinated with HER-2(9_{828}) plus IFA and GM-CSF were incubated for 6 h with each of the indicated stimulators at an E:T = 1:1 ratio in the presence of an anti-CD107a/b-FITC mAb mix. The intracellular staining protocol for IFNγ is described in Sect. "Materials and methods". Five mice ($n = 5$) were immunized per group and the mean values ± SD from the pooled data are shown.*$P < 0.05$, *** $P < 0.001$

responder $CD8^+$ T cells cross-reacting with protein epitopes expressed by these cell lines.

Moreover, using a CFSE staining protocol we were able to track the peptide-specific CTL function in vivo by monitoring the presence and lysis of the peptide-pulsed target syngeneic splenocytes. HER-2(9_{828})-pulsed or HER-2(9_{657}) control peptide-pulsed (both groups labeled with a high concentration of CFSE; $CFSE^{high}$) and unpulsed (labeled with a low concentration of CFSE; $CFSE^{low}$) target HHD splenocytes were co-administered to HHD mice previously immunized with HER-2(9_{828}). As shown in Fig. 4a, HHD mice ($n = 4$) vaccinated with HER-2(9_{828}) exhibited high levels of in vivo cytotoxic responses against HER-2(9_{828})-pulsed syngeneic splenocytes, the mean cytotoxic response being 50.2 ± 19.2% (range 28.15% to 74.43%). The specificity of this response was shown by the significantly lower levels of cytotoxicity (mean cytotoxic response was 10.7 ± 6.9%) observed against inoculated HER-2(9_{657}) control peptide-pulsed syngeneic splenocytes (Fig. 4b; $P < 0.01$). In a second control experiment, by vaccinating HHD mice with IFA plus GM-CSF alone, we observed low levels of induced in vivo cytotoxicity against HER-2(9_{828})-pulsed syngeneic splenocytes (mean cytotoxic response of 8 ± 6.9%) (Fig. 4c; $P < 0.01$).

In vivo therapeutic mouse model

Furthermore, we explored the therapeutic efficacy of HER-2(9_{828}) in the potential treatment of established tumors. According to the protocol described in Sect. "Materials and methods", HHD mice were inoculated with syngeneic ALC.A2.1.HER tumor cells and, once tumors were palpable, mice were injected thrice with HER-2(9_{828}) in IFA and GM-CSF [18]. As shown in Fig. 5d, a highly pronounced delay of tumor growth was observed when mice were treated this way. Three out of six (50%) mice receiving the HER-2(9_{828}) plus IFA vaccine co-administered with GM-CSF were completely cured, whereas tumor growth in the remainders was significantly delayed compared to untreated mice (Fig. 5a, $P < 0.05$) or mice treated only with IFA (Fig. 5b, $P < 0.01$) or with the control peptide gp(9_{154}) in the presence of GM-CSF (Fig. 5c, $P < 0.05$).

Ex vivo detection of HER-2(9_{828}) reactive T cells in patients with breast cancer

To verify recognition of the HER-2(9_{828}) epitope by human specific $CD8^+$ T cells, PBMC from HLA-A*0201$^+$ breast cancer patients the tumors of which expressed HER-2/*neu* were tested ex vivo for recognition of HER-2(9_{828}) synthetic peptide, using flow cytometric HLA-A*0201$^+$ pentamer analysis. Among the HER-2/*neu*$^+$ HLA-A*0201$^+$ patients examined ($n = 20$), positive were defined as those with ex vivo frequencies higher than 0.015% which is calculated from the highest frequency detected in healthy donors' $CD8^+$ T cells (i.e., 0.009%) plus 2 SD of the mean value for this group (i.e., 2 × 0.003%) (Fig. 6a). Out of the 20 patients tested, 12 were considered negative having fewer than 0.015% $PENT^+/CD8^+$ cells while the percentage of HER-2(9_{828}) $PENT^+/CD8^+$ in the positive ones ($n = 8$) ranged from 0.02% to 0.045% (Fig. 6a). In contrast, the frequencies of HER-2(9_{828}) $PENT^+/CD8^+$ cells among all HER-2/*neu*$^+$, HLA-A*0201$^-$ breast cancer patients ($n = 10$) and HLA-A*0201$^+$ healthy donors ($n = 10$) tested, were equal or below 0.014% and 0.009%, respectively (Fig. 6a). Figure 6b shows one representative dot plot from the ex vivo analysis of $PENT^+/CD8^+$ T cells in each group of examined individuals.

In vitro generation of HER-2(9_{828})-specific CTL from PBMC

To assess whether HER-2(9_{828}) was also capable of generating CTL responses in humans, we applied an in vitro sensitization protocol recently published by us [18] using Treg ($CD4^+CD25^+$)-depleted PBMC from patients who had been scored as positive in the previous figure (Fig. 6a). As evident from previous studies [18, 36, 37], depletion of Tregs was proven necessary so as to obtain increased frequencies of tumor-peptide specific CTL. Data from Fig. 6c signify the capacity of patients' CTLs, generated after in vitro stimulation as described in Sect. "Materials and methods",

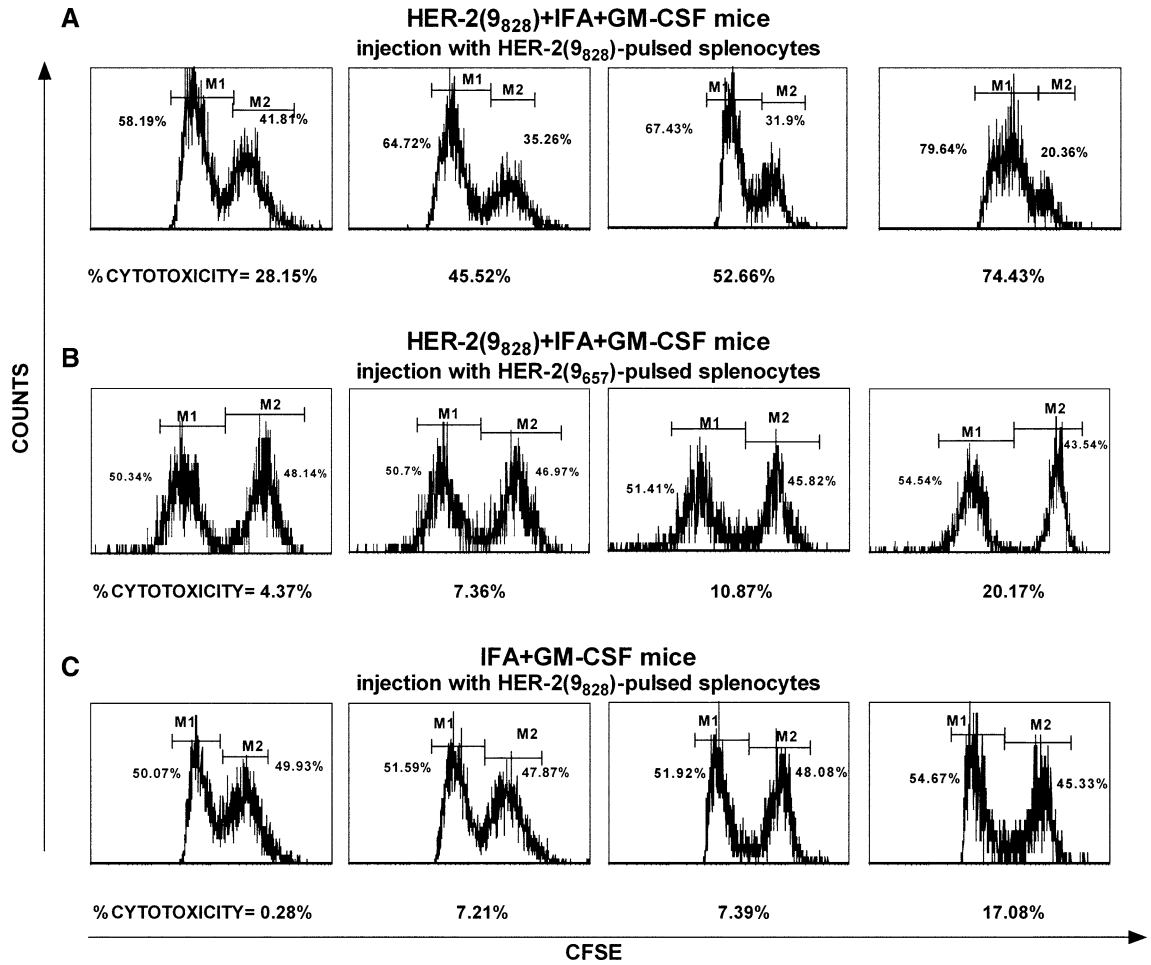

Fig. 4 In vivo cytotoxicity of HHD mice immunized with HER-2(9_{828}). **a**, **b** Individual histograms from HHD mice immunized with HER-2(9_{828}) peptide and injected i.v. with a mix of 15×10^6 syngeneic splenocytes that consisted of 7.5×10^6 unpulsed cells which were stained with 0.5 μmol/L CFSE ($CFSE^{low}$ cells) and (**a**) 7.5×10^6 HER-2(9_{828})-pulsed cells or (**b**) 7.5×10^6 HER-2(9_{657})-pulsed cells both stained with 5 μmol/L CFSE ($CFSE^{high}$ cells). Histograms of individual animals vaccinated with IFA plus GM-CSF, but in the absence of peptide, are shown (**c**). 48 h after injections, splenocytes were isolated and immunofluorescence was evaluated by flow cytometry. The percentage of cytotoxicity for CFSE-labeled splenocytes for each animal was calculated as follows: 100 × (percentage $CFSE^{low}$ cells – percentage $CFSE^{high}$ cells)/percentage $CFSE^{low}$ cells. M1 and M2 markers refer to $CFSE^{low}$ and $CFSE^{high}$ cells, respectively. $P < 0.01$ in all cases

to effectively lyse HER-2(9_{828})-pulsed T2 cells (up to 46.34% for patient 1 and 45% for patient 2) as opposed to a minimal lysing effect against T2 cells loaded with the control HER-2(9_{657}) peptide (up to 4.73% for patient 1 and 10.75% for patient 2) ($P < 0.05$ in both cases). Moreover, the same CTLs also lysed HER-2/*neu* overexpressing human tumor cell line transfected to express HLA-A*0201 (SKOV3.A2) (cytotoxicity levels raising up to 46.38% for patient 1 and 27.4% for patient 2), but not its non-transfectant (SKOV3) (specific lysis up to 12.73% for patient 1 and 11% for patient 2) ($P < 0.05$ in both cases). Finally, upon encounter of the CTL with the RS.A2.1.DR1 primary cell line, cytotoxicity levels raised up to 41.62% for patient 1 and up to 51% for patient 2. This strongly suggests that HER-2(9_{828}) was not only recognized by in vitro sensitized CTLs, but these CTLs could also bring about the killing of primary tumor cells. The above findings are in accordance with previous data (Fig. 2a, b) regarding natural expression of this peptide epitope on the surface of HER-2/neu^+ HLA-A*0201$^+$ tumor cell lines.

Discussion

The search for new CTL epitopes derived from the amino acid sequence of well-identified tumor antigens through the utilization of algorithm-based databases predicting binding to MHC Class I alleles has led to the identification of numerous T-cell epitopes, most of them restricted to HLA-A*0201. Combining the information obtained by this approach together with that gained by proteasomal digestion pattern analysis we could identify a new HLA-

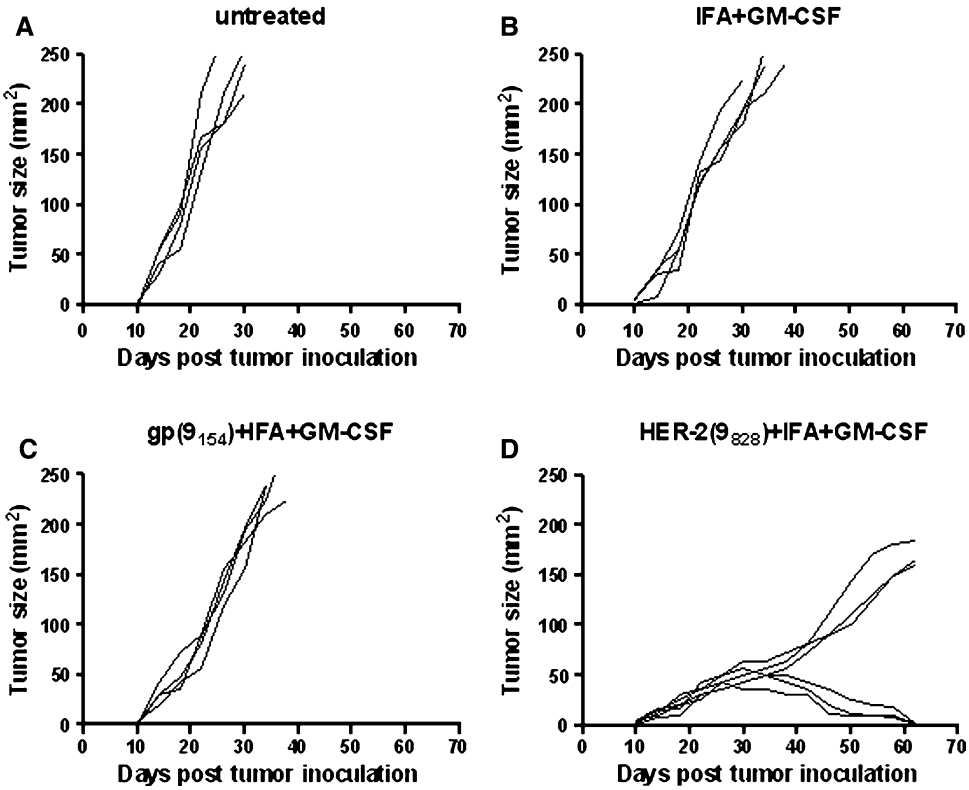

Fig. 5 Active immunization with HER-2(9_{828}) induces strong antitumor activity in mice. HHD mice were inoculated s.c. with 5×10^4 ALC.A2.1.HER transplantable tumor cells and 10–12 days later when tumor was palpable these were vaccinated as described in Sect. "Materials and methods" either with HER-2(9_{828}) in IFA plus GM-CSF (**d**), or with control peptide gp(9_{154}) in IFA plus GM-CSF (**c**), or with IFA and GM-CSF alone (**b**), or left untreated (**a**). Tumor growth thereafter was observed regularly every 4 days. *Curves* individual mice. $P < 0.05$ in all cases

A*0201-binding epitope derived from the tumor antigen HER-2/*neu*, namely HER-2(9_{828}). Through a series of in vivo and in vitro assays we could demonstrate that HER-2(9_{828}) is an immunogenic and naturally processed and expressed epitope on various HLA-A*0201$^+$ and HER-2/*neu*-expressing tumor cell lines, capable of generating peptide-specific and functionally potent CTL in HHD transgenic mice. HER-2(9_{828})-specific CTL were also generated in HLA-A*0201$^+$ HER-2/*neu*$^+$ breast cancer patients with pre-existing immunity to this peptide.

When tested in functional assays, the affinity of HER-2(9_{828}) for binding to HLA-A*0201 molecule as well as its capacity to form stable MHC/peptide complexes was intermediate compared to that of HER-2(9_{369}), which has been classified as a good binder due to its high-affinity for binding to HLA molecules and its strong immunogenicity [33, 34]. Moreover, similarities in the kinetic profiles of HER-2(9_{828}) and HER-2(9_{767}), the latter being already attributed properties of an intermediate binder [12], reinforce the classification of HER-2(9_{828}) as an intermediate binder with the HLA-A*0201 molecule. Slow release from its anchor amino acids maybe a significant factor influencing the immunogenicity of a peptide. To this end, Vertuani et al. [34] found that the high biological activity of a peptide analog of HER-2(9_{369}) was associated with a slower dissociation profile compared to the native HER-2(9_{369}) peptide, resulting in an epitope with greater HLA-A*0201 stability. Moreover, the dissociation rate of peptide gp100(9_{209}) modified at an HLA-A*0201 anchor residue was significantly slower compared to the native peptide, resulting in robust anti-tumor responses in melanoma patients vaccinated with the modified peptide [38, 39]. However, high-affinity binding of tumor peptides to MHC Class I molecules does not always coincide with increased peptide immunogenicity and generation of robust anti-tumor responses. To support this, Andersen et al. [40] reported that even the strongest immunogenic peptides from overexpressed tumor (self) proteins on p53$^{-/-}$ mice displayed an intermediate MHC Class I-binding and stabilization capacity. The authors explained their findings by postulating the absence of tolerance toward MHC Class I-restricted self peptides having an intermediate affinity for MHC Class I alleles. In another study, Mc Mahan et al. [41], through a series of experiments, attempted to determine the optimal binding requirements for mimotopes of the gp70 peptide aiming at a more effective anti-tumor immunity. In this study, it was shown that MHC/peptide complexes that formed a high-affinity interaction with TCR elicited the generation of T cells that were not functional in vivo and failed to enhance anti-tumor immunity. In contrast, vaccination with mimotopes exhibiting an intermediate affinity range, elicited functional T cells and provided protection against tumor growth in vivo. Thus, the immunogenicity of HER-2(9_{828}) might be explained by its ability to remain

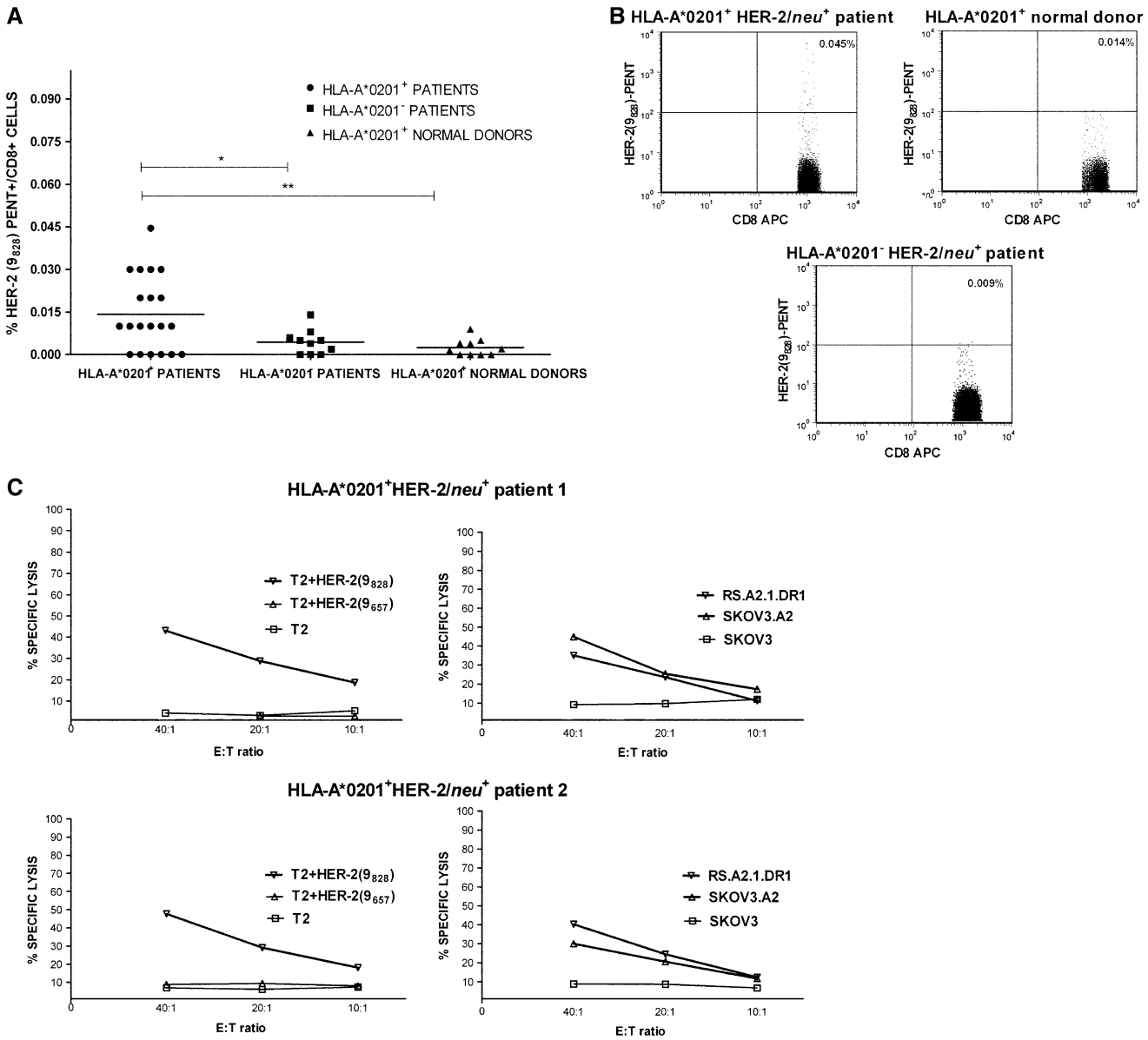

Fig. 6 Ex vivo identification of PENT$^+$/CD8$^+$ T-cell frequencies in HER-2/*neu*$^+$ and HLA-A*0201$^+$ or HLA-A*0201$^-$ breast cancer patients and HLA-A*0201$^+$ healthy donors using flow cytometry (**a**). Frequencies were detected by staining with PE-labeled HLA-A*0201 pentamer presenting the HER-2 (9_{828}) epitope as described in Sect. "Materials and Methods" (*$P < 0.05$, **$P < 0.01$). **b** Indicative dot plots of the HER-2(9_{828})-PENT$^+$/CD8$^+$ frequencies in the PBMCs of the above mentioned groups using HLA-A*0201 pentamer binding analysis. **c** Immunogenicity of the HER-2(9_{828}) epitope in vitro. HER-2(9_{828})-specific CTL were generated from patients' PBMC after CD4$^+$ CD25$^+$ regulatory T-cell depletion and stimulation with peptide HER-2(9_{828}), as described in Sect. "Materials and methods". Effector CTL were used thereafter in a standard ^{51}Cr release cytotoxicity assay and effectively lysed HER-2(9_{828})-pulsed T2 cells and the human HER-2/*neu*$^+$ HLA-A*0201$^+$ cell lines SKOV3.A2 and RS.A2.1.DR1. Each point represents the mean value from triplicates. SD was negligible and thus omitted. $P < 0.05$ in all cases

bound to MHC Class I molecules on APC long enough to allow an increased stability of the TCR/MHC/peptide complex [42]. This in turn would result in a robust signaling downstream the nucleus with stronger activation of the peptide-specific T cells.

In animal studies, we could deduce that immunization of HHD transgenic mice with HER-2(9_{828}) resulted in the generation of peptide-specific and functionally active CTL. We have used the HHD model which is mostly relevant at identifying HLA-A*0201-restricted peptides as vaccine candidates. The ALC.A2.1.HER is a fast growing tumor in HHD mice and therefore represents a powerful tool for assessing the efficiency of HLA-A*0201-restricted HER-2/*neu* peptide-based vaccines [18, 26]. According to our active immunization results, 50% of HHD mice therapeutically vaccinated with HER-2(9_{828}) rejected its ALC.A2.1.HER

tumors and became long-term survivors, whereas the survival of the remainders was significantly prolonged.

Furthermore, in humans, HER-2(9_{828}) exhibited high spontaneous immunogenicity as it was recognized by 8 out of 20 HLA-A*0201$^+$ HER-2/*neu*$^+$ breast cancer patients. Pre-existent immunity in these patients to HER-2(9_{828}) could be a result of autovaccination, induced by the release of tumor antigen by the dying apoptotic/necrotic tumor cells during or after chemotherapy [18]. Accordingly, the degree of response to chemotherapy may have an impact on the frequency levels of HER-2(9_{828})-specific CTL in vivo [18]. Another possibility would be that detectable peptide-specific CTL expansions are controlled by regulatory T cells (Tregs) the percentages of which may differ among patients, as this has been shown in various types of cancer [43–49]. Thus, the genetic background of the patients (i.e., HLA-DR alleles) represents also an important parameter influencing the *ex vivo* frequencies of tumor peptide-specific CTL, since inducible Tregs generated after recognition of tumor peptides have been demonstrated in experimental tumor models [50–52] and patients with melanoma [53, 54]. Most importantly, in vitro generated peptide-specific CTL from two HLA-A*0201$^+$ HER-2/*neu*$^+$ breast cancer patients exhibiting pre-existent immunity to HER-2(9_{828}) were able to recognize the peptide in vitro and exhibited potent cytotoxic properties upon encountering the HER-2/*neu*$^+$ HLA-A*0201$^+$ SKOV3.A2 tumor targets as well as the primary RS.A2.1.DR1 tumor cell line, reinforcing the statement that HER-2(9_{828}) is a naturally expressed epitope on the surface of these HER-2/*neu*$^+$ HLA-A*0201$^+$ cell lines.

Nonetheless, irrespective of the nature of underlying mechanisms, our data point to the fact that HER-2(9_{828}) is an immunodominant peptide, which, combined with its ability to induce a substantially long-lasting anti-tumor immunity can be considered as a preferable vaccine candidate. This is greatly supported by recent data demonstrating that patients who have pre-existent peptide-specific immune response, detectable at the time of vaccination, achieve higher levels of tumor-specific T-cell immunity overall most likely due to the boosting of the memory response [55]. The inclusion of HER-2(9_{828}) in multipeptide vaccines [for example together with HER-2(9_{369}) and HER-2(9_{654}) both of which are already in clinical trials [56, 57] or with HER-2(10_{85}) which has been recently reported by us [18] or with immunogenic peptides from other tumor proteins expressed on breast tumor cells (i.e., MUC1)] offers another possibility for its potential use in peptide-based vaccination protocols.

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