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A degenerate HLA-DR epitope pool of HER-2/neu reveals a novel *in vivo* immunodominant epitope, HER-2/neu₈₈₋₁₀₂

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Abstract

Purpose—Over the past two decades, there has been significant interest in targeting HER-2/neu in immune-based approaches for the treatment of HER-2/neu+ cancers. For example, peptide vaccination using a CD8 T cell activating HER-2/neu epitope (amino acids 369-377) is an approach that is being considered in advanced phase clinical trials. Studies have suggested that the persistence of HER-2/neu-specific CD8 T cells could be improved by incorporating HLA class II epitopes in the vaccine. Our goal in this study was to identify broad coverage HLA-DR epitopes of HER-2/neu, an antigen which is highly expressed in a variety of carcinomas.

Experimental Design—A combination of algorithms and HLA-DR binding assays was used to identify HLA-DR epitopes of HER-2/neu antigen. Evidence of pre-existent immunity in cancer patients against the identified epitopes was determined using IFN- γ ELISpot assay.

Results—Eighty-four HLA-DR epitopes of HER-2/neu were predicted, 15 of which had high binding affinity for 11 or more common HLA-DR molecules. A degenerate pool of four HLA-DR restricted 15-amino acid epitopes (p59, p88, p422 and p885) was identified against which greater than 58% of breast and ovarian cancer patients had pre-existent T cell immunity. All four epitopes are naturally processed by antigen-presenting cells. Hardy-Weinberg analysis showed that the pool is useful in ~84% of population. Lastly, in this degenerate pool we identified a novel *in vivo* immunodominant HLA-DR epitope HER-2/neu₈₈₋₁₀₂ (p88).

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TRANSLATIONAL RELEVANCE

HER-2/neu is expressed in approximately 20–30% of breast and ovarian cancers. Immune based approaches targeting HER-2/neu (e.g. trastuzumab) have become standard of care for patients with breast cancer. Others, such as the E75 vaccine, are on the horizon. Thus, continued research into HER-2/neu immunology seems warranted in order to improve therapies. In this study we report a pool of degenerate HLA-DR epitopes of HER-2/neu against which breast and ovarian cancer patients had elevated T cell immunity. This study also revealed a novel *in vivo* immunodominant HLA-DR epitope of HER-2/neu, p88. The pool of epitopes covers approximately 84% of population. Given its immunogenicity and degeneracy, we anticipate that in future translational studies, it will be possible to use this pool as a vaccine component.

Conclusion—The broad coverage and natural immunity to this epitope pool suggests potential usefulness in HER-2/neu targeting immune based therapies such as vaccines.

Keywords

MHC class II; HLA-DR; Helper T cells; Vaccines; Peptides

INTRODUCTION

HER-2/neu is a 185-kDa transmembrane protein homologous to epidermal growth factor receptor (1). Dimerization of this protein (homodimers or heterodimers with other erb family proteins) results in downstream signaling events leading to proliferation, migration, adhesion and transformation of cells. It is overexpressed in several cancers of epithelial origin including breast, colorectal, ovarian and pancreatic carcinomas (2). Overexpression of this protein is required for the maintenance of malignant phenotype of several tumors and is associated with poor prognosis in cancer patients (3). HER-2/neu is widely used as a target antigen in breast and ovarian cancer vaccines (2–5).

Generally, vaccines designed against cancers are aimed at eliciting effective cytotoxic T lymphocyte (CTL) responses as CTLs can directly kill the tumor cells. In previous studies, several MHC class I epitopes of different tumor antigens such as p53, carcinoembryonic antigen, MAGE2/3 and HER-2/neu were identified and many of these peptides are being or have been tested in clinical trials (6). Several HER-2/neu HLA class I peptides were shown to induce peptide specific CTLs which were capable of killing HER-2/neu⁺ tumor cells (7). Among all the HER-2/neu class I epitopes, p369-377 (also referred as E75) has been studied extensively (4,5,8,9). In fact, this peptide is under consideration by us for testing in human phase III clinical trials as a vaccine to prevent breast cancer recurrence. However, the results of phase I and phase II clinical trials using E75 suggest significant opportunities for improvement (8,10). Previous studies with E75 peptide suggest that immunization with this epitope alone doesn't result in long-lasting immunity but immunization with the HLA class II peptide, p369-384, which encompasses E75 elicits E75-specific T cell immunity that persists over one year (4,5). The results indicate that in order for HLA class I peptide vaccines to induce effective long-lasting immune responses it is important to have CD4 T cell help (11).

CD4 T cells are well known to have a fundamental role in tumor antigen (TA)- specific immunity (12). They enhance TA-specific immune responses a) by activating cytotoxic T cells through cytokines or by directly interacting with costimulatory molecules such as CD27, CD134 and MHC Class II antigen expressed on the surface of CTLs; b) by mediating cell death of tumor cells through apoptotic mechanisms such as Fas/FasL pathway and granzyme-perforin dependent pathway; and c) by activating effectors of the innate immune system such as macrophages and eosinophils (11). Previous studies showed that CD4 T cells when used along with CD8 T cells in adoptive T cell immunotherapy induced cancer regression in approximately 50% of melanoma patients (13). These important properties of CD4 T cells have led to considerable interest in identifying CD4 T cell defined TA epitopes with the aim of using them as vaccines. CD4 T cell epitopes of many different TAs such as carcinoembryonic antigen (CEA), folate receptor alpha, gp100, HER-2/neu, insulin, MART1/Melan-A, NY-ESO-1, p53, tyrosinase and insulin like growth factor binding protein 2 (IGFBP-2) have been identified in recent years (14–20).

Even though several CD4 T cell epitopes of different TAs have been reported, their utility as cancer vaccines is hindered by the broad polymorphism at the MHC class II locus. In addition to this, MHC class II gene frequency is usually low, typically <15% in any population compared to MHC class I gene frequency which is usually high (eg. 40–50% in case of HLA-A2). These

issues need to be considered when designing a CD4 T cell epitope vaccine to cover a large percentage of the population. Several HLA-DR variants overlap in their binding characteristics to different epitopes. Thus identification of degenerate epitopes which bind to several HLA-DR variants should increase the efficacy and scope of CD4 T cell epitope based immunotherapies. In our previous studies, we developed an approach that permits identification of broad coverage pools of degenerate epitopes of TAs such as IGFBP-2, CEA, and folate receptor alpha (15,20,21).

In the current study, a panel of 84 HLA-DR binding epitopes of HER-2/neu was predicted using a computer-based predictive algorithm called predicted IC₅₀ (PIC) (20). From this candidate panel a pool consisting of four HLA-DR epitopes of HER-2/neu was identified. HLA-DR epitopes that constitute this pool are immunogenic, naturally processed, and cover ~84 % of diverse population including Caucasians, African Americans and Asians (Hardy-Weinberg equilibrium analysis). A novel HER-2/neu HLA-DR-restricted epitope, p88, was identified in this degenerate pool as an *in vivo* immunodominant epitope.

MATERIALS AND METHODS

Reagents

The peptides used in this study were synthesized at Mayo Clinic Proteomics Core Facility or at Epimmune Inc. Purity of the peptides was determined by reversed phase high-performance liquid chromatography and amino acid analysis, sequencing and/or mass spectrometry. Lyophilized peptides were diluted in DMSO and PBS. Recombinant human ErbB2 protein, HER-2/neu cytoplasmic domain (676-1255 a.a) and partial ErbB2 recombinant protein (22-122 a.a) were obtained from In Vitrogen (Carlsbad, CA) and Novus Biologicals (Littleton, CO) respectively. HER-2/neu entire extracellular domain protein (1-627 a.a) was generously provided by Raphael Clynes (Columbia University, New York). CEF viral peptide pool was obtained from NIH AIDS Research and Reference Reagent Program.

Patients and Donors

This study was approved by Institutional Review Boards (IRB) at Mayo Clinic and University of Washington, Seattle, WA. Blood samples were obtained from 18 female healthy donors and 38 patients (9 breast and 29 ovarian) at Mayo Clinic. Samples of 10 breast cancer patients were obtained from the University of Washington, Seattle, WA. Samples from both institutions were processed and stored in a similar fashion. All the patients were disease-free at the time of sample collection. Formalin fixed, paraffin embedded (FFPE) tissue samples were obtained from the patients at the time of initial surgical procedure (breast tumor 6 and ovarian tumor 20). The mean ages of healthy donors and patients were 42 ± 11 and 55 ± 2 years, respectively ($p < 0.0001$). Tumor grade and stage information was available for patients as described previously (20). There were no differences in T cell responses between patients and healthy donors against non-specific stimulus, PMA/ionomycin and CEF viral antigens (20).

Preparation of PBMCs

PBMCs were isolated from blood by density gradient centrifugation as described previously (20). Cells were cryopreserved in liquid nitrogen in freezing medium (RPMI 1640 with FBS and dimethylsulfoxide) at a cell density of $25\text{--}50 \times 10^6$ cells/ml.

Epitope prediction

PIC (predicted IC₅₀), a modified linear coefficient and matrix-based method was used for predicting HLA-DR binding capacity of peptides (22,23). Lower PIC values indicate higher binding affinity of peptides to a HLA class II molecule.

HLA-DR purification

HLA-DR molecules were chosen for this study to allow balanced population coverage (24). HLA-DR molecules were purified from EBV-transformed homozygous cell lines or from transfected fibroblast cell lines (23,25). Cell lysates were passed through LB3.1 monoclonal antibody columns and HLA class II molecules were eluted at high pH (pH 11.5) followed by pH reduction to 8.0. Eluates were concentrated by centrifugation.

HLA-DR binding assays

The binding affinity of peptides to different HLA-DR molecules was determined by their ability to inhibit the binding of high-affinity radiolabeled probe peptides to specific HLA-DR molecules using a solid-phase capture radioimmunoassay (26). Briefly, purified HLA-DR molecules and radiolabeled peptides were incubated in the presence of the inhibitor peptide in a reaction vessel for 2 days either at room temperature or at 37°C in the presence of protease inhibitors. After incubation, the percentage of HLA-DR bound radioactivity was determined by capturing HLA-DR/peptide complexes on Optiplates (Packard Instruments) coated with the mAb LB3.1 and determining bound counts per minute followed by affinity calculations. As in previous studies, peptides with affinities for specific HLA-DR molecules of 1,000 nmol/L or better were defined as high-affinity binders.

ELIspot

A 10-day ELIspot assay was used to determine reactivity of low-frequency T cells to HER-2/neu peptides (Table 1) and was done in groups of two (two healthy donors, one healthy/one cancer patient, or two cancer patients) as previously described (20). A patient was considered as a responder to a specific peptide if the mean T cell frequency was higher than mean T cell frequency plus 2 standard deviations of control population (20). A peptide was considered naturally immunogenic if greater than 10% of the patients demonstrated significantly elevated immunity to that peptide relative to the controls.

Generation of HER-2/neu specific CD4⁺ T cells

Dendritic cells (DCs) were generated from PBMCs as described previously (20). Briefly, PBMCs were cultured in modified RPMI medium in 6 well tissue culture plates (6×10^6 cells/well) for 2 hrs at 37°C. Non-adherent cells were removed and adherent cells (monocytes) were cultured further in the presence of hGM-CSF (800U/ml) and hIL-4 (1000U/ml) at 37°C. On day 5, CpG (1µg/ml) was added to each well. On day 6, DCs were pulsed with peptide (10µg/ml) for 4hrs in the presence of B7-DC crosslinking antibody (A gift from Dr. Larry Pease, Mayo Clinic). Pure CD4 T cells isolated from PBMCs by isolation kit (Miltenyi Biotec Inc, Auburn, CA) were added to peptide pulsed DCs and the cultures were incubated at 37°C with periodic interleukin-2 (10U/ml) and interleukin-12 (10ng/ml) addition. On day 15, cells were assayed for reactivity with the HER-2/neu antigen (HER-2/neu peptides and HER-2/neu protein) and irrelevant antigens by ELIspot. For this assay, *in vitro* stimulated CD4 T cells (1×10^5 cells/well) and autologous irradiated PBMCs (1×10^5 cells/well) were added at 1:1 ratio in each well in 96 well NC-plates coated with anti-human IFN-γ Ab and incubated at 37°C at 5% CO₂ for 20–24 hours in the presence of different stimuli. Stimuli were respective HER-2/neu peptide (10 µg/ml) and either the HER-2/neu cytoplasmic domain (676-1255 a.a), a partial HER-2/neu extracellular domain recombinant protein (22-122 a.a) or HER-2/neu entire extracellular domain protein (1-627 a.a) (1 µg/ml). For the irrelevant peptide, Human collagen II peptide, HII.71 (PPGLTGPAGEPGRQGSPGAD) (10 µg/ml) was used and for irrelevant protein, ovalbumin was used. Results are expressed as antigen-specific CD4 T cells per 1×10^5 CD4 T cells. To determine HLA class II restriction of peptide-specific CD4 T cells, HLA-DR and HLA-DP, DQ, DR specific antibodies (BD Biosciences, San Jose, CA) (10 µg/ml) were used in ELIspot assay.

Immunohistochemistry

Immunohistochemical staining was performed using FDA approved Hercep Test kit (DAKO K5207). Briefly, formalin fixed paraffin embedded tissue sections were deparaffinized and antigen retrieval was carried out using citrate retrieval buffer. Slides were treated with peroxidase blocking reagent followed by incubation with prediluted rabbit polyclonal primary antibody for 30 minutes at room temperature. Visualization reagent was applied for 30 minutes at room temperature followed by a 10-minute incubation at room temperature with diaminobenzidine. Sections were counterstained with hematoxylin. Staining intensity was graded on a 0–3 scale: 0 and 1 grades were considered as low expression, and 2 and 3 grades were considered as high expression. Staining was performed in a blinded fashion.

Statistical analysis

Statistical analyses were performed using GraphPad InStat Software or GraphPad prism software. Two-tailed Mann-Whitney tests or student's t-tests were used to analyze the data unless otherwise stated. $P < 0.05$ was considered as significant.

RESULTS

HER-2/neu peptides have high binding affinity to HLA-DR molecules

Eighty-four HER-2/neu HLA-DR epitopes were predicted using PIC. All 84 peptides were synthesized and tested for binding to HLA-DR molecules as described in the Materials and Methods. Fifteen of the 84 peptides were selected based on high affinity degenerate ($\leq 1000\text{nM}$) binding to at least 11 out of 15 HLA-DR molecules (Table 1). These peptides were re-synthesized at high purity and tested for reactivity in peripheral blood samples from both normal healthy donors and breast or ovarian cancer patients using IFN- γ ELISpot assays. As predicted, these peptides bound with high affinity to DRB1 *0101 molecule (Table 1).

Breast and ovarian cancer patients have elevated HER-2/neu epitope pool-specific T cell immunity

PBMCs from breast and ovarian cancer patients were used in ELISpot assays to determine the frequency of T cells against the 15 peptides selected on the basis of binding assay results. A pool of four peptides (p59, p88, p422 and p885) was identified against which $\geq 15\%$ of patients had elevated T cell responses. Mean T cell frequencies of responders were in the range of 314 ± 64 to 635 ± 172 (Fig. 1), higher than T cell frequencies detected among healthy donors. There were no significant differences ($p > 0.05$) in epitope-specific T cell responses between breast and ovarian cancer patients. Also, cumulative T cell frequency against the pool observed in patients was significantly higher than in controls ($p = 0.006$) (Fig. 2A). Fifty-eight percent of the patients and 22% of the controls responded to the pool ($p = 0.009$) (Fig. 2B). Although our results show that only 58% of patients responded to peptide pool challenge, Hardy-Weinberg analysis suggests that this pool would be useful in 84% of patients (Table 2).

T cell responses against the peptide pool are HER-2/neu expression dependent

Tumor tissues were obtained from breast and ovarian cancer patients and immunohistochemical staining was performed to determine whether T cell responses observed in patients correlated with HER-2/neu expression in tissue samples. In our previous studies, we reported that breast cancer patients treated with trastuzumab and chemotherapy have increased HER-2/neu-specific CD4 T cell responses (27). As the breast cancer patients selected for this study were under active treatment at some point before the collection of blood samples, we have excluded the T cell response results from these patients in correlation analysis in order to avoid the bias. Based on the staining intensity, patient samples were divided into high and low expression categories (Fig. 2C). As shown in Fig. 2D, patients with high levels of HER-2/

neu expression (n = 6) in their tumors had elevated T cell responses against the pool compared to T cell responses observed in patients with low levels of HER-2/neu expression (n = 16) (p=0.04).

Peptides that constitute the epitope pool are naturally processed

To determine whether HLA-DR epitopes of the pool are naturally processed, peptide-specific CD4 T cells were generated as described in Materials and Methods. These CD4 T cells were tested for their reactivity against HER-2/neu protein and individual HER-2/neu HLA class II peptides. As shown in Fig. 3, peptide specific CD4 T cells responded to HER-2/neu protein and peptides. There were no discernable differences between the reactivity of CD4 T cells against peptides and HER-2/neu protein (p>0.05). These results indicate that the four epitopes that constitute the pool are naturally processed and presented by antigen presenting cells to CD4 T cells. These experiments were repeated three times using CD4 T cells isolated from three different donors.

p88 is an *in vivo* immunodominant epitope of HER-2/neu

We also correlated HER-2/neu expression in tumors with T cell responses against individual peptides constituting the pool. As shown in Fig. 4A, patients with HER-2/neu^{hi} tumors had elevated T cell responses against p88 peptide. This response is significantly greater than p88-specific T cell responses seen in patients with HER-2/neu^{lo} tumors. However, the differences in p59, p422 and p885 specific T cell responses among patients with HER-2/neu^{hi} tumors and HER-2/neu^{lo} tumors were not statistically significant (data not shown). Patients with HER-2/neu^{hi} tumors had significantly higher p88 specific T cell responses compared to the T cell responses against the other epitopes (p59, p422 and p885) comprising the pool (Fig. 4B). No significant differences (p>0.05) in T cell responses were observed among patients with IHC0, IHC1+ and IHC2+ tumors. These results suggest that T cell responses observed in the patients correlate with HER-2/neu expression in tumors of the patients with the immunodominant T cell responses being p88-specific. Thus we conclude that p88 is an *in vivo* immunodominant epitope of this pool. To verify that the p88 epitope was HLA-DR restricted, p88-specific T cells were generated and tested for reactivity against p88 peptide in the presence or absence of HLA-DR antigen-specific mAb. As shown in Fig. 4C, epitope-specific responses were completely blocked by the inclusion of two different antibodies recognizing HLA-DR, but not of an isotype control antibody.

DISCUSSION

HER-2/neu-based peptide vaccines have been used for the past several years to treat different types of cancers. Much focus has been on the development of HLA class I peptide vaccines which have the ability to elicit TA-specific CTLs. Recently, there has been considerable interest in incorporating peptides that also activate CD4 T cells since they have a prominent role in prolonging effector CD8 T cell responses and maintaining immunologic memory. Thus, identification of novel HLA class II epitopes of HER-2/neu may facilitate the development of effective multi-epitope vaccination strategies against different cancers using HER-2/neu based peptide vaccines. The genetic diversity at the HLA-class II locus poses a significant problem, however, requiring the identification of degenerate epitopes that can be used in broader patient populations. In this study we identified a pool of four naturally processed degenerate HLA-DR epitopes of HER-2/neu antigen against which breast and ovarian cancer patients had elevated T cell immunity. This pool is predicted to be recognized by up to ~84% of the population and each peptide covers a minimum of 12 of 15 major HLA-DR alleles. Characterization of the immunogenicity of this pool led to the identification of a novel *in vivo* immunodominant epitope, p88. Although other CD4 T cell epitope pools for HER-2/neu have been established, the pool in the current report appears to be superior in terms of coverage.

For example, using an older algorithm (TSites), in the 1990's Disis and colleagues developed 3 different HER-2/neu pools, an intracellular domain pool (p776, p927, and p1166), an extracellular domain pool (p42, p98, and p328), and an HLA-A2 pool (p369, p688, and p971) (28). Together these 9 peptides cover 11 of 15 HLA-DRs examined with only a mean coverage of 2 epitopes per allele. In contrast, the pool of 4 epitopes identified in the current study covers 14 of 15 alleles examined with a mean coverage of 4 epitopes per allele.

Some self proteins or TAs are overexpressed in tumor cells compared to their expression in normal cells. These TAs can be immunogenic if they are presented along with costimulatory molecules and other danger signals in the tumor microenvironment or regional lymph nodes. Thus, it is possible to observe correlations between expression of TAs in tumor cells and endogenous immune responses against these TAs in cancer patients. Goodell and colleagues (29) reported that overexpression of HER-2/neu protein in tumors correlates with endogenous T cell responses observed in breast cancer patients. By immunohistochemical analyses we determined that patients with HER-2/neu^{hi} tumors had elevated T cell responses against the pool compared to patients with HER-2/neu^{lo} tumors, and the majority of T cell responses observed in patients with HER-2/neu^{hi} tumors were p88 specific. Also, it was observed that the proportion of patients responding to p88 epitope (23%) was higher than that of patients responding to other epitopes (p59 (16%), p422 (16%) or p885 (15%)) in the pool. Based on their findings Perez et al (30) suggested that HER-2/neu protein might have lysosomal targeting sequences which would allow it to be processed in endogenous MHC class II pathway. Our data indicate that all the epitopes in the pool including p88 are derived from natural processing of HER-2/neu protein, suggesting that the p88 sequence in the HER-2/neu protein could be targeted by lysosomes and ultimately presented by MHC class II molecules after being processed in endogenous MHC class II pathway. Moreover, inhibition experiments confirm HLA-DR restriction of p88 peptide. Thus we speculate that with the increase in the expression of HER-2/neu in tumor cells, p88 epitope is presented on the surface of tumor cells at a higher rate compared to other epitopes in the pool. Taking into consideration all the above facts we concluded that p88 is an *in vivo* immunodominant epitope. As immunodominance of the epitope is influenced by several factors such as HLA binding, TCR binding, stability, and abundance of the peptide (20), in the future, it will be necessary to design studies including this p88 peptide as a component in HER-2/neu cancer vaccines to address the significance of our finding.

In addition to incorporation with HLA class I epitopes, there are other potential applications of the degenerate epitope pool described in the current study, such as including it with trastuzumab therapy. Trastuzumab (Herceptin) is a humanized monoclonal antibody against HER-2/neu protein and it is a drug of choice for the treatment of HER-2/neu+ breast cancer patients with metastatic disease (31). It has been suggested that trastuzumab acts on cells by enhancing the internalization of HER-2/neu protein and ultimately resulting in increased surface expression of HLA class I peptides on the surface of tumor cells, thereby increasing their susceptibility to the lytic effects of cytotoxic T cells (32,33). Taylor and colleagues also showed that trastuzumab treatment enhanced CD4 T cell and antibody responses in breast cancer patients, immunity which correlated well with clinical responses to therapy (27). Given the importance of CD4 T cells in CTL and antibody responses (11), the use of HER-2/neu CD4 T cell epitopes along with trastuzumab may boost clinical efficacy. Given the degeneracy of these epitopes, we speculate that the vaccine regimen should be useful in large population. Also, the identified pool of epitopes may be useful as biomarkers to predict patient's responsiveness to trastuzumab-based therapy.

Even though we reported a pool of only four HLA-DR epitopes of HER-2/neu antigen which might be useful in future studies as a cancer vaccine regimen, the potential use of the other HLA-DR epitopes reported in this study should not be overlooked. For example, our HLA-DR

binding affinity data indicates that epitopes such as p83, p432 and p783 binds at least 13 out of 15 HLA-DR variants, but elevated peptide-specific T cell responses were not observed in the current group of patients. Several factors such as lack of natural processing of these epitopes despite their high binding affinity to several HLA-DR variants, natural deletion mechanism of T cells responding to these epitopes, and inhibition of T cells responding to these epitopes by regulatory T cells (T_{reg}) could account for the lack of responsiveness by the patients (15, 20). Future studies addressing these issues could determine whether these epitopes also constitute an immunogenic, degenerate HLA-DR epitope pool of HER-2/neu antigen. In fact, these epitopes (p432 and p783) encompass HLA class I epitopes p435 and p785 which were reported as potential CTL epitopes in previous studies (7,34). Also, we predicted that 84% of patients could respond to the degenerate pool, but we observed only 58% of patients had elevated immunity against the pool. Several factors such as immunodominance, immunosuppression and HER-2/neu expression in the patients selected for this study can be attributed to the differences in predicted and observed responses (15).

In summary, in this study we identified a pool of degenerate HLA-DR epitopes of HER-2/neu antigen against which breast and ovarian cancer patients have endogenous immunity, and which consists of a novel *in vivo* immunodominant HLA class II epitope p88. We predict that in future studies, this pool would be useful in the development of successful peptide based immunotherapeutic strategies against HER-2/neu⁺ cancer patients and also would serve as an effective tool in determining immune responses of cancer patients treated with HER-2/neu based vaccine regimen.

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Figure 1. Detection of HER-2/neu peptide-specific immunity in breast and ovarian cancer patients
Shown are scattergrams (Panels A, B, C, D) of IFN- γ production by T cells derived from patients and healthy donors. Results are shown as T cell frequency which represents peptide-specific T cells per million PBMCs. Percentages represent proportion of patients responding to peptides above the gray area, which represents mean + 2 SD of the healthy control responses. Each dot represents an individual (patient or healthy donor). Mean epitope specific T cell frequencies of the responders (patients groups above gray area) are indicated in each panel.

Figure 2. Breast and ovarian cancer patients demonstrate elevated HER-2/neu-specific T cell immunity compared to normal healthy individuals

A) Cumulative T cell frequency against the pool in all controls and patients. Each bar is the mean of T cell frequency against the pool in all patients and controls. *B)* Column graph showing proportion of patients and healthy donors (control) responding to the pool of HER-2/neu peptides. *C)* Immunohistochemical analysis of HER-2/neu expression in tumor tissues obtained from breast and ovarian cancer patients. Images are representative of low (upper panel) and high levels (lower panel) of HER-2/neu expression in tumor tissues (40 x magnifications). *D)* Correlation of T cell responses against the pool observed in the patients with high HER-2/neu expression (n=6; IHC3+ = 3, IHC2+ = 3) and low HER-2/neu expression (n = 16; IHC 1+ = 9, IHC 2+ = 7) in the tumor tissues. Each bar is the mean of cumulative T cell frequency

against the pool in the patients with low and high levels of HER-2/neu expression in tumor tissues. *P* values were calculated using Mann-Whitney test and Fisher's exact test.

Figure 3. Peptides in the pool are derived from natural processing of HER-2/neu protein
 ELISpot (Panels A, B, C, D) assay results of peptide-specific CD4 T cells derived from short term culture. Peptide-specific CD4 T cells were tested for their response against relevant peptides (p59, p88, p422 and p885), HER-2/neu protein, irrelevant peptide (HII.71) and irrelevant protein (ovalbumin). Results are shown as peptide-specific CD4 T cells per 10⁵ CD4 T cells. Each column is the mean $\pm$ SE of three replicates. In all panels, the CD4 T cell responses against HER-2/neu peptides and protein are significantly higher ($P < 0.05$) than the responses against irrelevant peptide and irrelevant protein. P values were calculated using Mann-Whitney test

Figure 4. p88 is an *in vivo* immunodominant epitope of the HER-2/neu protein

A) p88 specific T cell responses among the patients with low and high levels of HER-2/neu expression in tumor tissues. Results are shown as peptide-specific T cells per 1×10^6 PBMCs. B) Comparison of T cell responses against each epitope in the pool among the patients with high levels of HER-2/neu expression in tumor tissues. Each bar is the mean of epitope-specific T cell frequencies in the patients with HER-2/neu^{hi} tumors. C) ELISPOT results of p88-specific CD4 T cells derived from short term culture. p88 specific CD4 T cells were tested for their response against relevant peptide and irrelevant peptide in the presence or absence of anti HLA-DR antigen-specific mAb and isotype matched mAb.

TABLE 1

Binding affinities of HER-2/neu peptides to purified HLA-DR

Sequence	Peptide Name	Position [¶]	IC ₅₀ nM to purified HLA															
			DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1302	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101	
NLELYLPTNASLSF	HER-2/neu.59	59	4.9	7356	6.2	2.7	38	7.2	94	3055	30	141	105	23	ND	29	189	
LTYLPTNASLSFLQD	HER-2/neu.62	62	9.7	3364	19	16	80	15	426	4081	213	150	47	132	141	1633	173	
IQEVQGYVLLAHNQV	HER-2/neu.77	77	57	7763	111	178	102	35	213	302	165	3438	103	75	13,508	546	1361	
YVLLAHNQVRQVPLQ	HER-2/neu.83	83	28	454	53	104	1185	92	300	358	208	302	1.9	679	649	124	18	
HNQVRQVPLQRLRIV	HER-2/neu.88	88	950	971	840	78	1303	80	85	6644	21	42	270	340	ND	18	173	
MEHLREVRAVTSANI	HER-2/neu.347	347	9.6	2970	533	12	200	9.7	95	4345	262	221	23	86	ND	81	216	
LREVRAVTSANIQEF	HER-2/neu.350	350	17	3913	43	8.2	50	12	456	5187	661	161	1.5	27	ND	163	94	
LSVFQNLQVIRGRIL	HER-2/neu.422	422	1.3	345	6.3	33	26	7.1	148	859	9.6	486	80	33	ND	67	17	
RGRILHNGAYSLTLQ	HER-2/neu.432	432	2.4	710	480	129	2845	5.6	5077	430	773	40	1.3	5.4	358	562	82	
LRLSLRELGSGLALIH	HER-2/neu.455	455	7.1	ND	896	14	603	142	1075	594	309	498	16	24	16,142	549	726	
VLGVVFGILIKRRQQ	HER-2/neu.666	666	67	2449	177	335	101	17	35	ND	12	268	17	185	ND	958	38	
SRLLGICLTSTVQLV	HER-2/neu.783	783	80	2923	85	13	90	9.0	634	137	80	446	4.7	39	3567	481	392	
PIKWMALESILRRRF	HER-2/neu.885	885	12	30	14	250	161	664	312	3620	133	66	349	3.3	ND	62	3.4	
IKWMALESILRRRFT	HER-2/neu.886	886	16	10	37	1075	435	1795	515	9282	136	241	1118	11	ND	340	3.3	
FSRMARDPQRFVVIQ	HER-2/neu.976	976	29	35	512	2224	855	1423	798	1481	49	6867	240	1408	901	227	45	

[¶]Position of N-terminal amino acid; ND=not determined; Peptides that constitute degenerate pool are in bold

TABLE 2
HLA class II frequencies and number of degenerate HER-2/neu peptides of pool that bind to each allele's gene product

Allele	HLA-DRB1										HLA-DRB 3-5			
	DRB1 *0101- 06	DRB1 *0301- 13	DRB1 *0401- 32	DRB1 *0701- 04	DRB1 *0802- 21	DRB1 *0901	DRB1 *1101- 35	DRB1 *1201- 06	DRB1 *1301- 34	DRB1 *1501- 08	Total Coverage DRB1	DRB5 *0101	Total Coverage DRB 4-5	Predicted response rate (%) [§]
Caucasoids	9.4 [†]	11.1	12.8	13.2	3.7	2.0	13.4	2.3	10.2	10.8	69.2	23.9	67.2	90
Blacks	5.5	14	10.5	9.23	4.8	2.0	15.7	4.4	14.3	9.9	69.0	16.5	35.7	75
Asian	3.0	5	13	5.8	6.5	9.4	7.8	13.5	4.9	14.4	58.7	22.0	64.2	85
Estimated Average	6	10	12	9	5	5	12	7	10	12	65.8	20.8	56.1	84
# of epitopes	4 [¶]	3	4	4	4	1	4	4	4	4	4	4	4	

[†] Frequency of allele in the population.

[¶] Number of epitopes in the HER-2/neu pool that bind to this HLA-DR variant.

[§] Predicted percentage of population that could respond to at least one epitope.