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Identification of a broad coverage HLA-DR-degenerate epitope pool derived from carcinoembryonic antigen

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Abstract

CD4 T cells are important for anti-tumor immune responses. Aside from their role in the activation of CD8 T cells, CD4 T cells also mediate anti-tumor immune responses by recruiting innate immune effectors into the tumor microenvironment. Thus, the search for strategies to boost CD4 T cell immunity is an active area of research. Our goal in this study was to identify HLA-DR epitopes of carcinoembryonic antigen (CEA), a commonly over-expressed tumor antigen. HLA-DR epitopes of CEA were identified using the epitope prediction program, PIC (predicted IC_{50}) and tested using *in vitro* HLA-DR binding assays. Following CEA epitope confirmation, IFN-γ ELIspot assays were used to detect existing immunity against the HLA-DR epitope panel of CEA in breast and ovarian cancer patients. *In vitro* generated peptide-specific CD4 T cells were used to determine whether the epitopes are naturally processed from CEA protein. Forty-three epitopes of CEA were predicted, 15 of which had high binding affinity for 8 or more common HLA-DR molecules. A degenerate pool of four, HLA-DR restricted 15-amino acid epitopes (CEA.24, CEA. 176/354, CEA.488 and CEA.653) consisting of two novel epitopes (CEA.24 and CEA.488) was identified against which 40% 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 ~94% of patients. Patients with breast or ovarian cancer demonstrate pre-existent immune responses to the tumor antigen CEA. The degenerate pool of CEA peptides may be useful for augmenting CD4 T cell immunity.

Keywords

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

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INTRODUCTION

In recent years T cell (CD8 and CD4) targeted immunotherapy against cancer has generated increasing interest. Results associated with several ongoing clinical trials, particularly adoptive T cell therapy, have been quite dramatic [1]. Cytotoxic CD8 T cells lyse tumor cells directly and because of this property, these cells have frequently been targeted when designing vaccines for the treatment of cancer. Helper CD4 T cells play a central role in adaptive anti-tumor immunity in several pathways, including: 1) production of cytokines that have important roles in the longevity and effector functions of antigen-activated CD8 T cells; 2) activation of antigen-presenting cells (APCs) by CD40 and nCD40L interactions which enables improved priming of T cell immunity, and 3) activation of CD8 T cell-independent mechanisms of tumor eradication [2]. This latter effector function is multi-dimensional and includes potential anti-angiogenic properties of IFN-γ and the intratumoral recruitment of innate immune effector cells such as macrophages and eosinophils [2].

Strategies using defined peptide epitopes have been used to generate anti-tumor T cell responses, either *in vivo* (e.g. vaccination) or *in vitro* (e.g. for adoptive T cell therapy), due to advantages such as chemical definition, ease of synthesis, long-term stability and targeting specificity (e.g. MHC class I). Recently, increasing attention has focused on identifying CD4 T cell-activating MHC class II epitopes from a variety of different tumor antigens, including carcinoembryonic antigen (CEA), folate receptor alpha, tyrosinase, gp100, MART1/Melan-A, NY-ESO-1, p53, HER-2/neu, and insulin like growth factor binding protein 2 (IGFBP-2) [3-12]. However, the human MHC class II locus is very polymorphic making it difficult to develop effective strategies that can be tested in the majority of patients. Despite the polymorphism, however the peptide binding characteristics of each variant do not differ significantly making it possible to identify degenerate peptides capable of binding multiple allelic variants of HLA-DR. Identification of degenerate peptides of TAAs may lead to effective vaccination strategies against cancer.

CEA is a membrane glycoprotein and is overexpressed in several human malignancies, such as colorectal, gastric, pancreatic, non-small-cell lung, breast, cervical, ovarian, prostrate, and head and neck cancers [13-15]. CEA is shown to have an important role in the development of metastatic disease by inhibiting cell death [16] and cooperating in cellular transformation with several proto-oncogenes such as *BCL2* and *c-myc* [17]. The utility of this protein as a target antigen for immunotherapies is well documented. Several CD8 T cell epitopes of CEA have been identified so far [18-20] and DNA encoding the whole protein has been used in advanced vaccine clinical trials [21]. Lastly, it has been reported by Bos and colleagues that CD4 T cells are important in eliciting protective immunity against CEA in murine models [22]. Thus the identification of a degenerate MHC class II-binding pool from CEA is important for use as a vaccine, alone or in combination with MHC class I epitopes, and for monitoring immune responses in whole antigen approaches.

We used the PIC (predicted IC_{50}) epitope prediction program, as previously described [5], to identify a pool of 43 potential HLA-DR binding epitopes of CEA. Four of these CEA peptides were naturally targeted in patients previously diagnosed with either breast or ovarian cancer. This pool of four epitopes is calculated to bind to HLA-DR in ~94 % of people suggesting its potential utility as a broad coverage CD4 T cell-activating vaccine component.

MATERIALS AND METHODS

Reagents

Purified CEA protein was obtained from Abcam (Cambridge, MA). CEA peptides were synthesized either at Mayo Proteomics Research Center or by Pharmexa-Epimmune, Inc. (San Diego, CA). The purity (>95%) and identity of peptides were determined by reverse-phase HPLC and mass spectrometry analysis, respectively. The synthetic peptides were lyophilized, resuspended in DMSO and then diluted in PBS.

Epitope prediction

A modified linear coefficient or matrix-based method called PIC (predicted IC_{50}) was used for predicting peptides with HLA-DR binding capacity [23, 24]. PIC is proprietary to pharmexa-Epimmune, Inc., (San Diego, CA) but another prediction algorithm (developed by Dr. Alessandro Sette) similar to PIC is available at NIH-supported public website, www.iedb.org. PIC is predicated on the assumption that each residue along a peptide molecule can independently contribute to binding affinity. PIC generates a score for individual peptides that is derived from polynomial coefficients describing the relative binding associated with each of the 20 naturally occurring amino acid residues for each peptide position. Next, mathematical transformations are performed, including linear polynomial scaling, an experimental power transformation, and a further linear correction based on minimizing the deviation of predicted values from experimental values. Based on these operations the algorithm yields a predicted IC50 value (designated as PIC) for the corresponding input sequence. PIC converts coefficient-based scores into an IC50 prediction and enables prioritization of peptides for further screening based on the predicted strength of HLA-DR binding. Lower PIC values indicate higher binding affinity to HLA. The program analyzes 15 amino acid long sequences offset by 3 residues encompassing the entire protein.

Subjects

Blood specimens were obtained from 18 healthy donors and 38 (9 breast and 29 ovarian) disease-free cancer patients from Mayo Clinic. Ten breast cancer patient samples were obtained from University of Washington (Seattle, WA) and were processed and stored using the same procedures and protocols as Mayo Clinic samples. FFPE tissue samples were obtained from patients at the time of initial surgical procedure. This study was approved by Institutional Review Boards at both the Mayo Clinic and the University of Washington. Patients were free from active treatment for at least 30 days when blood was collected. For T cell studies, the mean (± s.e.m) ages of healthy donors and patients were 42 ± 11 and 55 ± 2 years, respectively ($p<0.0001$). Tumor grade information was available for 34 patients (1 grade 2, 2 grade 7, 3 grade 5 and 4 grade 20) and stage information was available for 35 patients (12 stage I, 7 stage II, 15 Stage III and 1 stage IV). Tumor tissues for CEA staining were available for 26 patients (breast tumor 6, ovarian tumor 20). The patient and healthy donor populations, as described in our previously published study, had no differences of T cell reactivity to non-specific mitogen (PMA/ionomycin) or viral antigens between the groups [5].

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from blood by density gradient centrifugation as described previously [25]. Cells were cryopreserved in liquid nitrogen in freezing medium (RPMI with 45% FBS and 10% dimethylsulfoxide) at a cell density of 25-50 × 10^6 cells/ml.

Affinity purification of HLA-DR molecules

HLA-DR molecules used in this study (list in Table 1) were chosen to allow balanced population coverage [26]. HLA-DR molecules were purified from EBV-transformed homozygous cell lines or from transfected fibroblasts by affinity chromatography as previously described [24] using mAb LB3.1 coupled to Sepharose CL-4B beads. Eluates containing HLA-DR molecules were obtained after passing cell lysates through an anti-DR column. The eluate was then concentrated by centrifugation.

HLA-DR binding assay

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 gel-filtration radioimmunoassay [27]. 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 LB3.1 antibody 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.

Enzyme-linked immunosorbent spot assay (ELISpot assay)

A 10-day ELIspot for detecting low-frequency T cells in PBMCs of healthy donors and breast/ovarian cancer patients was used to determine reactivity to the CEA derived peptides (Table 1) and was done in groups of two (two healthy donors, one healthy/one cancer patient, or two cancer patients) essentially as previously described [25]. A patient was identified as having an immune response to a specific peptide if the calculated T cell frequency to that peptide exceeded the mean frequency of the control population plus two standard deviations [5]. 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 antigen-specific $CD4^+$ T cells

Dendritic cells were generated from PBMCs as described previously [5]. Briefly, PBMCs were seeded into six-well plates (6×10^6 cells/well) in culture medium (complete RPMI medium with human AB serum) containing granulocyte macrophage colony-stimulating factor and interleukin-4. On day five, bacterial CpG was added to the cultures at the concentration of 1μg/ml. On day six, peptide (10 μg/ml) and B7-DC crosslinking antibody (10 μg/ml) (A gift from Dr. Larry Pease, Mayo Clinic) were added to the DC cultures. After 4 hours incubation, pure CD4 T cells isolated from PBMCs by magnetic separation (purity ~99%) were added and the cultures were incubated at 37°C with periodic interleukin-2 and interleukin-12 addition

Determination of HLA class II restriction and reactivity of peptide-specific $CD4^+$ T cells against CEA protein

On day 15 of *in vitro* stimulation, peptide-specific CD4 T cells were assayed for reactivity with the CEA antigen (CEA peptides and CEA protein) and irrelevant antigens by ELIspot and T-cell proliferation assays. For these assays, *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 plates for proliferation assay or in 96 well NC-plates coated with anti-human IFN-γ Ab for ELIspot assay) and incubated at 37°C at 5% CO_2 for 20-24 hours in the presence of different stimulants. Stimulants were each CEA peptide (10 μg/ml) and CEA

protein (1 μg/ml). For the irrelevant peptide, C140, cyclin D1 peptide (MELLLVNKLKWNLAA) (10 μg/ml) was used and for irrelevant protein, PKC nu (1μg/ml), a protein of similar preparation and size to CEA was used. To determine HLA class II restriction of peptide-specific CD4 T cells, anti human HLA-DR and HLA-DP, DQ, DR antibodies (BD biosciences, San Jose, CA) (10 μg/ml) were used in ELIspot and proliferation assays. Wells with CD4 T cells and irradiated PBMCs alone were considered as background. ELIspot and proliferation assay results (background subtracted) are expressed as antigen-specific CD4 T cells per million PBMCs and CPM respectively. These experiments were repeated three times using CD4 T cells isolated from different healthy donors.

Immunohistochemistry

Formalin fixed paraffin embedded tissue sections were deparaffinized and antigen retrieval was carried out using EDTA. Slides were treated with peroxidase blocking reagent followed by incubation with protein block for 5 minutes. Mouse monoclonal anti-CEA antibody (Cell Signaling Technology, Inc. Danvers, MA) was applied at a 1/500 dilution for 60 minutes. Visualization was carried out using DAKO's Dual + Envision link followed by incubation 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, 2 and 3 grades were considered as high expression.

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

Identification of CEA peptides with high binding affinity for HLA-DR molecules

Using the epitope prediction program PIC, 43 candidate CEA HLA-DR binding peptides were identified (data not shown). These peptides were tested for their binding to 15 different HLA-DR molecules as described in Materials and Methods. Fifteen (35%) out of 43 peptides (Table 1), which bound to at least eight different HLA-DR molecules with IC_{50} binding affinity of ≤1,000nM, were selected for further analysis.

Detection of elevated levels of peptide specific T cell immunity in cancer patients

A 10-day IFN-γ ELIspot assay as described in Materials and Methods was used to determine whether the individuals previously diagnosed with breast or ovarian cancer had generated natural immunity to any of the peptides. Immunity to four of the fifteen peptides (CEA.24, CEA.176/354, CEA.488, CEA.653) was detected in patients with either breast or ovarian cancer as shown in the scattergrams in Fig. 1a-d. T cell frequencies to each of the peptides ranged from 593 ± 124 (± SE) to 989 ± 150 peptide-specific T cells/million PBMCs. There were no discernable differences ($p>0.05$) in epitope-specific T cell frequencies between breast and ovarian cancer patients (data not shown). As shown in Fig. 2a, the cumulative T cell frequency of the three peptides CEA.24, CEA.176 and CEA.653 was increased in patients, whereas for one peptide, CEA.488, a smaller increase was observed. The difference in cumulative T cell frequency against the pool observed in patients and controls was statistically significant ($p=0.01$). Based on the available data describing the allelic frequencies, Hardy-Weinberg calculations estimated that the CEA pool of four peptides covers ~94% of individuals (Table 2) [28, 29]. As shown in Fig. 2b, 40% of

patients demonstrated immunity to the pool and this is significantly (p=0.006) higher than the proportion of healthy donors responding to the pool.

T cell responses do not correlate with patient clinical features

Blood was drawn from female volunteers without major exclusion criteria other than that they had not been previously diagnosed with cancer. Patients who had been diagnosed and treated for breast or ovarian cancers or both but who were currently disease free were selected for immune assessment in this study. Although this unimpeded enrollment resulted in an age difference between the healthy control donors and the patients, statistical analysis showed that age did not explain the elevated immunity to CEA ($p > 0.05$). Neither stage nor grade of tumor correlated with the levels of immunity ($p > 0.05$). Immunohistochemical staining results showed that 38% of patients had high levels of CEA expression while 62% had little or no expression (data not shown). These staining results are consistent with previous reports demonstrating that 10-50% of breast and ovarian cancer patients have CEA positive tumors [30, 31]. Sixty percent of patients with CEA^{hi} tumors and 38% of patients with CEA^{lo} tumors had detectable immune responses ($P > 0.05$).

CEA.24, CEA.176/354, and CEA.488 peptides are derived from naturally processed whole CEA protein

Even though elevated T cell responses were observed against the CEA pool in breast and ovarian cancer patients, only those that are naturally processed are relevant to immunotherapy. CEA.653 has already been identified in previous studies as naturally processed and presented [3]. To determine whether the remaining three peptides in CEA pool are naturally processed from whole proteins, we generated peptide-specific CD4 T cells by incubating them with CEA.24, CEA176/354, or CEA.488 and then testing for reactivity against whole CEA protein. As shown in Fig. 3, we found that CD4 T cells specific to CEA. 24, CEA.176/354 and CEA.488 peptides responded to both whole protein and respective peptide but did not respond to irrelevant protein (PKC nu protein) of similar size and irrelevant peptide (cyclin D1 peptide C140). There were no discernable differences between the reactivity of CD4 T cells against peptides and CEA protein ($p > 0.05$). Lastly, to confirm the HLA class II restriction of peptide-specific CD4 T cells, inhibition experiments were performed as described in Materials and Methods. As shown in Fig. 4, the reactivity of peptide-specific CD4 T cells was blocked using anti human HLA class II antibodies, whereas use of isotype control didn't effect the reactivity of these CD4 T cells. These results confirm that the pool of peptides (CEA.24, CEA.176/354, CEA.488 and CEA.653) against which breast and ovarian cancer patients had elevated immunity were naturally processed, presented and peptide-specific CD4 T cells are HLA class II restricted.

DISCUSSION

The importance of CD4 T cells in anti-tumor immunity has led to development of strategies to identify HLA-class II epitopes contained within different tumor-associated antigens [2, 32]. Recently there has been interest in defining CD4 T cell epitopes in CEA because it is a tumor antigen that is highly expressed in different types of cancers and has a role in tumorigenesis of cancers. Identification of several CD4 T cell epitopes from tumor antigens such as HER-2/neu, IGFBP-2 and folate receptor alpha provides evidence of pre-existing immunity against tumor antigens [5, 33]. Based on these prior works demonstrating elevated tumor-specific immunity, we took a comprehensive approach not only evaluating many potential CEA epitopes but also many different HLA-DR variants in order to capture a pool of epitopes that would be useful in the majority of patients. In summary, the novel findings of this study are 1) patients have elevated Th1 CD4 T cell immunity to pool of CEA epitopes CEA.24, CEA.488, CEA.176 and CEA.653 and 2) a pool of 4 degenerate CEA

epitopes consisting of two novel epitopes (CEA.24 and CEA.488) was established that is potentially useful in 94% of patients.

An important finding, which confirms the validity of our approach to epitope discovery, is that two of the four epitopes within the HLA-DR degenerate pool have been previously identified, namely CEA.176/354 and CEA.653. The assumption was made that CEA.176/354 is essentially the same as the previously discovered epitope $CEA_{177\text{-}189/355\text{-}367}$ [4]. CEA.176/354 fully encompasses this peptide. At the time of discovery, Campi and colleagues found that, when used during *ex vivo* priming amongst a pool of CEA epitopes, that $CEA_{177\text{-}189/355\text{-}367}$ was immunodominant. While our results support the conclusion that this epitope is immunodominant, we observed that 17%, 13% and 25% of breast and ovarian cancer patients responded to the peptides CEA.24, CEA.488 and CEA.653, respectively which are either equal or greater proportions than the proportion (13%) responding to CEA.176/354. Thus, we speculate that all the peptides that constitute the CEA HLA-DR degenerate pool identified in this study might be co-dominant. In that prior work, $CEA_{177\text{-}189/355\text{-}367}$ was found to be restricted by several HLA-DR1 variants (DRB1*03, DRB1*13, DRB1*07, DRB1*14, DRB1*1101, DRB1*1104, DRB1*0405, DRB1*14) demonstrating its profound degeneracy. The present study extends these prior studies by further demonstrating binding to DRB1*0101, DRB1*0401, DRB1*0404, DRB1*0802, DRB1*1501, DRB3*0101, and DRB4*0101. The other peptide that was previously identified, $CEA_{653\text{-}667}$ (i.e. CEA.653), was identified by Kobayashi and colleagues using a similar algorithm [3]. In that study it was found that CEA.653 binds to three HLA-DR molecules HLA-DRB1*04, -DRB1*07 and –DRB1*09, which we extended in the current study binds, with high affinity, to include at least five additional HLA-DR molecules, DR1 (DRB1*0101), DR3 (DRB1*0301), DR11 (DRB1*1101), DR13 (DRB1*1302) and DRB5 *0101. Thus, based on these findings, our estimate that the HLA-DR degenerate pool of CEA.24, CEA.176/354, CEA.488, and CEA.653 is useful in more than 94% of patients may be an underestimate. The true utility may approach 100%, if considering the alleles examined in our studies along with the others examined in prior studies.

The use of HLA-class II epitopes that contain, fully within their sequences, HLA class I epitopes to induce both CD4 and CD8 T cell responses simultaneously is an effective vaccination strategy [25, 34, 35]. The CEA-derived HLA-A2-restricted CAP-1 peptide had been used as a vaccine in several studies [36, 37] but the weak results of clinical trials using this vaccine have dampened enthusiasm of moving CEA peptides further into clinical trails. Recently it was shown by Saha and colleagues that incorporating a component that activates helper T cells in CEA HLA class I peptide vaccine is important for increasing the efficacy of vaccine [38], perhaps by increasing CD8 T cell longevity. Thus identification of new CEA peptides that encompasses both HLA class I and class II epitopes might address some of the causes of these previous failures. Of the four epitopes that constitute the pool used in the current study, CEA.24-38, CEA.176-190/354-370 and CEA.653-667 all encompass previously reported HLA-A2 motifs, CEA.24-32, CEA.176-184/354-362 and CEA.652-680 [20]. Use of this pool, therefore, as a vaccine might induce both CD4 and CD8 T cell responses. Alternatively, the current pool could be used as a CD4 T cell-activating component when mixed with other unrelated CEA HLA class I epitopes.

Although our epitope identification paradigm resulted in the establishment of a promiscuous pool of four epitopes that is potentially useful in a broad population, we cannot rule out that the possibility that the other epitopes that were not chosen are not biologically relevant and potentially useful. For example, our binding assay results showed that peptides such as CEA.50 and CEA.116 bind 14 and 13 HLA-DRs respectively out of 15 HLA-DR molecules, but elevated T cell immunity against these peptides was not seen in the patients (data not shown). Furthermore, in previous studies, CEA.116 was reported as a naturally presented

CD4 T cell epitope of CEA and shown to be immunogenic in HLA-DR4 transgenic mice [39]. Despite its promiscuous binding, the lack of response in the patients to this epitope is likely attributable to immunodominance by the other peptides and impairment of the T cell repertoire specific for CEA.116 by peripheral tolerization mechanisms. Tassi and colleagues have recently reported that patients with pancreatic cancer demonstrate impaired immunity (e.g. Th2 immunity) to CEA relative to normal healthy controls [40]. In our study, some important CEA HLA-DR epitopes reported in previous studies [41] may have been overlooked because the basis of our discovery process was elevated IFNγ (i.e. Th1) immunity. Thus, our study may have focused on the discovery of epitopes for which the effects of tolerance are minimal. The central problem that remains to be answered is whether it is better, in terms of clinical efficacy, to boost existing anti-tumor immunity or to reverse (i.e. break tolerance) impaired immune responses. Although we have shown that cancer patients have endogenous T cell immunity against the pool of CEA HLA-DR epitopes, the role of humoral responses in CEA specific immunity remains unanswered in this study. But, previous studies demonstrating elevated levels of CEA-specific IgG antibodies in breast cancer patients suggests the presence of CEA-specific CD4 T cells which is consistent with our data [42]. Thus our results support the previous findings that tolerance against CEA is not complete and we speculate that it is possible to boost the pre-existing immunity (both humoral and cellular immunity) against CEA using the pool of peptides reported in this study.

Despite our prediction that 94% of patients could respond to the degenerate pool, we observed only 40% of patients had elevated immunity against the pool. Several factors such as immunodominance, immunosuppression and CEA expression in the patients selected for this study can be attributed to the differences in predicted and observed responses [5]. The finding of anti-CEA T cell immunity in patients without detectable CEA expression would suggest that the immune system may have selected for antigen-negative variants. Indeed, recent murine and human studies have shown that antigen-specific immune effectors (T cell and antibody) can select for antigen negative variants i,e. immunoediting [1, 43]. For example, adoptive transfer of MART1/MelanA specific CD8 T cells into patients with metastatic melanoma resulted in the appearance of antigen-negative tumor variants [1]. At present it remains unclear if CEA-negative tumors can develop from CEA-positive tumors. Although CEA is associated with a more aggressive tumor, the fact that it is absent in a high proportion of patients suggests that it is dispensable. Thus CEA should subject to immunoediting.

Lastly, given the fact that CEA is highly expressed in several carcinomas [30], we presume that identification of this degenerate pool of CEA HLA-DR epitopes against which cancer patients have elevated endogenous T cell immunity might be useful in developing a multiepitope based CEA vaccine which would cover large population of cancer patients.

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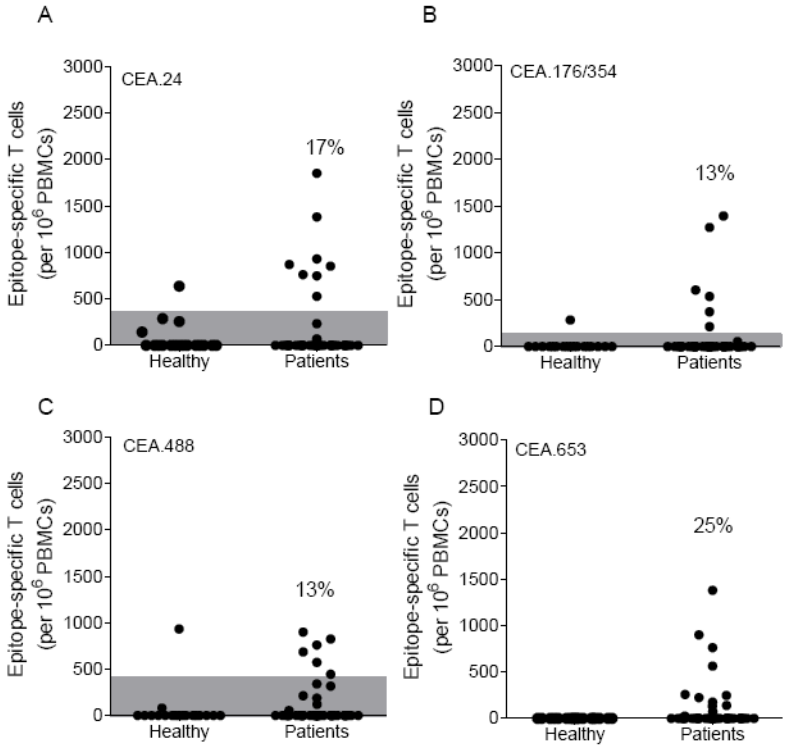

Fig. 1.

Detection of CEA peptide-specific immunity in breast and ovarian cancer patients. Shown are scattergrams showing IFN-γ production by T cells derived from patients and healthy donors. Results are represented as peptide-specific T cells per million PBMCs. Percentages represent proportion of patients responded 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).

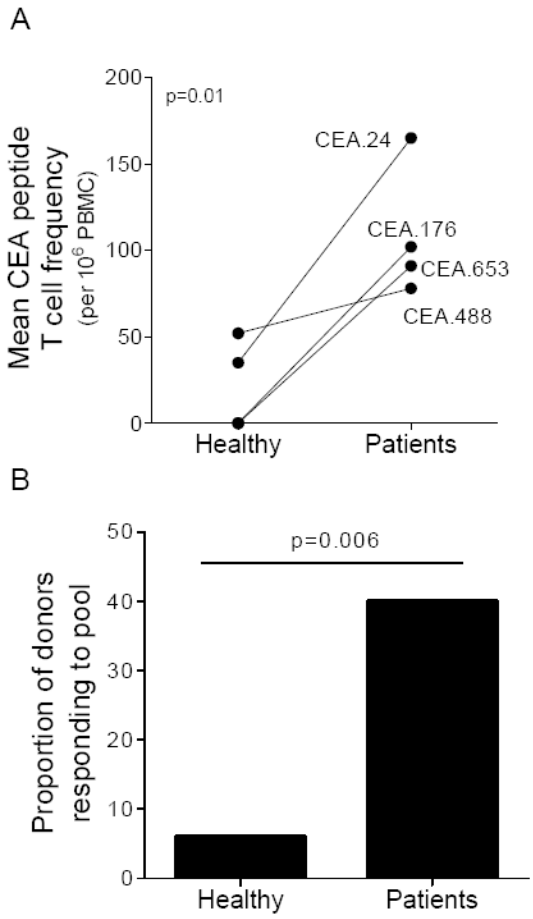

Fig. 2.

Breast and ovarian cancer patients demonstrate elevated CEA-specific T cell immunity compared to normal healthy individuals. **a** Relational diagram comparing cumulative T cell frequencies of each peptide between control and patients. **b** Column graph showing proportion of patients and healthy donors (control) responded to pool of CEA peptides. *P* values were calculated using Mann-Whitney test.

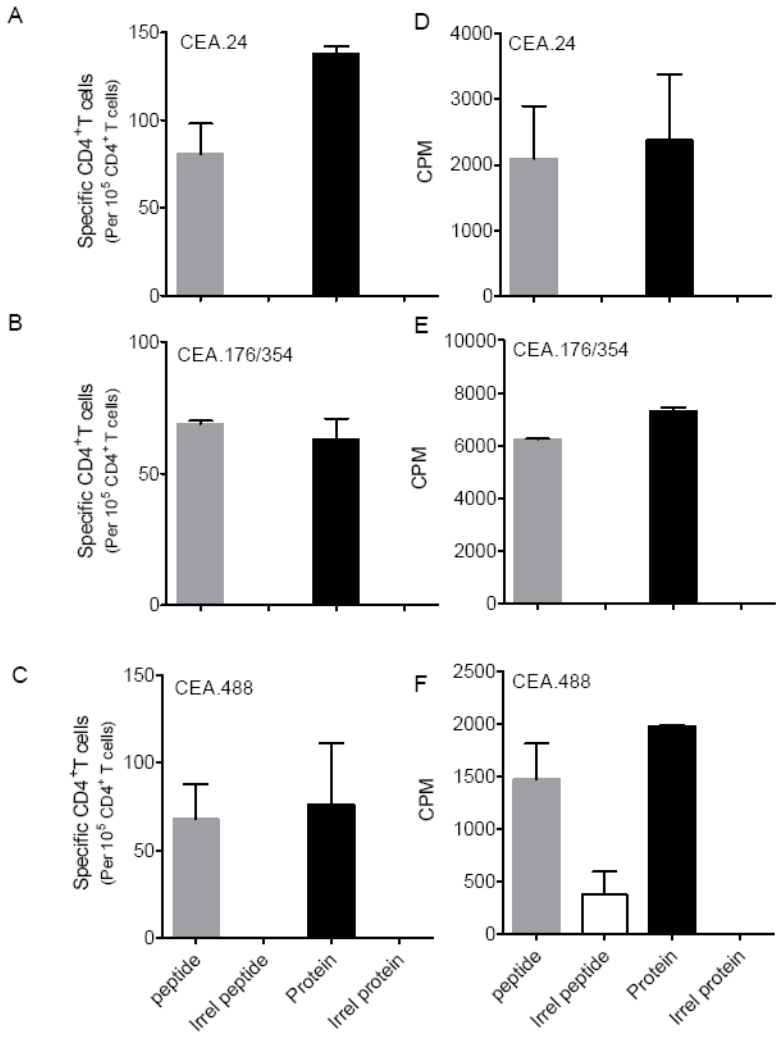

Fig. 3.

Peptides in the pool are derived from natural processing of CEA protein. **a,b,c** ELIspot and **d,e,f** Proliferation 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 peptide (CEA.24, CEA.176, CEA.488), CEA protein, irrelevant peptide (C140) and irrelevant protein (PKC nu protein). ELIspot results are shown as peptide-specific CD4 T cells per 10^5 CD4 T cells. Proliferation assay results are shown as counts per minute (CPM). Each columns are the mean ± SE of three replicates. In all panels, the CD4 T cell responses against CEA peptides and protein are significantly higher ($P<0.05$) than the responses against irrelevant peptide and irrelevant protein. Columns without any values indicate that the mean values of the triplicates are lower than the background. Data shown are the representative of one of three repeated experiments with similar results.

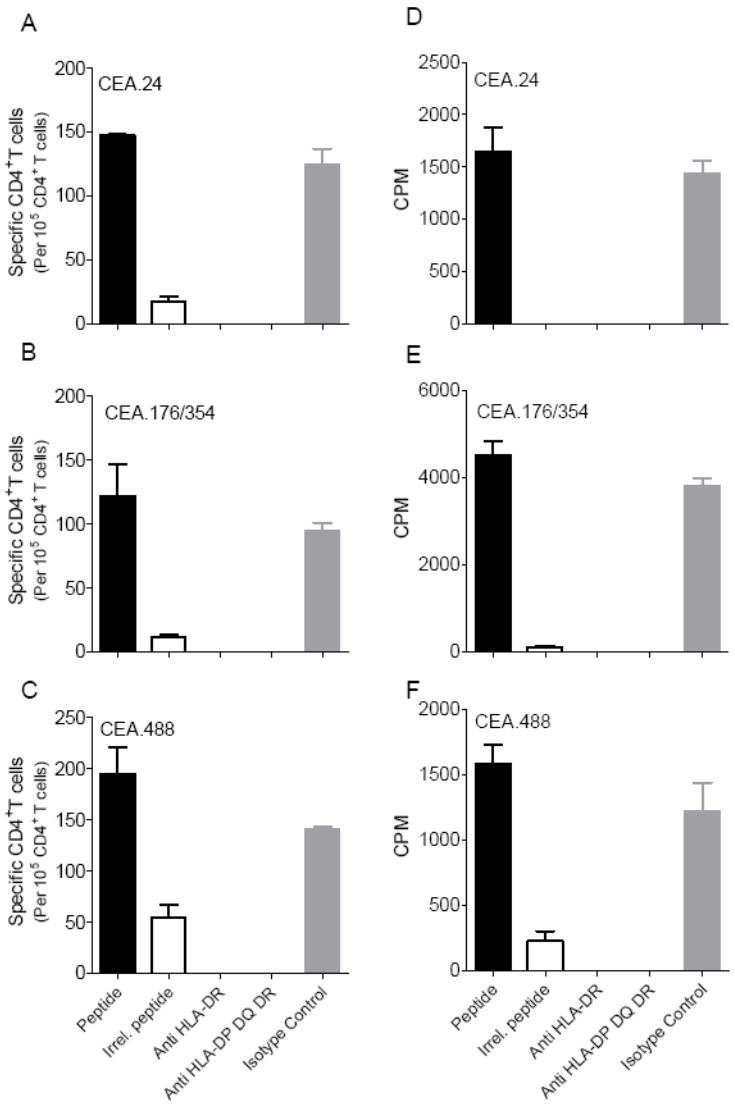

Fig. 4.

Peptide-specific CD4 T cells are HLA class II restricted. **a,b,c** ELIspot and **d,e,f** Proliferation 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 peptide (CEA. 24, CEA.176, CEA.488) and irrelevant peptide (C140) in the presence or absence of anti HLA-DR, HLA-DP, DQ, DR antibodies and isotype control. ELIspot results are shown as peptide-specific CD4 T cells per 10^5 CD4 T cells. Proliferation assay results are shown as counts per minute (CPM). Each columns are the mean ± SE of three replicates. Columns without any values indicate that the mean values of the triplicates are lower than the background. Data shown are the representative of one of three repeated experiments with similar results.

TABLE 1

Binding affinities of CEA 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
LLTFWNPPTTAKLTI	**CEA.24**	**24**	**6.9**	**16,313**	**273**	**52**	**258**	**3.7**	**174**	**5779**	**52**	**4995**	**245**	**46**	**ND**	**2171**	**31**
TAKLTIESTPFNVAE	CEA.33	33	72	613	106	41	383	70	1736	4019	5977	2907	35	140	ND	53	3350
EVLLLVHNLPQHLFG	CEA.50	50	2.7	830	3.4	1.7	30	5.4	59	989	40	5.4	0.36	4.7	334	50	1088
YSWYKGERVDGNRQI	CEA.65	65	511	ND	34	585	360	866	8432	ND	1840	ND	533	306	3002	453	1043
NRQIIGYVIGTQQAT	CEA.76	76	216	ND	108	1.5	129	46	46	345	36	4351	990	2.6	ND	5.2	2230
GREIIYPNASLLIQN	CEA.97	97	62	433	251	88	550	29	1959	1355	2209	212	24	49	4035	43	10,612
DTGFYTLHVIKSDLV	CEA.116	116	64	984	84	260	95	23	90	83	174	174	1072	65	14,943	18	564
FYTLHVIKSDLVNEE	CEA.119	119	101	80	184	169	41	56	514	718	6385	616	1340	14	14,343	22	4501
YLWWVNNQSLPVSPR	**CEA.176/354**	**176/354**	**563**	**167**	**422**	**226**	**84**	**18**	**181**	**1105**	**28**	**1316**	**971**	**172**	**668**	**708**	**1606**
QELFIPNITVNNSGS	CEA.282	282	147	644	25	227	379	1658	293	1086	358	1299	26	740	ND	3880	3869
SYTYYRPGVNLSLSC	CEA.423	423	1.6	4425	6.8	4036	300	5.4	21	1372	776	371	7.2	46	2626	1784	135
RTTVKTITVSAELPK	**CEA.488**	**488**	**89**	**1267**	**58**	**54**	**11**	**4.2**	**3763**	**367**	**6988**	**2758**	**96**	**29**	**ND**	**1263**	**1917**
NGTYACFVSNLATGR	CEA.650	650	839	818	11	558	30	20	70	377	2174	2052	30	2351	6963	3247	37
YACFVSNLATGRNNS	**CEA.653**	**653**	**183**	**774**	**225**	**41**	**327**	**531**	**1774**	**4520**	**217**	**2399**	**107**	**1237**	**ND**	**1569**	**17**
NNSIVKSITVSASGT	CEA.665	665	34	103	43	1.8	128	34	184	469	209	1328	4.4	274	15,808	26	2280

¶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 CEA peptides that bind to each allele's gene product

	HLA-DRB1										HLA-DRB 3-5				
Allele	**DRB1 *0101-06**	**DRB1 *0301-13**	**DRB1 *0401-32**	**DRB1 *0701-04**	**DRB1 *0802-21**	**DRB1 *0901**	**DRB1 *1101-35**	**DRB1 *1301-34**	**DRB1 *1501-08**	**Total Coverage DRB1**	**DRB3 *0101**	**DRB4 *0101**	**DRB5 *0101**	**Total Coverage DRB 3-5**	**Predicted response rate (%) §**
Caucasoids	9.4†	11.1	12.8	13.2	3.7	2.0	13.4	10.2	10.8	**69.2**	53.2	50.9	23.9	**93.4**	**97**
Blacks	5.5	14	10.5	9.23	4.8	2.0	15.7	14.3	9.9	**69.0**	55.2	21.1	16.5	**77.8**	**84**
Asian	3.0	5	13	5.8	6.5	9.4	7.8	4.9	14.4	**58.7**	54.2	48.9	22.0	**92.4**	**98**
Estimated Average	6	10	12	9	5	5	12	10	12	**65.8**	54.2	40.3	20.8	**88.5**	**94**
# of epitopes	4¶	2	4	4	2	1	3	4	3	**3**	1	1	2	**1**	

† Frequency of allele the population.

¶ Number of epitopes in the CEA pool that bind to this HLA-DR variant.

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