An HLA-DR–Degenerate Epitope Pool Detects Insulin-like Growth Factor Binding Protein 2–Specific Immunity in Patients with Cancer

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Abstract

Recent studies have shown the importance of helper CD4 T cells in initiating and sustaining tumor-specific CD8 T-cell immunity. This has paved the way for identifying MHC class II epitopes that could be incorporated into class I–based vaccines. In this study, the goal was to identify an HLA-DR–degenerate epitope pool derived from insulin-like growth factor binding protein 2 (IGFBP-2). IGFBP-2, a regulator of insulin-like growth factor action, is overexpressed in the majority of breast and ovarian cancers. Using algorithms, we predicted 29 HLA-DR1–binding epitopes. Binding assays targeting 15 different HLA-DRs revealed that 10 epitopes were degenerate, binding to at least four different HLA-DR variants. An IFN-γ enzyme-linked immunosorbent spot assay was used to assess immunity to these 10 epitopes in 48 patients with either breast or ovarian cancer and 18 controls. Elevated T-cell immunity in patients was detected in 4 of the 10 epitopes (IGFBP2.17, IGFBP2.22, IGFBP2.249, and IGFBP2.293). The cumulative T-cell frequency of these four epitopes was elevated in patients relative to controls. All four peptides are naturally processed and presented to CD4 T-cells. The degenerate pool of peptides covers nearly 80% of patients and may be useful for augmenting CD4 T-cell immunity in patients undergoing immunization. [Cancer Res 2008;68(12):4893–901]

Introduction

Because CD8 T cells can directly kill tumor cells, cancer vaccines have historically focused on eliciting CD8 T-cell responses. The identification of the minimal epitopes that are presented to CD8 T cells by MHC class I has led to clinical trials examining the immunogenicity and clinical activity of class I peptide-based vaccines in patients with cancer. Despite predicted successes, in general, the clinical translation of this approach has been largely unsuccessful, due in part to weak immunogenicity and lack of sustained responses. One explanation for the weak immunogenicity is that CD8 T cells that are able to mediate regression are depleted by central tolerance. However, this notion was dispelled by recent T-cell therapy studies which show that melanoma-derived tumor-infiltrating CD8 T cells can cause tumor regression when activated appropriately, *ex vivo*, and subsequently reinfused (1–3). Thus, the problem seems to be that vaccines are not optimally designed to generate or reactivate endogenous latent or tolerized CD8 T cells. A notable problem is that many vaccines are not designed to elicit CD4 T cells which are required for optimal CD8 T-cell induction.

CD4 T cells are critical during natural immune responses for protection against infection and malignancy and they coordinate effective immunity by inducing or recruiting cells of the innate (e.g., macrophages and dendritic cells) and the adaptive (e.g., CD8 T cells and B cells) immune systems (4–10). In the absence of CD4 T-cell help, vaccines may induce CD8 T cells which, regardless of their numbers or specificity, are unable to mediate the regression of tumor due to defects (e.g., exhaustion and senescence) and are rapidly eliminated (11, 12). This paradigm has initiated interest in supplementing class I peptide vaccines with class II epitopes to elicit concurrent CD4 T-cell help. Two general strategies could be employed. The first is to include foreign class II epitopes which, because of their foreign nature, can generate strong T-cell help. One example of this strategy is PADRE, a synthetic HLA-DR–degenerate epitope (13). The alternative approach is to include class II peptides derived from tumor antigens. There are advantages and weaknesses for each approach. Because of its foreign nature, PADRE has the potential to be a strong immunogen but the response is only relevant during the active immunization but not secondary expansion of CD8 T cells. In contrast, tumor antigen–derived class II epitopes may be weaker immunogens, but the induction of cognate CD4 T cells would be beneficial to secondary immune responses important to preventing relapse in the minimal disease setting. This latter point was recently shown in preclinical studies by Hwang and colleagues, who showed that although not required for secondary activation of antigen-specific CD8 T cells, cognate CD4 T-cell help led to a 2-fold increase in the frequency of secondary CD8 T cells resulting in better tumor control (14). The problem of employing cognate class II epitopes within vaccines, however, is that very few have been identified that could be useful in clinical trials. One problem that limits utility is the diversity at the *HLA-DR* locus in humans. Although class I alleles can be fairly frequent (e.g., 40–50% in the case of HLA-A2), specific *HLA-DR* allelic frequencies are much lower and typically <15%, which makes informative peptide vaccine trials difficult. However, research into the epitope binding properties of the different allelic variants of *HLA-DR* has shown that, despite broad genetic variation, the epitope repertoire that each variant binds has significant overlap, thus increasing the possibility that epitopes can be identified that would be useful in larger cohorts of patients. In fact, with newer algorithms which have increased predictive power, it should be possible to establish pools of class II peptides that would be potentially useful in vaccines.

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Table 1. Binding affinities of IGFBP-2 peptides to purified HLA-DR

Sequence	Peptide name	Position	IC_{50} nmol/L to purified HLA					
			DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701
ALPLPPPPLLPLLPL	IGFBP2.9	9	174	ND	18	4.2	20	336
PPPLLPLLPLLLLLL	IGFBP2.14	14	15	5,816	15	16	65	13
LLPLLPLLLLLLGAS	IGFBP2.17	17	119	ND	337	35	674	964
PLLLLLLGASGGGGG	IGFBP2.22	22	20	18,033	339	2,144	1,422	5,882
PPPVAPPAAVAAVAG	IGFBP2.58	58	178	ND	380	81	ND	2,684
LKSGMKELAVFREKV	IGFBP2.184	184	607	ND	881	862	34	ND
LDQVLERISTMRLPD	IGFBP2.234	234	795	452	25	20	18	5.2
ERGPLEHLYSLHIPN	IGFBP2.249	249	7.7	ND	497	29	110	18
GLYNLKQCKMSLNGQ	IGFBP2.268	268	923	ND	743	2,835	5,589	1,791
TGKLIQGAPTIRGDP	IGFBP2.293	293	148	9,554	40	27	608	883

Abbreviation: ND, not determined.

Our goal in the current study was to establish a pool of peptides derived from a frequently overexpressed tumor protein that could have potential to elicit tumor antigen–specific CD4 T-cell immunity in a broad population of patients. We chose insulin-like growth factor binding protein 2 (IGFBP-2) because it is a secreted protein likely to be recognized by CD4 T cells due to its uptake into the class II antigen presentation pathway. IGFBP-2 is overexpressed in a number of malignancies, including ovarian, breast, lung, colon, and prostate cancer (15–18). Several studies show that most (>80%) breast and ovarian cancers overexpress IGFBP-2, suggesting that it could make a choice vaccine antigen in these patient populations (15, 17, 19–23). IGFBP-2 modulates the bioavailability of IGF-I and IGF-II (24, 25). Increased expression of IGFBP-2 is associated with increased stage and grade of ovarian cancer and is a negative prognostic factor for progression-free and overall survival, suggesting its importance in initiating or promoting tumor development (17, 26).

An algorithm was used to predict class II (HLA-DR)–binding peptides from IGFBP-2, followed by class II–binding studies, patient screening, and *in vitro* confirmation of natural processing. The end result was the development of a pool of four HLA-DR–degenerate epitopes that may be useful for inclusion in vaccines to stimulate cognate tumor-specific CD4 T-cell immunity in most patients regardless of the HLA-DR variants they express.

Materials and Methods

Reagents. The CEF viral peptide pool was from the NIH AIDS Research and Reference Reagent Program (27). Purified IGFBP-2 was from R&D Systems. All peptides were synthesized by either the Mayo Clinic Protein Chemistry and Proteomics Core or by Epimmune, Inc. and purified to >95% homogeneity by reversed phase high-performance liquid chromatography as previously described (28). The purity of the peptides was determined with reversed phase high-performance liquid chromatography and amino acid analysis, sequencing, and/or mass spectrometry. Lyophilized peptides were resuspended in DMSO and then diluted in PBS.

Epitope prediction. The prediction program used, PIC (predicted IC_{50}), is a modified linear coefficient or matrix-based method for predicting peptides with HLA-DR binding capacity (29, 30). The algorithm yields a predicted IC_{50} value (designated as PIC), and lower PIC values indicate a higher probability of binding to HLA.

Patients and donors. The study was approved by the Institutional Review Boards at both the Mayo Clinic and the University of Washington. Eighteen healthy donors and 38 patient samples were obtained from the Mayo Clinic. Ten patient samples were from the University of Washington and were processed and stored using the same procedures and protocols as the Mayo Clinic samples. Patients were free from active treatment for at least 30 days when blood was collected. For the T-cell studies, the mean ($\pm$SE) ages of the healthy donors and patients were 42 $\pm$ 11 and 55 $\pm$ 2 years, respectively ($P < 0.0001$). Due to sera unavailability, not all of the controls used in the T-cell studies were examined for IGFBP-2 antibodies in their sera. However, additional control and patient sera were available for antibody assessment. Thus, for the IGFBP-2 antibody studies, the mean ($\pm$SE) ages of the healthy donors and patients were 42 $\pm$ 1 and 59 $\pm$ 1 years, respectively ($P < 0.0001$). Additional healthy donor sera was obtained from Bioreclamation.

Preparation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from blood as described (31), and cryopreserved in liquid nitrogen (20×10^6/mL cells) in freezing medium (RPMI with human serum albumin and DMSO; ref. 32).

HLA-DR purification. The HLA-DR molecules (see Table 1 for list) were chosen for this study to allow balanced population coverage (33). The HLA molecules were purified from EBV-transformed homozygous cell lines or single MHC allele–transfected cell lines using antibody-based affinity chromatography. Briefly, HLA-DR molecules were captured by the passage of cell lysates over LB3.1 monoclonal antibody (anti–HLA-DRA) columns. MHC molecules were then eluted with diethylamine and *n*-octylglucoside in NaC1 (pH 11.5). The pH was reduced to 8.0 and the eluates were concentrated by centrifugation.

HLA-DR binding assays. Binding of peptide to HLA-DR was done with radioligand binding inhibition assays as described previously (34). Briefly, 1 to 10 nmol/L of radiolabeled peptide was coincubated for 2 days at either room temperature or 37°C with 1 μmol/L to 1 nmol/L of purified HLA-DR molecules in the presence of a cocktail of protease inhibitors and in the absence or presence of known competitive binding peptides. 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 measurements. As in previous studies, peptides with affinities for specific HLA-DR molecules of 1,000 nmol/L or better were defined as binders for the respective antigens (34).

Enzyme-linked immunosorbent spot assay. A 10-day enzyme-linked immunosorbent spot (ELIspot) assay for detecting low-frequency T cells was used to determine reactivity to the IGFBP-2–derived peptides (Table 1)

Table 1. Binding affinities of IGFBP-2 peptides to purified HLA-DR (Cont'd)

IC_{50} nmol/L to purified HLA								
DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1201	DRB1 *1302	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101
1,087	ND	41	1,337	1,524	262	ND	40	9,665
86	307	121	23	1,322	5.0	16,239	2.1	121
213	5,893	458	320	2,022	182	ND	19	2,390
5,696	ND	12,818	18,255	2,164	253	ND	316	ND
ND	317	ND	ND	237	1,378	ND	1,888	ND
260	ND	163	1,768	4,974	91	ND	417	843
1,607	52	467	314	1,052	84	ND	367	223
9,361	638	1,993	50	1,149	23	ND	1,648	4,000
204	9,434	2,600	413	561	223	ND	1,613	5,609
5,160	700	7,989	2,691	927	1,733	2,191	465	36

and was done in groups of two (two healthy donors, one healthy/one cancer patient, or two cancer patients) exactly as previously described (27). A 15–amino acid degenerate HLA-DR binding peptide (amino acids 140–154, KISQAVHAAHAEINEAG) derived from cyclin D1, produced in the same fashion as the IGFBP-2 peptides, was used as a negative control peptide. This peptide was shown to bind with high affinity (<1,000 nmol/L) to HLA-DRB1*0101, *0401, *0404, *0802, *1101, *1201, *1302, *1501, DRB4*0101, and DRB5*0101. A pool of two previously identified naturally processed carcinoembryonic antigen (CEA) epitopes were used as a positive control because CEA is frequently overexpressed (45–50%) in breast and ovarian cancers (35, 36). These peptides were CEA.176 (amino acids 176–190, YLWWVNNQSLPVSPR) and CEA.653 (653–667, YACFVSLATGRNNS; refs. 37, 38). Collectively, both of these peptides bind with high affinity (i.e., IC_{50} < 1,000 nmol/L) to HLA-DRB1*0101, *0301, *0401, *0404, *0405, *0701, *0802, *1101, *1302, *1501, DR3*0101, and DR4*0101. Phorbol 12-myristate 13-acetate (PMA)/ionomycin and the CEF pool were used as positive non–tumor-related controls as previously described (27, 39).

ELISA. ELISAs were done as previously described (27). Briefly, 96-well plates were coated with IGFBP-2 protein, tetanus toxin, or 1 μg/mL of bovine serum albumin followed by washing and blocking. Human sera were added to the plate at a 1:40 dilution in triplicate and plates were incubated for 2 h at room temperature. After washing, horseradish peroxidase–conjugated antihuman IgG was added for 1 h at room temperature. After a final wash, each well was developed with 100 μL of a tetramethylbenzadine substrate (BD Bioscience).

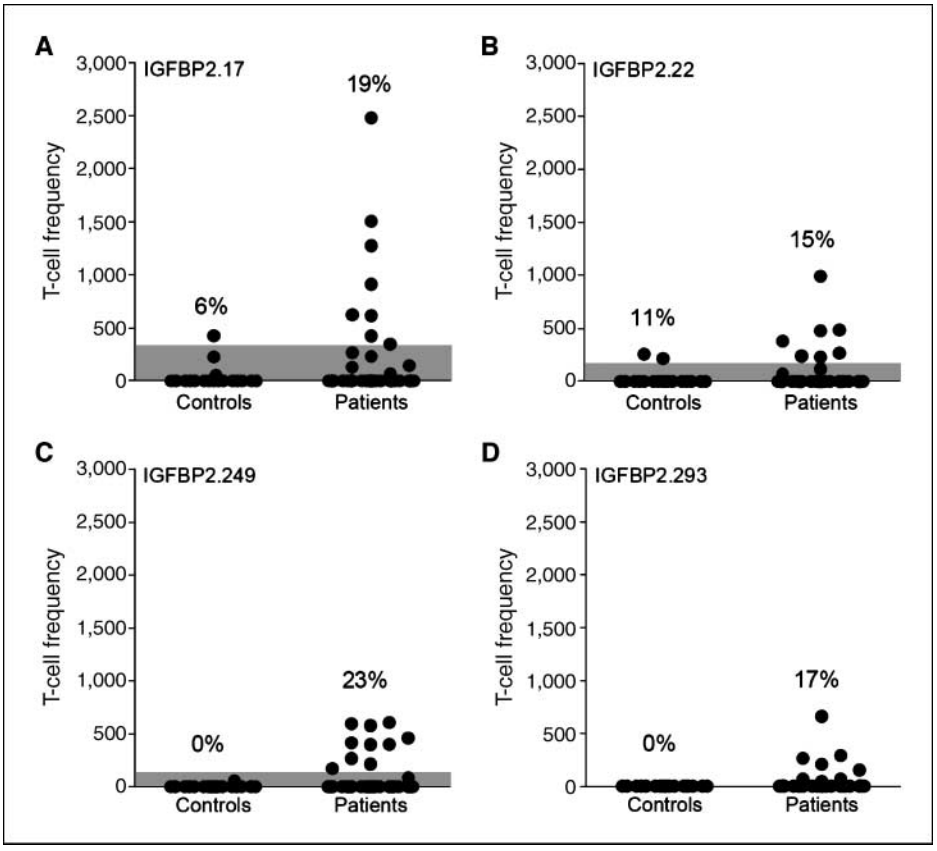

Figure 1. Detection of IGFBP-2 peptide–specific immunity in patients with breast and ovarian cancer. *A* to *D,* scattergrams of IFN-γ ELIspots from controls and patients. *Percentages,* fractions responding above the mean + 2 SD (*area outlined in gray*). *Points,* values calculated for triplicate determinations from unique individuals (representative of a unique IGFBP-2 peptide). The T-cell frequencies are presented as T cells per million PBMCs.

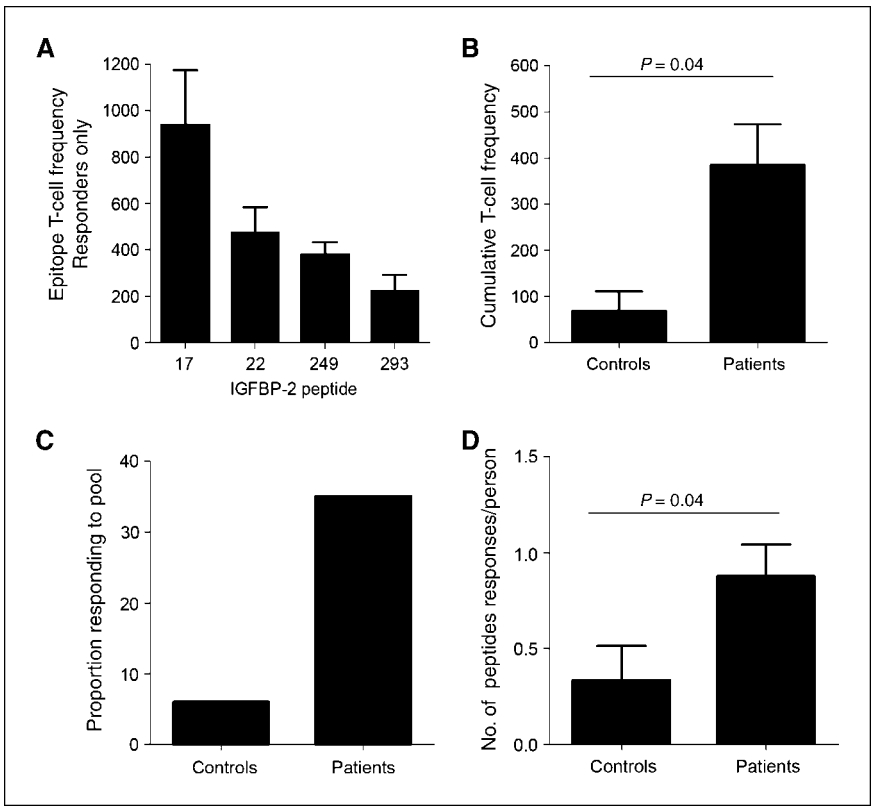

Figure 2. Patients with breast and ovarian cancer have elevated T-cell immunity to HLA-DR–degenerate epitopes in IGFBP-2. *A, columns,* mean epitope T-cell frequency in the responding patients only for each of the four IGFBP-2 peptides; *bars,* SE. *B,* the proportion of controls and patients responding to the pool of four IGFBP-2 peptides. Each control or patient had a statistically elevated ($P < 0.01$) response to the pool compared with the background established by the healthy controls. *C,* the cumulative T-cell frequency (*columns,* mean from all four peptides; *bars,* SE) in all controls and all patients. Values from individual patients were significant at $P < 0.01$. *D,* the mean number of peptides in the pool that the controls or patients responded to. *Columns,* mean for either all of the controls or for all of the patients; *bars,* SE; the *P* value was calculated using a one-tailed Student's *t* test. The T-cell frequencies are presented as T cells per million PBMCs.

Generation of antigen-specific human CD4+ T cells. Dendritic cells were generated by seeding PBMC in six-well plates (3×10^6/well) in culture medium (RPMI 1640, human AB serum, sodium bicarbonate, MEM nonessential amino acids, sodium pyruvate, L-glutamine, and 2-mercaptoethanol) with granulocyte macrophage colony-stimulating factor and interleukin 4. On day 5, bacterial CpG was added to the cultures at 1 μg/mL and the cells were ready for T-cell induction on day 6 at which time the dendritic cells were pulsed with protein or peptide antigen and B7-DC–crosslinking antibody (a gift from Dr. Larry Pease, Department of Immunology, Mayo Clinic, Rochester, MN) for 4 h. Magnetically purified CD4+ T cells were added and incubated at 37°C with periodic interleukin 2 additions. On day 15, cells were assayed for reactivity to cognate antigens (i.e., IGFBP-2 peptide and protein) or to irrelevant antigens. For the irrelevant peptide, human collagen II peptide, HII.71 (PPGLTGPA-GEPGRQGSPGAD) was used, and for an irrelevant protein of similar size, ovalbumin was used.

Statistical analyses. Statistical analyses were performed using InStat (GraphPad Software). Data were analyzed using two-tailed Mann-Whitney (nonparametric data) or Student's *t* tests unless otherwise stated, and the results were considered statistically significant if $P < 0.05$. The cumulative *HLA-DR* frequencies and the estimated percentage of patients that would respond to the IGFBP-2 peptide pool were calculated using Hardy-Weinberg equilibrium properties. Population allele frequencies were derived per Hardy-Weinberg equilibrium from the *HLA-DR* population estimates. The percentage of alleles in the population that responded to the IGFBP-2 peptide pool was then determined and used to calculate the percentage of patients with at least one responding allele.

Results

Identification of HLA-DR–degenerate epitopes of IGFBP-2. Twenty-nine epitopes were predicted from the full-length IGFBP-2 sequence (data not shown). Following determination of binding to 15 distinct HLA-DR variants, 10 were selected to test in ELIspot screening assays based on an ability to bind to at least four distinct variants with an IC_{50} binding affinity of at least 1,000 nm (Table 1). All 10 bound to HLA-DRB*0101 with high affinity as predicted with the PIC algorithm.

Patients with breast and ovarian cancer have elevated T-cell immunity to HLA-DR–degenerate epitopes of IGFBP-2. Using an ELIspot assay with a limit of detection of ~1:100,000 antigen-specific T cells per million PBMC, patient-derived PBMC were screened for reactivity against all of the HLA-DR binding peptides (27, 40). Fifteen percent or greater of patients responded to 4 of the 10 epitopes (IGFBP2.17, IGFBP2.22, IGFBP2.249, and IGFBP2.293). Scattergrams depicting the ELIspot results for these four peptides are shown in Fig. 1*A–D*. The T-cell frequencies to each of the peptides in the responders ranged from 220 ± 71 to 936 ± 238 (mean ± SE) peptide-specific T cells/million PBMC (Fig. 2*A*). When evaluated as a pool the cumulative T-cell frequencies were (mean ± SE) 394 ± 110 T cells/million PBMC for all patients and 68 ± 43 T cells/million PBMC for the healthy controls (Fig. 2*B*). There was no statistical difference ($P > 0.05$) in the cumulative T-cell frequencies between the patients with breast (368 ± 164 T cells) and ovarian (393 ± 110 T cells) cancers. Additionally, there were no differences in the responses to the individual peptides among the two patient groups (data not shown).

Based on the available data describing the allelic frequencies of the HLA-DR homologues (including the paralogues), this pool of four IGFBP-2 peptides should cover nearly 80% of the population (Table 2; refs. 41, 42). However, the total proportion of patients responding to the pool (i.e., to at least one peptide) at a significance level of $\alpha = 0.01$ was only 35% (Fig. 2*C*), which may indicate tumor-induced immunosuppression or levels of T-cell reactivity below the limits of detection of the ELIspot assay. The proportion of controls

that responded to the pool was 6%. Also, it was predicted that the average patient would likely respond to at least 2.3 peptides in the pool. However, the number of IGFBP-2 peptides that the patient population responded to per person was only 0.9 ± 0.2 peptides/person (Fig. 2*D*). This was, however, statistically elevated compared with 0.3 ± 0.2 peptides in the control population ($P = 0.04$). Again, there were no significant differences between the patients with ovarian and breast cancers ($P > 0.05$).

Elevated immunity in patients is confined to tumor antigens and is independent of age. A number of different control variables were examined in order to determine if the elevated responses were due to some inherent differences in the samples. First, the ability of the samples to respond to the nonspecific stimulus, PMA/ionomycin, was examined (Fig. 3*A*). There were no significant differences ($P = 0.4$) between the number of spots obtained in the controls (2,212 ± 366 spots) and the patients (2,049 ± 300 spots). Second, the overall response to the CEF viral class I peptide pool was not different ($P = 0.4$), with a T-cell frequency of 192 ± 76 T cells/million PBMC in the controls and 133 ± 36 T cells/million PBMC in the patients. As another control, the response to a degenerate HLA-DR binding epitope of cyclin D1 was examined (see Materials and Methods). Cyclin D1 is highly overexpressed in breast and ovarian cancers, but because of its intracellular localization, it is not expected to be as readily presented through HLA-DR, which requires that the protein be released from the cell and taken up and presented by antigen-presenting cells (i.e., B cells, macrophages, and dendritic cells; refs. 43, 44). As shown in Fig. 3*A*, despite high-affinity binding to 9 of 15 alleles, there was little response in either population (controls, 23 ± 16; patients, 21 ± 13 T cells/million PBMC, $P = 1$).

To avoid selection bias, recruitment was essentially unimpeded by selection criteria, except for the requirement that either an individual be a healthy female with no history of breast or ovarian cancer or have been diagnosed with one of the two cancers and be disease-free. This recruitment resulted in an age difference (see Materials and Methods). Thus, regression analyses of the data were done to determine if age had a role in the differential response to the IGFBP-2 pool. As shown in Fig. 3*B* and *C*, age seemed to play no role in the immune responses observed to the IGFBP-2 pool as the slopes of the regression lines were not significantly different ($P > 0.5$) from a slope of zero. In addition to these controls, immune responses to a pool of two previously described naturally processed CEA peptides, CEA.176 and CEA.653, were also examined as a positive control. In this study, HLA-DR indiscriminancy of these peptides was observed with CEA.176 binding with high affinity to 13 of 15 HLA-DRs examined and with 9 of 15 HLA-DRs using CEA.653 (data not shown). As shown in Fig. 3*D*, at a significance level of $\alpha = 0.01$, 23% of patients responded to the pool, whereas only 6% of the controls responded, a finding consistent with aberrant expression of this protein in cancer patients but not in healthy individuals. Collectively, these data show that the sample qualities were similar and suggest that the T-cell responses to IGFBP-2 were due to antigen exposure *in vivo* and not due to some artifact. Correlations of the IGFBP-2 T-cell responses to other tumor and treatment characteristics were also examined and included stage, grade, time from diagnosis, and time of the blood draw from last chemotherapy. These clinical data were available on 67% to 94% of patients, depending on the criterion. Stage information was available for 45 patients (16 stage I, 10 stage II, 17 stage III, and

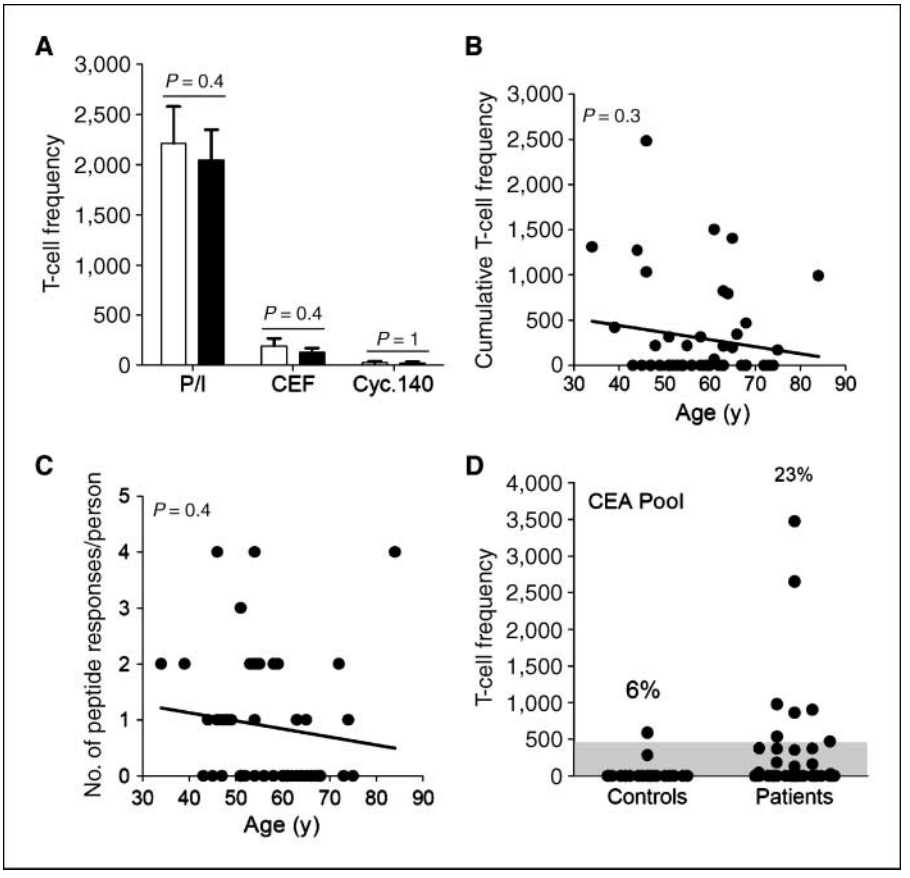

Figure 3. Elevated immunity in patients is confined to tumor antigens and is independent of age. *A,* T-cell responses to PMA/ionomycin (*P/I*), the CEF peptide pool, and cyc.140 in both controls (*open columns*) and patients (*filled columns*). *Columns,* mean obtained from 18 control donors and 48 patients; *bars,* SE. *B,* linear regression analysis comparing age and the cumulative T-cell frequency to the pool of four IGFBP-2 epitopes in patients. *Points,* unique patients. *C,* a similar regression analysis comparing age with the number of peptides in the pool that each patient responded to. *D,* T-cell frequencies to the CEA pool. *Points,* sum of the responses to CEA.176 and CEA.653; each represents the response from a single individual. *Percentages,* the fraction of individuals in each group that had an elevated response relative to the mean ± 2 SD of the healthy controls (*gray shaded area*) at a significance levels of $\alpha = 0.01$. The T-cell frequencies are presented as T cells per million PBMCs.

Table 2. HLA class II gene frequencies and number of IGFBP-2 peptides that bind to each allele's gene product

Sequence	HLA-DRB1						
	*DRB1 *0101-06*	*DRB1 *0401-32*	*DRB1 *0701-04*	*DRB1 *0802-21*	*DRB1 *0901*	*DRB1 *1101-35*	*DRB1 *1201-06*
Caucasoids	9.4	12.8	13.2	3.7	2.0	13.4	2.3
Blacks	5.5	10.5	9.23	4.8	2.0	15.7	4.4
Asian	3.0	13	5.8	6.5	9.4	7.8	13.5
Estimated Average	6	12	9	5	5	12	7
No. of epitopes*	4	4	3	1	2	1	2

NOTE: Values are shown as a percentage of the population, except for the final row.
*The total number of epitopes in the pool that each HLA-DR is able to bind.
† Mean number of epitopes the average individual might respond to.

2 stage IV patients). Stage did not correlate with the cumulative T-cell response (P = 0.74). Tumor grade was available for 33 patients (1 grade 1, 7 grade 2, 5 grade 3, and 20 grade 4) which also did not correlate with the cumulative T-cell response (P = 0.96). At the time of blood draw, the mean time (months) from diagnosis (n = 36) and the mean time from the last chemotherapy (n = 32) was 54.8 months (range, 8.7–174.4) and 33.2 months (range, 4.1–105.8), respectively. Neither of these variables were correlated with the cumulative response to the IGFBP-2 peptide pool (P = 0.17 and P = 0.22, respectively).

The IGFBP-2 peptides are naturally processed and presented by dendritic cells to CD4 T cells. Having shown that the four IGFBP-2 epitopes bind to multiple HLA-DRs and that patients have elevated immunity to each one, the next step was to confirm that the epitopes activated CD4 T cells and were naturally processed from whole protein. IGFBP-2 peptides in the pool were naturally processed from whole proteins by autologous antigen-presenting cells, and pure $CD4^+$ short-term (i.e., 9 days old) peptide-specific T-cell lines were prepared from the peripheral blood of patients. The lines were evaluated by IFN-γ ELIspot for

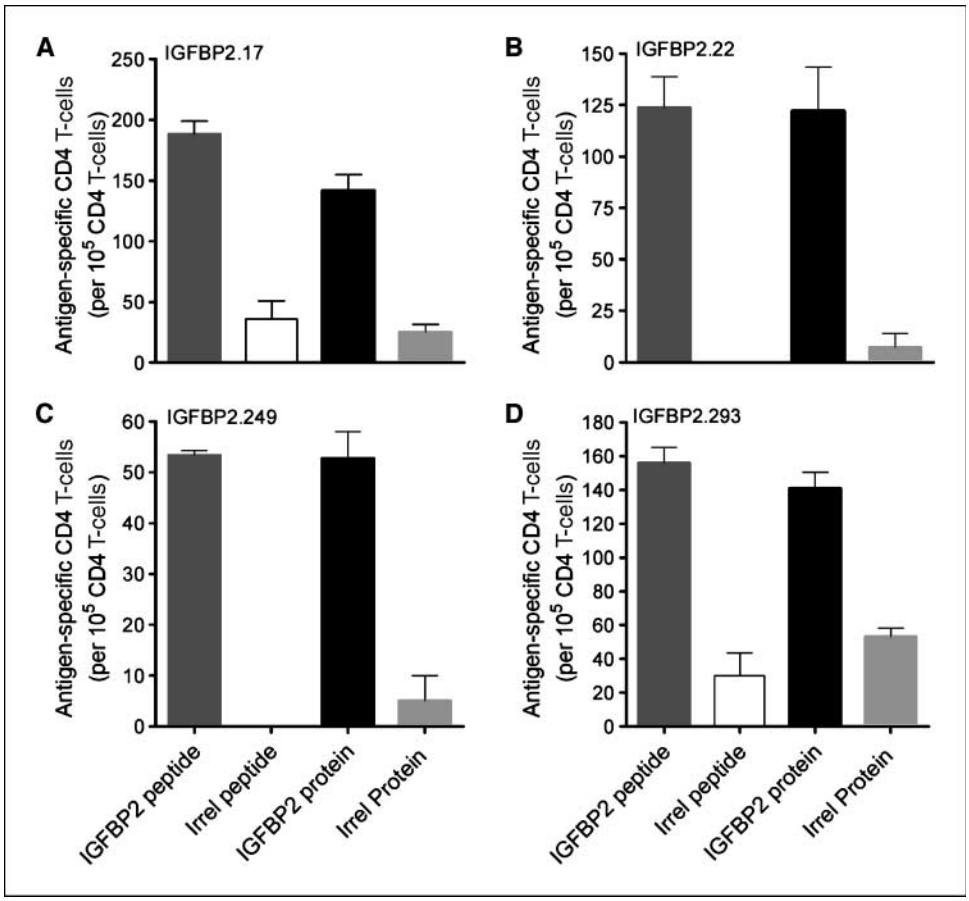

Figure 4. The IGFBP-2 peptides were naturally processed and presented by dendritic cells to CD4 T cells. ELIspot results obtained by stimulating short-term IGFBP-2 peptide–specific T-cell lines with relevant peptides, IGFBP-2 whole proteins, control ovalbumin proteins, and an irrelevant 15-mer peptide. Each panel represents a T-cell line specific for one of the IGFBP-2 peptides. *Columns,* mean of three replicates; *bars,* SE. In all cases, the cognate peptide or protein response was significantly higher ($P < 0.05$) than the irrelevant response.

Table 2. HLA class II gene frequencies and number of IGFBP-2 peptides that bind to each allele's gene product (Cont'd)

HLA-DRB1			HLA-DRB4/5			Predicted percentage of patients that could respond
DRB1 *1301-34	DRB1 *1501-08	Total Coverage DRB1	DRB4 *0101	DRB5 *0101	Total coverage DRB4/5	
10.2	10.8	64.0	50.9	23.9	67.1	86
14.3	9.9	63.1	21.1	16.5	35.7	67
4.9	14.4	64.3	48.9	22.0	64.2	83
10	12	64.0	40.3	20.8	56.1	79
1	3	2.3 †	3	1	2 †	

responses to cognate and irrelevant antigen. Representative results are shown in Fig. 4 for each of the IGFBP-2 pool peptides. In all cases, the IGFBP-2 protein response was commensurate (and statistically indistinguishable $P > 0.05$) with the cognate peptide frequency of the CD4 T-cell line. These experiments were repeated twice or thrice for each peptide using T cells derived from three different patient donors. The results verify that all of the IGFBP-2 peptides in the pool are recognized by CD4 T cells and are naturally processed and presented by antigen-presenting cells.

IGFBP-2–specific antibodies are not increased in patients relative to controls. In many cases, increased antibody responses are observed against tumor antigens. Considering that CD4 T-cell responses can lead to the generation of antibody responses, IGFBP-2–specific antibody responses were assessed in the patient population relative to normal healthy controls. Patient sera had a mean (±SE) level of IGFBP-2 antibody of 37 ± 7 ng/mL, which was not significantly different ($P > 0.05$) from the control population (53 ± 10 ng/mL). In both populations, the levels of antibodies specific for tetanus toxoid were significantly elevated relative to the IGFBP-2 antibody levels ($P < 0.0001$) and were statistically similar ($P = 0.18$) between the control (1,034 ± 133 ng/mL) and the patient (1,104 ± 113 ng/mL) populations. These results show that although patients were able to generate IgG against antigens (i.e., tetanus toxoid), they did not generate antibodies to IGFBP-2 relative to the controls.

Discussion

The CD4 T cell plays an initiating role in the development of an immune response targeting extracellular antigens and can become activated by many antigen-presenting cell types such as B cells, dendritic cells, and macrophages in many different tissues such as lymphoid tissue, connective tissue, and epithelia (45). The CD4 T cell then has a central role in guiding the immune response toward a cell-mediated Th1 immune response, an antibody-mediated Th2 response, or some intermediate. In addition to these immune response developmental roles, the generation of memory CD4 T-cell immunity is important for secondary immune responses to the same challenge antigen (e.g., either microbial or tumor). A better understanding of the critical role of CD4 T cells in coordinating comprehensive immune responses has led vaccine immunologists to consider incorporating CD4 T-cell–activating epitopes into vaccine preparations. Thus, there is a continued need for identifying class II epitopes. Ideal epitopes would be those (*a*) which are HLA-degenerate, (*b*) for which there is a T-cell repertoire, (*c*) which are derived from tumor antigens that are commonly encountered in the majority of tumors, and (*d*) which are readily processed from whole protein and presented on class II. These characteristics would likely enable easier clinical testing.

The recent observation that class II epitopes can be degenerate suggests that epitope pools can be designed to cover a large percentage of patients without having to tailor vaccines to accommodate diverse class II backgrounds. In the current study, the pool of four IGFBP-2 peptides may be useful in nearly 80% of patients whose tumors overexpress IGFBP-2, which includes nearly all breast and ovarian tumors, with an average predicted response of two or more peptides per individual. For many of the variants, such as HLA-DRB1*01 and *04, responses to all four of the peptides would be expected. Although HLA-DRB1*08, *11, and *13 recognize only one of the peptides in the pool, it is likely that patients would respond to more than one because of the fact that HLA-DR expression is codominant (i.e., not subject to allelic exclusion) and that homozygosity at the DRB allele is extremely rare (41). Despite these predictions, however, we found that the pool only detected immunity in 35% of patients and that average response rate was only about one epitope per person. Although there are many reasons that may explain this discrepancy, tumor-induced immunosuppression is highly suspect. In recent years, it has become clear that tumors, including breast and ovarian tumors, possess many mechanisms by which to suppress the development of full coordinated immunity. For example, both tumor types are known to induce or recruit CD4 regulatory T cells (Treg). In one key study, it was found that Tregs, as measured by immunohistochemistry, are associated with a high death hazard in ovarian cancer (46). *In vitro*, the Tregs isolated from the tumors were found to be capable of blocking tumor antigen–specific immune responses. Similar results have been obtained in recent breast cancer studies (47). As one other example, Hamanishi and colleagues found that ovarian cancer patients with tumors that expressed the immunoregulatory molecule B7-H1 had a markedly poorer prognosis than patients without expression (48). Immunosuppression may be the reason that immunity was not observed in the predicted number of patients. Furthermore, this may also explain why we were unable to detect measurable IGFBP-2–specific IgG responses which require CD4 T-cell responses for development (e.g., class switching, etc.). An alternative plausible explanation for the discrepancy between the observed and predicted frequency of IGFBP-2–specific T-cell immunity is immunodominance. Immunodominance is a phenomenon by which the responding T cells recognize only a tiny fraction of the potentially immunogenic peptides (49, 50). It is well known that breast and ovarian tumor cells can overexpress several other immunogenic proteins including HER-2/neu, MUC1, and the folate receptor α (27, 51, 52). One likely scenario is that the natural immune response to a specific antigen is influenced by the

abundance of one antigen relative to another. Indeed, as Yewdall has stated "Immunodominance reflects the final product of multitudinous positive and negative factors. . ." (49).

Although others have found evidence of tumor antigen–specific T cells in cancer patients, the current study is the first to show that IGFBP-2 is immunogenic and recognized by T cells *in vivo* (53, 54). Most studies identifying epitopes are based extensively on the studies in which immunity is driven *in vitro* with cultured T cells and dendritic cells. The demonstration of an endogenous response clearly shows that a T-cell repertoire potentially specific for IGFBP-2 can be activated *in vivo* by antigen that is naturally processed in the lymph node or tumor tissues. We speculate that this finding is potentially predictive of better responsiveness in patients in future vaccine settings as compared with those vaccine peptides that are discovered in systematic *in vitro* studies. Indeed, this may be why class II peptide-based vaccines targeting HER-2/neu, developed by Disis and colleagues, have been successful in reaching advanced phase clinical trials. In those studies, all of the HER-2/neu–derived peptides used in the vaccine preparations were those which had been previously shown to be naturally targeted by CD4 T cells in patients with breast cancer (51, 55, 56).

Lastly, whereas the design of the current studies favored the selection of peptides in which there was a substantial (and statistically significant) proportion of the patients responding, the importance of the other IGFBP-2 peptides, with high affinity binding, cannot be readily discounted. For example, the HLA-DR binding studies showed that IGFBP2.234 bound degenerately to 11 of 15 of the HLA-DR tested. There are a few key possibilities as to why responses to this and other peptides were not observed. First, given its high-affinity binding, natural deletional mechanisms may have minimized the T-cell repertoire that is capable of responding to this peptide. Second, it is possible that despite its binding affinity, the epitopes are not naturally processed by the proteasome. Lastly, it could be that this peptide is also recognized by antigen-specific regulatory T cells which could block the elicitation of effector T cells, as has recently been described by Hamdi and colleagues (57).

In conclusion, in this study, we identified IGFBP-2 as a naturally targeted antigen in patients with breast and ovarian cancer. Furthermore, we identified an HLA-DR–degenerate pool of peptide epitopes that could potentially be useful in nearly 80% of patients. Such a pool may be useful for providing essential helper CD4 T-cell immunity during vaccination.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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