Insulin-like Growth Factor–Binding Protein-2 Is a Target for the Immunomodulation of Breast Cancer

Kyong Hwa Park, Ekram Gad, Vivian Goodell, Yushe Dang, Thayer Wild, Doreen Higgins, Patty Fintak, Jennifer Childs, Corazon dela Rosa, and Mary L. Disis

Abstract

Breast cancer is immunogenic and well suited to treatment via immunomodulation. The disease is often treated to remission and time to relapse is generally measured in years in many cases. Immune-based therapies, such as cancer vaccines, may be able to affect the clinical progression of nonmetastatic disease. Immune targets must be identified that have the potential to inhibit tumor growth. Insulin-like growth factor–binding protein-2 (IGFBP-2) has direct effects on breast cancer proliferation via stimulation of critical signaling pathways. We questioned whether IGFBP-2 was an immune target in breast cancer. IGFBP-2–specific IgG antibody immunity was preferentially detected in breast cancer patients compared with controls (P = 0.0008). To evaluate for the presence of T-cell immunity, we identified potential pan-HLA-DR binding epitopes derived from IGFBP-2 and tested the peptides for immunogenicity. The majority of epitopes elicited peptide-specific T cells in both patients and controls and had high sequence homology to bacterial pathogens. IGFBP-2 peptide-specific T cells could respond to naturally processed and presented IGFBP-2 protein, indicating that these peptides were native epitopes of IGFBP-2. Finally, both immunization with IGFBP-2 peptides as well as adoptive transfer of IGFBP-2–competent T cells mediated an antitumor effect in a transgenic mouse model of breast cancer. This is the first report of IGFBP-2 as a human tumor antigen that may be a functional therapeutic target in breast cancer.

Introduction

Breast cancer is a disease that is theoretically well suited to immunomodulation, particularly active immunization. The majority of patients respond well to standard therapies, such as surgery, radiation, and chemotherapy, obtaining a complete remission or minimal residual disease state. Vaccination may be most effective with low tumor burden (1). The time to relapse after definitive treatment in high-risk patients can be measured in years rather than months, allowing the development of a potentially therapeutic immune response (2). Moreover, breast cancer is immunogenic, and patients with the disease have been found to have preexistent immunity to tumor-associated proteins (3). Recent studies have suggested that immunization against one of the most well-characterized breast cancer–associated tumor antigens, HER-2/neu, results in resolution of HER-2/neu–overexpressing ductal carcinomas in situ in some women and may prevent the development of disease relapse in patients whose invasive breast cancers overexpress the protein (4–6). Furthermore, HER-2/neu–specific antibody immunity induced by vaccination has been shown to inhibit protein phosphorylation and the growth of HER-2/neu–overexpressing breast cancer in vitro (7).

The success of any targeted cancer therapy depends on inhibiting the growth of cells that express the target (i.e., proteins that maintain or affect the malignant phenotype), and cancer vaccines are no exception (8). HER-2/neu is a biologically relevant tumor antigen and aberrant signaling via the receptor is an important growth regulator for breast cancers expressing the protein. However, multiple oncogenic pathways are implicated in breast cancer progression; thus, additional immunologic targets need to be defined to enhance the therapeutic efficacy of immunization and affect tumor growth.

The insulin-like growth factor (IGF) pathway is emerging as an important growth regulator in breast cancer. IGF signaling stimulates proliferation and inhibits apoptosis in cancer cells (9). In particular, IGF-binding protein-2 (IGFBP-2) is increasingly overexpressed during breast cancer progression (10). Recent studies have suggested that not only does IGFBP-2 have a direct proliferative effect on tumor growth but also that the protein is a regulator of phosphatidylinositol 3-kinase (PI3K)/Akt activation and may facilitate the malignant transformation (11–13). Stimulating immune eradication of IGFBP-2–overexpressing breast cancer cells may potentially affect cancer progression.

Studies presented here show that IGFBP-2 is a human tumor antigen eliciting both antibody and T-cell immunity in women with breast cancer. Moreover, in a transgenic mouse model of breast cancer, T cells specific for IGFBP-2 inhibit tumor growth. Thus, IGFBP-2 may represent a relevant target for the immunomodulation of breast cancer.

Materials and Methods

Human subjects. Serum samples were collected from 220 patients with breast cancer after written consent. Serum samples derived from 100 volunteer donors between the ages 18 and 75 y were obtained from the Puget Sound Blood Bank (Seattle, WA). The volunteers met all criteria for blood donation. All sera were aliquoted and stored at −80°C. For T-cell studies, peripheral blood mononuclear cells (PBMC) were obtained by leukapheresis, after informed consent, from 9 breast cancer patients, 1 ovarian cancer patient, and 10 volunteer donors. Cells were ficolled and cryopreserved as previously described (14).

Evaluation of humoral immunity specific for IGFBP-2. The human antibody response to IGFBP-2 was assessed by indirect ELISA as has been previously described (15). For murine serum samples, the same protocol was used except for the following substitutions: purified mouse recombinant insulin-like growth factor binding proteins were used as coating antigens.
IGFBP-2 protein (Sigma) and anti-mouse IgG/horseradish peroxidase conjugate (Zymed Laboratories). IGFBP-2 antibody immuity detected by ELISA was verified using Western blot analysis (15).

Scoring system for the prediction of MHC class II binding epitopes.

We have shown that high binding affinity across multiple class II alleles predicts immunogenic human epitopes (16). Other investigators have shown that predicted peptides that score highly across more than one algorithm are more likely to be natural epitopes (17). Therefore, to identify IGFBP-2–specific MHC class II epitopes that have optimal binding affinity and promiscuity across multiple alleles, we developed a combined scoring system using widely available algorithms for predicting class II binding. The following five algorithms were used for prediction of class II peptides derived from the IGFBP-2 protein sequence: SYFPEITHI (Institute for Cell Biology, Heidelberg, Germany), Propred (Institute of Microbial Technology, Chandigarh, India), MHC-Thread (University of Aberdeen, Aberdeen, United Kingdom), Average Binding matrix method, and Rankpep (Harvard, Boston, MA; ref. 18). Binding predictions were generated using each algorithm for the 15 most common MHC class II alleles: DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0701, DRB1*0802, DRB1*0901, DRB1*1101, DRB1*1201, DRB1*1302, DRB3*0101, DRB4*0101, and DRB5*0101.

The 14 peptides described in this study were selected as follows. For each available MHC class II allele from the five algorithms, 20 peptide sequences were initially selected solely based on the rank order of the predicted binding affinity. The sequences ranged from 9 to 15 amino acids in length. Individual amino acids for each selected peptide were assigned a score (from dark red to light blue) based on its final score, with dark red being the highest at >9,000, and light blue the lowest at 500 to 1,000. Twenty distinct 15–amino acid peptides were chosen, representing all potential “immunogenic hotspots.” Scores (S × N) of the amino acids ranged from 0 to 9,394. The sum of the scores of each selected 15-mer peptides ranged from 7,610 to 107,357. Of the 20, peptides scoring above the lower end of 99% confidence interval of the mean were chosen for construction and further analysis (n = 14). IGFBP-2 peptides were synthesized by Genemed Synthesis, Inc., purified by high-performance liquid chromatography, and characterized by mass spectrometry for use in all experiments. The 14 peptide sequences were compared in the National Center for Biotechnology Information databases using BLAST program to identify shared homology with other human proteins or with proteins from other species (Table 1).

Evaluation of T-cell responses to IGFBP-2 peptides and protein and highly homologous foreign antigens. PBMCs from 20 subjects were evaluated by enzyme-linked immunospot (ELISPOT) for antigen-specific IFN-γ production. Briefly, 96-well nitrocellulose plates (Millititer, Millipore) were coated overnight at 4 °C with 50 μL/well of 10 μg/mL anti-human IFN-γ monoclonal antibody (clone 1-D1K; MabTech) in Dulbecco’s PBS (DPBS; Life Technologies-Invitrogen). The plates were washed thrice for 5 min each with 200 μL DPBS/well and blocked with 100 μL/well of 2% bovine serum albumin (BSA) in DPBS for 2 h at room temperature. PBMCs were plated at 250,000 per well with 10 μg/mL of the various IGFBP-2 peptides, 1 μg/mL of IGFBP-2 recombinant human protein, 2.5 μg/mL of cytomegalovirus (CMV), or medium alone in a total volume of 200 μL/well for 96 h at 37 °C in 5% CO2. The plates were washed with 200 μL of 0.05% Tween/DPBS. Wells were incubated for 2.5 h at room temperature using 50 μL of 1 μg/mL anti-IFN-γ (clone 7-B6-1; MabTech) antibody diluted in 0.05% Tween/DPBS. After washing thrice with PBS, streptavidin-alkaline phosphatase (Bio-Rad) was diluted 1:1,000 and added at 50 μL/well for 2 h at room temperature. After another washing step with PBS, 100 μL/well of 5-bromo-4chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Bio-Rad) were added for up to 20 min. Color development was stopped by washing under running tap water. After drying overnight at room temperature, colored spots were counted using an AID ELISPOT High-Resolution Reader System and AID ELISPOT Software version 3.5 (Autoimmun Diagnostika GmbH). The mean number of spots and SE from six replicates at each dilution were reported for each antigen. Response to peptide antigens was considered to be positive when the mean number of spots in the experimental wells was statistically different (P < 0.05) from the mean number from no antigen control wells.

For the ELISPOT assay of the cultured T-cell lines, 1 × 104 of PBMCs, inactivated by irradiation at 3,000 rads, were added to the same number of cultured T cells per well and incubated for 24 h at 37 °C in 5% CO2. Peptide-specific T cells were assayed for IFN-γ production in the presence of 25 μg/mL of IGFBP-2 peptides, 2.5 μg/mL of IGFBP-2 protein, 200 μL/well of Candida albicans (clinical grade, obtained from Hollister-Stier Laboratories) diluted 1:1,000 in medium, 1 μg/mL of Pseudomonas aeruginosa (Sigma-Aldrich), and 1 μg/mL of phytohemagglutinin (PHA) as positive control. HER-2/neu peptide p328-342 (25 μg/mL), myoglobin (2.5 μg/mL),

Table 1. Homology of peptides derived from IGFBP-2 protein

<table>
<thead>
<tr>
<th>IGFBP-2 peptides</th>
<th>% Homology with mouse IGFBP-2</th>
<th>Homologous protein</th>
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<tr>
<td>p8-22</td>
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<tr>
<td>p17-31</td>
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<td>p109-123</td>
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<td>Trypanosoma cruzi</td>
</tr>
<tr>
<td>p121-135</td>
<td>80</td>
<td>Trypanosoma cruzi</td>
</tr>
<tr>
<td>p164-178</td>
<td>86</td>
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<td>p307-321</td>
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and medium alone served as negative controls. All assays were performed in six-well replicates.

To assess the antigen-specific T-cell responses in the vaccinated, tumorbearing mice, 2 x 10^7 splenocytes per well were used for the IFN-γ ELISPOT assay. Briefly, spleens were pressed through the 70-μm cell strainer (BD Labware). The cells were washed with RPMI 1640 (Invitrogen) and pelleted at 300 x g for 10 min. To lyse RBCs, the pellet was resuspended in 5 mL of ACK lysis buffer [0.15 mol/L NaCl, 10 mmol/L KHCO₃, 0.1 mmol/L Na₂EDTA (pH 7.4)] per spleen and incubated for 5 min at room temperature. Standard mouse T-cell medium (described below) was added to stop the lysis buffer, and the cells were pelleted. Resultant splenocytes were stimulated by different antigens: IGFBP-2 peptides mixture (10 μg/mL of each peptide p8-31, p251-265, and p291-305), murine IGFBP-2 protein (2.5 μg/mL), and PHA (1 μg/mL) as positive control. The same protocol for human IFN-γ ELISPOT was used except for the following substitutions: anti-mouse IFN-γ monoclonal antibody (clone AN18; MabTech) for coating and biotinylated anti-mouse IFN-γ antibody (clone Rh-642-biotin; MabTech) for detection.

**Generation of IGFBP-2–specific T-cell lines.** For the generation of human T-cell lines, cryopreserved PBMCs were thawed, washed, and resuspended at a concentration of 3 x 10^6/mL in T-cell medium. The cells were stimulated with 10 μg/mL of various IGFBP-2 peptides and expanded in culture as previously described (19, 20). For the generation of IGFBP-2–specific human T-cell lines, pooled splenocytes from IGFBP-2 peptide vaccinated mice were used. The same protocol as for human T-cell expansion was used, except that 10 ng/mL of recombinant mouse interleukin (IL)-7 and 5 ng/mL of recombinant human IL-15 were added to stop the lysis buffer, and the cells were pelleted. Resultant splenocytes were stimulated by different antigens: IGFBP-2 peptides mixture (10 μg/mL of each peptides p8-31, p251-265, and p291-305), murine IGFBP-2 protein (2.5 μg/mL), and PHA (1 μg/mL) as positive control. The same protocol for human IFN-γ ELISPOT was used except for the following substitutions: anti-mouse IFN-γ monoclonal antibody (clone AN18; MabTech) for coating and biotinylated anti-mouse IFN-γ antibody (clone Rh-642-biotin; MabTech) for detection.

**Phenotypic analysis of the T cells.** To phenotype the IGFBP-2 peptide-specific cultured human T cells, four-color flow cytometric analysis was performed using the following antibodies: FITC-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD8, PE-Cy5-conjugated CD3, and PE-Cy7-conjugated CD66 (all from Beckman Coulter). For extracellular staining, cells were incubated for 30 min at room temperature with optimal dilution of each antibody. For the analysis of FoxP3 expression in PBMCs and antigen-specific T-cell lines, intracellular staining of FoxP3 using a PE-conjugated anti-human FoxP3 antibody (clone 29D, mouse IgG1; Biolegend) together with surface staining with FITC anti-CD3, PE-Cy7 anti-CD4, and PE-Cy5 anti-CD25 was performed following the manufacturer’s protocol. Fluorescence-activated cell sorting analysis was performed using the Cytomics FC 500 MPL Flow Cytometry System with CXP software (Beckman Coulter). Typically, 50,000 to 100,000 events were collected per sample.

**Murine experiments.** Neu transgenic mice [strain name, FVB/N-TgN (MMTVneu)-202Mul] were obtained from Charles River Laboratory and bred under specific pathogen-free conditions at the University of Washington. Animal care and use were in accordance with institutional guidelines. Mice were either immunized s.c. with 50 μg of each IGFBP-2 peptide as a mixture in complete Freund’s adjuvant (CFA), phycoerythrin (PE)-conjugated anti-CD4, PE-Cy5-conjugated CD3, and PE-Cy7-conjugated CD66 (all from Beckman Coulter). For extracellular staining, cells were incubated for 30 min at room temperature with optimal dilution of each antibody. For the analysis of FoxP3 expression in PBMCs and antigen-specific T-cell lines, intracellular staining of FoxP3 using a PE-conjugated anti-human FoxP3 antibody (clone 29D, mouse IgG1; Biolegend) together with surface staining with FITC anti-CD3, PE-Cy7 anti-CD4, and PE-Cy5 anti-CD25 was performed following the manufacturer’s protocol. Fluorescence-activated cell sorting analysis was performed using the Cytomics FC 500 MPL Flow Cytometry System with CXP software (Beckman Coulter). Typically, 50,000 to 100,000 events were collected per sample.

**Reverse transcription-PCR analysis of IGFBP-2 mRNA expression.** Total RNA from the MMC cell line was isolated using RNA-Aqueous kit (Ambion). cDNA was generated from 5 μg RNA from SuperScript III reverse transcriptase (Invitrogen) with oligod(T) as primers according to the manufacturer’s protocol. Five microliters of 1:40 diluted cDNA were then used as a template for PCR analysis. The primer pair to amplify an 80 bp product was designed based on Genbank sequences of mouse IGFBP-2: 5′-GGG GCCGTACCCTGTGAAA-3′ (sense) and 5′-TCCCTCGAGGTGGTCTCATC-3′ (antisense). The cycling conditions were as follows: 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, 62°C for 60 s, followed by a final extension at 72°C for 4 min.

**Evaluation of IGFBP-2 protein expression in murine tumors and human cell lines.** Standard immunohistochemistry was performed. Briefly, 5-μm-thick sections of PBS/formalin-fixed and paraffin wax-embedded murine tumor specimens were deparaffinized in xylene, rehydrated in a graded series of ethanol, and washed in distilled water. Antigen retrieval was achieved by placing the tissues in 0.01 mol/L citrate buffer at pH 6.0 and exposing them to repeated microwave heating for periods of 10 min at high power. The specimens were cooled at room temperature for 15 min and washed in PBS (pH 7.6) thrice. Endogenous peroxidase or phosphatase activity was quenched by incubation in 3% H₂O₂/ PBS for 30 min followed by 3 consecutive days before the first vaccine. The treatment was repeated twice weekly until termination of the study. This regimen resulted in >95% CD4+ or CD8+ T-cell depletion (data not shown). For adoptive T-cell experiments, tumor was established in each mouse by injecting with 1 x 10⁶ MMC cells 10 d before the T-cell transfer. Tumors were measured every 2 to 3 d with Vernier calipers, and tumor volume was calculated as the product of length x width x height x 0.5226. In vivo data are presented as mean ± SE of 5 to 10 mice per group.

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blocking of nonspecific antibody binding in 10% goat serum for 30 min at room temperature. Tissue sections were then incubated overnight at 4°C in a humidifier with primary goat anti-IGFBP-2 (polyclonal antibody diluted 1:200 in 1% BSA; Santa Cruz Biotechnology). BSA (1%) in PBS was used to incubate negative controls. After washing with PBS, sections were incubated with a biotinylated anti-goat secondary antibody (Vector Laboratories) at room temperature for 1 h. Avidin-conjugated peroxidase (DAKO) was added at room temperature for 30 min after washing with PBS. Finally, slides were washed with PBS and developed with 3,3′-diaminobenzidine (DAKO). After terminating the reaction, sections were counterstained with freshly filtered hematoxylin and mounted.

MCF-7 and SKBR3 human breast cancer cell lines were obtained from the American Type Culture Collection. The MCF-7 cells were cultured in Eagle’s MEM (Life Technologies) supplemented by 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humidified 5% CO₂ atmosphere. The SKBR3 cells were cultured in RPMI 1640 containing 10% FBS and antibiotics. Cells were harvested at confluence, pelleted, and stored at −80°C until use. Anti-human IGFBP-2 antibody and anti-α-actin antibody were purchased from Santa Cruz Biotechnology for Western blot. Cell lysates were made as previously described for MCF-7, SKBR3, MMC, and syngeneic splenocytes (22). Commercially provided T98G cell lysate (Santa Cruz Biotechnology) was used as a positive control. Lysates, 10 μg/each, were resolved on 12% SDS-PAGE gel and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were blocked with 5% nonfat milk/TBS for 1 h and incubated overnight with each primary antibody (1:500) at 4°C. After washing, the membranes were incubated for 1 h with a peroxidase-labeled secondary antibody (1:5,000; Amersham Pharmacia Biotech) at room temperature. After rewashing, the bands were visualized using a peroxidase-linked enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**Statistical analysis.** The unpaired, two-tailed Student’s t test was used to evaluate differences in T-cell responses in ELISPOT assay, antibody responses between patients and volunteer donors, and differences in tumor growth between animal treatment groups. The relationship between the degree of homology and the immunogenicity of the peptides is shown in Figure 2.

Figure 2. The majority of IGFBP-2 peptides identified by a scoring system combining multiple MHC class II peptide binding algorithms can be recognized by human T cells. A, 14 peptides associated with highest binding affinity across multiple MHC class II alleles. Colors represent final scores from five algorithms for each amino acid from dark red to light blue in the order of rank scores. Color strata are as follows: dark red, ≥9,000; red, 8,000 to 9,000; orange, 7,000 to 8,000; light orange, 6,000 to 7,000; gold, 5,000 to 6,000; tan, 4,000 to 5,000; yellow, 3,000 to 4,000; light yellow, 2,000 to 3,000; light green, 1,000 to 2,000; light blue, 500 to 1,000. B, percent of volunteer donors (white columns) and cancer patients (black columns) showing T-cell responses to specific IGFBP-2 peptides.
was analyzed by the Spearman correlation analysis. The \( \chi^2 \) trend and the Student's \( t \) test were used to compare the magnitude and pattern of T-cell responses in cancer patients and volunteer donors. In all cases, a \( P \) value of <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 3.02 (GraphPad Software).

Results

Breast cancer patients can have antibody immunity to IGFBP-2. Sera from patients with breast cancer were more likely to have antibody immunity specific for IGFBP-2 than samples from volunteer donors (\( P = 0.0008 \); Fig. 1A). Moreover, the level of antibody response to IGFBP-2 was significantly higher in breast cancer patients (\( P = 0.0008 \)) when compared with volunteer donors. The mean level of IGFBP-2–specific IgG immunity for breast cancer patients was 0.3 \( \mu \)g/mL (range, 0–10.4 \( \mu \)g/mL) and for volunteer donors was 0.032 \( \mu \)g/mL (range, 0–1.6 \( \mu \)g/mL). Antibody responses were confirmed by Western blot. A representative example of two patient samples positive for IGFBP-2 antibodies (P1 and P3) in ELISA and one negative patient sample (P2) is shown in duplicates (Fig. 1B).

The majority of IGFBP-2 peptides identified by a scoring system combining multiple MHC class II peptide binding algorithms can be recognized by human T cells. Figure 2A depicts the entire IGFBP-2 protein sequence and the identified immunogenic "hotspots." Ten of the 14 (71%) evaluated peptides stimulated significant IFN-\( \gamma \) ELISPOT responses in volunteer donors and cancer patients (Fig. 2B). Of the 14 peptides, 36% elicited responses in cancer patients and 50% were immunogenic in volunteer donors. Fourteen percent of the peptides elicited responses in both cancer patients and volunteer donors. Due to the high incidence of T-cell responses to IGFBP-2 peptides in volunteer donors, we evaluated the peptides for sequence homology with other known proteins. The majority (13 of 14) of peptides displayed significant sequence homology with bacterial pathogens (Table 1). Eight of the 14 immunogenic IGFBP-2 peptides showed \( \geq60\% \) shared homology with common pathogens, such as \textit{P. aeruginosa} and \textit{Aspergillus oryzae} (Table 1). Moreover, several peptides had multiple homologies to infectious proteins (e.g., p8-22; see below). However, there was no relationship between the degree of homology and the immunogenicity of the peptides (Spearman\( r = 0.088; P = 0.76 \)).

Human T-cell responses specific for IGFBP-2 peptides can be restricted to a single peptide or show multiple specificities. Eight of 20 subjects (40%) showed IGFBP-2 peptide-specific IFN-\( \gamma \)–producing T-cell responses to one or more peptides. Representative examples are shown in Fig. 3. Twelve of 20 (60%) had no detectable immunity to any IGFBP-2 peptide (e.g., Fig. 3A). Half of the responding donors (\( n = 4 \)) showed immune responses restricted to single epitope (e.g., Fig. 3B) and the other half had polyclonal responses to multiple epitopes (e.g., Fig. 3C). There were no significant differences between cancer patients and volunteer donors in terms of pattern of response (\( \chi^2 = 3.125; P = 0.077 \)) or magnitude (\( P = 0.48 \)) of the IGFBP-2 peptide-specific T-cell response.

IGFBP-2 peptide-specific T cells respond to IGFBP-2 and highly homologous proteins. We questioned whether IGFBP-2 peptide-specific T cells could respond to IGFBP-2 protein and, thus, represent native epitopes. PBMCs from one breast cancer patient and two volunteer donors who showed peptide-specific T-cell
responses were selected for T-cell expansion. Each subject had a response to a different peptide: p8-22, p251-265, or p291-305. The IGFBP-2 p8-22 T-cell line responded significantly to IGFBP-2 protein compared with human myoglobin, a control protein ($P = 0.0022$; Fig. 4A). In addition to A. oryzae, the IGFBP-2 peptide p8-22 has a 60% homology to C. albicans and 47% homology to P. aeruginosa and also showed specificity to both those proteins ($P = 0.026$ and $0.039$, respectively; Fig. 4B). Similarly, IGFBP-2 p251-265 (Fig. 4C) and p291-305 (Fig. 4D) T-cell lines responded to the recombinant protein compared with control ($P = 0.002$ and $0.0022$, respectively). All of the peptide-specific T-cell lines showed specific reactivity by IFN-γ secretion in response to their stimulating IGFBP-2 peptide and not to HER-2/neu p328-342, a control 15-mer peptide ($P < 0.05$).

The IGFBP-2–specific T-cell lines were predominantly composed of CD4+ T cells (mean, 53.6%; range, 46.4–66.3%), CD8+ T cells (mean, 36.0%; range, 25.1–43.7%) and cells double negative for CD4+ and CD8+ (mean, 6.7%; range, 4.0–9.5%) accounted for the rest of the cell population. None of the cultured T-cell lines showed outgrowth of regulatory T cells. The mean percentage of T regulatory cells was 0.7% (range, 0.2–1.1%) after in vitro expansion.

IGFBP-2–specific immunity inhibits tumor growth in neu transgenic mice. Human IGFBP-2 has a high degree of homology with murine IGFBP-2 (82%), and for this reason, we questioned whether immunity to IGFBP-2 would affect tumor growth. IGFBP-2 peptides p8-22, p251-265, and p291-305 were chosen for in vivo study because they were shown to be native epitopes of human IGFBP-2, and all have significant homology with murine IGFBP-2 protein (Table 1). IGFBP-2 mRNA is expressed in both the MMC cell line and fresh spontaneous tumor from neu transgenic mice (data not shown), so we evaluated protein expression in the tumors. Immunohistochemical staining showed diffuse intracellular IGFBP-2 staining in MMC (Fig. 5B) compared with control (Fig. 5A). Western blot analysis was performed to compare the amount of IGFBP-2 protein in the MMC tumor with two commonly studied human breast cancer cell lines: MCF-7 and SKBR3 (Fig. 5C). IGFBP-2 protein was expressed in SKBR3 at approximately the same level as in MMC but could not be detected in murine splenocytes (Fig. 5C).

Animals were immunized with a vaccine composed of all three peptides. The peptides were immunogenic in the mice. The vaccine generated both peptide and murine IGFBP-2 protein-specific

Figure 4. IGFBP-2 peptide-specific T cells respond to IGFBP-2 protein. Antigen-specific responses of IGFBP-2 peptide-specific T-cell lines [p8-22 (A), p251-265 (C), and p291-305 (D)] were analyzed by IFN-γ ELISPOT. Antigens tested include IGFBP-2 peptides and protein [dark gray columns], PHA-positive control (black column), HER-2 p384-398 peptide and myoglobin as negative controls (light gray columns), and medium alone (white column). B, the p8-22 response (gray column) against P. aeruginosa (PA) and C. albicans (CA; black columns) with medium alone (white column). Columns, mean of IFN-γ-secreting spots for six replicates; bars, SD. *, $P < 0.05$ versus spots obtained from negative control wells.
IFN-γ–secreting T cells (Fig. 6A). IFN-γ ELISPOT responses were significantly higher to the peptide mix ($P = 0.0022$) and murine recombinant protein ($P = 0.002$) compared with no antigen wells.

IGFBP-2 peptide vaccination inhibited tumor growth by $\sim 50\%$ compared with control groups (Fig. 6B). The differences of mean tumor size between the IGFBP-2 peptide vaccinated group on day 21 (mean $\pm$ SD; $304 \pm 66 \text{ mm}^3$), tetanus toxoid peptide vaccinated group (mean $\pm$ SD; $881 \pm 126 \text{ mm}^3$), adjuvant alone (mean $\pm$ SD; $902 \pm 136 \text{ mm}^3$), and PBS control group (mean $\pm$ SD; $796 \pm 115 \text{ mm}^3$) were statistically significant (PBS versus IGFBP-2 vaccine, $P = 0.035$; adjuvant alone versus IGFBP-2 vaccine, $P = 0.035$; tetanus toxoid peptide versus IGFBP-2 vaccine, $P = 0.035$). To assess the therapeutic efficacy of IGFBP-2–specific T cells, $1 \times 10^6$ of in vitro cultured IGFBP-2 peptide-specific T cells were adoptively transferred to 10-day tumor-bearing mice. A single infusion of IGFBP-2–specific T cells inhibited tumor growth by $60\%$ (Fig. 6C).

Twenty-eight days after T-cell transfer, the mean tumor size of the IGFBP-2–specific T-cell–treated group (mean $\pm$ SD; $691 \pm 231 \text{ mm}^3$) was significantly different from that of animals receiving an equal dose infusion of naive splenocytes (mean $\pm$ SD; $1,751 \pm 90 \text{ mm}^3$; $P = 0.015$).

To further determine which T-cell subset was the mediator of the antitumor effect, mice were selectively depleted of CD4$^+$ and CD8$^+$ T cells just before and during IGFBP-2 immunization. As shown in Fig. 6D, the antitumor effect of vaccination was affected by CD8$^+$ T-cell depletion but not by CD4$^+$ T-cell depletion. The average tumor size in mice depleted of CD8$^+$ T cells ($1,062 \pm 90 \text{ mm}^3$), significantly larger than the tumors in mice depleted of CD4$^+$ T cells ($454 \pm 82 \text{ mm}^3$; $P = 0.02$) or nondepleted animals ($427 \pm 35 \text{ mm}^3$; $P = 0.02$).

**Discussion**

IGFBPs, such as IGFBP-2, transport IGFs from the circulation into tissues and are part of an important regulatory network controlling cell proliferation, migration, and apoptosis (23). IGFBP-2 is one of six IGFBPs and is found at elevated levels in the sera of cancer patients (24–26). Studies have shown that IGFBP-2 has an IGF-independent growth-stimulatory effect on tumor cells, directly promoting cell growth while inhibiting apoptosis (27). More recently, IGFBP-2 has been shown to act as a regulatory protein for PTEN in breast cancer cells (13). Elevated levels of IGFBP-2 prevent PTEN interaction with IGF receptor-II, thus resulting in enhanced cell proliferation via activation of the PI3K/Akt signaling pathway. In breast cancer, IGFBP-2 expression has not been found in normal glandular tissue but has been found in increasing levels in premalignant and malignant disease with the highest levels associated with invasive ductal carcinomas (10). We questioned whether IGFBP-2 might be a target for immunomodulation in breast cancer and whether the ability to recognize IGFBP-2 was within the realm of the human T-cell repertoire. Data presented here show that not only is IGFBP-2 a human tumor antigen but also that an IGFBP-2–specific T-cell response may inhibit tumor growth in vivo.

Initially, we evaluated whether patients with breast cancer developed IGFBP-2 IgG antibody immunity, which we theorized would be a marker for a potential cellular immune response as immunoglobulin class switching from IgM to IgG requires cognate CD4$^+$ T-cell help (28, 29). Moreover, it has been shown that tumor antigen-specific antibody immunity is positively associated with a concomitant antigen-specific T-cell response, indicating that IgG immunity may act as a marker for the presence of CD4$^+$ and CD8$^+$ T-cell immunity (30, 31). Previous work by our group showed that those peptides most likely to be native epitopes of the tumor antigen HER-2/neu bound at high affinity across multiple class II alleles (16). For this reason, we analyzed the IGFBP-2 protein sequence using five class II prediction algorithms across multiple class II alleles and developed a scoring system that would maximize the identification of peptides with predicted promiscuous high-affinity class II binding. The majority of peptides identified in this fashion elicited a T-cell response, indicating that this approach may be a useful tool in class II epitope prediction.
The identified IGFBP-2 class II peptides showed a high degree of homology with common bacterial pathogens. As a comparison, HER-2/neu peptides shown to elicit T-cell responses in vitro and in vivo did not show such structural similarity to bacterial antigens (data not shown), nor have these HER-2/neu peptides been shown to be immunogenic in non–tumor-bearing individuals (32). Structural similarities between sequences derived from micro-organisms and self-epitopes have been termed "molecular mimicry," which is a suggested explanation for some autoimmune diseases (33). Indeed, T-cell specificities that are cross-reactive with both self-antigens and bacterial antigens have been implicated in the pathogenesis of diabetes and multiple sclerosis (34, 35). Molecular mimicry of peptide sequences derived from foreign organisms with self-tumor antigens has been identified as one potential reason for the immunogenicity of melanoma antigens, such as MART-1 (36). A dominant HLA-A2 class I epitope derived from MART-1 and capable of eliciting CTLs with the ability to lyse tumor was highly homologous and cross-reactive with a herpes simplex virus-1 peptide. It is unknown what role molecular mimicry plays in the immunogenicity of IGFBP-2. Potentially, the bacterial sequences and self-sequences may share HLA class II binding anchor residues. The high incidence of detectable immunity in non–tumor-bearing individuals, however, would suggest that the T-cell responses observed may not be due entirely to autoimmunization via exposure to an IGFBP-2–expressing malignancy.

IGFBP-2 is immunogenic in patients with breast cancer, but does that immune response have an effect on tumor growth? We asked this question using the neu transgenic mouse model. Neu transgenic mice are engineered to express nontransforming rat neu on a mouse mammary tumor virus promoter (37). The breast cancer that occurs in these mice is histologically similar to breast cancer in humans. Hyperplastic lesions progress to infiltrating ductal carcinomas, which commonly metastasize to local lymph nodes and soft tissue sites. Moreover, the tumors that develop are estrogen receptor low and show tamoxifen resistance (38). IGFBP-2 is expressed in these tumors. Both active immunization with IGFBP-2 peptides as well as adoptive transfer of IGFBP-2–competent T cells mediated an antitumor response in treated mice compared with controls. Although results in mice cannot be directly comparable with humans, the model has many immunologic similarities with human breast cancer. T regulatory cells are operative in dampening immunity to the neu antigen; therefore, immunization against IGFBP-2 circumvented tolerance (39). T regulatory cells have been shown to play an important role in the progression of human breast cancer (40). Inflammatory infiltrates develop as the tumor progresses in neu transgenic mice just as such infiltrates develop in human breast cancers (41, 42). Finally, the antigenic repertoire in the neu transgenic mouse seems to be quite similar to that found in patients with malignancy (42). Thus, studies presented here may lay the foundation for the development of a vaccine targeting IGFBP-2 in patients with breast cancer.

**Figure 6.** IGFBP-2–specific immunity inhibits tumor growth in neu transgenic mice. A, IFN-γ ELISPOT using splenocytes from vaccinated mice. Columns, mean spots for six replicates; bars, SD. White column, medium; gray columns, IGFBP-2 peptides and protein; black column, PHA. *, P < 0.05 versus spots obtained from medium-only wells. B, tumor measurements from mice injected with IGFBP-2 peptide vaccines (■), tetanus toxoid peptide (▲), adjuvant alone (▲), or PBS (▲). Points, mean tumor measurement from 10 mice; bars, SD. C, tumor growth for mice treated with IGFBP-2 peptide-specific T cells (▲) or naive splenocytes (▲). Points, mean tumor measurement from five mice; bars, SD. D, tumor growth in mice injected with PBS alone (▲), IGFBP-2 vaccine and CD8+ depletion (▲), IGFBP-2 vaccine and CD4+ depletion (▲), and IGFBP-2 vaccination with no depletion (▲). Points, mean tumor measurement from five mice; bars, SD.
Of note, although mice were immunized with putative class II epitopes, the effector cell mediating the antitumor response was the CD8+ T cell. It has long been known that T-helper (Th) cells activate antigen-specific effector cells and recruit cells of the innate immune system, such as macrophages, eosinophils, and mast cells, which can enhance cross-presentation (43, 44). Moreover, antigen-primed Th cells can directly activate tumor antigen-specific CTLs. Investigations have shown that the infusion of Th cell clones into tumor-bearing animals can activate a CTL-mediated antitumor response (45). Other studies have shown that the function of tumor-specific CTL is enhanced by Th cells through costimulatory molecules present on the surface of the CTL, such as CD27, CD134, and MHC (46). In addition to direct contact, Th can activate CTL through cytokine secretion, which can stimulate the growth and expansion of effector T cells. For example, Th1 cells release IFN-γ, which activates antigen-presenting cells to up-regulate molecules that activate antigen-specific effector cells and recruit cells of the innate immune system, such as macrophages, eosinophils, and mast cells, which can enhance cross-presentation (43, 44). Moreover, antigen-primed Th cells can directly activate tumor antigen-specific CTLs.

Despite an increasing identification of human tumor antigens, there is still little insight as to which targets may potentially elicit an antitumor response. Indeed, evidence in murine models suggests that some tumor-associated proteins may actually serve to inhibit immunity by inducing the elaboration of T regulatory cells in an attempt to prevent an autoimmune response (48). There is a need for the identification of biologically relevant immunogenetic proteins that may ultimately serve as tumor rejection antigens. IGFBP-2 has a direct growth-stimulating effect on breast cancer cells, is expressed in most breast cancers, and is immunogenic in breast cancer patients, and immunity against the protein can significantly inhibit tumor growth in a biologically relevant animal model. Therefore, IGFBP-2 may be an important target for the immune modulation of breast cancer.

References

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