Elimination of IL-10–Inducing T-Helper Epitopes from an IGFBP-2 Vaccine Ensures Potent Antitumor Activity

Denise L. Cecil1, Gregory E. Holt2, Kyong Hwa Park3, Ekram Gad1, Lauren Rastetter1, Jennifer Childs1, Doreen Higgins1, and Mary L. Disis1

Abstract

Immunization against self-tumor antigens can induce T-regulatory cells, which inhibit proliferation of type I CD4+ T-helper (T_1) and CD8+ cytotoxic T cells. Type I T cells are required for potent antitumor immunity. We questioned whether immunosuppressive epitopes could be identified and deleted from a cancer vaccine targeting insulin-like growth factor–binding protein (IGFBP-2) and enhance vaccine efficacy. Screening breast cancer patient lymphocytes with IFN-γ and interleukin (IL)-10 ELISPOT, we found epitopes in the N-terminus of IGFBP-2 that elicited predominantly T_11 whereas the C-terminus stimulated T_102 and mixed T_11/T_102 responses. Epitope-specific T_102 demonstrated a higher functional avidity for antigen than epitopes, which induced IFN-γ (P = 0.014). We immunized TgMMTV-neu mice with DNA constructs encoding IGFBP-2 N-and C-termini. T cell lines expanded from the C-terminus vaccinated animals secreted significantly more type II cytokines than those vaccinated with the N-terminus and could not control tumor growth when infused into tumor-bearing animals. In contrast, N-terminus epitope–specific T cells secreted T_11 cytokines and significantly inhibited tumor growth, as compared with naïve T cells, when adoptively transferred (P = 0.005). To determine whether removal of T_102–inducing epitopes had any effect on the vaccinated antitumor response, we immunized mice with the N-terminus, C-terminus, and a mix of equivalent concentrations of both vaccines. The N-terminus vaccine significantly inhibited tumor growth (P < 0.001) as compared with the C-terminus vaccine, which had no antitumor effect. Mixing the C-terminus with the N-terminus vaccine abrogated the antitumor response of the N-terminus vaccine alone. The clinical efficacy of cancer vaccines targeting self-tumor antigens may be greatly improved by identification and removal of immunosuppressive epitopes. Cancer Res; 74(10); 2710–8. ©2014 AACR.

Introduction

Cancer vaccines have progressed slowly from clinical testing into standard-of-care use despite decades of development and evaluation. The majority of vaccines that have advanced to later stage clinical trials have been protein- or tumor cell-based. Both vaccine platforms supply intact antigen for presentation to the immune system. In general, phase III studies reported with these types of vaccines have shown little or modest clinical efficacy in definitive randomized trials (1–3). Obstacles to improved efficacy have included the poor immunogenicity of self-proteins, which are cancer-associated antigens, low-to-moderate immune responses elicited by vaccination, and the observation that active immunization against self-antigens can induce immunosuppressive cells, such as T-regulatory cells (Treg), to proliferate (4).

In the last several years, there has been an increased understanding of the type of immune response that is needed to elicit tumor destructive immunity. Patients with cancer who endogenously develop type I T cells (both IFN-γ-secreting CD4+ and activated cytotoxic T cells) capable of infiltrating their tumors have a significantly improved disease outcome as compared with individuals who do not mount such an immune response (5, 6). Recent studies of a variety of cancer vaccines have reported a correlation of vaccine-induced type I T-cell levels, specifically T-helper (T_1) cells, with disease resolution or overall survival benefit after immunization (7–9).

For cancer vaccines, the main mode of stimulating the development and activation of T_11 cells has been vaccine adjuvants to induce the appropriate co-stimulatory signals or alter the cytokine milieu of the vaccine environment to elicit T_11 (10, 11). There has been little exploration of the modulation of the antigenic epitope within the T-cell receptor (TCR; ref. 12). Investigations have well-documented that the nature of the T_11 epitope presented to the TCR is the first signal in the differentiation of a naïve T_11 cell into a specific T_11 phenotype (13). As T_11 cytokines have been shown to be much more effective than T_12 cytokines in activating antigen-presenting cells (APC) to stimulate tumor-specific adaptive immunity and...
T<sub>\text{H}2</sub> have been shown to suppress T<sub>\text{H}1</sub> cells, it would be a benefit to develop a vaccine that included only antigenic amino acid sequences that elicited primarily or exclusively T<sub>\text{H}1</sub> responses (14, 15).

We questioned whether screening epitopes derived from a self-tumor antigen for sequences that may induce antigen-specific Treg or T<sub>\text{H}2</sub> and then eliminating those epitopes from a subunit vaccine would enhance vaccine efficacy. Studies described below demonstrate that specific peptides of self-proteins can preferentially elicit IFN-\(\gamma\) or interleukin (IL)-10 secretion by T cells. Elimination of epitopes that elicit IL-10 secretion assures the antimumor potency of an insulin-like growth factor–binding protein (IGFBP-2)-directed vaccine.

Materials and Methods

Evaluation of antigen-specific T-cell phenotype and functional avidity

IGFBP-2 peptides, predicted to bind promiscuously to human MHCII, were selected using web-based algorithms as previously described (16). Peripheral blood mononuclear cells (PBMC) from 20 female patients with breast cancer, obtained by a University of Washington Institutional Review Board–approved informed consent, were cryopreserved and evaluated by ELISPOT for antigen-specific IFN-\(\gamma\) or IL-10 secretion, according to our published methods (17, 18). Data are reported as the mean number of spots for each experimental antigen minus the mean number of spots detected in no-antigen control wells (corrected spots per well, CSPW). Positive responses were defined by a statistically significant difference (\(P < 0.05\)) between the mean number of spots from 5 replicates in the experimental wells and the mean number from no-antigen control wells for an individual.

Some donors, who demonstrated either an IFN-\(\gamma\) restricted \((n = 5)\) or IL-10–restricted epitope-specific response \((n = 4)\), had their PBMCs assessed for the appropriate cytokine secretion induced by a single epitope at varying concentrations (10, 1, 0.1, and 0.01 \(\mu\text{g/mL}\); ref. 18). For each donor, data are graphed as best-fit nonlinear regression curves for the mean CSPW generated at all peptide concentrations. The mean half-maximal effective peptide concentration (EC<sub>50</sub>) is calculated for each curve using the "log(agonist) versus response -Find ECAnything" analysis in GraphPad Prism.

Antigen-specific IFN-\(\gamma\) production by mouse spleen cells was quantitated by ELISPOT as we have reported (18), with the modification that polyvinylidene difluoride (PVDF) plates (Millipore) were coated with 10 \(\mu\text{g/mL}\) anti-mouse IFN-\(\gamma\) (clone AN-18; Mabtech) and 5 \(\mu\text{g/mL}\) biotinylated anti-mouse IFN-\(\gamma\) (clone R4-6A2; Mabtech). Data are reported as CSPW as defined above.

Generation of human and murine IGFBP-2–specific T<sub>\text{H}1</sub> and T<sub>\text{H}2</sub> cell lines

Human antigen-specific T-cell lines were generated for phenotyping using our published methods (18). Spleen cells from IGFBP-2 (1–163) (N-terminus)-vaccinated mice were stimulated with a pool of peptides; p8–22, p17–31, p67–81, p99–113, p109–123, and p121–135 (10 \(\mu\text{g/mL}\) each) and IGFBP-2 (164–328) (C-terminus)-vaccinated mice were stimulated with p164–178, p190–204, p213–227, p235–249, p251–265, p266–280, p291–305, p307–321 (10 \(\mu\text{g/mL}\) each) peptides. The T cells were subjected to a second in vitro stimulation on day 8 by adding equivalent numbers of peptide-loaded (10 \(\mu\text{g/mL}\)) autologous-irradiated (3,000 rads) splenic cells to the original culture. A total of 10 ng/mL recombinant mouse IL-7 (R&D Systems), 5 ng/mL recombinant human IL-15 (PreproTech, Inc.), and 10 U/mL recombinant human IL-2 (Hoffman-La-Roche) were added on days 5 and 12, with additional IL-2 on days 15 and 18 for T-cell expansion (16).

Assessment of T-cell phenotype

Antibodies were obtained from ebioscience (murine) or Biolegend (human). Receptor expression was documented in the expanded T cells by adding phycoerythrin (PE)-Cy7–conjugated anti-mouse CD49b (clone: DX5; 0.5 \(\mu\text{g}\)), allophycocyanin (APC)-conjugated anti-mouse CD4 (clone: GK1.5; 0.2 \(\mu\text{g}\)), or APC-conjugated anti-human CD4 (clone: OKT4; 20 \(\mu\text{L}\)). PerCP-conjugated anti-mouse CD3 (clone: 145–2C11; 0.2 \(\mu\text{g}\)), or PE-Cy7–conjugated anti-human CD3 (clone: UCHT1; 20 \(\mu\text{L}\)) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD19 (clone: 1D3; 0.5 \(\mu\text{g}\)). For extracellular staining, cells were incubated 30 minutes with the receptor antibodies. Intracellular expression of FOXP3 was documented after permeabilization and fixation with the FOXP3 Buffer Set (Biolegend) according to the manufacturer’s instructions and staining with PE-conjugated anti-mouse FOXP3 (clone: 150D/1/E4; 0.5 \(\mu\text{g}\) and anti-mouse CD4 or PE-conjugated anti-human FOXP3 (clone: 26D6; 20 \(\mu\text{L}\)) and anti-human CD4. Flow cytometry was performed on the FACSCanto (BD Biosciences) and data analyzed using FlowJo software (BD Biosciences). Typically, 100,000 cells were collected per sample. Results are reported as a percentage of total cell number or a percentage of a specific cell population.

Cytokine levels in the murine T-cell cultures were assessed according to manufacturer’s instructions using the appropriate ELISA (ebioscience) on medium collected from the splenic T cell lines on day 10 of culture. Data are expressed as mean ng/mL ± SD of 3 separate expansions.

Vaccine construction

IGFBP-2 (1–163) (N-terminus) and IGFBP-2 (164–328) (C-terminus) were amplified using the primers and conditions listed in Supplementary Table S1 with the Herculase II polymerase (Stratagene). For the IGFBP-2 (1–328) (full-length) construct, cDNA was made from RNA extracted from the human breast cancer cell line, MCF-7 [American Type Culture Collection (ATCC)]. The cDNA was amplified using primers and conditions listed in Supplementary Table S1. The insert and eukaryotic expression vector, pUMVC3 (National Gene Vector Biorepository), were cut with EcoRI and BamHI restriction enzymes and ligated using Escherichia coli ligase (New England Biolabs). Transformation of XL1 blue competent bacteria (Stratagene) allowed kanamycin-resistant clone selection. Sequencing (Agencourt/Beckman Coulter Genomics) was performed on each clone and each large-scale DNA prep (Quagen) to confirm identity. All DNA plasmids were determined to express the
correct-sized protein in vitro by transfecting HEK-293 (ATCC) cells using Polyfect reagent (Qiagen) and Western blotting probing with anti-IGFBP-2 polyclonal antibodies (Santa Cruz Biotechnology, Inc.; data not shown).

**Sequence alignment**

N-terminal (amino acids 1–163) or C-terminal IGFBP-2 (amino acids 164–328) sequences were aligned with human, viral, bacterial, or fungal proteins searching the refseq protein database in NCBI's DELTA-BLAST algorithm using the default parameters. Alignments with less than 35% positivity (identical amino acids or conservative amino acid substitutions) more than 80 amino acids were excluded as insignificant homology (19). Thirty-five percent represents a conservative assessment for identification of potential cross-reactive sequences for allergens (19). Data are reported as the percentage positivity (number of positive amino acids/total amino acids examined × 100).

**Vaccination, adoptive transfer, and assessment of tumor growth**

Animal models were in accordance with institutional guidelines. Female FVB/N-TgN (MMTVneu)-202Mul mice (Tg-MMTVneu) (6–8 weeks old; mean weight, 18.5 g; range, 15.4–23.1 g; Jackson Laboratory) were immunized with IGFBP-2 DNA constructs or pUMVC3 vector alone (50 μg plasmid) as a mixture in complete Freund's adjuvant/incomplete Freund's adjuvant (Sigma). Three immunizations were given 2 weeks apart. For tumor challenge, a syngeneic mouse mammary tumor cell line, MMC (0.5 × 10⁶ cells), was implanted into the mammary fat pad 2 weeks after the last vaccine or 7 days before T-cell adoptive transfer (n = 10/group; ref. 20). Tumors were measured as previously described (16). Briefly, measurements on length, width, and depth were assessed by Vernier caliper 2 to 3 times per week by the same technician at each time point. The measurements were multiplied by the volume constant of an ellipsoid shape, or π/6, to calculate tumor volumes. All tumor growth is presented as mean tumor volume (mm³ ± SEM). Data are representative of two independent experiments.

For adoptive transfer, 5 × 10⁶ IGFBP-2 N-terminal- or C-terminal–specific T cells were transferred into tumor-bearing mice by i.v. tail vein injection. The same number of splenocytes derived from naïve mice were used as a control infusion. Data are representative of two independent experiments.

**In vivo T-cell depletion**

Depletions were performed as previously described (21). Briefly, mice were vaccinated with the N-terminal vaccine 3 times, 14 days apart as described above. MMC cells were implanted 2 weeks from the last vaccine. Monoclonal antibodies were used for in vivo depletion (250 μg of anti-CD4; clone GK1.5 and 100 μg of anti-CD8; clone 2.43, UCSC Monoclonal Antibody Core) via intraperitoneal injection of the specific antibody three consecutive days before MMC implant and twice per week until the experiment was terminated. Rat IgG2b was used as a control. Data are shown as the mean ± SEM of 5 mice per group.

**Statistical analysis**

The unpaired, 2-tailed Student’s t test, Fischer exact test, or χ² test was used to evaluate differences between groups. P < 0.05 was considered significant. In vivo studies were designed on the basis of a priori power calculations generated by in vivo modeling of previous experiments with the same dose of MMC (Sample Size Predictor, Stata 12.0 IC). When control tumors reached a size that achieved 80% power with a significance level of 0.05 using a 2-sided, 2-sample test to reject the null hypothesis (Stata IC), the experiments were terminated. All statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software).

**Results**

**The IGFBP-2 C-terminus is enriched for epitopes that induce IL-10–secreting T cells as compared with the N-terminus**

Investigations indicate the predominant cellular immune response in most patients with breast cancer is of a Th2 phenotype (22, 23). As epitope motifs have been shown to influence Th1 phenotype, we questioned whether we could identify sequences within a self-antigen that were specific for eliciting Th1 or Th2 or Treg for the purpose of excluding immune suppressive sequences from an epitope-based vaccine construct (13). We chose to analyze Th1/Th2/Treg by examining IL-10 secretion, as IL-10 has been shown to negatively regulate Th1 activity and enhance the expression of TGF-β, mediating the conversion of Th1 to Th2 (24, 25). Furthermore, IL-10 is also secreted by Tregs, which can proliferate via self-peptide stimulation (26). IGFBP-2 epitope–induced IL-10 and IFN-γ secretion was variable in breast cancer PBMCs (Fig. 1A). We noted that epitopes within the C-terminus (p190–p307) of the protein were more immunogenic, stimulating a greater magnitude IL-10 and IFN-γ response than epitopes in the N-terminus. The mean IL-10 epitope-specific response (18 CSPW; range, 0–129 CSPW) in the C-terminus was 6-fold greater than the mean IL-10 epitope-specific response in the N-terminus (3 CSPW; range, 0–44 CSPW; P < 0.001). The mean IFN-γ epitope-specific response (12 CSPW; range, 0–82 CSPW) in the C-terminus was 2-fold greater than the mean IFN-γ epitope-specific response in the N-terminus (6 CSPW; range, 0–70 CSPW; P = 0.022). Epitopes in the C-terminus equally elicited the same magnitude of IFN-γ and IL-10–secreting cells by ELISPOT (P = 0.132). In contrast, epitopes derived from the N-terminus of IGFBP-2 induced 3-fold more IFN-γ–secreting cells than IL-10–secreting cells by ELISPOT (P = 0.012).

Figure 1B shows the percentage of patients with a specific response to individual epitopes. In some patients, individual epitopes were shown to induce exclusively IFN-γ or IL-10–secreting cells by ELISPOT. In some patients, individual epitopes induced a mixed response with evidence of both IFN-γ and IL-10–secreting cells. When responses were pooled and grouped by N- and C-terminus rather than by individual epitopes, a significantly greater number of patients responded to the C-terminus of the protein (mean responder, 42%), compared with the N-terminus (mean responder, 31%; P = 0.007). The C-terminal epitopes induced a mix of both IL-10 and IFN-γ secretion...
secretion in response to antigen in a higher percentage of patients (mean responder, 20%) than that induced by the N-terminal epitopes (mean responder, 7%; \( P = 0.000 \)) where responses appeared to be more restricted to either Th1 or Th2.

To assess whether the T cells elicited were Th2 or FOXP3+ Tregs, we evaluated the phenotype of cultured T cell lines. T cells generated were CD3+ (mean, 90%; range, 86%–94%) composed primarily of CD4+ (mean, 73%; range, 70%–77%) with fewer CD8+ (mean, 27%; range, 24%–30%). No culture demonstrated an outgrowth of Treg (mean CD4+ FOXP3+, 1.7%; range, 0.9%–2.5%) as compared with baseline.

**IGFBP-2 epitope-specific Th2 demonstrates a higher functional avidity and homology to a greater number of bacterial and self-proteins than IGFBP-2 epitope-specific Th1**

Titration studies documented that the peptides that induced IL-10 secretion were recognized by T cells with a higher functional avidity (mean EC50 concentration, 2.4 ± 0.56 \( \mu \)g/mL; Fig. 2A) than those peptides that induced an IFN-\( \gamma \) response (mean EC50 concentration, 0.11 ± 0.04 \( \mu \)g/mL; Fig. 2A) those peptides that induced an IFN-\( \gamma \) response (mean EC50 concentration, 2.4 ± 0.56 \( \mu \)g/mL; \( P = 0.014 \); Fig. 2B).

Our previous studies, which identified IGFBP-2 as a human antigen, had demonstrated that some epitopes derived from murine IGFBP-2 shared a high degree of homology with bacterial proteins (16). Indeed, T cells specific for IGFBP-2 sequences were demonstrated as cross-reactive with highly homologous sequences from antigens such as *Candida albicans* and *Pseudomonas aeruginosa* (16). We questioned whether this homology was associated with the Th2 induction observed with the use of the C-terminus vaccine. The N- and C-terminus differed in the amount of sequence homologies shared with foreign antigens. We identified 157 bacterial species that demonstrated 35% shared amino acid positivity over 80 or more amino acids (range, 35%–43%) for the human IGFBP-2 C-terminus (Fig. 2C; Supplementary Table S2). In contrast, the N-terminus demonstrated no sequence homology with bacterial proteins, a difference of more than 100-fold. There was no difference in the number of viral homologies between the 2 termini (N-term, 0 and C-term, 0).

The IGFBP-2 N-terminus shared significant homology with other IGFBP proteins, and only one additional self-protein, CYR61 (Fig. 2C; Supplementary Table S3). The C-terminus also demonstrated significant homology with other IGFBP proteins but also to 9 additional self-proteins, including thyroglobulin, nidogens, and testicans (Fig. 2C; Supplementary Table S4). Only 16% of all homologous sequences for the N-terminus were non–IGFBP-related whereas 64% of homologous sequences for the C-terminus were self-proteins other than IGFBP family members.

**An N-terminus, but not IGFBP-2 C-terminus, vaccine both stimulates type I immunity and inhibits tumor growth**

Human and murine IGFBP-2 are highly homologous (82%) and tumors that arise in the TgMMTV-neu overexpress IGFBP-2 (21). We immunized mice with DNA constructs encoding the N-terminus (1–163), the C-terminus (164–328), and the full length (1–328) of IGFBP-2. The N-terminus vaccine could elicit peptide-specific Th1 (mean, 73 CSPW; range, 0–190 CSPW) compared with the C-terminus vaccine (mean, 10 CSPW; range, 0–89 CSPW; \( P = 0.023 \)) or the IGFBP-2 full-length sequence (mean, 0 CSPW; \( P = 0.007 \); Fig. 3A). The mean tumor volume of N-terminus–vaccinated mice (104.2 ± 8.4 mm³) was significantly less than that observed in the empty vector control.
and IGFBP-2 full-length (278.3 ± 33 mm$^3$) vaccinated mice ($P < 0.001$ for all; Fig. 3B). Indeed, tumor growth after vaccination with the C-terminus and full-length constructs was no different than control ($P > 0.15$ for all). We also found that hIGFBP-2 (1–163) tumor volumes were significantly smaller than the vector control group, as determined by a 2-way ANOVA with repeated measures with a Bonferroni posttest, at multiple time points. Significance was observed as early as 22 days after tumor implant ($P < 0.001$) and additionally at 26 and 28 days after tumor implant ($P < 0.0001$).

Tumor inhibition was mediated by CD4$^+$ T cells (Fig. 3C). Elimination of CD8$^+$ T cells had no impact on the antitumor response elicited by vaccination with isotype control ($P = 0.291$). Depletion of CD4$^+$ T cells within 2 weeks of completing immunizations, however, resulted in a significant loss of the tumor-inhibitory effect of the N-terminus vaccine ($P = 0.027$) compared with vaccine without depletion and was no different than the administration of empty vector alone ($P = 0.291$).

**IGFBP-2 vaccine–induced T$_{H2}$ can abrogate the antitumor effect of IGFBP-2–specific T$_{H1}$**

To confirm that the inability of the C-terminus vaccine to inhibit tumor growth was not due to the lack of an immune response, we evaluated cytokine secretion from T cell lines generated after vaccination. T cell lines derived from mice vaccinated with the N-terminus (mean, 77% CD3$^+$ cells) were divided equally between CD4$^+$ (mean, 50%) and CD8$^+$ (mean,
of B cells, natural killer (NK) cells, or FOXP3+CD4+ T cells were detected in any culture. Expanded T cell lines from the C-terminus secreted significantly more of the type II cytokines IL-4 (mean, 42.4 ± 5.4 pg/mL; P < 0.001) and IL-10 (mean, 1.011 ± 154 pg/mL; P = 0.002) than those from the N-terminus (mean IL-4, 5.5 ± 1.9 pg/mL; mean IL-10, 368.8 ± 45.5 pg/mL; Fig. 4A). T cell lines from mice vaccinated with the N-terminus construct secreted significantly more T(H)1 cytokines, IFN-γ (mean, 702.5 ± 125.7 pg/mL; P = 0.008) and TNFα (mean, 926 ± 244 pg/mL; P = 0.015) than T cells from mice vaccinated with the C-terminus construct (mean IFN-γ, 135.8 ± 33.4 pg/mL; mean TNFα, 186.5 ± 64.4 pg/mL; Fig. 4B). T cells from mice vaccinated with the N-terminus adoptively transferred into tumor-bearing mice inhibited tumor growth (mean, 76.1 ± 23.6 mm³) compared with naive T cells (mean, 195 ± 14.4 mm³; P = 0.005; Fig. 4C). Conversely, tumor growth in mice treated with T cells derived from animals vaccinated with the C-terminus construct (mean, 149.2 ± 18.3 mm³) was not statistically different than the naive T-cell–treated mice (P = 0.09). Immunization with a vaccine that mixed both N- and C-terminus constructs in equivalent amounts abrogated the antitumor effect (mean, 292.3 ± 16.7 mm³) of the N-terminus construct when used alone (mean, 178.7 ± 16.6 mm³; P = 0.001). Mean tumor growth after immunization with the combination vaccine was not significantly different than the empty vector control (313 ± 41.3 mm³; P = 0.712) or the C-terminus vaccine alone (300.4 ± 23.4 mm³; P = 0.409; Fig. 4D).

Discussion

The generation of tumor-specific T(H)1, via vaccination, can result in the activation of both innate immune cells and CD8+ cytotoxic T cells (CTL). Vaccine-stimulated antigen-specific T(H)1 secretes type I cytokines, such as IFN-γ, which enhance the function of local antigen-presenting cells and augment endogenous antigen presentation (27). An increased processing of tumor cells by the antigen-presenting cells results in epitope spreading, which is associated with tissue destruction. Many current cancer vaccine approaches, especially those that employ the use of whole intact antigen, elicit T(H)2 or mixed T(H)1/T(H)2 immunity (28). Subunit or epitope-based vaccines may be much more effective for preferentially inducing T(H)1 than whole antigen approaches. Data presented here demonstrate that a self-tumor antigen contains sequences that are capable of specifically stimulating either a T(H)1 or T(H)2 response. Moreover, the T(H)2 generated by such epitopes are of a higher functional avidity than the T(H)1 cells elicited, thus may compete more effectively for antigen/MHC complexes at the site of the tumor. Removal of T(H)2–inducing sequences from a vaccine construct, however, will allow T(H)1 dominance and an effective antitumor response.

The differentiation of a naive T(H) cell into one with a mature phenotype is influenced by the binding of a particular peptide to the MHC (signal 1), the co-stimulation provided at the time of antigen recognition (signal 2), and the cytokine environment in which the immune response is generated (signal 3; ref. 29). Signals 2 and 3 can be influenced by the adjuvants provided with vaccination. We chose CFA/IFA as a vaccine adjuvant as

![Figure 3. An N-terminus, but not C-terminus, IGFBP-2 vaccine both stimulates type I immunity and inhibits tumor growth. A, IFN-γ ELISPOT in splenocytes from mice immunized with the indicated vaccine. The data are presented as CSPW. The horizontal bar indicates the mean CSPW ± SEM. n = 10 mice per group. *, P < 0.01. B, mean tumor volume (mm³ ± SEM) from mice injected with pUMVC3 alone (○), pUMVC3-HIGFBP2 (1–328) (■), pUMVC3-HIGFBP2 (164–328) (●), or pUMVC3-HIGFBP2 (1–163) (○). n = 5 mice per group. **, P < 0.001. C, mean tumor volume (mm³ ± SEM) from mice injected with pUMVC3 (○) or pUMVC3-HIGFBP2 (1–163) with isotype control IgG (○), CD8⁺ depletion (●), or CD4⁺ depletion (▲). #, P < 0.05 compared with pUMVC3-HIGFBP2 (1–163) vaccine with isotype control IgG.

50%) cells. T cell lines generated from mice vaccinated with the C-terminus (mean, 65% CD3⁺ cells) were predominantly CD4⁺ (mean, 64%) with fewer CD8⁺ (mean, 36%) cells. Less than 0.5%
this agent has been shown to stimulate both T_{H1} and T_{H2} responses, thus would be less likely to bias to a specific T_{H} phenotype (30). We sought to influence signal 1 by determining whether immunosuppressive epitopes could be identified within a tumor antigen protein sequence and then removed. There have been several reports of tumor antigen-specific Tregs present in the peripheral blood of patients with cancer and volunteer donors. Two recent studies identified both mammoglobin-specific (31) and p53-specific Treg (32) in the blood of patients with breast cancer and volunteers, respectively. The Treg in both these models had common characteristics: they recognized specific class II binding peptide sequences and suppressed the response of conventional T cells and CTL. Presumably, p53 Treg in volunteer donor blood represents one mechanism of peripheral tolerance. The mammoglobin-specific Tregs were enriched in the blood of patients with breast cancer compared with controls, indicating that those cells were educated and stimulated to proliferate by endogenously expressed antigen. Similarly, antigen-specific Tregs can be induced by active immunization against a cancer antigen. Investigators, immunizing patients with melanoma, demonstrated Tregs specific for a 17-amino acid peptide derived from NY-ESO expanded in vitro with administration of an NY-ESO targeting vaccine (4). In some cases, both effector T cells and Tregs could be stimulated by the same NY-ESO peptide sequence. We did not identify IGFBP-2–specific Treg in our T-cell expansions, rather conventional T_{H2}. Vaccine-induced T_{H2} can have the same immunosuppressive effects as Tregs. T_{H2} cytokines have been shown to markedly reduce the secretion of IFN-γ by leukemic-specific T_{H1} when present in the same co-culture (15). Similar to NY-ESO Treg, we also found IGFBP-2 sequences that stimulated both IFN-γ and IL-10. However, it was possible to identify sequences that elicited predominantly IFN-γ secretion in response to antigen, allowing epitopes that generated mixed responses to be removed from the vaccine construct.

The IGFBP-2 N- and C-termini differed significantly in the prevalence of T_{H1}- versus T_{H2}-inducing epitopes. We had previously reported that individual epitopes of IGFBP-2 shared significant sequence homology with foreign proteins, in particular bacterial antigens (16). As the primary T_{H1} response to bacteria is T_{H2} with type II cytokine secretion driving the development of antibodies needed to bind to and clear bacteria, we questioned whether the C-terminus may contain greater areas of homology with bacteria than the N-terminus. We used a standard methodology for predicting the potential for cross-reactivity to allergens, which requires a minimum of 35% identity over 80 amino acid sequences to define risk for cross-interaction (19, 33). The IGFBP-2 C-terminus harbored more than 100-fold greater sequences with potential cross-reactivity to bacterial antigens than the N-terminus. Potentially, the sequence similarity of the C-terminus to bacteria could be steering T_{H1} differentiation to type II. We also identified that the C-terminus had a greater sequence homology
with numerous self-proteins outside of the insulin like growth factor receptor family, in contrast to the N-terminus whose homology was restricted. It has recently been reported that memory Tregs differentiate into T1r2 after downregulation of FOXP3 following in vitro expansion (34). The C-terminus T1r2 could represent a pool of higher avidity T cells that have been stimulated by numerous self-antigens presented endogenously in the periphery and may play a role in maintaining self-tolerance.

IGFBP-2 T1r2 demonstrated a higher functional avidity than T1r1 responding to antigen at lower concentrations. In all likelihood, tumor antigen is presented in the context of MHC in low concentrations in the tumor bed. Subsequently, vaccine-induced T1r2 will more effectively compete for those peptide/MHC complexes than lower avidity T1r1. Type II cytokines secreted in response to antigen stimulation will prevent APC activation and the development of CTLs. Removing the T1r2 dominant sequences and allowing a T1r1 dominant response resulted in inhibition of tumor growth after administration of an IGFBP-2 vaccine. In humans, although of a lower avidity, the T1r1 population boosted with active immunization may still be quite functional. Recent evidence suggests that CD4+ T cells with lower functional avidity produce cytokines for longer periods of time and are less prone to experiencing exhaustion in the face of chronic antigen exposure (35). In contrast, higher avidity T cells have been shown to be more likely to experience antigen-induced cell death, which could be a particular problem in situations of chronic antigen stimulation such as with cancer (36). Furthermore, higher avidity T cells can become tolerized in the tumor microenvironment (37).

Clinical studies of cancer vaccines often use constructs encoding the full-length self-protein or immunize with the complete antigenic self-protein in adjuvant and rarely monitor both the T1r1 and T1r2 immune response generated with active immunization. Studies described here raise the question as to whether the lack of efficacy seen with many cancer vaccines might lie in the augmentation of T1r2 concurrent with T1r1. Selection of particular portions of a tumor antigen that specifically stimulate type II, but not type I, T cells provides the potent capability of developing vaccines that preferentially elicit T1r1 and CTL. Combination of a T1r1-generating cancer vaccine with potent adjuvants and/or checkpoint-blocking agents can have the potential to drive the type I immune response to therapeutic levels in vivo.

Disclosure of Potential Conflicts of Interest

M.L. Disis has commercial research grant from Seattle Genetics, has ownership interest (including patents) from Ventriks and Epigenomics, and is a consultant/advisory board member of Ventriks, EMD Serono, and Celgene. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.L. Cecil, G.E. Holt, E. Gad, M.L. Disis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.L. Cecil, G.E. Holt, L. Bastetter, J. Childs, D. Higgins, M.L. Disis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.L. Cecil, G.E. Holt, M.L. Disis
Writing, review, and/or revision of the manuscript: D.L. Cecil, K.H. Park, M.L. Disis
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Denise L. Cecil, Gregory E. Holt, Kyong Hwa Park, et al.