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14. ABSTRACT The insulin-like growth factor (IGF) pathway plays an important role in breast cancer growth and metastasis. The IGF-I receptor (IGF-IR) is over-expressed in almost 50% of triple negative breast cancers (TNBC). Thus, therapeutically targeting tumor cells which have upregulated IGF-IR may be a promising approach to treat TNBC. IGF-IR is immunogenic in breast cancer and is a potential target for active immunization. We sought to develop a vaccine that will elicit Th1 immunity to IGF-IR. Ninety-five percent of the peptides predicted to bind with high affinity to MHCII induced a Th1 immune response in human PBMC. However, since IGF-IR is a "self" tumor antigen, Th epitopes could potentially elicit either an inflammatory Th1 (i.e. IFN-g) or immunosuppressive Th2 (i.e. IL-10) response. A ratio of magnitude and frequency of ELISPOT responses for IFN-g and IL-10 was calculated. The peptides that demonstrated a preference to secrete IFN-g over IL-10 were located primarily in the C-terminus of IGF-IR. Vaccination with those C-terminal peptides in a mouse model of TNBC demonstrated a robust Th1 response and concomitant inhibition of tumor growth. These data suggest that more effective peptide-based vaccines could be designed when both Th1 epitopes and immunosuppressive epitopes are screened simultaneously.					
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INTRODUCTION

The insulin-like growth factor (IGF) pathway plays an important role in breast cancer growth and metastasis. The IGF-I receptor (IGF-IR) is overexpressed in almost 50% of triple negative breast cancers (TNBC), defined as estrogen (ER) and progesterone (PR) receptor and HER-2/neu receptor (HER2) negative. We have determined that IGF-IR is immunogenic in breast cancer and is a potential target for active immunization. Immunologic eradication of tumor cells overexpressing IGF-IR could be beneficial in preventing disease relapse in a patient population with no targeted therapy. We hypothesize that vaccination with MHCII IGF-IR-specific peptides will induce an anti-tumor immune response in breast cancer.

The specific aims of this proposal are to: (1) To identify putative Class II epitopes, derived from IGF-IR, that stimulate IGF-IR-specific T cells in patients with breast cancer; (2) To evaluate the immunogenicity, clinical efficacy, and safety of an IGF-IR class II polyepitope vaccine in a mouse model of TNBC.

BODY

Table 1. IGF-IR specific Type I T cells are readily detected in the peripheral blood of breast cancer patients and volunteer donors.

	Control		Cancer	
	Mean	Range	Mean	Range
p7-21	11.2	0-63.2	10.4	0-73.3
p24-38	23.8	0-85.8	13.9	0-62.8
p39-53	12.8	0-79.7	20.7	0-65
p76-90	31.8	0-91.3	35.1	0-160
p354-368	14.6	0-92.5	12	0-80.1
p363-377	0		0	
p388-402	9.8	0-54.3	6	0-72
p554-559	4.8	0-46	8.7	0-76.8
p787-801	8.5	0-47.1	6.1	0-49.3
p891-905	33.6	0-126.6	15.8	0-68.8
p906-920	24	0-118.9	20	0-94
p921-935	20.1	0-118	15.6	0-39
p1028-1042	33	0-91.6	20.8	0-93
p1080-1094	7	0-57.8	5.3	0-81.6
p1092-1106	10.4	0-69.8	13	0-101.6
p1166-1180	20.5	0-114	5.5	0-50
p1212-1226	20.3	0-134	10.7	0-85.7
p1302-1316	24.3	0-112.6	22.4	0-81.8
p1307-1321	34.8	0-134.7	29.8	0-97
p1311-1325	34.9	124.3	24.5	0-91

48%), compared to epitopes in the ECD (median percent responding: 27%; $p=0.035$) (Fig 2).

Aim 1.b. To determine whether IGF-IR-peptide specific T cell lines can recognize human recombinant IGF-IR protein presented endogenously by autologous antigen presenting cells (APC).

Aim 1. To identify putative Class II epitopes, derived from IGF-IR, that stimulate IGF-IR-specific T cells in patients with breast cancer.

Aim 1.a. To identify IGF-IR peptides based on predicted high avidity binding across multiple class II alleles.

The 20 IGF-IR peptides predicted to bind with high affinity to multiple MHC class II alleles are listed in Table 1.^{1,2} The majority of the peptides, 95%, induced significant antigen specific IFN-g secretion in both breast cancer and control PBMC (Table 1), as measured by ELISPOT.² Seven percent of donors did not respond to any peptide, 23% of donors responded to 1-3 peptides, 54% of donors responded to 4-10 peptides and 16% of donors responded to >10 peptides. There was no significant difference in the magnitude of response of any individual IGF-IR peptide between patients and controls and both populations had a similar incidence and magnitude of response to CEF peptides ($p=0.357$) (Fig. 1). The CEF peptide pool is derived from cytomegalovirus, Epstein Barr virus and Influenza virus and used here as a positive control.

Since there were no differences between cancer and controls, all 43 donors were considered together for further statistical analyses. The peptides were grouped into domains of IGF-IR. There was a significant increase in the magnitude of response in the C-terminal domain (CTD) compared to the extracellular domain (ECD) ($p<0.001$) or the kinase domain (KD) ($p=0.001$) (Fig 1). Significantly more subjects responded to epitopes in the CTD of the protein (median percent responding:

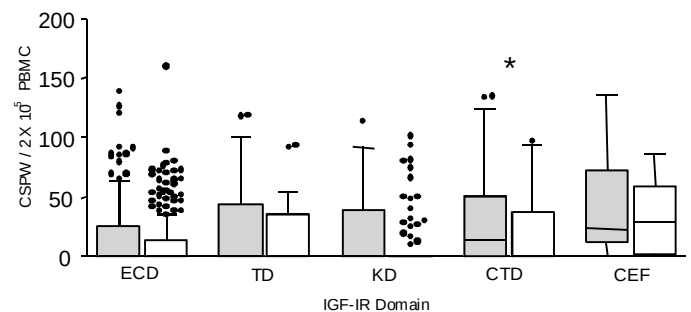


Figure 1. IGF-IR peptides in the CTD induce a higher magnitude of IFN-g response than the other domains. IFN-g ELISPOT for volunteer (n=23; gray bars) and breast cancer (n=20; white bars) PBMC for IGF-IR peptides in the extracellular domain (ECD), transmembrane domain (TD), kinase domain (KD), C-terminal domain (CTD) and CEF peptides. The data are presented as interquartile box plots with Tukey whiskers. Median corrected spots per well (CSPW) are indicated by the horizontal bar; * $p<0.01$ compared to ECD and TM.

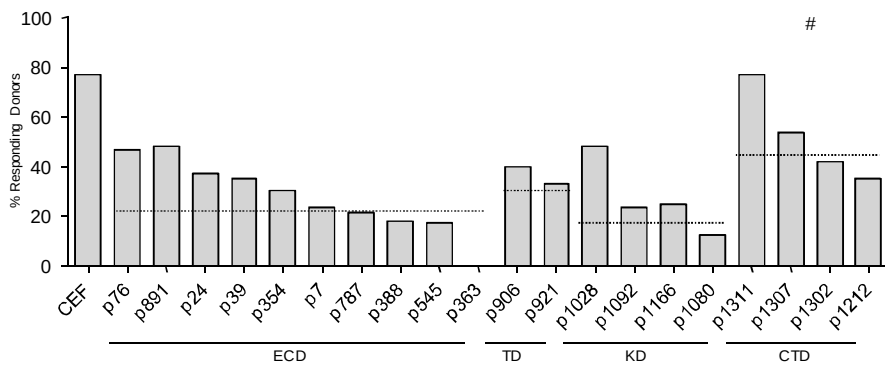


Figure 2. IGF-IR CTD peptides induce IFN-g secretion in significantly more donors. Horizontal dashed line indicates the median percent responding in each domain; #p<0.05 compared to all other domains.

To determine whether responding peptides were native epitopes of IGF-IR, peptide specific T cell lines were generated from three control and two cancer donor's PBMC and evaluated for specificity to human IGF-IR.² The peptides were randomly chosen, two peptides from the ECD, and one peptide each from the TM, KD and CTD. Each peptide was 100% homologous with murine IGF-IR. The T cell lines (mean, 98.4% CD3⁺ cells) were predominantly CD4⁺ (mean, 67.8%; range 59.8-73.6%), with CD8⁺ (mean, 25.5%; range, 23.3-28.4%) and CD4⁺CD8⁺ (mean, 1.2%;

range 0.68-1.88%) cells. The T cell lines generated were both IGF-IR peptide (p354-368, p=0.001; p545-559, p=0.013; p921-935, p=0.041; p1092-1210, p=0.002; p1307-1321, p=0.04 compared to HIV p17) and IGF-IR protein specific (p354-368, p=0.001; p545-559, p=0.001; p921-935, p=0.04; p1092-1210, p=0.03; p1307-1321, p=0.04 compared to mock transfectants) (Fig. 3a-e). All the IGF-IR specific T cell lines secreted Type 1 cytokines; TNF α (mean, 1,028 pg/ml; range 101-2,370 pg/ml) and IFN-g (mean, 68,419 pg/ml; range, 37,030-99,106 pg/ml). Additionally, the T cell lines secreted the Type 2 cytokine, IL-10 (mean, 823 pg/ml; range 71-2,154 pg/ml). IFN-g secretion was significantly greater than TNF α (p=0.002) or IL-10 secretion (p=0.003) (Fig. 3f). Minimal IL-2 (mean, 26 pg/ml; range, 0-132 pg/ml) and no IL-4 (mean, 0) was detected.

Aim 1.c. To determine whether identified IGF-IR peptides stimulate T regulatory (Treg) cell proliferation.

Tregs can modulate the immune response by secreting the immunosuppressive cytokines IL-10 and TGF β .^{4,5} Given that Tregs can proliferate in the peripheral blood in response to stimulation with 15-mer peptides specific for common tumor antigens^{5,6}, we identified class II epitopes in IGF-IR that might preferentially enhance the growth of Tregs. IL-10 ELISPOT was performed on 20 volunteer and 20 breast cancer donor PBMC.⁷ Similarly to that observed with IFN-g secretion, there was no significant difference in response between cancer and controls for any individual peptide. Thirty-seven percent of donors did not respond to any peptide, 5% of donors responded to 1-3 peptides, 17% of donors responded to 4-10 peptides and 6% of donors responded to >10 peptides.

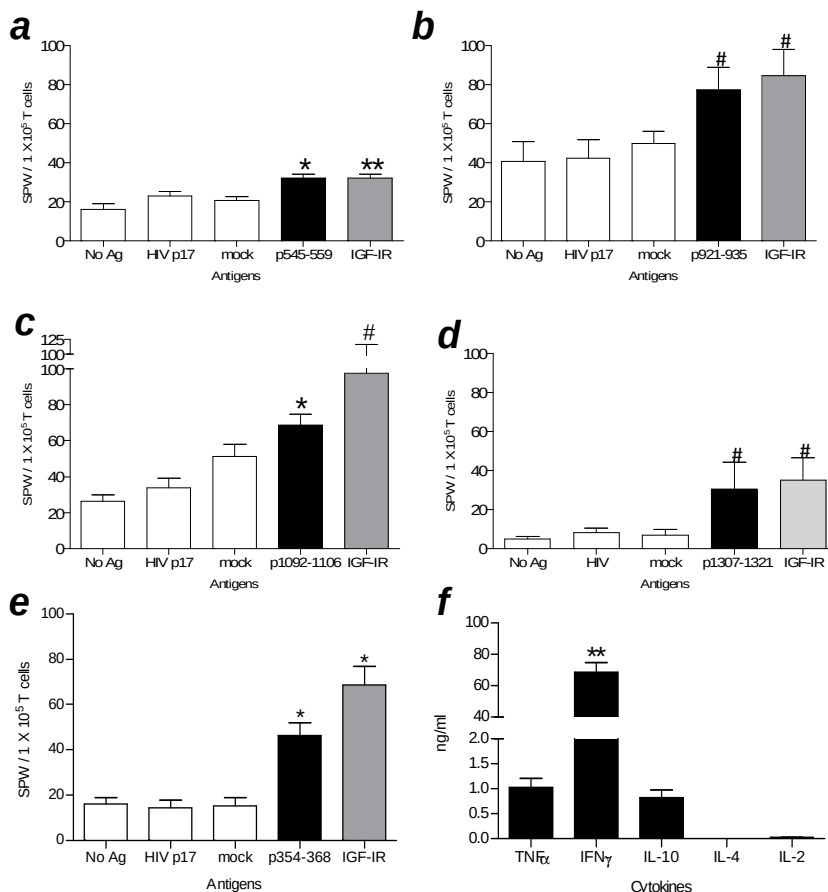


Figure 3. IGF-IR peptides are native MHCII epitopes. (a-e) IFN-g ELISPOT for IGF-IR peptide-specific T cell lines. Antigens include IGF-IR peptides, cos-1 cell lysate transfected with pcDNA3 encoding IGF-IR, and HIV p17 and cos-1 lysate transfected with empty pcDNA (mock) are used as negative controls. Data are expressed as mean spots per well $\pm$ SD; **p<0.001, *p<0.01, #p<0.05. (f) Cytokine secretion from IGF-IR T cell lines pooled from 5 different donors expanded with peptides p354, p545, p921, or p1092; **p<0.001 compared to TNF α and IL-10 secretion.

There was a significant increase in the magnitude of response in the ECD ($p=0.012$), TMD ($p=0.006$) and KD (0.008) compared to the CTD (Fig. 4). Significantly more subjects responded to epitopes in the TD of the protein (median percent responding: 38%), compared to epitopes in the CTD (median percent responding: 15%; $p=0.001$) (Fig 5).

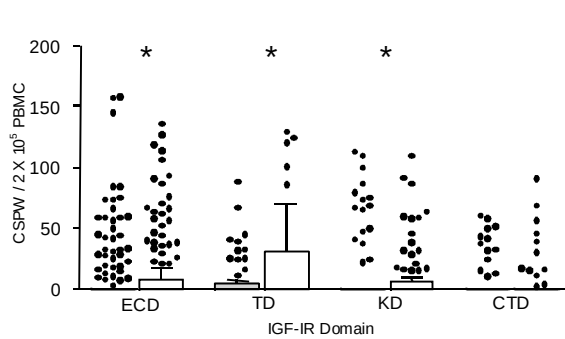


Figure 4. Some IGF-IR peptides are potentially immunosuppressive. IL-10 ELISPOT for volunteer ($n=20$; gray bars) and breast cancer ($n=20$; white bars) in each of the IGF-IR domains. The data are presented as interquartile box plots with Tukey whiskers. Median corrected spots per well (CSPW) are indicated by the horizontal bar; $*p<0.01$ compared to CTD.

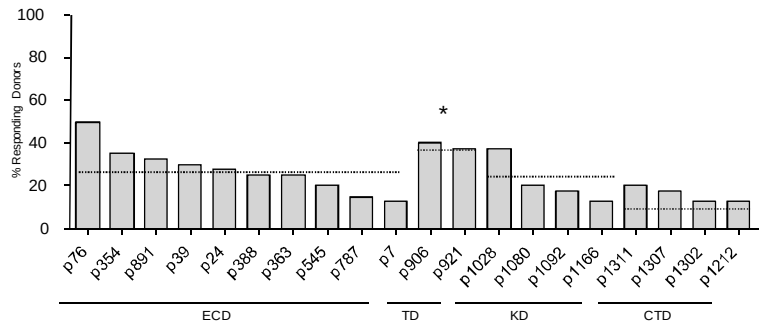


Figure 5. A greater percentage of donors have T cells that secrete IL-10 when stimulated with IGF-IR peptides in the TD. Horizontal dashed line indicates the median percent responding in each domain; $*p<0.01$ compared to CTD.

To choose peptides for the multi-epitope vaccine that induced increased IFN-g without inducing IL-10, we created a ratio of IFN-g to IL-10 that analyzed both the magnitude and frequency of ELISPOT response using the following algorithm: corrected mean spots per well $\times$ percent of responding donors. Although there were no differences observed in any individual peptides for both IL-10 and IFN-g secretion, we used the magnitude and frequency of responses for cancer patients only. The peptides were ranked from highest IL-10 response to highest IFN-g response, where high IL-10 compared to IFN-g is shown below 1 and high IFN-g compared to IL-10 is shown above 1 (Fig 6). The top three peptides (p1301-1316, p1307-1321, and p1212-1226) are in the CTD and the fourth peptide (p1166-1180) is in the kinase domain. These peptides, along with p1311-1325 were chosen for the multi-epitope vaccine. Though not in the top four peptides, p1311-1325 was chosen to be included since it was very similar to p1307-1321 and predominantly induced secretion of IFN-g.

Aim 2. To evaluate the immunogenicity, clinical efficacy, and safety of an IGF-IR class II polypeptide vaccine in a mouse model of TNBC.

Aim 2.a. To determine the immunogenicity and therapeutic efficacy of IGF-IR immunization.

Breast cancers derived from TgC3(l)-Tag mice express IGF-IR (data not shown). Non-tumor bearing parental FVB/N mice were immunized with IGF-IR-CTD peptide pool (p1301-1316, p1307-1321, p1311-1325 and p1212-1226).² All peptides were 100% homologous to the mouse protein. Antigen specific T cells were generated after vaccination ($p=0.026$ compared to HIV peptide) (Fig. 7a). TgC3(l)-Tag mice were also vaccinated with the IGF-IR-CTD peptide pool. The syngeneic tumor cell line M6⁸ was implanted subcutaneously in the flank of the mouse.² After 32 days of growth, the mean tumor volume of the IGF-IR-CTD vaccinated group was 17 ± 3 mm³ compared to 161 ± 17 mm³

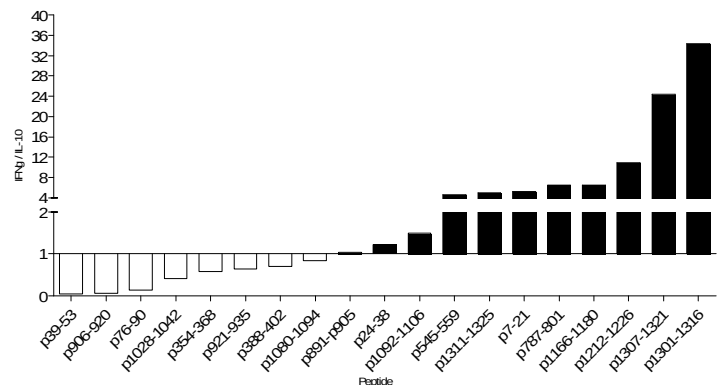
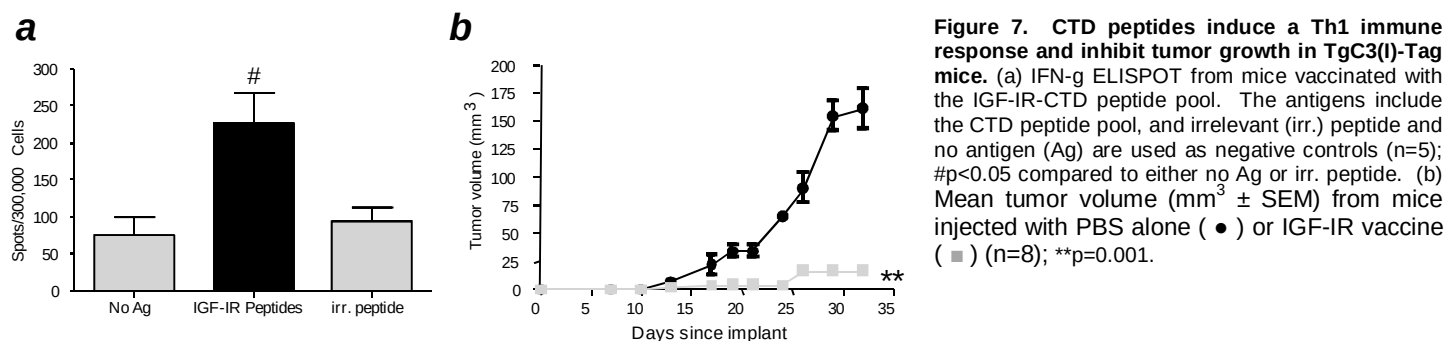


Figure 6. CTD peptides stimulate a Th1 immune response with limited immunosuppression. IGF-IR peptides were analyzed by the magnitude and frequency of response to both IFN-g and IL-10 ELISPOT in breast cancer donors. Peptides were ranked from preference to secrete IL-10 to preference to secrete IFN-g. No preference to secrete either cytokine is indicated at 1 on the y-axis.

in adjuvant control mice ($p=0.001$) (Fig. 7b).



Aim 2.b. To determine which immune effector arm is essential for mediating therapeutic efficacy after immunization.

Work on this sub-aim has not been started.

Aim 2.c. To determine whether IGF-IR vaccination induces diabetes or other toxicities in immunized mice.

Work on this sub-aim has not been started.

KEY RESEARCH ACCOMPLISHMENTS

- Ninety-five percent of the IGF-IR peptides predicted to bind to MHCII with high affinity can stimulate a Th1 immune response in normal volunteer and breast cancer PBMC.
- There was no difference in the magnitude and incidence of the Th1 immune response to any individual IGF-IR peptide between cancer and controls.
- At least five IGF-IR peptides are native MHCII epitopes.
- CTD peptides induce a significantly greater inflammatory Th1 response with significantly less immunosuppression in most patients tested.
- Vaccination with the IGF-IR-CTD peptide pool stimulates a Th1 immune response and inhibits tumor growth in the TgC3(I)-Tag mouse model of TNBC.

REPORTABLE OUTCOMES

Abstract: Vaccination with peptides in IGF-IR that induce robust Th1 immunity with limited immunosuppression significantly inhibit tumor growth in a model of triple-negative breast cancer.

This abstract was presented orally at the 2011 Era of Hope Meeting

CONCLUSION

Epitope-based vaccines rely on the fact that subdominant epitopes, not normally presented, will be presented by an inflammation-induced increase in antigen presenting cell function or because an overexpression of a protein on the target cell exposes aberrant epitopes.^{9,10} Given that these epitopes are usually ignored or have never been seen, they may have the ability to circumvent tolerance. Identification of subdominant epitopes has been achieved from several tumor antigens, eliciting T cell responses across multiple MHC class II alleles.^{1,2,11-15} Although some clinical benefit has been described, it is clear that these subdominant MHCII-restricted epitopes can still be further optimized for increased immunogenicity, as immunosuppressive mechanisms can still dominate.¹⁶ Most studies have examined the role of non-antigen-specific immunosuppression by Tregs, but recent ex vivo analyses of human PBMC have described the presence of peptide-specific CD4⁺ Tregs in cancer patients that were not detected in healthy individuals. These Tregs,

which express high levels of FOXP3, secreted IL-10 when stimulated with specific 15-mer peptides from gp100, TRP1, NY-ESO-1 and survivin in patients with metastatic melanoma.³ Peptide specific Tregs were also detected in a large cohort of colon cancer patients responding to synthetic long peptides from MUC-1, Her-2, telomerase, CEA, and EGFR.⁶ Since peptide-specific immunosuppression can be detected in current vaccines, more immunogenic vaccines could be designed that monitored not only for inflammatory T cell responses (e.g. IFN- γ secretion), but also for immunosuppressive responses (e.g. IL-10 secretion).¹⁷ In the first year of this award, we have identified peptides in IGF-IR that induce a inflammatory T cell response in cancer patients with limited immunosuppression. Additionally, active vaccination with these peptides can significantly inhibit breast cancer growth. Experiments are currently underway to determine if a vaccine that contained peptides with a predominant immunosuppressive response, as measured by IL-10 secretion, would enhance tumor growth.

TNBC is considered more clinically aggressive than other breast cancer phenotypes: patients who develop metastatic TNBC have a shorter survival than patients with metastatic breast cancer of other subtypes and the majority of deaths occur within the first 5 years after therapy is completed.¹⁸ But, if a TNBC patient can achieve a complete remission with standard therapy, their chance at survival is similar to other better prognosis breast cancer subtypes.¹⁹ Data generated in this first year has paved the way for a more effective vaccine targeting this aggressive breast cancer phenotype.

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APPENDICES

2011 Era of Hope Meeting Abstract:

The insulin-like growth factor (IGF) pathway plays an important role in breast cancer growth and metastasis. The IGF-I receptor (IGF-IR) is overexpressed in almost 50% of triple negative breast cancers (TNBC), defined as estrogen (ER) and progesterone (PR) receptor and HER-2/neu receptor (HER2) negative. Thus, therapeutically targeting tumor cells which have upregulated IGF-IR may be a promising approach to treat TNBC. We have determined that IGF-IR is immunogenic in breast cancer and is a potential target for active immunization. Our aim is to develop vaccines that will elicit Th1 immunity to IGF-IR. Antigen specific Th1 can modulate the tumor microenvironment to enhance cross priming, supporting the proliferation of cytotoxic T cells which are capable of eradicating breast cancer cells. Since it has been demonstrated that natural immunogenic human epitopes can be predicted by high binding affinity across multiple class II alleles, we used a combined scoring system from five algorithms for predicting class II binding to determine Th epitopes of IGF-IR and identified 20 potentially immunogenic peptides. We observed that 95% of the peptides predicted induced a Th1 immune response as measured by IFN-gamma ELISPOT in human PBMC. However, IGF-IR is a “self” tumor antigen, thus, Th epitopes could potentially elicit either an inflammatory Th1 or immunosuppressive Th2 response, characterized by secretion of cytokines such as IL-10. To determine the propensity of a peptide to induce a Th1 or Th2 response, we created a ratio of IFN-gamma to IL-10 that analyzed both the magnitude and frequency of ELISPOT responses for each peptide. We demonstrated that 60% of the peptides show a preference to secrete IFN-gamma over IL-10 and those peptides were located primarily in the C-terminal intracellular portion of the protein. Thus, this area would likely be ideal for a multi-epitope vaccine. Vaccination with peptides p1166-1181, p1212-1227, p1301-1316, 1307-1322 and p1311-1326 in C3T(ag) mice demonstrated a robust Th1 response and concomitant inhibition of tumor growth by 85% compared to adjuvant only control animals. These data suggest that more effective peptide-based vaccines can be designed when both Th1 epitopes and immunosuppressive epitopes are screened simultaneously and epitopes that are most likely to induce robust Th1 responses in the majority of individuals can be identified and included as vaccine components.