LINEAR EPITOPES THAT GENERATE ANTI-HER-2 ANTIBODY RESPONSES WITH TRASTUZUMAB- (HERCEPTIN) LIKE BIOLOGICAL ACTIVITY

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List of Abbreviations

A Disintegrin and Metalloproteinase (ADAM)

Antibody (Ab)

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Cluster of Differentiation 20 (CD20)

Cluster of Differentiation 3 (CD3)

Complement –Dependent Cytotoxicity (CDC)

Cyclin-Dependent Kinase (CdK)

Dendritic Cell (DC)

Ductal Carcinoma In Situ (DCIS)

Epidermal Growth Factor Receptor (EGFR)

Extracellular Signal-Regulated Kinase (ERK)

Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)

Healthy Donor (HD)

Human Epidermal Growth Factor Receptor (HER)

Immunoglobulin Alpha (IgA)

Immunoglobulin Delta (IgD)

Immunoglobulin Epsilon (IgE)

Immunoglobulin Gamma (IgG)
Immunoglobulin Mu (IgM)
Interferon Gamma (γ-INF)
Interleukin 12 (IL-12)
Interleukin 2 (IL-2)
Interleukin 4 (IL-4)
Invasive Breast Cancer (IBC)
Mitogen Activating Protein Kinase (MAPK)
Monoclonal Antibody (mAb)
Natural Killer Cell (NK cell)
Phosphoinositide 3-Kinase (PI3K)
Receptor Tyrosine Kinase (RTK)
T-helper Cell (Th Cell)
Toll-like Receptor (TLR)
Variable, Diverse, and Joining (VDJ)
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Chapter 1

Introduction into Immunotherapy

Immunotherapy:

The field of Tumor Immunotherapy has a long history; one that has been characterized by sporadic successes followed mostly by disappointing failures to realize promises. The immune system’s potential to clear or control malignancies has been demonstrated for centuries (Hobohm 2001; Hobohm 2005). The ancient Greek philosopher Parmenides stated “give me the power to induce fever, and I cure all diseases.” Since his time, many have been witness to the spontaneous regression of tumors following infection. Physicians have been observing and recording such regressions since the mid 1700’s (Stephenson et al. 1971; STEWART 1952).

William B. Coley was one of the first physicians to attempt to harness the power of the immune system for the purpose cancer treatment by strategically inducing infection from Streptococcus pyogenes and Serratia marcescens at the site of disease in
his patients (Hoption Cann, van Netten, van Netten 2003). He attempted to reduce risk of death through infection by either heat-killing or filtering the infectious agents. He then bottled these modified agents, creating thirteen different variations during his career. Collectively these formulations are referred to as “Coley’s Toxins”. After his passing, his daughter, Helen Coley Nauts, complied records from patients treated with Coley’s Toxins, either by her father or other physicians. This retrospective analysis resulted in surprising statistics that demonstrate the power of the immune system in clearing cancerous cells (Table 1.1). In 1983, the University of Texas Center for Alternative Medicine did a meta-analysis in which they compared 128 patients treated with Coley’s Toxins from 1890-1960 to 1,675 patients treated with standard-of-care medical treatment for the time. Survival rates were shown to be similar for both groups (Richardson et al. 1999).

End Results of 484 Cases of Malignant Disease With Histologic Confirmation in Which Coley’s Toxins Were Used

<table>
<thead>
<tr>
<th></th>
<th>Inoperable</th>
<th></th>
<th>Operable</th>
<th></th>
<th>Total</th>
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<tr>
<td></td>
<td>Total No. of Cases</td>
<td>5 yr. Survival</td>
<td>Percent with 5 year Survival</td>
<td>Total No. of Cases</td>
<td>5 yr. Survival</td>
<td>Percent with 5 year Survival</td>
</tr>
<tr>
<td>Carcinoma</td>
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<td>21</td>
<td>88%</td>
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<tr>
<td>Malignant Melanoma</td>
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<td>4</td>
<td>21%</td>
<td>5</td>
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<td>60%</td>
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<tr>
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<td>38%</td>
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<tr>
<td>Lymphosarcoma</td>
<td>45</td>
<td>24</td>
<td>53%</td>
<td>4</td>
<td>4</td>
<td>100%</td>
</tr>
<tr>
<td>Hodgkin’s Disease</td>
<td>14</td>
<td>1</td>
<td>7%</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Total</td>
<td>312</td>
<td>134</td>
<td>43%</td>
<td>172</td>
<td>105</td>
<td>61%</td>
</tr>
</tbody>
</table>

Table 1. This table was adapted from Nauts, H.C. et. Al., 1946
Research into the mechanisms contributing to the success of these toxins has led to valuable progress in the field of immunotherapy. In 1993, Arthur Kreig discovered that unmethylated bacterial DNA could evoke an immune response (Krieg et al. 1995). In addition, Havas et. al. 1990, showed that purified Lipopolysaccharide (LPS) found in the cell wall of bacteria could provoke regression of tumors in mice but only at concentrations that were lethal 30% of the time. However, mixed bacterial toxins were able to cause similar regression, but at concentrations that were lethal 15% of the time, hence demonstrating the potential of using multiplexed stimulating signals to enhance immunity. (Havas et al. 1990) Furthering the understanding of the immune system’s role in tumor regression, investigators showed that interleukin-12 (IL-12) was the mediator between the activation of an immune response via toll-like receptor (TLR) and tumor regression. (Iwasaaki et al. 2000; Nastala et al. 1994; Tsung and Norton 2006; Zou et al. 1995).

Collectively, these works along with others, have advanced the knowledge of mechanisms that drive immune responses. Hence the stage is set for further developments that will release the potential of immunotherapy.

**Monoclonal Antibodies:**

Currently, there are a number of immunotherapeutic strategies to treat cancer. These include cytokine therapy, vaccine therapy (conventional and cellular), adoptive T cell therapy, and monoclonal antibody therapy. The lines demarcating the different
approaches are not always completely clear in each case and a number of overlaps and overt combinational approaches exist. We will discuss several of these. Currently, the most highly developed and economically viable approach is monoclonal antibody-based therapy. Monoclonal antibody therapy became possible in 1975 when Köhler and Milstein developed hybridoma technology, the ability to fuse a specific antibody-producing B cell with a cancerous B cell myeloma. This allowed for the industrial-scale generation of antibodies specific for a single target epitope. As the technology progressed, it allowed for rapid advancements in monoclonal antibody therapeutics. A precedent was set in 1997 when the FDA approved Rituximab, a monoclonal antibody for the treatment of follicular lymphoma. Following this monumental breakthrough in monoclonal antibody based immunotherapy would be: Trastuzumab which was FDA approved in 1998 to treat HER-2 positive breast cancer, Gemtuzumab ozogamicin in 2000 for acute myelogenous leukemia, Alemtuzumab in 2001 for chronic lymphocytic leukemia, Ibritumomab tiuxetan in 2002 for B cell non-Hodgkin’s lymphoma, Tositumomab in 2003 for follicular lymphoma, Cetuximab in 2004 for colorectal, head and neck cancer, Bevacizumab in 2004 an angiogenesis inhibitor, Panitumumab in 2006 against EGFR for colorectal cancer, Ofatumumab in 2009 for chronic lymphocytic leukemia, Ipilimumab in 2011 for melanoma, Brentuximab vedotin in 2011 for Hodgkin’s lymphoma, and Pertuzumab in 2012 for HER-2 positive breast cancer.

Monoclonal antibodies directed against malignancies probably all work through a combination of three possible mechanisms: antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and cell signaling
alterations. *(Fig. 1.1)* Rituzimab, Ofatumumab, Trastuzumab, Cetuximab, and Alemtuzumab all appear to use ADCC as at least part of their mechanism of action (Golay and Introna 2012). Recent studies have shown the importance of not only the binding of antibody to the Fc receptor, but in addition it appears that binding to the specific FcYRIIIA enhances ADCC (Clynes et al. 2000a). A new generation of antibodies will include an altered Fc portion that binds specifically to FcYRIIIA thereby increasing the cytotoxic effects (Schmiedel et al. 2013).

Activating the complement system is another way in which monoclonal antibodies can have an effect on cancer cells. The classical complement pathway is activated when circulating pro-proteins from the complement system bind to antibodies complexed with antigen (Crist and Tauber 1997). The complement cascade is initiated and a series of cleavages by complement proteases result in chemotaxis of phagocytes and neutrophils, cell membrane lysis, clumping, and enhanced opsonization and thereby phagocytosis of antigen-bearing cells.

The third way in which monoclonal antibodies exert their effect is through cell signaling. Cell signaling is specific and complex with minute differences eliciting polarizing effects such as proliferation and apoptosis. Antibodies are able to create,
**Figure 1.1: Ways in Which Antibodies Affect Tumor Cells.** This figure illustrates five ways in which antibodies can have an effect on tumor cells. A. Illustrates antibody-dependent cell-mediated cytotoxicity (ADCC) in which the tumor cell is marked by bound antibody for lyses by an effector cell (NK cell). B. Illustrates complement dependent cytotoxicity (CDC) in which antibody marks the tumor cell for complement dependent lyses. C. Illustrates how antibodies can sterically hinder the dimerization of receptors, thereby blocking subsequent signaling. D. Illustrates how antibodies can block the binding of ligand, thereby blocking subsequent signaling. E. Illustrates how antibody can induce receptor internalization making it unavailable for signaling.
block and alter signals through a plethora of mechanisms such as blocking dimerization (Junntila et al. 2009), acting as ligand (Walker et al. 1995), buffering ligand concentrations (O’Hear and Foote 2005), and altering receptor conformations (Raffai et al. 1995). In addition to the above mentioned “naked” antibody mechanism of actions, monoclonal antibodies are now being engineered with toxic or radioactive molecules to enhance specificity of cytotoxic drugs and minimize collateral damage to nearby healthy cells (Gibiansky and Gibiansky 2014; Schechter et al. 1987).

**Cell Based Approaches:**

One of the other branches of immunotherapy uses cellular components of the immune system, rather than recombinant forms of secreted proteins such as monoclonal antibodies or cytokine therapy. There are basic types of cell based approaches; adoptive T-cell therapy and dendritic cell (DC) vaccines (Fig. 1.2). Both types of immunotherapy require the extraction of the patients own cells, followed by in vitro activation and possibly expansion of the cells. MHC differences require that this type of therapy be tailored to the individual patient. Adoptive T-cell therapy requires that cells be taken from the tumor site or from peripheral blood. These cells are either already antigen specific, or attain enhanced antigen specificity through in vitro expansion or genetic engineering of antigen receptors. The cells are then either injected into the site of tumor or into peripheral blood. Although much research has been done
**Figure 1.2: Cell Based Immunotherapy.** This figure illustrates two cell based immunotherapies: T cell Based in which the patients T cells are expanded and treated in vitro and then returned to the patient and DC based Immunotherapy in which monocytes from the patient’s peripheral blood are extracted, expanded and differentiated into DC’s, treated in vitro and returned to the patient.
using this approach and many clinical trials have been performed with occasional sporadic successes, there are currently no FDA approved adoptive T-cell therapies.

DC therapy employs a similar technique to adoptive T-cell therapy in that it uses cells from peripheral blood of the patients. The cells from the peripheral blood are sorted, and the monocytes are used as precursors to DCs which are activated and antigen-loaded in vitro. The cells are then administered back to the patient at various possible anatomical locations. The first, and currently only, DC immunotherapy was FDA approved for prostate cancer on April 29, 2010. Sipuleucel-T was able to increase median survival rates in hormone-refractory prostate cancer (Kantoff et al. 2010). The results are particularly encouraging because the patients had all advanced to late stage, metastatic prostate cancer, which is a high bar to set for an immune-based therapy. For example, such late stage cancers often times alter their surrounding environment to tamp down anti-tumor immune responses (Orimo et al. 2005; Polanska and Orimo 2013). The results for Sipuleucel-T, along with its FDA approval, will help to advance the field of DC immunotherapy.

**Breast cancer vaccine approach based on the HER-2 protooncogene:**

In order to create specific anti-tumor immune response, a defined target antigen must be determined. Ideally, the antigen would be expressed in a large proportion of tumor cells, but relatively absent from normal healthy tissue. This differential distribution of target antigen is essential for the immune system to attack and destroy
the abnormal tumor cells, while minimizing damage to normal, healthy tissues. It would also be advantageous if the selected tumor target was critical to the cancer cell’s survival, or capacity to cause disease. Because of these requirements, human epidermal growth factor receptor 2 (HER-2) has been put forth as a candidate tumor antigen for breast cancer. HER-2 has in fact become a major focus for many immunotherapy based cancer treatments including a number of attempts to produce vaccines (Disis et al. 1994; Disis et al. 1996; Disis et al. 1997; Disis et al. 2004; Disis et al. 2009; Mittendorf et al. 2011), as well as a fairly successful monoclonal antibody-based drug Trastuzumab (Herceptin) (Swain et al. 2013). Another highly promising dendritic cell based HER-2 vaccine against early breast cancer (Ductal carcinoma in situ) has been developed in collaboration between our laboratory and the laboratory of Dr. Brian Czerniecki at the University of Pennsylvania. Therefore, there appears to be a consensus that HER-2-based immunotherapy is a productive avenue of investigation.

**HER-2 and the HER Family of Receptor Tyrosine Kinases:**

The main function of a cellular receptor is to transmit information from the exterior of the cell to the interior of the cell. In the case of receptor tyrosine kinases (RTK) the transmitted information codes for primordial signals that determine growth, proliferation, differentiation, migration, and apoptosis (Robinson, Wu, Lin 2000). RTKs are allosteric enzymes that contain an extracellular ligand binding domain, a transmembrane domain, and an intracellular kinase domain responsible for recruitment of adapter proteins and subsequent initiation of cellular signaling cascades (Fig. 1.3).
Figure 1.3: The Structure of a Receptor Tyrosine Kinase. This figure illustrates the structure of a receptor tyrosine kinase (RTK) showing the external domain which is responsible acquiring the outside signal via ligand binding and dimerization, the transmembrane portion, the tyrosine kinase portions with becomes phosphorylated via dimerization, and the cytosolic tail capable of autophosphorylation and the phosphorylation of specific adapter proteins. The signal is then carried out through a phosphorylation cascade resulting in growth and proliferation transcription factors entering the nucleus.
The HER family of RTKs consists of four receptors: EGFR or HER-1, HER-2, HER-3 and HER-4 (Yarden and Sliwkowski 2001). Each member of the HER family plays a specific role in the development of organisms but their expression tends to dissipate with maturation. However, these receptors have been shown to be up-regulated in many cancers of epithelial origin, and have therefore been thought to play a role in the pro-proliferation, anti-apoptotic, and metastatic nature of cancerous cells (Yarden and Sliwkowski 2001).

EGFR, or HER-1, was the first member of the HER family to be discovered. EGFR is essential for epithelial development, proliferation, and organogenesis. EGFR receptor knockout mice typically result in perinatal mortality; survivors exhibit abnormalities of the gastrointestinal tract, lungs, brain skin, eyes, kidneys, and liver. (Miettinen et al. 1995; Threadgill et al. 1995) If animals receive enhanced amounts of EGF, early eye opening and tooth eruption are observed. (COHEN 1962; COHEN and ELLIOTT 1963) In normal epithelial cells the presence of EGFR diminishes with age; however, in certain cancers the receptor becomes upregulated once again. (Nanney et al. 1990)

The HER-2 protoonocogene codes for a 185 kDa transmembrane tyrosine kinase that serves as a growth factor signaling molecule (Cho et al. 2003) Mice defective of the erbb-2 (homolog of HER-2) gene die at an embryonic stage due to heart defects (Harari and Yarden 2000). It is unique amongst its HER family because not only does it have no known ligand, it also does not require the presence of ligand to convert it to its open conformation (Cho et al. 2003). It is therefore an easy dimerization partner for not only
other HER-2 proteins, but also other HER family members. It is over-expressed in approximately 30% of all breast cancers, but is absent at high levels in non-cancerous adult tissues with the exception of low level expression on some cardiac cells (Paik, Kim, Wolmark 2008). HER-2 over-expression in breast cancer is considered a poor prognostic indicator because it is associated with invasion, metastasis and resistance to some front-line chemotherapeutic agents (Menard et al. 2001).

HER-3 is also a unique family member in that it contains no phosphorylation sites within its receptor tyrosine kinase portion and therefore is unable to homodimerize (Yarden and Sliwkowski 2001). However, it is the most potent mitogenic activator with comparatively large numbers of adapter proteins that interact with tyrosines in its C-terminal domain (Sassen et al. 2008) HER-4 appears to be equally as important as HER-2 for cardiac development but less is known about the role of this receptor in the adult tissue. It is of current belief that the presence of HER-4 in breast tumors correlates with prolonged survival (Abd El-Rehim et al. 2004; Junttila et al. 2005; Pawlowski et al. 2000; Witton et al. 2003). In estrogen receptor-positive tumors the prolonged patient survival appears to be due to growth inhibition and cell differentiation (Feng et al. 2007; Naresh et al. 2006).

The HER family has been implicated in many different cancers of epithelial origin (Yarden and Sliwkowski 2001). However, the complexity of these receptors, their interactions with other family members, and diverse and overlapping signaling pathways has made data regarding the relative roles of these receptors difficult to
interpret. Conflicting data pertaining to the role of HER-3 in breast tumors is an example of inconclusive data most likely due to the complex mechanisms that govern these receptors, such as ligand variations, endogenous antibodies with a wide range of epitopes and isotypes, varying dimerizations partners, and number of adapter proteins (Fig. 1.4). Some studies have shown the presence of HER-3 to have adverse outcomes pertaining to survival (Abd El-Rehim et al. 2004; Sassen et al. 2008; Xue et al. 2006) whereas other have found HER-3 to correlate with a better prognosis (Lee et al. 2007; Pawlowski et al. 2000) In vitro data suggests that HER-3 is involved in tumor growth and resistance to endocrine and HER-directed treatments (Frogne et al. 2009). It is clear that the presence or absence of the receptor itself is only one piece to the puzzle.

The corresponding partner with which HER family members dimerize determines which signaling cascades are activated, and with what intensity. The cytosolic tail, which is responsible for the initiation of the signaling cascades that follow receptor activation, varies amongst HER family members (Fig. 1.5). HER family members activate different mitogenic pathways and with varying intensities and durations. Therefore, there is great variation in receptor signaling depending on the dimer makeup. Homodimeric combinations appear to be less mitogenic in general than heterodimeric combinations. Dimers containing HER-2 seem to elicit the strongest mitogenic responses (Pinkas-Kramarski et al. 1996). More specifically dimers of HER-2 and
Figure 1.4: Receptor Tyrosine Kinase Action. This figure illustrates the complexity and numerous variables that are involved in receptor tyrosine kinase signaling. Ligand variability determines if the receptor can dimerize and possibly to what extent. The availability of various dimer partners and the partner’s unique characteristics also play a significant role in signaling.
**Figure 1.5: Adapter Proteins Associated with HER-2 and HER3.** HER-2/neu and HER3 form the most potent anti-apoptotic and pro-proliferation dimer within the HER family. Both are unusual members as HER-2/neu has no known ligand and is constitutively in an open conformation and HER3 has no receptor tyrosine kinase portion, relying on its dimerization partner for phosphorylation. HER3 aggressively targets the Akt pathway with 6 tyrosines that recruit adapter proteins capable of activating the Akt pathway.
HER-3 makeup are the most intense mitogenic signal with the longest duration (Lee-Hoeflich et al. 2008; Vaught et al. 2012)

The signal initiated by the dimerized receptors is determined by a plethora of factors including cellular context, specific ligand binding, and the dimerization partner. It is reasonable to conclude that the intricacy and complexity noted is similar for all HER family members and that more work must be done to identify such conditions so that control and modification of extenuating variables can lead to enhanced advantageous clinical outcomes.

**Herceptin (Trastuzumab) a monoclonal antibody-based therapeutic agent for HER-2 positive breast Cancer:**

Given the importance of HER-2 in growth factor signaling, tumor survival and relationship with poor prognosis, it is perhaps not surprising that therapies already exist that target this molecule. Trastuzumab (Herceptin) is a monoclonal antibody created and distributed by Genentech as a HER-2 based therapy for breast cancer. The first version of trastuzumab was developed in 1990 by vaccinating Balb/c mice with NIH 3T3/HER-2-3400 cells, which are embryonic mouse fibroblasts trasfected with p185 HER-2. The resulting monoclonals were screened for recognition of HER-2pos SKBR-3 breast cancer cell lines (Fendly et al. 1990; Hudziak, Schlessinger, Ullrich 1987). A panel of 10 monoclonal antibodies was selected and characterized for their ability to inhibit growth in the SKBR-3 cells. Some antibodies enhanced cellular growth, others had no effect, and
some were capable of inhibiting cellular growth in SKBR-3 cells. The monoclonal antibody 4D5 (murine trastuzumab) was found to be the most effective in inhibiting the growth of SKBR-3 cells (Hudziak, Schlessinger, Ullrich 1987). In an attempt to make the murine mAb more clinically useful, the antibody was “humanized” by substituting mouse-specific IgG sequences with their human counterpart. Specifically, the binding loop of mAb4D5 was inserted on human variable region framework and human IgG1 constant domains. In addition, point mutations were made in the human framework to enhance both binding and capacity to inhibit target cell proliferation. The humAb4D5-8, “Trastuzumab”, was the result (Carter et al. 1992).

On September 25, 1998, trastuzumab became the first HER-2 based therapy to be approved by the USDA (FDA) for the treatment of HER-2 positive breast cancer in both early and late stages. Since then trastuzumab has shown significant impact on patient outcome (Slamon et al. 1987) and provided impetus for the development of other HER-2 based therapies. However, mechanism of action continues to be a debate and acquired resistance and tolerance to trastuzumab therapy remains a concern to breast cancer patients and the oncologists that treat them.

Trastuzumab is hypothesized to have several mechanisms of action. One mechanistic effect pertains to signaling through the PI3K/Akt pathway. Stimulation of the PI3K/Akt pathway leads to enhanced proliferation, migration, and differentiation (Chen et al. 2013). It is thought that the reduction in signaling, via trastuzumab’s binding to HER-2, leads to an upregulation in p27 which leads to inhibition of cyclin-
dependent kinase (Cdk) and subsequent cell cycle arrest (Chu, Hengst, Slingerland 2008). The increased synthesis and protein stability via post translation regulation of p27 inhibits cyclin D kinase 2 which in return results in the induction of G\textsubscript{1} cell cycle arrest (Lane et al. 2000; Le et al. 2000a; Le et al. 2000b; Neve et al. 2000; Pietras et al. 1998; Sliwkowski et al. 1999; Yakes et al. 2002).

Another mechanistic hypothesis postulates that trastuzumab’s proximity to the cytoplasmic membrane results in a unique ability to block proteolytic cleavage of the extracellular domain by metalloproteinases and a disintegrin and metalloproteinases (ADAMs) (Baselga 2000; Codony-Servat et al. 1999; Liu et al. 1999; Molina et al. 2001). The cleavage of the extracellular domain of HER-2 results in a constitutively active isoform of the receptor tyrosine kinase resulting in an enhanced pro-proliferation, anti-apoptotic signaling cascade. Since both metalloproteinases and ADAMs are upregulated in cancer (Duffy et al. 2011; Egeblad and Werb 2002; Mochizuki and Okada 2007), it is plausible that cleavage of the ECD of HER-2 is one mechanism in which HER-2 positive cells exert their ligand-independent malignant phenotype, and that interruption by trastuzumab may result in signaling down-regulation. (Fig. 1.6)

Another hypothesis regarding Herceptin’s mechanism of action is that it blocks dimerization, especially that of the HER-2/HER3 heterodimer (Hermanto, Zong, Wang 2001; Le et al. 2000a; Le et al. 2000b; Yakes et al. 2002).
Figure 1.6: Trastuzumab (Herceptin) May Block the Cleavage of the ECD of HER-2.

HER-2 in its full length canonical form is a 187 kDa protein consisting of 1255 amino acids. It contains an extracellular domain, a transmembrane domain, a receptor tyrosine kinase domain, and a C-terminus domain with multiple phosphotyrosines capable of initiating signaling cascades. A site between aa 647 and 648 is subject to cleavage by metalloproteinase and ADAMs, which are both up-regulated in many cancers. The cleavage of the ECD results in a constitutently active intracellular domain.
Dimerization occurs in domains II and IV of the ECD; domain IV being the binding site of Herceptin. HER-2 requires dimerization in order for there to be cross-phosphorylation of the RTK portion of the protein and subsequent autophosphorylation capable of sequestering adapter proteins, and resulting in the initiation of a cellular signaling cascade. The ligand-independent open conformation of the HER-2 protein makes it an easy target for dimerization with other HER-2 proteins or other HER family members. The heterodimerization of HER-2 and HER-3 creates a particularly potent pro-proliferation and anti-apoptotic signal by utilizing the AKT pathway. This is due to the numerous tyrosine docking sites present on HER-3 capable of sequestering adapter proteins that initiate the PI3K/Akt signaling cascade as well as the MAP/ERK pathway (Lee-Hoeflich et al. 2008). Over-stimulation of both pathways is associated with proliferation and the avoidance of apoptosis. Blocking the dimerization of this heterodimer could be beneficial in slowing the malignant phenotype potentiated by the overstimulation of these pathways (Fig. 1.7).

Some studies attempting to show Herceptin’s ability to block HER-2 dimerization with HER-3 have been inconclusive. Inconsistencies seen amongst studies may be due to the change in conformation seen when HER-3 becomes ligand-bound. If certain ligands are present, Herceptin will be ineffective in blocking dimerization, but in the absence of these ligands, Herceptin will in theory be able to effectively block dimerization and therefore down-regulate AKT phosphorylation (Fig. 1.8). Ligands complicate the situation resulting in questions regarding not only presence of the ligand, but concentration and possible splice variants. For example, the bound ligand is...
Figure 1.7: HER-2/neu is the preferred dimerizing partner for the HER family. The different heterodimers as well as the homodimerization result in the stimulation of the MAPK and Akt signaling pathways. The kinetics of the pathway stimulation changes with the number of phosphorylated tyrosines and the adapter proteins associated with the tyrosines.
Figure 1.8: Inhibition of Her-2 and Her-3 heterodimerization by Trastuzumab and Pertuzumab and the signaling cascade that occurs upon active heterodimerization. Domains II and IV are involved in the dimerization of both HER-2 and HER-3. Pertuzumab binds to region II, which is unavailable in the absence of heregulin whereby HER-3 maintains its closed position. However upon binding, heregulin is able to convert HER-3 into the open conformation in which region II is available to either dimerization or partake in the inhibitory mechanism of Pertuzumab. Trastuzumab on the other hand is capable of binding to region IV of the HER-2 receptor. When HER-3 is in the closed conformation, Trastuzumab is sufficient for inhibition of heterodimerization, however, upon heregulin induced conformation of HER-3, Trastuzumab loses its inhibitory effect. The signaling cascade that follows dimerization utilizes PI3K as a mode of phosphorylating AKT, which has shown to be a powerful activator of tumor survival and invasiveness.
capable of changing the conformation of the receptor, making some antibody epitopes more accessible and others either less or non-accessible. Although HER-2/neu has no family members (which form dimer partners with HER-2) have multiple known ligands. Each bound ligand recruits its own unique pattern of adapter proteins which define possible downstream signaling events. Also, the presence of splice variants among the ligands has the potential to introduce additional variation of ligand-dependent receptor signaling activity.

Furthermore, trastuzumab has been hypothesized to disrupt DNA repair (Pietras et al. 1999), down-regulate angiogenesis and participate in antibody-dependent cellular cytotoxicity (Baselga 2000; Kono et al. 2002) Some of these mechanisms may be regulated through Fc receptors on lymphocytes. Mice deficient for FcγRIII showed a loss of trastuzumab-mediated tumor inhibition, whereas mice deficient for inhibitory FcγRIIB showed a marked increase in tumor inhibition (Clynes et al. 2000b).

Thus, there are multiple proposed mechanisms that attempt to explain the effects of trastuzumab on proliferation and apoptosis of cells expressing HER-2. With the passing of nearly a quarter of a decade since its development, there is still considerable confusion as to which fraction of clinical activity is contributed by each of the known mechanisms of action. It is clear, however, that the observed effects of trastuzumab are complex, and dependent upon a number of variables that may manifest themselves both in in vitro studies, as well as within the treated individual.
The incorporation of a humoral response could theoretically increase efficacy of the current vaccination process:

The principal investigator of this laboratory, and his collaborators, have worked together to devise a HER-2-based dendritic cell vaccination platform to treat patients with early HER-2 overexpressing breast cancer (Czerniecki et al. 2007; Koski et al. 2012). The vaccine was designed to recruit CD4<sup>pos</sup> helper T lymphocytes and CD8<sup>pos</sup> cytotoxic T lymphocytes, without a specific intention to elicit antibody responses. It is possible that some antibodies are produced incidentally, but archived patient sera have not yet been systematically assayed for antibody production. Nonetheless, given the success of trastuzumab, it may be highly advisable to plan for future modifications of the current vaccine that in addition to strong T cell immunity, will also induce powerful antibody responses.

Although redesigning the vaccine so that it also induced antibody responses will not be technically difficult in theory, it must be kept in mind that not all anti-HER-2 antibodies can be expected to behave equally well, or provide benefit to the vaccinated subject. Antibodies are multifaceted and complex in their mechanisms of action. They can coat (opsonize) targets and render them susceptible to phagocytosis, they can bind to surface receptors of targets and interfere with signal transduction, they can activate the complement system and damage the cell membrane of the target, and they can elicit antibody-dependent cellular cytotoxicity from Natural Killer (NK) and other cell types. Indeed, many studies have shown the presence of naturally occurring antibodies
capable of recognizing the HER-2 protein in cancer patients with tumors that overexpress HER-2; however, this response does not appear to be adequate to control the disease (Bei et al. 1999; Disis et al. 1997; Disis et al. 1999; Disis et al. 2004). To the contrary, the presence of endogenous antibodies has traditionally been correlated with poor prognosis. This could be because endogenous HER-2/neu specific humoral immunity correlates with patients with the highest level of HER-2/neu protein overexpression in their primary tumors (Goodell et al. 2008; Ward et al. 1999). This means that the only the patients who already have the most aggressive and dangerous tumors will naturally develop strong antibody responses against HER-2. The contradiction seen with endogenous antibodies being a poor prognostic indicator, and the use of antibodies as tumor control may be due not only to the degree of overexpression of the HER-2/neu protein, but also to the specific properties defining individual antibodies produced against HER-2. Predominating antibody classes (IgG, IgA, IgM) as well as specific epitope binding contribute to the activity an antibody will possess against tumors. These range from the actual enhancement of proliferation (for example by acting as a pseudoligand) to induction of cell death via apoptosis (Kim et al. 2002) Some antibodies have been shown to stimulate growth and proliferation (Stancovski et al. 1991), while others have shown to block the binding of growth factors, interfere with a necessary dimerization altering internal cellular signaling (Junttila et al. 2009; Molina et al. 2001; Nagata et al. 2004; Zhang et al. 2011), or block cleavage of an extracellular domain (Molina et al. 2001). Therefore, the specific epitope through which the antibody binds to the target protein will determine the signal the cell
receives and how it is processed. The other component that has shown equal
importance is that of the Fc portion (Kim et al. 2002), which varies between different
antibody classes and isotypes. The heavy chain, which contains the Fc portion of the
antibody determines its ability to bind complement, participate in antibody dependent
cell cytotoxicity and initiate recruitment of other effector cells. Both components of an
antibody should be considered of equal importance.

Therefore, designing an anti-cancer vaccine that will elicit humoral immunity
requires thorough consideration. The specific epitope within the receptor in which the
induced antibodies bind determines if the humoral response is pro-proliferation/anti-
apoptotic or anti-proliferation/pro-apoptotic. Many approaches have been taken to
address this component. Some have opted to vaccinate with whole protein resulting in a
milieu of polyclonal antibodies (Dela Cruz et al. 2003). Others have attempted to target
a specific epitope by vaccinating with peptides of varying lengths and conformations
(Disis et al. 1999). With this kind of precision a great deal of investigation is required to
determine if the peptide is capable of inducing antibody and if so, whether the antibody
binding to the epitope elicits desirable biological responses.

The manner in which the humoral response is induced will impact the isotype of
the resulting antibodies. There are 5 different human (and murine) immunoglobulin
(antibodies) classes: IgG, IgA, IgM, IgE, and IgD. Within these, the IgG class has 4
subtypes and the IgA class has 2 subtypes. Each class and subtype plays a different and
essential role in immunity (Fig. 1.9). The presence of cytokines, secreted by helper T
<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Sub-Isotypes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>4</td>
<td>In its four forms, provides the majority of antibody-based immunity against invading pathogens. The only antibody capable of crossing the placenta to give passive immunity to the fetus.</td>
</tr>
<tr>
<td>IgA</td>
<td>2</td>
<td>Found in mucosal areas, such as the gut, respiratory tract and urogenital tract, and prevents colonization by pathogens. Also found in saliva, tears, and breast milk.</td>
</tr>
<tr>
<td>IgM</td>
<td>1</td>
<td>Expressed on the surface of B cells as a monomer and in a secreted form as a pentamer. Eliminates pathogens in the early stages of humoral immunity before there is sufficient IgG.</td>
</tr>
<tr>
<td>IgE</td>
<td>1</td>
<td>Binds to allergens and triggers histamine release from mast cells and basophils. Also protects against parasitic worms.</td>
</tr>
<tr>
<td>IgD</td>
<td>1</td>
<td>Functions mainly as an antigen receptor on B cells that have not been exposed to antigens. It has been shown to activate basophils and mast cells to produce antimicrobial factors.</td>
</tr>
</tbody>
</table>

**Figure 1.9: Immunoglobulin Isotypes.** This figure illustrates the 5 main immunoglobulin isotypes and shows the number of sub-isotypes associated with each. A brief description of each isotype’s role in adaptive immunity is listed on the right.
cells, determines which isotypes are secreted from plasma cells (Harris et al. 2000; Lund 2008). The isotype class typically targeted in immunotherapy is IgG, which composes approximately 75% of serum immunoglobulins in humans. It is the predominating classes and subtypes of antibody and the cytokines that determine them that dictate the functional qualities of the humoral response.

Recently, the Temporal Model was developed to explain how these Immunoglobulin classes and subtypes work in coordination (Collins and Jackson 2013a); (Fig. 1.10). In the genomic sequence IgG3 is first followed by IgG1, IgG2, and IgG4 respectively. The model suggests that the genomic sequence correlates with the amount of time each antibody isotype stays in the germinal center accruing a greater number of point mutations in the VDJ gene, which increasing the affinity for antigen. The IgG3 contains the lowest affinity for antigen, yet is the most potent complement fixer, and has highest affinity for phagocytic Fc receptors (FcγRs). It has a relatively short half-life of approximately 7 days and comprises only about 7% of the total IgG in circulating sera. In contrast, B cells that stay in the germinal center for the longest period of time tend to secrete IgG4 antibodies that are responsible for shutting down the immune response. IgG4 has the greatest affinity for antigen, yet does not bind complement and binds only to the FcγRIIB which is an inhibitory FcγR. The model suggests that IgG3 emerges early in the humoral response with robust activity but with less specificity. On the contrary, IgG4 emerges near the final stages of the humoral response acting to shut down any remaining inflammatory responses. The two remaining isotypes, IgG1 and IgG2, act as
Figure 1.10: Adapted from Collins et. al., 2013. The Temporal Model of Human IgG Antibody Function. This figure illustrates the immunoregulation that occurs via the four IgG isotypes. Each isotype has a distinct function that aids in the rapid initiation, well-balanced sustained response, and the shutting down of the humoral portion of the overall immune response. (Collins and Jackson 2013b)
the primary humoral classes. IgG1 emerges slightly earlier than IgG2 and tends create pro-inflammatory conditions, whereas IgG2 tamps down the inflammatory response. It is the ratio of these two isotypes that ultimately keeps the humoral response in check.

It is therefore essential to consider the fine epitope specificity and isotype of the generated antibodies when designing and antitumor humoral based vaccine. Ideally, anti-tumor humoral activity would be robust with epitope specificity capable of blocking cell signaling cascades that lead to the proliferation and anti-apoptosis signals commonly seen in cancerous cells. Additionally, it would be advantageous if the antibody were of an isotype that would subject the targeted cell to complement dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) via binding of complement and FcγRs to the Fc portion of the antibody.

**Designing a next-generation vaccine that elicits both cell-mediated and humoral immunity**

It has been suggested that the stimulation of both T-cell and B-cell mediated immunity may act in a manner that is not simply additive, but synergistic, thereby leading to a potentiated and potent tumor clearing capability (Nanni et al. 2004; Reilly et al. 2001). Hence it is possible that a vaccine strategy recruiting both cellular and humoral effectors may enable superior anti-tumor immunity. Our current vaccine platform, already tested in Phase I and II clinical trials generates the former but not the latter. In order to achieve both, we would propose a prime-boost strategy where
individuals are vaccinated first with the dendritic cell-based component to recruit helper and cytotoxic T cells in the relative absence of antibody. After this priming step, the individual would be boosted with free peptides containing appropriate HER-2 non-conformational epitopes capable of generating high-titer antibodies with the biological activity of Trastuzumab. It is the goal of this dissertation to lay the groundwork for such a second-generation, antibody-inducing vaccine.

With this goal in mind, this dissertation has three Specific Aims:

Aim 1 will formally verify the expected modest capacity of the current dendritic cell vaccination platform to induced enhanced antibody responses against HER-2. We will also analyze naturally-occurring antibodies in unvaccinated individuals who are disease-free, who have early HER-2-expressing breast cancer (DCIS) and those who have more advanced HER-2-expressing invasive cancer (IBC) to determine whether or not there are differences in amounts or subtypes of IgG in these different disease states.

Aim 2 will identify short linear peptides on the HER-2 sequence capable of acting as a vaccine to induce in mice antibodies that recognize whole HER-2 protein or its recombinant extracellular domain.

Aim 3 will evaluate whether immunogenic peptides spanning the domain of HER-2 recognized by the therapeutic monoclonal antibody Trastuzumab are capable of eliciting antibodies with Trastuzumab-like activity as assessed in in vitro studies on HER-2 over-expressing breast cancer cell lines.
Chapter 2

Examination of antibody responses in early breast cancer patients treated with a dendritic cell-based HER-2 vaccine

Introduction

Immunotherapy has the potential to enhance and guide the immune response against tumor cells providing a robust, long-lasting immunity which can provide protection from future malignancies. With this in mind a DC based anti-HER-2 vaccine was created at the University of Pennsylvania (Czerniecki, Koski et al. 2007, Sharma, Koldovsky et al. 2012, Koski, Koldovsky et al. 2012) The vaccine was designed to treat breast cancers in the early stage known as ductal carcinoma in situ (DCIS). Treating cancers in their early stages is optimal for immunotherapy because the tumor load is low, the tumor has had less opportunity to alter and subvert immunity, and the patients have not yet experienced potentially immuno-compromising therapies such as chemo- and radiation treatments.

Patients enrolled in the DC based HER-2 vaccine trials were diagnosed as having HER-2 positive, early stage ductal carcinoma in situ (DCIS) and had received no previous treatment for the disease. The diagnoses were based on biopsies with >5% of the cells staining positive for HER-2 with an intensity of 2+ or 3+. Patients meeting the above criteria and consenting to participate in the trial underwent leukapheresis for the
purification of monocytic DC precursors. Patient sera was also obtained for testing pre- and post-vaccination to assess immune responses. The monocytes were then treated in vitro with GM-CSF and IL-4 to differentiate them into DCs. The DCs were then pulsed with 6 synthetic peptides base on the HER-2 sequence that are known to be broadly stimulatory to T helper cells, as well as 2 additional peptides stimulatory to cytotoxic T-cells in patients with the common HLA-A2 class I antigen. The DCs were then activated with the cytokine IFN-γ and a special clinical-grade preparation of bacterial lipopolysaccharide (LPS). This combination results in the rapid activation and maturation of the DC and enhances their capacity to produce the cytokine interleukin-12 (IL-12), which is critical for strong Th1-polarized T-cell-mediated immunity. The DCs were then injected directly into the lymph nodes of the breast cancer patients. The patients were vaccinated once a week for 4 weeks. Following the completed vaccination schedule patients received a mammogram and MRI followed by surgical resection. (Sharma, Koldovsky et al. 2012)

The first trial consisted of 27 patients. At the time of surgical resection, after completion of vaccination schedule, 5 patients had no signs of residual disease and 11 patients lost all apparent HER-2 expression. The anti-HER-2 response was better in patients with tumors that were estrogen receptor (ER) negative. This is not unexpected due to the cross-talk between HER-2 receptors and ERs which provides the cell with anti-apoptotic and pro-proliferative signals; therefore, in patients with both receptors the tumor cells tend to be less responsive to the treatment targeting only one growth factor receptor.
A second larger Phase I/II trial was conducted with similar results to the first (manuscripts in preparation). When considering both Phase I/II clinical trials, these vaccines appeared quite promising with evidence of long-term T-cell immunity, decreased levels of HER-2 expression, apparent reductions in tumor volume, and protection from recurrence (Czerniecki, Koski et al. 2007). Although strong T-cell immunity was assessed in these studies, the role of antibodies, if any, were not systematically explored.

T lymphocytes and antibody-secreting B lymphocytes encounter their specific antigen in two different ways. B cells acquire whole, free protein antigens through interactions with their surface immunoglobulin receptor. These captured proteins are internalized, proteolytically processed, and presented to T helper cells in conjunction with self MHC class II molecules. The T cells then supply “help” signals to the B cells that allow them to secrete IgG. T cells, on the other hand, only see processed and fragmentary peptide antigen bound to self MHC. T helper cells are presented to these processed antigens by dendritic cells, macrophages and B cells. The current DC-based vaccine uses peptide antigen that is pre-bound in vitro to the DCs; therefore no free peptide, or whole HER-2 protein, would be available for interaction with B cells. Hence no antibody response is expected, but rather an optimized T cell mediated immune response. Unexpectedly, immunohistochemistry (IHC) comparison of pre-vaccine tumor needle biopsies with post-vaccination surgical specimens revealed a large B-cell infiltration into the site of disease as an apparent consequence of vaccination (Czerniecki, Koski et al. 2007).
The large infiltration of B-cells to the site of disease would suggest that these cells have been “called in” to scour the tumor environment and possibly take up antigen from the tumor cells (including shed HER-2) so that they can present these antigens to T-cells. If this is the case, then it is possible that the B cells may actually be producing, contrary to original expectations, anti-HER-2 antibodies. Therefore, the B cells may be stimulated not by HER-2 vaccine peptide, but by HER-2 shed from the tumors. They could possibly present the resulting processed peptides to the T cells that were induced by the actual vaccine thereby gaining “help” for production and secretion of IgG.

Due to the previously observed infiltration of B cells, we hypothesized that the treatment of patients with the DC based anti-HER-2 vaccine indirectly enhanced anti-HER-2 antibodies. We also hypothesized that the vaccine-induced increase in the Th1 cell population would have an effect on the class of anti-HER-2 antibodies in the patient sera; presumably favoring a class of IgG1 which it thought to be associated with a Th1 response. With these expected changes in antibody titers and class we would expect to see a reduction in metabolic activity of HER-2-expressing breast cancer cells when treated in vitro with post-vaccination sera, as compared to pre-vaccination sera. This is because anti-HER-2 antibodies should at least partially block growth factor signaling initiated by the HER-2 receptor tyrosine kinase.

In order to test the stated hypotheses, we obtained pre- and post- immunization sera from patients that received the dendritic cell vaccine and analyzed them for differences in levels of anti-HER-2 antibodies as an apparent consequence of
vaccination. In addition, we obtained sera from presumed healthy donors, patients with HER-2 positive invasive breast cancer (IBC) that were untreated, patients with HER-2 positive IBC that were treated with the anti-HER-2 monoclonal antibody-based drug Herceptin, and patients with HER-2 negative invasive breast cancer (IBC).

Materials and Methods

Cell Lines

The SKBR3 cell line was isolated by the Memorial Sloan-Kettering Cancer Center in 1970. It was derived from a pleural effusion of a adenocarcinoma originating from the breast of a 43 year old, Caucasian female. The HER-2 overexpressing SKBR3 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultivated in McCoy’s 5A media supplemented with 10% FBS and 1% penicillin/streptomycin (Biowittaker) and incubated at 37\(^\circ\) C and 5% CO\(_2\).

ELISA for Total IgG of Pre and Post Sera

ELISA assays were used to test for the presence of antibodies reactive to recombinant HER-2 ECD in all sera. EIA/RIA plates were coated with 50ul of a recombinant HER-2/neu ECD from ACRO Biosystems at a concentration of 5ug/ml in bicarbonate buffer and allowed to incubate overnight at 4\(^\circ\) C. The plates were then washed three times with PBS with tween and then blocked with 200ul of 1% BSA in PBS for 1 hour. The plates were washed again three times and 50ul of sera was added at a dilution of 1:200 in 1% BSA. The plates were incubated at room temperature for 2 hours
and then washed three times. The presence of bound HER-2 antibodies was then detected by incubating the plate with 50ul of anti-human IgG secondary conjugated to HRP (Thermo Scientific) for 1 hour, washing three times, developing with 50ul of TMB peroxide substrate (KLP), stopping the reaction with 25ul of 1N HCL, and reading the optical density of the resulting reaction at 450nm.

ELISA assays for Antibody Class

An ELISA was performed as previously stated, to test all sera. The top half of the plates were coated with HER-2 ECD in bicarbonate buffer and the bottom half treated with bicarbonate buffer as a background control. The plates were incubated overnight at room temperature. The next day the plates were blocked with 1% Casein in PBS before the addition of sera (1:100) in blocking buffer. The samples were incubated for 2 hours, washed three times, and then secondary was added. Three different secondary anti-human, HRP conjugated antibodies were used. The first one was a pan anti-IgG capable of recognizing all IgG classes (Thermo Scientific), the second was specific for IgG1 (Life Technologies), and the third was specific for IgG4 (Life Technologies). The pan IgG antibody was diluted 1:50,000 and the IgG1 and IgG2 were diluted 1:500, per recommendations of the manufacturer’s instructions. The secondary antibody was allowed to incubate for 1 hour before the plates were washed 6 times and developed with TMB substrate solution. Each serum was assessed in four trials for accuracy and reactivity to HER-2 calculated as a ratio of the OD against HER-2 divided by the background. The trials were averaged for each serum and then a paired t-Test was run.
to determine whether or not there was a change in antibody class from pre vaccination to post vaccination.

**Biological Assays with Patient Sera**

The patient sera were also analyzed for their ability to inhibit metabolic activity as measured by the alamar blue assay. HER-2pos SKBR3 breast cancer cells (5X10^5), were plated in 96-well cluster plates in 100ul culture medium. After 48 hours incubation, human subject sera were added to a concentration of 5% and incubated an additional 24 hours. Then 10ul of a 560uM resazurin sodium salt (Sigma–Aldrich) stock solution was added to each well. The cells were then incubated and observed every 30 minutes for development of color. Generally around 4 hours post-addition of resazurin dye, sufficient color change was noted, and optical density read at 630nm.

**ELISA assay for Comparing Healthy Donors, HER-2 positive DCIS patients, and HER-2 Positive Invasive Breast Cancer Patients**

Again an ELISA was performed to test for endogenous antibodies in the four categories. The ELISA was performed identically to the previously stated protocol for isotyping with the exception that the serum was run eight times for accuracy. Again the trials were averaged and this time an ANOVA was run to determine if there was a significant difference between groups.
Statistical Analysis

Comparisons were made between sera from patients before vaccination to the same patient after vaccination using a Paired t-test. Results were considered statistically significant if $p<0.05$.

The presence of endogenous HER-2 antibodies was compared in HER-2 positive DCIS patient sera, healthy donor sera and HER-2 positive IBC patient sera. A single-classification ANOVA was performed to determine if there was a significant difference in the mean values of the three groups. Results were considered statistically significant if $p<0.05$.

Results

Comparison of Pre-Vaccination to Post-Vaccination Patient Sera

We hypothesized that the vaccination with the DC based anti-HER-2 vaccine would enhance overall anti-HER-2 IgG activity. ELISA data was analyzed for change in pre- to post-vaccination sera with the expectation that vaccination would enhance anti-HER-2 antibody levels. Analysis revealed that this was not typically the case. A paired T-test showed that out of 13 patient antiserum pairs, no more than 6 had a modest enhanced antibody production as a consequence of vaccination. The remaining 7
showed no enhancement at all or a decrease in overall anti-HER-2 antibody activity (Fig. 2.1). However, it is interesting to note that regardless of whether anti-HER-2 Ig levels increased after vaccination, most patients appeared to have endogenous elevated anti-HER-2 antibody levels.

Although we did not observe consistent increases in total IgG, we considered the possibility that there may be changes in individual Ig subclassess. There are four IgG classes, IgG1, IgG2, IgG3 and IgG4. IgG1 production is associated with immune responses polarized toward the Th1 T helper phenotype. IgG4 is associated with non-Th1 immunity. Since our DC-based vaccine is known to generate highly polarized Th1 immunity, we hypothesized that we might see an increase in IgG1 class at the expense of IgG4, which would not be apparent when only looking at total IgG. Ironically, when analyzed via ANOVA we saw a slight, yet significant, decrease in both IgG1 and IgG4.
Figure 2.1: Individuals Show a Difference in Ab Optical Densities to HER-2/neu Following Dendritic Cell Vaccination. Sera was obtained from thirteen patients that participated in the first Th1 polarizing dendritic cell based clinical trial. The deidentified, coded sera was taken both before vaccination (pre) and after vaccination (post). The sera was analyzed via ELISA for recognition of the extracellular domain of HER-2/neu. Optical densities ± SEM indicate either an increase or decrease in Ab capable of recognizing the extracellular domain of HER-2/neu. There was no clear pattern of Ab induction after vaccination with the polarizing dendritic cells.
Although we detected no sign that antibody levels were increasing post-vaccination, we nonetheless tested these sera for the ability to inhibit the metabolic activity of HER-2-expressing breast cancer cell line SKBR3 via the Alamar Blue assay. As expected, we detected no significant differences between pre- and post-vaccine sera.

Comparison of Pre-Existing Anti-HER-2 Antibody Levels in Healthy Donors, HER-2-positive DCIS Patients and HER-2-positive IBC Patients

After noting the apparent presence of anti-HER-2 antibodies in the DCIS patients even prior to vaccination, we hypothesized that these individuals may naturally develop antibodies against HER-2, compared with healthy controls. We wanted to learn whether the enhanced anti-HER-2 Ig levels would remain high or dissipate with the progression of the disease from DCIS to IBC. Unfortunately, a large number (9 out of 12) of our available HER-2-positive IBC patients had been previously treated with the monoclonal antibody-based drug Herceptin, and the residual antibody left a high background reactivity against HER-2 that overshadowed any measurable change in endogenously-produced antibodies. Therefore our sample size of IBC patients became smaller than anticipated (n=-3). Nonetheless, ANOVA analysis of ELISA data indicated a statistically-significant increase in anti-HER-2 antibody levels (both IgG1 and IgG4) in unvaccinated HER-2-positive DCIS patients (n=16) compared with normal donors (n=13), and HER-2
Figure 2.2: **Isotypes of Pre and Post Vaccination Sera.** Sera was obtained from sixteen patients that participated in the third Th1 polarizing dendritic cell based clinical trial. The sera was taken both before vaccination (pre) and after vaccination (post). The sera was analyzed via ELISA for specific antibody isotypes (IgG1, IgG4, and pan IgG) capable of recognizing the extracellular domain of HER-2/neu. Optical densities ± SEM indicate either an increase or decrease in Ab capable of recognizing the extracellular domain of HER-2/neu. There was no clear pattern of change in pan IgG Ab (as seen in patients from the first trial (Fig. 2.1)), but there was a significant decrease in IgG1 and IgG4 ($p=0.01$ and $p=0.006$) after vaccination with the polarizing dendritic cells.
Figure 2.3: Alamar Blue Analysis of Human Sera. An Alamar Blue assay was performed with SKBR-3 cells. Cells were incubated with sera from patients prior to receiving the dendritic cell vaccination (Dark Blue) and sera from the same patients after they had received the vaccination (Light Blue). The metabolic activity of the SKBR-3 cells was assessed via the change in colored as resazurin is oxidized to resorufin, subsequently changing the absorption properties resulting in a difference in optical density ± SEM.
positive IBC patients. Lower HER-2 antibody levels in the HER-2-positive IBC patients may indicate the possibility that the endogenous anti-HER-2 response is lost upon disease progression. (Fig 2.4 & 2.5)

Discussion

Due to a large infiltration of CD20+ B cells observed through immuno-histochemical analysis of excised tumors from HER-2 vaccine recipients, it was hypothesized that there would be an increase in antibodies against HER-2. However, we were not able to confirm this. In addition we hypothesized that the Th1 polarized cells would encourage the B cells to secrete immunoglobulins of the IgG1 class. Quite unexpectedly, we actually saw a reduction in IgG1 and IgG4 antibodies when comparing pre-vaccination sera to post-vaccination sera. Furthermore, the biological activity of pre- and post- sera showed no difference in the ability to regulate metabolism in SKBR3 cells. Given the paucity of induced antibodies, this latter finding was not surprising, and it thus appears that the DC-based vaccine is quite inefficient at enhancing anti-HER-2 humoral responses. The first Phase I/II clinical vaccine trial found that out of 27 vaccinated subjects, 5 had no evidence of disease after the full course of immunization. Of the remaining 22 subjects with residual disease, HER-2 expression levels were markedly reduced; for many subjects the remaining disease became essentially HER-2-negative (Sharma, Koldovsky et al. 2012). It is possible that the apparent elimination of HER-2-
Figure 2.4: Comparison of Anti-HER2 IgG1 Ab’s in Healthy Donors, HER2 Pos IBC, and HER2 Pos DCIS. Sera was obtained from 13 healthy donors, 3 HER-2 positive IBC patients, and 16 HER-2 positive DCIS patients prior to treatment. The sera was analyzed via ELISA for antibody isotype IgG1 capable of recognizing the extracellular domain of HER-2/neu. Optical densities indicate either an increase or decrease in Ab capable of recognizing the extracellular domain of HER-2/neu. A significant difference was noted between the three cohorts $F(2,29)=3.75$, $p=.036$. 
Figure 2.5: Comparison of Anti-HER2 IgG4 Ab’s in Healthy Donors, HER2 Pos IBC, and HER-2 Pos DCIS. Sera was obtained from 13 healthy donors, 3 HER-2 positive IBC patients, and 16 HER-2 positive DCIS patients prior to treatment. The sera was analyzed via ELISA for antibody isotype IgG4 capable of recognizing the extracellular domain of HER-2/neu. Optical densities indicate either an increase or decrease in Ab capable of recognizing the extracellular domain of HER-2/neu. A significant difference was noted between the three cohorts $F(2,29)=3.75, p=.036$. 
overexpressing cells could offer an explanation regarding the statistically-significant
decrease in HER-2-reactive antibody levels after vaccination. First, it should be noted
that antibodies have a defined half-life (typically around 21 days for IgG). If HER-2 is no
longer present, the immune system will cease production of new antibodies against the
protein. The levels of pre-existing antibody will then simply decay over time until it is no
longer detectable in the serum. In this case, it would be expected that the subjects with
the strongest drop in anti-HER-2 antibodies after vaccination would be those who
experienced the most precipitous reductions in levels of HER-2 expression on their
tumors. On the other hand, it is possible that HER-2-overexpressing tumors release HER-
2 protein, particularly if under stress or are being destroyed by a vaccine-induced T cell-
mediated immune responses. The HER-2 ECD could complex with anti-HER-2 antibodies
in the blood, and make them unavailable for detection in conventional ELISAs that
evaluate subject’s serum. In this case, tumors that retain much of their HER-2
expression could actually cause an apparent drop in HER-2 antibody levels. The first step
in testing either of these hypotheses would be to examine patient records to determine
which subjects experienced vaccine-induced loss of HER-2 expression, and compare
changes in pre-to post- levels of anti-HER-2 antibodies with those from individuals who
did not experience loss in HER-2 expression. Our current IRB protocol does not provide
for this evaluation, but an approved modification would make this analysis feasible in
the near future

Our observation that HER-2-overexpressing DCIS patients might have elevated
anti HER-2 antibody levels even prior to vaccination prompted us to formally compare
pre-vaccine DCIS antisera to normal donors, as well as HER-2-positive invasive breast cancer patients. We demonstrated that the DCIS patients had significantly higher levels of IgG1 and IgG4 subclass antibodies against HER-2 than did healthy controls. We also saw signs that these levels may drop once again with more advanced IBC. We are currently acquiring additional serum samples from untreated HER-2-positive IBC patients to enhance this analysis.

It is intriguing to speculate on the biology of natural immune responses to HER-2 protein. HER-2 is important in embryological development (Lee, Simon et al. 1995), and also is expressed transiently during breast development in mouse during puberty (Schroeder, Lee 1998). Apart from this, HER-2 is found on a number of epithelial tissues, however the gene amplification that leads to its high expression in breast tumors is not observed (Press, Cordon-Cardo et al. 1990). Therefore, the relatively sudden and high-level expression of HER-2 protein on developing breast tumors may be perceived as “foreign” by the immune system, triggering a response that is insufficient to control the tumor, but nonetheless induces the production of detectable anti-HER-2 antibodies. But what could potentially reduce antibody levels in more advanced disease that continues to over-express HER-2 protein? Studies have demonstrated that as breast cancer progresses from DCIS to IBC, a statistically-significant (p<.001) increase occurs in tumor-infiltrating CD25^{pos}/FoxP3^{pos} “regulatory” T cells (Bates, Fox et al. 2006). These regulatory T cells function to suppress autoimmune responses by inhibiting the activity of effector T cells (Thornton, Shevach 1998), and also are known to directly suppress IgG
production by B cells (Lim, Hillsamer et al. 2005). Thus it is conceivable that Treg could shut down the production of anti-HER-2 antibodies for the more advanced IBC patients.

We will follow up our observations on HER-2 antibody levels by acquiring additional IBC patient sera to further confirm our hypothesis that IBC patients demonstrate a loss of HER-2 antibody responses compared with DCIS patients. Our observation of high anti-HER-2 antibody levels for DCIS patients, particularly those of the IgG4 class, might also have applications for a screening diagnostic for early HER-2-expressing tumors. Learning of the humoral anti-HER-2 activity in patients even prior to vaccination, we decided to look for more global trends in antibody expression. It seems logical that if the immune system is to view the overexpression of HER-2 as a danger, it would mount a response that would include anti-HER-2 humoral activity. If the HER-2 is to dissipate or disappear, one would expect the humoral immune response to go down. Additionally over time, if the HER-2 remains consistently overexpressed, the immune system begins to view this as a “new normal” and begins the process of turning itself off to avoid a situation of chronic inflammation. In this situation as well, one would expect the humoral immune response to go down or class switch to a protective class such as IgG4. We tested this hypothesis by looking at the anti-HER-2 humoral response in healthy donors, HER-2 positive DCIS patients, HER-2 positive IBC patients, and HER-2 negative IBC patients. We were able to show a significant decrease in anti-HER-2 humoral activity in the HER-2 positive IBC cohort when compared to the HER-2 positive DCIS cohort. We were also able to determine a significant difference in anti-HER-2 humoral activity between the healthy donor cohort and the DCIS cohort. The
overexpression of HER-2 in the DCIS patients is most likely a newer situation for the immune system leading to a HER-2 mounted immune response. It seems logical that the immune system of the HER-2 positive IBC patients has learned to shut down the inflammatory response due to time of exposure; hence, explaining the reduction in anti-HER-2 antibodies. Although the anti-HER-2 immune response is rarely proven to be sufficient to clear the tumor, this study shows that an anti-HER-2 response is in fact mounted and that by enhancing this response at an early stage or attempting to turn it back on in a later stage we may be able to aid in the anti-tumor battle.
Chapter 3

Identification of Linear B-Cell Epitopes and T-Cell Helper Epitopes within the Extracellular Region of Rat Erbb2 (HER-2/neu homolog)

Introduction

The induction of an anti-tumor humoral (i.e. antibody) response requires a three-cell activation system. The cell that actually produces antibody is the Plasma Cell, which is an activated and terminally-differentiated B cell. The activation of the B cell in turn requires a properly activated helper T cell, which itself requires an appropriately activated DC. Therefore, the three-cell system necessary for the production of IgG consists of a DC, a helper T cell and a B cell acting either sequentially or in unison. (Fig. 3.1)

In order for the naïve T-cell to be activated and attain effector functions, a DC must be activated in a manner that allows it to supply the necessary signals to the T-cell. Naïve T-cell activation has been described through a 3 signal model. Signal one is comprised of peptide antigen (complexed to MCH class II molecules) supplied by the antigen-presenting DC and sensed by the T-cell through the T cell receptor (TCR).
Figure 3.1: A Three Cell System for Induction of Antibodies. In order to induce an IgG humoral response B cells must obtain direct help for T helper cells and indirect help from DC’s. DC’s act as a liaison between the innate immune system and the adaptive immune system by obtaining signals from the peripheral (antigen and PAMPs) and bringing them back to the lymph node. The DC then activates naïve T cells capable of recognition of the a particular antigen complexed to an MCH II molecule. The activated T helper cell can then provide necessary support to a B-cell that is capable of recognizing the T cell receptors specicity via its antigen complexed to a MHC II molecule. If the B cell receives appropriate support it may differentiate into a antibody secreting plasma cell.
(Williams, Weiner et al. 1988) The interaction of the MHC class II and TCR is stabilized by the CD4 molecule that defines helper T cells. The second signal is “co-stimulation”. This is achieved though the B7 molecules (CD80 and CD86) found on the surface of DCs or other antigen presenting cells, (Azuma, Cayabyab et al. 1992, Nestle, Thompson et al. 1994) and sensed through CD28 counter-receptors on the T cell. (Vandenberghe, Freeman et al. 1992) If this co-stimulatory signal is received, activation and IL-2 production is triggered. (Jenkins, Taylor et al. 1991) If it is not received, the T cell will enter a non-functional state known as anergy (Jenkins, Burrell et al. 1990) and will not participate in active immune responses. The third and final signal provides information regarding how the T cell should differentiate and which effector functions should be acquired. This 3rd signal usually comes not from a surface receptor, but rather are soluble factors such as cytokines released primarily by the activating DC, or other accessory cell. (Curtsinger, Schmidt et al. 1999) Such 3rd signals often determine which cytokines will be produced by the T lymphocyte when it encounters its target antigen in the periphery, and thus will determine the quality of the immune response (Tominaga, Yoshimoto et al. 2000) Thus, 3rd signals prepare T cells to meet different types of biological threats.

The production of high affinity IgG and generation of B cell memory is dependent on T cell help(Kehry, Hodgkin 1993, Tsuji, Nibu et al. 1994, Vieira, Rajewsky 1990) Therefore, when attempting to induce a lasting humoral response consisting of high-titer IgG, it is essential to have T helper cell stimulation. The T-cell-dependent activation of a B-cell requires three steps. The first step requires that antigen (usually in native
conformation) bind and crosslink B-cell receptors (surface bound immunoglobulins). The B-cell then internalizes the antigen via endocytosis, processes it, and displays it on its surface complexed to MHC class II. (Rock, Benacerraf et al. 1984, Lanzavecchia 1985) The B-cell can now bind a TCR through linked recognition. Through CD40L on the T-cell and CD40 on the B-cell, the T-cell is sustains growth and differentiation and the B-cell increases proliferation, class switching, and somatic hypermutation, increasing the immunoglobulin’s affinity for the antigen. (Spriggs, Armitage et al. 1992) In addition, the T-cells secrete cytokines that regulate proliferation and antibody production.

The major goal of these studies was to identify regions of the HER2 protein that could be represented as short synthetic peptides that were capable of inducing high-titer antibodies that were in turn able to recognize native-conformation HER2 protein. By necessity peptides capable of inducing IgG must simultaneously contain tandem or overlapping sequences capable of stimulating helper T cells. Identification of antibody epitopes and the analysis of induced antibodies will allow us to identify regions capable of inducing antibodies with activities similar to the monoclonal antibody based therapeutic agent Herceptin (trastuzumab). Simultaneous identification of T helper epitopes will allow us to construct DC-based vaccines in mouse models of HER2 overexpressing breast cancer. The high sequence homology between rat Erbb2 and HER2 means that most generated antibodies will be cross-reactive between mouse and human proteins.

Materials and Methods
Construction of a Peptide Library of the Extracellular Domain of HER2/neu

We had a synthetic peptide library constructed (20\textsuperscript{th} Century Biochemicals Marlboro MA) spanning the extracellular domain of rat HER-2/neu (ErbB2), which is 97% homologous with the human gene. The peptide library consisted of 62 peptides of 20 amino acids in length, each overlapping by 10 amino acid residues. The lyophilized, desalted peptides were reconstituted at a concentration of 10 mg/ml in DMSO, aliquoted into 50\mu l aliquots and stored at -80°C. The peptides were divided into 12 pools for vaccination, containing 5 peptides each, with the exception of pools VIII and XII, which contained 6 peptides. (Fig. 3.2 and 3.3)

Vaccination of Peptide Library and Sera Collection

Each peptide pool was assigned three Balb/c or C57bl/6 mice. The mice were vaccinated in each flank with 25\mu g of peptide emulsified in 100\mu l Freund’s Complete Adjuvant. After a 14 days, mice were sacrificed, exsanguinated, and spleens removed. The recovered blood was allowed to clot at 4°C for 1 hour and then centrifuged at 4600 g’s for 20 minutes. The sera from the three mice per pool was removed, pooled together, aliquoted into 50\mu l aliquots, and stored at -80°C for future assays.
Figure 3.2: An illustration of Peptide Pools I-VI. Peptide Pools I-VI incorporate amino acids 1-310 of the extracellular domain of HER2/neu. Peptides 1-19 (Blue) represent the corresponding Domain I (also blue) of the models of the ECD of HER2 on the right. Domain I (and III) would be the ligand binding region in other HER family members, but HER2 has no known ligands. Peptides 20-30 (green, and continuing to Peptide 31 on Fig. 3.3) correspond to Domain II (also green) in the models of HER2 on the right. Domain II (and IV) are responsible for dimerization.
Figure 3.3: Part II of II: An illustration of Peptide Pools VII-XII. Peptide Pools VII-XII incorporate amino acids 300-640 of the extracellular domain of HER2/neu. Peptides 32-48 (Yellow) represent the corresponding Domain III (also yellow) of the models of the ECD of HER2 on the right. Domain III (and I) would be the ligand binding region in other HER family members, but HER2 has no known ligands. Peptides 49-62 (red) correspond to Domain IV (also red) in the models of HER2 on the right. Domain IV (and II) are responsible for dimerization.
Cell Line

The SKBR3 cell line was isolated by the Memorial Sloan-Kettering Cancer Center in 1970. It was derived from a pleural effusion of an adenocarcinoma originating from the breast of a 43 year old, Caucasian female. The HER2 overexpressing SKBR3 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultivated in McCoy’s 5A media supplemented with 10% FBS and 1% penicillin/streptomycin (Biowittaker) and incubated at 37°C and 5% CO₂.

ELISA for Pooled Sera Recognition of Peptide

An ELISA was performed so that each pool was tested for recognition of its vaccinating peptide plus on peptide out of its pool for a control. For example, Pool I was tested for recognition of Pepides 1-5 and Peptide 53 for control. The other 11 pools were tested in a similar manner. EIA/RIA plates were coated with 50ul of a 5ug/ml peptide solution in bicarbonate buffer for 2 hours. The plates were then washed three times and blocked with 1% BSA for 1 hour. The plates were then washed three times before the addition of sera was added at dilutions of 1:50, 1:100, 1:200, and 1:500. The sera were allowed to incubate on the plate for 2 hours at room temperature. The plates were then washed three times, and 50µl per well of a 1:1000 goat anti-mouse HRP-conjugated secondary antibody (Santa Cruz) was added. The secondary antibody was allowed to incubate for 1 hour at room temperature. The plates were then washed
three times and the bound antibodies were detected with the addition of 50µl of TMB peroxidase developer solution (Kirkegaard and Perry Laboratories, Gaithersburg MD). The reaction was stopped with 25µl of HCL and the resulting optical densities were read at 450nm.

**ELISA for Recognition of the Recombinant Extracellular Domain of HER2**

An ELISA was performed to detect the ability of sera to recognize a recombinant human HER-2 extracellular domain (Speed Biosystems). EIA/RIA plates were coated with 50µl /well of 5ug/ml recombinant HER-2 ECD diluted in bicarbonate buffer and incubated overnight at 4°C. The plates were then washed 3 times and coated with 1% BSA in PBS for 1 hour at room temperature. The plates were then washed three times and 50µl of sera (diluted 1:200) was added to the plates and incubated for 2 hours at room temperature. The plates were washed 3 times and anti-mouse IgG conjugated to HRP (Santa Cruz) was added and allowed to incubate for 1 hour at room temperature. The plates were washed 3 times and TMB (Kirkegaard and Perry Laboratories, Gaithersburg MD) was used to detect the presence of bound antibody. The reaction was stopped with 25µl of 1N HCL and the optical density was read at 450nm.

**Immunocytochemistry (ICC) for Recognition of Native HER2**

In order to determine binding of the anitsera via immunocytochemistry, fluorescent microscopy, the HER-2 overexpressing breast cancer cell line SKBR3 was
employed. Cells were plated on fibronectin-coated coverslips and incubated in 6-well plates for 24 hours. The cover slips were then removed from the 6-well plates and bound cells fixed with equal parts of ice cold acetone and methanol. The cover slips were then incubated at -20°C for 20 minutes, blocked with 1% BSA for 30 minutes at room temperature, washed two times with PBS and incubated with 20ul of sera for 1 hour at room temperature. The cells were then washed 2 times with PBS and incubated in the dark with FITC-conjugated anti-mouse IgG, (Sigma Aldrich, St Louis, MO) for 1 hour at room temperature. After the one hour incubation period the cells were washed 2 times with PBS, and the cover slips were mounted to slides with VectaShield mounting medium (Vector Laboratories) and sealed with clear fingernail polish. The cells were visualized using an Olympus IX70 inverted microscope. The FITC fluorochrome was exited at 494mn which then emitted fluorescence at 518nm for visualization.

Flow Cytometry for the Recognition of Native HER2

The ability of sera to bind to native HER-2 protein was assessed by flow cytometry using HER-2 overexpressing SKBR3 breast cancer cells. In order to assess binding, 1X10^6 SKBR3 cells were placed into a 5ml polystyrene round bottom tube, washed once with FACS buffer (PBS containing 5%FBS and 5% sodium azide) and incubated with 20µl of sera in 20µl of FACS buffer for 30 minutes at 4°C. The cells were then washed with FACS buffer two times to remove all unbound antisera. The cells were then incubated with 25µl of 1:25 anti-mouse IgG conjugated to FITC (Sigma Aldrich) diluted in FACS buffer. The secondary antibody was allowed to incubate in the dark for
20 min at 4°C. The cells were then washed two times with FACS buffer and spun down at 1000 g’s for 10 min. The supernatant was removed and the cells were resuspended in 500µl of FACS buffer and visualized via FACS using a blue excitation laser of 488nm. Emission was read at approximately 520nm.

**Western Blot Analysis for the Recognition of Native HER2**

SKBR3 cell lysate provided a target protein for sera to bind in western blot analyses. SKBR3 cells were grown to confluency in a T75 flask. The cells were washed two times with cold PBS and lysed with RIPA buffer containing protease inhibitors (complete Protease Inhibitor Cocktail, Roche). A SDS-Page gel was used in the separation of 30µg of total protein from SKBR3 cell lysate via electrophoresis. The gel was then transferred to a PVDF membrane using 100V for 1 hour duration. The membrane was stained with Ponseau S and the lanes were cut so that each lane could be exposed to a different sera pool. The membrane strips were then blocked with skim milk for one hour and before being exposed to sera from Pools I-XII. The sera was diluted 1:100 in skim milk and the membranes were incubated overnight at 4°C. The membranes were then washed three times in TBS-T and an anti-mouse IgG-HRP conjugated antibody (Santa Cruz) was added at a dilution of 1:10,000 in skim milk. The membranes were incubated with the secondary for one hour at room temperature, washed three times in TBS-T, and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).
Results

Recognition of Peptides

The first ELISA performed on the sera from Balb/c mice was to determine the immunogenicity of each of the 62 peptides. Sera pools were tested via ELISA to see if they could recognize their corresponding vaccinating peptides. Analysis revealed that some HER-2 peptides were highly immunogenic in Balb/c mice while other peptides were completely non-immunogenic. No sera pools showed appreciable binding to the control peptide, suggesting that the results of the ELISA are valid and not confounded by background or cross-reactivity issues. We therefore defined positive peptide reactivity to be defined by optical densities greater than the control peptide with the highest optical density. Pools I, IV, V, VII, VIII, IX, X, XI, and XII each showed recognition of one or more peptides, while Pools II, III, and VI showed no measurable reactivity with any of the vaccinating peptides. Pool XI showed the greatest amount of recognition with all 5 peptides being recognized. Pools IV, V, and X were able to recognize 3 peptide within their pool, Pools VII, IX, and XII recognized 2 peptides, and Pools I and VIII recognized on peptide from their pool. (Table 3.1) Not all positive peptides were equally potent at inducing antibodies, as reflected in differences in optical densities in ELISAs. (Fig. 3.4)

Because the ability to immunologically recognize peptides is genetically determined by Major Histocompatibility Complex (MHC) alleles, we also immunized MHC-disparate C57Bl/6 (H-2b) mice to compare peptide responses to Balb/c (H-2d). This
strategy maximizes our ability to identify all possible B cell epitopes on HER-2. The results from vaccinated C57bl/6 mice are shown in Figure 3.5. The two strains are directly compared in Figure 3.6, where it can be seen that the differences in genetics reveals additional immunogenic peptides. For example, Balb/c recognize 38, 48 and 51, while C57bl/6 does not, while on the other hand, peptides 26, 27 and 59 are recognized by C57bl/6 but not Balb/c. (Table 3.1) Positive sera was identified by an optical density over 0.2.

Recognition of Recombinant and Native HER2

ELISA analysis for the recognition of a recombinant HER2 ECD showed that although some pools of sera from vaccinated Balb/c mice were able to recognize their respective vaccinating peptides, they were not necessarily able to recognize the recombinant HER2 ECD. We only counted 4 of the 12 sera pools (Pool IV, VII, XI, and XII) as positive for being able to recognize the ECD of HER2, whereas 9 of the 12 sera pools

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Table 3.1 Summary of Balb/c and C57bl/6 mouse sera Recognition of Vaccinating Peptides. A table was composed to compare and contrast sera from the Balb/c mice with H-2d haplotype to that of the C57bl/6 mice with H-2b haplotype. Similarities and differences can be seen between the two groups.
Figure 3.4: Recognition of Vaccinating Peptides by Sera Pools I-XII of Balb/c Mice (Haplotype 2-Hd). An ELISA was performed with the original vaccinating peptide bound to the plate as capture antigen. The sera obtained from the vaccinated Balb/c mice was then used to detect the presence of Ab’s to the bound peptides. An anti-mouse whole IgG secondary, conjugated to HRP, was used to detect the presence of Ab’s capable of recognizing peptides of the ECD of HER2/neu. TMB was used to develop the ELISA resulting in changes in optical density + SEM. Sera was considered positive for peptide recognition if it had an optical density greater than the highest negative control peptide. This is illustrated on the graph by a line at approximately .132 on the y-axis.
Figure 3.5: Recognition of Vaccinating Peptide Sera Pool I-XII from C57 Mice (Haplotype H-2b) An ELISA was performed with the original vaccinating peptides bound to the plate as capture antigen. The sera obtained from the vaccinated C57bl/6 mice was then used to detect the presence of Ab’s to the bound peptides. An ant-mouse whole IgG secondary, conjugated to HRP, was used to detect the presence and quantity of specific Ab’s capable of recognizing peptides of the ECD of HER2/neu peptide. TMB was used to develop the ELISA resulting in changes in optical density ± SEM. Sera was considered positive for peptide recognition if it had and optica density higher than .2 on the y-axis. This is represented by a line on the graph at the .2 y-axis.
Figure 3.6: Comparison of Peptide Recognition by Balb/c and C57 Sera. The graph shows differences in sera recognition of vaccinating peptides between the haplotype H-2d Balb/c mice and the haplotype H-2b C57bl/6 mice.
were able to recognize at least one of their respective vaccinating peptides. (Fig. 3.7) Sera was considered positive if it had an optical density 10x’s that of the control sera.

Immunocytochemistry was used to assess the ability of the sera pools from vaccinated Balb/c mice to bind to HER2 overexpressing SKBR3 cells. The microscopy images revealed that certain pools were capable of binding to the SKBR3 cells and others were not. (Fig. 3.8) The microscopy images were analyzed using Image J. The averaged background was subtracted from the fluorescence of 10 selected cells and averaged to obtain the corrected total cell fluorescence. The corrected total cell fluorescence was then used to create a chart for comparison. (Fig. 3.9) We considered sera to be positive if it the corrected mean fluorescence of the ten cells was greater than 10x’s the corrected mean fluorescence of the control sera. Analysis revealed that 5 of the 12 sera pools (Pools VI, V, X, XI, and XII) were capable of binding to native HER2 on SKBR3 cells as assessed by ICC. A line was drawn on the graph to represent the division between positive and negative assignments of sera.

Flow cytometry helped to confirm ICC results. As in ICC, sera from Balb/c mice vaccinated with peptide pools were analyzed using HER2 overexpressing SKBR3 cells as targets. (Fig. 3.9) Again there was variation among the pools of sera that made assigning a value of positive or negative somewhat subjective. A graphical representation of the flow cytometry data illustrates the variation and clearly shows superior binding of Pools IV, V, XI, and XII, with respectable binding from Pools VII and X, and minimal binding
Figure 3.7: Vaccinated Pools Show Recognition of the ECD of HER2. An ELISA using bound synthetic HER2 ECD was used to test Balb/c sera from Pools I-XII for ability to recognize HER2 protein in its more native conformation. Pools showing recognition that was 10x’s greater than the negative control were considered positive. This is represented by a line on the graph at .06 on the y-axis.
Figure 3.8: Immunocytochemistry Showing Binding Capability of Pools XII and XI Compared to Control Sera and Secondary Only to HER2 Overexpressing SKBR3 Cells. Immunocytochemistry was performed with the sera from Pools I-XII against SKBR3 cells, the above shows Pools XI, XII, control sera, and secondary only. A. The binding of Pool XI to SKBR3 cells. B. The binding of Pool XII to SKBR3 cells. C. The binding of sera from a mouse vaccinated with Freund’s Complete Adjuvant only. This was used as a negative control. D. The binding of anti-mouse whole IgG only.
Figure 3.9: Mean Total Fluorescence of Pools I-XII of Balb/c sera Shown in a Graphical Representation. Mean fluorescence was obtained by averaging the background and subtracting if from the fluorescence of 10 cells. The each adjusted cell fluorescence was then averaged for each pool. Pools considered positive showed a mean fluorescence 10x’s greater that the negative control sera. This is represented by the line drawn at .00004 on the y-axis.
from Pools II and VI. **(Fig. 3.10)** Again a line was drawn on the graph to depict the level at which each sera was assigned a positive or negative value.

Western blot analysis was used to further confirm that the binding seen in ICC and Flow cytometry was to that of HER2. Western blot analysis used HER2 overexpressing SKBR3 cell lysate as a target for sera from Balb/c mice vaccinate with peptide pools. Western blot analysis revealed weight appropriate bands (187 kD) in the lanes probed with sera form Pools X, XI, and XII. **(Fig. 3.11)**

**Discussion**

Our goal in these combined experiments was to find short linear peptides that contained T cell helper epitopes and could induce a humoral response capable of recognizing HER2 in its native conformation. Because high titer IgG requires T cell help, an anti-peptide ELISA also simultaneously identified peptides containing Helper T cell epitopes.

We were able to identify 22 short linear peptides that were immunogenic (contained a T cell helper epitope) in Balb/c mice and 29 in C57bl/6. Seven of the peptides recognized by Balb/c mice were unique to the H-2d haplotype, whereas 14 of the peptides recognized by C57bl/6 mice were unique to the H-2b haplotype. However,
Figure 3.10: Sera from Peptide Pools Show Binding to HER2 Overexpressing SKBR3 Cells via FACS. SKBR3 cells were used to test the recognition of HER2 by sera from Pools I-XII of Balb/c mice via BD FACs Aria. Results are expressed graphically as the number of positive events for each sera minus the number positive events seen with the control sera. Pools considered positive had approximately twice as many positive events as the control sera. This is represented by a line at approximately 700 events on the y-axis.
Figure 3.11: Western Blot Analysis with HER2 Overexpressing SKBR3 Cells Shows Binding of Sera Pools from Balb/c Mice. Western Blot Analysis with HER2 overexpressing SKBR3 cells reveals bands in lanes 10, 11, and 12 at 187 kDa which is consistent with the weight of HER2. Pools X, XI, and XII also show a band at approximately 100 kDa which may represent the cleaved ECD (p100).
due to secondary and tertiary folding of native proteins, recognition of the vaccinating peptides by the corresponding antisera could not guarantee that the antisera would recognize the HER-2 protein in its native conformation. Recognition of native protein on the tumor cell is essential for the antibodies to have anti-tumor activity. Therefore in order to identify sera, and consequently peptides, with the potential for generating anti-tumor antibodies, it was essential to rigorously screen for recognition of either the extracellular domain, or full-length HER-2 protein. In order to accomplish this with confidence, we employed several complementary techniques including ELISA, flow cytometry, western blot analysis, and immunofluorescence. According to the results of each technique utilized, we assigned a positive or negative value to each of Pools I-XII so that we could determine which immunogen scored positively in each category, and thus assign a rank-order for each pool/peptide that would guide selection and focus for follow-on biological studies. Although complementary, each technique had advantages and drawbacks that could provide us with unique information. Together the resulting data allowed for us to focus on certain peptides for biological assessment.

The advantage of using the recombinant protein in an ELISA system is that the ECD of HER-2 is the only target present for the antisera to bind. This reduces the chance that the antisera could be recognizing something else present on the cell (for example, other HER-family proteins which share some sequence homology with HER-2). However, potential drawbacks exist from binding a protein onto a plate surface. The conformation of the protein (HER2 ECD) can be altered by the binding making it either more or less accessible to antibodies. Additionally, the orientation in which the protein binds may
make certain epitopes unavailable for antibody binding. The pools capable of recognizing the recombinant HER-2 ECD were IV, VII, XI, XII. Pool XI showed the greatest recognition followed by IV, VII, and XII respectively. Interestingly, this appeared to correlate with the number of peptides recognized within each pool.

Immunofluorescent microscopy has the advantage of allowing us to determine which pools of sera were able to recognize HER2 in its presumed native conformation. It also allows us to visualize the binding so that we know that the antibodies are binding to the appropriate location on the cell, which is the surface of the cell in this case. However, possible drawbacks to this technique exist because the cells are fixed. Fixing cells crosslinks proteins which can make binding of antibody to its target protein difficult. In addition the treatment with acetone and methanol can denature the proteins altering the presumed native conformation. By ICC all pools showed greater fluorescence than the negative control sera, which was obtained from balb/c mice vaccinated with adjuvant but no HER-2 peptides. However, we assigned sera that showed fluorescence 10x’s greater than the control sera to a positive category. By this criterion, antisera against pools IV, V, X, XI, and XII were positive for native HER2 recognition on the HER2 overexpressing cell line SKBR3.

Flow cytometry was used to further confirm binding to native HER2 on SKBR3 cells. Flow cytometry provides the benefit of not having to fix the cells as in ICC and it can measure the fluorescence in numerous cells in a short period of time (for our experiments 10,000 cells). However, the cells are not viewable as they are in ICC so it is
hard to assure that the antibodies are binding to the proper location; hence, enhancing the likelihood of the proper target protein. **Figure 3.10** provides a summary of each pool’s ability to bind to SKBR3 cells as viewed by FACS. Pools II, IV, V, VI, VII, X, XI, and XII showed an increase in the number of fluorescing cells over control serum, with Pool XII showing the greatest increase. We considered sera to be positive if it was able to show fluorescence in 2x’s as many events as the control sera.

Although protein is not in its native conformation when being utilized as a target in a SDS-PAGE western blot, the analysis shows the molecular weight of the target proteins in which the sera is binding. This did two things; it showed that the target protein was of a molecular weight that appropriate for that of HER-2 and it demonstrated specificity by not showing non-specific bands. **(Figure 3.11)** Sera from Pools X, XI, and XII showed some binding capability. A second band appeared in these same lanes at the 100 kD weight. This could be explained by the cleavage of the ECD which is often referred to as p100.

A table was constructed to summarize serum reactivity against HER-2 protein by the various methodologies utilized. **(Table 3.2)** The table helps to identify Pools XII, XI, IV, and possibly X as good candidates for further assessment due to the high consistency of whole HER-2 recognition across the various assays.
Table 3.2: A Summary of Antisera from Pools I-XII. A table was created to summarize the ability of Pools I-XII of Balb/c mouse sera to recognize either vaccinating peptides, ECD, or native protein via ELISA, Western Blot Analysis, FACS, or ICC. This table helps to identify Pools XI and XII as having the greatest binding, flowed by IV, V, and X.

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With these experiments we intended to identify peptides of interest that we felt would have the potential to induce biological activity. Although all immunogenic peptides capable of binding to the native protein hold potential for biological activity, Pool XII from the Balb/c mice was of particular interest due to it being immunogenic, capable of recognizing the whole protein, and being representative of the region in which the humanized 4D5 monoclonal antibody, trastuzumab, has been shown to bind (Cho, Mason et al. 2003) which would allow for a positive control for biological based assays. In addition, Pool VI from the C57 mouse antisera was determined be a good candidate for future testing for similar reasons to that of Pool XII; it too showed immunogenicity, recognition of whole protein, and it represents a region in which the humanized 2C4 monoclonal antibody, pertuzumab, has been shown to bind (Agus, Gordon et al. 2005). Figures 3.12 and 3.13 illustrate the putative regions on HER-2 where the pool-induced antibodies bind.
Figure 3.12: Binding Regions of Sera from Pools IV, VII, X, XI. This figure illustrates the regions on the HER2 ECD from which the vaccinating peptides were derived. It is assumed that the antibodies elicited from these peptides would bind to a similar region. The dark purple section of the ECD represents Domain I, which is typically a ligand binding region along with Domain III (light aqua) in other HER family members, but HER2 has no known ligand. Pool V binds within Domain I. The light purple region on the HER2 ECD represents Domain II which is responsible for dimerization along with Domain IV (dark aqua). Pool VII peptides represent a region that incorporates Domain II and III. Pool X peptides represent a region within Domain III, and Pool XI peptides reside mainly in Domain IV but span slightly into Domain III.
Figure 3.13: Regions of Interest within the ECD of HER2. This figure illustrates regions of interest within the HER2 protein. Pool XII peptides represent a region within Domain IV that is similar to the epitope in which trastuzumab (Herceptin) binds. The site cleaved by metalloproteinases and ADAMs is near in proximity to the location in which Pool XII and trastuzumab bind. One of trastuzumab’s proposed mechanisms of action is the protection from cleavage by proteases. Pool XII peptides may be capable of providing similar protection.
Chapter 4

Characterization of the In Vitro Biological Activity of anti-sera from Mice Vaccinated with HER2 library Peptide 61

Traditionally cancers are treated with surgery, radiation, or chemotherapy. Avoiding destruction of healthy tissue is impossible with these treatments. Immunotherapy offers a tissue-sparing alternative that constitutes less threat of damage and disfigurement to the patient. The challenge persists in finding ways to generate robust immune responses sufficient to overcome immune tolerance to “self” tissues, and finding appropriate targets against which do direct immunity.

The use of trastuzumab, an anti-HER-2 monoclonal antibody-based drug, as an immunotherapy agent has helped to shed light on the complexities involved in treating cancers with mAbs. With development beginning in 1987, trastuzumab has been used as a passive immunotherapy for approximately 20 years. Trastuzumab, which was originally FDA approved to treat HER2 positive metastatic breast malignancies, has evolved clinically to treat nearly all stages of HER2 positive breast cancer (Hudis 2007, Moja, Tagliabue et al. 2012). It has shown success when used alone, in conjunction with chemotherapy, and as a vector for the delivery of chemotoxic drugs to HER-2 positive...
cells (Baselga 2000). However, debates regarding trastuzumab’s mechanism of action have been ongoing since its initial discovery as a mouse mAb (4D5). (Hudziak, Lewis et al. 1989, Fendly, Winget et al. 1990) Countless numbers of researchers have attempted to pinpoint exactly how it works and how in order to better identify patient cohorts that would benefit from treatment with the drug.

It was originally thought that trastuzumab could down regulate cell surface expression of HER2 leading to a decrease in subsequent signaling. (Hudziak, Schlessinger et al. 1987) However, more recent data suggests this is not the case, and that there is no detectable change in cell surface HER2 expression (Austin, De Maziere et al. 2004, Hommelgaard, Lerdrup et al. 2004). Moreover, it has been suggested that HER2 cell surface expression may be protected from cleavage by metalloproteinases or ADAMs (Molina, Codony-Servat et al. 2001). The phosphorylation of HER2 has also been questioned, with some reporting a down regulation in pHER2 (Lane, Beuvink et al. 2000, Nagata, Lan et al. 2004(Kumar, Shepard et al. 1991)) and others seeing no change in phosphorylation (Junttila, Akita et al. 2009). Relative consistency has been reported in the overall down-regulation in the phosphorylation of Akt (Junttila, Akita et al. 2009, Lane, Beuvink et al. 2000, Yakes, Chinratanalab et al. 2002). However, Yakes et al. found the kinetics of pAkt to have an initial transient up regulation before being slowly down regulated. Junttila et al. did not see the transient up regulation, but rather an immediate decrease significant within the first 15 minutes. The phosphorylation of Akt correlates with data regarding the phosphorylation of HER3, which is known to phosphorylate Akt.
Knowing that Herceptin binds to region IV of HER2 and that Herceptin subsequently down regulates the phosphorylation of both HER3 and Akt, it was suggested that the mechanism of action of trastuzumab was to disrupt the heterodimerization of HER2 and HER3. (Junttila, Akita et al. 2009, Lee-Hoeflich, Crocker et al. 2008)

Another, more recently-developed therapeutic mAb (pertuzumab) that binds to region II of HER2 (Franklin, Carey et al. 2004) was able to shed further light on the dynamics of the HER2-HER3-trastuzumab complex. It was determined that pertuzumab was superior to trastuzumab in down regulating pAkt phosphorylation when ligand was present. However, in the absence of ligand, trastuzumab appeared to be superior (Agus, Akita et al. 2002). In addition, Junttta et al. was able to show the presence of ligand-independent heterodimerization of HER2 and HER3 when HER2 was overexpressed, resulting in a functionally different heterodimer than the canonical ligand dependent HER2-HER3 dimer. (Junttila, Akita et al. 2009)

Through these intensive studies focused on trastuzumab, and to a lesser extent pertuzumab, much has been learned about the potential biological activity HER2-directed antibodies. Trastuzumab, a passive immunotherapy agent, differs from active (e.g. vaccine) immunotherapy in many ways. Passive immunotherapy supplies immune system components (in this case antibodies) generated outside the body, but does not activate the immune system of the treated subject in a manner that would require their
immune system to produce any antibodies on its own. Passive immunotherapy has benefits since it can employ monoclonal antibodies that have been highly engineered for IgG class and with point mutations to artificially increase target binding. However, passive immunotherapy, since it does not elicit the patient’s own immune system, lacks memory and therefore lasting protection against future metastases. A course of Herceptin can also cost over $70,000.

In summary, trastuzumab is thought to exert its anti-tumor activity in four ways:

1) Blocking Dimerization with other HER family members thereby blocking subsequent cell signaling (Junttila, Akita et al. 2009)

2) Blocking cleavage by ADAMS or metalloproteinases resulting in a constitutively active tyrosine kinase portion (Molina, Codony-Servat et al. 2001)

3) Having the Fc portion of the Ab bind to Fc Receptors (FcRγ) on effector cells and (Mellor, Brown et al. 2013)

4) Opsonizing the tumor cell for identification and destruction by macrophage (Mamidi, Cinci et al. 2013)

Since the peptides forming Pool XII in our library subtend the region in which trastuzumab is known to bind, and Peptide 61 is the most immunogenic peptide within Pool XII (Fig. 4.1), we hypothesized that sera from mice vaccinated with P61 would display biological activity similarly to trastuzumab. It should be noted that our expectations were not that our antiserum would work better than trastuzumab since
Fig. 4.1: Immunogenic Peptides in Pool XII. Pool XII was vaccinated with Peptide 57-62 of the peptide library spanning the ECD of HER2. Pool XII peptides are from a region within the ECD that is similar to the epitope of trastuzumab, and was therefore selected for testing biological activity. Our goal was to vaccinate with single peptides, so because Peptide 61 appears to be the most immunogenic peptide within Pool XII, we chose Peptide 61 as the vaccinating peptide for biological testing.
this monoclonal Ab has been rigorously engineered for superior target binding, but rather we hypothesized that antisera capable of recognizing P61 should have similar qualitative properties to trastuzumab in contrast to non-immune control sera.

Materials and Methods

Cell lines

The SKBR3 cell line was isolated by the Memorial Sloan-Kettering Cancer Center in 1970. It was derived from a pleural effusion of a adenocarcinoma originating from the breast of a 43 year old, Caucasian female. This HER2 overexpressing SKBR3 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultivated in McCoy’s 5A media supplemented with 10% FBS and 1% of 100x penicillin/streptomycin mixture (Biowittaker) and incubated at 37°C and 5% CO₂.

Vaccinations and Sera Collections

Balb/c mice (5 per group) were vaccinated with 25µg of Peptide 61 in a 100µl emulsion of Freund’s Complete Adjuvant placed subcutaneously in each flank. Every two weeks mice were similarly boosted with 12.5 µg of peptide in Incomplete Freund’s Adjuvant. Blood samples were taken via submandibular bleeding the day prior to each boost. Mice were boosted a maximum of 4 times prior to sacrifice. Collected blood samples were allowed to coagulate at 4°C for 1 hour then spun down at 4600 g’s for 20
minutes. The sera were removed, apportioned into 50µl aliquots to avoid loss of activity through multiple freeze-thaw cycles, and stored at -80°C for later analysis.

Sera IgG Column Purification

IgG antibodies were purified from mouse sera with protein AG agarose spin columns (Thermo Scientific) via manufactures recommendations. Sera was added to columns, allowed to bind for 10 minutes, washed 3 times and eluted in 3 cycles. The three elution fractions were tested via ELISA to identify the fraction with the greatest ability to bind to the ECD of HER2, and only these high-activity fractions retained for further analysis.

Alamar Blue

HER-2 overexpressing SKBR3 cells were plated 96-well plates with 1 X 10^5 cells/well in 100µl of McCoy's media supplemented with 10% FBS and allowed to incubate overnight. The next day the cells were treated with 5% IgG purified anti-P61, 5% IgG purified control sera, or 40 µg/ml of trastuzumab. Four days after the addition of antibodies, 20µl of .15mg/ml of resazurin in PBS, was added to the culture. The cells were incubated for 4 hours allowing for the resazurin to be reduced to resorufin via NADH flux in the mitochondria, thereby indicating metabolic activity. After a 4 hour
incubation period the optical density could be read at 540nm via the Bioteck ELx800 absorbance microplate reader.

Annexin V and Propidium Iodide for Apoptosis

SKBR3 cells were grown in 6-well dishes and treated with either 5% P61 sera, 40 µg/ml of Herceptin, or 5% negative control sera. Three of the wells were harvested after 24 hours and the remaining three were harvested after 48 hours. After harvesting, the cells were washed 2 times in cold PBS, resuspended in binding buffer at a concentration of 1 x 10^6, and 100 µl of cell solution (1 x 10^5 cells) was transferred to a 15 ml falcon tube. Annexin V (5 µl, from BD Biosciences) and PI (2 µl from BD Biosciences) were added to each falcon tube and allowed to incubate at RT in the absence of light. After the 15 minutes of incubation, 400 µl of binding buffer was added to the tubes and then the cells were analyzed via FACS for binding.

Western Blot Analysis

SKBR3 cells were grown in 6-well plates in the presence or absence of 1nM of heregulin-1β (Sigma Aldrich) until approximately 80% confluent. At this point the cells were treated with 5% P61 anti-sera, 5% control sera or 40µg/ml of trastuzumab. The cells were allowed to incubate for an additional 30 minutes in the presence of their respective treatments and then harvested with 100µl of RIPA buffer supplemented with
Halt protease inhibitor cocktail (Thermo Scientific) and PhosStop phosphatase inhibitors cocktail (Roche). The lysates were centrifuged for 20 minutes at 13,000 g’s. The resulting supernatants were assayed for total protein concentration, via Bio-Rad DC Protein Assay, so that 30µg of total protein could be loaded into a 4-15% SDS-page gel (Bio-Rad). The gel was then transferred onto a PVDF membrane at 100V for 1 hour. The membrane was stained with ponceau for confirmation of protein transfer and blocked for 1 hour in 1% BSA. The membrane bound proteins were exposed to Rabbit anti-phospho-HER2 (Cell Signaling Technology), Rabbit anti-phospho-HER-3 (Cell Signaling Technology), Mouse anti-phospho-ERK1/2 (Cell Signaling Technology), or Rabbit anti-phospho-Akt (Cell Signaling Technology). The membrane was allowed to incubate overnight at 4°C with its respective primary antibody. The next day the membrane was washed three times in TBS-T and incubated in 1:10,000 anti-rabbit or anti-mouse IgG conjugated to HRP (Santa Cruz) for 1 hour at room temperature. The membrane was washed again three times in TBS-T and visualized with pico SuperSignal chemiluminescents from Pierce. The membrane was developed digitally with ImageQuant LAS 4000.

Statistical Analysis

Analysis of Variance (ANOVA) was done to compare anti-P61 sera to control sera in the ability to regulate the phosphorylation of HER-2, HER-3, Akt, and ERK1/2. A
separate ANOVA was done to compare trastuzumab to control sera. Results were considered to be significant at a $p$ value of 0.05.

**Results**

**Alamar Blue for Reduction in Metabolism**

Initial experiments tested the capacity of P61 antisera to inhibit metabolic activity of the HER2 over-expressing SKBR3 breast cancer cell line. Because this assay has a duration of several days, we took the unusual step of immunoaffinity purifying immune and control sera to obtain highly enriched IgG. By so doing, we were removing endogenous growth factors that might vary in concentration from one preparation of raw serum to another and thereby ensuring that any changes in metabolic activity of SKBR3 cells resulted only from antibody activity. This assay utilizes a dye that is chemically altered by cellular metabolic activity, and in the process changes spectral absorbance properties. These changes can be read spectrophotometrically. In these experiments, anti-P61 IgG was able to significantly reduce metabolism ($p<.001$) compared to IgG purified control sera, but trastuzumab proved superior to both anti-P61 IgG and IgG purified control sera ($p<.001$). (Fig. 4.2) Therefore, p61 antisera displayed suppressive properties similar to that of trastuzumab.
**Figure 4.2: Alamar Blue Assays Show a Downregulation in Metabolism of SKBR3 Cells Rx with Anti-P61 IgG.** Alamar Blue assays showed a significant metabolic down-regulation in SKBR-3 cells treated with purified anti-P61 sera and trastuzumab when compared to purified control sera as shown by optical density percent of control sera ± SEM. There was a significant difference between anti-P61 sera and control sera $F(1,33)=47.13, p<0.0001$ and between trastuzumab and control sera $F(1,30)=17.91, p<0.0001$. 
Annexin V and Propidium Iodide Staining to assess Apoptosis

Alamar Blue staining showed that both Trastuzumab and p61 antisera suppressed metabolic activity in SKBR3 cells, but it does not indicate whether loss of metabolic activity results from actual target cell death. We therefore sought to determine whether incubation with p61 antisera induced the external membrane expression of phosphatidylserine, an early apoptotic marker detected via Annexin V staining, as well as the capacity to take up propidium iodide (PI) stain, a late apoptotic marker. In SKBR3 cells incubated for 24 hours with the respective antibodies, we observed a significant (p<.05) difference between p61 and control serum, as well as between trastuzumab and control serum, in the number of SKBR3 cells displaying the Annexin V$^{\text{pos}}$/PI$^{\text{neg}}$ early apoptotic phenotype. When we observed cells at 48 hours post-incubation, we likewise observed a significant increase (p<.05) of cells displaying the Annexin V$^{\text{neg}}$/PI$^{\text{pos}}$ late apoptotic phenotype in the p61 sera-treated SKBR3 cells compared with the control serum group. Trastuzumab showed an increase in PI staining at the 48 hour mark, but it was not significant. These studies show that p61 antisera is capable of inducing apoptosis in HER-2-overexpressing SKBR3 cells and suggests that the suppression of metabolic activity seen in Alamar Blue assays is at least in part due to actual cell death. (Fig. 4.3)
Figure 4.3: Annexin V and Propidium Iodide Show Induced Apoptosis in SKBR3 Cells Treated with P61 Sera and trastuzumab (Herceptin). Annexin V was used to stain phosphatidylserines that flip from the unexposed inner leaflet to the exposed outer leaflet during the early stages of apoptosis. Annexin V was able to show significant binding (compared to control) to SKBR3 cells treated with P61 sera $F(1,5)=16.58$, $p=.01$ and trastuzumab $F(1,2)=31.22$, $p=.031$ after a 24 hour incubation period. Propidium Iodide (PI) intercalates with DNA and therefore stains cells that have lost membrane integrity which can happen in late stage apoptosis. SKBR3 cells treated with P61 sera showed a significant $F(1,4)=8.677$, $p=.042$ increase in PI staining (compared to control) after 48 hours of incubation. Trastuzumab (Herceptin) was also able to show an increase in PI staining over control sera, but it was not significant at the 48 hour mark.
P61 antisera inhibits HER2 signaling pathways

Having demonstrated that p61 antisera is capable of suppressing, over the course of several days, metabolic activity in treated SKBR3 cells, and that some portion of this suppression is likely through induction of apoptotic cell death, we next investigated the earliest biochemical events in HER2 signaling, to determine whether p61 antisera was capable of altering the activation of these pathways in a manner similar to Trastuzumab.

In these short-term assays, cultured SKBR3 cells were incubated with non-immune negative control mouse serum (ctl), P61 antiserum (P61) or trastuzumab for 30 minutes at 37°C in the presence or absence of ligand (heregulin), as indicated. After incubation, cells were harvested, lysed with 100µl RIPA buffer supplemented with HALT protease inhibitor and PhosStop phosphatase inhibitor, and 30µg of protein was separated by SDS-PAGE and then electro-transferred onto PVDF membranes. Blots were then probed with various antibodies to determine phosphorylation status of various HER-2 signaling pathway components. Western blot analysis revealed a statistically-significant (p<.001) suppression of HER-2 phosphorylation at tyrosine residue 1248 in the presence of either trastuzumab or P61 antiserum, compared with control serum. (Figure 4.4) When HER-3 phosphorylation status at tyrosine residue 1289 was examined, a similar picture emerged when heregulin was present. Compared with control antiserum, statistically-significant suppression of phosphorylation of HER3 was observed with trastuzumab
**Figure 4.4: P61 Anti-Sera Down-regulates HER2 Phosphorylation.** Western blot analysis shows that P61 sera is able to significantly $F(1,16)=43.229$, $p<0.0001$ downregulate HER2 phosphorylation (pHER2) as is trastuzumab $F(1,16)=64.196$, $p<0.0001$ after 30 minutes of incubation when compared to control sera. Western blot analysis of protein extracts from HER-2-overexpressing SKBR3 cells incubated for 30 minutes with negative control sera (dilution), P61 antisera (dilution) or trastuzumab (concentration here). Protein (30µg) was separated on 4-15% SDS-PAGE gradient gels, electro-transferred onto PVDF membranes and probed with antisera specific for HER-2 p1249. Specific bands were visualized with an enhanced chemiluminescent substrate and analyzed on ImageQuant LAS 4000. The pHER-2 bands were normalized to GAPDH and expressed as a percent of negative control + SEM. Left panel: Summary of 3 separate experiments. Right panel: Representative blot.
(p<.05) as well as with p61 antisera (p<.001) while in the presence of heregulin. **(Figure 4.5)** Therefore, with the initial events of HER-2 signaling, trastuzumab and P61 sera display similar phosphorylation-inhibiting activities.

We next turned our attention to events downstream from receptor phosphorylation, starting with the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) family of enzymes. When analyzing phosphorylation status of MAPK/ERK in mammalian cancer cells, it is customary to simultaneously observe ERK1 and ERK2. (Vantaggiato, Formentini et al. 2006) Results showed a more intense band at the 44 kD position (ERK1) than at the 42 kD position (ERK2). Bands were analyzed by summing the density for the two bands together for total ERK 1/2 phosphorylation. We found that total ERK 1/2 phosphorylation was significantly down-regulated by anti P61 sera (p<.05) and trastuzumab (p<.01) compared to control. **(Fig. 4.6)**

Finally, we turned our attention to Akt. Surprisingly, we found radically different activities of P61 antisera depending upon whether or not recombinant heregulin-β1 (a ligand for the HER-3 major heterodimerization partner of HER-2) was added. In the presence of heregulin, both trastuzumab and P61 antisera significantly decreased Akt phosphorylation, compared to negative control serum **(Figure 4.7, upper left panel).** A representative blot is illustrated in the lower left panel. However, when heregulin was present, trastuzumab suppressed Akt phosphorylation in a manner similar to when
Figure 4.5: P61 Anti-Sera is Capable of Downregulating HER3 Phosphorylation.
Western blot analysis shows that P61 sera is able to significantly $F(1,10)=14.637, p=.003$ downregulate HER2 phosphorylation (pHER2) as is trastuzumab $F(1,6)=9.639, p=.021$ after 30 minutes of incubation when compared to control sera. Western blot analysis of protein extracts from HER-2-overexpressing SKBR3 cells incubated for 30 minutes with negative control sera (dilution), P61 antisera (dilution) or trastuzumab (concentration here). Protein (30µg) was separated on 4-15% SDS-PAGE gradient gels, electro-transferred onto PVDF membranes and probed with antisera specific for HER-3 p1289. Specific bands were visualized with an enhanced chemiluminescent substrate and analyzed on ImageQuant LAS 4000. The pHER-3 bands were normalized to GAPDH and expressed as a percent of negative control ± SEM. Left panel: Summary of 3 separate experiments. Right panel: Representative blot.
Figure 4.6: P61 Anti-Sera is Capable of Downregulating HER3 Phosphorylation.
Western blot analysis shows that P61 sera is able to significantly $F(1,10)=6.408, p=.03$ downregulate HER2 phosphorylation (pHER2) as is trastuzumab $F(1,6)=24.136, p=.003$ after 30 minutes of incubation when compared to control sera. Western blot analysis of protein extracts from HER-2-overexpressing SKBR3 cells incubated for 30 minutes with negative control sera (dilution), P61 antisera (dilution) or trastuzumab (concentration here). Protein (30µg) was separated on 4-15% SDS-PAGE gradient gels, electro-transferred onto PVDF membranes and probed with antisera specific for pERK1/2. Specific bands were visualized with an enhanced chemiluminescent substrate and analyzed on ImageQuant LAS 4000. The pERK1/2 bands were normalized to GAPDH and expressed as a percent of negative control ± SEM. Left panel: Summary of 3 separate experiments. Right panel: Representative blot.
Figure 4.7: Western Blot Analysis Shows Phosphorylation of Akt when Treated With P61 Sera.  

A. Western blot analysis shows significant down regulation in the phosphorylation of Akt when SKBR3 cells were treated with P61 sera $F(1,3)=44.348, p=.007$ and trastuzumab $F(1,2)=69.230, p=.014$ in the presence of heregulin for 30 minutes.  

B. Western blot analysis shows down regulation in the phosphorylation of Akt when SKBR3 cells were treated with trastuzumab and increased phosphorylation of Akt when treated with P61 sera in the absence of heregulin for 30 minutes.  

C. Western Blot analysis shows the down regulation of pHER3 and pAkt in SKBR3 cells treated with trastuzumab and P61 sera.  

D. This graph illustrates the change in phosphorylation of Akt in SKBR3 cells when treated in the presence and absence of heregulin. The phosphorylation goes up (indicating loss of efficacy) for both trastuzumab and P61 sera when heregulin is absent, but the loss of efficacy for P61 sera is far greater than that of trastuzumab.
ligand was absent. On the other hand, P61 antisera actually caused a statistically-
significant enhancement of Akt phosphorylation (Figure 4.7 upper right panel). On the
average, Akt phosphorylation in the presence of heregulin was suppressed 68% as
compared to the control, while p61 enhanced Akt phosphorylation by 339% (Figure 4.7
lower right panel).

Discussion

We began our studies by determining whether IgG purified from p61 antisera
could suppress metabolic activity in HER-2-overexpressing SKBR3 breast cancer cell
lines. We found this to be the case, with immune serum demonstrating reductions in
cellular metabolism compared with negative control IgG. As expected, suppression was
not as strong as seen with the engineered monoclonal antibody trastuzumab, but it was
nonetheless significant (p<.001). Suppression of metabolic activity as demonstrated by
the Alamar Blue assay does not, however, provide any insight as to what deleterious
effect, if any, p61 antisera exerts on SKBR3 cells, and does not necessarily imply cell
death. For example, if p61 antisera merely slowed proliferation of SKBR3 cells, then
after several days of incubation with anti-p61 and control IgG, there could simply be
more SKBR3 cells in the control group when Alamar Blue dye was added. A greater
number of cells would more efficiently alter the dye, and account for the differences
between p61 and control groups. On the other hand, p61 antibodies could simply lower
the rate of oxidative metabolism without even affecting cell proliferation rates.

However, since it is our long-term goal to develop immune-based therapy against cancer, and since neither of these two possibilities promise a therapeutically-meaningful impact on disease, we sought direct evidence of cell death induced by anti-p61 antibodies.

To test whether or not the down regulation in metabolism was due to cell death we stained treated cells with Annexin V and propidium iodide (PI). The fluorescently-labeled Annexin V binds to phosphatidylserine that flips from the inner leaflet of the cellular membrane to the outside of the cell during the early stages of apoptosis (Vermes, Haanen et al. 1995, Koopman, Reutelingsperger et al. 1994). PI is a fluorescent compound that intercalates with DNA and therefore stains the nucleic acids. PI, however, cannot cross intact plasma membranes of healthy cells, and can only enter the cell when membrane integrity is lost or cells are dead (Lecoeur 2002). By using both stains it is possible to distinguish healthy intact cells (Annexin V<sub>neg</sub> and PI<sub>neg</sub>), from cells in early stage apoptosis (Annexin V<sub>pos</sub> and PI<sub>neg</sub>), as well as those in late stage apoptosis or are dead (Annexin V<sub>pos</sub> and PI<sub>pos</sub>)(Koopman, Reutelingsperger et al. 1994, Vermes, Haanen et al. 1995). We were able to see a significant difference between SKBR3 cells treated with P61 sera and control sera in that cells treated with P61 sera, as well as trastuzumab, seemed to be more accessible to Annexin V staining within 24 hours and PI staining after 48 hours. This would suggest that P61 sera induces apoptosis in a manner similar to that of trastuzumab.
HER2 phosphorylation is associated with higher tumor grade and shorter disease-free overall survival. (Frogne, Laenkholm et al. 2009) One study showed that among HER2 positive patients, 96% showed active HER2 phosphorylation, but more interesting was the fact that 39% of patients showing strong HER2 phosphorylation were classified as being negative for HER2, but concomitantly expressed HER1 or HER3. (Frogne et al. R11) This would suggest that low levels of HER2 (undetectable by conventional immunohistochemical staining) are enough to establish a potent mitogenic signaling cascade and that the measurement of pHER2 may be a better indicator of an anti-apoptotic, pro-proliferation signal. Comparing pHER2 to total expression of HER2 poses many problems as the regulation of HER2 is intricate in that an apparent increase in presence may be due to the lack of cleavage (Molina, Codony-Servat et al. 2001) therefore showing protection or up regulation of expression, or a down regulation may be due a decrease in expression, endocytosis (Hurwitz, Stancovski et al. 1995) or cleavage (Codony-Servat, Albanell et al. 1999). Typically, fluorescent in situ hybridization (FISH) is performed in addition to immunohistochemistry (IHC) in human biopsies to aid in this dilemma, under the assumption that gene amplification always results in significant HER2 overexpression.

The homodimer of HER2 or the heterdimer of HER2-HER3 or HER2-EGFR are capable of phosphorylating the MAP/ERK pathway. The mitogen-activated protein regulated kinases (MAP), formerly referred to as the extracellular signal-regulated kinases (ERK), provides a pro-proliferation signal by phosphorylating several
transcription factors. There are five known ERK homologs: ERK1, ERK2, ERK5, ERK7, and ERK8. (Abe, Saelzler et al. 2002) ERK1 and ERK2 have an 85% sequence identity (Boulton, Cobb 1991) and function similarly in that they both tend to ultimately trigger transcription providing the cells with proliferation, growth, differentiation and migration signals. (Meloche, Pouyssegur 2007, Bai, Luo et al. 2011) (Fig. 4.8) ERK 1 (a.k.a. MAPK3) is a 44 kD kinase that must be dually phosphorylated on Thr 202 and Tyr 204 for complete activation. (English, Pearson et al. 1999) ERK 2 (a.k.a. MAPK1) is a 42 kD kinase that is also dually phosphorylated but on Thr 185 and Tyr 187. ERK 2 is typically expressed in greater quantity than ERK 1 and therefore will tend to show a thicker band at the 42 kD position, or lower band. (Vantaggiato, Formentini et al. 2006) The MAP/ERK pathway is typically defective in cancer cells allowing for uncontrolled growth and metastases. (Park, Jung et al. 2011) Total ERK1/2 phosphorylation is correlated with shortened disease free survival. (Mueller, Flury et al. 2000)

Since HER2 is known to activate the MAP/ERK pathway, it seemed logical that the down regulated phosphorylation of HER2 may subsequently lead to a down regulation in the phosphorylation of ERK1/2. Western blot analysis was able to show a significant down regulation in the total phosphorylation of ERK1/2, confirming this
Figure 4.8: The MAP/ERK Signaling Pathway Illicited by the Homodimerization of HER2 and the Heterodimerization of HER2 and HER3. This figure illustrates the signaling of ERK1 and ERK2 demonstrating their overlap in signaling and furthermore why both are considered together when assessing growth and proliferation.
hypothesis. Trastuzumab was also capable of down regulating the phosphorylation of upstream HER2 and downstream ERK1/2, again confirming that these immune sera possessed biological properties similar to trastuzumab.

The phosphorylation of Akt is part of the downstream signaling cascade initiated by the phosphorylation of HER3. Assuming that HER3 is ligand-dependent for dimerization, and attempting to look at environmental conditions that could contribute to results we decided to first probe for the phosphorylation of HER3 in the presence or absence of heregulin (one of its principal known ligands). Results showed that anti-P61 sera was able to down regulate pHER3 in the presence of heregulin, a property shared by Herceptin in this study, and consistent with previous reports. However, when heregulin was not added to the culture medium, anti-P61 sera was no longer able to down regulate pHER3, in fact it enhanced it over control sera, and likewise trastuzumab became less effective under these conditions. On average, Herceptin showed a 109% increase and anti-P61 sera showed a 442% increase in the phosphorylation of HER3 when comparing cells with heregulin in the media to cells without.

Being a downstream recipient of the phosphorylation of HER3, it would be logical that the phosphorylation of Akt would follow a similar pattern to that of HER3. Western blot analyses were again performed as previously stated with the primary antibody being rabbit anti-phospho-Akt (Cell Signaling). This was in fact the case, and pAkt was observed to be significantly down-regulated by both anti-P61 sera and Herceptin in
the presence of heregulin. However, in the absence of heregulin the effectiveness of trastuzumab was reduced, and these ligand-free conditions actually made anti-P61 sera increase the phosphorylation of Akt over that of the control sera.

It is possible to conceptualize a model in which pHER3 and pAkt are downregulated by trastuzumab or P61 in the presence of heregulin because pHER and pAkt are both thought to rely on the heterodimerization of HER3 with either HER2 or EGFR. If the dimerization of HER2 and HER3 is hindered by the binding of antibody to HER2, phosphorylation of HER3 and subsequent Akt would be downregulated. However, explaining how the absence of heregulin causes anti-P61 sera to actually enhance the phosphorylation of HER3 and Akt is more difficult. (Fig. 9 & 10) Recent research has shown that when heavily overexpressed, HER2 can heterodimerize with HER3 in a ligand independent manner. It is possible that the binding of anti-P61 sera can change the conformation of HER2 in a manner that allowes for closer contact with HER3 making it easier to dimerize. It is not possible at this time to definitively determine the mechanism behind this somewhat unusual observation.
Figure 4.9: Cell Signaling in HER2 Overexpressing Cells in the Presence and Absence of Heregulin. This figure illustrates the effects of ligand on HER2 and HER3 expressing cells. When heregulin is present, HER2 is able to homodimerize (signaling through the MAP/ERK pathway) and heterodimerize (signaling through the MAP/ERK and Akt pathways). When heregulin is absent, HER2 is still able to homodimerize, but it is no longer able to heterodimerize with HER3, which down regulates Akt phosphorylation and subsequent signaling.
Figure 4.10: Cell Signaling in HER2 Overexpressing Cells Treated with Anti-P61 sera or Trastuzumab in the Presence and Absence of Heregulin. This figure illustrates the effects of ligand on HER2 and HER3 expressing cells. When heregulin is present, Ab’s are able to block HER2 homodimerize and HER2/HER3 heterodimerization and subsequent signaling throught the MAP/ERK and Akt pathways. When heregulin is absent, Ab’s are still able to block HER2 homodimerize, but Akt phosphorylation is enhanced possible to to an altered conformation enabling ligand-independent heterodimerization.
Chapter 5

Conclusions

The strategy currently employed in a clinical trial using Th1-polarizing dendritic cells appears to elicit a clinically significant Th1 response, but is insufficient in deploying a robust humoral response. Comparing patient sera before vaccination to sera after vaccination showed no clear pattern of increase in antibody production. To the contrary, a small but statistically significant overall trend in reduction of IgG1 and IgG4 was observed. It has been hypothesized that activating both arms of the immune system (cellular and humoral) may produce synergistic effects, thereby enhancing tumor control. It was our intention to actively elicit a humoral anti-HER-2 response with biological activity within a mouse model so that we could test for synergy in the immune arms (humoral and cell based) within a mouse model.

In an attempt to obtain short linear epitopes within the HER-2/neu ECD capable of eliciting a humoral response, we created a rat HER-2/neu library consisting of 62 peptides spanning the length of the ECD of HER-2/neu. The peptides were 20 aa in length and overlapped by 10 aa. Balb/c mice were vaccinated with pools of peptide (5 or
6 peptides per pool) from the HER-2/neu library and immunogenic peptides capable of providing T-cell help and B-cell activation were identified.

Peptide 61 was chosen for biological assays because of its relatively pronounced immunogenicity, and the fact that it represented a region in the topography of HER-2 recognized by Trastuzumab. Sera from mice vaccinated with P61 was able to modify metabolism of HER-2 overexpressing SKBR-3 cells through mechanisms that appeared to utilize the MEK/ERK pathway independent of ligand, and the Akt pathway in the presence of ligand. This was not consistent with Junttila et al. model of Herceptin-mediated disruption of the HER2-HER3 heterodimer that they determined to be HER3 ligand independent. However, when we compared Herceptin’s performance in the presence or absence of HER3 ligand, Herceptin activity was consistent with the activity of anti-P61 sera in that it was better able to down regulate Akt phosphorylation in the presence of HER3 ligand (heregulin). We also saw a reduction in the phosphorylation of HER2 with anti-P61 sera and Herceptin as seen by Lane et al. and Nagata et al.

Understanding that P61 specific T-helper cells would be needed for the induction of an anti-P61 humoral response, we conducted a short pilot study to see if priming the immune system with DC’s pulsed with P61 would produce a more robust humoral response. The results suggested that the P61 pulsed DCs did in fact prime the immune system for an enhanced humoral response when compared to no priming or priming with unpulsed DCs. (Fig. 5.2)
Figure 5.1: A Pilot Study Showing DC’s Priming the Immune System for a Humoral Response. A pilot was performed to test if vaccination with DC’s pulsed with P61 could prime the immune system for the induction of an anti-P61 humoral response. The initial results suggest that this is the case.
It is unclear at this time whether or not the epitope of P61 is beneficial for tumor control in vivo, but other noted small linear peptides, either alone or in combination, may prove effective in creating a robust anti-tumor response. In addition, it has been shown that it is often beneficial to induce antibodies to multiple epitopes (polyclonal) against HER2 (Sharon, Liebman et al. 2005). It may be beneficial for us to test peptides in combination. When combined with the dendritic cell mediated, CD 4+ T-cell stimulating vaccine, the response elicited may more effectively control tumor cells.

Future Studies

Future studies will focus on in vitro biological activity of other contributing peptides in the identified Pools of the Balb/c and C57bl/6 mouse sera. Identified peptides with anti-tumor in vitro activity will then be validated in vivo experiments. In vivo experiments will determine efficacy in prevention and tumor control either with or without the addition of DCs. Ultimately data obtained would provide a mouse model for the DC based vaccine plus the addition of a humoral stimulating peptide adjuvant. This would indicate whether or not it would benefit patients to receive humoral stimulation or if the DC vaccine by itself is the best option thus far.
References


