ABSTRACT

Reprogramming T Cell Specific Immune Response to Cyclin B1
in Breast Cancer Patients Using a TLR8/7 Agonist

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Cyclin B1 is a cell cycle regulatory protein aberrantly overexpressed in a number of cancers, including breast cancer. While the current standards of care for breast cancer are sometimes curative, many patients suffer relapse. This necessitates novel therapeutic approaches. Therapeutic vaccination has become an increasingly attractive option because of the ability to expand and possibly correct the function of cancer antigen-specific T cells, expand memory T cells, and effectively control tumor antigen delivery. Therefore, we analyzed the immune repertoire to Cyclin B1 in patients with breast cancer in order to achieve these goals.

Healthy donor CD4+ and CD8+ T cells can express IFNγ in response to Cyclin B1 long peptides, thus confirming prior findings. Cyclin B1 long peptides were also able to stimulate antigen-specific cytokine secretion from breast cancer patients PBMCs (22 out of 25 patients studied). However, PBMCs from breast cancer patients secrete high amounts of type 2 cytokines (IL-4, IL-5, IL-13, TNFα) and low amounts of IFNγ when compared to healthy donors. These results suggest a Type 2 bias in breast cancer patients

PBMCs, similar to our earlier findings showing a pro-tumor, inflammatory Th2 microenvironment in tumor infiltrates.

We next analyzed whether this response could be modified, resulting in a block of Th2 cytokines, or increase in Th1 cytokines. Our earlier studies suggested that CL-075, a TLR8/7 agonist, generates Type I cytokine secretion and drives antigen-specific CD8+ T cell responses. In healthy donor PBMCs, we found this led to a significant increase in the amount of antigen-specific IFNγ expressed by both CD4+ and CD8+ T cells. In breast cancer patient PBMCs, 21 out of 22 patients showed a modified cytokine secretion signature that was antigen-specific with CL-075. Those patients were also able to either increase IFNγ-specific response or block Th2 cytokine response, while 6 out of 22 patients were able to do both.

Therefore, the inflammatory Th2 immune response to Cyclin B1 in breast cancer patients can be modified using a TLR8/7 agonist, thereby providing a rationale for a combination of Cyclin B1 long peptides and TLR8/7 agonists as a therapeutic possibility.

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in Breast Cancer Patients Using a TLR8/7 Agonist

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AA.....Amino acid

APC.....Allophycocyanin

BC or BRCA.....Breast cancer

BME.....2-Beta-mercaptoethanol

C.....Celsius

CB1.....Cyclin B1

CD.....Cell differentiation

CDK-1.....Cyclin depedent kinase -1

CEF.....CMV, EBV, Flu short peptide mix

CFSE.....Carboxyfluorescein diacetate succinimidyl ester

CMV.....Cytomegalovirus

cRPMI.....complete Roswell Park Memorial Institute medium

CTL.....Cytotoxic T lymphocyte

DC.....Dendritic cell

DCIR.....Dendritic cell immunoreceptor

DMSO.....dimethyl sulfoxide

DNA.....deoxyribonucleic acid

EBV.....Epstein Barr Virus

ER.....Endoplasmic reticulum

ERα.....Estrogen receptor alpha

FACS .....Fluorescence-activated cell sorting

FCS .....Fetal calf serum

FITC.....Fluorescein

Flu M1.....Influenza virus matrix 1

Flu MP ..... Influenza matrix protein

FSC .....Forward Scatter

GM-CSF.....Granulocyte macrophage colony stimulating factor

HD..... Healthy donor

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER.....Human Epidermal growth factor Receptor

HLA ..... Human leukocyte antigen

HPC..... Hematopoietic progenitor cell

HPV .....Human papillomavirus

HTS.....High-throughput sampler

HYOU/Orp150.....Hypoxia up-regulated protein 1/ 150 kDa oxygen-regulated protein

IAP .....Inhibitor of apoptosis

ICS ..... Intracellular staining

IDO ..... Indoleamine 2,3-dioxygenase

IFNα.....Interferon alpha

IFNγ .....Interferon gamma

IGFIR ..... Type 1 insulin-like growth factor receptor

IL.....Interleukin

IRF .....Interferon regulatory factor

IU ..... International units

kDa ..... kilo Daltons

LPS ..... Lipopolysaccharide

M ..... Molar

M1 ..... Type 1 macrophage

M2 ..... Type 2 macrophage

mAb ..... Monoclonal antibody

MAPK ..... Mitogen-activated protein kinase

MART-1 ..... Melanoma antigen recognized by T cells-1

mDC ..... Myeloid DC

MDC ..... Macrophage expression of the chemokines

MDSC ..... Myeloid derived suppressor cell

MFI ..... Mean fluorescence intensity

MHC ..... Major histocompatibility complex

MUC-1 ..... Mucin-1

ND ..... Normal (healthy) donor

NF-κB ..... Nuclear factor kappa B

NK ..... Natural killer

NLR ..... Nucleotide Oligomerization Domain receptor

NOD ..... Nucleotide Oligomerization Domain

PAMP ..... Pathogen associated molecular pattern

PBMC ..... Peripheral blood mononuclear cell

PBS ..... Phosphate buffered saline

PD-1 ..... Programmed death 1

pDC ..... Plasmacytoid DC

PE ..... Phycoerythrin

PerCP ..... Peridinin Chlorophyll

PMA ..... Phorbol 12-myristate 13-acetate

Poly I:C ..... Polyinosinic:polycytidylic acid

PR ..... Progesterone receptor

PSA ..... Prostate specific antigen

R10 ..... cRPMI + 10% FCS

RAb ..... cRPMI + 10% Ab serum

RNA ..... ribonucleic acid

RT ..... Room temperature

SD ..... Standard deviation

siRNA ..... silencing RNA

SSCHN ..... Squamous cell carcinoma of the head and neck

SSC ..... Side Scatter

T ..... Thymocyte

TAM ..... Tumor associated macrophage

Tcm ..... Central memory T cell

TCR ..... T cell receptor

Tem ..... Effector memory T cell

TGF-β ..... Transforming growth factor beta

TLR ..... Toll-like receptor

Th ..... T helper

TNFα ..... Tumor necrosis factor alpha

TSLP ..... Thymic stromal lymphopoietin

Treg ..... Regulatory T cell

VEGF ..... Vascular endothelial growth factor

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Thank you.

CHAPTER ONE

Introduction

Breast Cancer

Background

Breast cancer is the leading cause of cancer deaths in women, and globally responsible for 1% of deaths (World Health Organization). In the U.S., 1 in 8 women (12.5%) will develop invasive breast cancer, and 1 in 35 women (3%) will die from causes related to invasive breast cancer (National Cancer Institute). Last year, there were 1.45 million new cases of breast cancer: this number is expected to rise to 2.2 million by 2030. Healthcare costs attributed to breast cancer treatment in the United States alone was 16.5 billion last year, and is expected to increase by 32% by 2020 (American Cancer Society). Furthermore, while the five year survival rate of diagnosed stage I breast cancer is 98% and locally advanced cancers is 84%, the survival rate for metastasized cancers (23%) is much worse (National Cancer Institute). 1/2 to 2/3 of women that are initially diagnosed as having locally advanced or large tumor cancers will develop metastases, and 90% of breast cancer-related deaths are due to metastasis. The current gold standard treatment is usually a combination of lumpectomy or mastectomy, radiation treatment, and chemotherapy. These treatments can be successful for many women with early stage disease, however late stage patients and those with more aggressive cancers have to endure years of therapy that decrease their quality of life. Despite improved detection methods and treatments, the overall rate of deaths and mortality has not

changed significantly over time. The development of therapies such as Herceptin that specifically target cancer cells has shown clinical effectiveness in a subset of patients (Finn 2008). However, the limitation of this class of drugs is that they are restricted to those patients whose tumors express the target antigen. Additionally, these drugs poorly address the problem of residual disease and relapse, and are not adequately effective against other molecules besides the ones they have been chemically manufactured to recognize. 30% of women diagnosed with breast cancer will experience recurrence of disease in their lifetime. Therefore, there is a clear need for more comprehensive, inclusive, and effective treatments for those who suffer from breast cancer.

Stages and Types of Cancer and Advanced Disease

Following a breast cancer diagnosis, the severity of disease is classified using a series of stages (0-IV) and grades (1-3). The tumor is also classified by its resident tissue. Stage 0 indicates that a tumor has not spread beyond its resident tissue, and will not metastasize or move into other parts of the breast: these tumors are often referred to as benign. Stage I indicates that the tumor is invasive and has spread to other surrounding breast tissue, but not nearby lymph nodes, and grown up to 2cm with no lymph node involvement. Stage II is divided into grades A and B. Stage IIA describes tumors that have spread locally to the axillary lymph node in the armpit but are still small (2cm), or alternatively, larger tumors (2-5cm) that have not spread. Stage IIB includes tumors that are 2-5cm and have spread to axillary lymph node, or a tumor that is more than 5cm but has not spread to the lymph nodes. Tumors that have further metastasized to nearby tissues and/or lymph nodes comprise Stage III, which is separated into A, B, and C. Stage IIIA includes cancers that have been found in axillary lymph nodes or the breastbone,

whose cells are clumping together or sticking to resident structures. Stage IIIB describes cancer that has spread to the chest wall, breast skin or lymph nodes near the breastbone, as well as exhibit clumping in the axillary lymph nodes. Stage IIIB is also reserved for tumors that are inflammatory and might make the tissue appear red and warm to the touch. Stage IIIC includes cancer that has spread to all neighboring lymph nodes and several nearby tissues previously mentioned in Stage III, in addition to lymph nodes around the collarbone. Stage IV is the most serious classification, and indicates that the cancer has spread to areas besides those that are in direct contact with breast tissue, such as major organs or distant lymph nodes. The majority of patients used in this study were either Stage II or III.

Tumors can be classified into 3 grades. Grade 1 tumors have cells that look most like normal cells, and grow slowly in regular patterns. Grade 2 tumors have cells that look slightly different than normal cells, and that are actively growing and dividing faster than normal cells. Grade 3, the most severe, has cells that look highly irregular and are growing quickly in a disorganized way. Classification of patients used in this study are found in chapter two.

Breast Cancer Therapies, Treatments, and Pitfalls

Surgery

Lumpectomy is a procedure that takes out only the portion of the breast that has cancerous tissue. Sometimes this tissue might be difficult to distinguish from normal tissue, so a small amount of surrounding normal tissue is removed. The alternative, for those with particularly invasive cancer, or for those at high risk of recurrence, is

mastectomy, which removes the entire affected breast(s). This practice is more advanced than it has been in the past, with skin sparing surgery available that creates tissue almost identical to the tissue removed using your own skin with minimal appearance of scars. However, it is still an invasive surgery and comes with risks and complications. Surgery, however, is rarely the only treatment employed in breast cancer treatment. It is usually coupled with radiation and/or chemotherapy.

Radiation

A non-lethal dose of radiation is delivered to areas affected with cancer in order to cause DNA damage in the cancer cells. It is especially effective at slowing tumor growth and cancer cell division. Normal cells have better cellular and DNA repair mechanisms than cancer cells, so it is believed that any damage caused to normal tissue would be minimal over time. However, normal cells are initially adversely affected as well. The radiation is either delivered to the cancer site internally, using pellets, or externally, via a highly focused linear accelerator.

Some statistics have shown that radiation can reduce the risk of recurrence by up to 70%, and 60% of patients that do not opt for radiation after surgery have a recurrence in the same breast. Side effects of radiation include redness and soreness on the skin above the area that is being treated similar to a sunburn. If lymph nodes are being treated, they can also become sore as well as the skin around them. Sometimes discoloration can last for years after treatment. A majority of patients experience at least some discomfort as a result of radiation treatment.

Chemotherapy

Chemotherapy, like radiation, is designed to slow the growth of cancer and kill cancerous cells. Unlike other treatments, it is systemic: the chemotherapy medicine travels throughout the body and thus has an opportunity to affect many more healthy cells in the process of treating the cancer. Cells that can divide quickly, like cells in the blood, mouth, intestines, nose, nails, and hair, are especially prone. Chemotherapy is usually administered to patients of low stage immediately after surgery to kill remaining cells and prevent recurrence. This is called adjuvant chemotherapy. Neoadjuvant chemotherapy, on the other hand, is given to patients to shrink the size of the tumor before surgery so that less tissue has to be removed. Rarely is chemotherapy just one drug, but instead is a combination of 2-3 drugs, termed a regimen. Many chemotherapy drugs are given for a specific amount of time, usually 2-3 weeks. Once that time is completed, this marks the end of a cycle. Some medications may require multiple cycles to treat the cancer. If a patient does not respond to a particular regimen, a new regimen is prescribed until treatment options for that patient are exhausted. 30-60% of patients respond positively to chemotherapy treatment. While some chemotherapy drugs have mild side effects, others negatively impact quality of life. Fatigue, hair loss, nausea, and susceptibility to infection are the most common side effects, depending on the patient's overall health and the particular drug used. There are many possible side effects, ranging in severity, for all chemotherapy drugs. Medication can be given in addition to chemotherapy to lessen the impact of side effects, but they cannot be completely mitigated. These side effects can be compounded if combined with hormone therapy or a targeted therapy such as Herceptin.

Hormone Therapy

Some tumors express hormone receptors on their surface. These patients can benefit from hormone therapy that targets these receptors. Hormones can allow tumors to grow and divide. Hormone therapies that target progesterone receptor (PR) or estrogen receptor (ER) work by lowering the levels of those specific hormones in the body, or directly blocking the action of those hormones on the tumor. In extreme cases doctors might recommend the removal of ovaries to permanently eliminate the presence of estrogen in the body. Side effects from hormone therapy are similar to that of chemotherapy, with more possibility of sexual or fertility side effects.

Immunotherapy

The goal of immunotherapy is to use cells or products of the immune system against cancer. Several factors determine whether an immune response in this context will be therapeutic, including the quality of generated T cells, the control of Treg responses, and overcoming suppression in the cancer microenvironment (Palucka *et al.* 2010). This is generally accomplished through two types of immunotherapy: passive and active.

Examples of passive immunotherapy include the use of monoclonal antibody treatments and adoptive T cell transfer. Only one monoclonal antibody, Trastuzumab (Herceptin) has been approved for use in breast cancer, and works by binding Human epidermal growth factor receptor 2 (HER2) on breast cancer cells. This action causes a break in the normal functioning of the protein that makes cancer cells more prone to destruction through other means. HER proteins regulate cell growth, survival, adhesion, migration, and differentiation in all cells. In breast cancer, HER2 is commonly over-

expressed, and causes uncontrolled cell reproduction (Hudis 2007). It is also a very expensive treatment, costing $100,000 a year for treatment of one patient. Herceptin has side effects that mimic flu-like symptoms and symptoms similar to mild chemotherapy treatments, but also can cause cardiac dysfunction in a subset of patients. Additionally, cancer cells can develop resistance to Herceptin or lose expression of HER and still survive (Kute *et al.* 2004). Other antibody therapies rely on immune cells recognizing bound antibodies and targeting those cells for destruction. However, this method is sometimes not effective by itself to render objective responses, and does not usually result in any adaptive response or immunological memory. There is evidence that monoclonal antibody therapy might result in adaptive immune response through NK cell, dendritic cell (DC) cross talk and subsequent T cell priming, but more research is needed to verify these results (Lee *et al.* 2011).

Adoptive T cell transfer has received more attention in recent years because of the moderate success it has in cancer destruction. In some studies it has even achieved clinically objective responses in up to 50% of patients (Besser *et al.* 2010, Disis 2010). T cells, either CD4+ or CD8+, can be activated, primed against tumor antigens, and expanded ex vivo, and reinjected into the patient. However, isolation of T cells is not successful in every patient, and in fact, in a majority of patients, the immune response did not result in complete destruction of tumor (Disis *et al.* 2009, Disis 2010). There is some disagreement as to which method of activation should be used to generate both potent effector cells and memory cells that are long lasting (Yee *et al.* 2005). Butler *et al.* 2011 created artificial antigen presenting cells that expressed HLA-A201, CD80, and CD83 which expanded CD8+ T cells that could migrate to the tumor and have tumoricidal

effects that were long lasting. However, they were unable to achieve relevant clinical responses, and we hypothesize that initiating and priming these responses with patient derived DCs and expanding effector and memory T cells in vivo is superior to using artificial systems of activation, and once optimized will result in objective clinical responses. While some of these studies have had promising results, questions remain about the best method of delivery, T cell survival once they enter the body, migration to the tumor site form the injection site, their method of action against the tumor, and efficacy. In addition, tumor infiltrating lymphocyte (TIL) generation is labor intensive, and the therapy itself has a high dropout rate among enrolled patients (Besser *et al.* 2010).

These therapies are considered 'passive immunotherapy' since the immune system in the host has not itself been activated, only infused with activated cells or molecules that are immune system derived.

'Active immunotherapy' is different in that it uses various methods to utilize and activate the host's immune system. These vaccines rely on DCs, injectable vectors or plasmids containing antigenic sequences, or adjuvant/peptide combinations to generate an immune response. Peptide-based vaccines include epitopes that are seen by the immune system and can be processed by DCs and presented to T cells. However, peptides in current use do not include all immunogenic regions that might be important in generating immunity, or are restricted by HLA type. A vaccine consisting of tumor lysates, which might include more immunogenic epitopes, is difficult to reproduce and might include tolerogenic sequences (Disis 2010 and Palucka *et al.* 2010). Plasmid-based vaccines that contain DNA or RNA of tumor proteins are simple to generate and inexpensive to

manufacture, but by themselves are poorly immunogenic. Viral vector vaccines, on the other hand, can be strongly immunogenic, but are much more difficult to produce and might not be effective in immunocompromised patients (Disis *et al.* 2009). Some of these vaccines are not targeted to a specific cell subset, and are instead injected into patients and engulfed by any DC that is resident in the injection site or draining lymph node. Allowing any and every DC to come in contact with these vaccines might be counterproductive, because not every DC subset is optimal for generating an optimal immune response that would be effective against cancer, and capture of antigen by immature DCs might induce tolerance instead of immunity (Palucka *et al.* 2007, Koski *et al.* 2008).

Dendritic cell-based vaccines have the potential to address many of the drawbacks of other active immunotherapy treatments. Ex vivo patient DCs or monocytes extracted and cultured from the blood, loaded and activated, then reinjected to the patient to initiate an immune response is the typical format for DC-based vaccines. While these vaccines must currently be tailor-made for each individual patient, this also allows the selection of cell subsets that would be most effective in generating an anti-tumor response (Koski *et al.* 2008). In addition, DC-based vaccines have been shown to elicit Th1 responses in tumors, modulate the microenvironment, result in tumor antigen cross priming, and even result in epitope spreading, a broadening of antigens that are seen by the immune system present on tumor cells (Disis 2010). While there have been some good clinical outcomes, the rate of success is below what was expected (Palucka *et al.* 2007). Ideally, DCs that are excellent stimulators and proliferators of T cells, especially CD8+ T cells, would be the type of DCs needed to treat cancer. Recent studies from our lab have shown that

vaccination through DCs can elicit antigen-specific CD8+ T cells, and that these T cell survive in patients for long periods of time (Melief 2008, Palucka *et al.* 2010). More data that becomes available continually changes the prevailing theories on the ideal DC subset for use in this setting and the activators required to generate appropriate T cell responses. Current therapies have not yet produced a vaccine that can work in a broad range of patients, nor is it able to induce immunity in all patients (Disis 2010). Also, the peptide/antigen chosen for delivery to T cells through DCs is in question and varies with each disease. The selection of optimal antigen or peptide for use in this system is an important one (Palucka *et al.* 2010). We will discuss peptides and antigen chosen for this study at the end of the Introduction.

Because ex vivo DC generation is labor intensive, many groups, including ours, are looking to deliver antigen through engineered monoclonal antibodies that are specific to DC surface molecules (anti-DC fusion proteins), and include a payload of cancer antigen and proper activating signals for the DC. This direct delivery to DCs in the patient could lead to fast, effective, and relatively inexpensive cancer immunotherapy if successful. Our ultimate goal is to use the data obtained in this study to develop a breast cancer vaccine that could be delivered to DCs, which would properly activate and load DCs with antigens, and that would expand CD4+ and CD8+ effector and memory cells that could migrate and carry out their functions against cancer cells.

Breast Cancer and the Immune System

Overview of the Immune System

The human immune system is the first line of defense that protects the body from injury and infection. This protection can be separated into a few different categories: protection against parasites, bacteria, fungi, or virus; elimination of aged, damaged, or dying cells; and surveillance of tumors. Without the immune system, we would chronically fall victim to any one of these potential problems. However, an overactive immune system can be equally harmful in some cases, causing chronic allergies or even autoimmune disease. Therefore, a careful balance must be maintained in the host to satisfy these competing needs.

An immune response can be divided into two broad types: innate and adaptive. In an innate immune response, cells in the area of infection or injury act quickly to contain and control pathogens, while secreting cytokines that both combat pathogens in the area and recruit other immune cells. These cells are immediately available to act; they do not require prior exposure to the pathogen to be able to respond. They recognize pathogens through pathogen-associated molecular patterns (PAMPs) and toll-like receptors (TLRs). Important cell types in this phase include macrophages, granulocytes, and natural killer cells, among others. These cells coordinate with local tissues to limit the amount of damage done by the pathogen through protein secretion and phagocytosis.

Adaptive immune response relies on repeated or prior exposure to pathogen antigens to generate an efficient, specific response geared towards eliminating that specific pathogen. In the event a new pathogen is encountered, an immune response can be 'primed' if the host has never encountered it before: T cells are selected by their

affinity and specificity to antigens associated with that pathogen by DCs. Antigens carried to the lymph nodes by dendritic cells are used to expand thousands of T cells from only a few precursor cells that recognized the antigen. The T cells then travel back to the infection site to mediate specific killing of their targets. This is called cell-mediated immunity. B cells are generated in a similar manner: they proliferate and mature into plasma cells that secrete antibodies specific to the pathogen antigens, a process called humoral immunity. After the infection has cleared, some T and B cells will remain in the body as memory cells, and can be activated to assist in an immune response in the future if their antigen is encountered again. It is the goal of immunology to study all of these cells types and their interactions with each other and the host. This paper will primarily address topics on cells of the cell-mediated adaptive immune response.

Dendritic Cells

First characterized by Paul Langerhans in 1868, dendritic cells (DCs) were first thought to be nerve cells, mistaking their long dendrites for neurons. They were eventually recognized as the centerpiece of an immune response after their isolation, and were found to be potent stimulators of B and T lymphocytes. Since in vitro methods for their culture were discovered using GM-CSF and TNFα, their importance in research and medicine in generating specific, adaptive immune responses has been undeniable (Steinman *et al.* 1973, Caux *et al.* 1992, Banchereau and Steinman 1998, Steinman and Banchereau 2007).

Dendritic cells originate from hematopoietic progenitor cells (HPCs) and develop along pathways that determine whether they will become myeloid DCs (mDCs) or plasmacytoid DCs (pDCs). Both of these DCs are found circulating in the blood and in

secondary tissues (Ueno *et al.* 2007). Lymphocytes such as B cells and T cells rely on DCs for the direction and control of immune responses (Cao *et al.* 2007, Dubsky *et al.* 2005, Palucka and Banchereau 2006, Ueno *et al.* 2007). They are recognized as the centerpiece of an immune response – therefore any vaccination strategy should involve DCs. The immunogenicity of various antigens delivered to DCs in the context of vaccination has been shown in numerous studies (Banchereau and Paulcka 2005, Palucka *et al.* 2007, Melief *et al.* 2008). One unique feature of DCs that aids in their ability to generate potent immune responses is their plasticity and the plasticity of their precursors. Any number of different DC subsets can be created through culture with different combinations of cytokines. These subsets of DCs enable distinct types of immunity that allow the immune system to react appropriately to any pathogen it comes in contact with while still maintaining tolerance to self antigens (Palucka *et al.* 2009).

Immature DCs circulate in the blood and reside in the tissues, constantly sampling their surroundings. They become activated by coming in contact with a pathogen or protein that binds to and triggers one of the many danger signals present on DCs (PAMPs, TLRs, Fc regions, etc.) (Steinman and Banchereau 2007). DCs can also receive maturation signals through C-type lectins, NOD-like receptors (NLRs), other immune cells such as T cells, NK cells, NKT cells, and γδ T cells, CD40 ligand, proinflammatory cytokines, or by coming into contact with dying cells (Palucka *et al.* 2009). The DCs then mature to an antigen presenting cell (APC) and upregulate genes that are key to its function: markers for migration to secondary lymph tissues; morphology changes; secretion of cytokines and chemokines that attract and polarize innate and effector cells;

and upregulation of costimulatory molecules. They then facilitate the generation of antigen-specific T and B cells in the secondary lymph tissues.

The blood acts as a "pipeline for the immune system," containing a number of circulating and migrating cells that are being transported to and from injury sites or lymph tissues, including naïve, effector, or memory T cells, B cells, plasma cells, NK cells, macrophages, DCs, monocytes, and neutrophils (Chaussabel *et al.* 2010). These cells can be separated from blood taken from patients or healthy donors, and taken together are called peripheral blood mononuclear cells (PBMCs). After being antigen educated in the lymph nodes or lymphatic tissues, immune cells re-enter the blood to be transported back to tissue sites where they utilize their effector functions. Circulating cells express adhesion molecules that help guide them to injury or infection sites, along with receptors that are sensitive to chemotactic molecules in the blood. Circulating cells are also susceptible to any factors that are released systemically. Therefore, one could say that the populations of lymphocytes found in the blood are mostly likely a good indication of the overall immune environment in the body, and are ideal for studying global immune responses.

When studying any kind of cancer, one must also take into consideration the difficulty in obtaining numerous tissue samples for study, and in particular with breast cancer, the size of the samples obtained. Both of these obstacles make it difficult to study human immune responses in breast cancer. Luckily, immune cells flowing through the blood have likely encountered either the microenvironment itself, been subjected to systemic cytokines secreted from the tumor or surrounding cells, or have been in direct contact with the tumor.

Toll-like Receptors and TLR8/7 (CL-075)

Toll-like receptors (TLRs) are a family of transmembrane receptors that recognize microbial molecular patterns (PAMPs). All microorganisms possess these molecular patterns. Many artificial ligands have been created that mimic these natural signals. Each TLR (1-11) has a specific set of microbial product they recognize, which has been well characterized (Rakoff-Nahoum and Medzhitov 2009). TLRs have a variety of functions depending on which tissues are being studied. In mucosal sites they help defend against microorganisms, enhance the uptake of microorganisms in phagocytic cells and lead to the generation of chemicals that act against them, upregulate molecules on endothelium that can help with leukocyte trafficking, assist with tissue repair and regeneration, and act directly on immune cells to help regulate immune responses. Their action on DCs mediates T cell activation, differentiation, and maintenance through IL-12, processing and presenting antigen, upregulation of co-stimulatory molecules like CD80 and CD86, inhibition of Tregs, and the activation and maturation of B cells. TLRs signal through signal transduction pathways to activate Nuclear factor kappa B (NF-κB), Interferon regulatory factors (IRF), and Mitogen activated protein kinase (MAPK) signaling. Together, these activate genes essential to the generation of innate and adaptive immune responses. Studies have shown that addition of TLR agonists can enhance antigen-specific immune responses in vitro and in vivo. In the context of cancer, TLR activation of the immune system has been shown to help break tolerance to self antigen, and has been shown to have both pro- and anti-tumor effects (Rakoff-Nahoum and Medzhitov 2009).

TLR 8 in particular has been shown to have potent effects on DCs to generate a cytotoxic T lymphocyte (CTL)/Th1 polarizing effect on T cells (Klechevsky *et al.* 2010, Spranger *et al.* 2010). Recognition of patterns by TLR 7 or 8 activates pathways that induce proinflammatory cytokines, chemokines, type I interferon, and upregulation of costimulatory molecules, all of which are important for generating a tumor-specific immune response (Smits *et al.* 2008 and in Figure 1). TLR 7 and 8, unlike most other TLRs, are located in endosomal vesicles that travel between the Golgi apparatus and the cell membrane, transporting proteins and peptides. Wille-Reece *et al.* 2005 (a and b) showed that a TLR 8 agonist in combination with HIV peptide was able to confer immunity in non-human primates, and also generate more HIV-specific Th1 cells and antigen-specific CD8+ T cells compared to HIV peptide alone. They also found that when HIV peptide and TLR8 agonist were conjugated together, the effect was enhanced. Peng *et al.* 2005 showed that CD4+ regulatory T cells stimulated with TLR8 reversed their function and were able to assist in anti-tumor-specific immune responses. While this effect seemed to be independent of DCs, it is important to note that TLR8 has an effect on T cells and DCs. Memory T cells are especially sensitive to this effect (Smits *et al.* 2008). Spranger *et al.* 2010 showed that TLR 8 agonist CL-075 was most optimal in generating activated NK cells, Th1 polarized CD4+ T cells, and CD8+ T cells capable of secreting IFNγ that have cytotoxic function. Kastenmueller *et al.* 2011 showed that a conjugate TLR7/8 agonist/peptide vaccine led to the migration of DCs to lymph nodes and increased antigen uptake compared to peptide alone. These DCs were able to produce OVA-specific Th1 CD4+ T cells and CD8+ T cells that were more potent in terms of cytokine secretion. Other studies have shown that TLR7/8 treatment can

enhance the migration capacity of CD4+ and CD8+ T cells towards the area of vaccination and to infection sites (Smits *et al.* 2008). Figure 1 shows the effects of TLR7/8 activation on various cell types of the immune system.

Our institute conducted comparison studies with multiple TLR ligands to find the optimal TLR agonist for generating type I responses, and TLR8/7 agonist CL-075 was found to be most potent at activating and expanding antigen-specific T cells and polarizing helper T cells to a Th1 phenotype (Klechevsky *et al.* 2010 and in Figure 2). Therefore, CL-075, a TLR8 agonist will be used in conjunction with peptides in this study to elicit antigen-specific responses in patients.

DCs and T Cells

During a typical immune response, a pathogen will be encountered by epithelial cells and innate effector cells in the blood or tissues. If this pathogen binds to or activates danger signals, these cells will secrete cytokines and chemokines that will attract other innate cells. DCs also recognize pathogens and take up cells or proteins from the pathogen via phagocytosis or pinocytosis, or direct receptor mediated uptake. They mature while migrating to lymphoid tissues to present antigen to CD4+ or CD8+ T cells via their Major Histocomaptibility Complexes (MHC) that engage T cell receptor molecules (TCR) on T cells. Once a T cell has been encountered that is specific for the presented antigen, costimulatory signals are given to the T cell, which proliferates and differentiates into effector cells that can return to the site of infection. It is important to note that for both CD4+ and CD8+ T cells a second costimulatory signal is required after recognition of antigen that can only be given by an APC. The CD28 molecule on T cells

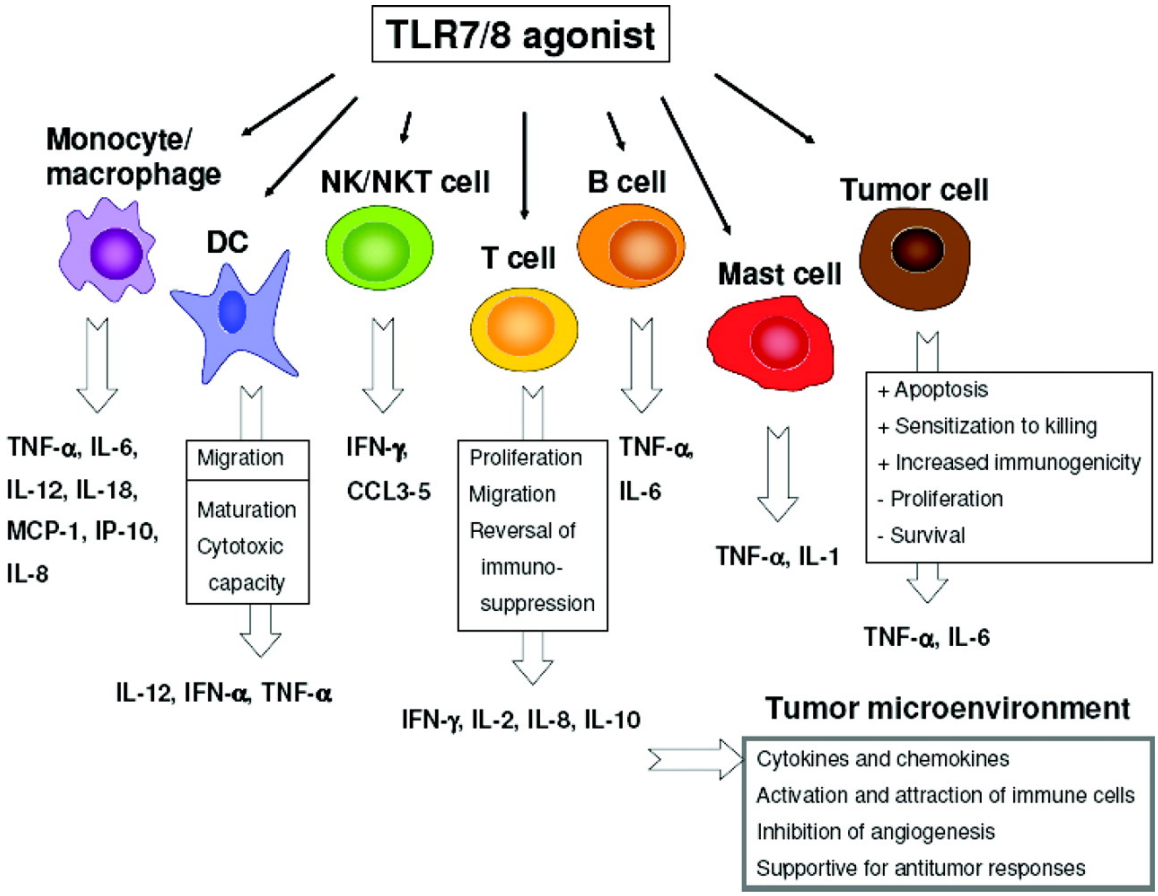

Figure 1. Diagram of effects of TLR7/8 agonist. From Smits *et al.* 2008. Black arrows, TLR7/8 agonists activate different components of the antitumor response; white arrows, cytokine and chemokine production by the activated cells. Abbreviations: CCL, chemokine (C-C motif) ligand; DC, dendritic cell; IFN-γ; interferon-γ; IL, interleukin; IP-10, interferon-inducible protein 10; MCP-1, monocyte chemotactic protein 1; NK, natural killer; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α.

interacts with CD80/CD86 on APCs, and this interaction allows T cells to become activated. If the CD28 – CD80/86 interaction does not take place, this usually causes the T cell to anergize (Pufnock *et al.* 2011). The strength of association between the TCR and MHC is also an important factor in T cell polarization and differentiation. Once the T cell receives these signals, signal transduction pathways activate and transcription factors bind directly to T cell effector cytokine gene promoters, enhancers, and locus control regions, thus signaling commitment to a T cell lineage and phenotype. During this time,

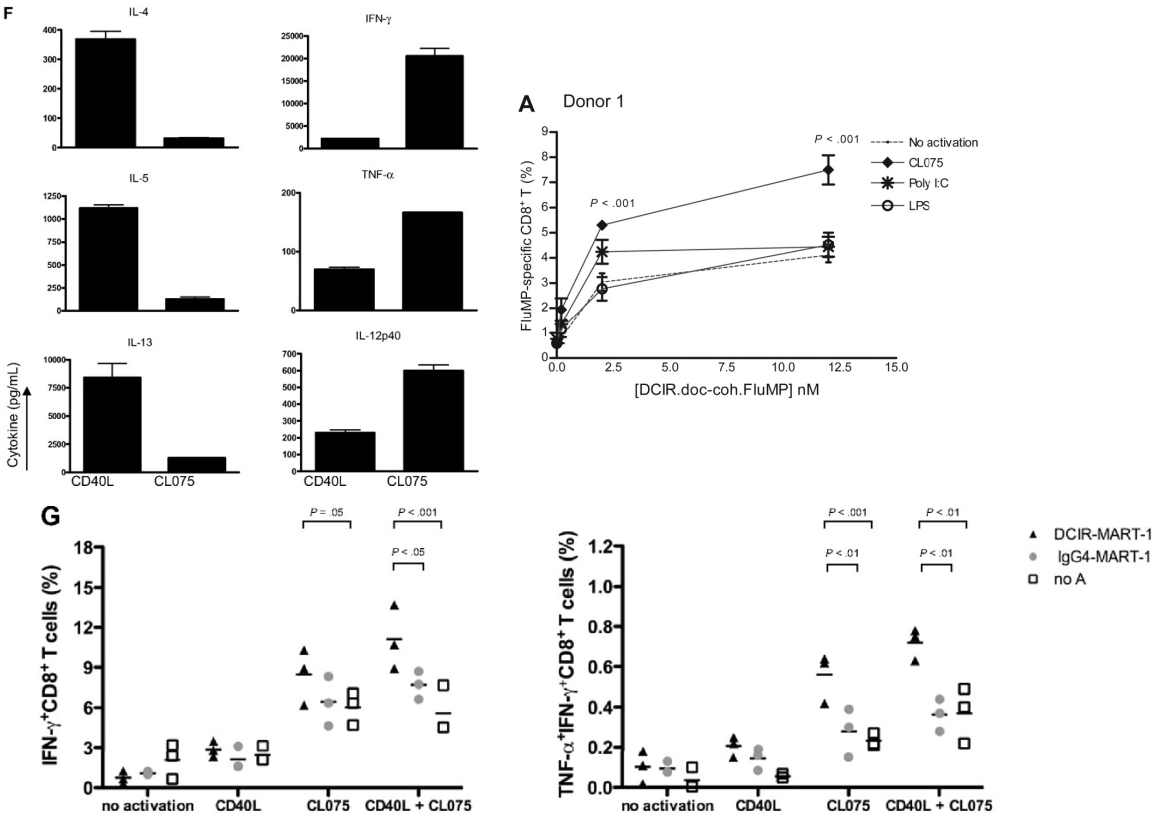

Figure 2. Effects of TLR 8/7 agonist CL-075 on cultures with CD8+ T cells. From Klechevsky *et al.* 2010.
F) IFN-α DCs were targeted with 113nM anti–DCIR-MART-1 fusion protein activated with either CD40L (100 ng/mL) or CL075 (1 µg/mL) and cocultured with autologous naive CD8+ T cells. Ten days later, cells were restimulated with fresh DCs that were loaded with 15mer overlapping peptides derived from the MART-1 protein. The levels of IL-4, IL-5, IL-13, IFN-γ, TNF-α, and IL-12p40 were measured by Luminex in the culture supernatant after 24 hours. The graph represents mean ± SD; n = 3.
A) Blood-derived mDCs from an HLA-A201+ donor were targeted with 12nM, 2nM, or 200pM of anti–DCIR.doc-coh.FluMP complex mAb, activated with either TLR3, TLR4, or TLR7/8 agonists (poly I:C, LPS, or CL-075) and cocultured with autologous CD8+ T cells for 10 days. Graph represents the percentage of FluMP-specific CD8+ T cells measured with a specific HLA-A201–FluMP(58-66) tetramer for each amount of anti–DCIR.doc-coh.FluMP complex mAb and with each DC-activator tested. DCs with no activation were used as a control: no activation (—), TLR7/8 (♦), TLR3 (*), and TLR4 (○) agonists; CL-075, poly I:C, and LPS, respectively. Data are representative of 4 independent experiments with 4 different donors. The graph represents mean ± SD; n = 3.
G) IFN-α DCs were targeted with 10nM anti–DCIR-MART-1 or a control IgG4-MART-1 fusion proteins activated with either CD40L (100 ng/mL) or CL-075 (1 µg/mL), or a combination of CD40L and CL-075 and cocultured with autologous naive CD8+ T cells. Coculture in the absence of an antigen served as an additional control (□). Ten days later, cells were restimulated with fresh IFN-α DCs that were loaded with MART-1 fusion protein and analyzed by flow cytometry for their intracellular cytokine production. Graphs show the frequency of IFN-γ (left panel) and IFN-γ+TNF-α+ (right panel) producing CD8+ T cells primed by DCIR-targeted, or control IFN-α DCs after 5-hour restimulation in the presence of monensin and 0.25 µg/mL of anti-CD28/CD49d mAb (n = 3).

DCs secrete cytokines and chemokines that are important for the polarization of T cells for their specific function.

While this is the classical model of an adaptive immune response to infection, it is important to keep in mind that the cytokine environment of the infection or inflammation site might cause changes to this model, such as in cancer. There are also many other cell types that can secrete cytokines that will affect DC function or maturation as well as the cytokine milieu, like natural killer cells (NKs), NK-T cells (NKTs), basophils, mast cells, myeloid suppressor cells, tissue epithelial cells, and stromal cells.

Long Peptides

Peptide vaccine studies have previously been focused on loading dendritic cells with short peptides (8-10 amino acids) for specific HLA types derived from tumor antigens (HLA-A201, not expressed in all patients). While some studies have found success with this method, cancer has the ability to change some of its antigens through mutation in order to avoid the immune system. Therefore, while using short peptides may confer immunity, there is no guarantee that the tumor will remain susceptible (Melief and van der Burg 2008). Short peptides can be exogenously loaded on any cells with MHC class I molecules, and in the absence of stimulatory signals from professional APCs, can lead to tolerance, an inadequate T cell response that causes clonal deletion, or worse, a tumor-protective response (Bijker *et al.* 2008). In this context it is also important to have peptide-loaded APCs present antigen in the inflamed draining lymph node in the presence of appropriate cytokines to further help clonal expansion and the generation of interferon-producing T cells. However, many times, researchers don't know precisely which sequences will result from processing and be presented naturally.

Allowing DCs to process whole protein or dead tumor cells allows for the possibility of epitope presentation that might lead to further tolerance instead of immunity (Palucka *et al.* 2009).

One solution to this problem is to use long peptides that cover a range of HLA types and immunogenic regions that the DCs can process naturally, but that are short enough to be used in conjunction with adjuvants or targeting antibodies and be used in a clinical setting. Allowing DCs to process the antigen naturally will increase the chances of having epitopes presented that can effectively prime T cells, and the selection of specific regions can limit the possibility of generating Tregs instead of cytotoxic lymphocytes. It will also allow both CD4+ and CD8+ T cell induction, and be of benefit to more patients through the presentation on multiple HLA molecules. This type of vaccination has already seen success in Human papillomavirus (HPV), cancer, and malaria (Melief and van der Burg 2008). As further proof that long peptides are superior to short peptides in the context of DC loading with cancer antigens, Faure *et al.* 2009 compared short peptide and long peptide loading and observed the resulting T cell stimulation and antigen presentation. They found that after longer incubation times (2-3 days), long peptide-loaded DCs had a longer duration of antigen presentation, which led to stimulation of T cells more efficiently than short peptide-loaded DCs. This was due to longer lasting presentation from a persistent antigen pool that was readily available for presentation even after 3 days (Faure *et al.* 2009). Therefore, we have decided to use long peptides in this study.

Selection and Polarization of T Cells by DCs

Researchers have been aware of the ability to prime and proliferate both CD4+ and CD8+ T cells using DCs loaded with peptides for some time. Generally, this process takes advantage of the ability of DCs to both activate and costimulate T cells, as well as a researcher's ability to control the sequence and size of peptides that are allowed to bind to MHC complexes. It is important to remember for this study that the most efficacious T cell response occurs when peptide, APC activating signals, T cell activating signals, and proinflammatory cytokines are all present at the same time and place in draining lymph nodes. The absence of any of these key factors can lead to immunosuppression, clonal deletion of T cells, or tolerance (Melief and van der Burg 2008).

CD8+ T cells, or CTLs, are mainly responsible for cell-mediated immunity. Naïve CD8+ T cells encounter antigen-presenting DCs in secondary lymph tissues. These interactions are transient at first, but once a stable connection is formed, it can last for hours (Germain *et al.* 2006). The CD8+ T cell will receive TCR-MHC signal, costimulation signal, and cytokine signals that program the response of CTLs (Harty and Badovinac 2008). The T cell receptor (TCR) is a heterodimeric surface molecule composed of alpha (α), beta (β), gamma (γ), and delta (δ) chains, and binds short peptide carrying MHC class I. The CD8+ T cells proliferate, creating thousands of daughter cells that are all specific to the antigen originally presented. These proliferated CTLs can release cytotoxic mediators such as granzymes, perforin, TNFα, and IFNγ when they encounter a target cell after leaving the lymphoid organs and surveying the body for corresponding antigen. Perforin forms pores in the cell membrane of the target cell, which allows granzymes to enter the cell and begin cellular cascades that result in the

target cell's apoptosis (Lieberman 2003). IFNγ has direct action against cells by causing cellular cascades that result in apoptosis, and causes susceptibility to action by other cytotoxic molecules. These cytotoxins are key for any immunotherapy targeting cancer because ideally, one would like to penetrate and destroy cancer cells. Also important to CTL function is the inclusion of CD4+ T cell help in the initial priming and expansion phase, and will be discussed later.

The MHC class I binding groove can ideally hold 9-10 amino acid (AA) peptides, and are recognized by TCRs of CD8+ T cells. MHC class I is present on most nucleated cells in the body, and acquire their peptides from the numerous proteins and antigens that have been degraded by the proteasomes within the cytoplasm of the cell. After a protein is digested, it is translocated to the lumen of the Endoplasmic reticulum (ER) where loading of the peptide occurs on the MHC I complex. Haplotypes for MHC I include HLA-A, B, C, E, F, G, K, and L. Some of these genotypes are found more frequently in the general population, or among certain ethnicities. Since MHC class I molecules acquire their peptides from proteins that were found in the cytoplasm, this method of presentation is typically called the endogenous presentation pathway, and is the typical response seen for intracellular pathogens, like a virus. CTLs can also be primed by exogenous peptides in a process called cross presentation.

Cross presentation occurs when extracellular proteins and their subsequent peptides end up on MHC I complexes and are presented to CD8+ T cells, instead of MHC II complexes, the normal presentation route for extracellular protein, described later. While the exact process of this is not specifically known, it is mostly DCs that are able to perform this function (Kurts *et al.* 2001). The current theory is that endocytosed

proteins that are not fused with MHC II vesicles are taken to a specialized compartment that acts similar to the ER (Guermonprez *et al.* 2003, Burgdorf *et al*. 2007). Here, the compartment is either transported to the lumen for MHC I loading in the endosome, or the peptides are released into the cytosol, and the natural intracellular MHC I loading process takes place after further peptide digestion (Guermonprez *et al.* 2003, Burgdorf *et al.* 2008). This mechanism is key for the immune system reaction to viruses that do not infect APCs, bacteria, or tumors. Related to cross presentation, cross priming describes the education of naïve CD8+ T cells by DCs through cross presentation, which is especially important for cancer immunotherapy and this study. Because tumor antigens are often self antigens, they will also be susceptible to a similar idea called cross tolerance. Cross tolerance occurs when self antigens are cross presented to T cells, and any autoreactive T cells are eventually eliminated through anergy and apoptosis (Kurts *et al.* 1997). This process eliminates T cells that might be specific for cancer antigens that are also self antigens, thereby making the frequency of cancer antigen-specific T cells quite low. This low frequency is the result of negative selection in the thymus: cells that react too strongly to self antigen are removed before being released into the body. Cross tolerance can also remove these cells in the tissues. A severe break in this process can lead to autoimmunity. This is something that must be overcome for peptide-based cancer immunotherapy to succeed.

MHC class II molecules are less prevalent, being found only on professional antigen presenting cells (APCs) such as DCs, macrophages, and B cells. Class II peptides can be anywhere from 15-24 AAs in length, and are recognized by CD4+ T helper cells (Th). The Th cells then initiate an appropriate immune response based on the

peptide they are presented and the surrounding immune environment. This could include recruitment of other phagocytes and innate immune cells, or they could provide stimulation and engagement with B cells or CD8+ T cells. MHC II molecules can hold larger peptides because their binding grooves are open ended, as opposed to MHC I molecules, which are closed. Peptides for MHC II are acquired from extracellular proteins. APCs sample the surrounding environment and endocytose proteins and antigens. The encytosed material becomes a vesicle which fuses with a lysosomal compartment in the cell that degrades the proteins and antigens further before its fusion with a vesicle that contains the MHC II heterodimer. The MHC II heterodimer is constructed in the lumen of the ER and fitted with a place-holding invariant chain in the future location of the peptide. It is then transported in a vesicle to the late endosome containing the degraded peptides. The MHC II complex invariant chain is degraded and removed by specialized proteins, and the peptide is placed into the groove, followed by transport back to the cell surface for presentation. Haplotypes for MHC II include HLA-DM, DO, DP, DQ, and DR.

MHC II complexes are recognized by TCRs on CD4+ T cells. CD4+ T cells, or Th cells, can be characterized into several different categories: Th1, Th2, Th3, Th9, Th17, Th22, and Tfh. In this study we will focus on Th1 and Th2. Th cells can also differentiate into effector, memory, or regulatory T cells of each type (Zhu *et al.* 2010). The cytokine environment around the parent Th cell, the affinity of the TCR for antigen, and the amount of costimulation available all have a dramatic effect on which type of helper cell it becomes (Zhu and Paul 2010). Based on these signals and their downstream signaling pathways, at least one transcription factor regulates the type of Th cell into

which a CD4+ T cell will develop, but these will not be discussed here. Recently activated Th cells will become Th0 cells, which secrete IL-2, IL-4 and IFNγ. If the surrounding cytokine environment has predominantly IFNγ and the strength of the TCR signal is very strong, the Th0 cells will most likely become Th1 cells (Paul and Zhu 2010). Th1 cells act on macrophages and CTLs to help maximize their killing ability. They produce IL-2, IFNγ and in some cases IL-10, which can be a suppressive cytokine. These cytokines aid CTLs in development and proliferation, eventually allowing them to find their target cells and release their cytotoxic molecules. This is typically called a type 1 immune response. IFNγ can increase production of IL-12 by dendritic cells, which further aids in Th1 cell mediated immunity. IFNγ also inhibits the production of some Th2 cytokines. If the environment around the Th0 cell has predominantly IL-4 and IL-10, and the TCR signal strength is relatively low, the Th cells will most likely become Th2 cells and secrete type 2 cytokines (Paul and Zhu 2010). These cells are important for humoral immunity, and interact with B cells to induce class switching and proliferation. They are also important for controlling immunity to extracellular parasites and allergic inflammation. Th2 cells can secrete IL-4, IL-5, IL-6, IL-10, and IL-13. This is known as a type 2 immune response. IL-4 can act on other Th cells to induce a Th2 phenotype, while IL-10 can suppress the secretion of type 1 cytokines IFNγ and IL-12 (Paul and Zhu 2010). IL-2, IL-7, IL-25, IL-33 and TSLP secreted from other Th cells or other cell types can also act on Th0 cells to skew them towards a Th2 phenotype, or strengthen their association with Th2 phenotype (Paul and Zhu 2010). Therefore, Th2 cells can preserve their decision to secrete type 2 cytokines, and can act on other Th0 cells to do the same

via a feedback loop. It is important to remember that more than one cytokine is required for all Th cells to differentiate into their respective subset (Zhu and Paul 2010).

In the body, Th0 cells are surrounded by a complex mix of cytokines at the moment of decision to commit to a helper phenotype, and after differentiation, these environments persist. It has been suggested that helper T cells are plastic and have the ability to re-differentiate to different and even opposite phenotypes that secrete different cytokines, but this still needs further investigation. Cells that produce both IFNγ and IL-4 can be detected ex vivo (Wan 2010). Th cell differentiation is therefore a complex process that is controlled by a combination of cytokines, encountered cells, cell signaling, signal strength, and transcription factors.

There is speculation as to whether CD4+ T cell help is necessary for generating potent CD8+ T cell responses. Early studies indicated that IL-2 was an important growth factor for CD8+ T cells, but when IL-2-deficient mice were created, they were not lacking in T cell responses, as was expected, but rather suffered from autoimmunity (Bevan 2004). Scientists initially thought that since IL-2 was produced by CD4+ T cells, that CD4+ T cells aided CD8+ T cells in their proliferation. It is now understood that a lack of IL-2 exposure led to many more CD8+ T cells that could survive after encounters with self antigen because of a lack of Treg control over homeostasis (D'Souza and Lefrancois 2003, Wan 2010). Regardless, there is also evidence that IL-2 signals are required for prolonged CD8+ T cell proliferation in non-lymphoid tissues and for memory T cell survival (D'Souza and Lefrancois 2003). Furthermore, experiments in vivo with DCs showed that CD8+ T cell priming was most efficient and robust when both CD4+ and CD8+ T cells recognized the same antigenic epitopes on APCs (Bennett

et al. 1997). Interaction of CD40-CD40L between CD4+ T cells and APC is an important signal to APCs that aids in CD8+ T cell priming (Schoenberger *et al.* 1998). This is especially the case for non-inflammatory antigens, such as cancer antigens (Bevan 2004). In response to infectious agents, CD8+ T cell responses can be generated regardless of CD4+ T cell help, most likely because the pathogen stimulates toll-like receptors (TLRs) which acts as a signal for maturation to DCs, enabling signaling and secretion of inflammatory cytokines. However, these responses, without CD4+ T cell help, do not generate long-lived memory T cells (Shedlock and Shen 2003, Bevan 2004).

In the case of cancer, a recently published study by Bos and Sherman (2010) showed that certain immune activators, like polyinosinic : polycytidylic acid (polyI:C), could substitute for CD4+ T cell help in the priming stage. However, tumor-specific CD8+ T cells could not traffic to the site of the tumor, or even the tissue of tumor origin. When they provided non-antigen-specific CD4+ T cell help during priming, it produced similar results. Inclusion of antigen-specific CD4+ T cells rescued the ability of CTLs to traffic to the tumor site. They also found that antigen-specific CD4+ T cells played a positive role in the recruitment, proliferation, accumulation of cells in the tumor, and survival of the antigen-specific CTLs. IFNγ secreted from CD4+ T cells was responsible for improvements in migration and trafficking: when IFNγ or chemokines that were induced by IFNγ were blocked, far fewer numbers of CTLs were found in tumors. This group also showed that CD4+ T cells can secrete IFNγ and IL-2 while in the tumor milieu. IL-2 contributed to sustaining the CTL response by enhancing proliferation and effector function. Specifically, they found low levels of perforin and granzyme B in the periphery, but saw a 3-4 fold increase in these cytotoxic molecules at the site of the tumor

when IL-2 was present. Another recent study confirmed the need for CD4+ T cell help in allowing CTLs to traffic to an infection site using a Herpes Simplex Virus vaginal infection model in mice (Nakaniski *et al.* 2009). CD4+ T cells were important in directing CD8+ T cells to sites of infection through secretion of IFNγ, which caused tissue resident cells and other cell subsets to secrete trafficking cytokines and chemokines. These results are crucial for consideration of an effective vaccination strategy. CD4+ T cell help is clearly important for appropriate and robust CD8+ T cell activation and CD8+ memory development (Cox and Zajac 2010, Wu *et al*. 2010, Nakanishi *et al.* 2009, Sun and Bevan 2003, Shedlock and Shen 2003, Bennett 1997).

Another important type of T cell deserves mentioning here because they are important in the context of cancer. Regulatory T cells (Tregs) are a type of T cell that can be induced from the complex regulatory networks of cytokines and cell signaling pathways. Tregs can be induced from naïve CD4+ T cells by the presence of TGFβ or by tolerogenic signals from DCs, and usually have some characteristics in common with other Th cells. They can also be formed naturally in the thymus. Their primary function is to keep the immune system in check; that is, they regulate Th1, Th2, and Th17 immune responses so they do not lead to autoimmunity or allergy. They also maintain tolerance to self antigens. Tumors often express self antigen as their primary identifying markers, and these are presented at the cell surface (Palucka *et al.* 2009). Many different cancers, and even some pathogens, have developed ways of evading natural immune responses by inducing Treg development in the areas of infection or at the tumor site. Tumors can even convert myeloid DCs into DCs that secrete TGFβ, which induce Treg proliferation (Ghiringhelli *et al.* 2005).

Some studies have suggested that a Treg phenotype is malleable - that is, it is possible to change its phenotype and function to Th1 or Th2 - however, these results are controversial (Wan 2010, Zhu *et al.* 2010, Sharma *et al.* 2010). There is additional supporting evidence that a helper T cell's phenotype is not completely set once it is differentiated, and that certain cytokine environments, especially the presence of excessive IL-2 and other polarizing cytokines, can change the phenotype of helper T cells (Wan 2010, Sharma *et al.* 2010). Some results of this have been mixed, generating T cells that secrete Th1, Th2, and Th17 cytokines simultaneously (Wan 2010), but others have seen reprogrammed Tregs aid in CD8+ T cell expansion and contribute to vaccine efficacy (Sharma *et al.* 2010).

DCs play a central role in the development of Tregs and in tolerance. DC functions depend on their maturation state, the tissue microenvironment, the immunosuppressive agents, and inflammatory stimuli present (Steinman and Banchereau 2007). For example, immature DCs that express low amounts of MHC II and costimulatory molecules on their surface can generate T cell tolerance to antigens expressed by tumors (Melief 2008). Tumor microenvironments can also be wrought with immunosuppressive and inflammatory cytokines that can lead to tolerogenic DCs (Melief 2008, Mantovani *et al.* 2008). DCs that have engulfed dead cells, such as dead tumor cells, can also become tolerogenic promoters. Tolerogenic DCs might be useful in situations such as controlling immune responses to gut microorganisms that are beneficial to the body, but in the context of cancer, become detrimental (Pulendran *et al.* 2010). A related group of cells, myeloid-derived suppressor cells, are a more varied mix of cells that can expand during cancer and suppress T cell responses, as well as lead to increased

presence of tolerogenic DCs (Gabrilovich *et al.* 2004, Gabrilovich *et al.* 2009). In addition, DCs respond to Thymic Stromal Lymphopoietin (TSLP) from both epithelial cells and cancer cells that leads to subsequent induction of Tregs and inflammatory Th2 cells (Pulendran *et al.* 2010, Aspord *et al.* 2007, Pedroza-Gonzalez *et al.* 2011). However, Tregs can be reprogrammed to produce proinflammatory cytokines through the use of antigen, TLR ligands, and CD40L, which then provide help in priming of CTLs (Sharma *et al.* 2010). Therefore, it might be possible using the tools of this study to have an effect on the prevalence of Tregs in the cancer microenvironment.

Another important subset of T cells is memory T cells. Relapse is a common occurrence in cancer patients, therefore a vaccination strategy that appropriately considers the generation of memory T cells to combat recurrence is essential. Memory T cells have already been exposed to antigen, whether it be through infection, through a prior vaccination, or encounter with cancer. Both CD4+ and CD8+ T cell expansions create memory T cells. After a naïve T cell encounters antigen for the first time (through infection or vaccination), the T cell proliferates into many daughter cells. While many become effector T cells, some continue to live inactively in various tissues in the body after the initial immune response and contraction phase as memory T cells, and can survive for long periods of time, even 10 years or longer (Sallusto *et al.* 2004). When they encounter their antigen again, they can proliferate and respond much faster than a naïve T cell.

There are two types of memory T cells: Central (Tcm) and Effector (Tem). Tcms have higher sensitivity to antigen, do not need as much costimulation as naïve T cells, and upregulate CD40L for signaling DCs and B cells. Tcms produce IL-2 immediately

after seeing antigen, and then later produce robust amounts of IFNγ or IL-4 (Sallusto *et al.* 2004). Tems have more rapid effector functions than Tcm cells. CD8+ Tems have large amounts of perforin, and both CD4+ and CD8+ Tems can produce IFNγ, IL-4, and IL-5 within hours of encountering antigen. Tem cells can be classified as CTLs, Th1 and Th2 cells based on their cytokine profiles (Sallusto *et al.* 2004). Effector memory cells express molecules that are important to CTL function and cytotoxic granule release. It has been shown that CD8+ Tcm cells are superior to CD8+ Tem cells for eradicating tumors after adoptive transfer into mice (Klebanoff *et al.* 2005). As previously mentioned, CD8+ T cells have improved function as memory T cells if CD4+ T cells provided help at their initial stimulation, even to the point that memory CD8+ T cells are dysfunctional if CD4+ help is not provided (Janssen *et al.* 2003, Sun and Bevan 2003, Shedlock and Shen 2003). Additionally, there have been reports that the method of vaccination can have an effect on the quality of memory T cells generated. A careful balance must be achieved, according to Harty and Badovinac 2008: too much inflammation or exposure to too much inflammatory cytokine at the onset of vaccination will result in early contraction and a slow acquisition of memory T cells. Too little inflammation or activating signals will not allow full maturation of DCs capable of sending all the signals necessary to promote an immune response, and might lead to suppression or tolerance. We keep these ideas in mind in the hopes that it will help us reprogram the immune system's natural response to cancer to favor initial eradication and future protection against relapse.

In the context of cancer, one must remember that, unlike an infection where the antigen/pathogen is cleared after a period of time, cancer persists. There is evidence that

when antigen is persistent, fully functional and long-lived memory cells are not developed that express CD62L and CCR7, and are therefore unable to traffic (Bevan 2004, Pulderan *et al.* 2010). The persistent antigen leads to these cells most likely becoming anergic with a gradual inability to secrete cytokines (Bevan 2004). In this case, CD4+ T cell help is especially important for the maintenance of memory CD8+ T cells, but might be subdued in cancer patients, especially those that have undergone chemotherapy or radiation treatments.

Cancer and the Immune System

The breast cancer microenvironment contains many factors that can act on cells of the immune system. Tumors are considered to have chronic inflammation, and thus attract these cells. However, once cells arrive, they are subjected to chronic inflammatory and suppressive signals that either dampen an appropriate immune response or render it completely inert. Some of these signals include cell products like TSLP, Transforming growth factor beta (TGFβ), indoleamine 2,3-dioxygenase (IDO), vascular endothelial growth factor (VEGF), Mucin-1 (MUC-1), IL-6, IL-10, IL-13, and arginase. Cell surface molecules that contribute include Fas-L and Programmed Death-1 (PD-1). Tregs and myeloid derived suppressor cells are also present and contribute to cancer immune escape.

Cancer immunotherapy is difficult due to the low immunogenicity of tumors and the immunosuppressive environment that surrounds the tumor: most tumor antigens are self antigens; tumors express low levels of HLA and stimulatory signals on their surface; and tumors can actively inhibit the immune system (Melief 2008). Tumors express many molecules on their surface, but without sufficient costimulatory signals, DCs present this

antigen to T cells in a non-activated state, leading to clonal exhaustion of T cells and tolerizing of DCs (Mantovani *et al.* 2008). Nevertheless, studies have shown that cancer patients have memory T cells and antibodies specific to cancer antigens, and yet cancer persists in these patients due to the suppressive environment around the tumor, which prevents the action of these cells and cell products (Mantovani *et al.* 2008). Infiltration of tumors by myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) creates an immunosuppressive environment that contributes to DC-mediated suppression of CD4+ and CD8+ effector cells and the generation of Tregs (Melief 2008). MDSCs are mostly immature myeloid cells whose differentiation into mature cells has been blocked (Gabrilovich *et al.* 2009, Disis *et al.* 2010). Cytokines secreted by tumors, MDSCs, or TAMs act directly on tumor antigen bearing DCs in the tumor environment or tumor draining lymph nodes, altering their phenotype and function. All of these contribute to effector T cell suppression. The activation level of DCs vary – immature DCs are found in the tumor mass with infiltrating cells, while mature DCs are confined to peritumoral areas in breast cancer (Aspord *et al.* 2007, Mantovani *et al.* 2008). The vast majority of DCs found are resting, nonactivated, or immature, all of which contribute to immune tolerance or suppression. DCs found in tumor draining lymph nodes have similar phenotypes. Antigen presentation to CD4+ or CD8+ T cells by immature DCs leads to clonal deletion, tolerance, or induction of Tregs, among others (Melief 2008).

There are two other types of myeloid cells that have an effect on T cell polarization in the tumor microenvironment: M1 and M2 macrophages. Like the classification of Th1 and Th2, M1 macrophages facilitate Th1 and CTL-mediated immunity, and M2 mediates Th2. Most TAMs are M2 macrophages (Mantovani *et al*.

2008). One of the most important differences between these types of macrophages is level of IL-12 secretion. M1s secrete high levels of IL-12, which is important for generation and maintenance of Th1 responses through DCs. M2s secrete low levels of this, and instead secrete IL-10. M2s contribute to the generation of Th2 cells, and have characteristics that are tumor promoting (Mantovani *et al.* 2008, Disis 2010). They also secrete proteases that contribute to increased invasion of tumors and metastasis, cytokines that block adaptive immune responses, and factors that increase angiogenesis. There are other innate cells that can contribute to tumor inflammation, such as neutrophils, mast cells, and eosinophils. B cells have also been shown to recruit inflammatory cells to the tumor environment (Disis 2010). These cells are not the focus of this study, and will not be discussed here.

In order to produce an effective vaccine for breast cancer, we must consider the environment in which vaccinated cells will be arriving. Our lab has studied the breast cancer microenvironment and concluded that tumors help program DCs to direct the development of CD4+ T cells secreting Th2 cytokines, especially IL-13, that facilitate tumor development and survival (Aspord *et al.* 2007, Pedroza-Gonzalez *et al.* 2011 and in Figure 3). Thus, it would be logical to avoid this T cell phenotype when generating T cells through vaccination, and also helpful to study whether vaccination can change the makeup of cells and cellular environment from facilitating Th2 (type 2), to Th1 (type 1). Type 1 immune responses include cytokines such as IFNγ and IFNα. IFNα in particular has a positive effect on cross presentation to CD8+ T cell through both DC activation and stimulation of CTLs. IFNγ contributes to the suppression of type 2 responses, and facilitates further IFNγ production in CD4+ and CD8+ T cells, encourages cytotoxic

function in CTLs, and upregulates MHC class I and II on DCs. This type of environment is much more beneficial for tumor destruction than a type 2 environment (Mantovani *et al*. 2008, Melief 2008).

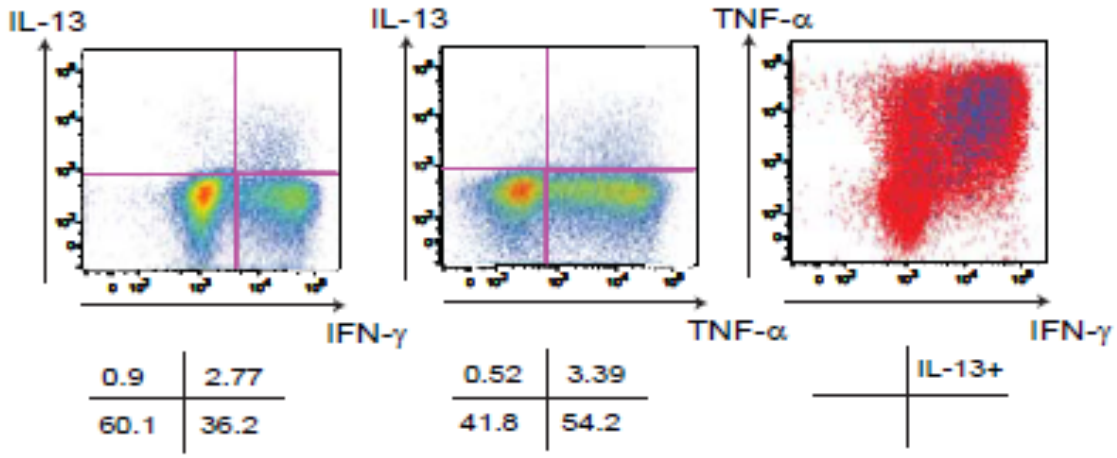

Figure 3. Inflammatory type 2 T cells in breast cancer patient tumor infiltrates. From Pedroza-Gonzalez *et al.* 2011. Single-cell suspensions from tumor samples were stimulated for 5 h with PMA and ionomycin. Cytokine production was measured by flow cytometry. Dot plots are gated on CD3+ CD4+ T cells. Blue indicates gate on CD3+CD4+IL-13+ T cells that coexpress IFNγ and TNFα. Representative of four different patients.

Inflammatory Th2 Driven by TSLP-OX40L Axis Persists in Breast Cancer Microenvironment

T cells infiltrating the tumor site secrete both type 1 and type 2 cytokines, specifically IFNγ, IL-4, and IL-13. It has been shown that CD4+ T cells in the tumor site can also secrete TNFα , the addition of which changes the classification to inflammatory Th2 (Liu *et al.* 2007, Aspord *et al.* 2007). But it is the DCs that are programmed by the tumor to generate and maintain these cells. Humice from our lab were implanted with tumors, and given DCs + CD4+ T cells, DC's alone, T cells alone, or PBS, and only the mice with both DCs and T cells showed significant tumor growth. DCs conditioned from primary tumor supernatant were able to induce a Th2 cytokine profile when cultured with CD4+ T cells. Additionally, in another experiment, mice were injected with either CD4+

or CD8+ T cells, and only those mice with CD4+ T cells showed significantly increased tumor growth. Tumor growth was significantly lowered with the administration of an IL-13 antagonist, suggesting that this process depends on IL-13. Therefore, these Th2 CD4+ T cells can facilitate tumor growth through secretion of IL-13, and they are dependent on the presence of DCs (Aspord *et al.* 2007).

To further investigate the molecular mechanisms of this Th2 inflammation, our lab focused on factors that contributed to this inflammation coming from breast cancer tumors and its effect on dendritic cells. We found that breast cancer tumors express, produce, and secrete TSLP, which induces expression of OX40L on dendritic cells. Blood mDCs that were cultured with sonicated breast cancer tumors acquired OX40L, and induced generation of inflammatory Th2 CD4+ T cells that secreted IL-13 and TNFα; blocking OX40L prevented the expansion of these T cells. Blocking TSLP in co-culture experiments also prevented the expansion of inflammatory Th2 cells. Expression of OX40L on DCs had already been shown to drive the differentiation of inflammatory CD4+ Th2 cells (Ito *et al.* 2005). OX40L positive DCs were found in the peritumoral areas of breast cancer tumors from patients. Indeed, when anti-TSLP or anti-OX40L was injected into humice bearing tumors and T cells, tumor growth was inhibited and the amount of IL-13 found was significantly less (Pedroza-Gonzalez *et al.* 2011). Therefore, breast cancer tumors secreting TSLP facilitate their own growth and survival by inducing expression of OX40L on DCs, which in turn differentiate CD4+ T cells into IL-13 secreting inflammatory Th2 cells.

TSLP-DCs were also shown to be able to attract Tregs to the lung cancer microenvironment by Li *et al.* 2011, who also found a correlation with the expression of

TSLP in lung tumor tissue and prevalence of Tregs, which would further lead to a suppressive environment. TSLP is also secreted by stromal cells in pancreatic cancer (De Monte *et al.* 2011). Therefore, this story has implications for other types of cancer. Clearly, creating a vaccine that could reprogram this skewing of CD4+ T cells into Th2 cells via TSLP-DCs in already established breast cancer tumor microenvironments would be advantageous.

Breast Cancer Antigens

Antigens

There are a number of antigens that are highly- or over-expressed in breast cancer tumors. These include but are not limited to: human epidermal growth factor receptor-2 (HER2/*neu*), Mucin 1 (MUC-1), insulin-like growth factor 1 receptor (IGFIR), heat shock protein Hypoxia up-regulated protein 1/ Oxygen-regulated protein 150 kDa (HYOU1/Orp150), Estrogen receptor (ERα), progesterone receptor (PR), Survivin, and Cyclin B1. Most of the antigens that are over-expressed in tumors are self antigens. In a recent study, it was shown that 46/75 possible tumor antigens could elicit immunity in clinical trials, and 20 out of those 46 had clinical efficacy. 80% of those 20 antigens were self antigens (Disis 2010). This highlights the difficulty of generating clinically effective immunotherapies, because self regulatory mechanisms that guard against autoimmunity must be overcome for therapies to be successful.

Tumors that over-express HER2/*neu* (about 25-35% of tumors) are associated with more aggressive disease and often correlate to a worse prognosis (Menard *et al.* 2004). HER2/*neu* over-expression on breast cancer can indicate susceptibility to certain

chemotherapy treatments: the recent drug development of Herceptin and other clinical trials centered around disruption of HER2/*neu* together highlight the importance of this receptor (Woll *et al.* 2004, Hueman *et al.* 2006, Hueman *et al.* 2007).

MUC-1, another known tumor antigen, is expressed in over 90% of breast cancers, as well as several other types of cancer (Singh and Bandyopadhyay 2007). MUC-1 is expressed in an extracellular, under-glycosylated form in cancer cells, but is found as a transmembrane, highly glycosylated glycoprotein in normal cells. Two epitopes that can elicit a MUC-1-specific immune response have been found (Broussart *et al.* 1999). Several studies using pulsed/loaded DCs with killed cancer cells or peptides have shown that MUC-1-specific CD8+ immunity can be created (Wierecky *et al.* 2006, Saito *et al.* 2006, Koido *et al.* 2005, Bohenkamp *et al.* 2004).

Other studies have shown that insulin-like growth factor 1 receptor (IGFIR) and heat shock protein HYOU1/Orp150 are also important candidates for breast cancer therapy because of their expression profiles on aggressive tumors, but extensive studies have yet to be completed (Chakraborty *et al.* 2008, Stojadinovic *et al.* 2007).

While there is much interest in the development of therapies targeting cancer antigens such as HER2 and MUC-1, it is important to note that cancer cells have the ability to mutate over time, and if expression of the target antigen is lost, the therapy will cease to be effective. One way to decrease the chance of this occurring is to select antigens that are vital to the survival of the cell, and therefore could not be lost or significantly mutated without cell death. One such protein is survivin. Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins that is generally known for its role in homeostasis and in cell division. In the context of cancer, it is one of the most

tumor-specific molecules found to date, and antagonizes apoptosis and promotes tumor-specific angiogenesis (Altieri 2008). Survivin is virtually undetectable in adult tissues (low expression in thymocytes and bone marrow derived stem cells during certain cell cycle phases), but has been identified as being over-expressed in a subset of all human cancers: lung, colon, breast, pancreas, stomach, liver, ovary, prostate, melanoma, lymphoma, and leukemia (Andersen *et al.* 2007). The over-expression of survivin in any cancer is a negative prognostic indicator, and is consistently associated with shorter life span, advanced disease state, resistance to therapy, and accelerated recurrences (Andersen *et al.* 2007). Experiments in our laboratory have shown that DCs loaded with killed allogeneic breast cancer cells presented survivin and were able to induce the generation of cytotoxic CD8+ T cells that were able to kill breast cancer cells in vitro (Saito *et al.* 2006). Survivin is a good candidate for further study, but there has been limited attention given to this protein in the context of immunotherapy. While we know that survivin can be presented by DCs, extensive work has not been done to address memory T cell repertoire or immunogenicity of this protein in breast cancer patients.

Cyclin B1

Cyclin B1 (CB1) is upregulated during the cell cycle, and is necessary for cell division. CB1 has been found to be over-expressed in multiple forms of cancer, including breast cancer, esophageal cancer, non-small cell lung cancer, renal cell cancer, and in many other cancers and related cancer cell lines (Aaltonen *et al.* 2009). Several studies have been published on the immunogenicity and T cell repertoire of CB1. However, there has been limited success when attempting to generate antigen-specific immunity. CB1 has been chosen as the model cancer antigen for this study because of the immune

system's ability to develop natural immunity to its antigens, established memory T cell repertoire in patients and healthy donors, its necessity to cancer cell survival, and its negative correlation with prognosis and treatment.

CB1 is a regulatory protein that is an essential component of the mitotic cell cycle. p53 regulates the G2-M transition of the cell cycle through CB1 expression (Innocente *et al.* 1999, Yu *et al.* 2002). CB1/Cyclin dependent kinase 1 (CDK-1) protein expression peaks at this stage of the cell cycle and is reduced to near zero during other stages. While small amounts of CB1 protein are produced in the cytoplasm of normal cells just prior to the G2 phase, it is transported to the nucleus shortly thereafter, and the remainder is degraded after it is used in mitosis. Therefore, CB1 is exclusively found in the nucleus during mitosis in normal cells. Disregulation of this checkpoint, by loss of p53 functionality or other means, leads to continuous and unscheduled CB1 expression in the cytoplasm, which in turn leads to continual substrate phosphorylation culminating in uncontrolled cell growth, as is seen in many cancers (Gorczyca *et al.* 1997, Surgue *et al.* 1997, Yu *et al.* 2002).

Abnormal CB1 expression was first discovered by Gong *et al.* 1994 in leukemic, breast, and colonic cancer cells lines. Kawamoto *et al.* 1997 later found that CB1 was expressed in all breast lesions examined. In cancer, CB1 protein is over-expressed during all phases of the cell cycle including G1, and reaches peak expression during the G2-M transition as in normal cells (Shen *et al.* 2004). Theoretically, because CB1 is expressed continuously in the cytoplasm of cancer cells, it may be processed and presented on the cell surface (Egloff *et al.* 2006, Kao *et al.* 2001).

In a study of 779 breast tumors and 53 cell lines, CB1 protein levels were found to be highest in breast cancers that are triple negative (those that do not express HER2, ER, or PR) and generally higher in tumors of advanced grade (Agarwal *et al.* 2009). CB1 was most highly correlated with tumors that showed highest cell proliferation and were therefore more aggressive. When CB1 expression was correlated with prognosis in hormone receptor positive breast cancers, it was shown to have the most significant correlation out of Cyclin B1, D1, and E1, suggesting that it can be an indicator of tumor recurrence and death (Agarwal *et al.* 2009). Another study by Aaltonen *et al.* 2009 analyzing 1348 invasive breast tumors showed that high CB1 expression was correlated with high tumor grade, large tumor size, positive node status, ERα and PR negativity, HER2 and p53 positivity, young age at diagnosis, high Cyclin E1 and A expression, high cell proliferation, shorter overall survival, and shorter metastasis free survival – in other words, cancers that are considered more aggressive. A study by Suzuki *et al.* 2007 showed that CB1 could be an independent prognostic factor (used by itself to indicate prognosis, independent of any other factors), and was confirmed by both Aaltonen *et al.* 2009 and Agarwal *et al.* 2009.

A few studies have attempted to disrupt CB1 to affect cancer cell survival. Androic *et al.* 2008 created CB1 siRNA that inhibited growth of cancer cell lines in vitro and in vivo. When this effect was combined with taxol, there was a marked increase in tumor cell apoptosis. However, this approach is currently not clinically feasible. CB1 is an essential cell cycle protein needed in normal cells to complete mitosis. However, without CB1, cancer cells are sure to die; therefore, a cancer cell mutating CB1 to escape the immune system is not a possibility.

Several studies have also shown that CB1 interacts with the adaptive immune system. CB1-specific antibodies are found in the blood of cancer patients, at both the premalignant and established phases (Covini *et al.* 1997, Suzuki *et al.* 2005 and Figure 4).

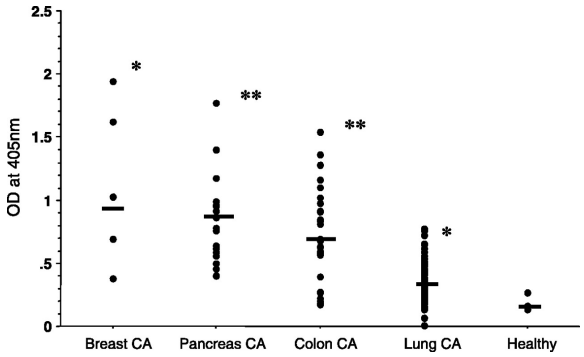

Figure 4. Cyclin B1 antibodies in cancer patients. From Suzuki *et al.* 2005. Sera from cancer patients and healthy donor controls were tested for their reactivity to recombinant cyclin B1 protein using an ELISA assay. Single patient's serum (•). Absorbance at 405 nm. *, $P < 0.05$; **, $P < 0.01$ (all values from patients with cancer were significantly higher than those from healthy middle age donors).

Certain CB1 HLA-A201 epitopes are capable of priming A2 matched healthy donor and breast cancer patient CD8+ T cells (Kao *et al.* 2001). The epitope sequences were eluted from HLA Class I molecules found on an A201+ epithelial cancer cell line, and then loaded onto DCs of an A201 healthy donor to prime CD8+ T cell responses in vitro. Kao *et al.* found antigen-specific IFNγ secretion by CD8+ T cells as measured by Enzyme-linked immunosorbent spot (ELIspot) assay after four rounds of stimulation. However, these T cells were not able to kill tumors from which the peptides were derived, suggesting that the peptides were of low affinity, or that the T cells were incapable of recognizing low level CB1 presentation by the tumors. In the same study, they were able to elicit memory responses in breast cancer patients and squamous cell carcinoma patients. The mutated peptides and CB1 derived peptides were able to elicit memory responses in 4/6 breast cancer patients using ex vivo PBMCs or PBMCS following one stimulation; however, only in A201 patients. Cells from 4/5 squamous cell

carcinoma patients had memory responses against CB1 peptides, and one of these patient's cells had the ability to lyse tumors from which the peptides were derived with additional CD80 costimulation in an A2 dependent manner (Figure 5). Without CD80 costimulation, the T cells were able to lyse tumor cells to a lesser extent. CB1 over-expression in tumors correlated with presence of CB1 memory T cells in patients, suggesting a link between the amount of peptide available and priming of CB1 T cells in patients.

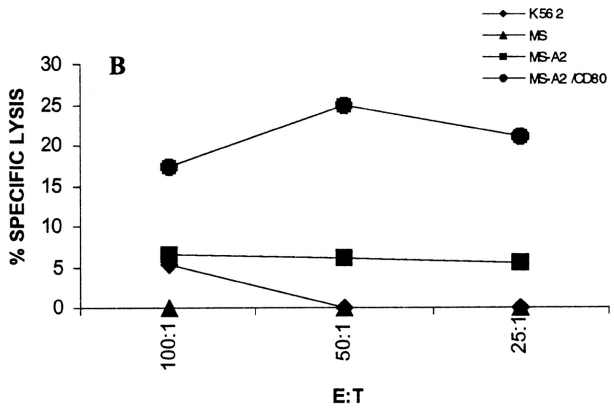

Figure 5. T cells from an HLA-A2.1+ Squamous cell carcinoma of the head and neck patient restimulated to cyclin B1 peptides in vitro are able to kill the original tumor. From Kao *et al.* 2001. T cells were restimulated with Cyclin B1 9mer for 5 days and tested in a CTL tumor killing assay.

It is important to note that the patients used in this study were post-operative, but had not received chemotherapy and were therefore not immunocompromised. This subject group is therefore not ideal as the vast majority of prospective patients, including those patients in which all other therapies had failed, have been exposed to chemotherapy and/or radiotherapy, and are at least somewhat immunocompromised. Nevertheless, this study provided evidence that it was possible to prime T cells or induce memory T cell responses with CB1-specific epitopes in an A2 dependent manner.

Taking into account the study above and the fact that Suzuki *et al.* 2005 studied correlation of CB1 antibody titers, it can be concluded that both CD4+ and CD8+ T cell responses are possible in vivo in cancer patients, including breast cancer.

Additionally, previous studies at our institute have found that CB1 is processed and presented on DCs loaded with killed allogeneic breast cancer cells, and these DCs can generate CTLs capable of killing CB1 expressing cell lines (Saito *et al.* 2006). DCs loaded with killed HLA-A*0201^{neg} T47D breast cancer cells, which express CB1, induce differentiation of CB1-specific T cells (Saito *et al.* 2006). CD8+ T cells primed in 3- one week cultures with DCs loaded with breast cancer cells were able to kill CB1 9mer peptide-pulsed T2 cells (Figure 6). The observed lysis was substantially higher than that of control PSA peptide-pulsed T2 cells or NK-sensitive K562 cells, therefore suggesting the presence of CB1-specific CTLs.

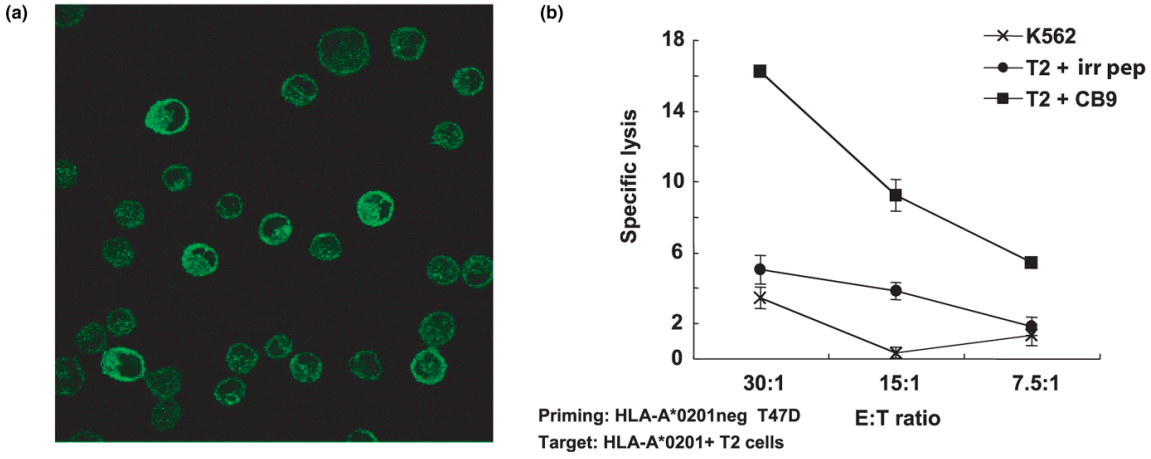

Figure 6. Cross priming of Cyclin B1-specific T cells. From Saito *et al.* 2006. A. Fluorescence microscopy analysis of Cyclin B1 staining with T47D breast cancer cells. B. Sorted Naïve CD8+ T cells were cultured for 3 weeks with autologous DCs loaded with HLA-A201 negative T47D breast cancer cells. CTLs killed T2 cells pulsed with Cyclin B1 derived 9mer peptide, but not T2 cells pulsed with irrelevant peptide (PSA), indicating the presence of Cyclin B1-specific CTLs. Representative of three experiments. Bars are standard deviation of triplicate wells.

This demonstration of priming breast cancer-specific CTLs was carried out with "classical" monocyte-derived DCs generated with GM-CSF and IL-4. However, myeloid DCs generated in the presence of type I interferon (IFN-DCs), TNFα (TNF-DCs) or IL-15 (IL-15-DCs) were found superior to IL4-DCs in cross-priming breast cancer-specific CD8+ T cell immunity (Ueno *et al.* 2007). This study provided evidence that sequences within the CB1 protein were immunogenic, available for processing from breast cancer cells, and able to prime T cells that subsequently killed CB1 target cells. While this data is important, it relied on an HLA-A201 positive donor, a haplotype that is not found in every individual with breast cancer. We will be relying on long peptides that contain a variety of different HLA immunogenic epitopes, with the hope that many more patients will benefit from it, regardless of HLA type.

Sorenson *et al.* 2009 conducted studies on breast cancer patient samples utilizing CB1 HLA-A2 mutated epitopes and were able to induce specific immunity in CD8+ T cells. Andersen *et al.* took this study a step further by using algorithms to determine sequences in CB1 that would bind to HLA molecules, and used the ones with highest affinity to load DCs and create T cell clones that killed cancer cell lines (Andersen *et al.* 2010). Cancer patient PBMCs responded to these peptides in an IFNγ ELIspot assay. However, again, this was in an HLA-A2-dependent manner, and did not incorporate other HLA types.

Another study was able to vaccinate mice with CB1 DNA-prime protein-boost vaccine to prevent growth of a transplanted CB1+ tumor (Vella *et al.* 2009a). This same group used p53-/- mice, which grow CB1+ tumor spontaneously, to show that vaccination could delay tumor growth and prolong survival.

Vella *et al.* 2009b also established that immunity to CB1 is found in healthy donors. They found CB1 antibodies and CB1-specific memory CD4+ and CD8+ T cells in circulation after restimulation with peptides comprising an immunogenic region of CB1. The T cells proliferated and produced IFNγ in response to CB1 peptide (Figure 7 and 8). The peptides used in this study were medium length peptides included regions that have HLA epitopes other than HLA-A2 (Figure 8), providing evidence that immunity can be induced quite readily even when A2 epitopes are not utilized.

Specific Aims of Dissertation

As mentioned previously, dendritic cells are the cornerstone of a robust immune response. Therefore, it makes sense to utilize a vaccine that will induce immunity through DCs. When properly activated, DCs loaded with an appropriate amount of antigen are delivered to the body, migrate to lymph nodes draining from tumors, and expand cancer-specific CD4+ and CD8+ T cells. These cells will ideally have immediate tumoricidal effects, and also create a memory population that will become sentinels against recurrence. Although much research has been done with dendritic cell vaccines in various forms of cancer, and despite partial successes and even clinical regressions, the results have not been as good as expected. There are many hurdles to overcome in cancer immunotherapy: suppressive microenvironment with both cell products and suppressive immune cells; immunoediting of cancer epitopes as a means of immune escape; various structural barriers to effector T cells entering the tumor area; and other factors relating to tumor cell biology and its interaction with the stromal

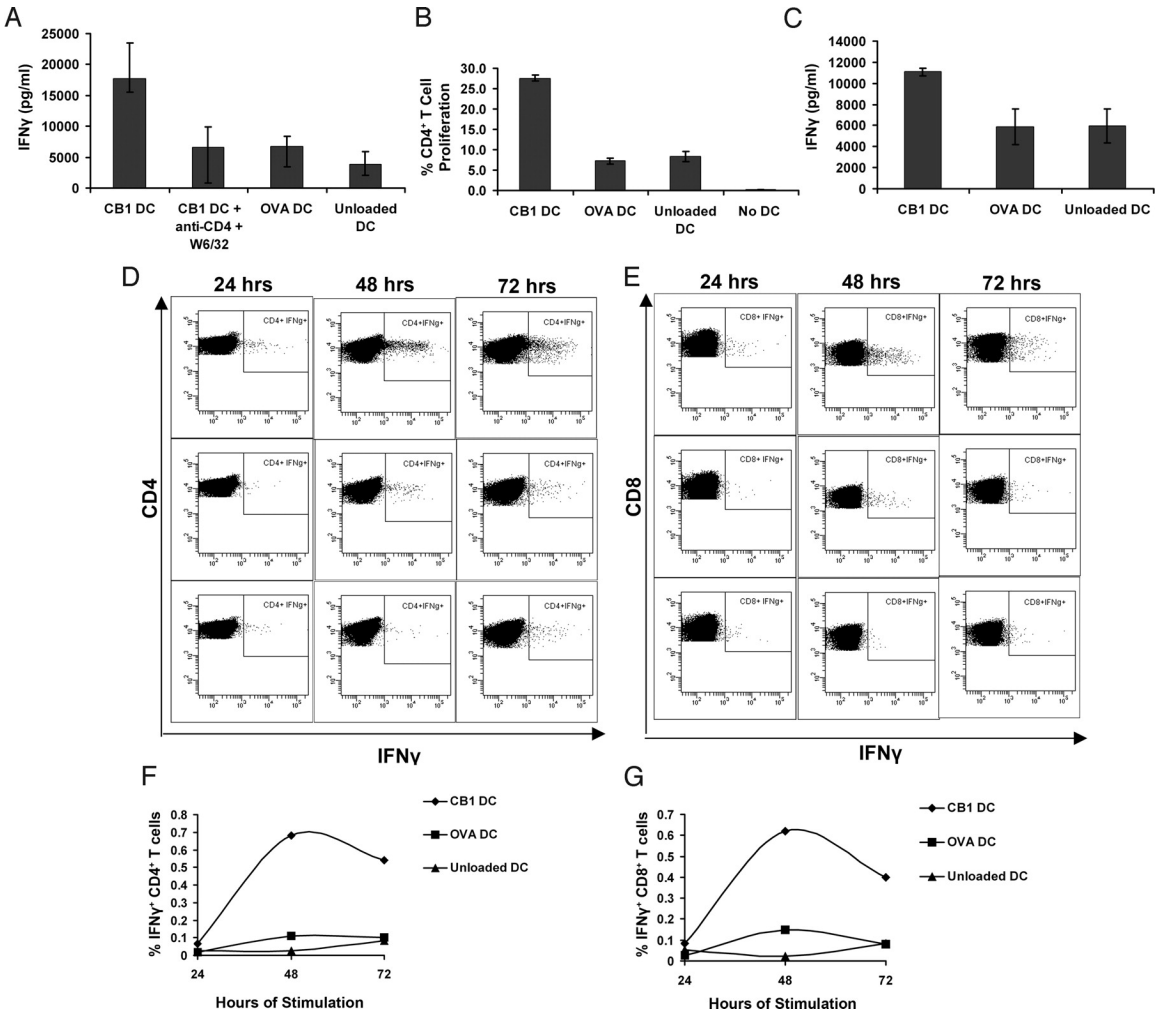

Figure 7. Healthy individuals have memory T cells specific for cyclin B1. From Vella *et al.* 2009b. (A) All T cells: Monocyte-depleted PBMC were cultured with autologous DCs that were loaded with ovalbumin (OVA), cyclin B1 (CB1), or unloaded. Supernatant from the seventh day of culture was tested by ELISA for IFNγ. W6/32: MHC class I blocking antibody. (B) CD4+ T cells: PBMC from the same donor as in A were labeled with CFSE and cultured with autologous DCs in the presence or absence of indicated antigen or without DC. Percentage of proliferating CD4+ T cells was assessed after seven days. (C) CD8+ T cells: CD8+ T cells were purified from PBMC (a second donor) and cultured with autologous DCs with and without indicated antigens. Supernatants were tested for IFNγ after 10 days. Bars indicate standard deviation. (D–G) Brefeldin A was added for 11 h to one set of a triplicate culture at 6, 30, and 54 h after combination of DCs with PBL. After the incubation periods, CD4+ T cells (D and F) and CD8+ T cells (E and G) were assessed for intracellular IFNγ. (D and E) Flow cytometric measurement of IFNγ. (Top) PBL stimulated with cyclin B1-loaded DCs. (Center) PBL stimulated with OVA-loaded DCs. (Lower) PBL stimulated with unloaded DCs. (F and G) show a graphical representation of the percentage of IFNγ-positive cells for CD4+ (F) and CD8+ (G) T cells.

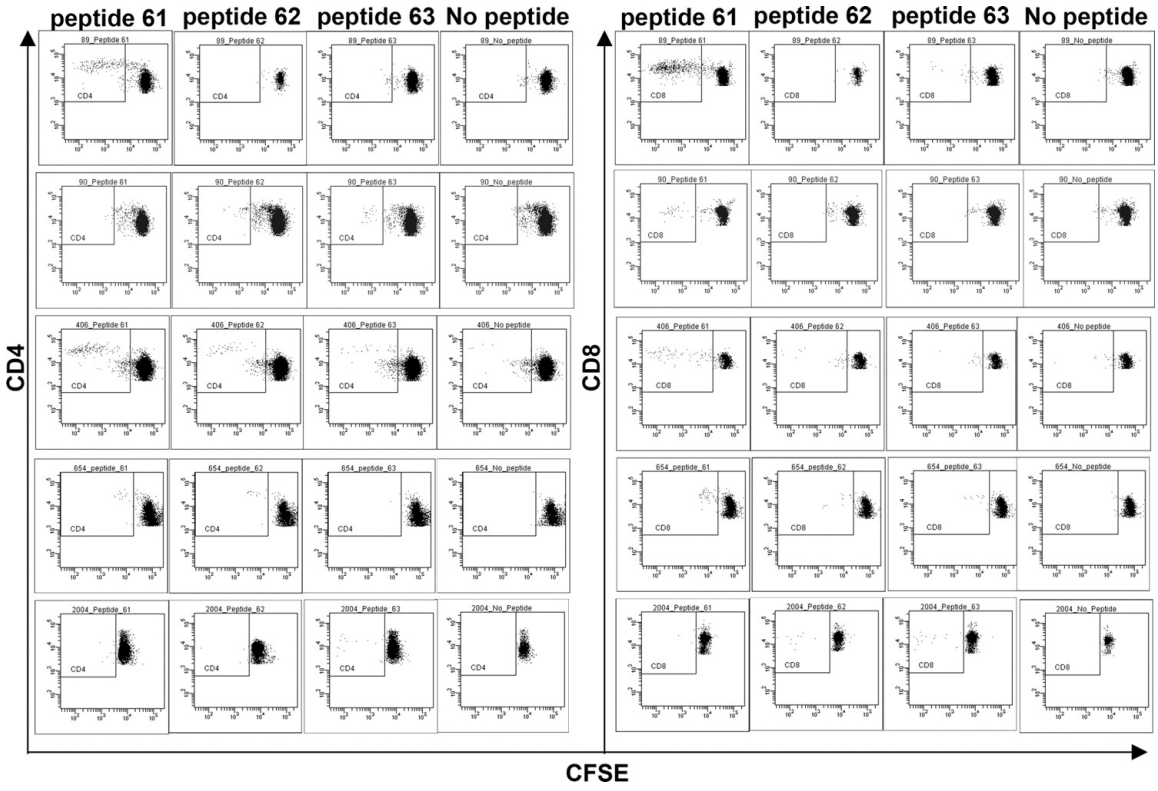

Figure 8. Identification of commonly recognized cyclin B1 peptides. From Vella *et al.* 2009b. PBMC were labeled with CFSE and stimulated with 2 µg/mL recombinant cyclin B1 peptides. After six days of culture, PBMC were stained with cell surface markers and proliferation was assessed by flow cytometry. peptide 61: KFRLLQETMYMTVSI; peptide 62: LQETMYMTVSIIDRF; and peptide 63: MYMTVSIIDRFM.

environment. On the experimental side, finding the correct dose of antigen and proper method of activating DCs has also been challenging. DC cancer vaccines to date have been limited in the types of HLA epitopes and length of peptides. This could limit the types of cells that are generated: robust CD8+ T cell responses require equally strong Th1 CD4+-specific T cells responses, and are considered necessary for an effective immune response against cancer (Melief and van der Berg 2008). Thus, there are many improvements that can be made to current cancer immunotherapies. We will address many of the experimental challenges here, as well as give thought to the environment in which generated T cells will be exposed to in cancer patients.

Specific Aims

The aim of my dissertation is to develop a vaccine that has the ability to reprogram or augment the natural T cell-specific immune response to CB1 antigen in breast cancer, with the intent of eventually using this vaccine to prove efficacy in vivo and in clinical trials. The breast cancer microenvironment is skewed towards a Th2-promoting milieu and encompasses several cell types that help create a feedback loop that encourages tumor growth, survival, and immune escape. CD8+ T cells are the preferred cell type to engage in destroying patient tumors, and these cells need strong Th1 CD4+ T cell help, as well as cooperation from other important cell types such as DCs and macrophages. We believe that inducing immunity to CB1 through the use of long peptides in T cells, in combination with CL-075, a TLR8/7 agonist that can act on other cell types to foster type I responses, can help change the breast cancer microenvironment and develop immunity to allow T cells the ability to perform their cytotoxic functions of tumor destruction. To accomplish this overall goal, we followed specific aims:

1. Establish CB1 immunity in healthy donors: using both short peptides and long peptides derived from the CB1 sequence through both DC/T cell co-cultures and PBMC cultures.
2. Establish CB1 immunity in breast cancer patients: through PBMC cultures and monitoring immune response by cytokine secretion.
3. Use TLR8/7 agonist CL-075 as an adjuvant in conjunction with long peptides to reprogam natural immune responses in healthy donors and breast cancer patients to CB1, in order to increase levels of type I cytokines or decrease type 2 cytokines.

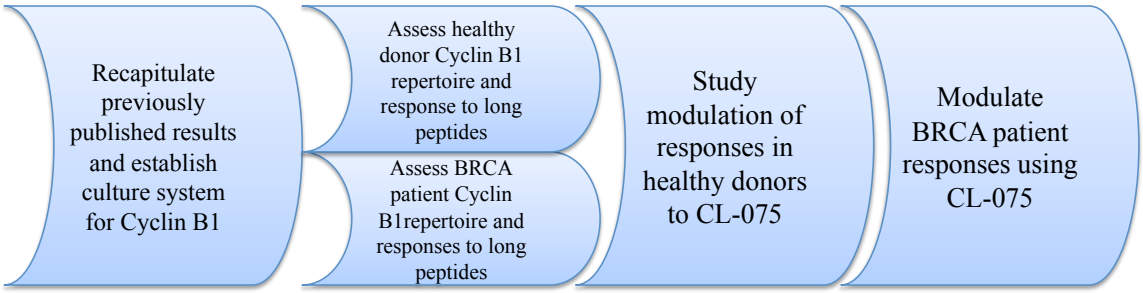

Figure 9. Visual representation of the flow of aims for this study.

With immunotherapy of breast cancer patients being the ultimate goal, we provide evidence here that our vaccination strategy is able to induce type I T cell immunity in both healthy donors and breast cancer patients, as well as modulate type 2 responses. We hope that these results will culminate in the generation of a vaccine that can be used in a clinical setting for breast cancer patients.

CHAPTER TWO

Materials and Methods

Experimental Outline

The overall experimental outline for this study that will be used to accomplish our aims is presented in the scheme in Figure 10.

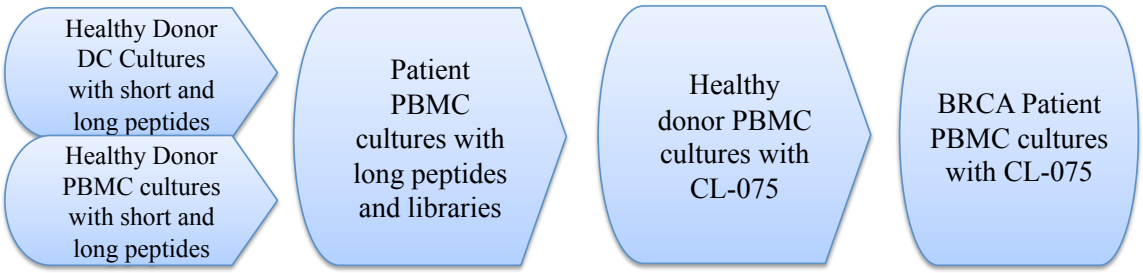

Figure 10. Overview of experimental scheme

Peptides and Targeting Reagents

Cyclin B1 Peptide Sequences

The Cyclin B1 36mer long peptide was derived from sequences provided by Olivera J. Finn. Sequence for Cyclin B1 50mer long peptide was derived from data from Saito *et al.* 2006, plus consideration of other immunogenic regions around the HLA-A2 9mer region by Hideki Ueno and A. Karolina Palucka. Cyclin B1 9mer and 10mers are from Saito *et al.* 2006 and Kao *et al.* 2001. Cyclin B1 36mer library 15mers are sequences from Vella *et al.* 2009b. Cyclin B1 50mer library 15mer sequences were derived by creating a library of 15mer sequences from the entire Cyclin B1 protein IFNγ sequence, and using all 15mers that were part of the 50mer sequence, from beginning to

end. Cyclin B1 36mer: DWLVQVQMKFRLLQETMYMTVSIIDRFMQNNCVPKK Cyclin B1 50mer: MEMKILRALNFGLGRPLPLHFLRRASKIGEVDVEQHTL AKYLMELTMLDY Cyclin B1 9mer: AKYLMELTM Cyclin B1 10mer: AKYLMELTML. Sequence of the entire Cyclin B1 protein with 36mer and 50mer sequences highlighted are in Figure 11. Sequences for Cyclin B1 long peptide 15mer libraries, along with cluster groupings, are found in Figures 12, 13, and Table 1. All peptides were manufactured by Biosynthesis (Lewisville, TX). Peptides are reconstituted in 50% acetonitrile (Fluka, Buchs Germany) 50% Water with Chromasolv (Sigma) solution (combination referred to from here as 'carrier') and stored at 1mM in -80C until use.

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1 malrvtrnsk inaenkakin magakrvpta paatskpglr prtalgdign kvseqlqakm
61 pmkkeakpsa tgkvidkklp kplekvpmlv pvpvsepvpe pepepepepv keeklspepi
121 lvdtaspspm etsgcapaee dlcqafsdvi lavndvdaed gadpnlcsey vkdiyaylrq
181 leeeqavrpk yllgrevtgn mrailidwlv qvqmkfrllq etmymtvsii drfmqnncvp
241 kkmlqlvgvt amfiaskyee myppeigdfa fvtdntytkh qirqmemkil ralnfglgrp
301 lplhflrras kigevdveqh tlakylmelt mldydmvhfp psqiaagafc lalkildnge
361 wtptlqhyls yteesllpvm qhlaknvvmv nqgltkhmtv knkyatskha kistlpqlns
421 alvqdlakav akv

- Blue = 36mer region
- Red = 50mer region

Figure 11. Cyclin B1 protein sequence and long peptide location. Numbers represent amino acid position.

Figure 12. Cyclin B1 36mer and 36mer library of 15mers. Figure demonstrates the overlapping nature of the 15mer library.

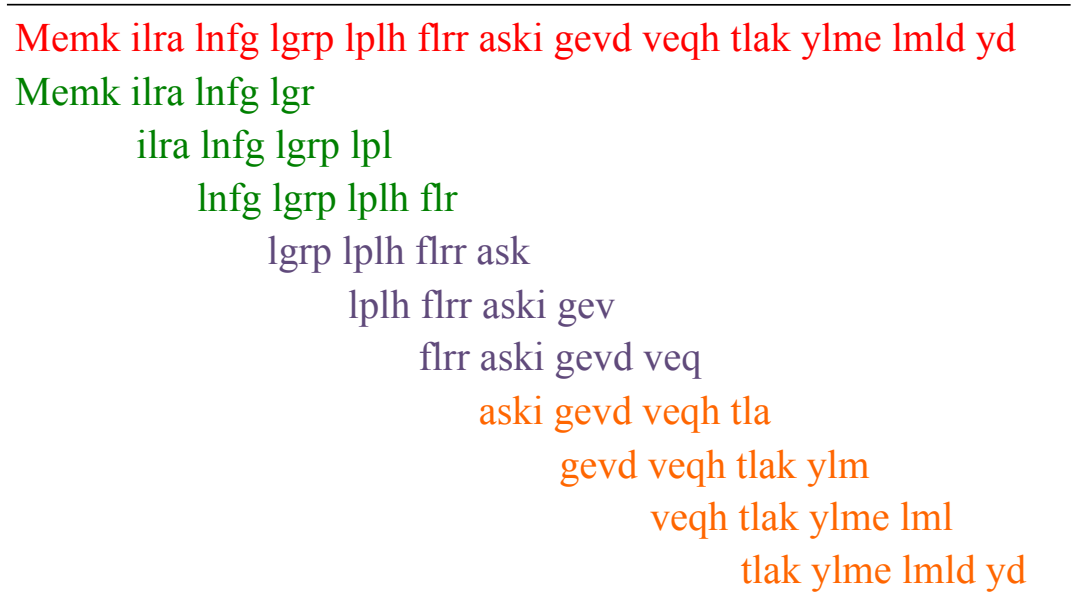

Figure 13. Cyclin B1 50mer and 50mer library of 15mers. Figure demonstrates the overlapping nature of the 15mer library.

Table 1: Cyclin B1 long peptide library sequences and cluster groupings.
Peptide is often shortened to "p" within text or figures, and cluster is shortened to "c".

36MER	C1	Peptide 1: DWLVQVQMKFRLLQE
		Peptide 2: QVQMKFRLLQETMYM
		Peptide 3: KFRLLQETMYMTVSI
	C2	Peptide 4: LQETMYMTVSIIDRF
		Peptide 5: MYMTVSIIDRFMQNN
		Peptide 6: VSIIDRFMQNNCVPK
		Peptide 7 (12mer): MYMTVSIIDRFM
50MER	C1	Peptide 72: MEMKILRALNFGLGR
		Peptide 73: ILRALNFGLGRPLPL
		Peptide 74: LNFGLGRPLPLHFLR
	C2	Peptide 75: LGRPLPLHFLRRASK
		Peptide 76: LPLHFLRRASKIGEV
		Peptide 77: FLRRASKIGEVDVEQ
	C3	Peptide 78: ASKIGEVDVEQHTLA
		Peptide 79: GEVDVEQHTLAKYLM
		Peptide 80: VEQHTLAKYLMELTM
		Peptide 81: TLAKYLMELTMLDYD

T2 Peptide Binding Assay

T2 cells were thawed from -180C by washing twice in Phosphate buffered saline (PBS) and once in complete Roswell Park Memorial Institue (cRPMI) 1640 medium [RPMI 1640, 1% penicillin/streptomycin (Pen/strep), 1% glutamine, 1% Sodium Pyruvate, 5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% Non-Essential Amino Acids, 0.1% 2-B-mercaptoethanol(2-BME)] plus 10% Fetal calf serum (FCS) or R-10. T2 cells were cultured for 2 days and harvested for binding experiment. In a 96 well U-bottom plate, 9mer, 10mer, Flu M1 peptide, and no peptide control wells were set up for serial dilution, starting at 80uM, and dividing by 2 until a concentration of 39nM was reached, 11 dilutions later. T2 cells were placed 100,000 cells per well to make the final volume 200ul and incubated overnight (18 hours). The plate was washed once with PBS to remove free peptide, and resuspended in staining media (PBS + 2%

FCS). HLA-A2 BB7.2 antibody (PE – BD) was added with media to make the final volume of each well 100ul, and stained for 30 mins on ice in the dark. Cells were washed with PBS and fixed with 1% Paraformaldehyde (PFA) and acquired on BD FACSCalibur Flow Cytometer (BD, San Jose, CA). The mean fluorescence intensity of the live T2 cells under the PE channel was calculated by the following formula: mean fluorescent index (MFI) of T2 cells stained with A2 antibody minus the MFI of unstained T2 cells without peptide, divided by the MFI of unstained T2 cells without peptide. A XY plot was constructed to determine the peptide with greater affinity. Higher FI equals higher affinity. Results are in Figure 14 below.

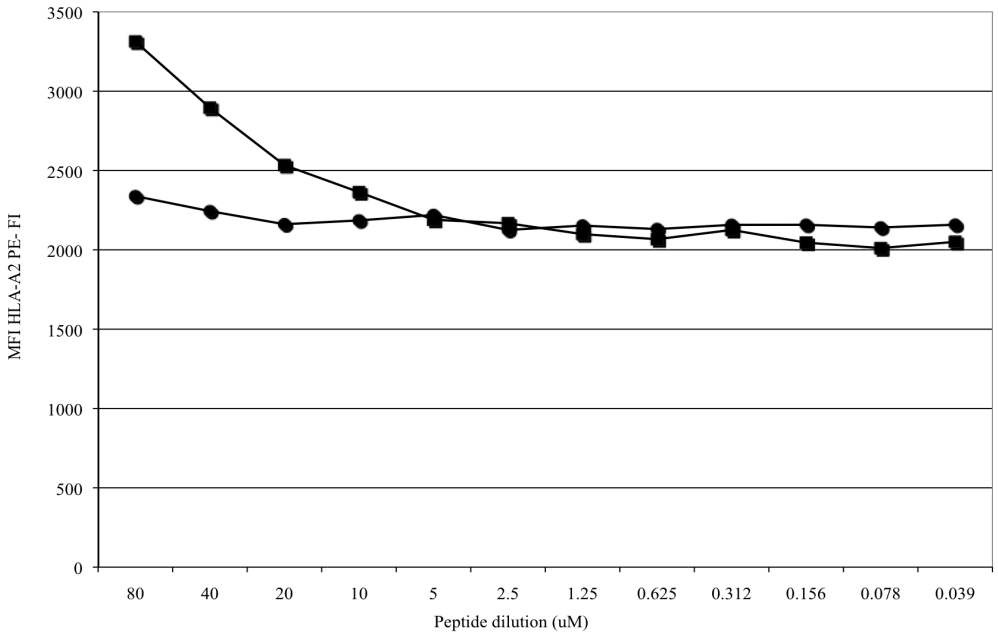

Figure 14. Peptide Binding Assay for HLA-A2 Cyclin B1 short peptides. Squares – Cyclin B1 10mer. Circles– Cyclin B1 9mer. Cyclin B1 10mer has better binding ability to HLA-A2 binding groove than 9mer above concentrations of 10uM.

Targeting Reagents

Anti-DC targeting reagents used in this study were obtained in house from Gerard and Sandy Zurawski through a proprietary method. They consist of a human IgG4 antibody genetically engineered to be specific for binding to CD40, a molecule found on the surface of DCs. Cyclin B1 peptides are engineered to be attached to the antibody via a unique flex region. The antibody used in this study was specific to CD40 and contained both Cyclin B1 long peptides, 36mer and 50mer.

Healthy Donor PBMC Cultures

Overall Scheme

The protocol used in this study for healthy donor PBMCs with an endpoint of intracellular staining is presented in Figure 15.

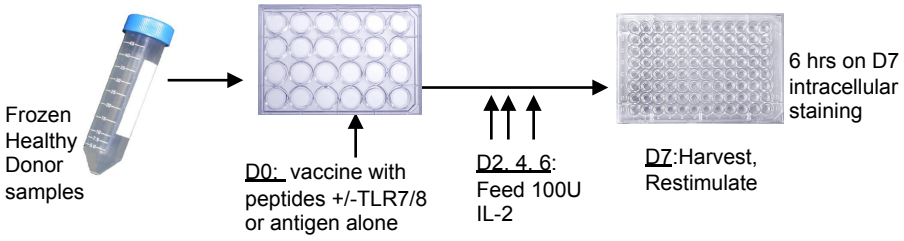

Figure 15. Protocol for healthy donor PBMC cultures D= day.

Cell Sample Collection and Storage

Apheresis blood draws were obtained through the cell and tissue core at BIIR. Samples were layered onto Ficoll (GE) and centrifuged at low speeds to isolate peripheral blood mononuclear cells (PBMCs). Alternatively, to obtain cell fractions, blood samples from leukapheresis are passed through counter-flow centrifugal elutration, which separates cells based on density and size. Peripheral blood monocytes were enriched

according to cellular density and size by elutriation (Elutra, CaridianBCT, Lakewood, CO) per manufacturers instructions. The automated program separates cells into 5 fractions using different flow rates and centrifugation speeds. Elutriation Fraction 5 consists mainly of monocytes, ~85% on average, with the remainder of the cells being granulocytes, lymphocytes, eosinophils and basophils as measured by diagnostic hemacytometry on ABX Pentra 60C+ (Horiba ABX Diagnostics, Montpellier, France), which incorporates cytochemistry, focused flow impedance, light absorbance and flow cytometry for calculating complete blood cell count with differential. Fractions that contain mostly T cells (Fraction 2/3) or mostly monocytes (Fraction 5) are used for this study. These samples are suspended in a solution of freezing media consisting of 90% FCS and 10% dimethyl sulfoxide (DMSO), and are stored in liquid nitrogen (-180C) until use.

PBMC Cultures

PBMC vials are removed from storage and thawed at 37C for five minutes. Cells are transferred to a 50mL conical vial with 1X PBS (Gibco) and centrifuged at 1200rpm for 8 minutes (mins). The PBS is then aspirated off of the cell pellet, and cells are resuspended in PBS, then centrifuged again at 1200rpm 8 mins. Cells are then resuspended in cell culture media cRPMI plus 10% human Ab Serum (R-Ab), at 10mLs per vial thawed. Cells are incubated (37C, 5%CO2) for one hour to rest, then centrifuged again at 1200rpm, 8 mins. Cells are resuspended for counting, either in PBS for CFSE labeling, or in R-Ab if not. Trypan Blue (Invitrogen) cell viability dye is used to dilute cells further for counting. Total cells were calculated by multiplying: a) the average of total viable cells counted on two 16-square grids of a hemacytometer, b) the dilution

factor, c) inverse of volume of sample in hemacytometer in mLs, and d) total volume of media cells are suspended in from which the aliquot was sampled. Recovery was calculated by dividing the total number of cells counted by trypan blue, divided by the number of cells frozen per vial, then multiplied by 100 for percentage. If CFSE labeling is not required, cells are then resuspended at a concentration of 1x10e6 per mL. Cells are plated 2x10e6 cells per well in a 24 well plate for 7 days. Cells receive IL-2 (R&D Systems, Minneapolis MN) at 100U/mL on days 2, 4, and 6 at the same time that half the media is changed. On day 7, media and cells are harvested from each well by thorough but gentle washing. Wells are covered in PBS and allowed to sit for 15 minutes, then re-harvested using same technique. Cells are washed in PBS, aspirated, and counted to determine concentration for restimulation. During most typical experiments, after peptides were titrated and optimal concentrations were determined, peptides were added in the following concentrations: 36mer long peptide at 3uM, 50mer long peptide at 0.3uM, Carrier solution at 1uL per mL, and Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Flu Matrix 1 virus (Flu-M1), i.e. CEF, peptide mix at 1uM.

CFSE Labeling

CFSE labeling is used to study the proliferation and cell division of cultures (Figure 16). Cells are labeled with Cell Trace CFSE (Carboxyfluorescein diacetate succinimidyl ester – Invitrogen) after cells rest, and before they are counted and resuspended in R-Ab for plating. Cells are labeled according to manufacturer's instruction. Cells are counted and resuspended in 10x10e6 cells per mL of PBS in a 50mL vial. CFSE is diluted and a 1uM solution is made in PBS. Cells are incubated in a 1 : 10 ratio of CFSE solution to cells in PBS for 8 minutes. R-Ab is added to the vial at a

volume twice that of the staining volume to quench the staining reaction. Cells are washed in R-Ab and resuspended for counting and plating.

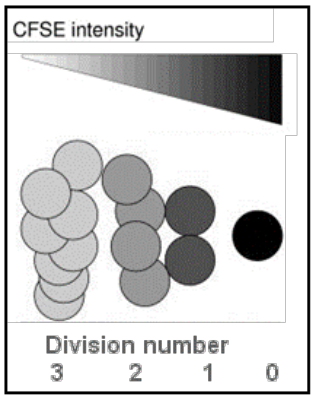

Figure 16. Representation of CFSE dilution as it relates to cell proliferation. Proliferation of labeled cells divides the amount of CFSE on cell in half for each cell division. Cells that express low amounts of CFSE have gone through more rounds of division.

Healthy Donor Dendritic Cell/ T Cell Cultures

Overall Scheme

The protocol used for cocluture of IFNα DCs and T cells with the endopoint of T cell intracellular staining is presented in Figure 17.

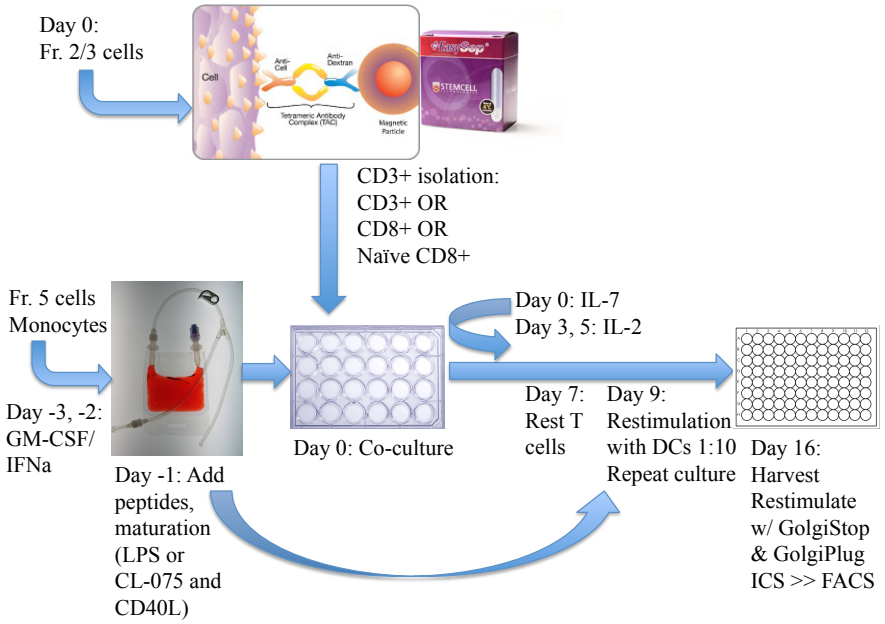

Figure17: IFNα DC/ T cell coculture protocol.

IFNα DC Culture

Frozen cells from Fraction 5 are thawed and washed in PBS twice. Cells are then washed in CellGenix (CellGenix Technologie Transfer GmbH, Germany) media and counted. Cells are added to cell culture bags (AFC, Gaithersburg, MD) at 1x10e6 cells/mL in CellGenix. Cells are cultured with 100ng/mL of GM-CSF (Leukine, Berlex, Wayne NJ) and 500 IU/mL of IFNα (Schering). On day 1, an additional dose of GM-CSF and IFNα is added in 1mL of CellGenix. On day 2, peptides are added overnight into the culture bags in the afternoon. The next morning on day 3, LPS (NIH, Bethesda MD) is added at 3ng/mL for 4 hours, followed by adding CD40L (R&D systems) at 100ng/mL for an additional 2 hours. Cells were then washed from the bag using PBS, and counted for plating with T cells. DCs that were not used immediately in culture were placed in freezing media and frozen in -80C for no longer than 1 month for use in restimulation. DCs were stained for DC phenotype via FACS and assessed for morphology via Geimsa staining. DCs are centrifuged by a Shandon cytospin (Thermo Shandon, Pittsburgh, PA), to adhere to a glass slide. The slide is then treated with Dif-Quick fixative, solution I, solution II (all from Siemans, IL), and rinsed. Once the glass slide was dry, a cover slip was fixed over the cells. Cells were observed under an Olympus light microscope at 20X and 40X magnification.

Autologous Naïve/Total CD8 Isolation

Naïve, or total, autologous CD8 T cells are isolated for culture with DCs. Cells are isolated on day 3 of DC culture for culture. Both cell population's isolation/harvest are timed so that they are ready to be plated in co-culture at the same time. Cells of Fraction 2/3 are thawed and washed in PBS-2%FCS twice. Cells are resuspended in

R-Ab with 100ug/mL of DNase I and incubated at room temperature (RT) for 15 minutes. Cells are strained for clumps, counted, then washed and resuspended at 50x10e6 cells per mL. anti-CD45RO Biotin (Stemcell) is added at 20ul/mL and incubated for 10 at RT in the dark if naïve cells are needed. Cells are washed and resuspended at 50x10e6 cells per mL. 100ul of anti-biotin TAC (Stemcell) is added, along with 50ul/mL of CD8 enrichment cocktail (Stemcell) and incubated for 10 mins at RT in the dark. 50ul/mL of CD8 nanoparticles (Stemcell) are added, followed by another incubation 10mins at RT in the dark. If total CD8 T cells are needed, only the CD8 enrichment cocktail and nanoparticles are used. Cells are then divided into 3mL aliquots for magnetic separation into polystyrene 12x75mm FACS tubes. Tubes are placed into Stemcell magnets for 5 mins and the cell suspension is then removed and placed in a new FACS tube. This process is repeated twice more to ensure highest purity of cells. A small sample of cells is taken for a purity check by staining for CD3, CD4, CD8, and CD45RA and analyzing by flow cytometry. Purity of cells was consistently above 95%.

DC-T Cell Coculture

DCs and T cells are cultured in R-Ab media. On day 0, 2x10e6 T cells and 200,000 DCs are plated in a 24 well plate in 2mLs with 10 IU/mL of recombinant human IL-7 (R&D). On day 3, 10 IU/mL of IL-7 is added along with 10 IU/mL of recombinant human IL-2 (R&D). Cells are split when necessary on days 3, 5, and 7, while adding 10 IU/mL of IL-2. On day 10, cells are harvested from wells, washed, and rested in 1% R-Ab for two days at 2x10e6 cells per well. At this point, cells are either restimulated for another round of culture with DCs and cultured for another 10 days, or restimulated for intracellular staining (ICS).

DC Culture Restimulation

DCs are made from thawed fraction 5 monocytes with GM-CSF and IFNα as previously described using the 3 day protocol. Alternatively, leftover DCs from day 1 of culture were resuspended 1x10e6 per mL, and stimulated for 2 hours with their respective peptides at 10uM. DCs were then plated 200,000 DCs per well. T cells are re-plated 2x10e6 cells per well. CD40L (100ng/mL) is added to each well, along with a dose of IL-7 and IL-2 (10 IU/mL). Total well volume is 2mLs. 10 day coculture is repeated.

Patient Cell Cultures

Overall Scheme for Patients

The overall experimental protocol for patient PBMCs and their healthy donor controls with an endpoint of Luminex is shown in Figures 19 and 20.

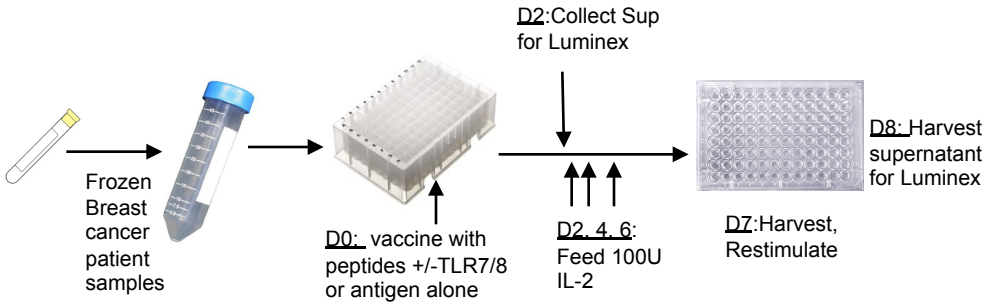

Figure 18. Protocol for breast cancer patient PBMC cultures. D = day.

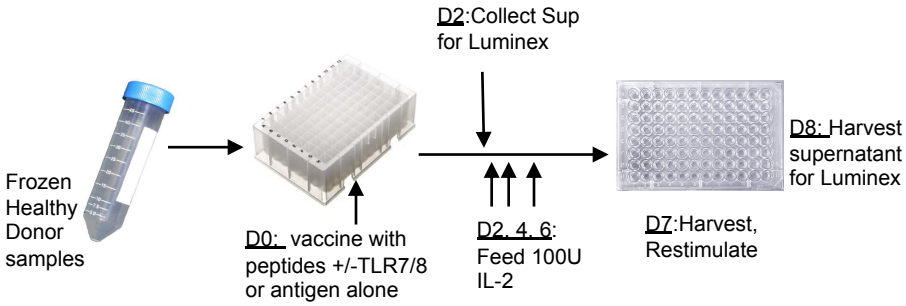

Figure 19. Healthy control PBMC protocol. D = day.

Patient Cell Sample Collection

Patient cells were collected from a breast cancer clinic on a voluntary basis following appropriate IRB protocols along with patient information. Patients samples used in this study were collected from Baylor Medical Center in Dallas, TX at the generous discretion of Joyce O'Shaughnessy, M.D., John E. Pippen, M.D., F.A.C.P., Joanne Blum, M.D., Ph.D., F.A.C.P., and with the help of Freda Murray N.P. and Dr. Luz Muniz. The average age of participants was 54.3 years (median 54.5 years). 19 patients were of Caucasian ethnicity and 5 were African American. Average date of surgery to remove primary tumor was 2005. 23 patients had cancer that originated in ductal cells of the breast, while 2 were lobular. Four patients had stage 4 breast cancer, nine had stage 3, nine had stage 2, and two patients had stage 1 cancer, with the mean cancer stage of 2.63. Patients varied in the number of lymph nodes cancer to which had spread, with the range being 0-17 and the mean 3.57. Patients had a heterogeneity of hormone receptor markers (ER, PR, and HER2) as summarized in Table 2. The average number of chemotherapy drugs that patients were actively receiving, or had received in the past, was 3.75, and average number of total cycles was 9. 17 patients were currently receiving some sort of chemotherapy regimen, while 8 were not. The average number of white blood cells in patient blood samples was 1.7x10e3/mm^3; average percentage of lymphocytes was 62.39%; average percentage of monocytes was 29.76%; average percentage of neutrophils was 7.12%; average number of platelets was 58.46x10e3/mm^3. A full description of all patients is in Table 2.

BIIR sample core isolated PBMCs. Cells were layered on Ficoll (GE Healthcare Life Sciences) and PBMC layer was removed and washed. PBMCs were aliquoted and frozen 5x10e6 cells per vial.

Patient PBMC Cultures

PBMC vials were removed from cryostorage and thawed at 37C for five minutes. Cells were transferred to a 50mL conical vial with PBS and washed twice. Cells were then resuspended in R-Ab, at 10mLs per vial thawed. Cells are incubated (37C, 5%CO2) for one hour, then washed. Cells are resuspended for counting either in R-Ab or PBS. Trypan Blue cell viability dye is used to dilute cells further for counting. Cells are then resuspended at a concentration of 2.5x10e6 per mL. Cells are plated 500,000 cells per well in 200uL in a 96 deep well plate for 7 days. On day 2, supernatant is removed for Luminex analysis and 1mL of media is added. Cells receive IL-2 at 100U/mL on days 2, 4, and 6 at the same time that half the media is changed. On day 7, media and cells are harvested from each well. Cells are washed in PBS, aspirated, and counted to determine concentration for restimulation. All conditions within one experiment are restimulated with the same number of cells. Cells are plated in 200uL in a 96 well U bottom plate for restimulation. 24 hours after restimulation, supernatants were taken from each well and prepared for Luminex analysis. Cell pellets were frozen in a 96 well V bottom plate for future use, or stained to determine T cell composition. At day 0, peptides were added in the following concentrations: 36mer long peptide at 3uM, 50mer long peptide at 0.3uM, Carrier solution at 1uL per mL, CEF peptide mix at 1uM, 36mer long peptide 15mer library clusters (1uM each), and 50mer long peptide 15mer library clusters (1uM each).

Table 2. Breast Cancer patients used in this study. WBC: white blood cells. WBC count and Platelets count in 10^3/mm^3. Lymphocytes, Monoctyes, and Neutrophils are expressed in percentages.

Patient	Age	Ethniticity	Yrs since surgery	Tumor Origin	Tumor Stage	Tumor Grade	# lymph nodes	ER	PR	Her2	#Chemo drugs	Cycles	Current Chemo	WBC	Lymphocytes	Monocytes	Neutrophils	Platelets
BRCA1	56	White	8	Ductal	3	c	10	no	no	no	4	8	N	1.2	73.1	24	1.9	114
BRCA2	55	White	3	Ductal	1		0	yes	no	no	2	4	N	1.5	76.2	20.9	2.4	94
BRCA3	84	White	0	Ductal	3	c	6	no	no		0	0	N	1.4	65.1	15.5	18.9	113
BRCA4	38	White	3	Ductal	3	b	6	no	no	no	5	11	Y	1.1	74	16.4	8.8	134
BRCA5	53	White	7	Ductal	3	a	6	yes	yes	no	4	8	Y	1	75.8	20.4	3	10
BRCA6	54	White	8	Lobular	3	a	4	yes	yes	no	6	8	Y	0.9	46.7	38.5	13.7	164
BRCA7	56	White	4	Ductal	3	c	17	yes	yes	no	3	4	Y	1.3	71.6	19.6	8.4	130
BRCA8	44	Black	1	Ductal	4		3	no	no	no	5	8	N	0.9	44.1	46	8.7	61
BRCA9	54	Black	3	Ductal	2	a		no	no	no	4	8	Y	1.6	42.6	52.1	4.8	19
BRCA10	48	White	12	Ductal	2	a		yes	no	yes	4	8	Y	1.1	48	26.2	25	29
BRCA11	56	White	5	Ductal	2	b	2	yes	no	no	1	4	Y	2.1	71.2	21.9	6.2	29
BRCA12	62	Black	5	Ductal	3	a	5	yes	yes	yes	5	12	Y	1.6	60.9	31.5	6.7	26
BRCA13	60	White	11	Ductal	1		0	yes	yes	yes	1	4	N	2.2	68.1	28.2	3.4	37
BRCA14	48	White	9	Ductal	2	a	3	yes	yes	no	6	14	Y	3.5	78.3	18.8	2.4	33
BRCA15	65	White	8	Ductal	4		0	no	no	no	1	29	Y	1.9	75.9	20.4	3.3	43
BRCA16	54	Black	2	Ductal	2	a	3	yes	yes	no	3	6	N	2	67.3	29	3.2	27
BRCA17	59	White	5	Ductal	2	a	2	yes	yes	no	4	8	Y	2.6	69.9	22.2	7.7	29
BRCA18	65	White	25	Ductal	3	a	5	yes	yes	no	6	11	Y	1.8	51.7	42.7	5	65
BRCA19	57	White	0	Ductal	2	a	0	no	no	no	3		Y	0.5	75.7	16.1	7.1	40
BRCA20	70	White	6	Ductal	2	b	1	yes	yes	no	3	8	N	3.8	28.1	67.6	3.7	27
BRCA21	39	White	1	Duct/Lob	2	b	0	no	no	no	3	8	N	1.5	61.4	33.3	4.8	54
BRCA22	45	White	7	Ductal	4			yes	yes	no	7	14	Y	1.8	68.1	27.2	3.5	58
BRCA23	40	Black	5	Ductal	3	b	1	no	no	no	7	22	Y	2.8	76.9	18.1	4.5	48
BRCA25	40	White	3	Ductal	4		1	no	no	no	3	9	Y	0.9	26.6	57.7	13.9	19
Mean	54.25		5.88				3.57					9.39		1.71	62.39	29.76	7.13	58.46
Median	54.50		5.00				3.00					8.00		1.55	68.10	25.10	4.90	41.50

Other peptides and antigens added in different experiments include: Survivin long peptide, Survivin library 15mer mix, and Cyclin D1 long peptide. Results for these experiments are not shown here. Peptides were restimulated with 1uM peptide for each condition.

Restimulation, Experiment Readouts, and Analysis

Restimulation

Cells are restimulated in a 96 well U-bottom plate in 200ul R-Ab for 6 hours in the presence of Golgi Stop (BD - monensin - 0.6uL per mL) and Golgi Plug (BD - brefeldin A -1uL per mL). The number of cells restimulated per condition in each individual experiment remains the same: for example, in one experiment, all 8 conditions are restimulated at 800,000 cells per well. Cells per well never exceed 1x10e6, with a minimum of 200,000 cells per well. Exact cell concentration per experiment is determined by lowest cell count. Peptides are added to each well according to their condition at 1uM. For example, PBMC originally cultured with 50mer long peptide are restimulated with 50mer long peptide. Shorter peptides for restimulation are not used. If CD40L or CD107a staining is to be performed, CD40L Ab (APC-Cy7 Biolegend) or CD107a (FITC – BD) is added at the beginning of restimulation at 10ul per well. After 6 hours, cells are transferred to 96 well V-Bottom plates for intracellular staining.

Intracellular Staining

Protocol is adapted from protocol provided by BD in their Fixation Permeabilization Solution Kit. Cells are washed in PBS (wash cycle includes centrifuging the plate at 2100 rpm for 3 mins, and flipping the plate to aspirate excess

volume after spin) and resuspended in solution that contains LIVE/DEAD fixable Aqua dead cell stain (Invitrogen) at 1:400 in total volume of 100ul. After 10 minutes incubation at room temperature, cells are washed twice in PBS, and then resuspended in PBS-2%FCS for surface molecule staining in 50ul total volume. Cells are incubated for 15 minutes at room temperature in the dark, then washed twice in PBS. Cells are resuspended in BD Cytofix/Cytoperm solution 150ul per well, and allowed to sit for 15mins. Cells are washed twice in perm/wash solution, then resuspended in perm/wash solution that contains a mix of intracellular cytokine antibodies. Cells incubate for 30 mins at room temperature in the dark. Cells are washed again and left to sit in Perm/wash solution for 10 mins. Finally, cells are washed twice more in perm/wash solution, and resuspended 1x10e6 cells per 100ul 1% PFA for fixation.

Flow Cytometry and Analysis

Cell samples are transferred to 12x75mm polystyrene tubes, or are left in 96 well V bottom plates for reading on flow cytometers. Samples are acquired within 24 hours of staining. Unless otherwise indicated, the entire sample is acquired. Compensation was computed using BD compensation beads and BD FACS Diva and adjusted using FlowJo (Treestar, San Carlos, CA). Flow cytometers used in this study include BD FACS Calibur, BD FACS Canto, and Custom Built BD LSRII. High throughput sample (HTS) plate readers were used with the Canto and LSRII. Data is analyzed using FlowJo. Standard gating method (Figure 20) for analyzing T cells is: gating on lymphocytes using FSC-SSC, gating Aqua negative cells using FSC- Aqua, gating CD3+ cells using FSC-CD3, then gating on CD4+ population or CD8+ population using CD4-CD8. Cytokines are analyzed from the CD4+ population or CD8+ population unless otherwise

noted. IFNγ plots are sometimes divided into Total IFNγ (lower number in the plot) and IFNγ hi (upper number in the plot, between 10^4 – 10^5). Total IFNγ is the total amount of IFNγ that those cells of interest expressed. IFNγ hi population is higher than 10^4 in IFNγ fluorescence (Figure 20). Total ratios of CFSE negative cells are analyzed from CD3+ population, and CFSE-cytokine plots are analyzed from CD4+ or CD8+ populations.

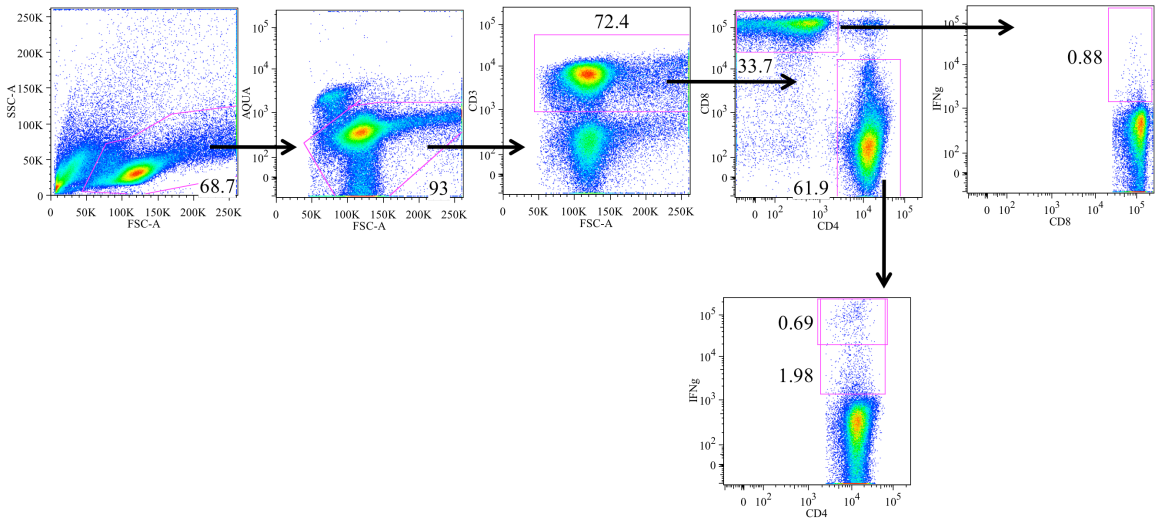

Figure 20: Standard gating method using FlowJo.

FACS Antibodies

A number of different antibodies were used depending on their availability at the time of the experiment. Following is a listing of the most common panels used, with rare exceptions. Panel 1 (used on Calibur): CD107a FITC (BD), IFNγ APC (BD), CD8 PE (BD), and CD3 PerCP (BD). Panel 2 (used on Canto and LSRII): CD3 PerCP (BD), CD4 PE-Cy7 (BD), CD8 PE (BD), IFNγ APC (BD), IL-2 FITC (BD), IL-10 Pacific Blue (eBioscience), Aqua (Invitrogen). Panel 3: CD3 PerCP (BD), CD4 FITC (BD), CD8 PE (BD), IFNγ APC (BD), TNFα PE-Cy7 (BD), Aqua. Panel 4: Same as panel 3 with

addition of CD40L APC-Cy7 (BioLegend). Panel 5: Same as panel 3 or 4 with addition of CFSE OR IL-2 FITC (BD). Panel 6: CFSE, IL-4 PerCP-Cy5.5 (BioLegend), IL-22 PE (R&D), CD8 ECD (Beckman Coulter), IL-10 PE-Cy7 (BioLegend), IL-5 APC (BD), IL-2 AlexaFluor 700 (BioLegend), CD3 APC-AF750 (Invitrogen), IFNγ Pacific Blue (BioLegend), Aqua. Panel 7: CFSE, IL-17 PerCP-Cy5.5 (BioLegend), IL-13 PE (BD), CD8 ECD (Beckman Coulter), TNFα PE-Cy7 (BD), IL-21 AlexaFluor 647 (BD), IFNγ AlexaFluor 700 (BD), CD3 APC-AlexaFluor 750 (Invitrogen), IL-10 eF450 (eBioscience), Aqua. Panel 8: CFSE, CD3 PerCP (BD), Perforin PE (eBioscience), CD8 ECD (Beckman Coulter), TNFα PE-Cy7 (BD), CD4 APC (BD), Granzyme B AF700 (BD), IFNγ Pacific Blue (BioLegend), Aqua. Panel 9: CFSE, CD3 PerCP (BD), CD8 APC-Cy7 (BD), CD4 Pacific Blue (BD), Granzyme B APC (Invitrogen), TNFα PE-Cy7 (BD), IFNγ AF700 (BD), Perforin PE (eBioscience), Aqua. Panel 10: Same as panel 9, when substitution of CD103 PE (BioLegend).

Luminex Analysis

Supernatants were removed from cultures to analyze the total cytokine makeup. Generally, around 80% of the supernatant was removed while taking care not to disturb the cell pellet. Samples were centrifuged to pellet any cells in the supernatant, then samples were placed in a 96 well U bottom plate and delivered to the BIIR Luminex core for analysis. Cytokines analyzed were: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, IL-21, IFNα, IFNγ , IP-10, TNFα , and MDC. Briefly, Luminex analysis incorporates the use of colored microbeads into samples that can bind to a variety of proteins, cytokines, and molecules. Microbeads will fluoresce a particular color, which is read by the analyzer, which is similar to a flow cytometer, and converted

to a numerical value. Information on the standard curve for the experiment is plotted, and compared to the values generated for each different kind of microbead to calculate a value in pg/mL of that cytokine in the supernatant. This is a high-throughput analysis technique: multiple cytokines can be analyzed in a single sample. Luminex is a cytokine multiplex bead-based platform using Bio Plex 200 and Bio Plex Manager 5.0 software (Bio Rad, CA).

Figures and Statistics

Figures were generated using Microsoft PowerPoint, Microsoft Excel, FlowJo, and GraphPad Prism. Statistics for healthy donors were computed using Prism. Advanced statistics for patient samples were done with the help of the Statistics Core at BIIR.

CHAPTER THREE

Results: Cyclin B1 Immunity in Healthy Donors and Breast Cancer Patients

Introduction and Rationale

As mentioned in the introduction, current therapeutic vaccines have had some success but have been limited in their ability to confer long term protection and are typically restricted by HLA type. Long peptide vaccines such as the ones used in this study have the ability to stimulate both CD4+ and CD8+ T cells of multiple HLA types, and since Cyclin B1 is found in a majority of breast cancer tissues, would be widely applicable. Additionally, there are many more cells and cell molecules in the cancer environment than just DCs and T cells. Therefore, culturing only these cells, however informative that might be, would not be a true representation of what might happen to these cells in a cancer environment. Therefore, we have decided to use PBMCs from the blood to truly study these responses to CB1.

PBMCs from the blood have been circulating and have most likely encountered the tumor microenvironment. Although resident cells in the tumor typically stay there, circulating cells have an opportunity to be exposed to the microenvironment. Therefore, we believe that studying PBMCs from the blood will give us a good indication of the global immune environment and reaction towards CB1 peptides.

Practically speaking, breast cancer patient blood samples are difficult to obtain in large quantities. Elutration and cell fractionation are only possible with large blood draws. While it is possible to culture DCs and isolate T cells using PBMCs, it would

result in cell waste with lower yields. Therefore to examine immunity to Cyclin B1 peptides, healthy donor blood samples were employed to answer some of our questions about Cyclin B1 immunity and repertoire. Early experiments we conducted as part of this study serve to validate the methods used in our protocols and those found by Vella *et al.* 2009b. Because Cyclin B1 is a viable immunological target and is expressed by tumors, we investigated Cyclin B1-specific immunity to long peptides in healthy donors.

The following chapter describes results obtained with Cyclin B1 peptides showing that healthy individuals and patients have immunological memory for Cyclin B1 that can be detected by recall assays, using both PBMC and DC-T cell cocultures. Type 1 immune responses are widely believed to be the most beneficial for cancer eradication because it involves the generation of cells that have specific, direct action against target cells. In contrast, inflammatory type 2 responses have been shown to be beneficial for cancer survival. Therefore, we also specifically characterize cytokine profiles in response to Cyclin B1 peptides to determine the immune environment present in breast cancer patients.

IFNγ Specific Response Can Be Detected in Cultures with DCs and-T Cells

Early experiments with Cyclin B1 long peptides were conducted with targeting molecules. These molecules are a monoclonal antibody specific to a certain receptor on dendritic cells, in this case CD40, and carry the long peptides directly to the DCs. We feel this is the most efficient way to deliver our long peptides to DCs for their processing and presentation. However, early experiments with targeting reagents yielded no significant results (not shown). All conditions at all concentrations were very similar to negative controls. We believe that a number of factors might have contributed to this, but

there are many aspects of DC targeting biology that were not specific aims of this project. We wanted to therefore validate that the long peptides used in this study were able to be processed and presented by DCs, and that certain epitopes that had been previously published were indeed capable of eliciting immunity using our culture systems before returning to the challenge of DC targeting biology.

The first step in accomplishing these goals was to utilize peptides that had been previously shown to be presented by DCs in other studies. Cyclin B1 9mer, 10mer, and 15mer (p80, p81) peptides were loaded onto GM-CSF/IFNα DCs using our 3 day DC culture protocol. Briefly, monocytes from fraction 5 are thawed and cultured in culture bags with IFNα and GM-CSF for 2 days. Cells are given peptide overnight on Day 2, and matured using LPS and CD40L on Day 3. DCs are then washed and plated with naïve CD8+ T cells for 7 days, given IL-7 on Day 0, and IL-2 on days 3 and 5. T cells are rested on Day 7 for 2 days, then restimulated with fresh DCs for another round of culture.

Results show that, in two separate experiments using two different healthy donors, IFNα DCs were able to prime naïve CD8+ T cells to express IFNγ in the presence of Cyclin B1 peptides (Figure 21 and 22). These responses are difficult to see, and only 2/6 donors were able to elicit IFNγ immune responses, and a total of 2/8 experiments were successful. Cyclin B1 is a self peptide, and thus the T cell repertoire for it is much lower than it would be for a viral antigen, for example. While the two donors recognized slightly different peptides, this shows that Cyclin B1 immunity can be achieved in the primary setting, further proving that Cyclin B1 repertoire exists in healthy donors, and validating that our system can reproduce results seen in others' published works (Kao *et al.* 2001, Saito *et al.* 2006).

Experiments were then performed loading DCs with long peptides (36mer and 50mer) and restimulating T cells with long or short peptides to test for generation of CB1-specific T cells. We were unable to generate an IFNγ-specific response using this protocol. Other cell types, DC subsets, or activation signals might be necessary to help generate this response. Therefore, we conducted further experiments with PBMCs since this was the primary cell type available from patients.

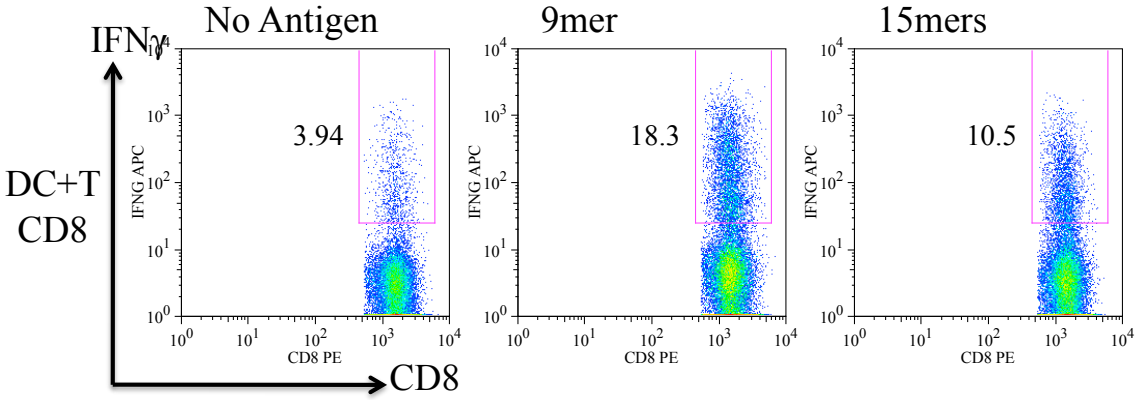

Figure 21. Cyclin B1 9mer and 15mer specificity in DC/T cell cocultures. Negative control condition, no antigen given to DCs - Unloaded DCs, T cells restimulated with 9mer. 9mer - DCs loaded with 9mer peptide, restimulated with 9mer. 15mers - DCs loaded with p80 and p81, restimulated with p80 and p81. Naïve CD8+T cells are cocultured with GM-CSF/IFNα DCs with addition of IL-2. Cells are rested on day 7, and restimulated on Day 9 for an additional round of culture with loaded DCs. Cells are then restimulated with respective peptides for 6 hours in the presence of GolgiSTOP and GolgiPLUG, and stained for surface and intracellular markers. Cells are gated on lymphocytes from FSC-SSC, then CD8+ cells. Percentage is of CD8+ T cells. DCs loaded with 9mer or 15mer peptides are able to present peptides and prime naïve CD8+ T cells, which expressed IFNγ in response to restimulation with the same peptides (b and c). Results after two cycles of culture.

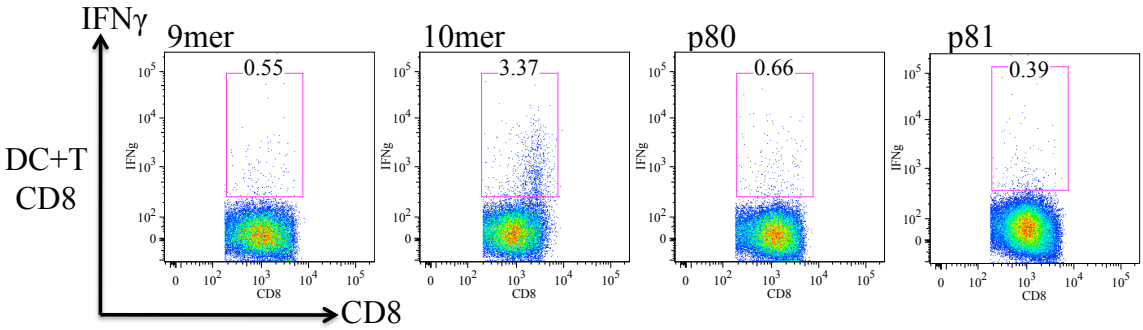

Figure 22. Cyclin B1 10mer specificity in DC/T cell cocultures. 9mer -DCs loaded with 9mer. 10mer - DCs loaded with 10mer. P80 - DCs loaded with p80. P81 - DCs loaded with p81. Naïve CD8+T cells are cocultured with GM-CSF/IFNα DCs with addition of IL-2. Cells are rested on day 7, and restimulated on Day 9 for an additional round of culture with loaded DCs. Cells are then restimulated with respective peptides for 6 hours in the presence of GolgiSTOP and GolgiPLUG, and stained for surface and intracellular markers. All T cells were restimulated with peptides respective to their loaded DC condition. Cells are gated on lymphocytes from FSC-SSC, the live cells from Aqua stain, CD3+ cells, then CD3 CD8+ double positive cells. Percentage is of CD8+ T cells. This healthy donor DCs presented Cyclin B1 10mer peptide to naïve CD8+ T cells, which were able to express IFNγ after restimulation with 10mer peptide. Results after two cycles of culture.

PBMC Experiments with Short Peptides

IFNγ-Specific Response to Short Peptides

PBMCs will be the cell type isolated from patients that will be used for this study. Therefore, in order to be able to compare healthy donor responses to responses of patients and to validate the immunogenicity of Cyclin B1 peptides using our culture systems, we tested responses of PBMCs to short peptides.

PBMCs were cultured for 7 days with IL-2, given at days 2, 4 and 6. Cells were restimulated with appropriate peptides on Day 7 for 6 hours with GolgiSTOP and GolgiPLUG, and then stained for surface and intracellular markers. We found some responses to short peptides: 2.35% IFNγ from CD8+ T cells for 9mer, 3.19% for p80, compared to 1.55% in controls (Figure 23). These responses are harder to detect than in previous DC experiments. This is perhaps due to the increased number of other cell types in culture, making T cells less abundant, and the already low frequency of cells becomes

even smaller. Low frequency itself, and the general lack of extensive repertoire due to Cyclin B1 being a self antigen might also be playing a role in making these responses hard to detect. In three donors tested, only one donor had responses to short peptides. However, there is clearly a response to Cyclin B1 short peptides in 1 donor, shown in Figure 23.

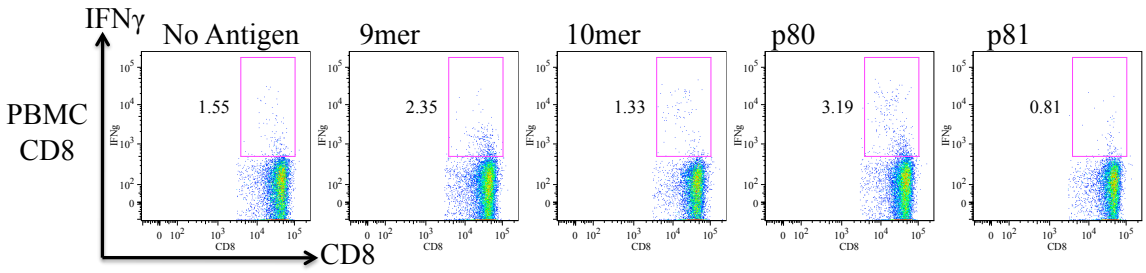

Figure 23. Cyclin B1 specificity to 9mer and p80 in PBMC cultures. PBMCs were cultured with the indicated peptides for 7 days and then restimulated with their respective peptides. Cells are gated according to the standard gating method described in Materials and Methods.

PBMCs Cultured with Long Peptides Elicit IFNγ-Specific Responses

Cyclin B1 50mer long peptide contains the HLA-A201 binding region that was tested with short peptides. To determine what Cyclin B1 epitopes were presented from long peptides, we stimulated PBMCs with 50mer long peptide, then restimulated with 9mer, 10mer and 15mer peptides, but did not elucidate any antigen specificity (data not shown). However, when long peptides conditions were restimulated with long peptides, some antigen-specific CD4+ T cells expressed IFNγ. This could be because the short peptides we used to restimulate T cells did not contain the epitopes that were presented on the DCs, not necessarily because there was not repertoire or that the peptides were not processed.

We then decided to optimize the culture conditions by titrating for concentration of the long peptide (Figure 24 and 25) and the number of cells cultured per well (Figure

26). A long peptide concentration of 0.3uM, with 2x10e6 cells per well was used for the remainder of normal healthy donor PBMC experiments. These experiments also served to validate antigen specificity with Cyclin B1 50mer. Clearly, this peptide has a range within which it operates best: concentrations of peptide that are too low do not stimulate the low frequency of cells present, and too much peptide may overstimulate cells. Similarly, too few cells do not seem to create enough of the cell to cell contact that is important for proliferating T cells, but too many causes overcrowding, and lowers the percentage of T cells that are proliferated in the culture that could make IFNγ. These experiments were representative of three independent experiments. We also optimized the restimulation conditions by titrating the peptide concentration and restimulation time (Figure 27 and 28). Optimal restimulation concentration was found to be 1uM (Figure 28) for both CD4+ and CD8+ responses, and restimulation time to be 6 hours, as opposed to overnight (18 hours – Figure 27). Further proof of antigen specificity was obtained by restimulating cultures that were started with 50mer with another long peptide from Cyclin B1, a 36mer, that contained no overlapping sequences with Cyclin B1 50mer (Figure 29). While some background staining can be detected, as seen with IFNγ expression when 50mer cells were restimulated with 36mer peptide, only 50mer cells restimulated with 50mer peptide can produce double positive IFNγ and IL-2 CD4+ T cells at levels higher than control condition with no antigen. Background in this experiment may be due to the earlier stimulation with peptide on day 0. Multiple experiments were performed with the same normal donor to test for reproducibility in detecting 50mer specific responses (Figure 30). While there was a range of responses, the results were significant. (50mer %IFNγ mean = 0.92 +/- 0.18; No antigen Carrier %IFNγ mean = 0.15 +/- 0.01; Wilcoxon

p value = 0.0002). While most of the experiment results fell within a range of 0.3-1% IFNγ, three experiments had very high percentages of IFNγ producing T cells for this antigen. This may be due to a larger number of precursor T cells in that particular culture. In any case, there is clear antigen specificity for Cyclin B1 50mer present in healthy donors.

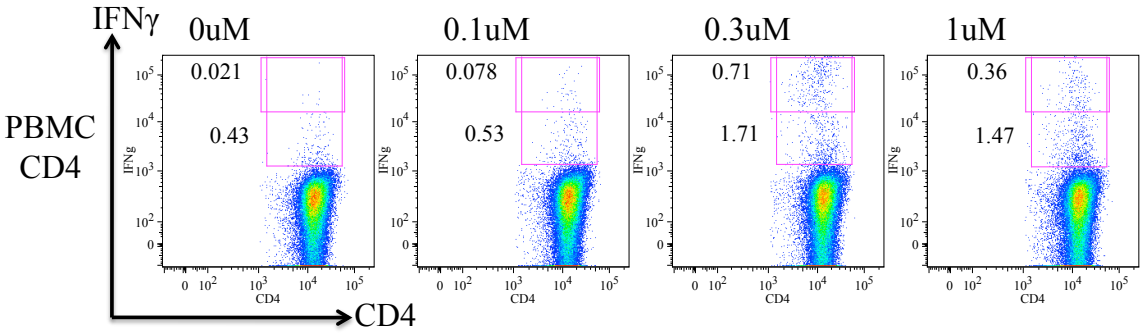

Figure 24. 50mer peptide titration. PBMCs were cultured for 7 days in the presence of 50mer, then restimulated on Day 7 with 50mer and stained. The optimal concentration of culture for 50mer peptide is 0.3uM based on IFNγ specific T cell stimulation.

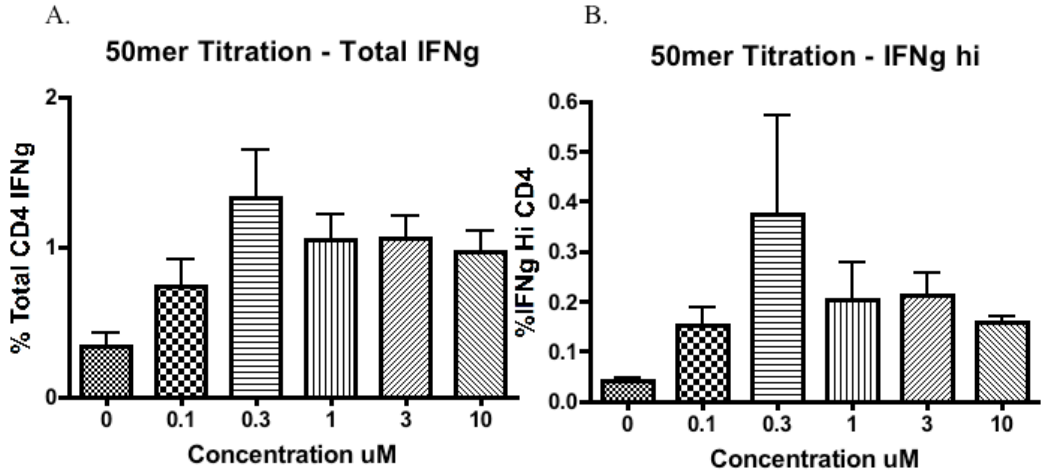

Figure 25. 50mer Titration: optimum concentration found from three experiments for 50mer is 0.3uM. PBMCs were cultured for 7 days in the presence of 50mer and restimulated with 50mer for 6 hours before staining. A. Total amount of IFNγ staining in CD4+ T cells for each concentration. B. Amount of cells staining 'hi' for IFNγ, between 10^4 - 10^5. These cells express higher amount of IFNγ.

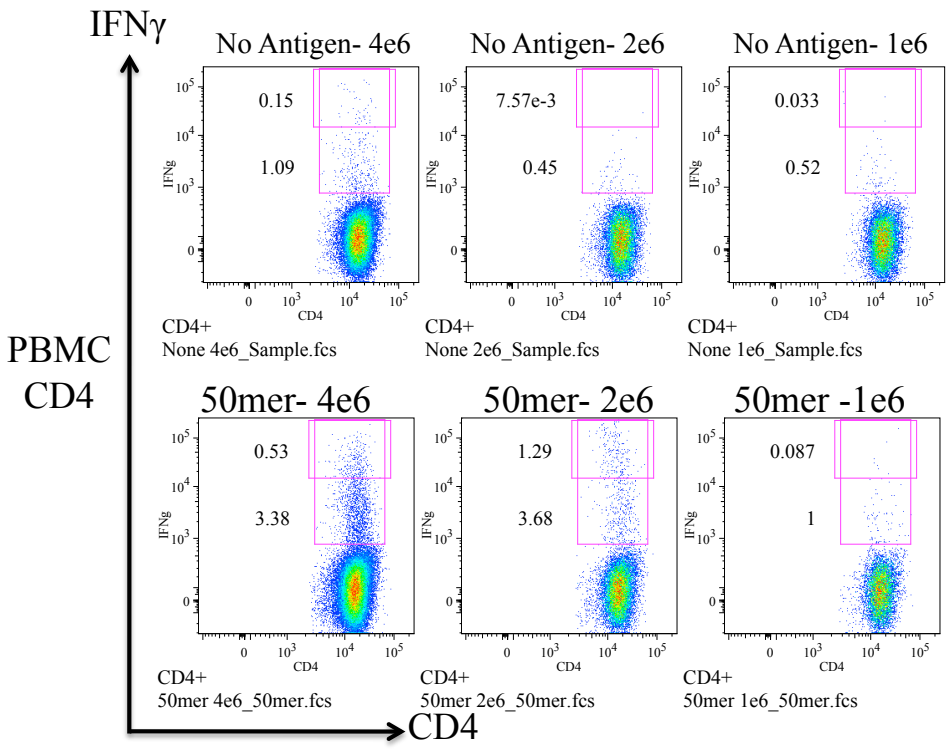

Figure 26. Cell number titration. 2x10e6 per well in 24 well plate is the optimal cell number for eliciting antigen specific responses to CB1 50mer. PBMCs were cultured for 7 days and then restimulated with 50mer peptide for 6 hours then stained. Cultures started with the cell numbers shown.

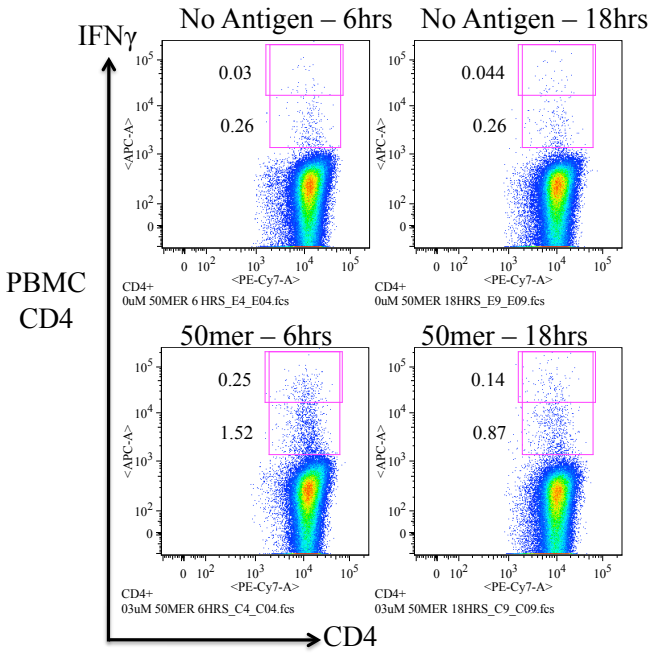

Figure 27. Kinetics: restimulation is optimal when performed for 6 hours. PBMCs were cultured for 7 days and then restimulated with 50mer peptide for either 6 hours or 18 hours, then stained. Higher antigen specificity was detected after 6 hours than 18 hours.

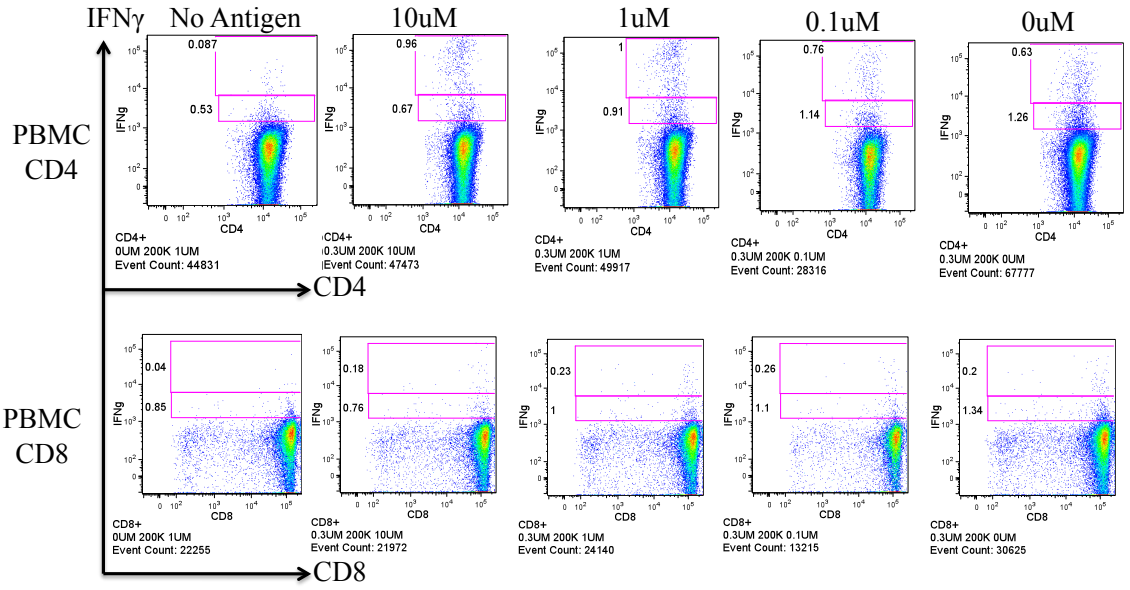

Figure 28. Optimal restimulation concentration for 50mer is 1uM. PBMCs were cultured with 50mer for 7 days and restimulated on Day 7 with 50mer peptide at concentrations of 10uM, 1uM, 0.1uM, and 0.01uM. Highest IFNγ response for CD4+ and CD8+ T cells was found at 1uM.

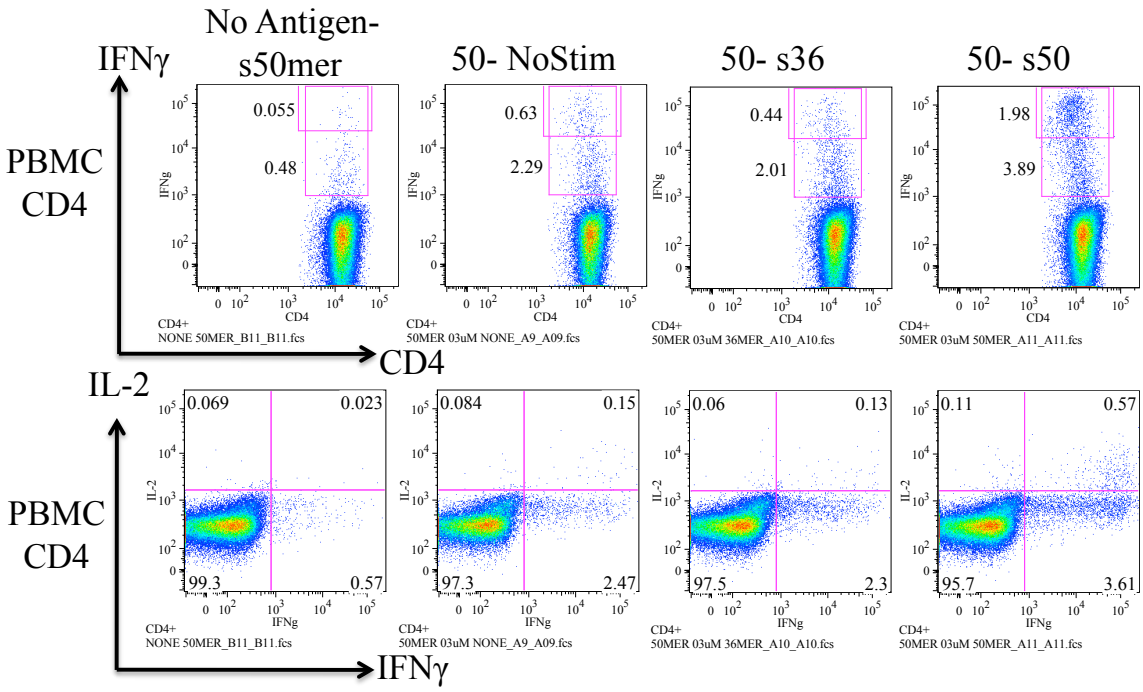

Figure 29. Specificity and quality of T cells: antigen specificity to 50mer can be detected by IFNγ + IL-2+ CD4+ T cells. PBMCs were cultured for 7 days in presence of peptide and restimulated with individual peptides on day 7 for 6 hours. First designation for label before the dash is the peptide added to culture on day 0, and label after the dash preceded by 's' denotes the restimulation peptide used. Top: Cyclin B1 specific IFNγ expression by CD4+ T cells. Cells gated on live, CD3+, CD4+ T cells. Bottom: CD4+ T cells expressing IL-2 and IFNγ. Cells gated on CD3+, CD4+ T cells.

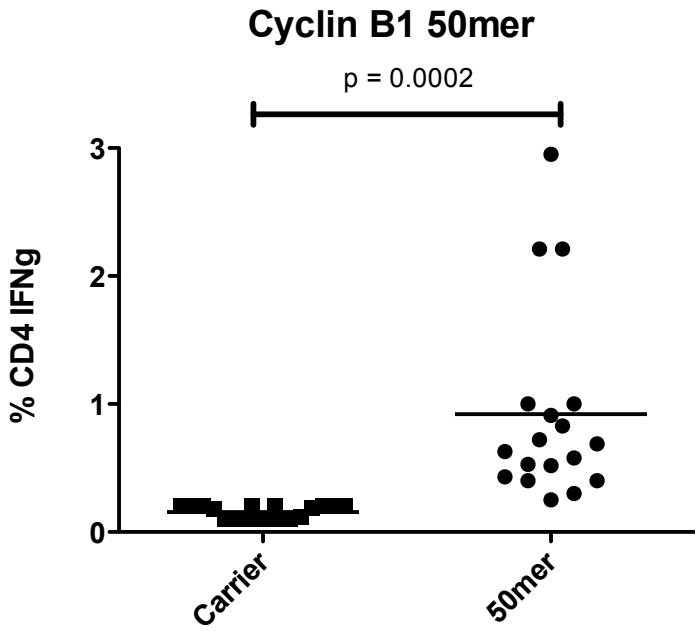

Figure 30. Reproducibility: repetitive testing of the same donor for 50mer IFNγ expression shows variance in responses due to precursor frequency. PBMCs were cultured for 7 days in presence of 50mer and restimulated for 6 hours before staining. Paired Wilcoxon test used for p value.

We have thus far shown responses to Cyclin B1 50mer. Most of these responses have been CD4+ T cells expressing IFNγ. CD8+ T cells have been more difficult to elicit using this peptide, but can be found in some experiments at very low percentages.

In addition to testing responses to Cyclin B1 50mer, we also looked at another immunogenic sequence from Cyclin B1, a 36mer that contains multiple HLA epitopes that had been previously reported as generating immunity using overlapping 15mer peptides (Vella *et al.* 2009b). Using the ideal culture conditions described here, we performed a titration to determine whether IFNγ-specific T cells could be detected, and if so, what concentration would be optimal for further study. We found the optimal concentration for the 36mer to be 3uM (Figure 31), with the same restimulation concentration as found with the 50mer (1uM, not shown). It is important to note that the optimal culture concentration for the long peptides is one log different (3uM vs. 0.3uM). This gives us reason to believe that in the context of Cyclin B1, and perhaps for other self

antigens or cancer antigens, that each individual peptide has its own concentration at which best results for IFNγ expression in T cells can occur. This may be due to the sensitive nature of the affinity of the T cells for the processed peptides.

We were also able to detect antigen-specific IL-2 secretion by CD4+ T cells after restimulation (Figure 32). Some CD4+ T cells that secrete IFNγ can also secrete IL-2 (0.035% vs. 0.27% for no antigen vs. CD4+ + IFNγ + IL-2+). These are the cells that would be most helpful in generating a CD8+T cell-specific response while also having some direct action against the tumor itself.

Overall, 2/5 PBMC donors responded to Cyclin B1 peptides. These are different donors than those used for DC/T cell experiments. One donor was used more frequently to do titration experiments and establish immunity. In this donor, 21/48 experiments had positive results. We have shown representative examples of experiments that yielded positive results. We attribute experiments that did not yield IFNγ expression from T cells to low frequency of precursor T cells, because experiments that did have IFNγ-expressing T cells are clear.

Responses to these peptides are primarily memory CD4+ T cell responses. This confirms previous data by O.J. Finn and colleagues that showed antibodies specific for Cyclin B1 in the blood of breast cancer patients. Therefore, our hypothesis that long peptides will be more beneficial to generate more epitopes presented on DCs is so far supported: since 8-10 AA sequences do not generate CD4+ T cell immunity, if we cultured only CD8+ T cells, we would miss potential signatures and epitopes that when stimulated, result in additional CD4+ immunity, as well as the possibility for CD4+ T cell help.

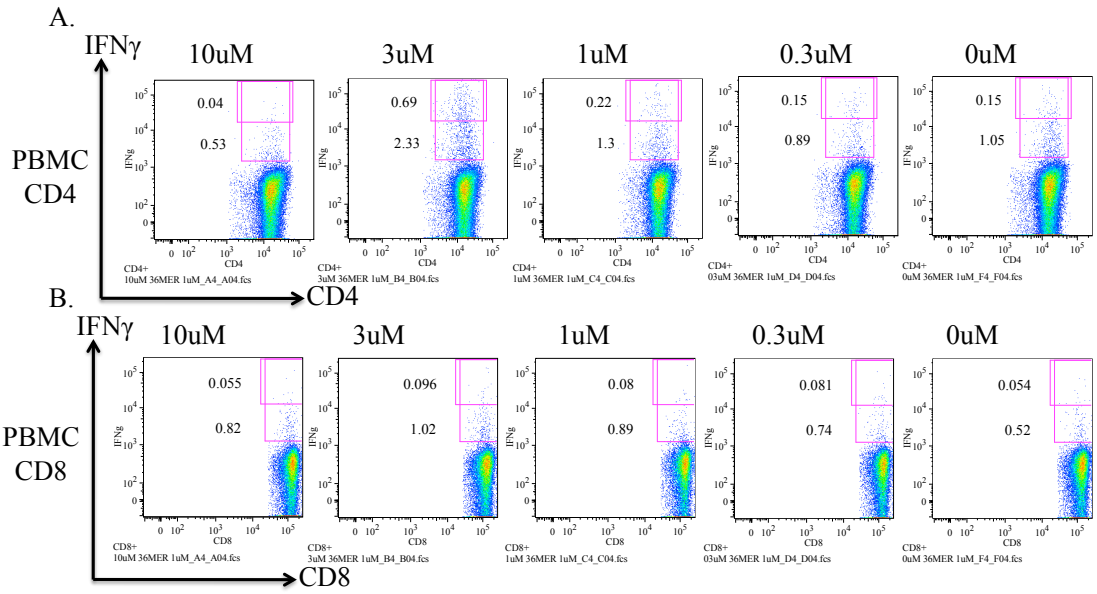

Figure 31. Optimal concentration for Cyclin B1 36mer is 3uM. PBMCs were cultured for 7 days in presence of peptide and restimulated on day 7 for 6 hours, then stained. Both CD4+ T cells and CD8+ T cells can secrete IFNγ in response to 36mer peptide.

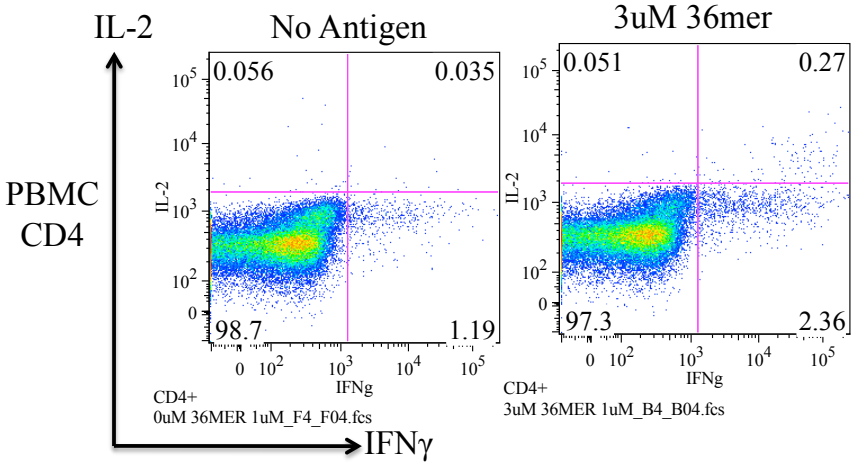

Figure 32. 36mer stimulates CD4+ T cells that express IFNγ and IL-2. PBMCs were cultured for 7 days in presence of peptide and restimulated with peptide on day 7 for 6 hours then stained. Cells are gated on CD3+ CD4+ T cells.

Ideally, we would like to have both of these peptides in a vaccine to have maximum efficacy and sequence coverage. This would allow maximum number of immunogenic epitopes to be seen by the immune system, and be of benefit to the most patients with different haplotypes. Additionally, all DC targeting reagents made so far that carry Cyclin B1 contain both sequences, so we wanted to test the effect on antigen

specificity when both peptides were added to culture to insure that cells were not being overstimulated with peptide, in addition to determining if antigen specific responses could still be detected. 36mer was tested at concentrations of 3uM and 1uM, and the 50mer at 1uM and 0.3uM. Maximum IFNγ production was achieved at 1uM for each peptide (Figure 33). Results were confirmed by a second, independent experiment (Figure 34).

Experiments with healthy donor PBMCs using long peptides have shown that repertoire exists for Cyclin B1 sequences both for CD4+ T cells and CD8+ T cells. These responses are sometimes difficult to generate, especially for CD8+ T cells.

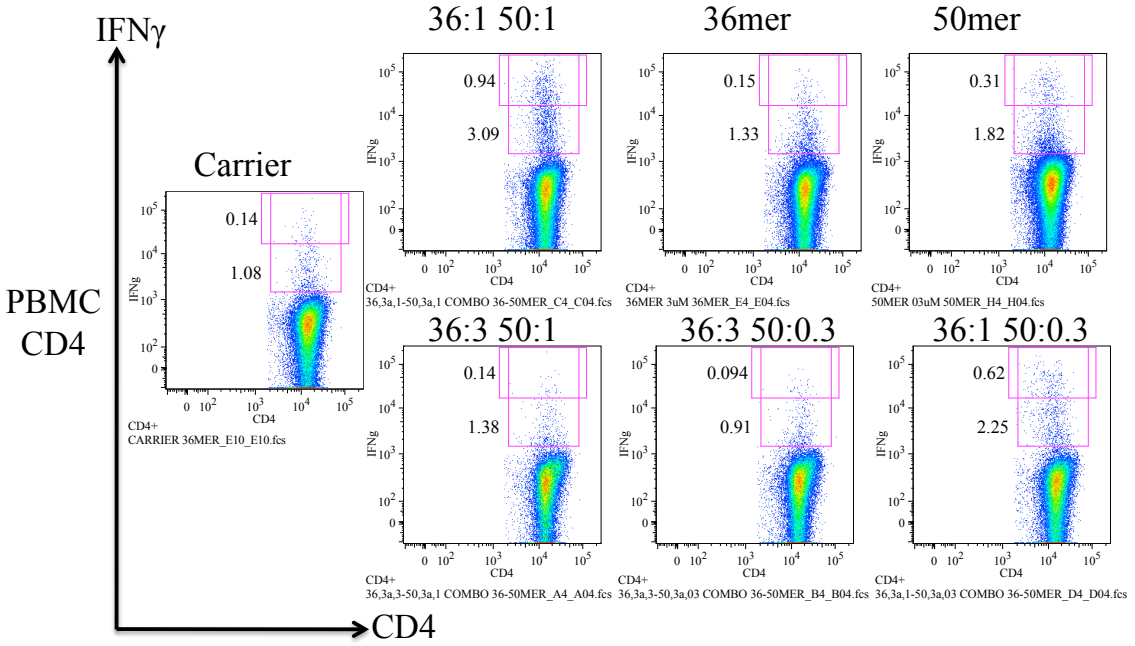

Figure 33. IFNγ-specific cells can be detected in peptide combination experiments. PBMCs were cultured for 7 days in presence of peptide, then restimulated on day 7 for 6 hours prior to staining. The first number indicates the peptide used, and the number after the colon, the corresponding concentration. Individual peptide cultures were restimulated only with individual peptide. Carrier – no antigen.

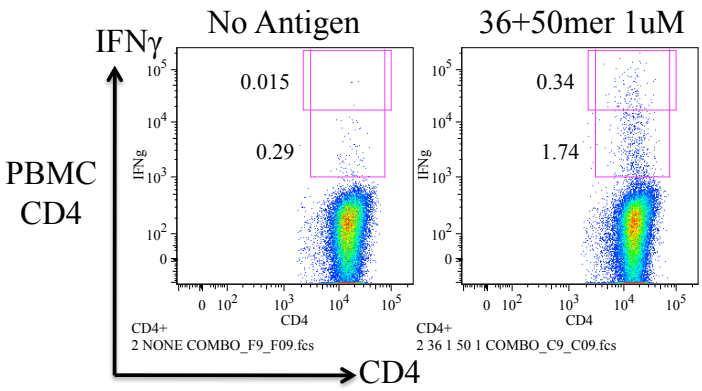

Figure 34. Combination of peptides can reliably generate peptide specific responses. PBMCs were cultured for 7 days in presence of peptide, then restimulated on day 7 for 6 hours prior to staining.

We think that this phenomenon is due to the low frequency of these T cells in the blood because Cyclin B1 is a self antigen, and therefore Cyclin B1-specific T cells that are of high affinity are probably deleted from the T cell repertoire. Having examined these responses at length, we moved on to assessing responses in breast cancer patients.

Patient Responses to Cyclin B1 Peptides

Using long peptides in a breast cancer vaccine is the ultimate goal of this study. Therefore, we must study patient responses to these antigens to determine immunity and to confirm that these peptides would be useful in this context. We utilized 7 day PBMC cultures, adding peptides on Day 0, taking sample supernatant on Day 2, adding IL-2 to facilitate T cell expansion, harvesting cells and restimulating on Day 7 with peptide, and collecting final supernatant after 24 hours. We analyzed the samples using Luminex to examine a wide range of cytokines. Intracellular staining could not be performed on these patients because of limiting cell numbers, but will be used in a future study to validate T cell-specific responses. Healthy donors used for comparison were cultured using the same method as patients.

To identify the global immune environment present in patient samples, and determine a baseline for comparison in our study, we looked at cytokine secretion of

breast cancer patient PBMCs and healthy donor PBMCs to Carrier (No antigen) (Figure 35).

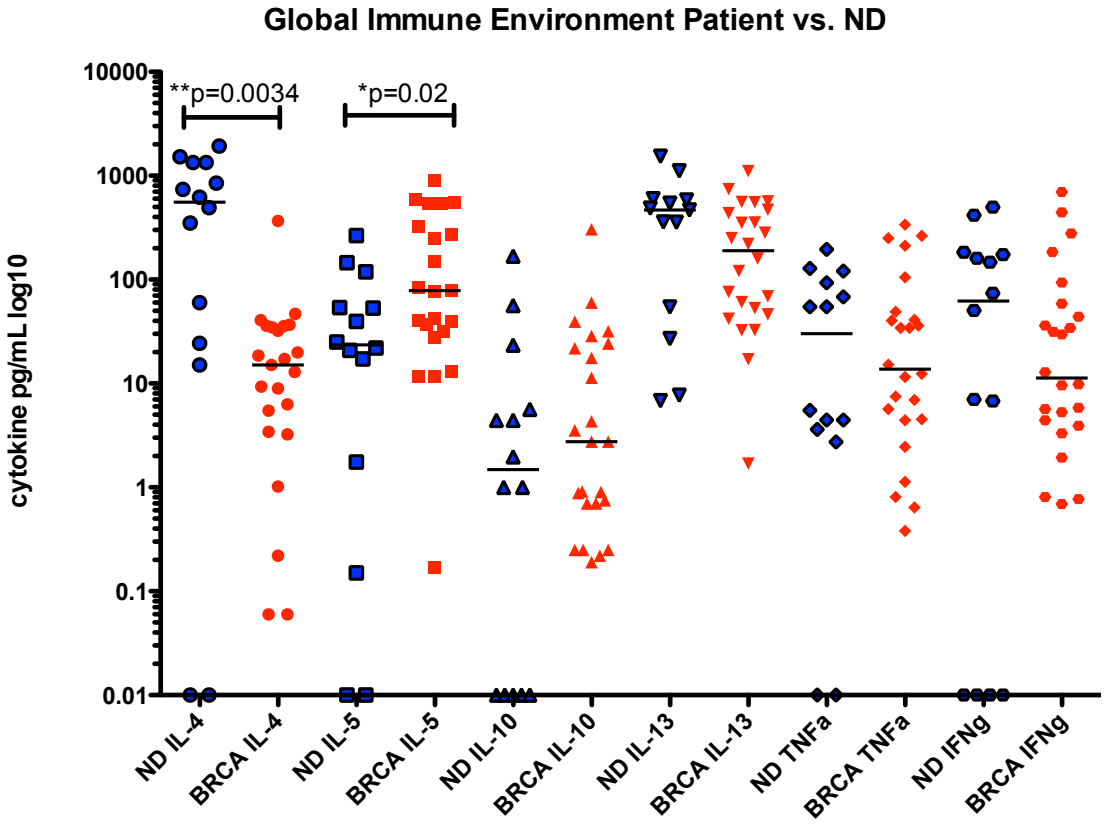

Figure 35. Global Immune response: patient and normal donor responses to no antigen control. PBMCs were cultured for 7 days with Carrier (no antigen), harvested and restimulated with Carrier on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. P value Mann Whitney test. ND – normal donor. BRCA – breast cancer patient.

Breast cancer patients and healthy donors had different global immune environment cytokine profiles. Healthy donors secreted significantly more IL-4 ($p = 0.0034$), and had trends of secreting more IL-13, TNFα and IFNγ, although none were statistically significant. BRCA donors secreted significantly more IL-5 ($p = 0.02$) and generally more IL-10, although not significant. This shows that the overall global immune environment prior to stimulation in BRCA samples was not especially skewed

towards Th2, except in the case of IL-5, compared to these healthy donors. They also have higher amounts of IL-10, which is an immunosuppressive cytokine. Overall, it appears that BRCA patient PBMCs secrete lower amounts of cytokines than healthy donors.

We also tested patient and healthy donor responses to viral antigen control peptides using CEF on Day 2 (Figure 36). For all cytokines tested, healthy donor PBMCs secreted more cytokines in response to CEF than BRCA cells (p=0.02 for IL-13, p= 0.0051 for IFNγ).

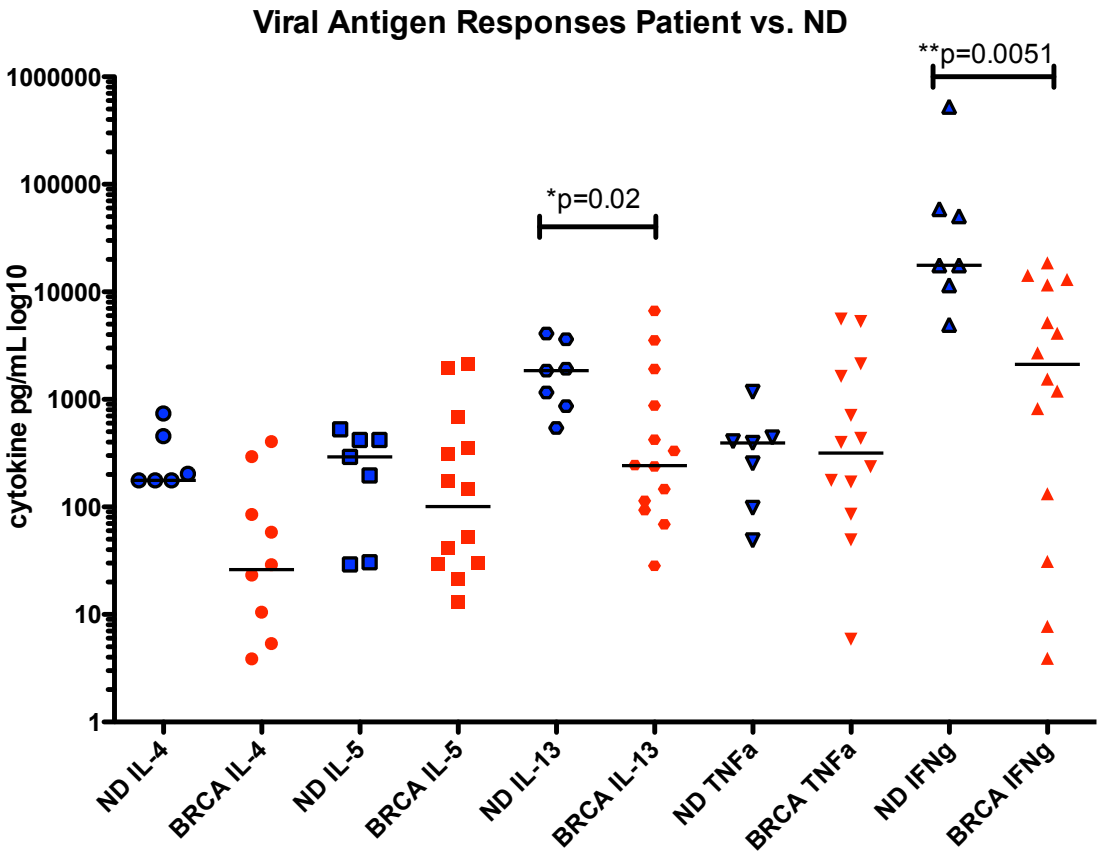

Figure 36. Healthy donors secrete higher levels of cytokines than BRCA patients in response to viral antigens. Viral antigens = CEF (mix of CMV, EBV and Flu short peptides) peptides. PBMCs were cultured for 7 days with CEF, harvested and restimulated with no antigen CEF on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. P values from Mann Whitney test. ND – normal donor. BRCA – breast cancer patient.

To assess the global immune response to all Cyclin B1 antigens, we used both long peptides and peptide libraries (Figure 37 and 38). Breast cancer patient PBMCs secrete more IL-4 ($p=0.02$) and IL-5, while healthy donor PBMCs secrete more IL-10, IL-13 ($p=0.0003$), TNFα, and IFNγ ($p=0.0031$) to these peptides. Therefore, the addition of breast cancer antigens by themselves changes the global immune response and induced cytokine environment to produce more Th2 cytokines than in healthy donors. When patient responses to antigen are compared to global Cyclin B1 peptides, they induce higher cytokine secretion, significantly for IL-13 ($p=0.01$), and IFNγ ($p=0.02$) (Figure 39).

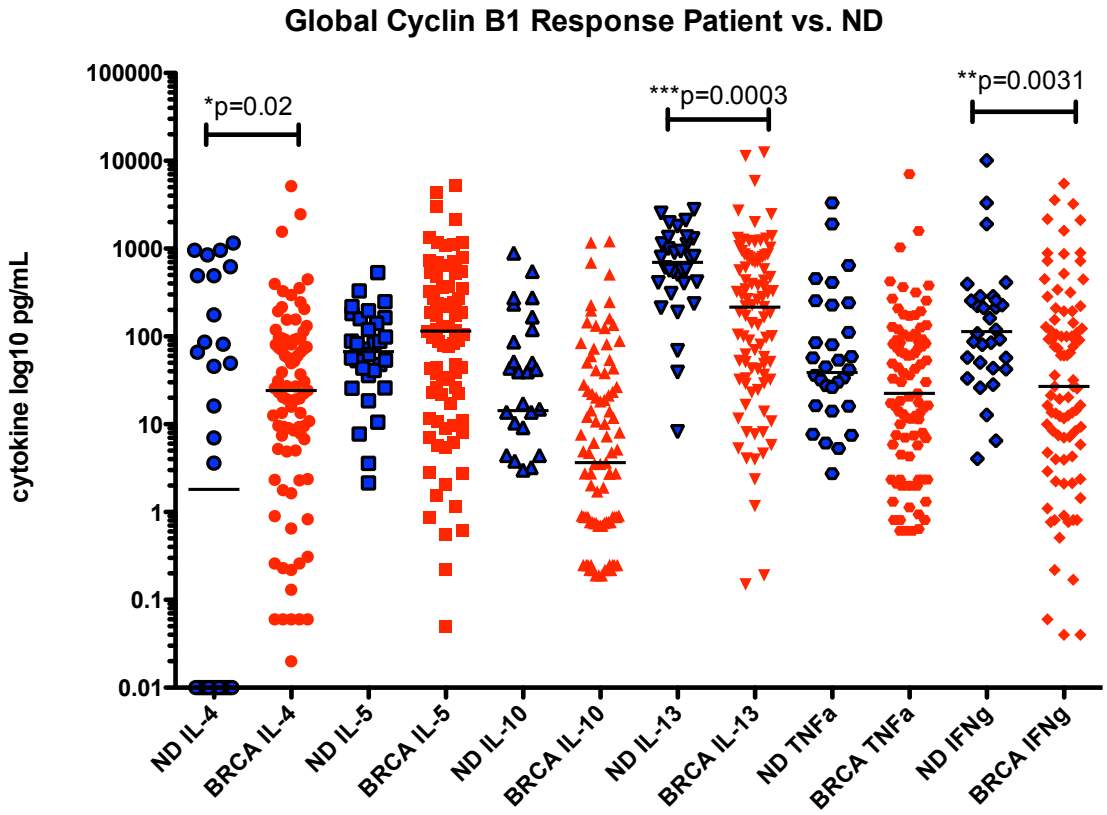

Figure 37. BRCA patients secrete more IL-4, and healthy donors secrete more IL-13 and IFNγ in response to all Cyclin B1 peptides. PBMCs were cultured for 7 days with Cyclin B1 long peptides and 15mer libraries in separate conditions, harvested and restimulated with peptides on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. P values from Mann Whitney test. Each data point represents one peptide specific response for one patient.

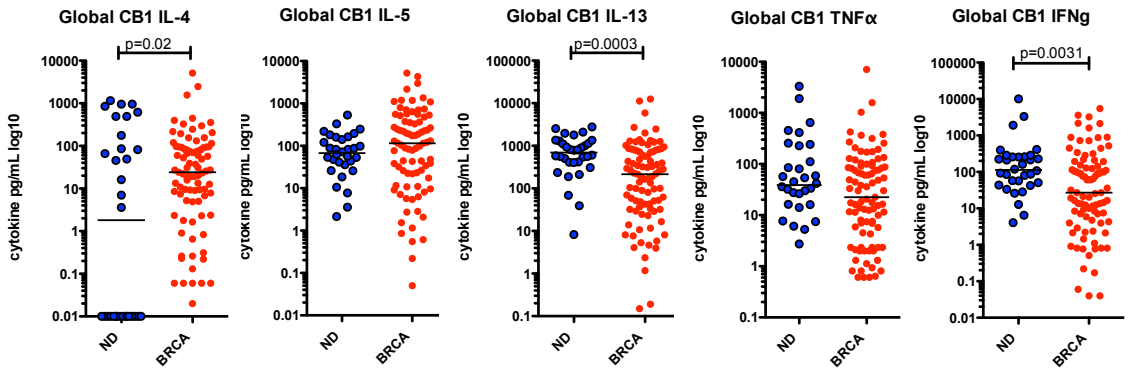

Figure 38. Individual cytokine analysis of patient and healthy donor responses to global Cyclin B1 peptides. PBMCs were cultured for 7 days with Cyclin B1 long peptides and 15mer libraries in separate conditions, harvested and restimulated with peptides on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. P values from Mann-Whitney Test. ND – normal donor. BRCA – breast cancer patient.

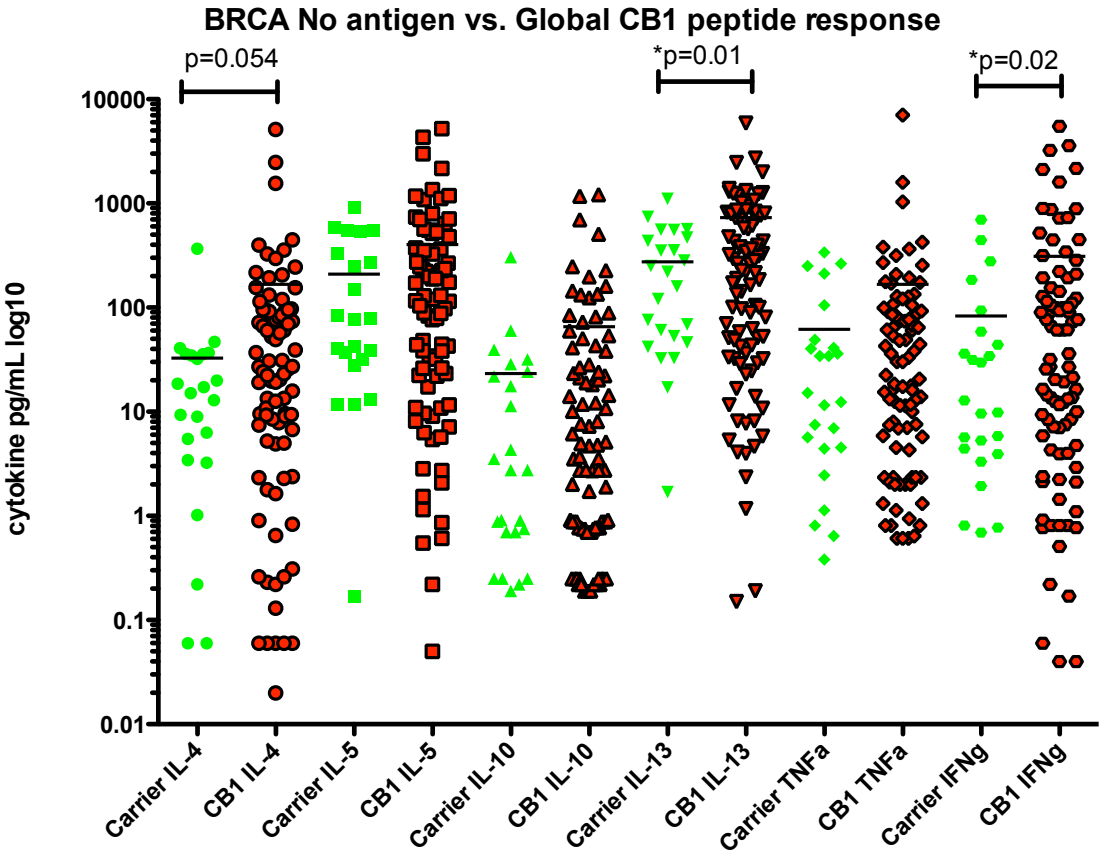

Figure 39. Patient responses to no antigen control vs global Cyclin B1 peptides. PBMCs were cultured for 7 days with Carrier (no antigen) or Cyclin B1 peptides, harvested and restimulated with respective peptides or carrier on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent mean values. P value Mann Whitney test. CB1 – Cyclin B1.

To have a more accurate picture of how the presence of individual antigens affects the cytokine environment, we analyzed individual peptides from this same experiment. While most other peptides had very similar profiles, Cyclin B1 50mer had a couple of key differences in its profile (Figure 40 and 41), especially when compared with carrier (Figure 42). Levels of IL-4 and IL-5 secretion were still higher than normal donors, IL-10 secretion was lower, and notably, TNFα secretion was higher than healthy donors. Patients secreted less TNFα than healthy donors when no antigen was present, however inflammatory TNFα is higher in patients than in healthy donors only with the addition of Cyclin B1 50mer.

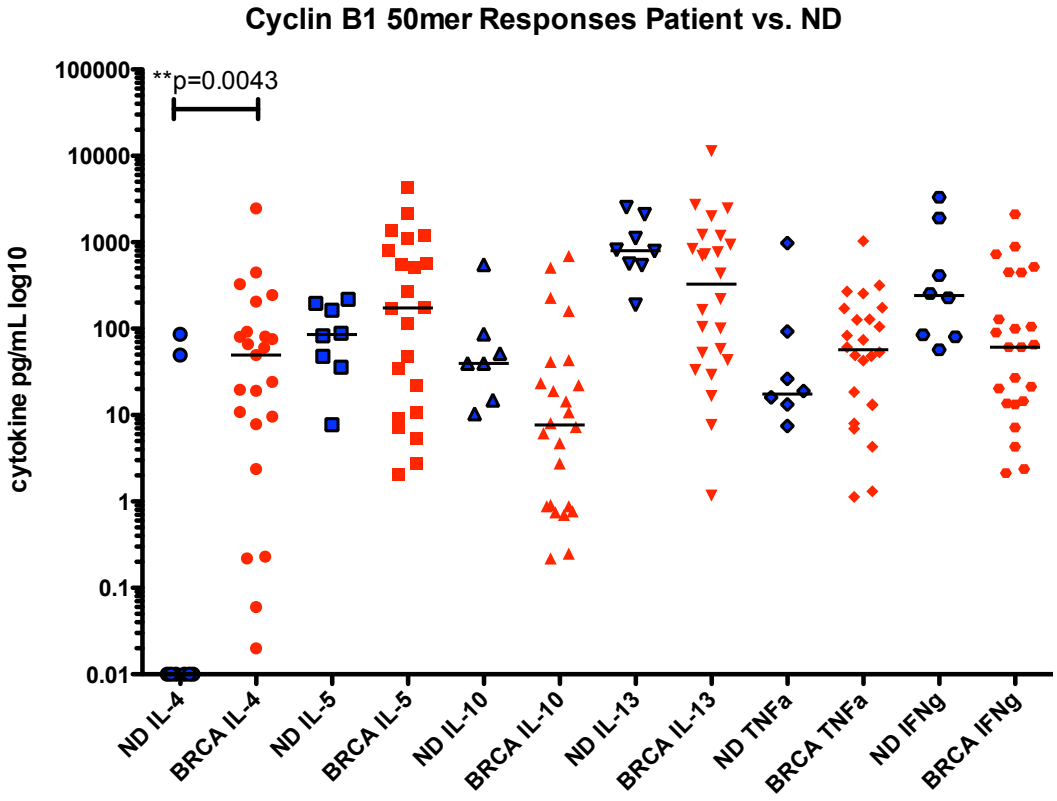

Figure 40. Cytokine profile of patient PBMCs with Cyclin B1 50mer is more skewed towards Th2 than healthy donors (ND). PBMCs were cultured for 7 days with Cyclin B1 long peptide 50mer, harvested and restimulated with peptide on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. P value from Mann-Whitney test.

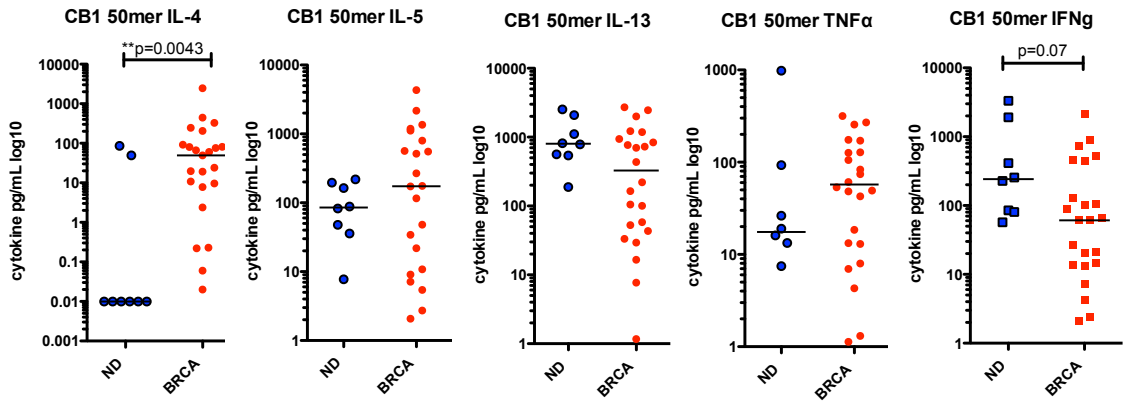

Figure 41. Individual cytokine profile of patient PBMCs with Cyclin B1 50mer. PBMCs were cultured for 7 days with Cyclin B1 long peptide 50mer, harvested and restimulated with peptide on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. P values are from Mann-Whitney Test. ND = healthy donor comparison.

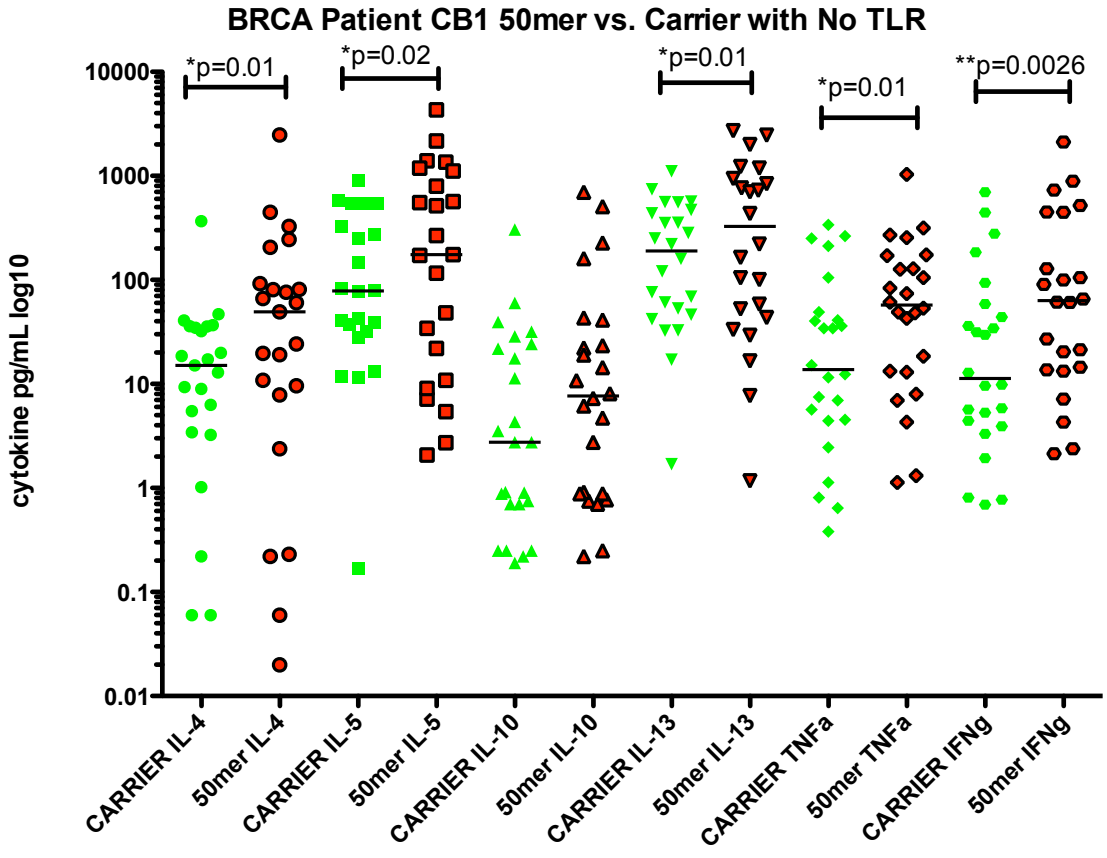

Figure 42. Patient responses to no antigen control vs Cyclin B1 50mer. PBMCs were cultured for 7 days with Carrier (no antigen), harvested and restimulated with no antigen Carrier or 50mer on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent mean values. P value Mann Whitney test.

Patients therefore secrete higher amounts of IL-4, IL-5, and TNFα than healthy donors, a change form previous results. BRCA patients still secrete high amounts of IL-13, although not more than healthy donors. When values for 50mer responses are compared to that of no antigen form the same patients, patients secrete significantly more cytokines (p value significant in 5/6 cytokines) in response to 50mer than to no antigen (Figure 42).

The same trends were true for Cyclin B1 36mer (Figure 43), although no values were significant. When compared to no antigen, patient PBMCs secreted more cytokines in response to 36mer than no antigen (Figure 44), significantly for IL-4 (p=0.008) and nearly significant for IFNγ (p = 0.06).

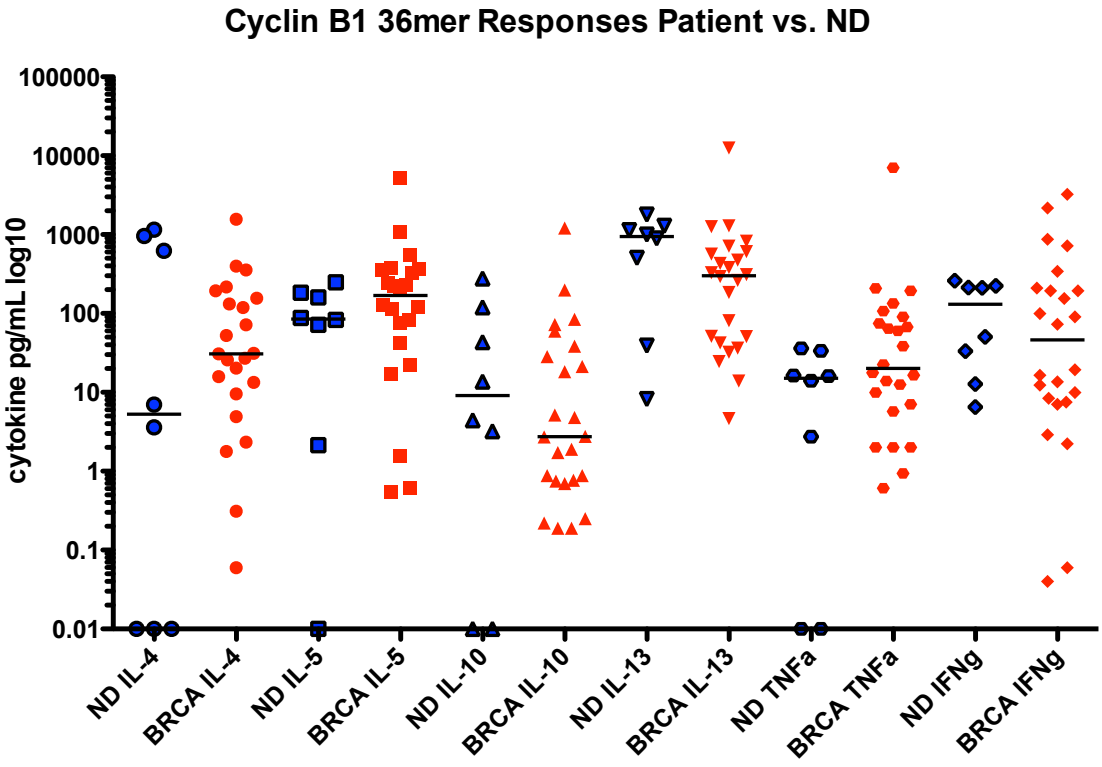

Figure 43. Cytokine profile of patient PBMCs with Cyclin B1 36mer. PBMCs were cultured for 7 days with Cyclin B1 long peptide 50mer, harvested and restimulated with peptide on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent median values. ND = healthy donor comparison.

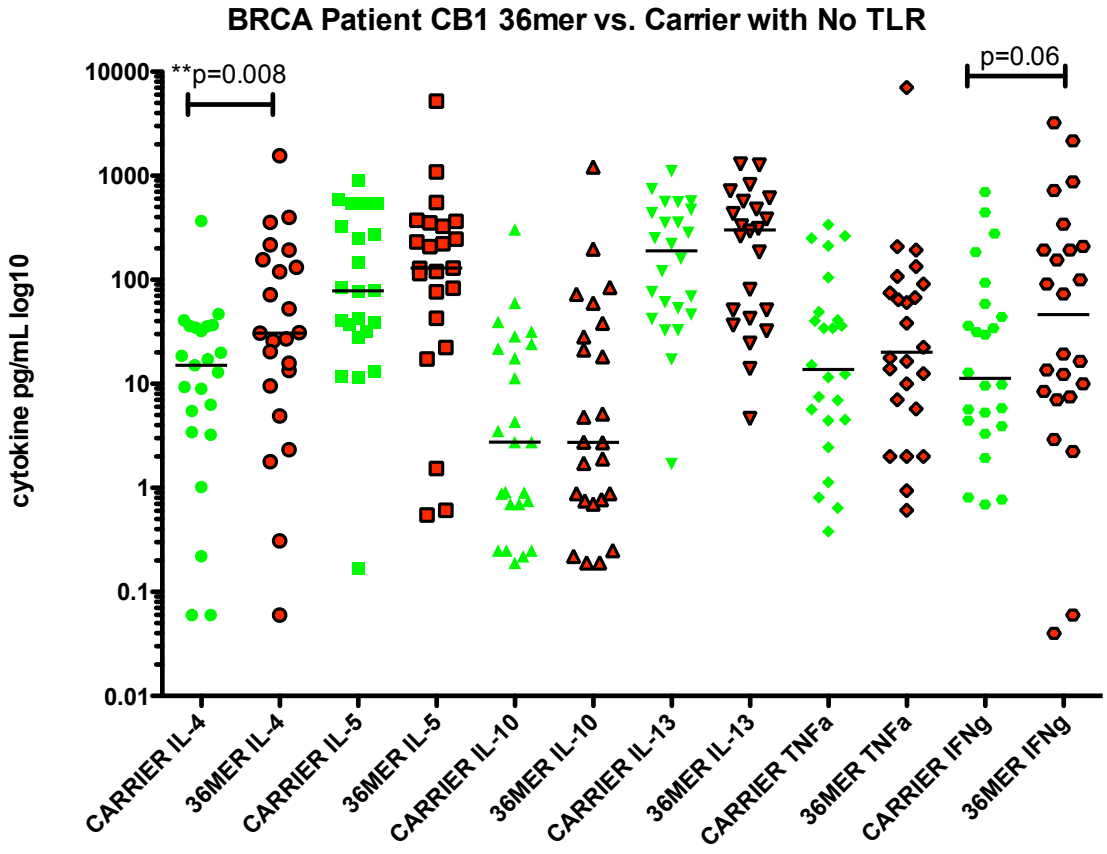

Figure 44. Patient responses to no antigen control vs Cyclin B1 36mer. PBMCs were cultured for 7 days with Carrier (no antigen) or 36mer, harvested and restimulated with respective peptides on Day 7, and supernatant was taken for Luminex cytokine secretion analysis after 24 hours. Bars represent mean values. P value Mann Whitney test.

This overall signature of Th2 cytokines found in patient samples, especially when exposed to Cyclin B1 peptides, mirrors the type of signature found in breast cancer tissues, showing that this signature affects more than the environment around the tumor, and can even extend to the bloodstream. We did not find any differences in the behavior of IL-13, however, and this is the cytokine that is most important in fostering an inflammatory Th2 environment in the tumor.

So while a Th2 bias is present in these samples with regards so IL-4, IL-5, and TNFα, it does not directly coincide with the environment in the tumor. A summary of patient responses is in Table 3.

Table 3. Comparison table of patient vs. healthy donor or no antigen control immune responses. CB1: Cyclin B1. NA: no antigen. H: healthy donor. B: breast cancer patient. Columns of cytokines indicate which donor or condition secreted more of that particular cytokine. Asterisk indicates significant p value.

Condition	IL-4	IL-5	IL-10	IL-13	TNFα	IFNγ
No antigen	H*	B*	B	H	H	H
Viral	H	H	H	H*	H	H*
Global CB1	B*	B	H	H*	H	H*
NA vs. CB1	CB1	CB1	CB1	CB1*	CB1	CB1*
50mer	B*	B	H	H	B	H
NA vs. 50mer	50*	50*	50	50*	50*	50*
36mer	B	B	H	H	B	H
NA vs. 36mer	36*	36	36	36	36	36

Discussion

One of the benefits of cancer vaccination over and above current therapies is that there is the potential for the patient to not only develop a strong primary T cell response against specific cancer antigens, but also acquire T cell memory, ensuring ongoing protection against relapse. Here, we have shown that healthy donors are able to elicit T cell responses against Cyclin B1 antigens indicating the presence of a T cell memory repertoire. Furthermore, DC/T cell cocultures with CB1 short peptides showed the ability to prime naïve CD8+ T cells.

When T cells were initially stimulated with long peptides and restimulated with short peptides, no T cell response were observed (data not shown) suggesting that the sequences being presented to T cells are not always the sequences that have been found to elicit immunity in previous studies. We consider this to be a further demonstration of our hypothesis that long peptides are better suited for generating T cell immunity in the

context of cancer. Data from previous studies suggest that it is essential to titrate both long and short peptides to determine the optimal concentration for use. We therefore titrated the CB1 long peptides and determined the optimal concentration to be in the range of 0.3 – 3uM, depending on the peptide used.

CD8+ T cell responses were strongest in the DC/T cell cocultures. However, CD4+ responses were predominant in PBMC experiments, supporting previous studies that found CB1 antibodies in cancer patient serum (Suzuki *et al*. 2005). The CD4+ T cells that did respond to CB1 long peptides in this study produced a small but clearly positive amount of IFNγ, suggesting a low precursor frequency of these T cells in the blood. The generation of Cyclin B1-specific IFNγ -producing T cells may rely on other cell types besides DCs and CD8+/ CD4+ T cells. The results observed when using PBMC cultures suggest that this might be the case. Also, the IFN*a* DCs used in the cocultures may not be as potent as the mDCs and pDCs present in the PBMC cultures at establishing CB1 immunity. It is clear from these experiments that DCs, with the aid of other cell types in PBMC cultures, can expand antigen-specific T cells, especially CD4+ T cells, and can therefore process and present antigen from long peptides. Cyclin B1 is a self antigen, and it is therefore not unexpected that T cells specific for this antigen should be rare. So much so that the T cell frequency is so low that measurable T cell responses were not observed in all experiments. T cells that are highly reactive against Cyclin B1 are most likely eliminated in the thymus during T cell negative selection. Cells that remain in circulation are most likely of low affinity, and are low in frequency (Alanio *et al.* 2010, Rizzuto *et al.* 2009). It is therefore not surprising that generating a Cyclin B1 response to peptides in healthy donors is challenging. PBMCs were cultured at 2x10e6 cells per well suggesting

that the frequency is equal to or lower than 1 in 2 million cells, assuming that any T cell in culture that was specific for CB1 would be expanded (Rizzuto *et al.* 2009). Vella *et al.* (2009b) showed that in spite of the low frequency, such responses were possible in an antigen-specific manner to Cyclin B1 15mer peptide. Here, we confirm these findings, and show that healthy donors are also able to elicit antigen-specific responses to Cyclin B1 long peptides. Healthy donors have had very little exposure to Cyclin B1 as a peptide presented in the context of danger signals, yet memory T cells from healthy donor blood are able to respond and produce IFNγ. When considered in the context of cancer, this means that at the onset of tumor establishment, there are memory T cells already in circulation that can encounter Cyclin B1 expressing tumor and engage in an immune response if the environment is suitable and they are properly activated. Future work should include elucidation of improved methods of generating Cyclin B1-specific T cells and using DC-targeted fusion protein monoclonal antibodies.

Long lasting, protective T cell responses to cancer antigens is the end goal of this study. Here we have shown that patients responded to long peptides within the short timeframe of 7 days, suggesting pre-exisiting memory. The patients in this study were chosen at random and would most likely have a variety of different HLA types. Overall, patients secrete more Th2 cytokines, specifically IL-4, IL-5, and TNFα, than healthy donors. IL-13, a key cytokine in the pro-tumor environment, is produced, but not at significantly higher levels than healthy controls. Conversely, healthy donors secrete more IFNγ in response to the same peptide, and patients more Th2 cytokines, suggesting that breast cancer patients are naturally skewed towards a type 2 response and a dampened production of IFNγ. Skewing the T cell response away form this Th2 bias is important in

modulating in the breast cancer microenvironment. Therefore, inclusion of CL-075, a TLR8/7 agonist, with propensity to lower Th2 cytokines and increase Th1 cytokines in cell cultures in a vaccination strategy could be a good way to achieve the goal of reprogramming the global immune response to Cyclin B1 in the context of breast cancer. We examine this possibility in Chapter 4 along with healthy donor responses to Cyclin B1 peptides with CL-075.

CHAPTER FOUR

Results: Reprogramming Cyclin B1 Immunity in Healthy Donors and Breast Cancer Patients

Introduction and Rationale

We have shown in the previous chapter that healthy donors have IFNγ-specific responses to Cyclin B1 peptides. We have also shown that patients have antigen-specific responses to Cyclin B1 peptides with a Th2 bias. It is our desire to augment the response in healthy donors and reprogram the Th2 response in patients. Our institute has previously shown that a TLR 8/7 adjuvant, CL-075, has the ability to enhance type 1 immune responses in an antigen-specific manner (Klechevsky *et al*. 2010). Type 1 immune responses are widely believed to be the most beneficial for cancer eradication because it involves the generation of cells that have specific, direct action against target cells. CL-075 also has the ability to decrease cytokines involved with a type 2 immune response, one that persists in the breast cancer microenvironment, is self propagating, and is immunosuppressive. Therefore, we have endeavored to utilize CL-075 to modulate and reprogram the immune responses against Cyclin B1 in breast cancer patients to one that could benefit tumor eradication. We test this first on healthy donors to learn about T cell-specific responses, then focus on modulating patient immune responses, both in the context of Cyclin B1 immune responses.

Antigen-Specific T Cells Express More IFNγ in Response to CL-075 in Healthy Donors

For a breast cancer vaccine, we would like to elicit stronger IFNγ responses from both CD4+ and CD8+ T cells. As discussed in the introduction, we believe that CL-075 has the potential to have this effect. Therefore, we cultured healthy donor PBMCs for 7 days and restimulated on Day 7 with peptide for 6 hours, followed by intracellular staining. We titrated CL-075 by adding it to the cells at day 0 to determine optimum concentration for use (not shown). We verified the manufacturers recommendation of 1ug/mL, and this is the concentration used henceforth.

The addition of CL-075 to PBMC cultures was found to increase the percentage of IFNγ expressing CD4+ and CD8+ T cells (Figures 45, 46, and 47). Repeated experiments with the same donor showed some variability depending on precursor frequency, however, we can observe a difference in the CD4+ and CD8+ antigen-specific IFNγ expression in these healthy donors (Figure 50). CL-075 can also increase the expression of CD40L, an important stimulatory ligand for DCs and CD8+ T cell expansion (Figure 48, 49). Double expression of IFNγ and CD40L on the same cells is also increased, as seen in Figure 48 (13.7% IFNγ + CD40L+ for CL-075 Carrier response vs. 22.1% IFNγ+ CD40L+ for CL-075 36mer response, and compared to 15% IFNγ+ CD40L+ for 36mer without CL-075). Carrier IFNγ T cell responses are also increased, but since CL-075 is known to generally increase type 1 cytokines, this is not a surprising finding. The differences between antigen-specific responses and carrier responses are considerable enough to convince us that the expression we observe is real and significant.

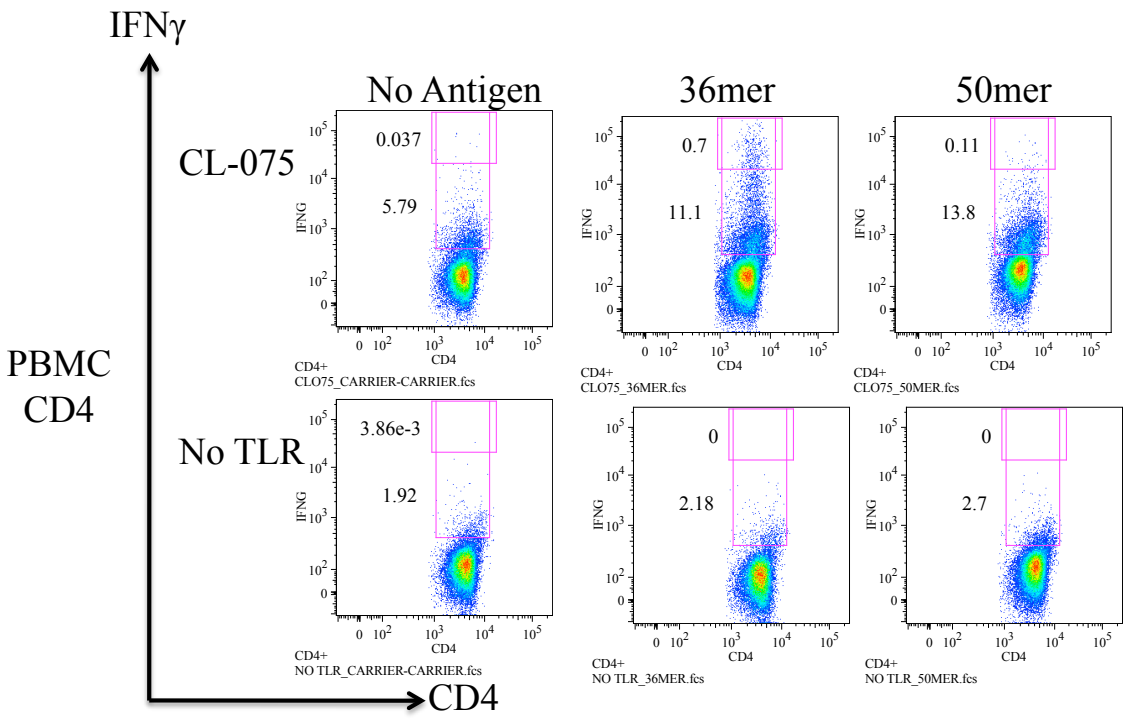

Figure 45. CL-075 increases antigen-specific expression of IFNγ in CD4+ T cells. PBMCs were cultured for 7 days in the presence of 36mer and 50mer peptide and CL-075 before restimulation for 6 hours on day 7. Cells are gated on CD3+ CD4+ live cells. Cells stimulated with either 36mer or 50mer and CL-075 express more IFNγ than carrier (no antigen) control, or cells not stimulated with CL-075 (No TLR).

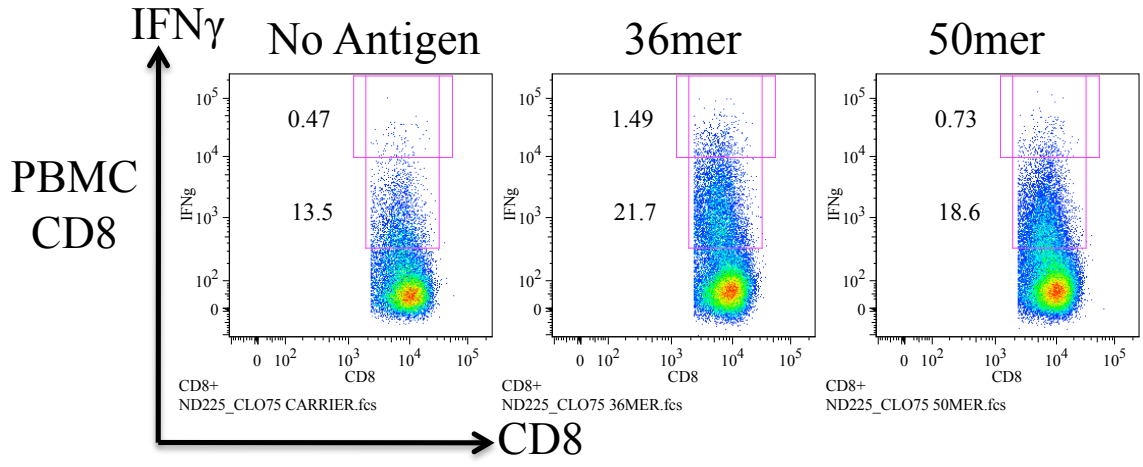

Figure 46. CL-075 increases antigen-specific expression of IFNγ in CD8+ T cells. PBMCs were cultured for 7 days in the presence of 36mer 50mer peptide and CL-075 before restimulation for 6 hours on day 7 followed by intracellular staining. Cells are gated on CD3+ CD8+ live cells. Cells stimulated with either 36mer or 50mer and CL-075 express more IFNγ than carrier (no antigen) control.

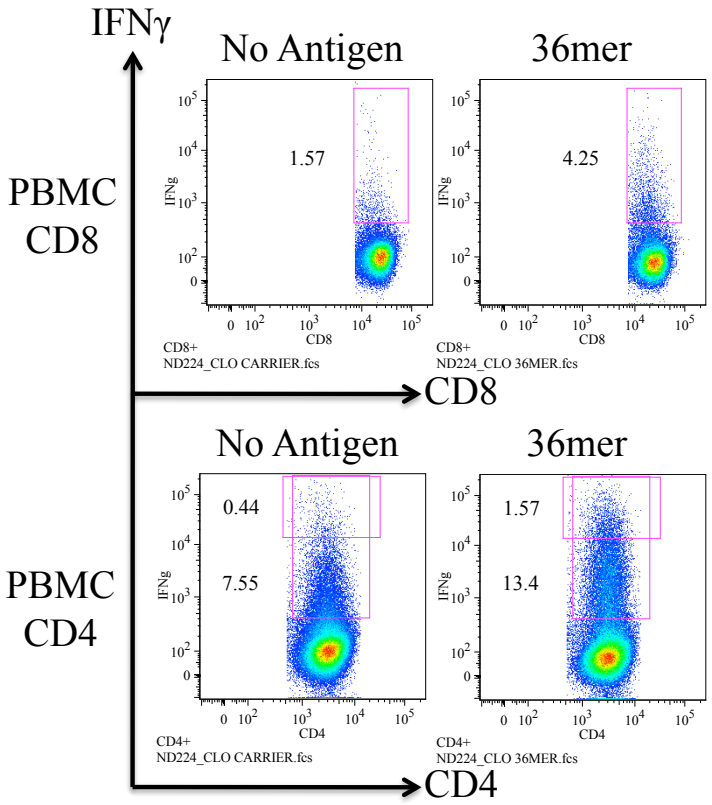

Figure 47. CL-075 can augment IFNγ antigen-specific response to long peptide in CD4+ and CD8+ T cells of the same donor. PBMCs were cultured for 7 days in the presence of 36mer peptide and CL-075 before restimulation for 6 hours on day 7. Cells are either gated on CD3+ CD8+ (top) live cells or CD3+ CD4+ (bottom) live cells. Cells stimulated with 36mer and CL-075 express more IFNγ than no antigen Carrier control.

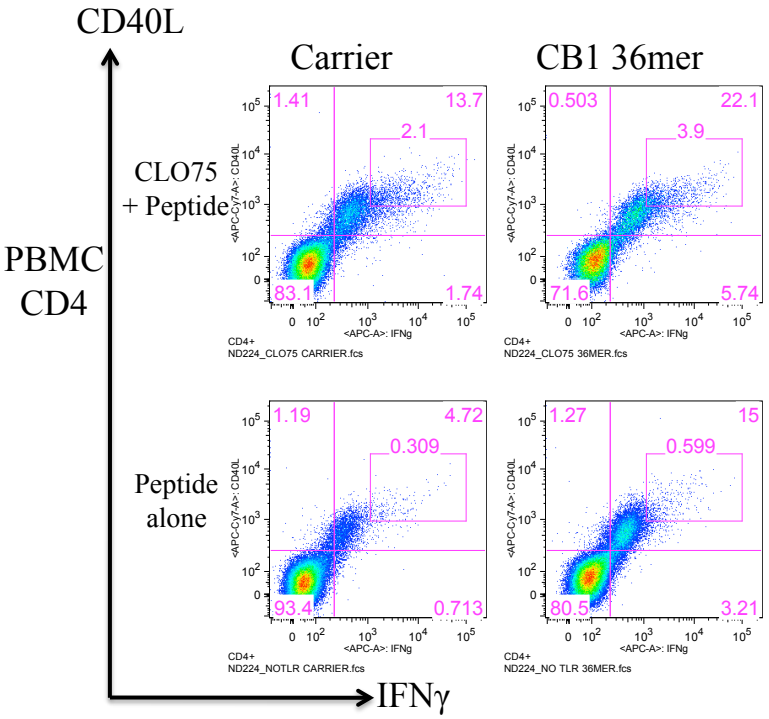

Figure 48. CL-075 increases peptide-specific expression of double positive IFNγ and CD40L on CD4+ T cells. PBMCs were cultured for 7 days in the presence of Carrier (no antigen) or 36mer peptide and CL-075 before restimulation for 6 hours on day 7 followed by intracellular staining. IFNγ and CD40L double positive cells from the same gating, either CL-075 treated (top) or with peptide alone (bottom). Figure demonstrates both peptide-specific expression of CD40L and increase of this response with the addition of CL-075.

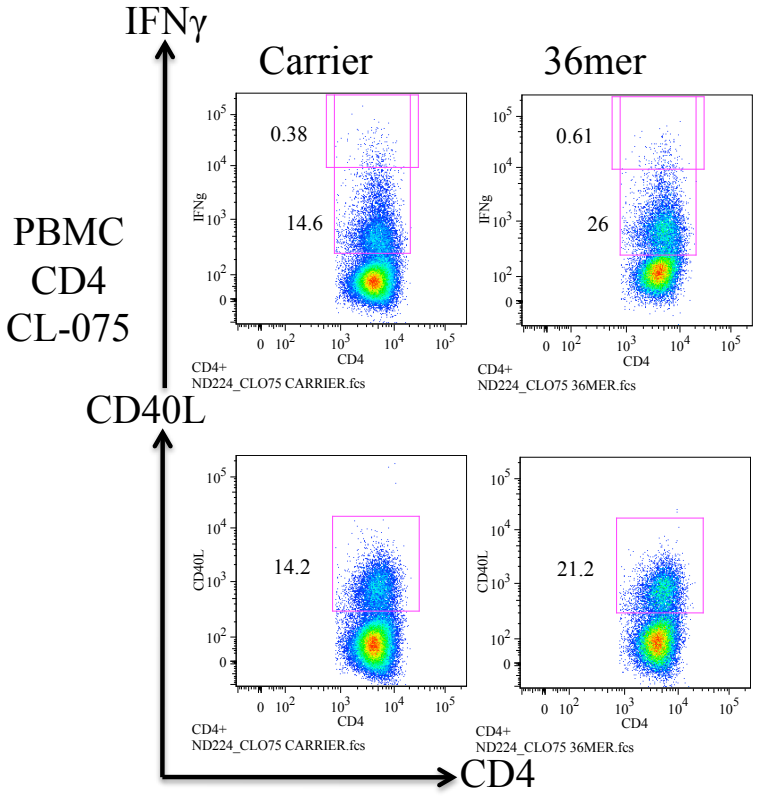

Figure 49. CL-075 increases peptide-specific expression of IFNγ and CD40L on CD4+ T cells. PBMCs were cultured for 7 days in the presence of Carrier (no antigen) or 36mer peptide and CL-075 before restimulation for 6 hours on day 7 followed by intracellular staining. Left panel shows cells gated on CD4+ T cells, and individual expression of either IFNγ (top) or CD40L (bottom).

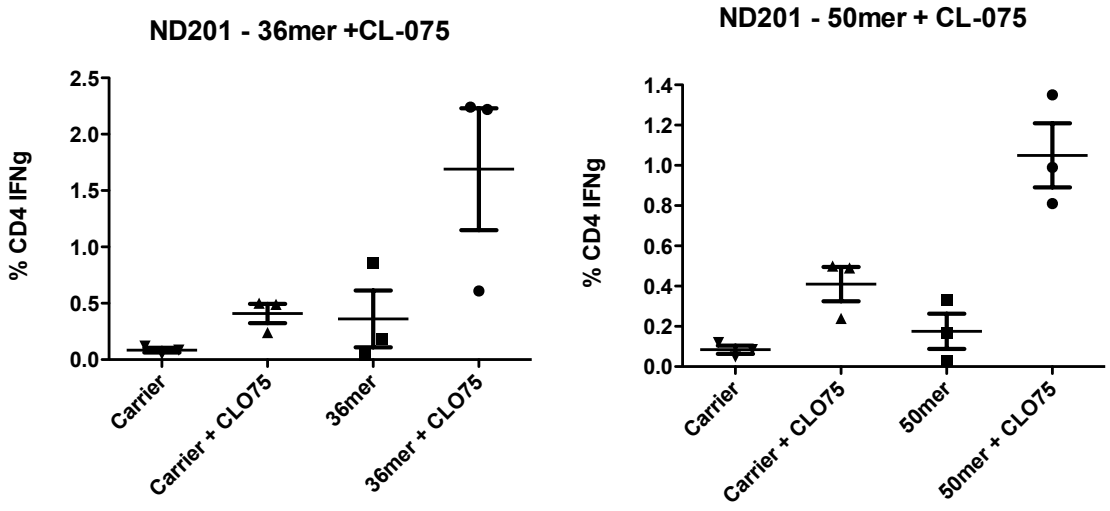

Figure 50. Reproducibility: repeated experiments with the same donor shows improvement in long peptide-specific IFNγ expression with addition of CL-075. PBMCs were cultured for 7 days in the presence of 36mer or 50mer peptide and CL-075 before restimulation for 6 hours on day 7. Results with 36mer peptide vs. Carrier (no antigen) control (left) and results with 50mer peptide vs. Carrier (right). Three independent experiments, bars represent mean +/- standard error of the mean.

To determine if CL-075 could augment the response to Cyclin B1 in multiple healthy donors, we tested 12 healthy donors for responses to Cyclin B1 long peptides with the addition of CL-075. Wilcoxon p value for CD4+ T cell IFNγ production was not significant for either 36mer or 50mer vs. Carrier with the addition of CL-075 (p=0.06), but there was enough evidence to suggest a trend (36mer mean %IFNγ = 8.19 +/- 1.91; 50mer mean %IFNγ 7.73 +/- 1.89; control 5.86 +/- 1.12). 8/12 donors saw improvement in IFNγ -specific response to Cyclin B1 peptides with the addition of CL-075 for CD4+ T cells. However, when looking at high IFNγ producing CD4+ T cells, CL-075 significantly increases IFNγ production (p- value for 36mer+ CL-075 vs. 36mer No TLR = 0.0005; p-value for 36 + CL-075 vs. Carrier + CL-075 = 0.03) (Figure 51). 10/12 donors saw a significant response in IFNγ hi-expressing cells.

In addition, CL-075 was able to significantly (Wilcoxon p value for 36mer = 0.0005, 50mer = 0.01) increase IFNγ production in CD8+ T cells for both 36mer (15.77 +/- 2.44) and 50mer (12.64 +/- 2.03) vs. Carrier control (9.33 +/- 1.70). 12/12 donors saw improvement in CD8+ T cell responses to 36mer, while 11/12 donors saw improvement to 50mer stimulated CD8+ T cells. Compared to conditions without CL-075, 36mer and 50mer CD4+ and CD8+ T cells produced significantly more IFNγ (all p-values significant) when compared to carrier with and without CL-075 (Figure 52). In summary, CL-075 was able to substantially increase the expression of IFNγ for both long peptides in virtually all healthy donor CD4+ and CD8+ T cells.

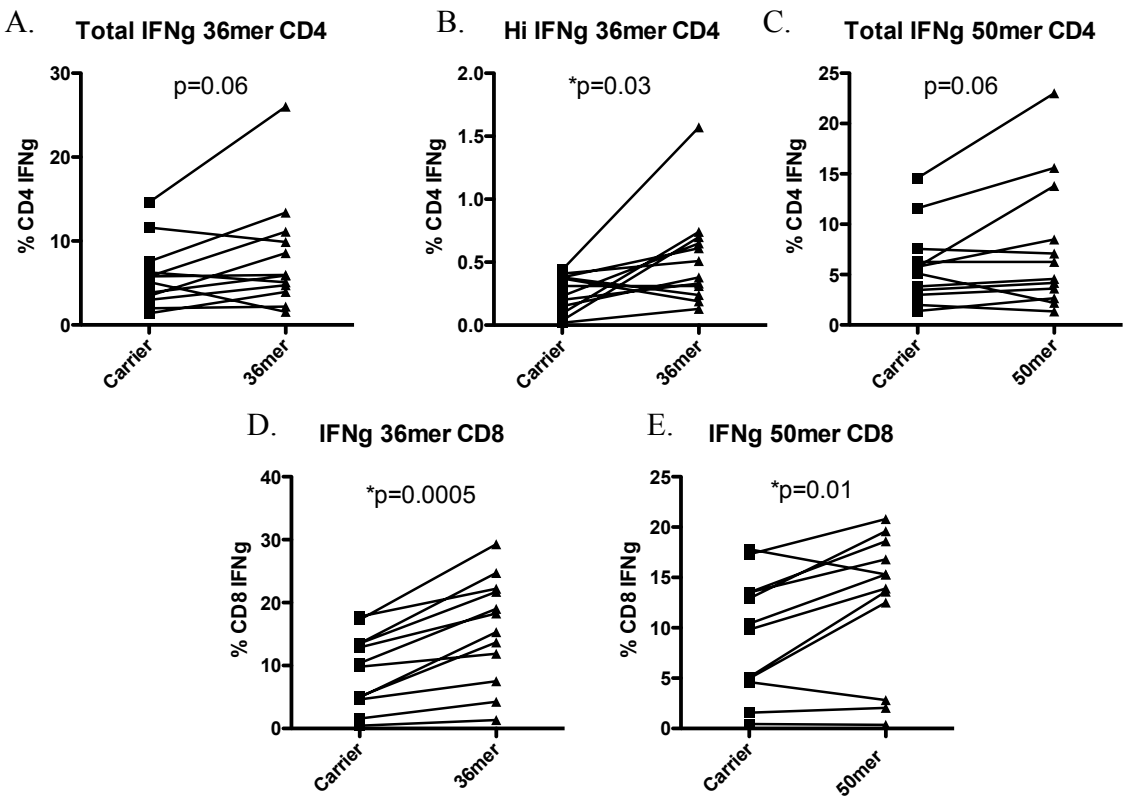

Figure 51. CL-075 increases IFNγ expression in T cells of 12 healthy donors compared to no antigen control carrier. PBMCs were cultured for 7 days in presence of peptide with CL-075 and restimulated on Day 7 with peptide for 6 hours. Results show graphical representation of flow cytometry data. Bars connect identical donors. A. Total amount of IFNγ expression in CD4+ T cells, 36mer vs. no antigen Carrier control. B. Amount of CD4+ T cells expression 'hi' levels of IFNγ , between 10^4 – 10^5. C. Total amount of IFNγ expression in CD4+ T cells cultured with either 50mer or Carrier. D. IFNγ expression in CD8+ T cells cultured with 36mer vs. Carrier. E. IFNγ expression in CD8+ T cells cultured with 50mer vs. Carrier. Statistical test used: Wilcoxon p values.

Patient Responses are Modulated with CL-075

IFNγ Responses on Day 2

In order to measure effector responses to CL-075 when added to PBMC cultures on Day 0, we took supernatant on Day 2 for Luminex analysis. BRCA patient IFNγ responses to peptides were significantly increased compared to conditions with no CL-075 (Figure 53). Responses with no antigen (carrier) were also increased, showing that in patients there is non-specific activity of CL-075 on all cells. CL-075 has the ability

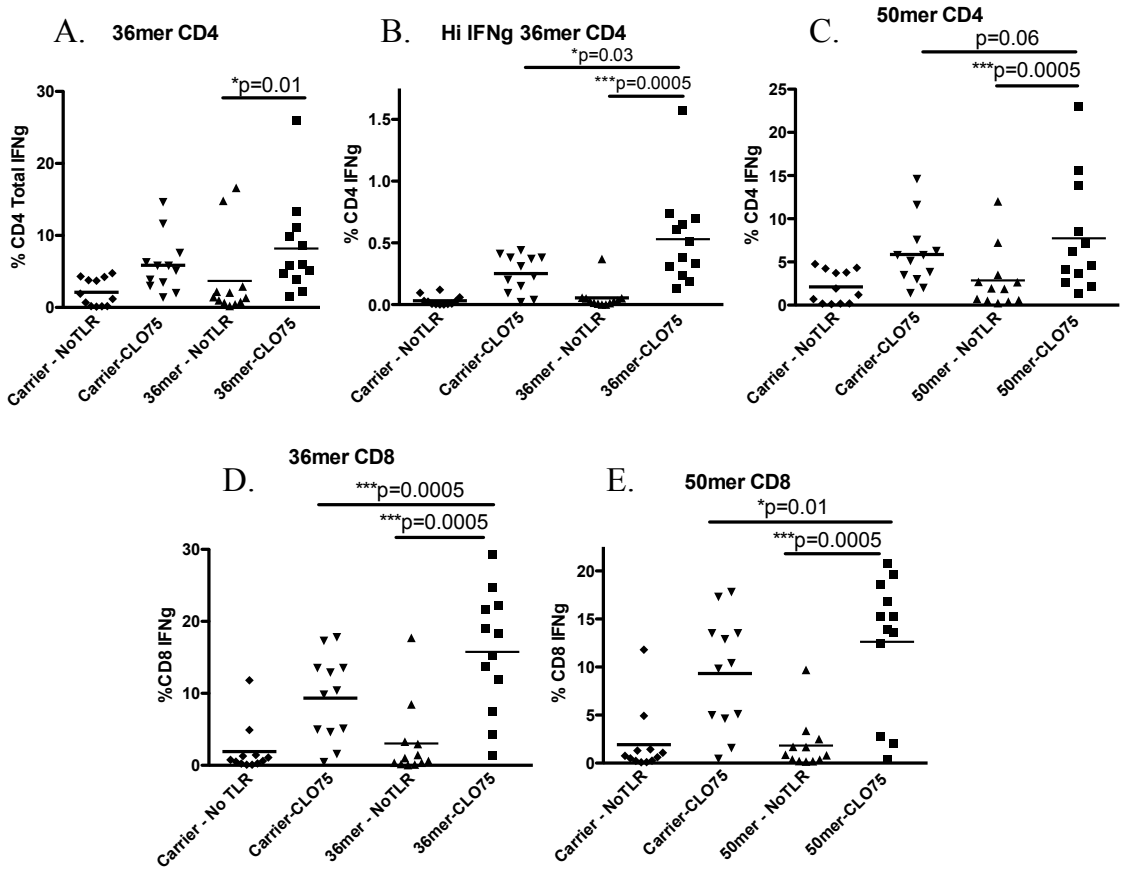

Figure 52. Responses to Cyclin B1 long peptides by healthy donors with CL-075 compared to no treatment and no antigen control carrier. PBMCs were cultured for 7 days in presence of peptide with CL-075 1ug/mL and restimulated on Day 7 with peptide for 6 hours. Results show graphical representation of flow cytometry data. A. Total CD4+ T cell responses to 36mer with comparison to Carrier and conditions without adjuvant. B. CD4+ T cell responses expressing high levels of IFNγ . All cells are in the 10^4 – 10^5 decade and therefore express 'hi' amounts of IFNγ . C. Total CD4+ T cell responses to 50mer. D. and E. CD8+ T cell responses to 36mer and 50mer, respectively, with comparison to Carrier and conditions without adjuvant. Wilcoxon p values. Bars represent mean values.

to modulate effector responses in a time period as short as 48 hours. Figure 54 also shows that patients still have responses to viral antigens on Day 2 without addition of CL-075. This level of IFNγ seen on day 2 has an effect on cell cultures, providing more Th1 cytokines than in conditions without CL-075.

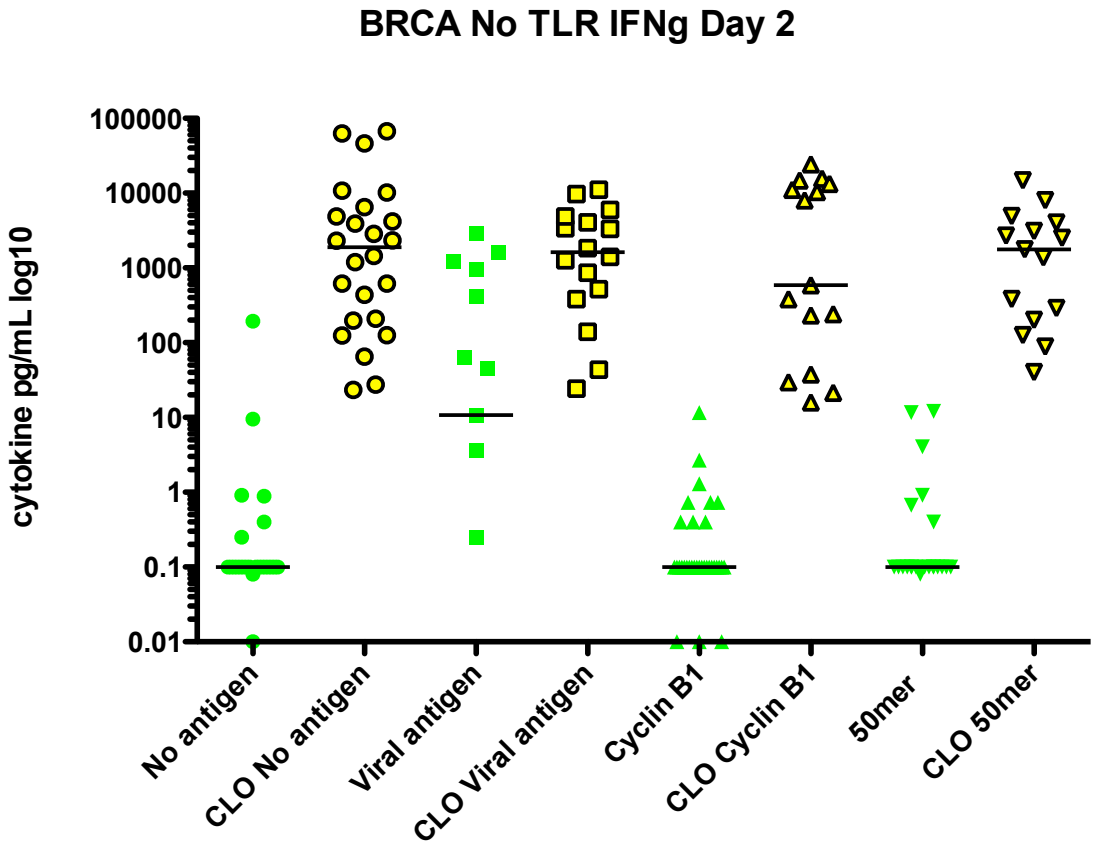

Figure 53. Patient responses to peptides on Day 2 No TLR vs. CL-075. PBMCs were cultured for 48 hours with Carrier (no antigen), CEF (viral antigens), all Cyclin B1 peptides, or long peptide 50mer. Supernatant was taken for Luminex cytokine secretion analysis. Patients have response to viral antigens after 48 hours, and IFNγ responses to all conditions tested increase with addition of CL-075. All condition comparisons between No TLR and CLO are significant (see Figure 54).

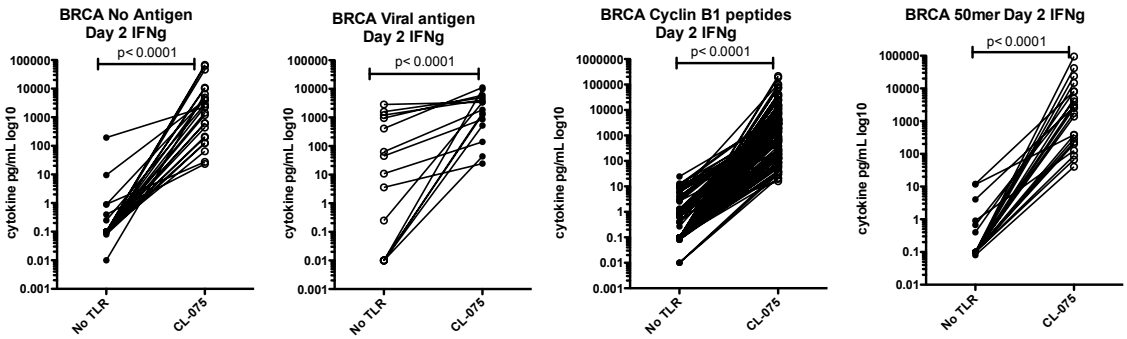

Figure 54. Patient responses to peptides on Day 2 with CL-075. PBMCs were cultured for 48 hours with Carrier (no antigen), CEF (viral antigens), all Cyclin B1 peptides, or long peptide 50mer. Supernatant was taken for Luminex cytokine secretion analysis. P values are from paired Wilcoxon test. Bars connect samples from the same patient.

Patient Responses on Day 8

BRCA patient cell PBMCs were cultured with CL-075 and Cyclin B1 peptides for 7 days, restimulated with the same corresponding peptide for 24 hours, followed by collection of supernatant and analysis by Luminex. The results were then analyzed with Excel and Prism. In the first round of analysis, we looked at whether an antigen-specific response was present in a particular patient. Although we had no strict guidelines for what constituted an antigen-specific response, in general, any response to a peptide at least two fold higher than a response to no antigen, in any cytokine, and across multiple cytokines for the same peptide, was considered an antigen-specific response. The cytokine profiles of all the cytokines measured for that patient peptide response was entered into a database and used to determine categorizations of patients into groups. Because data was organized in this way, some cytokines of some patient responses do not change much with the addition of CL-075, but it is important to remember that this patient did have a peptide/antigen-specific cytokine response across at least two different cytokines. Some patients had antigen-specific responses for more than one peptide, and sometimes the pattern of response was different not only between patients, but between peptides of the same patient, while remaining antigen-specific. An example of a full battery of peptides tested and what an antigen-specific response might look like that we would characterize as such is in Figure 55. After a significant number of patients were enrolled, we started to notice trends in types of responses typically demonstrated by patients. Overall, 23/24 patients had antigen-specific responses to at least one peptide. In those 23 patients, there were 43 total peptide-specific responses.

When all patients are considered, patient responses to Cyclin B1 50mer are significant (p=0.0059) compared to responses without CL-075. When compared to no antigen with CL-075, results are not significant (p=0.0574), but reveal a trend of antigen specificity. Overall patient responses to 36mer and 50mer with and without CL-075 are in Figure 56. 12/24 patients had a peptide-specific response to Cyclin B1 50mer (12 out of 43 total peptide responses). 10/24 patients had a peptide-specific response to 36mer. It is also significant that CL-075 could substantially raise IFNγ in response to carrier, but the trend for 50mer was not as clear. This speaks to the difficultly in working with self antigen cancer peptides. While the p value for Cyclin B1 50mer with and without CL-075 is still significant, there were some patients that had a decreased response to 50mer with CL-075. Further work will need to be done to determine a strategy for generating stronger IFNγ responses to these long peptides in these patients.

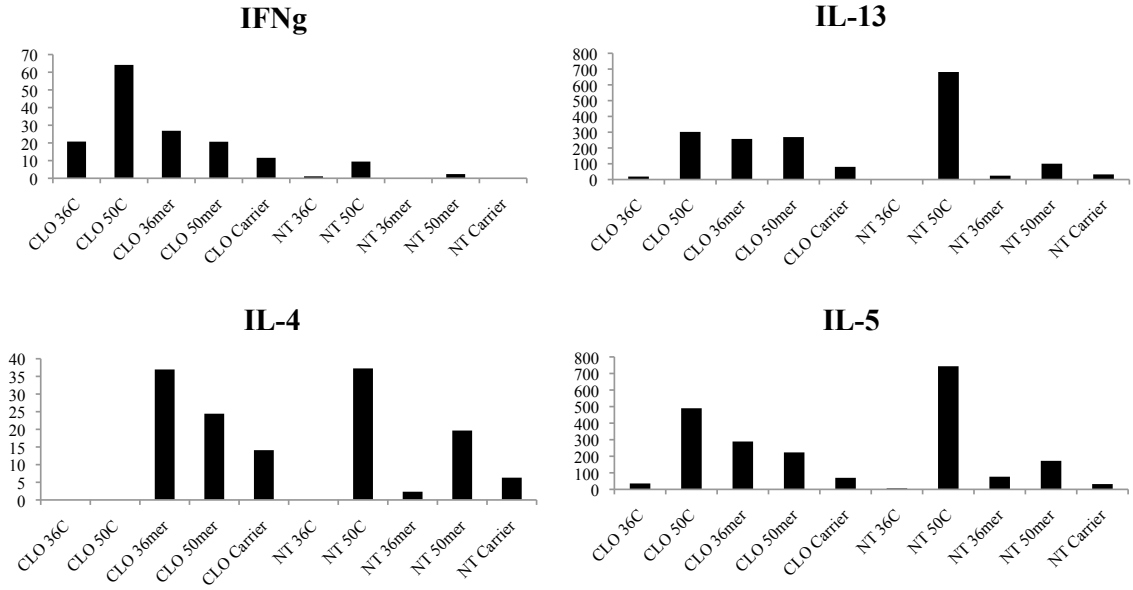

Figure 55. Example of an antigen-specific response from a patient. BRCA patient cell PBMCs were cultured with CL-075 and Cyclin B1 peptides for 7 days, restimulated with appropriate peptide for 24 hours, followed by collection of supernatant and analysis by Luminex. This donor has a peptide-specific response to 50mer peptide clusters. (50C). This patient can increase Th1 cytokines and block Th2 cytokines with the addition of CL-075.

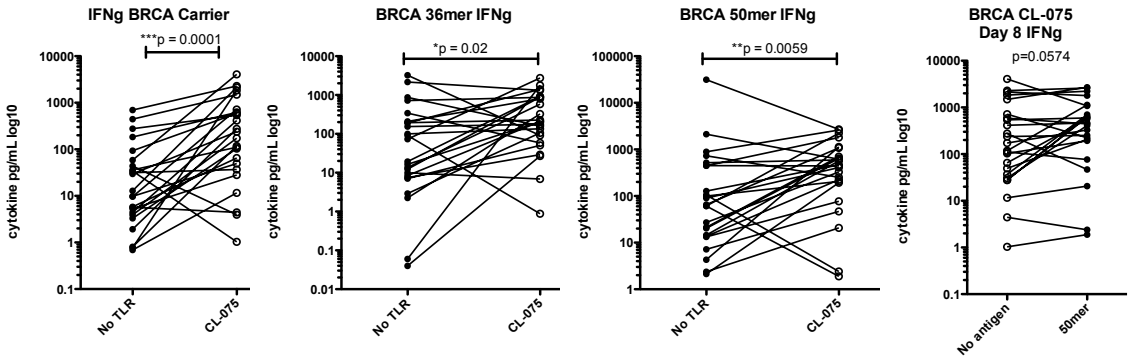

Figure 56. Responses by patients to CL-075 and Cyclin B1 peptides on Day 8. BRCA patient cell PBMCs were cultured with CL-075 and Cyclin B1 peptides for 7 days, restimulated with appropriate peptide for 24 hours, followed by collection of supernatant and analysis by Luminex. P values from paired Wilcoxon test. Figure demonstrates antigen specificity to Cyclin B1 50mer by all patients tested. Even though CL-075 upregulates overall IFNγ response, specificity to 50mer can still be detected.

Patient Responses Reveal Trends in Cyclin B1 Specificity in Response to CL-075

In the previous chapter we saw that patients had a decreased Th1 response to Cyclin B1 peptides than healthy donors, and an increased IL-4 and IL-5 response to Cyclin B1 peptides. Our goal is to reverse this trend in as many patients as possible in this study using CL-075. Patients were therefore categorized by two different grouping schemes: 1) Raise Th1 cytokines or Unmodulate Th1 cytokines; and 2) Block Th2 cytokines or Unmodulate Th2 cytokines. Some patients had antigen-specific responses to more than one peptide, so for the purpose of this categorization scheme, they are counted as separate data points because they are responses to a different peptide sequence.

In order to determine baseline or negative control immunity of patients, we measured cytokines in response to no antigen (carrier) with and without CL-075 (Figure 57). With the addition of CL-075, IL-5 increased ($p = 0.04$), IL-10 decreased ($p = 0.03$), and IL-13 ($p = 0.01$), TNFα and IFNγ ($p<0.0001$) all increased. The baseline response to CL-075 by these patients is to increase Th2 cytokines and Th1 cytokines. However, the IFNγ response was clearly the strongest of these.

When responses to individual peptides are considered, both 36mer and 50mer conditions did not see much change in the secretion of Th2 cytokines (Figure 58 and 59). However, the IFNγ secretion in both conditions was increased. Again, these are the responses of all patients, whether or not those patients showed antigen specificity for these long peptides.

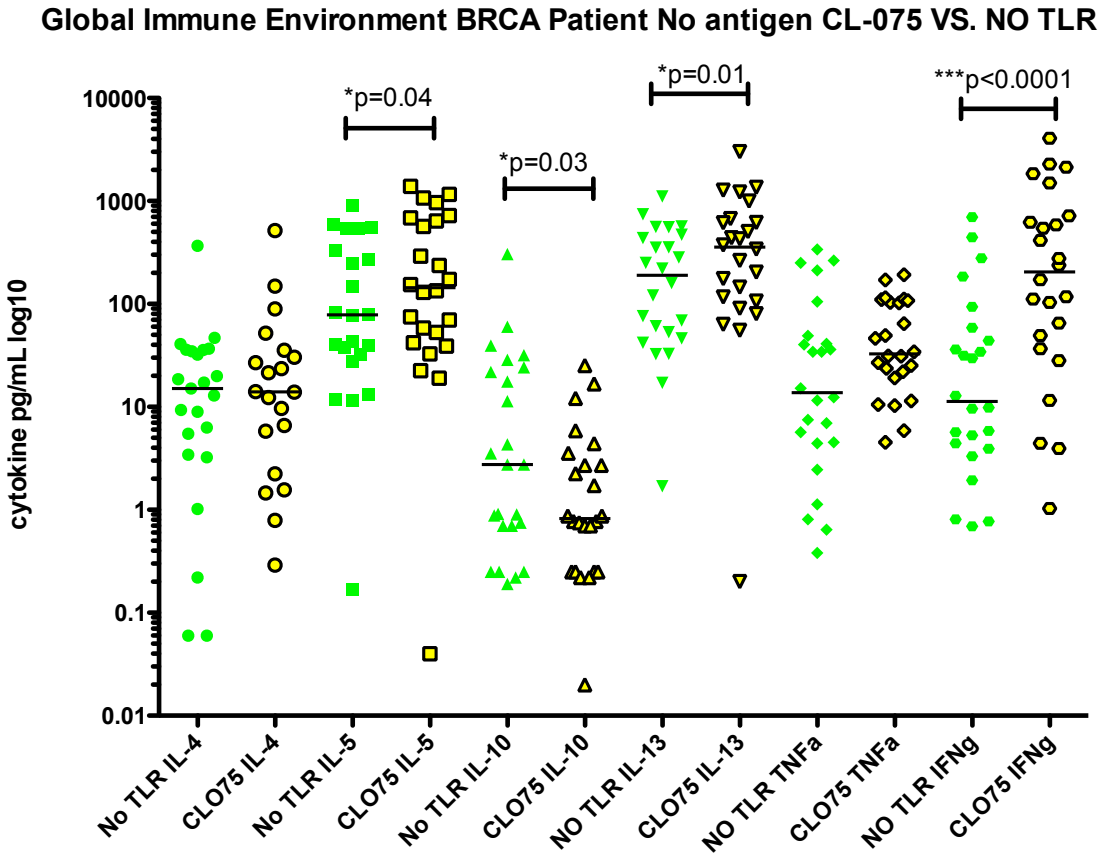

Figure 57. Overall immune environment in PBMCs of breast cancer patients with and without addition of CL-075. BRCA patient cell PBMCs were cultured with CL-075 and Carrier (no antigen) for 7 days, restimulated for 24 hours, followed by collection of supernatant and analysis by Luminex. Bars represent mean values. P value from paired Wilcoxon test.

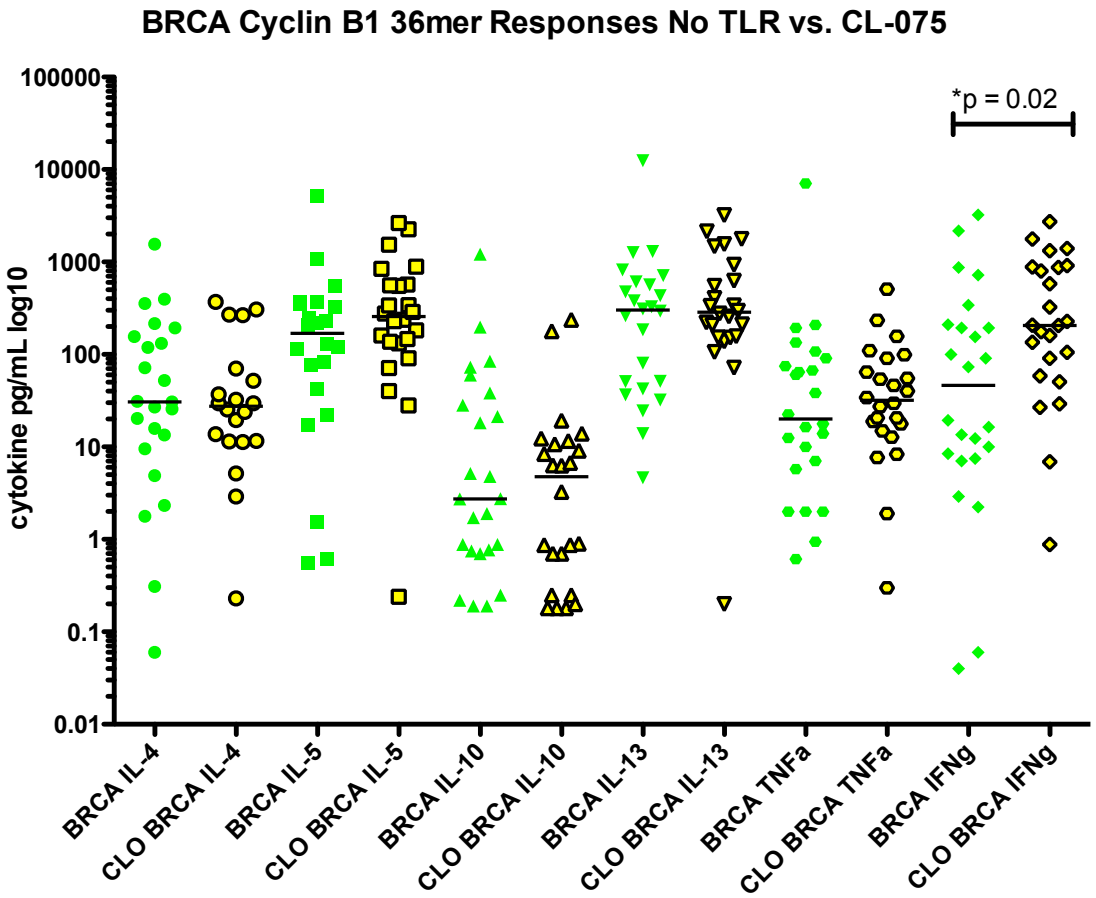

Figure 58. Immune environment in PBMCs of breast cancer patients to Cyclin B1 36mer with and without addition of CL-075. BRCA patient cell PBMCs were cultured with CL-075 and Cyclin B1 36mer for 7 days, restimulated for 24 hours, followed by collection of supernatant and analysis by Luminex. Bars represent mean values. P value from paired Wilcoxon test.

Figure 60 shows peptide-specific responses of patients that increased their Th1 cytokines, specifically IFNγ, and are in the 'Raise Th1' group. 31 peptide-specific responses are in this group representing 19 patients. The response of these patients to carrier was similar to that of the peptide-specific responses. However, we have already demonstrated that these patients have peptide-specific responses. Although the response to carrier is increased, this is expected, because at a baseline level, CL-075 is known to increase the levels of these cytokines. Since we only include antigen-specific responses

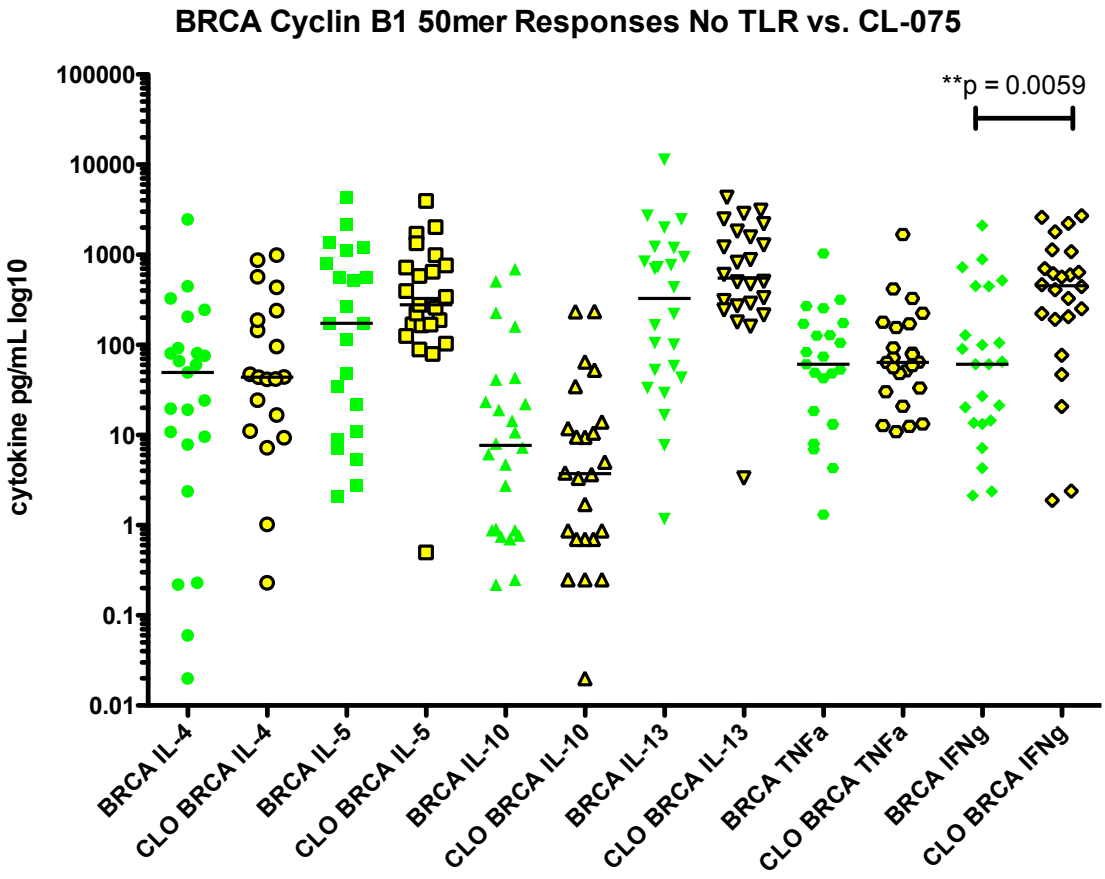

Figure 59. Overall immune environment in PBMCs of breast cancer patients to Cyclin B1 50mer with and without addition of CL-075. BRCA patient cell PBMCs were cultured with CL-075 and 50mer for 7 days, restimulated for 24 hours, followed by collection of supernatant and analysis by Luminex. Bars represent mean values. P value from paired Wilcoxon test.

in any of these groupings, the fact that CL-075 can also increase IFNγ in an antigen-specific manner is important to our study. Levels of Th2 cytokines in this group are on average unaffected comparing the untreated to the CL-075 group. There is a trend towards increasing the Th2 cytokines: only 6 patients raised IFNγ and lowered Th2 cytokines. However, when comparing CL-075 treated cells to no antigen controls with CL-075, levels of all cytokines increase, including IFNγ ($p = 0.04$). When compared to carrier

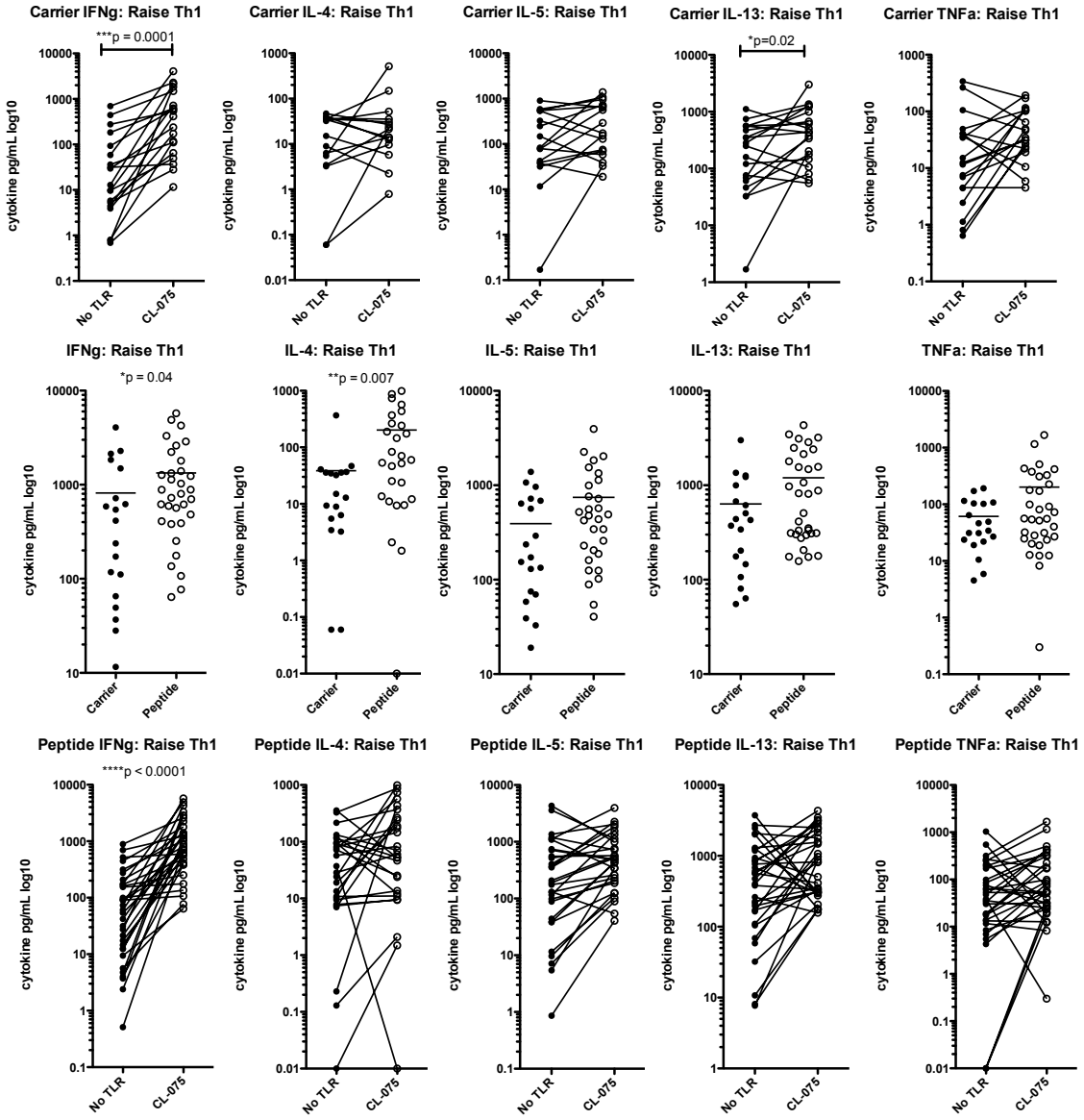

Figure 60 'Raise Th1': Peptide-specific responses by patients that increased Th1 responses with the addition of CL-075. Top row: Patient responses to carrier (no antigen) with no adjuvant vs. with the addition of CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. Middle row: comparison of patient responses to carrier or peptide with CL-075. All conditions were treated with CL-075. Bars represent mean values. P value from Mann-Whitney test. Bottom row: patient responses to peptide without adjuvant and with CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. 31 patient peptide-specific responses included in data set.

responses with CL-075, peptide responses are all higher than carrier responses across all cytokines.

The peptide-specific responses that were not included in the group that increased Th1 were placed into the 'unmodulated Th1' group (Figure 61). 12 peptide-specific responses of 8 patients fit into this group. When peptide-specific responses are compared to peptide responses, there is a less obvious trend of antigen specificity. As expected, responses in this group decrease IFNγ, but they also decrease all Th2 cytokines (all p values significant). Patients without an increased IFNγ-specific response, in general, lower the Th2 cytokines produced in response to CL-075.

Patients were regrouped according to their responses to Th2 cytokines. Patients that lowered secretion of at least one Th2 cytokine with the addition of CL-075 in a peptide-specific manner were put into the 'Block Th2' group (Figure 62). 16 peptide-specific responses of 13 patients were categorized in this group. Patients in this group significantly lowered secretion of all Th2 cytokines tested, although decrease of all Th2 cytokines was a not a requirement to be put in this group (all p values significant). Therefore, this suggests that the ability to lower the levels of more than one Th2 cytokine is a valid indicator of the ability to lower secretion of all inflammatory Th2 cytokines with the addition of CL-075. P value for secretion of IFNγ was not significant, but there was a clear trend in lowering IFNγ secretion in this group.

Patient peptide responses that were not in the 'Block Th2' group were put into the 'Unmodulated Th2' group (Figure 63). There were 27 peptide-specific responses representing 16 patients in this group. Patients in this group significantly increased their secretion of IFNγ, IL-5, IL-13, and TNFα. The antigen-specific nature of the

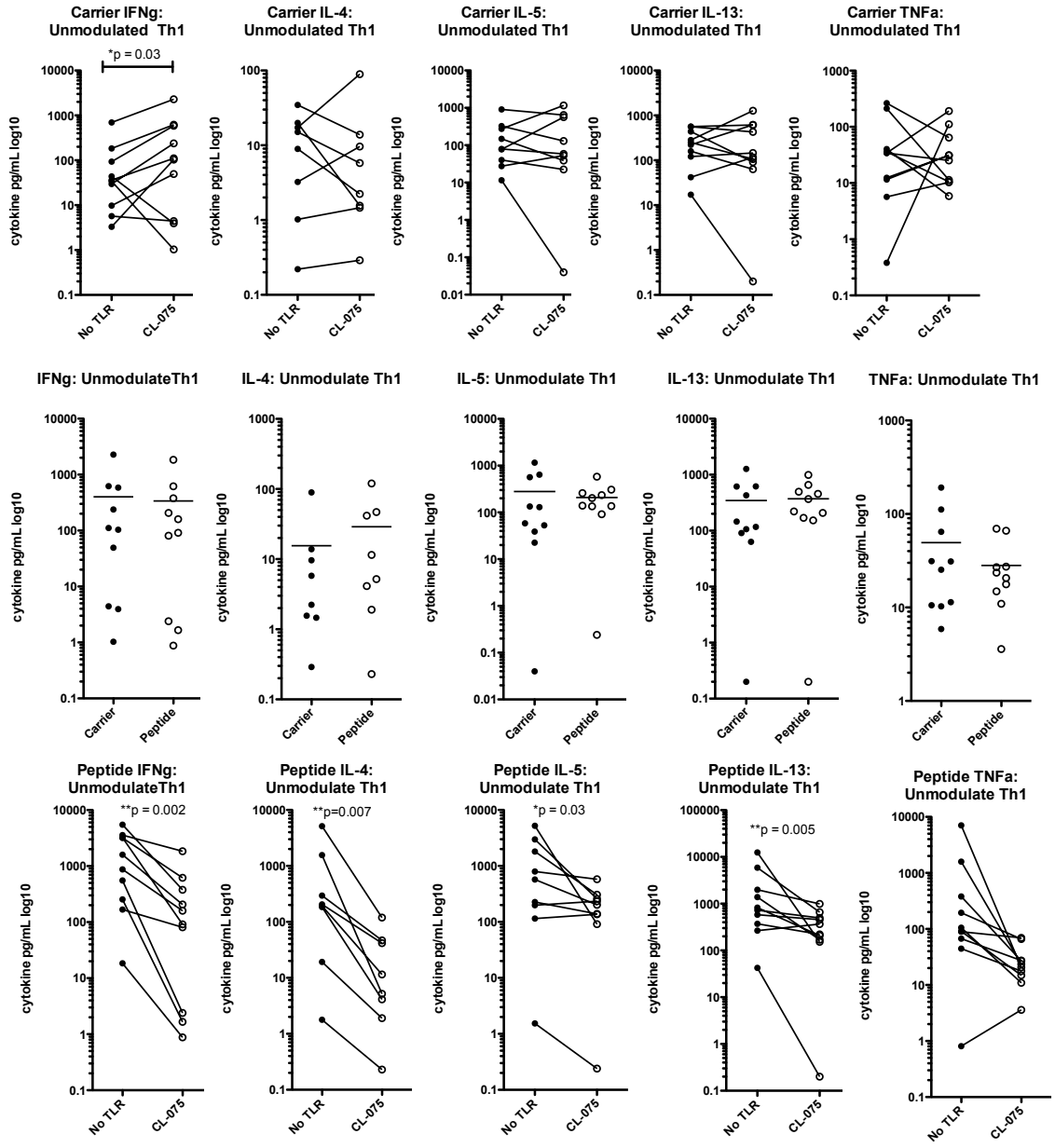

Figure 61. 'Unmodulate Th1':Peptide-specific responses by patients where Th1 responses were unmodulated with the addition of CL-075. Top row: Patient responses to carrier (no antigen) with no adjuvant vs. with the addition of CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. Middle row: comparison of patient responses to carrier or peptide with CL-075. All conditions were treated with CL-075. Bars represent mean values. P value from Mann-Whitney test. Bottom row: patient responses to peptide without adjuvant and with CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. 12 patient peptide-specific responses included in data set.

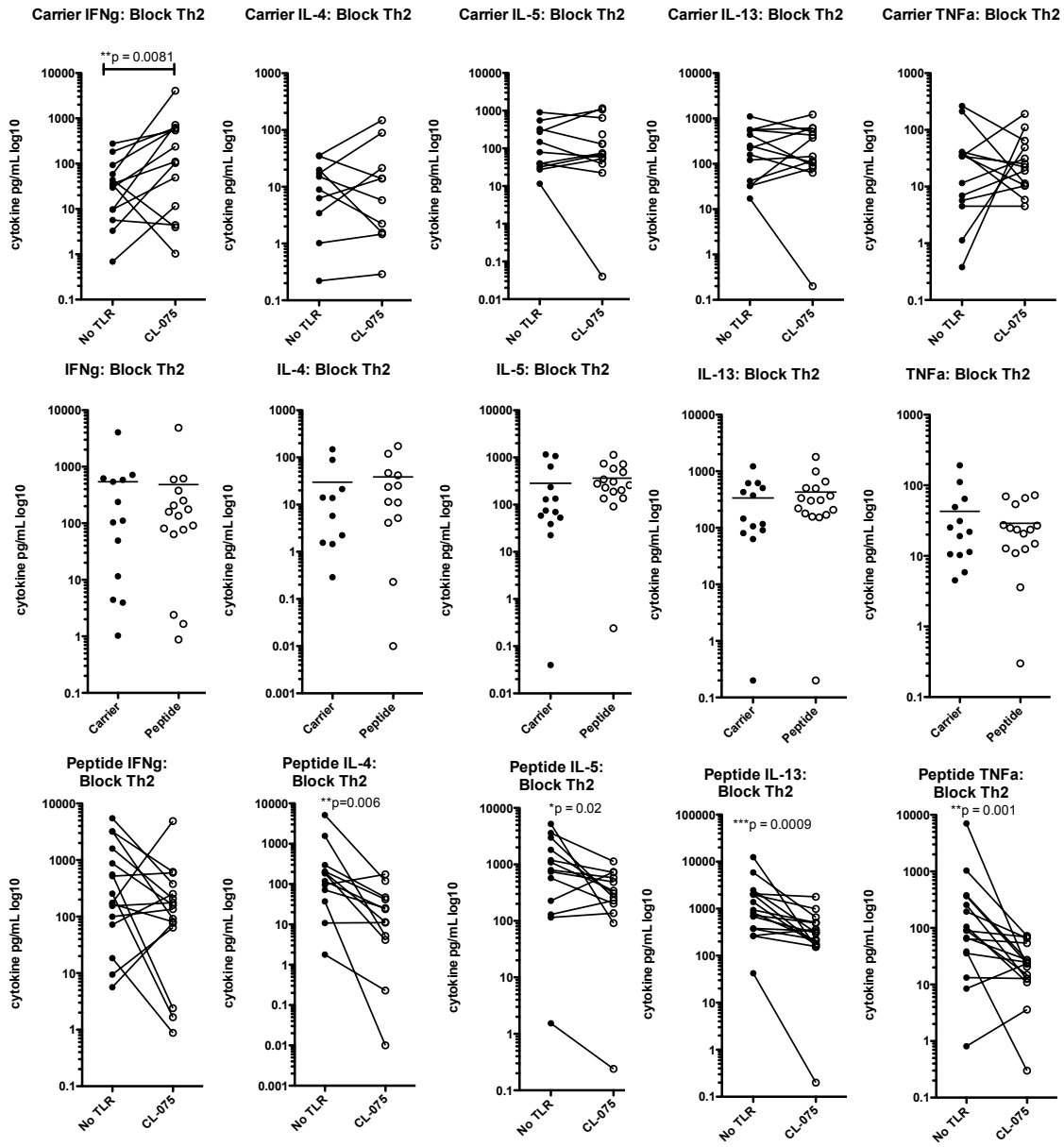

Figure 62. 'Block Th2': peptide-specific responses by patients that blocked Th2 responses with the addition of CL-075. Top row: Patient responses to carrier (no antigen) with no adjuvant vs. with the addition of CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. Middle row: comparison of patient responses to carrier or peptide with CL-075. All conditions were treated with CL-075. Bars represent mean values. P value from Mann-Whitney test. Bottom row: patient responses to peptide without adjuvant and with CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. 16 patient peptide-specific responses included in data set.

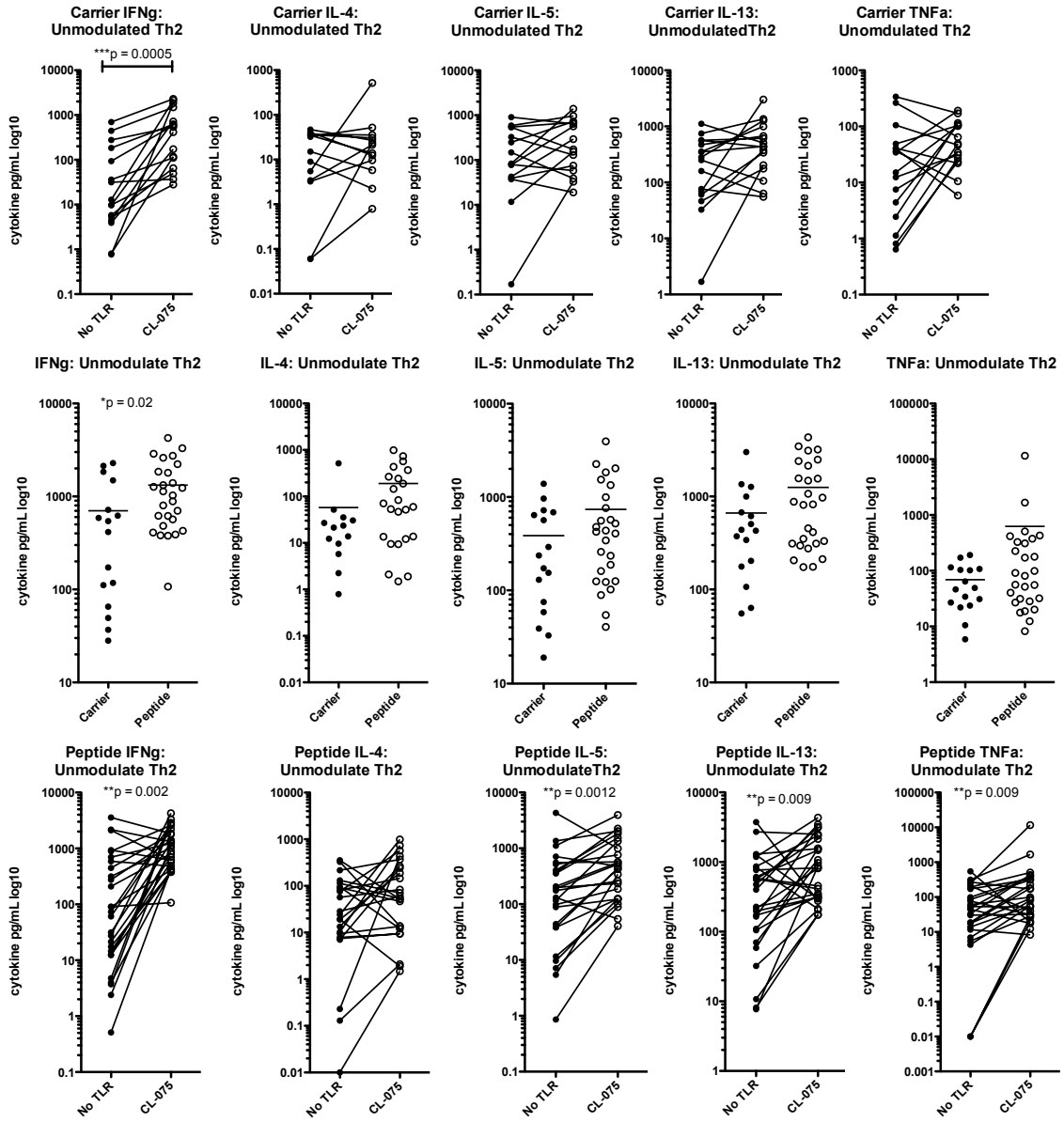

Figure 63. 'Unmodulate Th2': peptide-specific responses by patients where Th2 responses were unmodulated with the addition of CL-075. Top row: Patient responses to carrier (no antigen) with no adjuvant vs. with the addition of CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. Middle row: comparison of patient responses to carrier or peptide with CL-075. All conditions were treated with CL-075. Bars represent mean values. P value from Mann-Whitney test. Bottom row: patient responses to peptide without adjuvant and with CL-075. Bars connect peptide responses from the same patient. P value from paired Wilcoxon test. 27 patient peptide-specific responses included in data set.

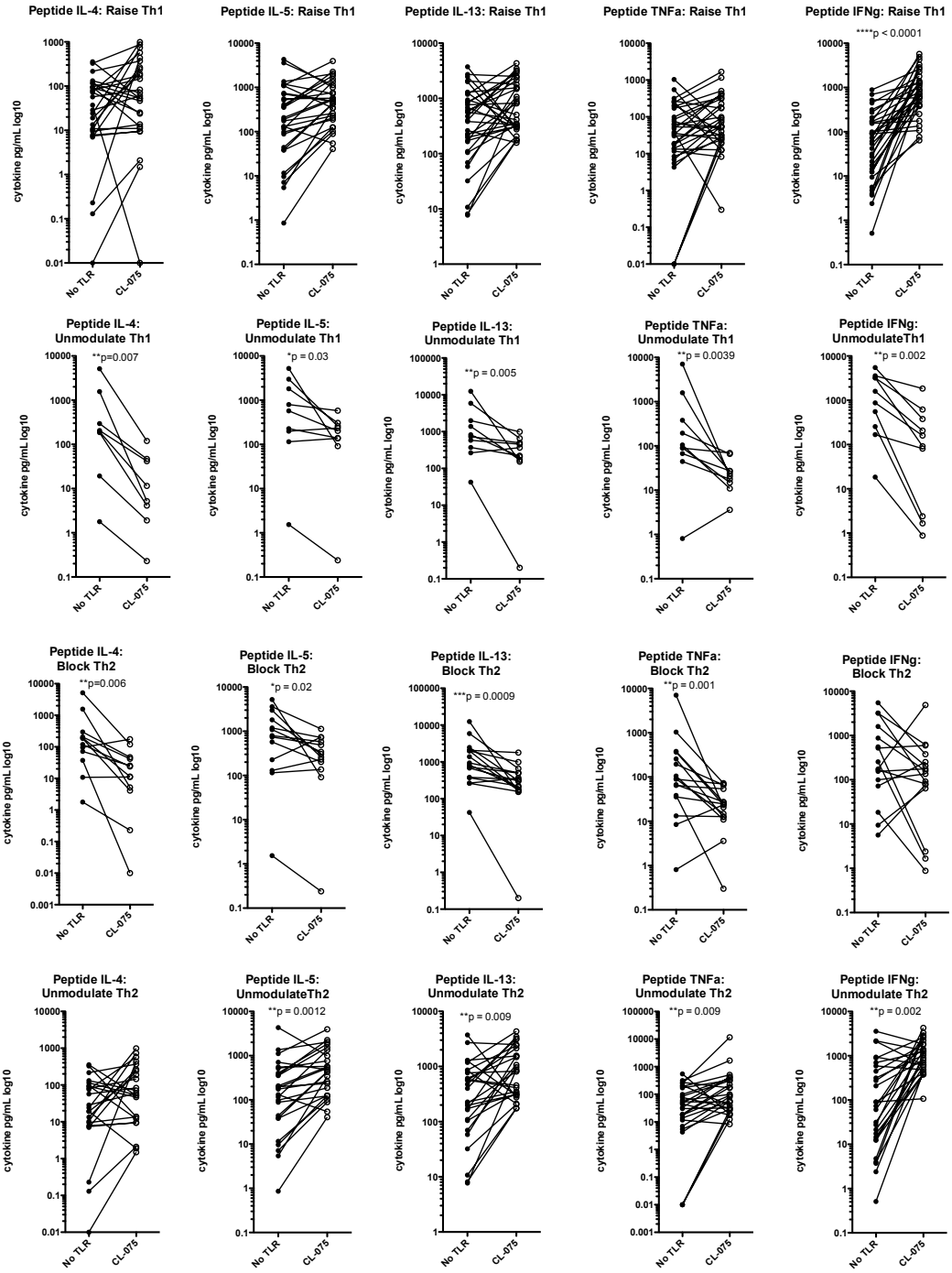

Figure 64. Peptide-specific responses of all patient groups showing No TLR vs. CL-075 treatment. Top row: Patient group 'Raise Th1'. Second row: Patient group 'Unmodulate Th1'. Third row: Patient group 'Block Th2'. Fourth row: Group 'Unmodulate Th2'. Bars represent values from same patient. P value from paired Wilcoxon test. These are identical graphs from the previous four Figures, with the bottom row of each of those Figures included in the present one to illustrate the overall trends present in antigen/CL-075 responses.

peptide-specific responses is more obvious to see in this group when compared to carrier. It is clear by looking at these last two groups that the ability to block Th2 cytokines through use of CL-075 comes as a group rather than individually to specific cytokines.

All peptide-specific responses of all groups before and after CL-075 treatment are in Figure 64. Together, we can see the increase of Th1 and Th2 cytokines vs. the blockade of these cytokines in two different subsets of patients, using two different grouping mechanisms.

A summary of the patient groupings with mean and median values for all cytokines tested is in Table 4.

Discussion

We saw in chapter three that healthy donors and breast cancer patients had memory T cells-specific for Cyclin B1. Here, we show that these responses can be enhanced through use of a TLR8/7 agonist, CL-075. The addition of CL-075 was able to significantly increase the antigen-specific IFNγ expression in both CD4+ and CD8+ T cells in 12/12 healthy donors. Even CD8+ T cells, in which it was difficult to see antigen-specific responses, were able to expand and specifically express IFNγ.

With this in mind, we attempted to elicit the same responses in breast cancer patients, while at the same time decreasing the Th2 cytokine response to Cyclin B1 peptides.

Patient responses to peptides were changed as early as day 2. The amount of IFNγ secreted was significantly increased. This is important because increased levels of IFNγ would then be present in the 7 day culture to skew the response to type 1, most likely through IL-12 secretion by DCs. Studies are ongoing to determine the specific cell types

Table 4. Summary of BRCA patient groupings. Cytokine values in pg/mL. CLO: CL-075.

Group	# Patients	# Peptides	Value	IFNg	IFNg CLO	IL-4	IL-4 CLO	IL-5	IL-5 CLO	IL-13	IL-13 CLO	TNFa	TNFa CLO
Raise Th1	19	31	Mean	145.2	1332.1	116.8	202.58	606.9	702.7	793.3	1199.3	120.0	200.8
			Median	60.9	801.7	69.1	59.79	189.8	424.4	520.5	810.9	38.4	55.4
Unmod Th1	8	12	Mean	1896.6	338.7	854.6	28.91	1325.6	208.5	2458.3	372.2	963.0	28.1
			Median	1237.0	125.6	193.5	8.33	573.8	171.2	774.1	294.1	98.2	22.1
Block Th2	13	16	Mean	1026.0	483.0	580.0	38.7	1421.8	364.7	2049.8	427.9	613.0	29.0
			Median	215.3	147.9	183.9	17.7	796.3	268.5	880.5	305.1	90.2	23.6
Unmod Th2	16	27	Mean	470.4	1326.5	104.6	190.3	441.8	738.8	638.6	1252.0	95.5	624.6
			Median	73.22	1028.2	42.7	59.79	175.2	424.4	401.5	821.7	44.5	81.2

that contribute most to this effect of increasing Th1 cytokines, however, it is suspected that DCs, through increased secretion of IL-12, have the greatest effect, especially since they interact directly with T cells. Combining these data with those in the previous chapter, we can hypothesize that CD8+ and CD4+ T cells are the primary producers of IFNγ in this system.

After 8 days without the addition of antigen, the global immune response, in the presence of CL-075 was significantly altered: Type 1 and 2 cytokines were increased. Of all the cytokines studied, peptide-specific IFNγ secretion by breast cancer patient PBMCs was affected the most. This suggests that CL-075 has the greatest effect on type 1 cytokines, specifically IFNγ, at least in a PBMC culture setting.

Grouping patients by ability to raise Th1 or block Th2 cytokines revealed patterns that suggest that increase or blockade of one cytokine may correlate with a similar response in other cytokines. While the desired response for this study was to increase Th1 and block Th2 cytokines, it appears that with these culture conditions, we can achieve one but not the other in most cases. 6 patients had this desired response, while 18 either increased all cytokines, or blocked all cytokines in a peptide-specific manner.

Antigen-specific responses were more difficult to see in patients after the addition of CL-075. This does not necessarily mean that antigen specificity is lost: those T cells specific for Cyclin B1 most likely still have the ability to carry out their cytotoxic functions in an antigen-specific manner. We have clearly established specificity towards Cyclin B1 in chapter three, and in healthy donors in this chapter. It is our hypothesis that additional T cells that secrete type 1 cytokines would be beneficial, not detrimental.

As seen in the introduction, CD4+ IL-13, TNFα and IFNγ secreting T cells are present in the breast cancer microenvironment. These cells are responsible for creating the Th2 environment that facilitates tumor growth and survival. It is therefore important to note that in many of the patients in this study, all three of these cytokines were blocked in addition to IL-4 and IL-5. This suggests that the action of these CD4+ T cells has been significantly altered by the addition of CL-075.

The next step in this study will be to confirm that triple positive CD4+ T cells were indeed the cell type blocked using CL-075 through use of flow cytometry. We will also analyze patient characteristics to find possible correlations with clinical factors and outcomes.

The data presented in this chapter combined with that of the previous chapter strongly suggests that the inclusion of CL-075 in a vaccination strategy could reprogram the global immune response to Cyclin B1, enhancing anti-tumor T cell responses in breast cancer.

CHAPTER FIVE

Conclusions

Breast cancer is a disease with which I am personally acquainted. Nearly all of the females on both sides of my family have been afflicted with breast cancer or related ovarian cancer, many of them dying due to the disease or its complications. I have seen first hand the emotional turmoil and physical stress that breast cancer treatment can engender, and it is a driving passion in my life to work towards eventual elimination of the inadequate current gold standards of care and the disease itself. Therefore, I work towards developing treatments that have fewer side effects, that does not affect the patient's quality of life., and that are clinically relevant.

I believe that immunotherapy, specifically those involving DC targeting of antigen and adjuvant, has this possibility. It is unique among most other treatments in that it would have virtually no side effects, and would be targeting the cell subset that directs immune responses both inside and outside the cancer microenvironment. Additionally, once the immune system is activated to recognize the particular antigen used in the study, the cytotoxic effects on tumors can happen systemically without the harsh treatments of chemotherapy. This activation can be channeled into a memory subset of cells that can defend against relapse. And, in the context of this study, it can work for a broad range of patients, not just those with certain HLA types. While some breast cancer treatments might work well when targeted towards those with certain HLA types, I believe that

including as many patients as possible for treatment is an endeavor that is worth the extra time and resources that it takes to research such therapies.

The best immunotherapy strategy relies on the use of dendritic cells to guide an immune response against cancer. It should: 1) induce robust effector CD8+ T cells that can traffic to the tumor and that are polyfuntional, of high avidity, and make cytotoxic molecules which kill tumors; 2) generate CD4+ T cells that express IFNγ in response to peptide to provide help to CD8+ T cells to create a type I microenvironment; 3) generate long lived memory CD4+ and CD8+ T cells that can be reactivated in the event of relapse; 4) skew DCs toward a phenotype that encourages type I driven immune responses to help overcome the Th2 microenvironment in breast cancer tissues; 5) avoid the expansion of Tregs, and ideally could reprogram existing Tregs to Th1 cells; and 6) allow T cells to have the ability to penetrate the tumor site and remain there to carry out their function.

The studies described here have advanced the knowledge of relevant treatment options by showing in this dissertation that:

- Healthy donors have T cell memory repertoire for both regions of Cyclin B1 epitopes presented in long peptides
- The repertoire and frequency of Cyclin B1 specific T cells is low
- The immune system in healthy donors can see epitopes from long peptides and expand T cells based on these epitopes
- The immune response to Cyclin B1 in healthy donors consists mostly of CD4+ T cells

- Both CD4+ and CD8+ T cells can express IFNγ in response to these CB1 long peptides, and this response can be increased by the addition of CL-075
- Breast cancer patient immune cells have the ability to see and respond to both CB1 long peptides
- Their natural responses to CB1 consisted of less type 1 cytokines and more type 2 cytokines than healthy donors
- We were able to modulate this response in a vast majority of these patients using CL-075
- Even though CL-075 increases cytokine production in conditions with no antigen, antigen specificity can still be seen to CB1 long peptides after treatment with CL-075
- As a whole, cytokine secretion was either increased or decreased unilaterally after CL-075 treatment; CL-075 can modulate both Th1 and Th2 responses
- Antigen-specific IFNγ secretion was increased in a majority of patients
- The data suggests that IFNγ +, IL-13+, TNFα + T cells are the cell subset whose cytokine profile is being enhanced or blocked through CL-075 treatment

The next steps in this study will include examining the responses of cell subsets to determine the primary contributor to this cytokine profile switch, along with looking specifically at the cytokines produced by CD4+ and CD8+ T cells.

CL-075 is a powerful modulator of immune responses, but it is not the only TLR agonist or activator at our disposal. Klechevsky *et al.* 2010 found that combining CL-075 with anti-CD40 increased the effect of generating a type 1 immune response. Others have used TLR3, TLR4, or TLR9 to also increase type 1 responses, limit type 2 responses, or control Treg and memory phenotypes of T cells (Sharma *et al.* 2010, Wu *et al.* 2010, Pufnock *et al.* 2011, Pantel *et al.* 2011). Combining TLR agonists might produce even more robust responses in these patients and must be considered.

There are more challenges in the future with regards to developing this vaccine for clinical use. We must determine the subset of DCs that are best to use in the setting of breast cancer. Immature DCs reside in the tumor microenvironment, constantly giving out tolerogenic signals to T cells and facilitating the Th2 environment. By activating the right subset of DCs, we might be able to generate T cells that would be better equipped to travel to tumor sites and carry out their functions without being affected by these tissue resident DCs. We must also determine if it is possible to change the phenotype of these immature DCs in the cancer microenvironment. This will require the right combination of adjuvants to use to activate the appropriate subset of DCs while still generating our desired T cell immunity.

Work from several groups has shown that DC targeting antibodies are relevant and have efficacy (Bonifaz *et al*. 2002, Bonifaz *et al*. 2004, Steinman *et al.* 2003, Trumpfheller *et al.* 2006, Tacken *et al.* 2005, Tacken *et al.* 2008, Cheong *et al.* 2010, Klechevsky *et al.* 2010, Ni *et al.* 2010). Additionally, recent studies have shown that Langerhans cells have increased capacity to generate CD8 and Th1 immunity (Ratzinger *et al*. 2004, Dubsky *et al.* 2007, Palucka *et al.* 2007, Klechevsky *et al.* 2008, Brewig *et al.*

2009, Palucka *et al.* 2010, Romano *et al.* 2011). Using our results and knowledge of these studies, we can create a vaccine that will deliver Cyclin B1 long peptides on an anti-DC antibody that carries activation signals for CD40 and TLR8/7, targeted to a specific DC subset that includes Langherans cells. Preliminary results using PBMCs to test an anti-CD40.Cyclin B1 fusion protein mAb show that this is possible (Figure 65), however the experiments are complex. We are still learning about ways to properly activate DCs in this setting and deliver antigen and adjuvant simultaneously.

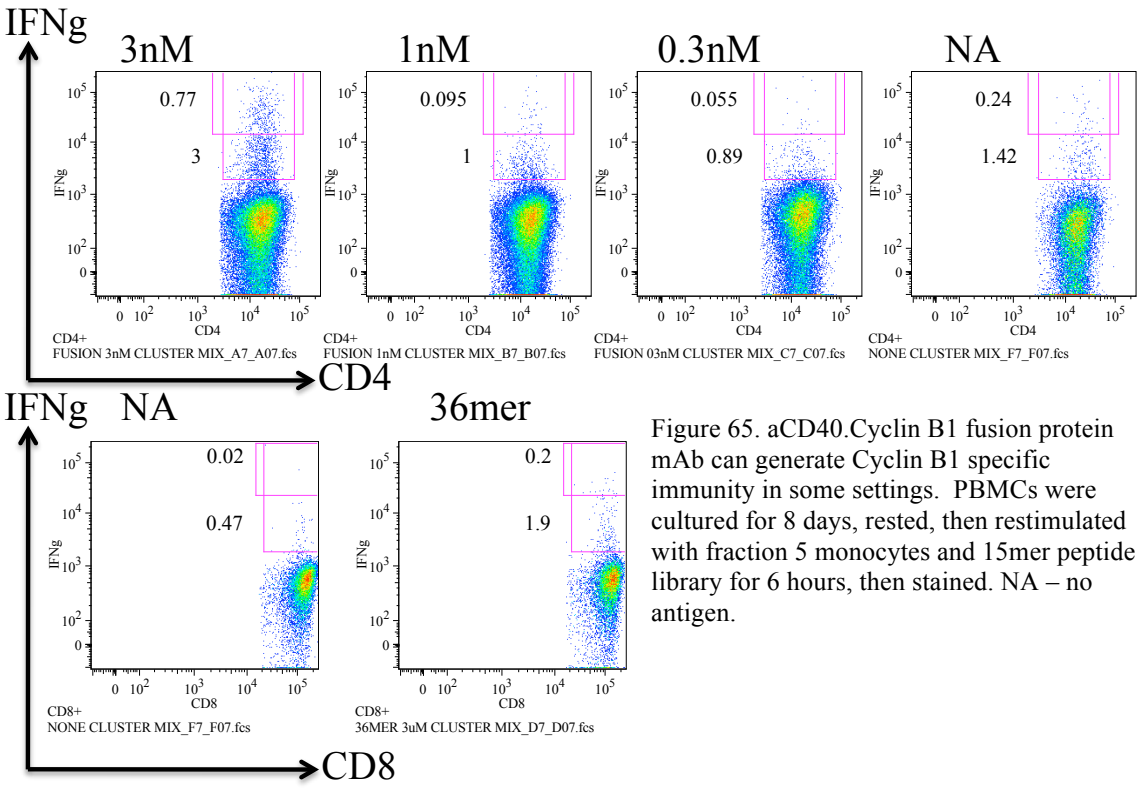

Figure 65. aCD40.Cyclin B1 fusion protein mAb can generate Cyclin B1 specific immunity in some settings. PBMCs were cultured for 8 days, rested, then restimulated with fraction 5 monocytes and 15mer peptide library for 6 hours, then stained. NA – no antigen.

We are in a unique position with tools such as Epimax, Luminex, gene microarrays, and polychromatic flow cytometry to study these responses in depth, and monitor patient responses to immunotherapy vaccines. Our knowledge of DC vaccines and anti-DC targeting antibodies should be combined with data obtained in this study

relating to Cyclin B1 specificity and the ability to reprogram immune responses in patients to develop a breast cancer immunotherapy vaccine that is beneficial for a large number of patients and has relevant clinical impact. It is my heartfelt wish to see this vaccine created using knowledge from this study and then progressed to clinical use after trials. My desire is to have an active part in designing this clinical trial, monitoring the patients, and helping guide research related to breast cancer vaccines. With all the advances being made in this field, I believe that a clinically effective vaccine using immunotherapy for breast cancer will be available sometime in the near future. For the sake of those everywhere that have been personally affected by this disease, including my family and I, I hope that the data in this study can aide in that effort.

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