Identification of Biomarkers in Non-Small Cell Lung Cancer Patients Treated with PD-1 Monoclonal Antibody Immunotherapy

By

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Cancer immunotherapy works by taking a patient's existing immune system and priming it to recognize cancer cells in order for immune cells to mount an effective response to the disease. This is a less invasive means of treating cancer for the patient. However current immunotherapy does come with its own unique side effects such as auto immune disorders that manifest in the patients' treatment due to the blocking of essential immune regulatory checkpoints. In this study, patients are treated with drugs nivolumab and pembrolizumab, both of which are PD-1 (Programmed Death Receptor 1) monoclonal antibodies. These antibodies bind to PD-1 and prevent ligand interaction with PD-L1. PD-1 is a receptor expressed on the surface of activated B-cells, macrophages and T-cells. When PD-1 is activated by PD-L1 a signal propagates from the receptor to inside the cell that results in the apoptosis of the cell that expresses PD-1. The activation of PD-1 on activated T-cells ultimately results in a reduction of T-cell proliferation and IFN-γ secretion. An apoptotic signal occurs through the inhibition of the cell survival signal that is propagated through the PI3K pathway. While there is knowledge on how the expression and activation of PD-1 on immune cells regulates the progression of cancer, there is a lack of evidence to suggest biomarkers in non-small cell lung cancer patients for

optimizing immunotherapy. This study serves to identify biomarkers in non-small cell lung cancer patients undergoing PD-1 monoclonal antibody immunotherapy. To accomplish this, blood samples were collected from non-small cell lung cancer patients undergoing the immunotherapy treatment and the cell counts were taken. Cell types of interest include cytotoxic T-cells, helper T-cells, B-cells, and granulocytes. Cytotoxic T-cells were identified by CD8 expression, a known marker of cytotoxic T-cells. Helper T-cells were identified by CD4 expression and B-cells were identified by CD19 expression, both of which are known markers of helper T-cells and B-cells, respectively. Secondly, this study investigated the expression levels of known immune regulatory genes and how these changed over the course of the immunotherapy treatment. Known immune regulatory genes included PD-L1, PD-1, CTLA4, CD28, A2A, CD80 and CD86. The expression levels of the proton sensing family of G-protein coupled receptors (G2A, GPR4, OGR1 and TDAG8) were also investigated. Thirdly, we investigated how tumor cell expression of PD-1 and PD-L1 was altered when introduced into an acidic environment. Due to the tumor microenvironment being characteristically acidic this would provide insight on how anti PD-1 and anti PD-L1 immunotherapies could potentially be used in various cancers and may also lead to the development of potential future combination therapies. Our study shows that approximately 90% of patients exhibited an increase in cytotoxic T-cell counts with 50% of patients achieving healthy donor cytotoxic T-cell counts after receiving immunotherapy. Additionally 2 patients out of the total 16 patients achieved and sustained cytotoxic T-cell counts above that of healthy donors. There was an observable trend that indicated a possible correlation that PD-1 levels at baseline could predict patient response to the PD-1 monoclonal antibody immunotherapy. In addition to our research into the clinical aspects of PD-1 monoclonal antibody immunotherapy, we also investigated the change in expression of PD-1 and PD-L1

mRNA in several cancer cell lines. We observed that there was a variation in how cancer cells responded to acidosis. PD-1 and PD-L1 mRNA expression was shown to be regulated through several variables such as the acidity of the media, duration of exposure to acidic conditions and cancer cell type. It was also observed that there was PD-1 and PD-L1 expression in these cancer cell lines, at 5 hour and 24 hour treatment times, with a prominent level of PD-L1 mRNA expression in most of these cancer cells.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF DIAGRAMS

LIST OF SYMBOLS AND ABREVIATIONS

A2A – Adenosine 2A Receptor

APC – Antigen Presenting Cell

AMP – Adenosine Monophospate

ATP – Adenosine Triphosphate

cAMP – Cyclic Adenosine Monophosphate

CCR7 – C-C Chemokine Receptor Type 7

cDNA – complementary Deoxyribose Nucleic Acid

CT – Cycle Threshold

DNA – Deoxyribose Nucleic Acid

ER – Endoplasmic Reticulum

FBS – Fetal Bovine Serum

G2A – G2 Accumulation

GAP – GTPase Activating Protein

GDP – Guanine Diphosphate

GEF – Guanine Nucleotide Exchange Factor

GPCR – G-Protein Coupled Receptor

GPR4 – G-Protein Receptor 4

GTP – Guanine Triphosphate

HIF1-α – Hypoxia Inducible Factor 1 - alpha

IFN-γ – Interferon gamma

IL-2 – Interleukin 2

IL-4 – Interleukin 4

IL-5 – Interleukin 5

IL-6 – Interleukin 6

LSA –Lineage Specific Antigen

mAbs – Monoclonal Antibodies

MCT – Monocarboxylate Transporter

MHC – Major Histocompatibility Complex

MNC – Mononuclear Cell

mRNA – Messenger RNA

NADH - Nicotinamide adenine dinucleotide

NLSA –Non Lineage Specific Antigen

NSCLC – Non Small Cell Lung Cancer

OGR-1 – Ovarian Growth Receptor

PAMP –Pathogen Associated Molecular Pattern

PCR – Polymerase Chain Reaction

PD-1 – Programmed Death Associated Gene 1

PD-L1 –Programmed Death Associated Gene 1 Ligand

Pen/ Strep – Penicillin/ Streptomycin

PI3K - Phosphoinositide 3-kinase

PKA - Protein Kinase A

PKC – Protein Kinase C

PLC – Phospholipase C

qPCR – quantitative Polymerase Chain Reaction

RBC –Red Blood Cell

RNA – Ribose Nucleic Acid

RT –Reverse Transcription

TCR – T-cell Receptor

TDAG8 – T-cell Death Associated Gene 8

TEC – Tumor Associated Endothelial Cell

TME – Tumor Micro Environment

TNF – Tumor Necrosis Factor

VEGF –Vascular Endothelial Growth Factor

Chapter 1: Introduction

The Tumor Microenvironment

Cancer is a disease that develops when healthy cells become malignant and continuously divide and spread. This causes dysfunction in the tissue that cancer presents itself in. To understand the Tumor Microenvironment (TME) it is crucial to first recognize that cancer cells are cells that do not follow normal cell protocol which dictates what conditions a cell can divide and the number of times a cell can divide. Instead, cancer cells operate in a nearly autonomous manner. There are many forms of cancers and each one is unique to the organ in which it metastasizes in based off of the proteins that these cancer cells display and the pathways they elicit to survive and proliferate. However, though there are differences amongst various cancer types there are 6 characteristics displayed in all cancers. These characteristics have come to be referred to as the "Hallmarks of Cancer" and include the following: the ability to sustain proliferative signaling, evasion of growth suppressors, the ability to invade other tissues and metastasize, and displaying replicative immortality, induction of angiogenesis and resistance of cell death[45]. Proliferation is a closely regulated trait in all cell types as it requires the consumption of resources to achieve this division of cells. Cell growth is maintained through the cell cycle which requires key proteins to initiate and sustain cell survival signals in order to achieve cell homeostasis[45]. Cancer cells have evolved in a fashion so that they can bypass key regulators in these proliferative growth steps that then allow the cancer cells to function in an independent manner. By hijacking these signals that control the cell cycle, cancer cells are able to dictate when to divide despite whether or not there are adequate enough resources to do so. Cancer cells possess the capacity to continuously replicate in an exponential manner, allowing for the growth of cancerous masses that have come to be referred to as tumors. In addition to this

increased proliferation, cancer cells are also able to resist cell death through the inhibition of apoptotic signals and appear to be immortal as they divide indefinitely where normal cells can pass through a limited number of successive cell growth-and-division cycles[45]. A cancer cell is a cell that is able to bypass any inhibitory checkpoints on cell growth and can then divide continuously until the needs of the cancer cells impede physiological conditions within the body and result in organ failure in the tissue that they have metastasized to[1-3,24]. It is believed that this ability for cancer cells to divide indefinitely in an immortal fashion is due to a lack of telomere shortening as ~90% of immortalized cells express telomerase, the enzyme that prevents telomerase shortening, at significantly high levels [27]. Cancer cells have also displayed the ability to induce the formation of blood vessels, thus initially increasing blood perfusion to the tumor although this increased perfusion is not sustained in the tumor. Tumor induced angiogenesis is not as efficient as physiological angiogenesis. Tumor associated endothelial cells (TEC) often do not adhere as well to each other as normal endothelial cells of a blood vessel do and therefore result in blood vessels that are characterized as unstable or leaky[5].

Previously it has been observed that when producing the cellular energy source adenosine triphosphate (ATP), cancer cells will avoid oxidative phosphorylation and instead enter into glycolysis[5]. By shifting into glycolysis as opposed to the higher energy yielding oxidative phosphorylation, the tumor produces energy at a lower rate and produces byproducts such as lactic acid. An abundance of lactic acid will then result in a decrease in the pH of the tumor microenvironment. Decreases in pH in the tumor microenvironment have been attributed to features of cancer progression such as local tumor invasion and distant metastatic spread[45]. Additionally, an increase in the transcription of vascular endothelial growth factor (VEGF) was reported in acidic areas of human glioma cells implanted in mice[5]. Acidic conditions were

attributed to increased hypoxic conditions in the TME which resulted in increased production of Hypoxia Inducible Factor 1-α (HIF1-α) which is responsible for the upregulation of VEGF. Increased VEGF then resulted in the formation of the blood vessels to supply cancer cells with nutrients for glycolysis, producing lactic acid and yielding the characteristically low pH of the TME[5].

The Warburg Effect

It is a common fact of biology that a normal cell's means of energy production is through oxidative phosphorylation that occurs in the mitochondria of cells with the exception of more rapidly proliferative cells such as endothelial cells and lymphocytes[29]. Due to their high rate of proliferation, rapidly proliferative cells utilize anaerobic glycolysis to serve as their main energy source due to its faster, though inefficient, rate of energy production and the generation of metabolic intermediates for biosynthesis. The Warburg effect is a phenomenon observed in most cancer cells where these cancer cells utilize glycolysis to produce the mass of their energy despite a possible adequate oxygen supply. This pathway is less efficient than the citric acid cycle and also bypasses the cell's mitochondria which led to Warburg's original hypothesis that there was dysfunction associated with the energy producing processes of the mitochondria in these cancer cells and suggested that these malignant cells may depend on glycolysis for their ATP production rather than the more efficient citric acid cycle that occurs in mitochondria [25]. However, several studies have shown that most cancers have functional mitochondria but are being bypassed during energy production in these cancer cells[29]. Several oncogenes such as p53, were originally thought to only mitigate aspects of the cell cycle but have now been attributed to maintaining homeostasis of cell metabolism as well[5]. By not going through oxidative phosphorylation the end result of the glucose used to create energy in the form of ATP changes

as much less ATP is produced per glucose molecule and lactic acid is produced. Normal cells will take pyruvate, the byproduct of glycolysis, and process this through the citric acid cycle producing the energetic compound NADH which donates protons to be shuttled through the electron transport chain to yield higher amounts of ATP. Malignant cells that have a higher rate of glycolysis than normal cells, do not process glucose in the same metabolic manner as normal cells. Once malignant cells convert glucose to pyruvate it is then converted to lactic acid[28]. This lactic acid builds up in the tumor and aids in the acidic nature of the Tumor Microenvironment.

It was originally postulated by Otto Warburg that this change in metabolism was thought to contribute to malignancies in normal cells which came to later be referred to as the "Warburg Hypothesis." Today, mutations in genes that are responsible for maintaining normal cell homeostasis (otherwise referred to as oncogenes) are thought to be the cause of malignant formations and the observed metabolic changes are assumed to be a product of this and not a cause, as was originally believed [26,27].

Acidosis in Cancer

Due to Otto Warburg's original observations in 1924, it is now well known in the field of cancer that most cancer cells have altered metabolisms when compared to normal cells[25-27]. And it is also well known that a byproduct of this altered metabolism is lactic acid[28]. However, it is still debated whether or not the lactic acid produced by cancer cells is simply a byproduct of their energy production or if the lactic acid serves a specific role in cancer progression and metastasis. Some reports indicate that this increase in lactic acid serves to aid tumor cell growth as the increased lactic acid helps regulate cell growth pathways in a manner that favor cancer cell growth. Lactic acid has also been shown to decrease penetration of dendritic cells into tumor cell clusters, a phenomenon that was first noted by a decrease in cytokine release of TNF and IL-6[33].

Additionally, lactic acid has been observed to increase tumor cell motility and thus increase the rate of metastasis[33]. Prior to this observation that lactic acid was directly responsible for this increased metastasis, scientists had observed a high rate of glycolysis in cancer cells which was assumed to be solely for energy production[33]. It was later observed that this increased glycolysis was a means of achieving acidic conditions in the TME through the production of carbonic acid in addition to lactic acid. One of the means in which acidosis increases tumor growth is through the regulation of the Vascular Endothelial Growth Factor (VEGF) protein. By decreasing the pH in the tumor microenvironment, the production of VEGF is increased by HIF1-α which is upregulated in hypoxic conditions. This increase in VEGF allows for increased vascularization in the tumor which allows for a greater supply of nutrients throughout the tumor, allowing for greater tumor cell proliferation[30]. Furthermore, increases in the vascularization of the tumor increases tumor proliferation by increasing the abundance of glucose to the tumor, resulting in further acidification of the TME, thus creating a positive feedback loop[30]. It is possible that acidosis in the TME could have other effects on tumor growth through regulation of proteins involved in the regulation of cell division, metabolism, apoptosis and vascularization.

The Immune System and Cancer

The immune system is the body's defense against bacteria, viruses and various agents of disease. It can be divided into two categories, the innate and the adaptive immune systems. The innate immune system is the first responder to infection and is comprised of granulocytes, natural killer cells, mast cells, macrophages and dendritic cells. The adaptive immune system differs from the innate immune system in that the adaptive immune system, unlike the innate, can alter its immune profile so that it can detect pathogens that the adaptive immune cells have previously encountered. The adaptive immune system will grow to build up an immunity against

each pathogen that it encounters by learning to distinguish proteins that are readily expressed on each unique pathogen. The innate immune system is a first response defense that is less specialized at detecting and combating pathogens than the adaptive immune system. While the adaptive immune system is more critical on what is allowed to survive within the host through the recognition of proteins that distinguish healthy cells from pathogens or diseased cells the innate immune system does not have as thorough of a screening process and will instead attempt to attack and eliminate most foreign bodies it encounters. The cells of the adaptive immune system include cytotoxic T-cells, helper T-cells and B-cells. These three lymphocytes of the adaptive immune system work together to activate and prime effector cells to recognize and terminate any foreign body encountered. Each cell of the immune system serves its own specific role in detecting and combating diseases. Antigen presenting cells (APC) such as dendritic cells, B-lymphocytes and macrophages serve to aid in the priming of effector cells for attack against specific antigens of diseased cells. Cytotoxic T-cells and Natural Killer cells are responsible for directly combating these diseased cells. B-lymphocytes and other antigen presenting cells serve to internalize any pathogen that is encountered and bind identifiable proteins to a major histocompatibility complex (MHC) within the B-cell. However, B-cells are not the only cells responsible for antigen presentation and effector cell activation. Dendritic cells also serve the purpose of effector cell activation through antigen presentation, but do not belong to the adaptive immune system as dendritic cells are seen as members of the innate immune system. Both B-cells and dendritic cells take pathogenic proteins or antigens that they internalize from the external environment and complex the antigen to MHC molecules for antigen presentation. This MHC-protein complex is then localized to the surface of antigen presenting cells where it can then be presented to T-cells. T-cells will recognize certain MHC complexes with antigens unique

to each T-cell type while the T-cell Receptor (TCR) recognizes the antigen that is bound to the MHC complex. Upon presentation of the MHC-protein complex, T-cells will then receive costimulatory signals to prime and activate them to effector T-cell status. Different T-cells require different stimulatory signals. T-Helper cells receive stimulatory signals from the binding of CD4 to the MHC Complex as well as from CD28 and CD40 binding to their ligands expressed on the APC. Cytotoxic T-cells receive their costimulatory signal from CD8 binding to the MHC complex and through CD28 binding to its ligands CD80/ CD86 on the APC. Cytotoxic T-cells recognize MHCI complexes while T-Helper cells recognize MHCII complexes. Upon presentation of the MHC complexed with an antigen, the T-cells differentiate into T-helper cells, memory T-cells, regulatory T-cells and effector T-cells.

Effector T-cell Activation and Priming

As previously mentioned, the immune system can be broadly divided into two categories; the adaptive immune system and innate immune system. Most current immune therapies focus on the cells of the adaptive immune system and how best they can be utilized to diminish the metastasis of cancer and for this reason, the focus of this section will be directed towards the adaptive immune system and the roles that the various adaptive immune cells play in immune response. The following paragraphs serve to discuss the activation of the lymphocytes of the adaptive immune system as well as how these cells are primed to recognize and attack cancer cells and other pathogens.

The adaptive immune system is comprised of a variety of cells. These cells include: Cytotoxic T-cells, T-helper cells, B-cells and debatably dendritic cells and natural killer T-cells[37]. Dendritic cells are antigen presenting cells that serve to bridge the innate and adaptive immune system by taking antigens derived from pathogens internalized through cells of the

innate immune system and complexing these pathogenic antigens to MHC molecules[37, 39]. These MHC antigen complexes are then presented to T-cells to prime the T-cells to recognize and attack cells expressing this displayed antigen. Natural killer cells are effector cells of the innate immune system that work in a similar manner as effector T-cells but are not dependent on antigen presentation to elicit an effector cell response to a stressed cell and typically function without the need for antigen recognition[38]. Recently it has been shown that natural killer cells do respond more efficiently to secondary infections due to a small capacity to recognize antigens that natural killer cells had previously encountered on diseased cells, thus displaying immunological memory within natural killer cells[40]. Natural killer cells are differentiated from lymphoid progenitor cells that are responsible for producing B-lymphocytes and T-lymphocytes of the adaptive immune system[39]. It is through the adaptive immune system that vertebrates are able to recognize and build a defense to pathogens through the use of immunological memory. Immunological memory is the capacity of immune cells to catalog information within their cells in the form of antibodies[37]. These antibodies are specific for antigens that were previously encountered by cells of the innate and adaptive immune systems. These antibodies are released when a previously encountered pathogenic antigen is recognized. The released antibodies then bring about the activation of effector T-cells to rid the body of the present pathogen[37,39,41]. There are two types of cells in the adaptive immune system that are responsible for managing immunological memory, memory T-cells and memory B-cells[37]. This immunological memory then prevents these pathogens from afflicting the host's body so poorly in the future. However, this recognition and combating of disease is made possible through cells of the adaptive immune system being able to recognize pathogen associated molecular patterns (PAMPs) expressed on these pathogens that are unique to these agents of disease.

For the adaptive immune system to build a response to a certain pathogen, it must first encounter the pathogen and process it so that it can be recognized in the future and then be disposed of by effector T-cells. To achieve this goal, a pathogen must first encounter an antigen presenting cells[37]. Antigen presenting cells are cells that are responsible for internalizing proteins expressed on pathogens and pairing them with a Major Histocompatibility (MHC) complex. This MHC complex is a protein that is recognized by certain effector cell types and aids in the priming of these effector cells so the cells can then recognize pathogens that express the protein that is bound to the presented MHC complex. In the adaptive immune system dendritic cells, macrophages, and B-Lymphocytes fill the role of antigen presenting cells and interact with naïve CD4+ T-cells and naïve CD8+ T-cells, activating the T-cells, causing them to differentiate[37]. CD4 is a molecular marker expressed on helper T-cells. CD8+ is a protein marker expressed exclusively on cytotoxic T-cells. Naïve CD4+ T-cells can further differentiate into the sub categories: T-memory cells, regulatory T-cells and T-helper cells upon activation by B-cells. T-memory cells serve to retain the PAMP from the initial pathogen for future encounters. Depending on the type of T-memory cell there can be two possible responses to secondary infection. If the T-memory cell is CCR7 deficient it is referred to as an effector memory cell and will display a pro-inflammatory phenotype and secrete cytokines IFN-γ, IL-5 and IL-4[41]. These effector memory T-cells will migrate to inflamed tissue where the cells then assume an effector cell phenotype. If the T-memory cell does have CCR7 receptors than it does not display a pro-inflammatory phenotype but activates dendritic cells instead[41]. Regulatory T-cells negatively regulate cytotoxic T-cells in order to keep cytotoxic T-cell counts from growing excessive and prevent an auto immune response. T-helper cells aid the adaptive immune system by helping to activate effector T-cells to fight disease or infection via the secretion of cytokines such as IL-2

which has shown to aid in the maturation of naïve CD8+ T-cells to active effector T-cells[41,42]. When a naïve CD8+ T-cell encounters an antigen presenting cell with an antigen paired to its MHC complex it is activated in a similar fashion as the naïve CD4+ T-cells. These activated naïve CD8+ T-cells then differentiate into effector cytotoxic T-cells, responsible for directly attacking stressed cells or agents of disease, and memory CD8+ T-cells, which circulate throughout the lymphatic system indefinitely to retain this antigen specific response.

For the process of T-cell activation to take place and the subsequent differentiation into T-helper cells and Cytotoxic T-cells, a series of activating signals must first be received. Once an antigen presenting cell has internalized an identifying protein of a pathogen it then pairs antigen to the MHC complex. This antigen-MHC complex is then localized to the surface of the APC which in the adaptive immune system is represented by B-cells. These B-cells then pair with T-cells to prime and activate them. Antigens presented by B-cells are recognized by T-cell receptors that are expressed on the membranes of T-cells while the MHC complex is recognized and held in place by the proteins CD4 and CD8 which are expressed on T-helper cells and Cytotoxic T-cells, respectively[37-39]. CD4 recognizes MHCII and CD8 recognizes MHCI. In addition to the activation signals received through TCR binding there are costimulatory signals occurring during this activation process. In both CD4+ and CD8+ T-cells the protein CD28, a costimulatory protein, is expressed. This CD28 protein binds to CD80-CD86 complex (B7) expressed on the APC. This CD28/ B7 binding then results in the propagation of a costimulatory signal in the T-cell, aiding in its activation. In addition to CD28 T-helper cells have another costimulatory protein known as CD40. When CD40 binds to its ligand CD40L that is expressed on APC a costimulatory signal is produced to aid in the differentiation of naïve T-helper cells (Diagram 1).

Effector T-cells, much like tumor cells, utilize aerobic glycolysis as a means of producing energy. It has been observed that this transition of glucose to lactate in the presence of oxygen to produce energy is essential for effector T-cell activation[35]. The current theory is that this use of aerobic glycolysis to produce energy rather than the utilization of the much more economical oxidative phosphorylation is due to the high proliferation rate of T-cells that is necessary for these cells to be able to mount attacks against diseased cells such as cancer cells. The byproducts of the aerobic glycolysis are then shuttled out of the activated effector T-cells through monocarboxylate transporters (MCT) with the most frequently used lactate transporter being MCT-1, which functions in a gradient dependent manner[35]. This shuttling of lactate from the T-cell then allows for the lactate molecule to be converted back into pyruvate and then back into glucose at the cost of 6 ATP, thus completing the Cori Cycle. Because tumor cells also utilize aerobic glycolysis as a means of energy production to fuel their rapidly proliferating nature, they too produce byproducts of aerobic glycolysis such as lactate. This then results in an acidic Tumor Microenvironment (TME) as is characterized by the Warburg Effect. This increase of lactic acid in the TME results in the blocking of the gradient dependent lactate shuttle MCT-1 that is expressed on the effector T-cells. This inability to rid the T-cell of the produced lactate results in the cell being unable to perform necessary tasks by having reduced IL-2, IFN-γ, perforin and granzyme production and ultimately leads to apoptosis of the T-cell[35].

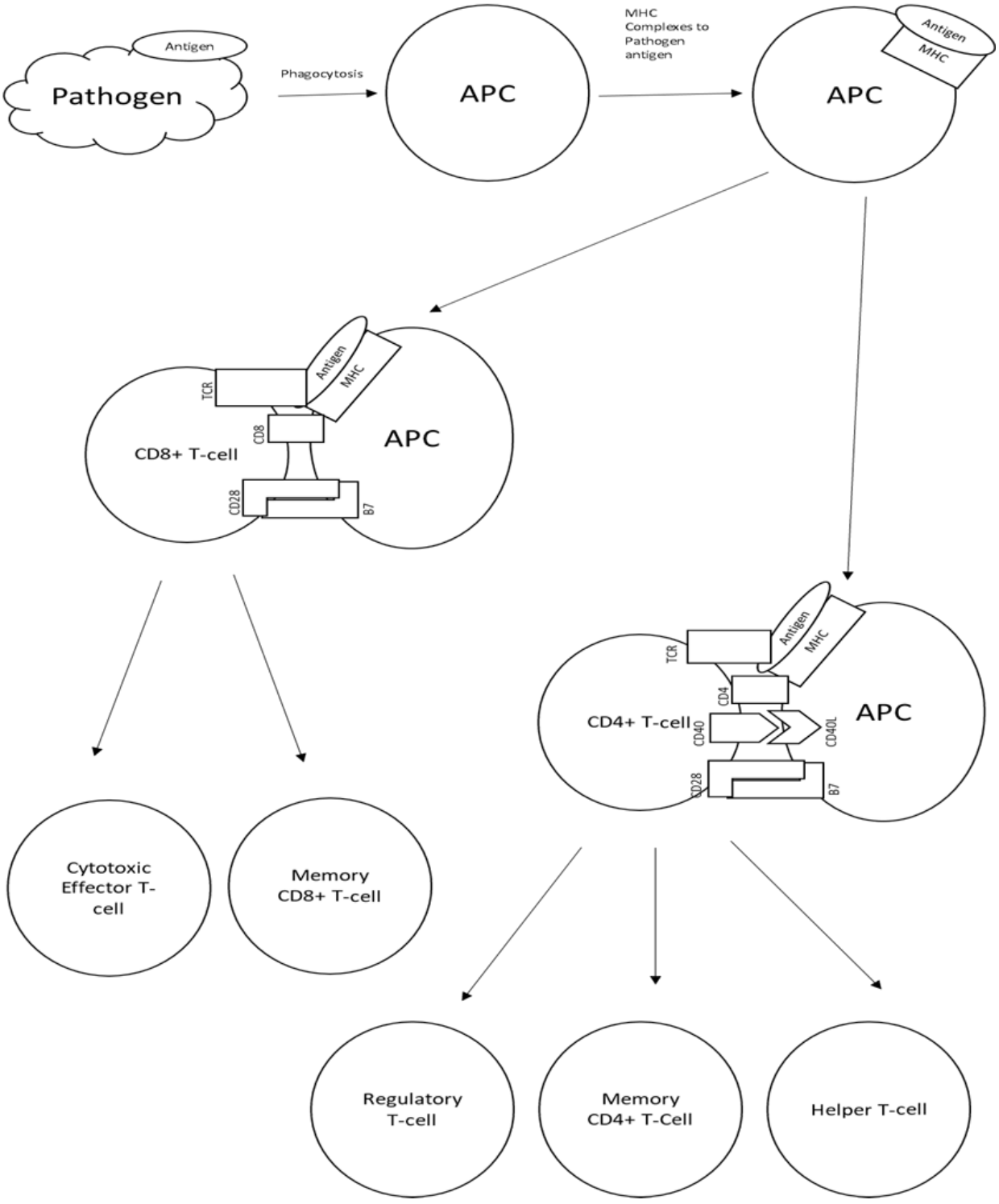


Diagram 1. Activation of adaptive immune cells

Role of Monoclonal Antibodies (mAbs) in Cancer Therapies

Antibodies are proteins that are mainly excreted by a subset of cells of the immune system known as plasma cells, a specialized B-lymphocyte. Antibodies can be soluble and thus circulate freely through the blood/ lymphatic system unbound to cells where they can directly bind antigens and inhibit disease progression through activation of the bound protein[37,39]. Antibodies can also be bound to cells and aid in immune cell response. These antibodies are responsible for the recognition of antigens as well as the presentation of these antigens to effector cells that are then primed for attack against cells that express these antigens. The epitope is a region on antigens that is recognized by antibodies via a region of their anatomy known as the paratope. This paratope is contained within the variable region of the Fab within the antibody. A collection of antibodies that can recognize multiple epitopes are referred to as polyclonal antibodies while a collection of antibodies that can only recognize one epitope due to there being only one type of paratope present are referred to as monoclonal antibodies. Additionally, monoclonal antibodies are derived from a single lineage of cells while polyclonal antibodies are produced and secreted by different B-lymphocyte lineages within the immune system.

Today the administration of antigen specific monoclonal antibodies (mAbs) to cancer patients has become a very common practice as can be seen in lymphoma and leukemia patients that are treated with the CD20 (a B-Lymphocyte antigen) antibody Rituximab[36]. Through the implementation of mAbs to fight cancer, immunotherapy has become a much more common practice for the treatment of cancer. Unlike chemotherapy that targets DNA replication and thus targets any dividing cell, mAbs work by specifically targeting the cells of the immune system and aid immune cells to diminish the tumorigenic qualities of cancer. Most mAbs work through

the recognition of certain lineage specific antigens (LSA) which are antigens whose expression is largely limited to a certain subset of leukocyte or tissue[34]. These mAbs bind to antigens and prevent interaction with other proteins expressed on cancer cells, thus preventing cancer cells from interacting with these immune cells in a negative fashion. However, there are also non-lineage specific antigens (NLSA) which are antigens that are not limited to only being expressed on a certain subset of cells[34]. These NLSA have shown to play various roles in the regulation of key cells that are involved in slowing cancer progression. It is for this reason that a large variety of mAbs have been designed to target each of these unique antigens. These NSLA mAbs serve to block several tumor promoting pathways and some have been designed to even deliver cytotoxic drugs by engineering these antibodies to attach to the drugs and then releasing the drug in the presence of cancer cells when certain cancer specific antigens are recognized by the mAbs[34].

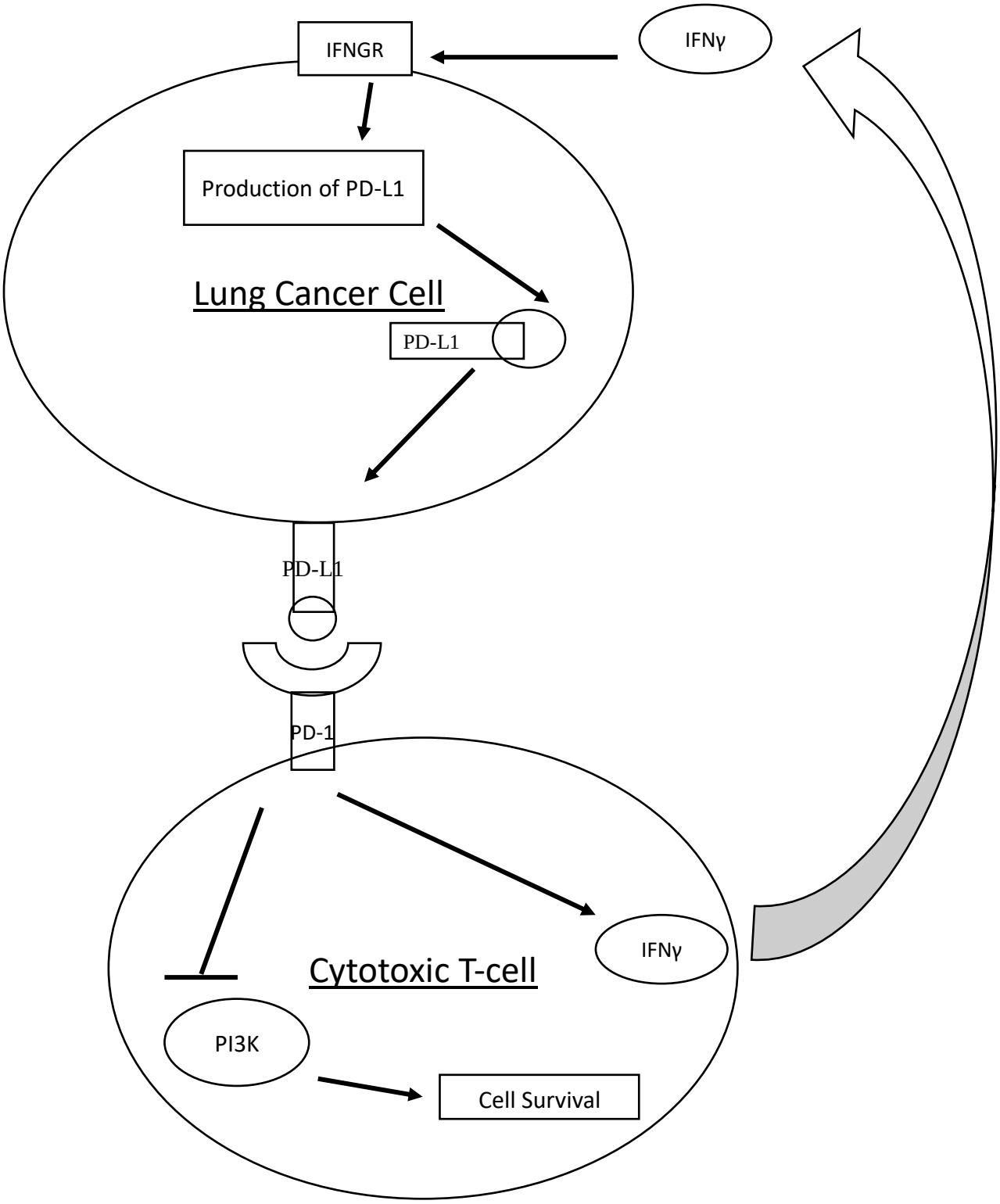


Diagram 2. Cancer cell immune regulation via the PD-1/ PD-L1 pathway

Role of PD-1/ PD-L1 in the Immune System Regulation of Cancer

As previously stated, one of the hallmarks of cancer is the ability to evade inhibitory growth signals. When inhibitory growth signals are not established in cancer cells, cells grow and proliferate in number in an exponential manner. One of the means that cancer cells avoid these inhibitory signals is by evasion and manipulation of cells of the immune system that are responsible for detecting cancer cells and ridding the body of them[45]. There are various mechanisms that cancer cells implement to avoid detection by the immune system. Within the last few years one of these immune regulators to as PD-1/ PD-L1 has gained a lot of recognition as an effective target for cancer treatment. The protein PD-1 (programed cell death protein 1) is referred to as an immune checkpoint receptor due to its ability to regulate the counts of any cells that it is expressed on through the induction of apoptosis within these PD-1 expressing cells[1-3, 24]. It has been shown that PD-1 has a high level of expression on cytotoxic T-cells which are the cells of the adaptive immune system that are responsible for the recognition and destruction of cancer cells. When PD-1 is activated by its ligand (PD-L1) an inhibitory signal is passed on through the cells possessing the activated PD-1. This inhibitory signal then suppresses the PI3K pathways which is responsible for cell survival. When the PI3K pathway is inhibited, cell survival declines and cell count decreases. In the case of cytotoxic T-cells this means that there are less effector T-cells to slow the metastasis of cancer and infections as the T-cells expression of PD-1 increases (Diagram 2).

G-Protein Coupled Receptors (GPCRs)

Cells utilize a variety of methods for communication with the surrounding environment. G-protein coupled receptors (GPCRs) are one of the means utilized by cells to communicate with and respond to the external environment. A GPCR is a protein that when activated results in the propagation of a signal to its coupled G-protein, resulting in the activation of the G-protein. G-proteins are hetero trimeric proteins whose activation depends on the presence or absence of GTP/ GDP. G-proteins are comprised of alpha, beta and gamma subunits. It is now known that both the alpha and beta-gamma subunits can transduce signals when activated by a receptor that they are coupled to[4-6]. When GTP is present the G-protein is referred to being in its "active" state. G-proteins are no longer in their active state when the GTP is converted to GDP. The conversion of GTP to GDP is catalyzed by the GAP (GTPase-activating proteins) which bind to activated G-proteins and activate GTPase activity. Conversion of a G-protein to its active state is achieved by the enzyme GEF (guanine nucleotide exchange factor) which exchanges a bound GDP for a GTP.

The responses that G-proteins trigger in cells depends on the type of alpha subunit that makes up the G-protein. There are four recognized classes of G-protein alpha subunits: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, $G_{\alpha 12/13}$. The G_s alpha subunit activates the cAMP-dependent pathway. This is achieved by activation of adenylyl cyclase, the protein responsible for cyclic AMP (cAMP) production. This cAMP then activates downstream effectors, one of these downstream effectors is protein kinase A (PKA). PKA phosphorylates key proteins such as AKT within cells to maintain homeostatic conditions. AKT is a protein that is crucial to glucose metabolism within cells. PKA is responsible for the regulation of sugar and lipid metabolism as well as cell survival signals such

as NF-κB and thusly correlates to PKA inducing cell dependent responses. The $G_{i/o}$ alpha subunit is the balance to the G_s alpha subunit because while the $G_{\alpha s}$ activates cAMP production, $G_{\alpha i/o}$ inhibits cAMP production by inhibiting adenylyl cyclase activation. Much like activation of PKA via the $G_{\alpha s}$, the G_q alpha subunit is responsible for the activation of phospholipase C (PLC) which has the downstream effect of activating protein kinase C (PKC). Protein Kinase C is very similar to Protein Kinase A from the $G_{\alpha s}$ pathway due to both proteins being able to regulate other proteins by modulating them when adding a phosphate group to these target proteins. Additionally, the G_q pathway also results in increased intracellular calcium levels due to IP_3, which is synthesized by activated PLC cleaving PIP_2 into DAG and IP_3, binding to calcium channels of the endoplasmic reticulum (ER) and resulting in a flux of calcium ions from the ER to the cytosol. The $G_{\alpha 12/13}$ subunits regulate actin cytoskeletal remodeling and have been shown to induce migration in endothelial cells through association with tyrosine kinase receptors[31,32].

Within the class of receptor proteins known as G-Protein Coupled Receptors (GPCRs) there is a subclass of GPCRs known as Class A GPCRs which account for 85 percent of GPCRs. Within the Class A GPCRs there are proteins that have shown an affinity to protons and have been rightly dubbed the proton sensing family of GPCRs. These GPCRs gain their affinity for protons through the ability of histidine residues on the receptors being protonated by free protons. This family of proton sensing GPCRs consists of the 4 GPCRs: TDAG8 (GPR65), OGR1(GPR68), GPR4 and G2A (GPR 132). TDAG8 (T-cell death associated gene 8) is a protein that was first discovered on T-cells and attributed to cell death on T-cells when it was activated. Since its initial discovery, TDAG8 has been observed to be expressed on other cells aside from just T-cells. In addition to the proton sensing GPCRs there is another GPCR that has shown some ability to regulate the immune system due to its presence on T-lymphocytes. A2A is

a GPCR that is responsible for the recognition of adenosine. A2A is activated upon encountering adenosine this then results in the receptor coupling to G_s and increasing adenylate cyclase activity. This then results in the completion of the G_s pathway which ends with activated PKA which is responsible for triggering various cell dependent responses [45-51].

Chapter 2: Materials and Methods

Research samples relied heavily on blood collected from non-small cell lung cancer patients undergoing anti PD-1 immunotherapy. Because of the use of human patients there was a clinical side which included administration of the drugs and the collection of the blood samples and there was the lab research aspect side of the data collection as well. The clinical aspect of this research was handled by certified health care professionals while I was responsible for the processing of the blood samples once they had been collected by the nurses on duty at the time. Additionally the cancer cell lines used: Jurkat human T-cell leukemia, PC-3 human prostate cancer, MCF7 human breast cancer, Caki-1 human kidney cancer, Hela human cervical cancer cells, NCI-H1299 human lung cancer, CRL-1596 Ramos Human B Lymphoma, CRL-1619 A375 human melanoma, SK-OV-3 human ovarian adenocarcinoma, and U937 human monocyte were all originally from American Type Culture Collection (ATCC).

Clinical Trial

Non-small cell lung cancer patients were recruited and scheduled to come in every 2 weeks for a 16 week period, yielding a total of 8 visits per patient. At each patient visit the on hand medical staff would draw a blood sample before administration of the anti PD-1 monoclonal antibodies. Patients were taken off the trial if the physicians in charge of that patient saw a decrease in the patients overall health or when the patient expired. If a patient successfully completed the full trial then, 8 blood samples would have been collected (averaging 1 sample per patient every 2 weeks) during the treatment and a final blood sample would be collected 2 weeks after the last dose was administered to the patient, bringing the total possible number of blood samples per patient to 9.

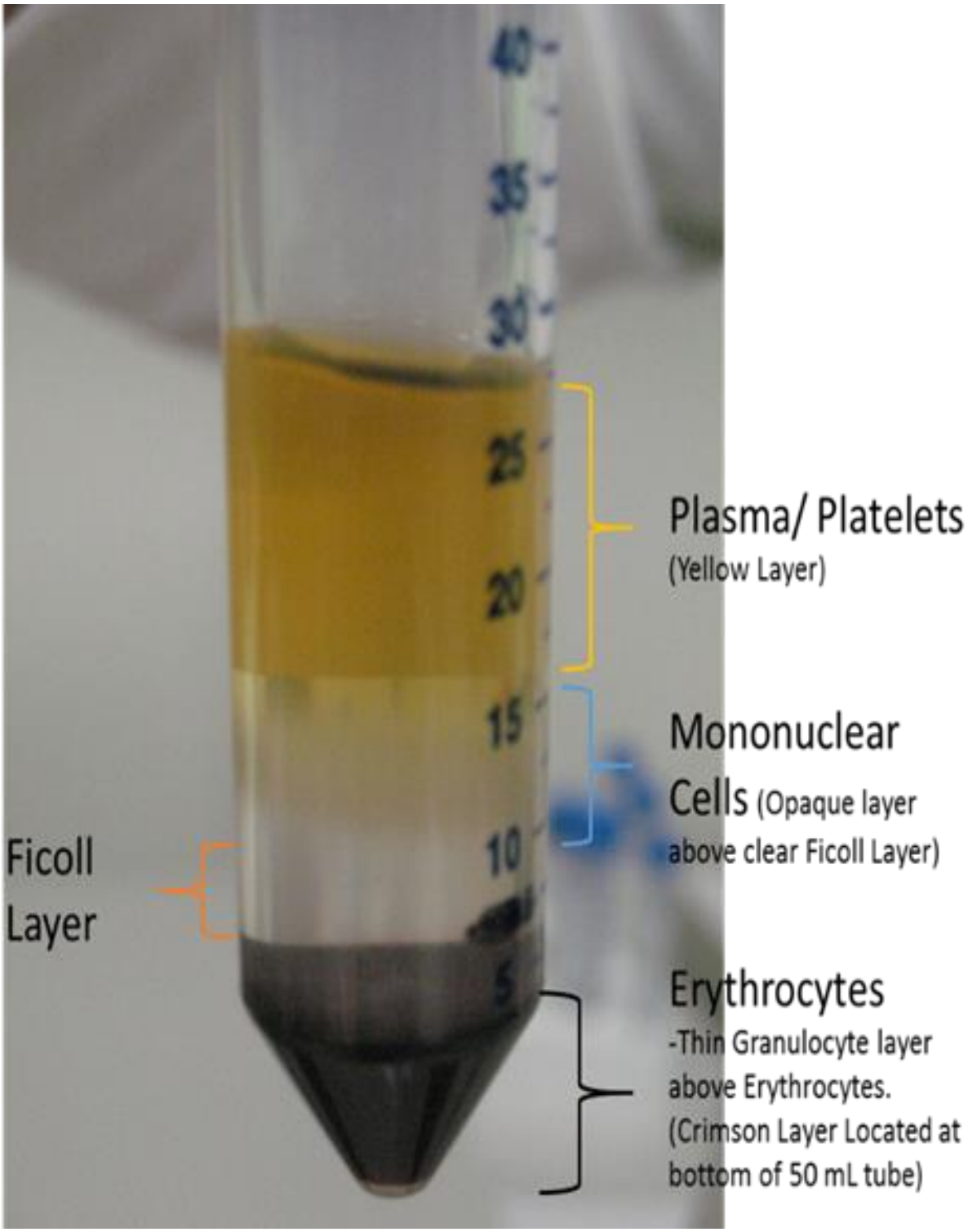


Diagram 3. Layering of Blood sample using Ficoll-Paque Centrifugation

Isolation of Immune Cells from Blood Samples

Blood samples were collected by medical professionals that were present on the days that each patient came in for their immunotherapy treatment. Up to 10 milliliters of blood was collected per patient sample for the cell isolation process. Once the blood samples were obtained they were transported in a sterile fashion to the lab. Once in a sterile environment a measured volume of the patient blood was taken and mixed with an equal volume of Phosphate Buffered Saline (PBS) (Gibco). This mixture of blood and PBS was then gently layered over a volume of Ficoll-Paque PLUS (GE Healthcare) in a 50ml conical tube. The Ficoll-Paque volume was ¾ the volume of the combined blood and PBS mixture. After layering there was a clear Ficoll layer at the bottom of the tube with the blood mixture suspended above this with no mixing of the two layers. The conical tube containing the layered ficoll and blood was then gently placed into a centrifuge, taking care not to mix the two layers, and then centrifuged for 40 minutes at 400x g. At the end of the 40 minute centrifugation there were 5 distinct bands present in the tube (Diagram 3). At the top was the plasma layer, below this was the Mononuclear cell (MNC) layer, below this was a clear ficoll layer (largely devoid of any cells), below the ficoll layer was a thin granulocyte layer that was then followed by a crimson red layer at the bottom of the tube which was largely comprised of erythrocytes (RBC). The plasma layer was removed first while taking care not to remove any cells from the MNC layer. This plasma was then frozen down in vials at -80 ^{o}C. Any residual plasma on top of the MNC layer was removed and discarded. The MNC layer was then placed in a 50ml conical tube and 3 volumes of PBS were added and the total cells were counted at this point using a hemocytometer. The MNC were then centrifuged for 15 minutes at 400*g and the supernatant was then removed. The pellet was again suspended with a volume of PBS equal to the previous total volume of the MNC. These cells were then again

counted using a hemocytometer. Then using the equation (y=x*25) with "x" being the number of $10e^7$ MNC and "y" being the volume (ul) of dynabeads needed to properly isolate that many cells, the volume of dynabeads was calculated. The MNC were then centrifuged again for 15 minutes at 400*g and then suspended in isolation buffer to a concentration of $10e^7$ cells/ml. The CD8+ dynabeads were washed in a 5ml test tube with 1ml of isolation buffer and then placed in a magnet for 1 minute at which point the supernatant was removed with the tube containing the washed beads still in the magnet. The dynabeads were then suspended to their original calculated volume using isolation buffer and the MNC were then added and left to incubate at 4ºC for 20 minutes with gentle rocking. Once incubation of CD8+ cells had concluded, the cells were placed in the magnet again for two minutes, following this 2 minute period, the supernatant was removed while the tube was still in the magnet and the supernatant was then added to CD4+ dynabeads that were washed during the 20 minute incubation. The CD4+ cells/ beads were then incubated for 20 minutes at 4°C with gentle rocking. The tube containing the CD8+ cells was then removed from the magnet and the bead bound cells were washed with 1ml of isolation buffer and placed in the magnet for 2 minutes at which point the supernatant was discarded with the tube still in the magnet and another 1ml of isolation buffer was added to the bead bound cells and placed in the magnet again for 2 minutes. Once the 2 minutes had passed, the supernatant was discarded and the cells were suspended in 1.5ml centrifuge tubes with 1ml of isolation buffer at which point they were then counted using a hemocytometer and smear slides were made. The cells were then placed back into the magnet for 2 minutes and the supernatant was discarded leaving just the bead bound cells. Once the supernatant was removed for a final time the bead bound cells were then promptly snap frozen and placed on metal beads at -80°C.

Once incubation of CD4+ cells had concluded, the cells were placed in the magnet again for two minutes, following this 2 minute period, the supernatant was removed while the tube was still in the magnet and the supernatant was then added to CD19+ dynabeads that were washed during the 20 minute incubation. The CD19+ cells/ beads were then incubated for 20 minutes at 4°C with gentle rocking. The tube containing the CD4+ cells was then removed from the magnet and the bead bound cells were washed with 1ml of isolation buffer and placed in the magnet for 2 minutes at which point the supernatant was discarded with the tube still in the magnet and another 1ml of isolation buffer was added to the bead bound cells and placed in the magnet again for 2 minutes. Once the 2 minutes had passed, the supernatant was discarded and the cells were suspended in 1.5ml centrifuge tubes with 1ml of isolation buffer at which point they were then counted using a hemocytometer and smear slides were made. The cells were then snap frozen and placed on metal beads at -80°C.

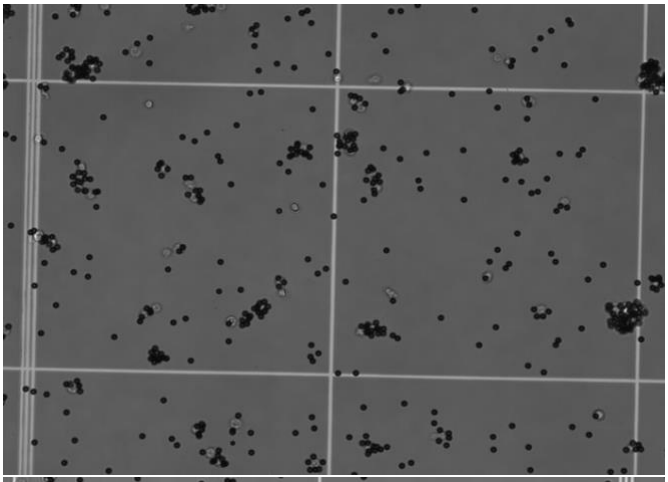
Once incubation of CD19+ cells had concluded, the cells were placed in the magnet again for two minutes, following this 2 minute period, the supernatant was removed while the tube was still in the magnet and the supernatant (containing the other MNC population) was then added to a 1.5ml centrifuge tube, these cells were then counted using a hemocytometer and smear slides were made using the cells. The other MNC population was then centrifuged for 10 minutes at 400*g, the supernatant was then removed and the pellet was snap frozen. The tube containing the CD19+ cells was then removed from the magnet and the bead bound cells were washed with 1ml of isolation buffer and placed in the magnet for 2 minutes at which point the supernatant was discarded with the tube still in the magnet and another 1ml of isolation buffer was added to the bead bound cells and placed in the magnet again for 2 minutes. Once the 2 minutes had passed, the supernatant was discarded and the cells were suspended in 1.5ml centrifuge tubes with 1ml of

isolation buffer at which point they were then counted using a hemocytometer and smear slides were made. The cells were then snap frozen and placed on metal beads at -80°C.

The remaining ficoll was discarded leaving only the granulocyte layer and the erythrocyte layer left in the conical tube. Red Blood Cell Lysis buffer was then added to the 50ml conical tube that contained the granulocytes and erythrocytes and the mixture was set to gently rock for 15 minutes at room temperature. The granulocyte/ erythrocyte mix was then centrifuged for 15 minutes at 400*g. After centrifugation the supernatant was aspirated, leaving behind a pellet of granulocytes and erythrocytes. This pellet was dissociated in another RBC Lysis and rocked for 15 minutes at room temperature after which it was centrifuged for 15 minutes at 400*g. After centrifugation, the supernatant was removed and the granulocyte pellet was suspended in 40ml of PBS and these cells were then counted using a hemocytometer. The cells were then spun down for a final time for 15 minutes at 400*g and snap frozen and placed on metal beads at -80°C.

Pictures were taken using an EVOS microscope while the cells were still on the hemocytometer used for counting (Diagram 4). Pictures were taken of CD8+ cells, CD4+ cells, CD19+ cells, other MNC and Granulocytes. Upon counting the bead bound cells the purity was assessed and 98-99 percent of cells represented were bound to magnetic beads. This purity remained consistent for all bead bound cell types for several patient samples that were chosen at random.

A.



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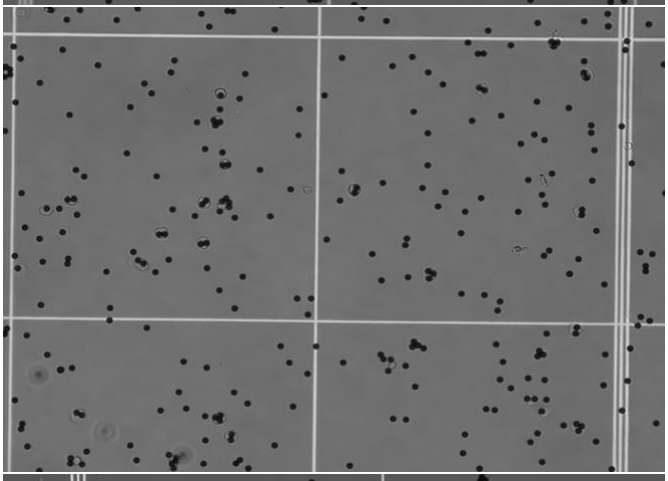


C.

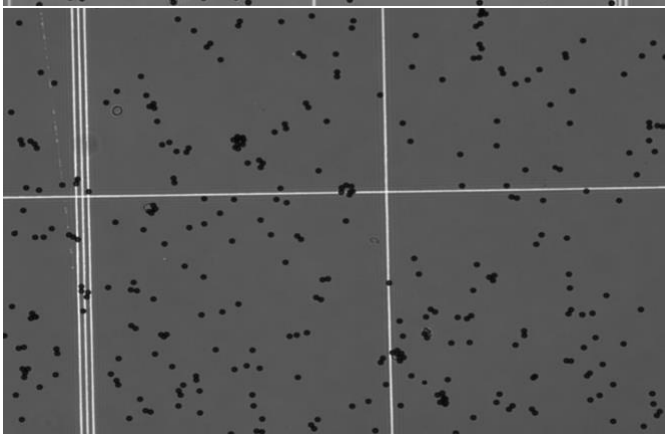


Diagram 4. Validation of antigen specific bead binding for isolation of specific lymphocytes from patient blood samples. CD8+ Cells from Patient J cycle 3 (A.). CD4+ Cells from Patient E cycle 6 (B.). CD19+ cells from patient B cycle 1 (C.). Pictures were taken at 20x magnification on an Evos Machine.

Statistical Analysis of Lymphocyte Counts from NSCLC Patients

Baseline cell counts for CD8+, CD4+ and CD19+ cells were analyzed independently of other cell types. All patient baseline cell counts were compiled into one group for each lymphocyte cell type and compared to the healthy donor counts for the respective cell type. To determine significance a non-paired two tailed Student's t-test was used. Analysis was done using the GraphPad Prism software.

pH Buffered Media

The media used to perform the pH treatments on the various cancer cell lines was an RPMI (Gibco) buffered media. It was found that rather than using the pKa buffers HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (2-(N-morpholino) ethanesulfonic acid) and EPPS, the RPMI media performed well enough with only Hepes and MES alone. Two separate RPMI medias were created with added 7.5mM Hepes and MES. These separate medias were then buffered so one had a pH of 6.4 and the other had a pH of 7.4 which served as our physiological pH. Once the desired pH was achieved in each of these medias, the medias were then filtered through a .22 micron filter and stored in a 4C fridge until they were needed for cell treatment.

Cell Culture

Cancer cell lines were cultured in media that contained 90% RPMI media with 10% Fetal Bovine Serum (FBS) and 1x Penicillin/ Streptomycin (Pen/ Strep). Cells were cultured to 70-90 percent confluence before splitting and being re-seeded in p60 plates. Adherent cells were treated with trypsin for 5 minutes at 37°C 5% CO_2 in order for the cells to detach from the plate. Cells were seeded in p60 plates at a total of $1x10^5$ cells per plate and left to incubate overnight. The

following day cells were treated with the buffered RPMI media at pH 6.4 and pH 7.4 and placed back in the incubator for 5 hours and 24 hours as there were two durations of exposure that were tested. Cells were incubated at 37°C with 5% CO_2. After a 5 hour period the 6.4 pH buffered media had increased in alkalinity to a pH of 6.47 and after 24 hours the 6.4 pH buffered media had increased to a pH of 6.53. The 7.4 pH buffered media showed no change in pH value at the 5 hour mark but by 24 hours the media had increased to a pH of 7.49.

Reverse Transcription Reaction (mRNA to cDNA)

RNA isolated from the cells of our cancer patients and from the cells of our pH treated cancer cell lines were then converted into cDNA through reverse transcription and their quality was confirmed through the implementation of PCR and gel electrophoresis using an 18S primer compatible with human cDNA. RT reactions were performed in an environment that was cleared of RNases before initiation of each of the individual experiments. RNA was measured using a nanodrop machine and then, using ultrapure RNase free water, mRNA was diluted to a concentration of 200 ng in 11 µl. This method of reverse transcription utilized a reverse transcriptase (superscript IV) (Invitrogen). Once the mRNA had been diluted in a total volume of 11 µl master mix containing equal parts of 50 µM random hexamers (Invitrogen) and 10 mM dNTP (ThermoFisher) was created. 2 µl of this first master mix were then added to each of the separate tubes containing cDNA. This cDNA mix was then centrifuged briefly and then placed in a PCR machine for 5 minutes at 65°C. Samples were then removed from the PCR machine and placed on ice for 1 minute. A second master mix was then created at the following ratios: 4 µl 5x Superscript IV Buffer (Invitrogen), 1ul 100 mM DTT (Invitrogen), 1 µl Ultrapure RNase/ DNase free water (Gibco) and 1 µl Superscript IV 200 U/ul. 7 µl of this second master mix was then added to each separate sample bringing the total reaction volume in each sample to 20 µl.

Samples were incubated at room temperature for 10 minutes and then placed into the PCR machine under RT4 protocol at 55℃ for 10 minutes and then 80℃ for 10 minutes. Samples were removed from the PCR machine once the machine had completed all of its required cycles and the samples were diluted 1:10 with Ultrapure RNase/ DNase free water.

Polymerase Chain Reaction (PCR)

Once mRNA had been reverse transcribed into cDNA it was then paired with primers for 18S, a ribosomal subunit that is expressed in most cell types. PCR was used to show expression of the genes of interest due to the ability of PCR to amplify any mRNA gene expression once the mRNA has been converted to cDNA and paired with primers. Since DNA cannot initiate synthesis on its own it must be paired with primers which bind to the DNA strand based off of a consensus sequence. Once bound to the DNA the primer acts as a foundation for DNA synthesis as nucleic acids are added onto it. By adding forward and reverse primers for a gene we only replicate a section of the cDNA that has that genes expression and this is done to an exact base pair match. Sense primers will first bind to the cDNA and code the sister strand for the entire length of that cDNA. The antisense primer will then bind to the newly synthesized sister strand and code the original cDNA but it cannot code past the point where the sense primer bound to the original cDNA. This process will then repeat and go on indefinitely, exponentially increasing the amount of gene expression on the cDNA for the primers it was paired with. PCR relies on thermal cycling, the act of heating and then cooling the cDNA and primers. First heat is applied to separate the double stranded DNA through denaturation. This is followed with a lower temperature that allows for the cDNA to bind to the introduced primers and then initiate transcription from the template strand, completing one cycle. This process then repeats a set

number of times which in our case for the 18S primers the PCR machine was set to cycle 32 times.

18S expression served as an indicator as to whether or not a successful reverse transcription reaction had occurred. The product of the PCR containing the transcribed cDNA and the forward and reverse 18S primers was then combined with 5 µl of 6X loading dye and then 15 µl of the dyed sample was loaded into a 1.5% agarose gel containing ethidium bromide at 0.5 µg/ 10 ml. A 100 base pair ladder was added alongside these samples to assess product base pair length. This gel was then administered a 100V current for 25 minutes using an electrophoresis tub. Once the 25 minutes had passed the gel was then placed under a UV light and the DNA band was photographed.

SYBR Green qPCR Analysis

Once cDNA was reverse transcribed from the mRNA derived from the patient immune cells as well as from the pH treated cancer cell lines we then utilized SYBR Green qPCR to determine gene expression at the mRNA level. For each gene I combined forward and reverse primers that bound to a series of nucleotides unique to each individual gene, and diluted with ultrapure RNase / DNase free water until a concentration of 2.5 µM was achieved for each separate primer in the solution. For qPCR analysis, PowerUP™ SYBR™ Green Master Mix from ThermoFisher Applied Biosystems was used. Separate master mixes were created for each of the genes investigated. Master mixes were created by combining 62.5% PowerUP™ SYBR™ Green Master Mix with 37.5 % of the 2.5 µM forward and reverse primers for the desired gene, bringing the concentration of the combined primers in the Master Mix to 1.5 µM. For the qPCR reaction, 3 µl of cDNA from each of the samples was added to the wells of 0.2ml 96 well qPCR plate and then 12 µl of the primer added mastermix was added to the wells containing the cDNA

for a total reaction volume of 15 µl. Once the cDNA and the Master Mix was combined, the 96 well plates were then covered with an optical adhesive cover and centrifuged for 3 minutes at -2000 rpm to collect all of the mixture to the bottom of the well. The plate was then placed in a QuantStudio3 machine and left there until the machine completed its runtime.

Once the machine had completed its cycles the CT values were given and used to calculate the relative expression. For patient samples and pH treated cancer cells the relative expression for a given gene was calculated by subtracting the CT value for the endogenous control gene (18S) for a specific treatment group from the CT value of a given gene from that same treatment group. The delta CT values were then used to calculate the delta delta CT (ΔΔCT) by subtracting the control group's delta CT value from the experimental groups CT value. For NSCLC patients the control group was the baseline point for each individual patient and the experimental groups were the different collection points from baseline to post cycle 2. Delta CT values were used to calculate the ΔΔCT values of the patients they were observed in. Physiological pH treated cancer cell lines served as the control groups for the pH treated cancer cell lines while the acidic pH treated cancer cell groups were the experimental values. Delta delta CT values were calculated by taking the relative expression of a given gene for one cancer cell line treated with 6.4 pH buffered media and subtracting the relative expression for the same gene in the same cancer cell line treated with 7.4 pH buffered media. The ΔΔCT values for 5 hours and 24 hours were calculated separately so not to use relative expression values from different durations of treatment. Fold values were then calculated from delta delta CT values using the equation X=2^-(ΔΔCT) with "X" representing fold value[52].

Gene Name	Forward Primer	Reverse Primer
G2A	GCAAAGAGGCTGATTGAGGAG	CTCAGTGCACAGGAACCAC
CTLA4	CTACCTGGGCATAGGCAACG	CCCCGAACTAACTGCTGCAA
PD-1	GGATGGTTCTTAGACTCCCCA	TGGAGAAGCTGCAGGTGAAG
PD-L1	TACTGTCACGGTTCCCAAGG	GTGCAGCCAGGTCTAATTGT
OGR1	CCCCTTCAGGCCCAAAGAT	CCAGCACGGTAACATAGACC
TDAG8	ACAAAGAGACAAGACTTCTCTGT	AGTGATCCAGGTCATGCTGT
CD80	ACCCTAAGCATCTGAAGCCA	AGTGAGAAAGACCAGCCAGC
CD86	GGAAGAGAGTGAACAGACCAAG	TCACTTTTGTCGCATGAAGATG
CD28	CGACTTCGCAGCCTATCG	TGGCGGTCATTTCCTATCCA
FOXP3	CACAACCTGAGTCTGCACAA	CTCTGGCTCCGTTTCTTGC
A2A	CATTGACCGCTACATTGCCA	GATGGCAAACGACAGCACC

Table 1. List of Genes and the forward and reverse primers used in qPCR analysis to determine mRNA concentration levels.

Cell line	Medium Used
205: Jurkat T cells	RPMI+10% FBS+7.5mM HM
206: PC-3, human prostate cancer cell	RPMI+10% FBS+7.5mM HM
207: Hela cells, human cervical cancer	RPMI+10% FBS+7.5mM HM
208: MCF7 cells, human breast cancer	RPMI+10% FBS+7.5mM HM
209: Caki-1 cells, human kidney cancer	RPMI+10% FBS+7.5mM HM
211: NCI-H1299 cells, human lung cancer	RPMI+10% FBS+7.5mM HM
215: CRL-1596, Ramos cells, human B lymphocyte	RPMI+10% FBS+7.5mM HM
216: CRL-1619, A375 cells, human melanoma cell	RPMI+10% FBS+7.5mM HM
219: SK-OV-3, Ovarian Adenocarcinoma Human	RPMI+10% FBS+7.5mM HM
25: U937 wildtype Human monocyte	RPMI+10% FBS+7.5mM HM

Table 2. List of cancer cell lines used for pH treatments and the media used to perform the treatments.

Chapter 3: Results

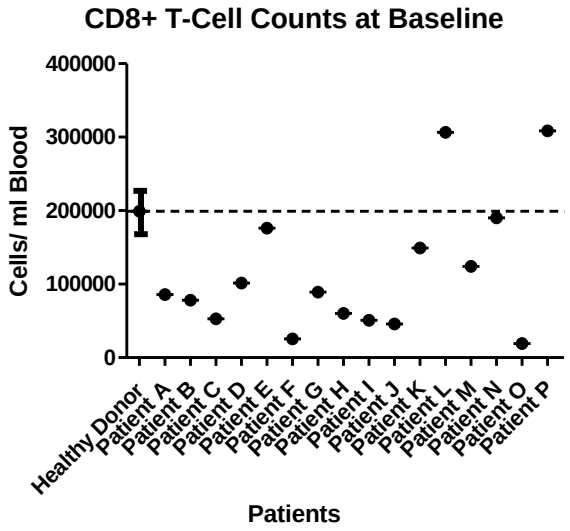
The results section of this thesis serves to describe what effects were observed in non-small cell lung cancer patients that were undergoing anti PD-1 immunotherapy. Results observed were viewed as a response to the treatment in these patients which were evaluated at cellular and molecular levels. First we observed how certain cells of the innate and adaptive immune system changed over the course of immunotherapy and we then followed these results with an investigation into how the mRNA levels of known immune regulatory genes and potential immune regulatory genes as well as GPCRs changed within cytotoxic T-cells of the adaptive immune system.

In addition to the cellular and molecular observations made in the patients of the clinical trial, we also began researching how changes in pH in the tumor microenvironment of various cancer types can alter the expression of PD-1 and its ligand PD-L1. To achieve this goal a wide range of human cancer cell types were utilized.

Lung Cancer Patients Show Lower Cytotoxic T-cell counts at Baseline When Compared to Healthy Donors.

We first examined immune cell counts in 5 healthy donors to determine cell counts for cytotoxic T-cells, T-helper cells, B-lymphocytes, granulocytes, total MNC and other MNC. Once there was an established average for each of these cell populations in the healthy donor groups we then shifted the focus onto looking at the counts of these cell populations in patient samples. Isolation of the three investigated lymphocyte cell types of the adaptive immune system was achieved through a Ficoll centrifugation and an antigen specific magnetic bead binding

procedure that allowed us to isolate each of the cell types by their expression of the cell specific markers CD8, CD4 and CD19 which represent cytotoxic T-cells, Helper T-cells and B-Lymphocytes respectively. Initial observation of patients before beginning the anti PD-1 immunotherapy is that the majority of these patients have low lymphocyte counts with 14 out of 16 patients' cytotoxic T-cell counts being below healthy donor levels at baseline (Figure 1). Statistical analysis of healthy donor CD8+ cell counts and patient baseline CD8+ cell counts showed no statistically significant difference with a p-value of 0.0627.



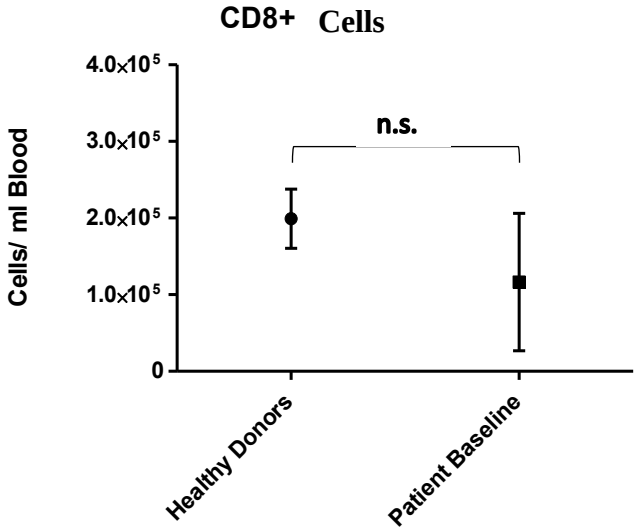


Figure 1. Baseline cytotoxic T-cell counts of Non-Small Cell Lung Cancer Patients that underwent anti PD-1 immunotherapy. Average cytotoxic cell count for healthy donors is represented by a dotted line. n.s.: not significant.

NSCLC Patients Show Lower CD19+ B-cell Counts when Compared to Healthy Donor Counts

Using a method very similar to that used to isolate out CD8+ T-cells, we were able to isolate out CD19+ B-cells. To achieve this isolation of B-lymphocytes we used the same Ficoll gradient centrifugation previously used to layer the blood samples into the 5 distinct cell layers mentioned above and then we introduced CD19 antigen specific beads to these samples after having introduced the CD8+ and CD4+ antigen specific beads to isolate out cytotoxic T-cells and T-helper cells, respectively. The data shows that out of the 16 patients that were evaluated, 12 of these patients had B-lymphocyte counts that were below that of healthy donor levels at baseline (Figure 2). Statistical analysis of healthy donor CD19+ cell counts and patient baseline CD19+ cell counts showed no statistically significant difference with a p-value of 0.2481.

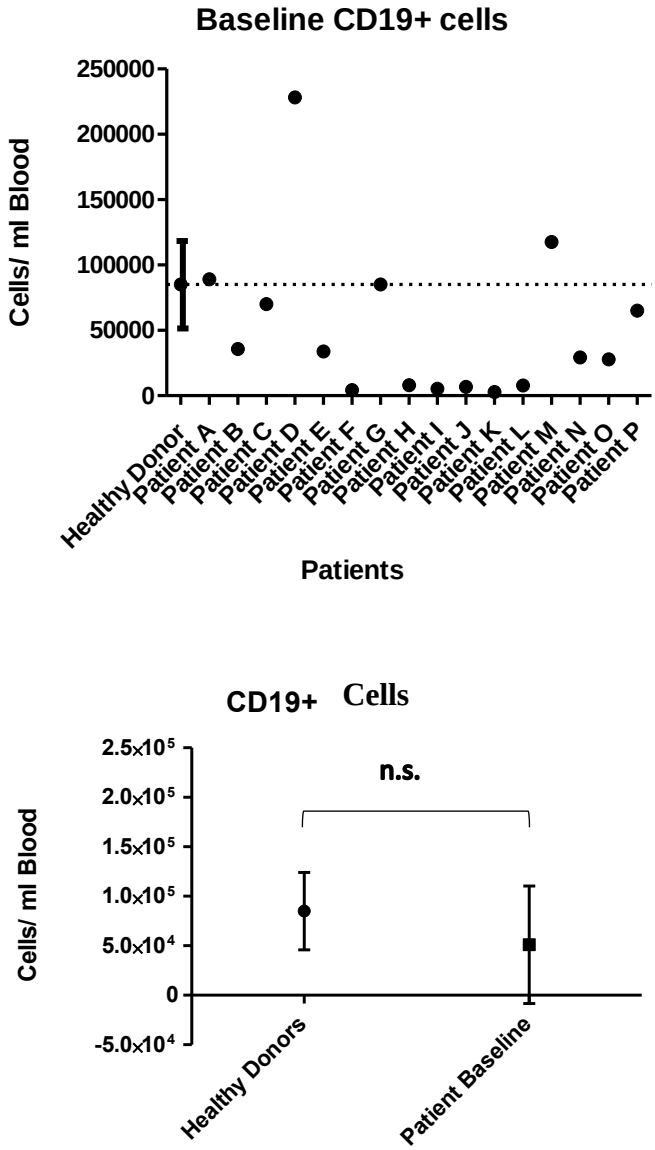


Figure 2. Baseline B-Lymphocyte counts of Non-Small Cell Lung Cancer Patients that underwent anti PD-1 immunotherapy. Average B-Lymphocyte count for healthy donors is represented by a dotted line. n.s.: not significant.

Lower CD4+ Lymphocyte Levels Observed in NSCLC Patients when Compared to Healthy Donors

To determine the levels of CD4+ cells present in the peripheral blood of our patients, we implemented a method much like the methods used to isolate our CD8+ Lymphocytes and CD19+ Lymphocytes. First blood was separated using the previously the Ficoll centrifugation technique and then the CD4+ beads were isolate from the total mononuclear cell population after the CD8+ cells were isolated out and before the CD19+ cells were isolated. This isolation of CD4+ lymphocytes from the blood of these cancer patients showed us that the majority of these patients entered immunotherapy with already low CD4+ cell counts, 11 out of 16 patients began their treatment with CD4+ cell counts below that of the average healthy donor counts indicated by the dotted line on figure 3. Statistical analysis of healthy donor CD4+ cell counts and patient baseline CD4+ cell counts showed no statistically significant difference with a p-value of 0.2651.

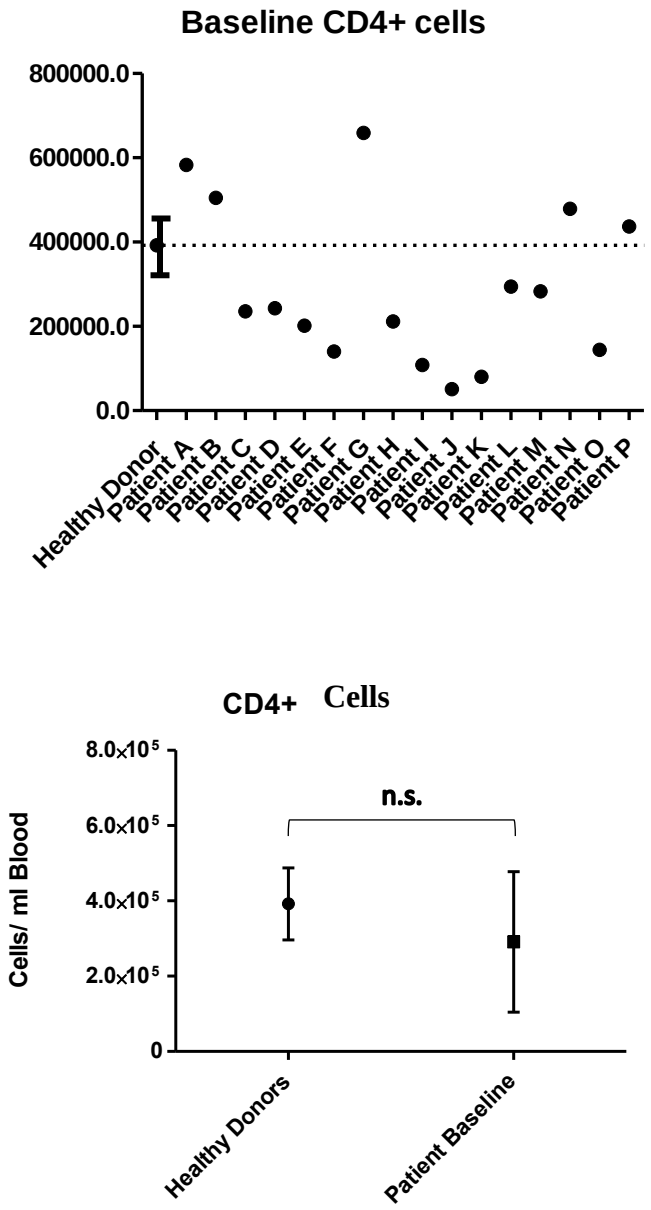


Figure 3. Baseline CD4+ Lymphocyte counts of Non-Small Cell Lung Cancer Patients that underwent anti PD-1 immunotherapy. Average CD4+ Lymphocyte count for healthy donors is represented by a dotted line. n.s.: not significant.

Patients Displayed an Increase in Cytotoxic T-cell Counts from Baseline within the First Three Cycles of Immunotherapy.

While patient cytotoxic T-cell counts were initially low at baseline, majority of patients saw an increase in their cytotoxic T-cell counts within the first three cycles of immunotherapy. From the entire patient population, 11 of the 16 patients showed an increase in cytotoxic T-cell counts within the first three cycles. Of the 16 patients, 2 patients were removed from the trial for health complications before they were administered a second dose of the monoclonal antibody. Additionally one patient that had an increase in cytotoxic T-cell counts at cycle one did not continue to cycle two and patients O and P are still continuing the trial and have not had their cell counts measured past post cycle 1. Meaning that 11 patients out of the 13 patients that reached a third cycle of immunotherapy displayed an increase in cytotoxic T-cell counts. Patients B and E exhibited cytotoxic T-cell counts at post cycle 2 that were lower than their initial counts at baseline while patients F and K were removed from the trial due to inabilities to continue the trial. (Figure 4)

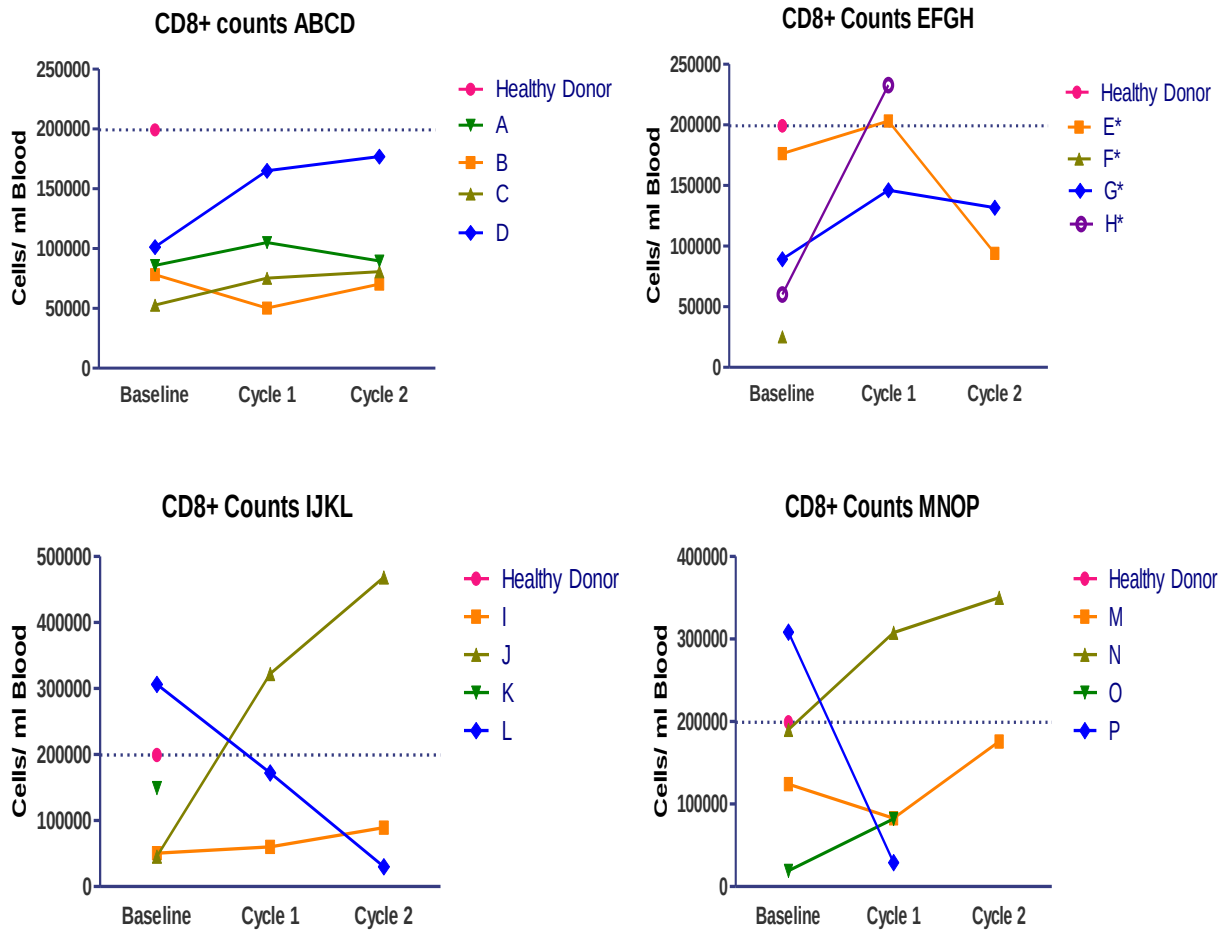
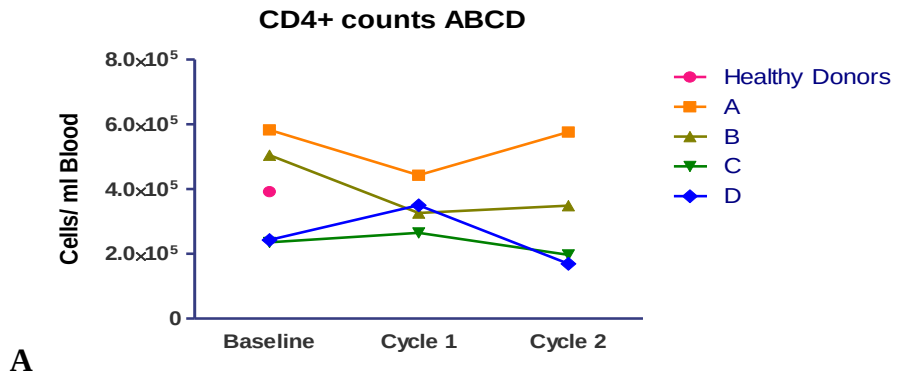


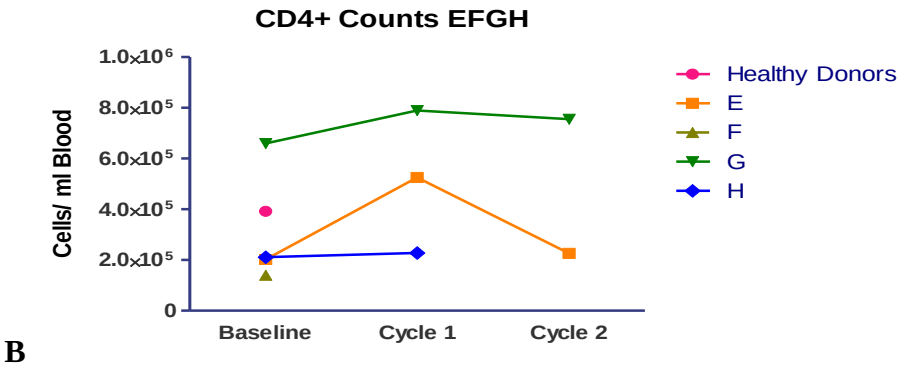
Figure 4. CD8+ T-cell counts for all patients of the first three cycles of their treatments. (A.) Cytotoxic T-cell counts for patients A-D. (B.) Cytotoxic T-cell counts for patients E-H. (C.) Cytotoxic T-cell counts for patients I-L. (D.) Cytotoxic T-cell counts for patients M-P.

NSCLC Patients Treated with PD-1 Monoclonal Antibody Immune Therapy Display CD4+ Growth Patterns that Resemble Each Patient's CD8+ Cell Counts

Much like cytotoxic T-cell counts in the NSCLC patients, CD4+ cell counts in these patients change over the course of immune therapy. Some patients displayed increases in CD4+ cells while others had decreases. However, patients seemed to follow a trend where if they displayed an increase in their CD8+ cell counts we would observe a corresponding increase in CD4+ cells and likewise, a decrease in CD8+ cells often revealed a decrease in CD4+ cell counts for the same patient. Although these corresponding changes were seldom to the same scale in the two different cell types, the corresponding changes were still exhibited. (Figure 5)



A



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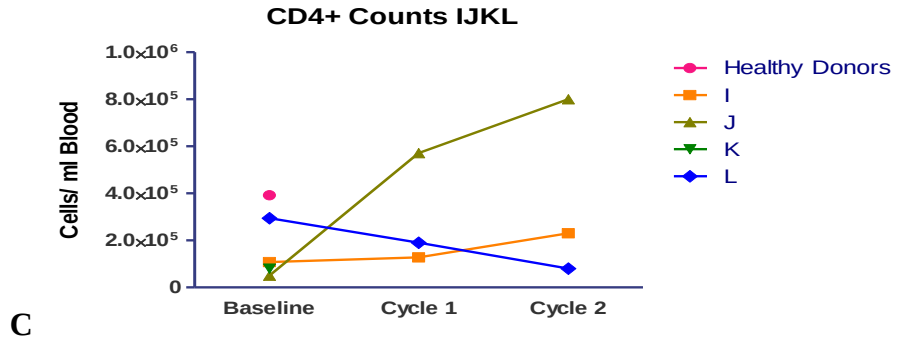

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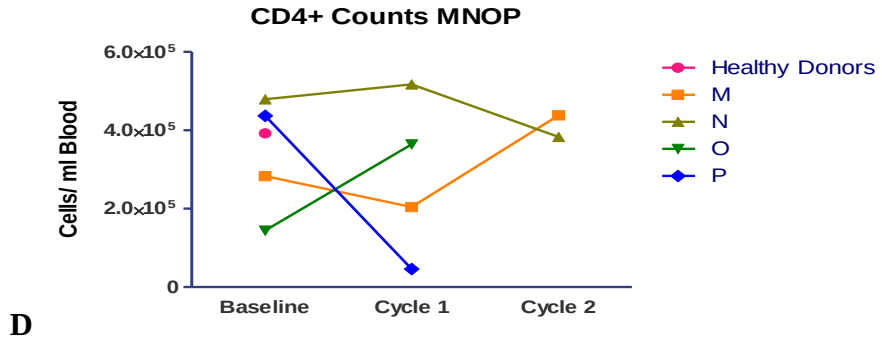

D

Figure 5. Helper T-cell counts of NSCLC patients within the first three doses of immunotherapy. (A.) patients A-D. (B.) patients E-H. (C.) patients I-L. (D.) patients M-P.

Response in Cytotoxic T-cell counts in NSCLC Patients undergoing anti PD-1 Immunotherapy is Highly Varied after the First 3 Cycles

As stated previously in the results, the majority of our patients undergoing anti PD-1 immunotherapy show an increase in their cytotoxic T-cell counts within the first 3 cycles of treatment. However, after this point these patients show high variation in their cytotoxic T-cell growth rates. Some patients continue this growth in CD8+ cell counts while others will slow in their growth rates or even start to decline. And of patients that continue to increase their cytotoxic T-cell counts some exhibit a cyclical manner of proliferation while others will show a constant, nearly linear pattern of growth. The two patients to complete the full length of the trial, patients “C” and “J”, displayed similar growth patterns in their cytotoxic T-cell counts but to different scales (Figure 6).

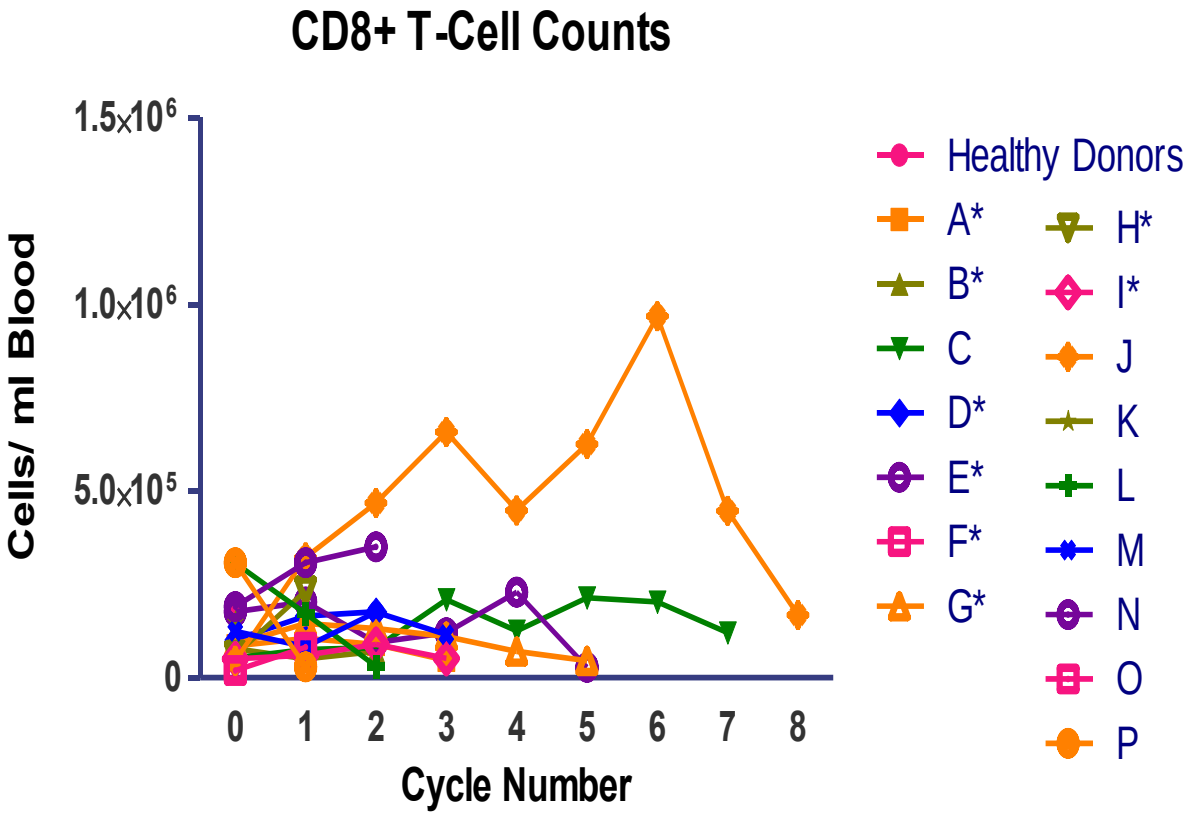

Figure 6. Cytotoxic T-cell counts of Non-Small Cell Lung Cancer Patients that underwent anti PD-1 immunotherapy.

High Variability of mRNA Expression in Cytotoxic T-cells of NSCLC Patients Undergoing Anti PD-1 Immunotherapy

To further our analysis of potential biomarkers in NSCLC patients we began to direct our focus towards mRNA expression of known immune regulatory genes in cytotoxic T-cells (Figure 7). Additionally, we looked at expression levels of a family of proton sensing GPCRs in these isolated patient cytotoxic T-cells due to these GPCRs having previously shown to have some immune regulatory capacity as well as some tumorigenic capacity as well[4]. As can be observed in figure 7 of this thesis, there is a high degree of variability in mRNA expression of the investigated genes among cancer patients. Figure 7 shows the change in fold values for each specific gene from baseline and then compare among patients. Figure 7 in addition to Figure 8 allowed for us to see which genes would warrant further investigation as can be seen in the following figures (10, 11, 12 and 13) which show relative expression of genes that were observed to have some coinciding trend with positive patient outcome.

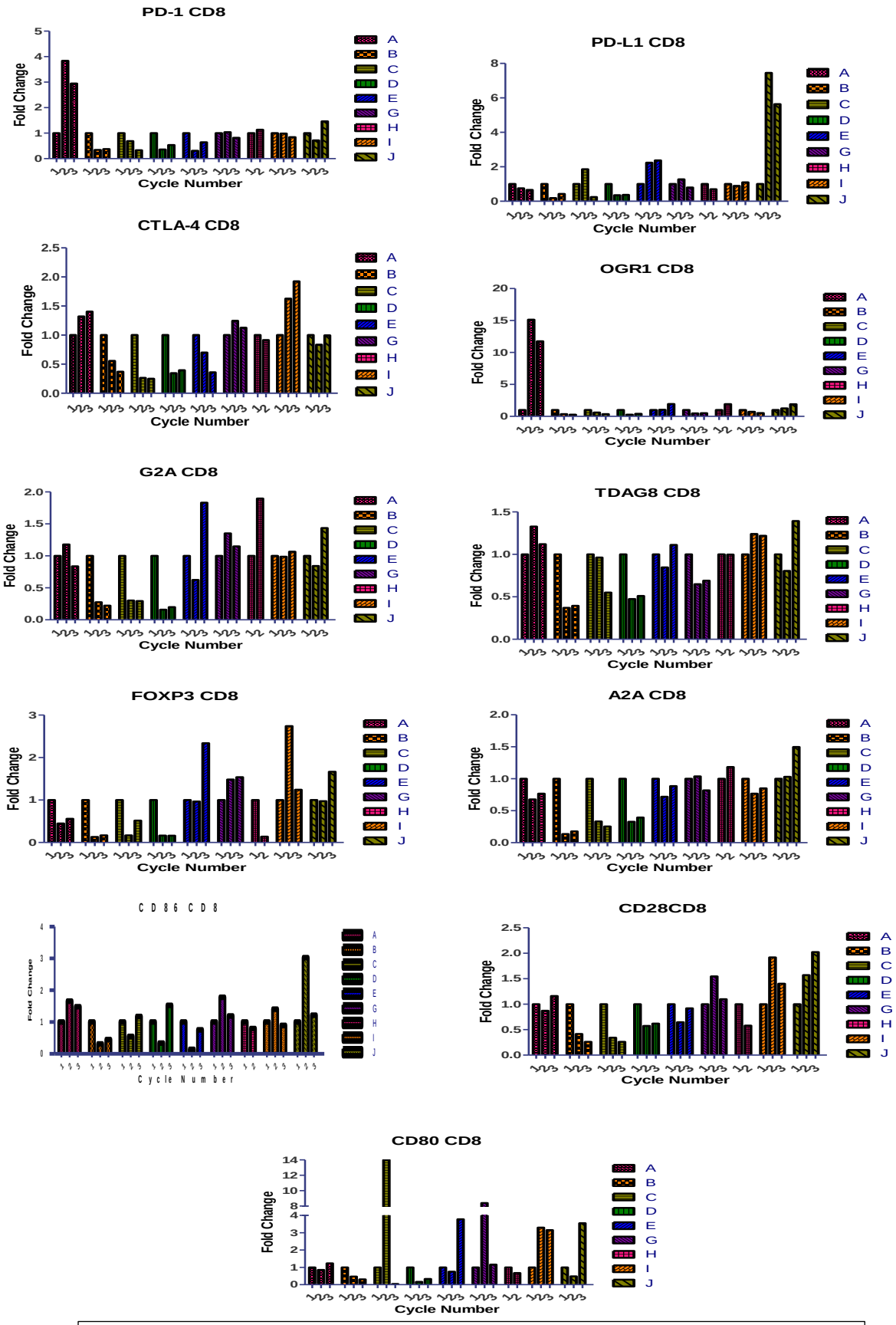

Figure 7. mRNA Gene Expression (fold change is normalized to baseline mRNA expression) in Cytotoxic T-lymphocytes Isolated from Patient Blood Samples.

Changes in the Relative Expression of Genes Expressed in the Cytotoxic T-cells of NSCLC Patients Display Similar Trends in Cytotoxic T-cell Responsive and Unresponsive Patients

When examining the relative expression of the genes of interest, we observed that there were no distinct variations in relative expression between patients that had increases in cytotoxic T-cells and those that had decreases or no change in their cytotoxic T-cell counts (Figure 8). Higher delta CT values correspond to lower gene expression. For all genes shown in Figure 8 of this thesis (CD28, TDAG8, A2A, OGR1, G2A, FOXP3 and CD86) all patients came in with similar relative expression levels of these genes. While some of these patients elicited unique trends in the expression levels of these genes over the course of immunotherapy they showed no correlation to cytotoxic T-cell growth nor did they indicate patient survivability.

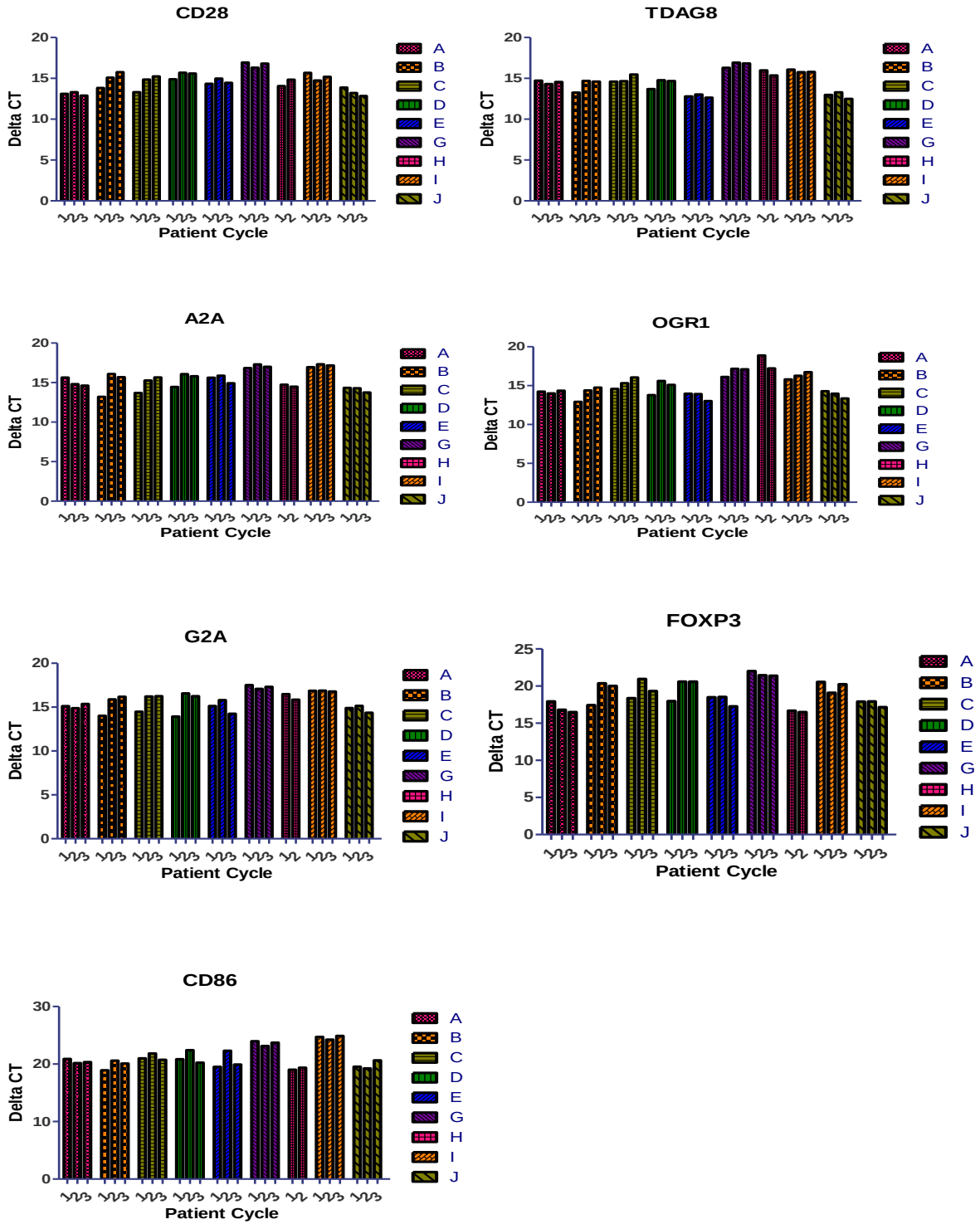

Figure 8. Relative Gene Expression (Delta CT) in Cytotoxic T-lymphocytes Isolated from Patient Blood Samples.

There is a "sweet spot" for Baseline PD-1 Expression in NSCLC patients undergoing anti PD-1 immunotherapy

Of the patients represented in Figure 9 of the results section of this thesis, 3 patients had very similar levels of relative expression of PD-1 in their cytotoxic T-cells. Additionally these three patients had some of the more noticeable levels of increased cytotoxic T-cell counts and two of these three patients had the longest survivability of all of the patients undergoing the trial. Additionally, patients with lower PD-1 expression (patient B and patient E) were patients that had decreased cytotoxic T-cell counts from when they first came into the trial. Patients A, G, H and I all had higher levels of relative expression of PD-1 when compared to patients C, D and J. However, Patients A, G, H and I did not show the same magnitude of increased cytotoxic cell counts as patients C, D and J with the exception of patient H who had an initial sharp increase in cytotoxic t-cell counts from baseline to cycle 1 but then was removed from the trial due to health complications.

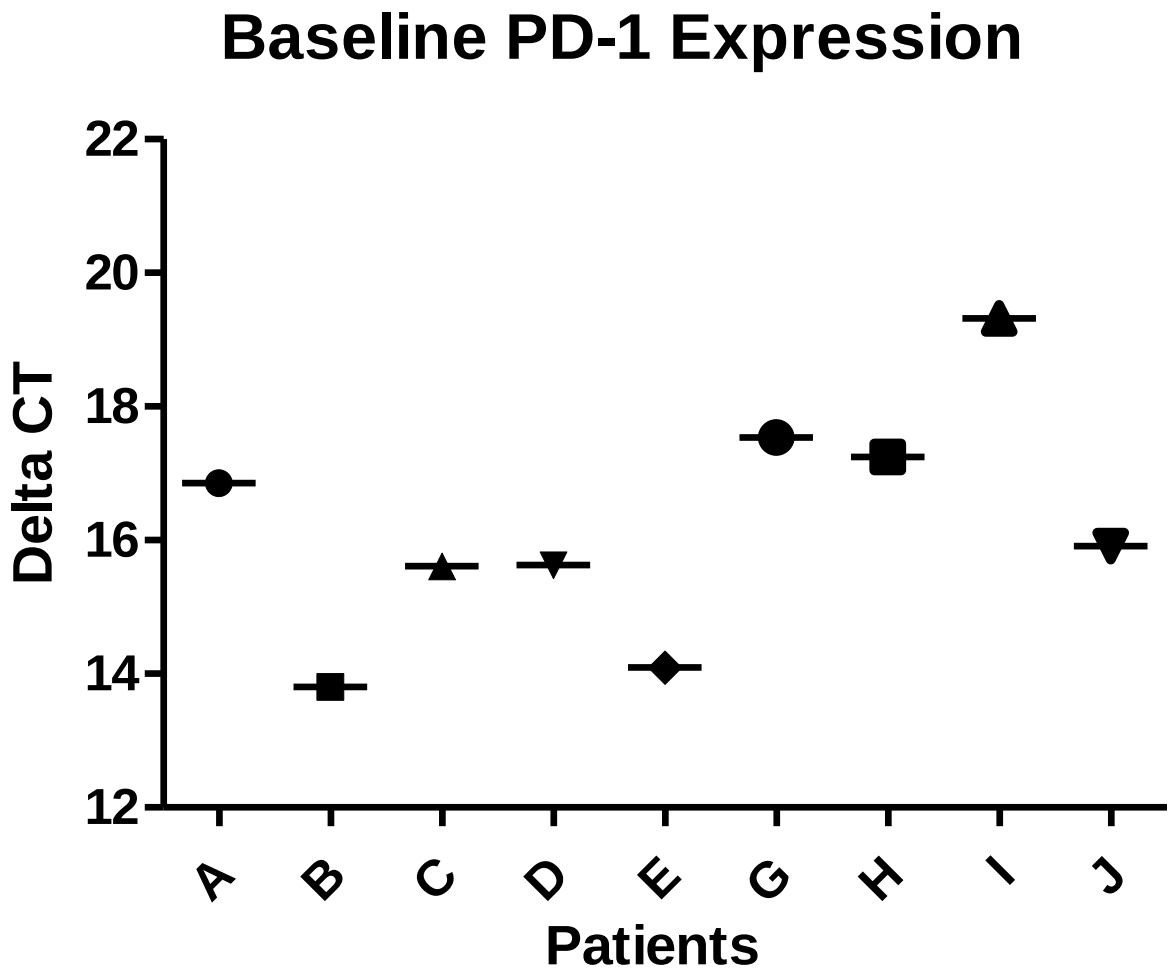

Figure 9. PD-1 mRNA expression of NSCLC patients at baseline.

Treatment of NSCLC Patients with anti PD-1 Antibodies Result in Varied Changes in levels of PD-1 mRNA Expression in the Cytotoxic T-cells of These Patients

Of the 9 patients that were investigated for their mRNA levels of various immune regulatory genes, 5 showed a decrease in their relative levels of PD-1 expression. Of these 5 patients that showed a decrease in relative PD-1 expression, 3 had increases in their cytotoxic T-cell counts. The 4 patients that displayed increases in their levels of relative expression of PD-1 all had increased cytotoxic T-cell counts. The 5 patients with decreased PD-1 mRNA expression had more varied cytotoxic T-cell counts with 2 of the 5 patients having an overall decrease in their cytotoxic T-cell counts (Figure 10).

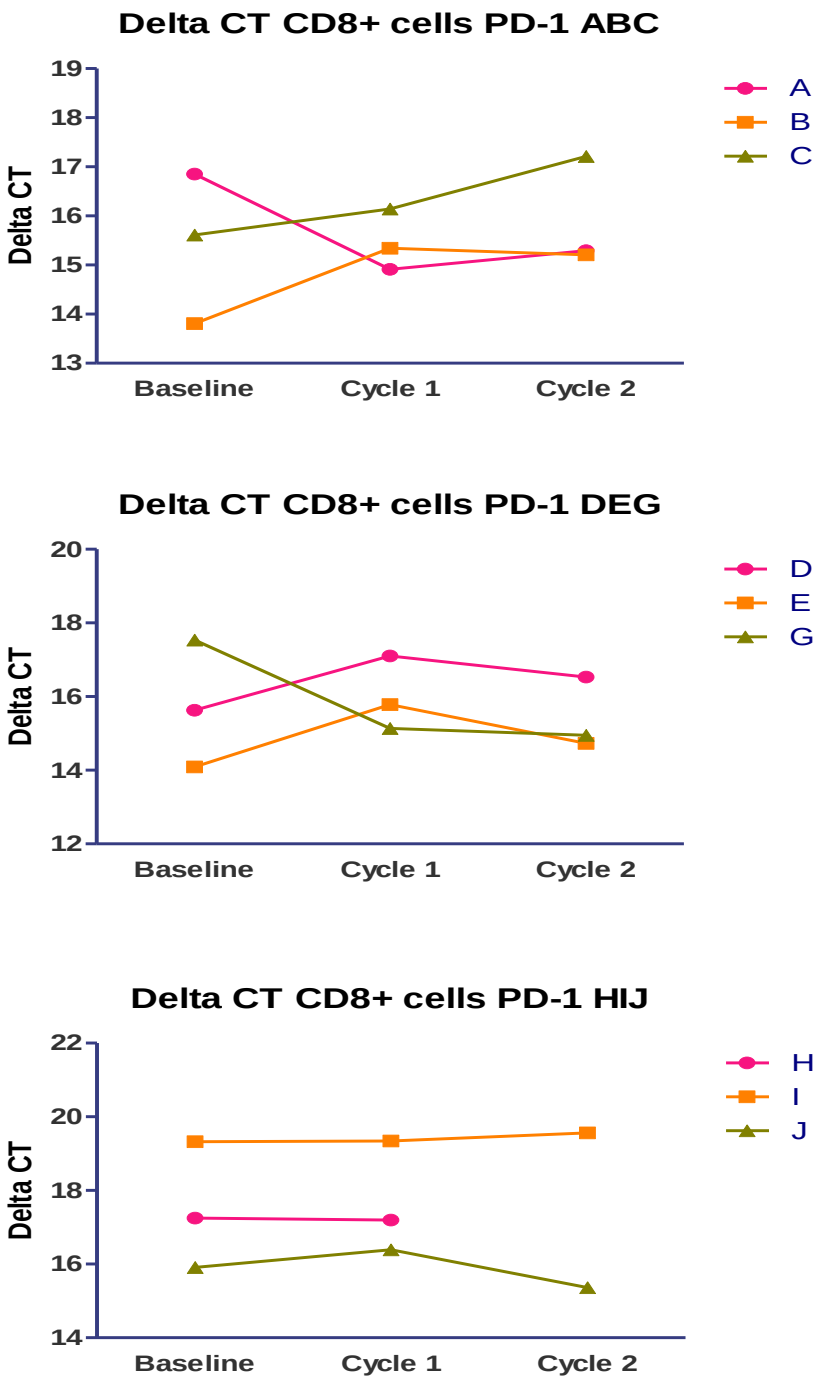

Figure 10. PD-1 mRNA expression of NSCLC patients for each of the first three cycles of the immunotherapy treatment.

NSCLC Patients Displayed Varied Changes in CD80 Expression in Cytotoxic T-cells

Of the patients that were kept on the trial for at least 3 cycles, only 3 patients saw a decrease in CD80 expression out of the 9 patients that were investigated for mRNA expression levels of the previously mentioned genes. Of these 3 patients that showed a decrease in CD80 expression, 2 of the patients had increases in their cytotoxic T-cell counts. It should be noted here that patient H also exhibited decreases in relative mRNA expression of CD80 but did not complete a full 3 cycles of immunotherapy. The 5 other patients that had increases in CD80 expression however had increases in their cytotoxic T-cell counts. Of the two patients that completed the trial and had the largest increases in cytotoxic T-cell counts from baseline, one patient had an overall increase in CD80 mRNA expression while the other patient had a decrease in CD80 mRNA expression (Figure 11).

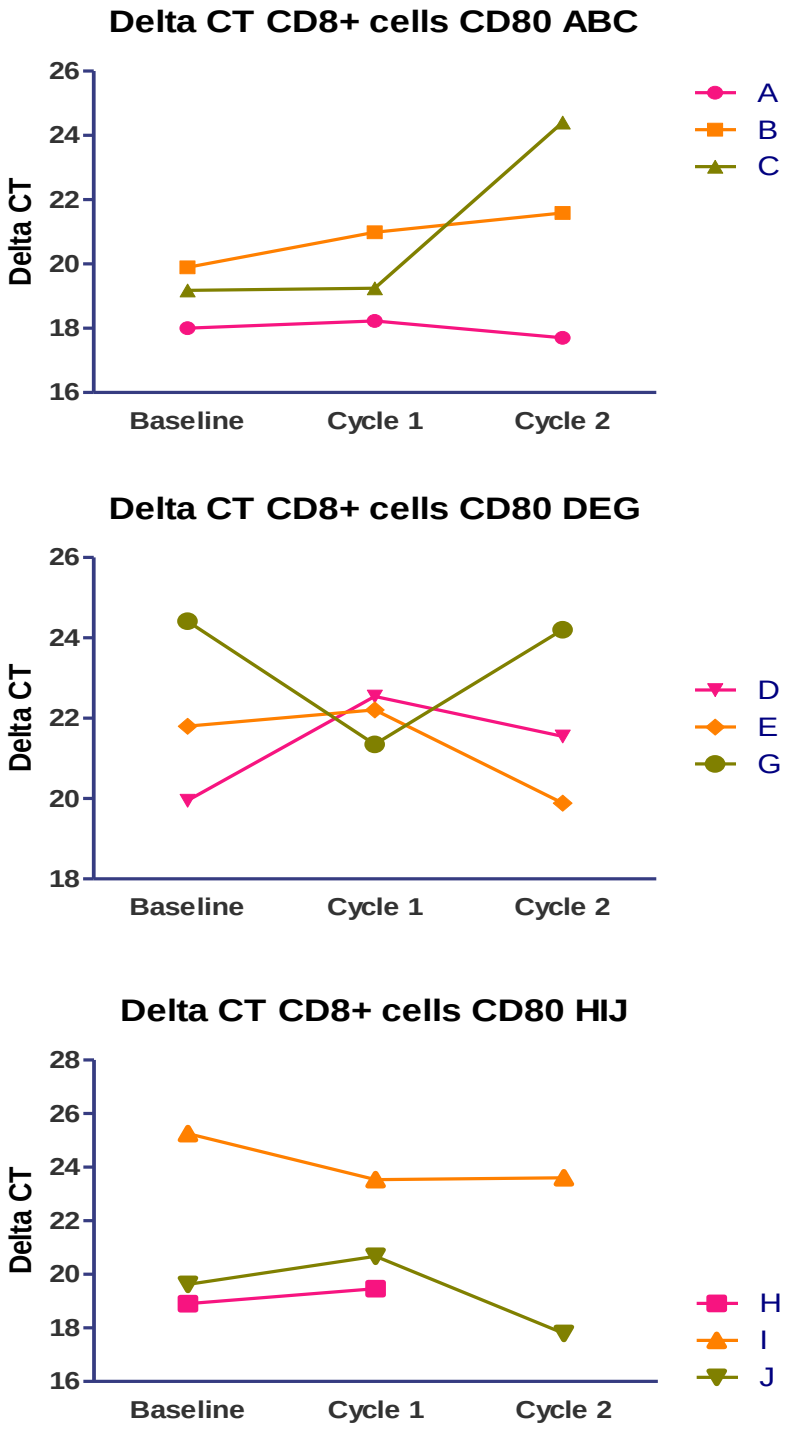

Figure 11. CD80 mRNA expression of NSCLC patients for each of the first three cycles of the immunotherapy treatment.

NSCLC Patients treated with Anti PD-1 Immunotherapy Display a Varied Response in Relative PD-L1 Expression in Cytotoxic T-cells

Next we furthered our investigation into gene expression by looking at PD-L1 mRNA expression within cytotoxic t-cells of NSCLC patients undergoing anti PD-1 immunotherapy. It was observed that there was a tendency for these patients to display a decrease in PD-L1 on cytotoxic T-cells with continued immunotherapy. Out of the total 9 patients that were used to determine molecular biomarkers, 6 of these patients showed a decrease in the relative expression of PD-L1 and of these 6 patients 5 showed increases in cytotoxic t-cell counts. The three patients that showed increases in relative PD-L1 expression however had increases in cytotoxic T-cell counts as well with the exception of patient E who had a severe drop in cytotoxic T-cell counts by cycle 3. Patient J which had the sharpest sustained rise in cytotoxic t-cell counts was among the patients that had an increase in the relative expression of PD-L1 (Figure 12).

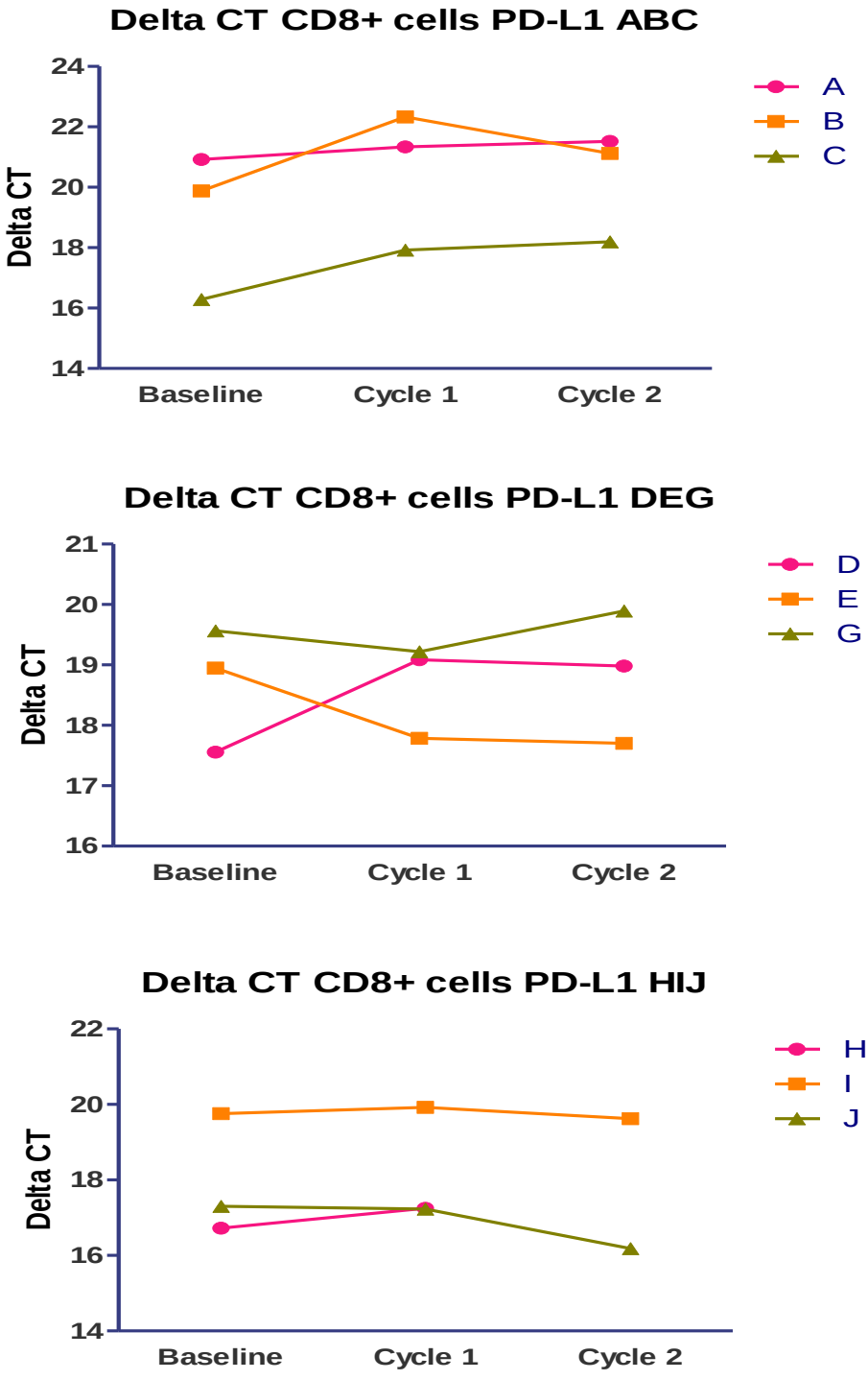

Figure 12. PD-L1 mRNA expression of NSCLC patients for each of the first three cycles of the immunotherapy treatment.

NSCLC Patients Treated with Monoclonal PD-1 Antibodies show Varied Changes in CTLA4 Expression on Cytotoxic T-cells

Continuing our investigation into immune regulatory protein expression on cytotoxic T-cells in immune therapy treated NSCLC patients we turned our focus to the protein CTLA4. When looking at the mRNA levels of expression of the immune regulator CTLA4 on cytotoxic T-cells we observed 6 of the 9 patients examined had overall decreased CTLA4 mRNA expression. Of these 6 patients with decreased CTLA4 mRNA expression, 2 had decreased cytotoxic T-cell counts from baseline. And all patients that had increased CTLA4 mRNA expression had increased cytotoxic T-cell counts from baseline values (Figure 13).

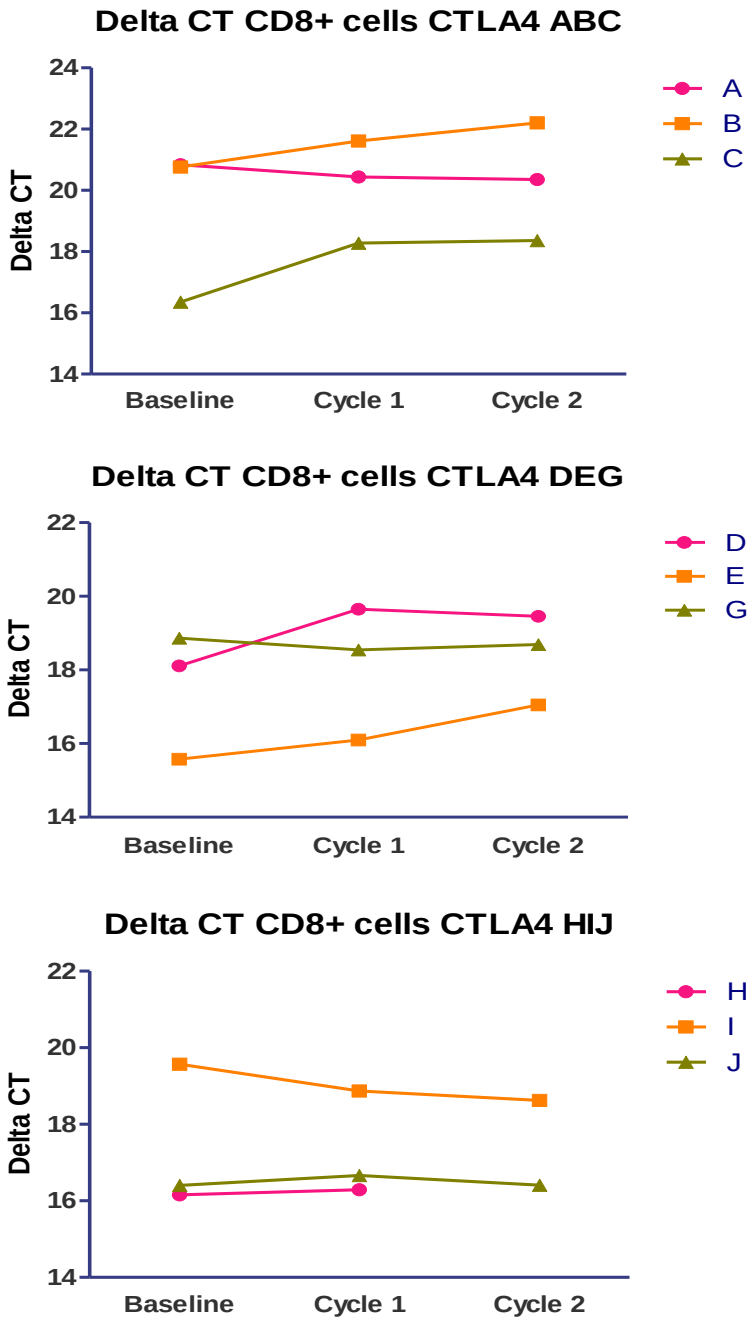

Figure 13. CTLA4 mRNA expression of NSCLC patients for each of the first three cycles of the immunotherapy treatment.

Human Cancer Cell Lines Treated with Acidic and Physiological Media for 5 hours Display PD-1 and PD-L1 Expression Levels that are Dependent on the Cancer Type

After extraction of the mRNA from the acidosis treated cancer cell lines and careful conversion of this mRNA to cDNA through the implementation of reverse transcription, we were then able to identify the expression levels of our genes of interest. We observed that after 5 hours of treatment, the expression of PD-1 and PD-L1 is highly varied among cancer types. However, there was still a slight trend when comparing PD-1 expression levels in these cancer cells to their PD-L1 levels. It was observed that the majority of the cancer cells tested (7/10) had higher mRNA expression levels for PD-L1 than PD-1 after 5 hours at physiological pH. Additionally, of the 10 cancer cell lines treated with 6.4 pH buffered media nine of the cancer cells had greater relative expression of PD-L1 than PD-1. Although it should be observed that both of these genes were well represented in these cancer cell lines but the expression levels were unique to each cancer type (Figure 14).

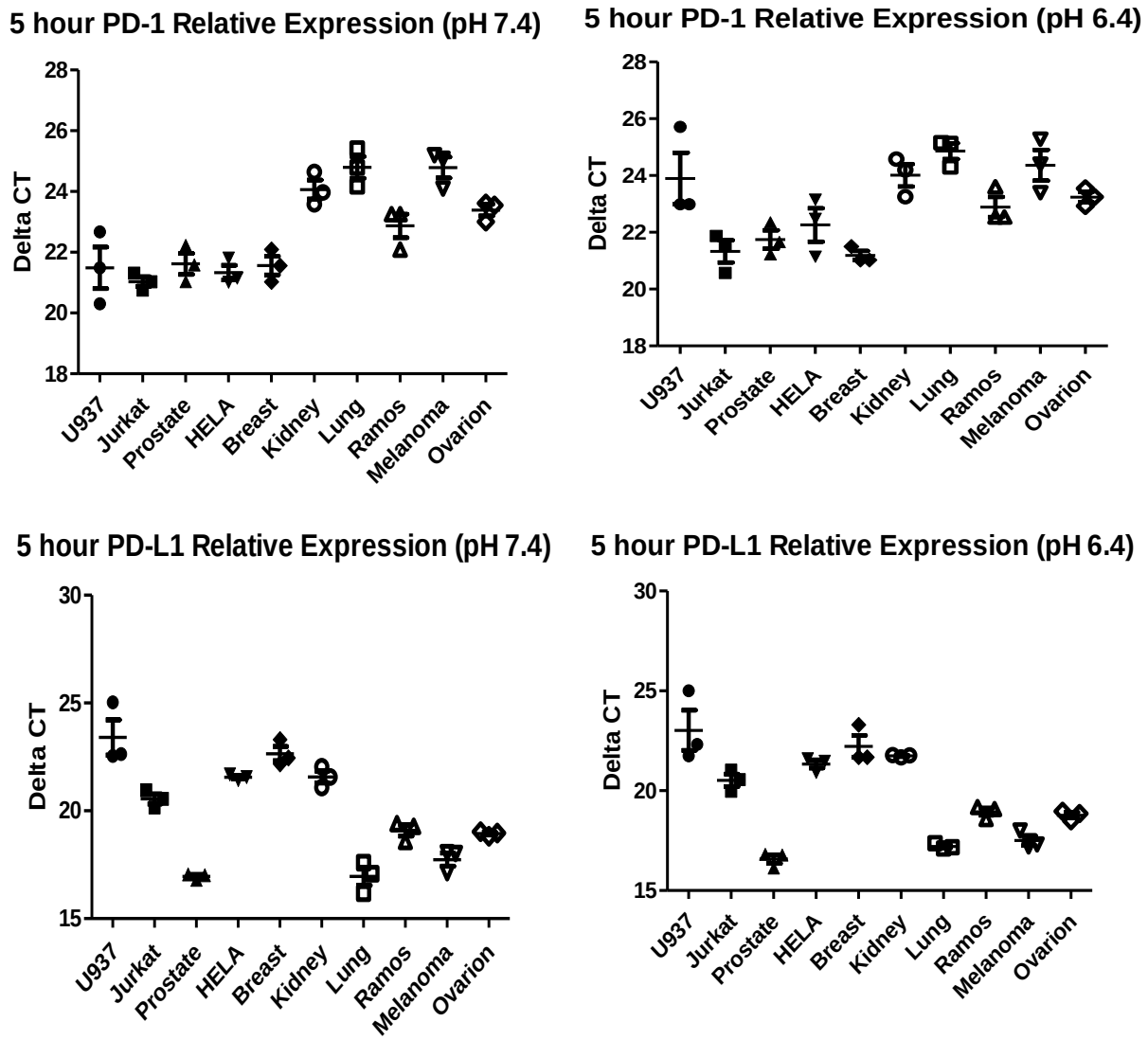

Figure 14. Relative expression of PD-1 and PD-L1 in cancer cell lines treated for 5 hours with acidic media (pH 6.4).

Human Cancer Cell Lines Treated with Acidic Media for 24 hours Display PD-1 and PD-L1 Expression Levels that are Dependent on the Cancer Type

When comparing PD-L1 expression to PD-1 expression within individual cancer cell lines at 24 hours of continuous treatment physiological pH buffered media we see that 8 of the 10 cancer cell types display PD-L1 expression that is greater than PD-1 expression after being treated with physiological pH buffered media. When treated with acidic pH buffered media, 8 of the 10 cancer cell lines showed PD-L1 expression levels that were greater than their individual PD-1 expression levels (Figure 15). Noticing these differences in expression levels with 5 hour and 24 hour treatments we then calculated the fold change for the mRNA expression of these genes which, normalizing to the physiological pH media treated cell lines as observed in figures 16 and 17 of the results section.

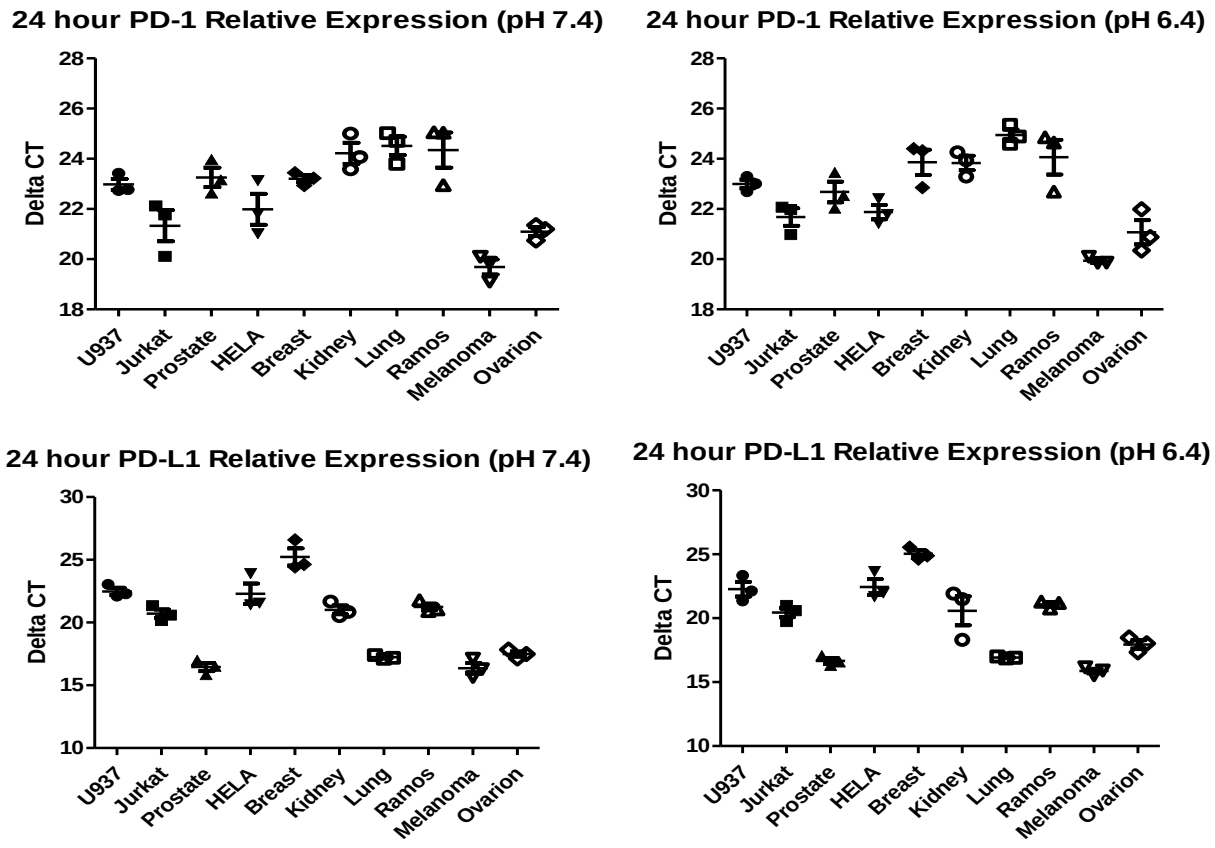

Figure 15. Relative expression of PD-1 and PD-L1 in cancer cell lines treated for 24 hours with acidic media (pH 6.4) and physiological pH buffered media (pH 7.4).

Changes in the Fold Values of PD-1 and PD-L1 Appear to be Linked to Each Other as the Two Genes Increase or Decrease Together in Each Cancer Cell Line

Similar to what was observed in Figures 14 and 15, the fold values for PD-1 and PD-L1 vary among cancer cell lines. However, it was observed in the 5 hour pH treated cancer cell lines that the PD-1 and PD-L1 fold values increased and decreased in similar manners within each individual cancer cell line. Meaning that if an increase in one gene was observed in an individual cancer cell line then it was likely that an increase the other gene would be observed in the same cancer cell line. This pattern was observed in 6 out of the 10 cancer cell lines in the 5 hour pH treatments and 5 out of the 10 cancer cell lines in the 24 hour treatment (Figure 16).

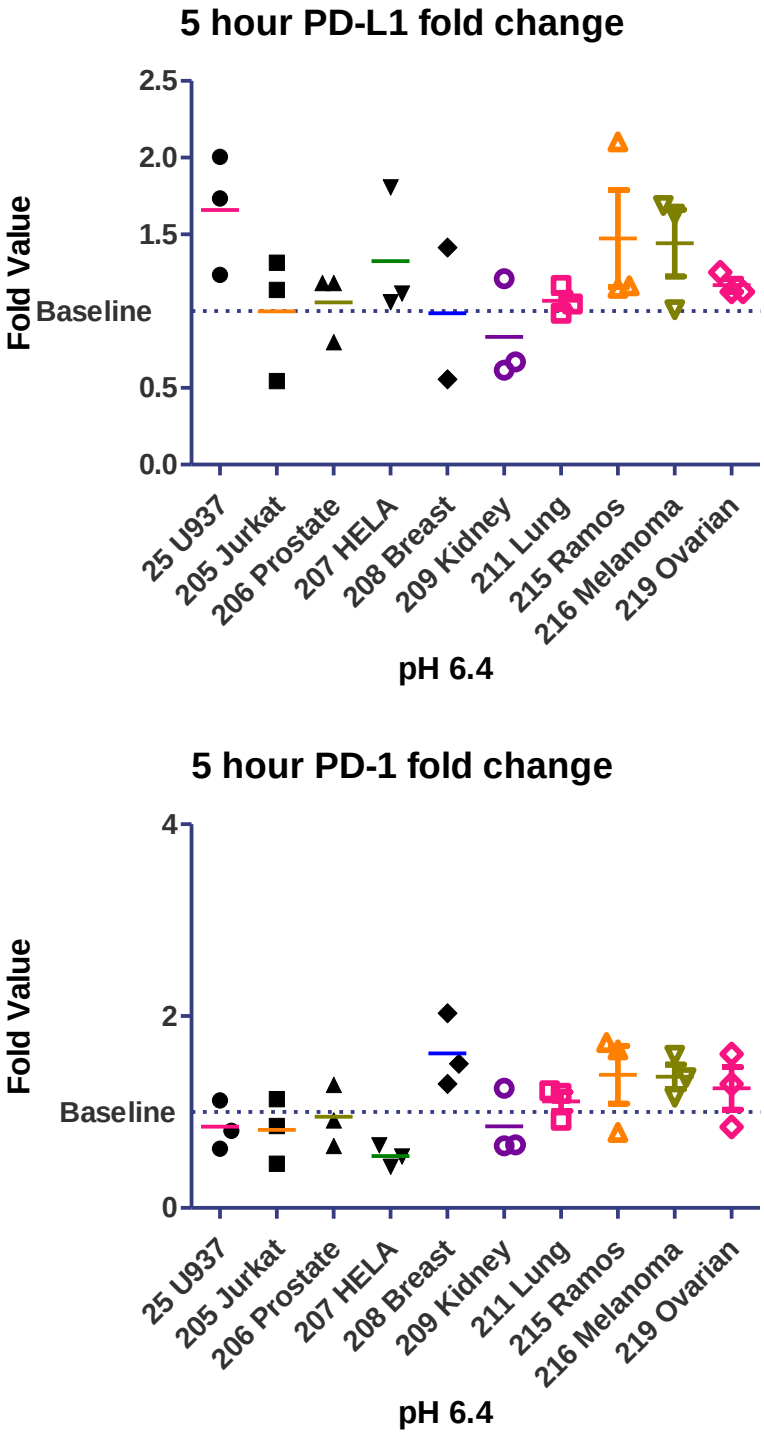

Figure 16. PD-L1 and PD-1 expression in human cancer cell lines after 5 hour acidosis treatment

Acidosis Induces Changes in PD-1 mRNA Expression that is Cancer Cell Dependent after 24 Hours of Treatment

To further evaluate the role of the PD-1/ PD-L1 checkpoint pathway on immune regulation and cancer prognosis we began to investigate how the expression of these proteins changes on cancer types when exposed to certain conditions. To start, we investigated how acidosis impacts the expression of these proteins in 10 different cancer cell types. Using RPMI media with 10% FBS and 1x Pen/ Strep with 7.5mM HEPES and MES added and then buffered to pH 6.4 and pH 7.4 we were able to grow these cells at an acidic pH and a physiological pH. Each cell line was treated in triplicates at 6.4 and 7.4 pH for 5 hours and 24 hours. I then extracted the RNA and protein from these treated cells and used the RNA in a reverse transcription reaction that yielded cDNA which was then used in qPCR to determine the mRNA expression of these genes at different levels of acidity at different time points. Figure 18 shows the difference of PD-L1 and PD-1 expression in various acidic media treated cancer cell lines normalized to physiological pH buffered media treated cancer cells at 24 hours. We observed that at 24 hours the change in PD-L1 expression levels between acidic and physiological pH levels was minimal. PD-1 expression was however highly varied depending on the cancer cell type. Visible increases can be seen in U937, Prostate, HELA and Lung cancer cell lines but all other cancer cell lines show minimal to no changes in expression with acidosis treatment (Figure 17).

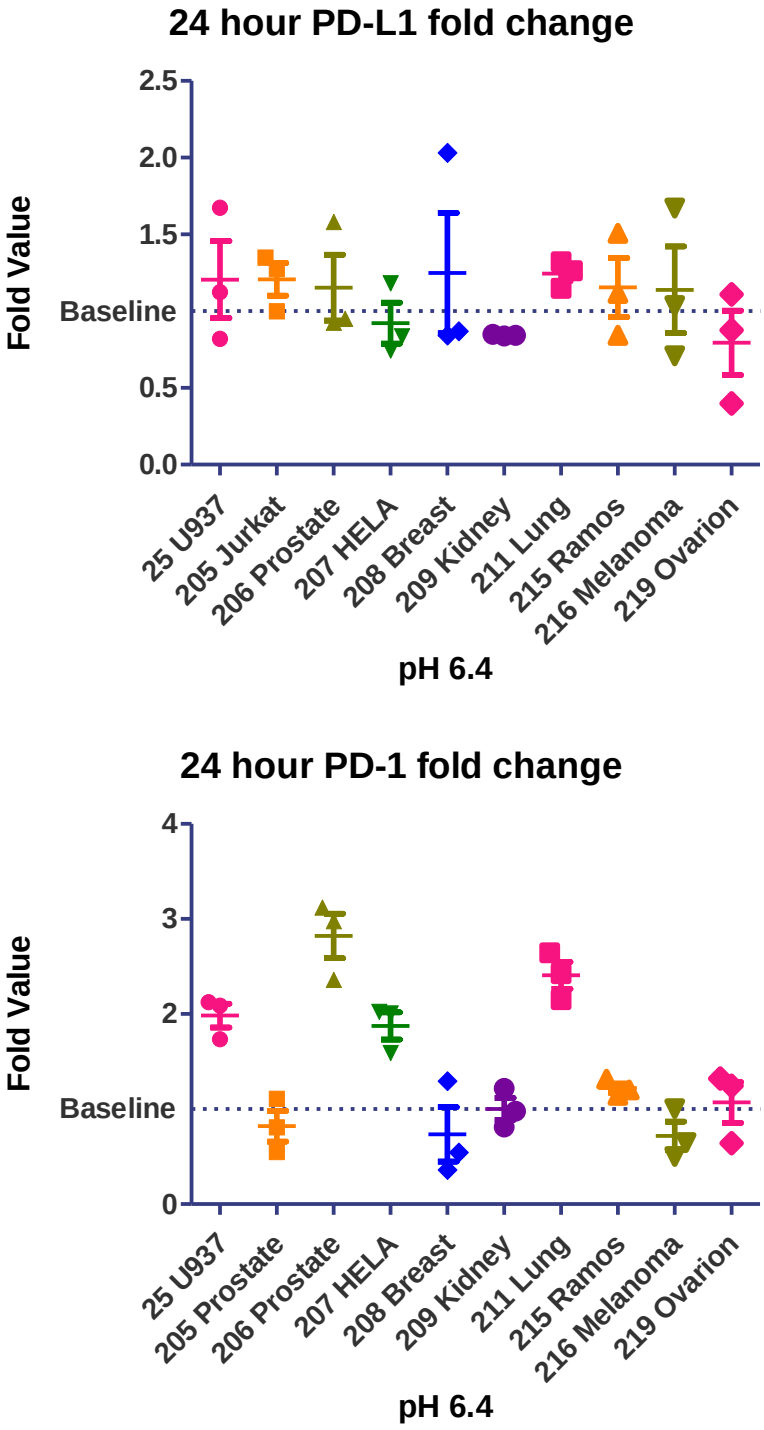

Figure 17. PD-L1 and PD- expression in human cancer cell lines after 24 hour acidosis treatment.

PD-L1 mRNA Expression Levels in pH Treated Cancer Cell Lines Vary with the Duration of Exposure to Acidic Conditions

When comparing the mRNA expression levels of the human cancer cell lines used in this study we not only see varying changes in expression for PD-L1 among cancer cells but we also see variation in the same cancer cells at different time points. This change in PD-L1 expression between different durations of treatment do not appear to show any pattern. Cell lines that show an increase in expression at 5 hours show a decrease at 24 hours while another cell line may have an increase at 5 hours but then has a smaller increase in PD-L1 expression at 24 hours. However, both lung cancer and melanoma cell lines displayed minimal increases in PD-L1 expression at both 5 hours and 24 hours (Figures 16 and 17).

PD-1 mRNA Expression Levels in pH Treated Cancer Cell Lines Vary with the Duration of Exposure to Acidic Conditions

Much like the PD-L1 mRNA expression in the acidosis treated cancer cell lines, the mRNA expression levels of PD-1 in the cancer cell lines also had high variation between the 5 hour and 24 hour treatments. Furthering on this likeness between the data represented in figures 17 and 18, there was an increase in PD-1 mRNA expression in melanoma cells at 5 hours but a decrease in expression of PD-1 was observed in melanoma cells at 24 hours of treatment while lung cancer cells still had an increase in PD-1 expression after 24 hours of treatment (Figures 16 and 17).

Chapter 4: Discussion

Our goal for this study was to identify biomarkers in non-small cell lung cancer patients undergoing anti PD-1 immunotherapy in hopes that we would be able to identify which patients would respond to the treatment and at the same time pull back the metaphorical curtains on the role of PD-1 in cancer in order to better understand the role the receptor plays in cancer and lay the groundwork to further developing more cancer treatment options. To achieve this task of identifying biomarkers in these patients we looked at cell counts of the innate immune system and how these changed over the course of continued immunotherapy. Additionally we looked at the expression levels of known genes that had known immune regulatory capabilities. Previous studies investigate cell counts of cytotoxic T-lymphocytes in NSCLC patients undergoing anti PD-1 immunotherapy but did not report findings past the second cycle of immunotherapy and did not report on the cell growth of other lymphocytes of the adaptive immune system. It has been observed that when treated with anti PD-1 monoclonal antibodies, both melanoma and NSCLC patients show increased cytotoxic T-cell counts[1-2]. However, little genetic analysis has been added to this information and as stated, these cell counts in previous clinical experiments do not span the entire duration of the immunotherapy treatment for each of these patients as our study does.

When addressing the possibility of cytotoxic T-cell counts serving as possible biomarkers in NSCLC patients we looked at three different qualifications; cytotoxic T-cell counts of the patients at baseline, the change in cytotoxic T-cell counts within the first three cycles of immunotherapy for each patient and cytotoxic T-cell growth rates and fluctuations in our patients. We observed that patients that had cytotoxic T-cell counts at or above healthy donor

levels typically responded poorly to immunotherapy by exhibiting decreases in cytotoxic T-cell counts with continued immunotherapy as can be seen in patients E and L (Figure 1 and Figure 5). On the other hand, most patients that had cytotoxic T-cell counts lower than healthy donors and completed a minimum of three cycles of immunotherapy showed an increase in cytotoxic T-cell counts (Figure 1 and Figure 5). We then followed our investigation on the counts of cytotoxic T-cells in our immune therapy treated NSCLC patients by looking at the counts of CD4+ T-cells. We observed that CD4+ cell counts also display increases and decreases in these NSCLC patients but in addition to this, the CD4+ cell counts showed similar growth trends with cytotoxic T-cells over the course of immune therapy with both cell lines increasing together in each individual patient or decreasing together (Figure 5 and Figure 6). Additionally, it is observed that the average ratio of CD4+ cells to CD8+ cells in an average healthy person is 2:1[42]. It is well known that CD4+ cells otherwise known as T-helper cells are directly responsible for the activation and survival of cytotoxic T-cells[42-43]. So it stands to reason that these similar growth curves between CD8+ and CD4+ cells are biologically significant in patient samples. Without CD4+ cells present in the adaptive immune system the immune system's ability to display immunological memory suffers[42-43]. However, the current belief in immune therapy is that PD-1 monoclonal antibodies bind to PD-1 expressed on cytotoxic T-cells and thus prevent PD-1 activation induced apoptosis on these cells[1-3]. It is possible that this increase in CD4+ T-cells with increased CD8+ cells is an increase in the sub population of CD4+ T-cells known as T-regulatory cells which are responsible for the suppression of CD8+ T-cell proliferation[44]. Future protein analysis of the CD4+ cell populations isolated from patients will have to be performed to accurately evaluate this potential presence of regulatory T-cells. Continuing with the processing of patient samples will help us to draw more definitive conclusions on how the

immune therapy regulates cell counts and help in our goal to identify biomarkers for these patients. Currently we have processed 16 patients of our total 20 patient goal. Having a sample size this small in number means that any observations made will have to followed in the future with more in depth experimentation, possibly through the recruitment of more cancer patients. Due to time constrains this thesis was constructed with the data collected from these 16 NSCLC patients. Statistical analysis was also limited due to the manner that the data was examined. Since each patient sample was treated as an individual data point at each treatment cycle longitudinally and was not added into an average, statistical analysis was impossible.

Since anti PD-1 mAb immunotherapy serves to block cancer's immune regulatory effects on cytotoxic T-cells and cause an increase in cytotoxic T-cells this may explain why some patients had an increase in cytotoxic T-cell counts while others did not. When looking at the relative expression levels of PD-1 in NSCLC patients undergoing anti PD-1 immunotherapy a trend can be seen. Patients with lower increases in their cytotoxic T-cell counts had higher baseline relative PD-1 expression values while the patients that had decreases in their cytotoxic T-cell counts had the lowest baseline relative PD-1 expression and patients that had the largest increases in cytotoxic T-cell counts are found in between these high and low spectrums of PD-1 mRNA expression in cytotoxic T-cells extracted from baseline (Figure 10). This data seems to suggest a trend where the relative expression of PD-1 in the cytotoxic T-cells of NSCLC patients could potentially be used to determine patient outcome and thus, PD-1 mRNA expression in cytotoxic T-cells may serve as a biomarker for NSCLC patient response.

Following the investigation into PD-1 we then turned our focus to its ligand PD-L1 and its expression in cytotoxic T-cells. PD-L1 expression on cytotoxic T-cells serves the same immune regulatory purpose that it does on cancer cells and also allows T-cells to induce cell

death in other cells expressing PD-1. However, because PD-L1 and PD-1 are both expressed on cytotoxic T-cells this allows the cells to self-regulate their numbers. Interestingly when we looked at the PD-L1 expression on the cytotoxic T-cells of these NSCLC patients undergoing the anti PD-1 immunotherapy we saw that 6 of the 9 evaluated patients had decreases in their PD-L1 expression and of these 6 patients, 5 had increases in their cytotoxic T-cell counts. However, the additional 3 patients that had increased PD-L1 expression displayed increases in the cytotoxic T-cell counts of 2 patients within the first three cycles of immunotherapy. Additionally, PD-1 and CTLA4 expression were observed to have decreased in 5 of the patients. However since the sample size was so small this roughly equaled to half of the patients having increases in PD-1 and CTLA-4 expression and the other half having an increase in the expression. Future experiments will be necessary in order to accurately determine if and then how PD-1 immune therapy regulates the expression of these genes in NSCLC patients.

Decreasing the expression of negative immune regulators PD-1, PD-L1 and CTLA-4 on cytotoxic T-cells as was observed in some of the patients may have some significance to immunological response. By PD-1 blockade decreasing the expression of these genes, it can then be perceived that cells are operating on a possible negative feedback loop. By anti PD-1 monoclonal antibodies binding to PD-1 proteins expressed on cytotoxic T-cells, the PD-1 is compromised and incapable of activation which then appears to have the potential result of decreased expression of other negative immune regulatory proteins. This negative feedback loop may serve as a mechanism within cytotoxic T-cells to signal for an increase in their activation. However, this negative feedback loop will have to be proved by additional research utilizing protein analysis techniques such as western blotting or ICC. These techniques when used on protein extracted from cytotoxic T-cells of patients would show actual changes in the level of

expression of the immune regulatory genes at the protein level and if the other immune regulator genes that did not show a trend are evaluated some significance may be found.

Additionally, some patients showed an increase in the gene CD80 that is responsible for the activation of receptors CTLA4, responsible for the induction of cell death on T-cells, and CD28, responsible for T-cell activation and survival. CD80 is largely expressed on antigen presenting cells but in the presence of immune therapy it appears that they are upregulated on cytotoxic T-cells, perhaps to aid in self-regulation. However, since CD80 can be attributed to decreases and increases in T-cell activation due to the proteins in interacts with it is difficult to determine the role CD80 plays in anti PD-1 immune therapy.

Evaluation of PD-1/PD-L1 mRNA expression in cancer cell lines treated with acidic buffered media showed varying cellular responses that appear to be dependent on several variables. Some cancer cell lines had increases in PD-1 or PD-L1 mRNA expression at 5 hours of treatment but when examined at 24 hours of exposure were found to have reverted back to physiological pH levels of expression for the genes or showed to have decreased their expression to below that of physiological levels. Additionally different cancer cell lines exhibited different regulation patterns for these genes when compared to other cells leading me to believe that the expression of PD-1 and PD-L1 and how an acidic TME regulates these expression levels is dependent on the cancer cell type.

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Appendix A

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
4N-70 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office 252-744-2914 · Fax 252-744-2284 · www.ecu.edu/irb

Notification of Amendment Approval

From: Biomedical IRB
To: Li Yang
CC: Sue Joyner
Date: 9/26/2016
Re: Ame1 UMCIRB 16-000719
UMCIRB 16-000719
Yang Protocol

Your Amendment has been reviewed and approved using expedited review for the period of 9/24/2016 to 7/25/2017. It was the determination of the UMCIRB Chairperson (or designee) that this revision does not impact the overall risk/benefit ratio of the study and is appropriate for the population and procedures proposed.

Please note that any further changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. A continuing or final review must be submitted to the UMCIRB prior to the date of study expiration.The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Description
Changes to Study Team/Personnel

The Chairperson (or designee) does not have a potential for conflict of interest on this study.

Appendix B

East Carolina University

Date: Friday, September 23, 2016 1:11:28 PM

Print Close

View: SF - Amendment Information

Amendment Type: Amendment

Instructions

An amendment request includes two parts:
the Amendment form **and** modifications to the Study SmartForm. Any modified documents that need to be uploaded within the Study SmartForm should simply be added to the list of existing documents.

Only one amendment request is allowed at any given time, i.e: amendment 1 must be approved, denied or withdrawn before amendment 2 can be created. However, multiple changes can be included on one amendment submission.

Study ID: UMCIRB 16-000719

Study Title: Yang Protocol

1.0 Amendment Name:
Amendment 1 for IRB Study #UMCIRB 16-000719

View: SF - Amendment Cover Sheet

Amendment Request

- An amendment request includes two parts: the Amendment form and modifications to the Study form
- Only one amendment request is allowed at any given time, i.e: amendment 1 must be approved, denied or withdrawn before amendment 2 can be created.

1.0 *** Type of change this amendment is making** (check all that apply):
Changes to Study Team/Personnel

2.0 **Fund number for IRB fee collection (applies to all for-profit, private industry or pharmaceutical company sponsored project revisions requiring review by the convened UMCIRB committee). If you are a non-ECU entity, payment is required at the time of submission.**

3.0 **Level of IRB review required by sponsor:**
Expedited

4.0 *** Description of Changes** - briefly summarize the changes:
Druid Atwell is being added to the study team. He completed his human protections modules on 9/21/2016.

Denise Brigham, MPH, RN is also being added to the study team. She completed her human protections modules on 8/1/2017.

Brittany Brown, Nancy Leffler, and Melanie Morgan are being removed from the study team.

5.0 **Upload sponsor memo describing changes**:

Upload any general information about this Amendment (specific changes must be added within the modified smartform):
There are no items to display

6.0 * **Current Subjects** - are any participants currently enrolled in the study?: ◉ **Yes** ○ No

7.0 **Subject Notification** - how will participants be notified of changes (if necessary):

N/A

View: SF - Study Team Changes

Study Team/Personnel Changes

Update the study personnel below. A change of Principal Investigator must be submitted as its own amendment. **Newly added study team members must also be added to the modified study smartform (page 1).**

1.0 **Study Coordinator:**

2.0 **Study Coordinator IRB Certification Renewal Deadline:**

3.0 **Subinvestigators:**
Last Name First Name Organization Profile IRB Certification Renewal Deadline
There are no items to display

4.0 **Other Study Staff:**

	Name	Organization	Profile	Role	IRB Certification Renewal Deadline
View	Druid Atwell	Internal Medicine, Department of	Druid Atwell's Profile	Performs lab tests in Dr. Li Yang's lab	
View	Denise Brigham	Internal Medicine, Department of	Denise Brigham's Profile	Back-up study coordinator	8/1/2017

View: SF - Final Page

Summary of Changes and Next Steps

1.0 You indicated this Amendment is changing/updating the items below. Please ensure you have updated these sections on the modified study application smartform. If you are uploading documents that are new, do not remove older documents. Instead, add them to the list so this addition can be tracked with each Amendment.

Amendment Type
Changes to Study Team/Personnel

2.0 If "Other Changes" is listed, please ensure you have updated the appropriate items on the modified

study application smartform.
SmartForm

3.0 If PI or other study personnel changes have been made, the newly added individuals will need to be added to page 1 of the parent study smartform. They will then need to "Agree to Participate" before the submission can be forwarded to the IRB office.

4.0 Click the 'Finish' button below, then follow the onscreen instructions to **Submit** the Amendment to the IRB office

Appendix C

Yang, Li

From:	umcirb@ecu.edu
Sent:	Wednesday, July 27, 2016 10:47 AM
To:	Yang, Li
Subject:	RX: Your study has been approved

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
4N-70 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** Fax **252-744-2284** **www.ecu.edu/irb**

Notification of Initial Approval: Expedited

From:	Biomedical IRB
To:	Li Yang
CC:	Sue Joyner
Date:	7/27/2016
Re:	UMCIRB 16-000719 Yang Protocol

I am pleased to inform you that your Expedited Application was approved. Approval of the study and any consent form(s) is for the period of 7/26/2016 to 7/25/2017. The research study is eligible for review under expedited category #2,5. The Chairperson (or designee) deemed this study no more than minimal risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The Investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Name	Description
BSOM HIPAA for Future Research dated 5/31/2016	HIPAA Authorization
BSOM Informed Consent for Yang Protocol dated 7/20/2016-clean copy	Consent Forms
BSOM Main Study HIPAA Authorization dated 4/26/2016	HIPAA Authorization
Yang Protocol dated 6/27/2016-clean copy	Study Protocol or Grant Application
Yang Protocol dated 6/27/2016-tracked changes	Study Protocol or Grant Application
Yang Protocol Healthy Volunteers Informed Consent Form dated 7/20/2016-clean copy	Consent Forms
Yang Protocol Request for Review of Protected Health Information in Preparation for Research signed by Dr. Yang on 4/27/2016	HIPAA Authorization
Yang Protocol UMCIRB Application for Waiver of Authorization signed by Dr. Yang on 6/1/2016	HIPAA Authorization

The Chairperson (or designee) does not have a potential for conflict of interest on this study.

IRB00000705 East Carolina U IRB #1 (Biomedical) IORG0000418
IRB00003781 East Carolina U IRB #2 (Behavioral/SS) IORG0000418

Study.PI Name:
Study.Co-Investigators: