Spring 5-6-2019

Effects of Treatment on Melanoma with Checkpoint Inhibitor, Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Anti-SEMA4D Pepinemab Combination Treatment

Lauren E. Fitzgerald
lauren.fitzgerald@student.shu.edu

Effects of Treatment on Melanoma with Checkpoint Inhibitor, Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Anti-SEMA4D Pepinemab Combination Treatment

By

LAUREN E. FITZGERALD

Submitted in partial fulfillment of the requirements for the
degree of Master of Science in Biology from the
Department of Biological Sciences of Seton Hall University
May, 2019

APPROVED BY

MENTOR

COMMITTEE MEMBER

DIRECTOR OF GRADUATE STUDIES

Acknowledgements

First and foremost, I would like to thank Dr. Joseph Gulfo for his mentorship during this three-year endeavor. In January of 2016, I made the best decision to take a course entitled Cancer Biology. I had no idea what I was getting myself into not having taken any graduate level classes prior to this course, but all of these major, in-depth topics were so eloquently explained by Dr. Gulfo. So much so, I found myself looking back to my cancer biology notes to help with other classes! You certainly set the foundation for my career at Seton Hall University. I will forever be in awe of this topic thanks to you, Dr. Gulfo, and I will forever be grateful for my time at Seton Hall because of you.

I would also like to take the time to acknowledge my committee member, Dr. Roberta Moldow, for all of her help and guidance throughout my time at Seton Hall. Dr. Moldow, you were always a friendly and knowledgeable mentor who helped guide me through the logistics of my thesis work. Thank you for your time and patience.

I would be remiss if I did not take this time to thank my family and friends who are perhaps the most encouraging and loving people that I am fortunate enough to have in my life. Thank you to my parents for always encouraging me to keep striving and for believing in me even when I did not believe in myself. Thank you to my fiancé for your unending love and support especially during the busiest and hardest times. Thank you to my coworkers, especially Erika and Sue, for your encouragement, guidance, and help both at work and grad school. And a very special thank you to my Uncle John for inspiring me to work and learn in this field. Every day, whether I am in the classroom or the lab, I hope to be like you. I love you all so much!

Table of Contents

List of Tables

List of Figures

Abstract

Traditionally, cancer therapies are generally non-specific treatments meant to eradicate cancer cells. This can result in death of healthy tissue, which can significantly affect the overall well-being of the patient during and after treatment. Immunotherapy offers a more targeted approach by using immune cells to specifically identify and kill cancer cells through various neoantigens presented on the surface of the cancer cell. Drugs that target and block PD-(L)1 and CTLA-4, known as checkpoint inhibitors, act to "cut the breaks" on the immune response and keep it actively seeking out and killing cancer cells. Many patients fail on these treatments due to a lack of $CD8^+$ T cells, and other cytotoxic immune cells, being called into the tumor microenvironment. Many therapies exist to work in combination with checkpoint inhibitors to turn these immunologically "cold" tumors "hot" and prolong an immune response. I postulated that blocking PD-1, CD47, and SEMA4D in a novel triple combination treatment will allow for a greater presence of CD8+ T cells into the tumor microenvironment and double the overall response rate of patients. In a clinical trial consisting of 200 patients, half were placed into the control receiving Pembrolizumab (anti-PD-1) and TTI-621 (anti-CD47), while the experimental group received the triple treatment of Pembrolizumab, TTI-621, and Pepinemab (anti-SEMA4D). Results indicated that the triple combination treatment was effective in improving progression-free survival, overall survival, and overall response rate compared to that of the control. This clinical trial supports that this triple combination treatment can be potentially used as a cancer therapeutic in the future.

Introduction

Summary of immuno-oncology and cold tumor concept

For decades, the leading treatment for a majority of cancers has been a triad of surgery, chemotherapy, and radiation. These treatment options are generally non-specific towards cancer cells and result in a massive deterioration of the patient's healthy cells and overall quality of life. While this blanket approach has been effective in eliminating many individuals' cancers and improving survivorship, there are a number of cancers that remain elusive to treatments or end up resurging years later. Throughout the history of cancer research, there have been marked advancements that support the fact that not every cancer is the same. Each cancer has a different combination of multiple mutations that affect an array of aspects of the cell cycle, angiogenesis, and anchorage dependence. Essentially, every tumor has a different mode of attack that can vary from patient to patient, or even within the tumor itself. Because of this cancer diversity, cancer researchers have been attempting to create more targeted therapies that will be special to a patient's specific type of cancer.

The human body has an incredible arsenal against cancerous growths. Heavy regulation of the cell cycle, DNA repair mechanisms, and pro-apoptotic mechanisms all seem to exist in order to thwart any rogue cell from establishing itself and harming the host organism. Despite the body's defenses against cancer, the cancerous cells can still manage to evolve and find a way to bypass these innate checks and balances. A normal cell can become transformed via the accumulation of mutations. Mutations specifically affecting some aspect of the cell cycle and cell growth aide in the transformed cell securing an advantage. Cancers will also accrue more mutations over time

allowing them to change their tactics along the way or add in their repertoire against the body's defenses. This wide array of potential cancer phenotypes can seem like a perpetual arms race to beat the cancer first thus making the development of effective treatments very difficult. In the 1980s, a relatively novel approach surfaced: Immuno-oncology **(**Decker et. al., 2017). Cancer Immunotherapy is a class of treatment options that uses the host's immune system to target, kill, and eliminate tumor cells just as it would a foreign bacterial or viral invader. Compared to other more traditional and broad-spectrum methods of cancer treatment, immunotherapy offers a more targeted approach, which would improve the quality of life for the patient by reducing negative, and potentially dangerous, side effects. Chemotherapy is a nonspecific treatment type that attacks rapidly dividing cells. While this may effectively kill cancer cells, it can also result in severe off-target affects – the killing of the patients' healthy, rapidly dividing cells such as skin cells and the lining of the digestive tract. Immunotherapy acts to target *neoantigens* – proteins unique to cancer cells – recruiting various immune cell types to effectively kill and eliminate cancer cells all over the body. Over the past three decades, the modern immuno-oncology movement has quickly exploded and the mechanisms behind its efficacy are being researched, treatments are being developed, and clinical trials are underway.

The Human Immune System

The human immune system utilizes an arsenal of molecular and cellular responses that work to recognize, target, and kill foreign entities. The immune response is comprised of two major branches – innate immune response and the adaptive immune response. Both aspects work in tandem and are responsible for the protection of the host from foreign, pathogenic invaders, such as bacteria and viruses, but achieve this through different mechanisms. The innate immune

system guards against general invaders and requires no prior exposure to learn what constitutes as a foreign invader. Elements of the innate immune system, such as macrophages, natural killer (NK) cells, and neutrophils are pre-programmed to recognize substances that are not native to the host body and eliminate them through phagocytosis. NK cells are essentially pre-programmed to target cancerous cells with the ability to recognize various configurations of cell surface proteins that identify the cancer cell. This system has a relatively quick response to non-native entities and releases molecules, like cytokines and chemokines, to trigger inflammation. This inflammation attracts other cell types – particularly ones involved in the adaptive immune response.

The adaptive immune response is more of a targeted system that acts on specific pathogens and is involved with immune memory. It is initiated by the phagocytosis of foreign pathogens. Key phagocytic cells, namely macrophages and dendritic cells, recognize foreign antigens – proteins presented on the surface of cells – phagocytize the invader, and cleave the proteins into small oligopeptides (Weinberg, 2014). Once cleaved, these small protein sequences (8-11 residues in length) are presented on a specific antigen presenting region on a Major Histocompatibility Complex I or II (MHC-I or MHC-II) complex. (Weinberg, 2014) These proteins on the surface of antigen presenting cells (APCs) act to bind with receptors on $CD4^+$ T Helper cells (T_H) or $CD8^+$ Cytotoxic T cells (T_C/ CTLs). T_H cells activate B cells initiating their replication and activating their maturation. Once activated, B cells produce antibodies that can then recognize the specific antigens throughout the body. This represents the humoral response of the adaptive immune system. Antibodies can recognize the antigen sequences and opsonize the invader or infected cell. This opsonization first neutralizes the target and allows for the subsequent phagocytosis by macrophages, attraction of complement molecules, or death by

cytotoxic T cells. $CD8^+$ Cytotoxic T cells represent another branch of the adaptive immune system – the cell-mediated response. Once mature, CTLs have T-cell receptors (TCRs) that allow them to target the antigen that was presented to them. Whenever a CTL comes in contact with an antigen recognized by its TCR, it can kill the cell triggering pro-apoptotic cascades. If the immune system can be manipulated to target cancer cells, researchers can exploit that as a potential treatment option.

Another aspect of the immune system, and one of the areas for promising immunotherapy targets, involves blocking the "off" switch for the immune system. The immune system has a natural homeostasis, so when "on" signals (such as cytokines and chemokines) are being released, they are also stimulating the "off" signals, through negative feedback, in order to avoid a severe overreaction of the immune system (Weinberg, 2014). These "off" signals can induce the activation of immunosuppressive cells or immunosuppressive proteins. Regulatory T cells (T_{Reg}) have the same antigen-specific TCRs that are also expressed by CTLs. This allows for the T_{Reg}'s to compete with the CTLs for the MHC-I proteins on the outside of cells. In addition, T_{eg}'s can secrete compounds to suppress the proliferation of T_H and CTLs, which enhances the immunosuppressive action.

Regulatory T cells are not the only immunosuppressive mechanism the human immune system can employ. Programmed Cell Death Protein -1 (PD-1) and its ligand, PD-L1, interact in order to induce what is known as T cell exhaustion, which results in an inhibition of T cell activation and proliferation. PD-L1 is generally not detectable in normal tissue, but an IFN gamma response can cause normal cells to upregulate its expression (Simon and Labarriere, 2017). Some tumor cells have evolved to express PD-L1 in order to block T cell activation/proliferation and go unnoticed by T cells thus evading destruction.

Checkpoint inhibitors, such as anti-PD-(L)1 and anti-CTLA-4, act to block the pathway that regulate the "off" switches of the immune system. This allows the attack cells of the immune system to continue working at targeting the tumor cells without a negative regulator. Current FDA approved checkpoint inhibitors currently on the market include Anti-PD-1 drugs, such as Nivolumab (Opdivo ®) and Pembrolizumab (Keytruda ®), Anti-PD-L1 drugs like Atezolizumab, and Anti CTLA-4 drugs like Ipilimumab (Yervoy ®).

Since there are many players in the immune system, there are many potential targets for immunotherapy. However, just as with other molecular or cellular defenses, cancer evolves yet another way to circumvent the body's defenses. Unfortunately, not all patients respond to checkpoint inhibitors. The efficacy of the checkpoint inhibitor treatment is dependent on the tumor's microenvironment (TME), particularly the presence of certain immune cells, such as CD8+ Tumor-Infiltrating Lymphocytes (TILs) and PD-L1 expression (Gulfo, 2018). Without these cells or protein markers present in the tumor environment, the tumor will not be recognized by the body's immune system. This is referred to as an immunologically "cold" tumor and will therefore escape destruction. The TME can be classified into four main types: Type I – IV. An immunologically "hot" tumor is one that exhibits a Type I phenotype (Gulfo, 2018). This TME is TIL+ and PD-L1+ providing an ideal environment for a checkpoint inhibitor. Blocking PD-(L)1 keeps the immune system revved up and continually targeting and attacking tumor cells. This is possible with TILs already present in the TME – they will continually attack the tumor cells, effectively shutting off the negative regulation of an immune response. An immunologically "cold" tumor does not have a TME that attracts TILs. Patients that do not respond to checkpoint inhibitors typically fall into the Type II- Type IV profiles. The key to an effective checkpoint inhibitor is maintaining or triggering a "hot" TME (Gulfo, 2018).

How to turn a cold tumor hot – different approaches

The immune system has a natural homeostasis that regulates its response. It takes a combination of signals from various cell types to elicit an immune response as well as numerous negative regulators to turn off a response. Because of this natural system of checks and balances, it can be difficult to manipulate a particular response with only one approach. Combination approaches (Checkpoint inhibitors + another immune target) are being researched for efficacy in cancer treatments (Gulfo, 2018). Different targets can be used to initiate a type I interferon response, while the checkpoint inhibitors prevent the negative regulation of the natural immune system. This continual immune response can then be used to target cancer cells throughout the body. Major therapy types include other checkpoint inhibitors (immunomodulators), cancer vaccines, oncolytic viruses, CD3 Targets bispecific monoclonal antibodies (mAb), adoptive T cell transfer, Monoclonal antibodies, or targeted fusion proteins.

LAG-3

LAG-3 is a surface receptor expressed on activated T Cells (Burugu et al., 2018). Binding of LAG-3 with its ligand will act as an immune suppressant by influencing the activity of Tregs and cytotoxic T cells. When bound to Major Histocompatibility Complex II (MHCII) class proteins, Treg activity and proliferation is enhanced. When bound with LAG-3 on Cytotoxic T cells, proliferation and cytokine production are reduced, thus supporting an immunosuppressive action (ct). Cancer cells can increase their production of MHCII production in order to exploit this immunosuppressive mechanism. Blocking LAG-3 diminishes the number of Tregs and restores the CD8+ effector T cells, counteracting the immunosuppressive activity and maintaining the type I tumor microenvironment for effective checkpoint inhibition. When working in

combination, the effects are enhanced with anti PD-1 as evidenced in various clinical trials (Burugu et al., 2018).

TIM-3

TIM-3 is an immune-inhibitory molecule first found on CD4+ T Helper cells and CD8+ T Helper cells. Binding of its ligand, galectin-9, leads to effector T cell death resulting in immune tolerance – exhausted T cells. Conversely, ligand binding on Regulatory T cells results in an enhanced immunosuppressive action. Blocking of this pathway, when combined with anti PD-1 therapy, has produced effective results (Burugu et al., 2018).

TIGIT

TIGIT is a transmembrane protein receptor that regulates the immune checkpoint on T and NK cells. When ligands (CD155, CD112, and Nectin 2) bind, effector functions are inhibited in part by the production of IL-10. This immunosuppressive cytokine is responsible for the expression of the M2 macrophage phenotype, which is representative of more of an anti-inflammatory profile (Chen et al., 2016 and He et al., 2017).

B7-H3 (CD 276)

This ligand belonging to the B7 family of molecules that are frequently overexpressed in many types of cancers (Burugu et al., 2018). This can be a critical and desirable cancer target/marker since it is lacking in healthy tissue. Recent studies have shown that this family of molecules is involved in immunosuppressive activity correlated to the increase in production of Interleukin – 10 (IL-10) and TGF-β as evidenced by Sai Han *et al* in cervical cancers (Sai Han et al., 2018). Functioning in tandem with CTLA-4 and PD-1, the B7-H3 class of molecules can also work to suppress T cell activation and proliferation further supporting it as an ideal checkpoint target. In addition to its immunosuppressive qualities, B7-H3 molecules have also been found to

upregulate the cell cycle in tumor cells, support angiogenesis, and allow for metastasis of various tumor types (Burugu et al., 2018 and Castellanos et al., 2017).

GITR

GITR is a type II transmembrane receptor found extensively on regulatory T cells, and in lower amounts on other immune cells such as effector T cells (Knee et al., 2016). Binding to its ligand (GITR-L) inhibits regulatory T cell function by inducing depletion of these cell types and activates effector T cells (Burugu et al., 2018 and Knee et al., 2016).

CD47 and SIRPα

CD47 is a cell surface immunoglobulin that negatively regulates anti-tumor immunity through suppression of phagocytosis via the SIRPα receptor on macrophages (Burugu et al., 2018). CD47 is expressed on nearly all cell lines, including cancer cells, and is used and exploited by cancers as a "don't eat me" signal. When CD47 found on the surface of a red blood cell binds to the SIRPα receptor on the macrophage, it triggers intracellular downstream effectors that inhibit phagocytosis (Burugu et al., 2018). To the macrophage, that cell has identified itself as something not to "eat." Cancer cells can exploit this mechanism and express CD47 on their cells. This tells the innate immune system to pass by, thus evading destruction. Blockade of the CD47/SIRPα interaction through the synthesis of monoclonal antibodies induces phagocytosis of cancer cells (Murata et al., 2018).

IDO

IDO is an intracellular enzyme that is found in macrophages and dendritic cells that converts tryptophan to kynurenine (Burugu et al., 2018). Less cytosolic tryptophan translates to less T cell proliferation. More kynurenine induces apoptosis of TH1 cells and promotes differentiation of TH1 cells to T regs. Blocking of IDO results in an increase in T cell response and inhibits tumor progression (Burugu et al., 2018). BIN1 is a tumor suppressor that controls expression of IDO and is deficient in numerous cancers.

KIR Family (Killer immune-globulin-like receptor)

This family of receptors is expressed on most NK cells and some T cells (Burugu et al., 2018). Some are inhibitory when they bind to MHC molecules (HLA-C/HLA-B), while those on NK cells, are used to identify against self-recognition (Burugu et al., 2018). This is an effective tool for the cancer since cancer cells are considered to be "self" thus making the targeting of these rogue cells difficult. Despite this, inhibiting the KIR receptors and HLA ligands that elicit an inhibitory response, the NK activation signals can be bypassed (Burugu et al., 2018).

CD94/NKG2A

On T cells, this receptor functions as an inhibitory checkpoint and blocking it with The IPH2201 antibody, can improve antibody-dependent cell cytotoxicity (Burugu et al., 2018). Many cancers have elevated $NKG2A^+$ NK cells such as an increase in NKG2A in lung and cervical tumors compared to the periphery (Burugu et al., 2018). The highest expression is found in cancers with low CD8+ TILs and Ki67 (Burugu et al., 2018).

TLR-9

TLR-9 recognizes a known pathogen signatures (PAMPs) by binding to single stranded unmethylated CpG-DNA and induces an inflammatory response in an effort to enhance cytotoxicity by way of IFNα expression (Gulfo, 2018). It also induces antigen expression that is needed to attract CD8+ TILs making it a desirable target to turn an immunologically cold tumor hot. (Gulfo, 2018).

DKK

Promotes an immune-suppressive tumor microenvironment by increasing the MDSC (Myeloid derived tumor suppressor cells) resulting in a suppressed T cell response (D'Amico et al., 2016). Another immunosuppressive action of DKK includes a decrease in NK activating ligands on tumor cells (Malladi et al., 2016). A decrease in these ligands will reduce interaction with tumor-targeting NK cells. DKK also increases Th2 polarization and decreases IFNα production all to achieve an immunosuppressive tumor microenvironment (Chae et al., 2016).

Agnostic mAbs targeting APCs (Antigen presenting Cells) – CD40

CD40 is expressed broadly on APCs including DCs, B cells, and monocytes. Antibodies targeting CD40 promotes DC maturation and cross-presentation of antigens to T cells. It also induces apoptosis of tumor cells and TAM conversion to M1-like macrophages (Ishihara et al., 2018).

DC vaccines

Dendritic cells are antigen-presenting cells that can be utilized in the regulation of the immune response. This provides a workable mechanism to derive vaccines that can be used to enhance tumor antigen presentation to T cells (Mastelic-Gavillet et al., 2019). They are generated *ex vivo* and pulsed with specific peptides (protein or whole tumor lysate) and can be used in combination with anti-PD-(L)1 or anti-CTLA4 (Mastelic-Gavillet et al., 2019).

Macrophage reprogramming

Tumor Associated Macrophages are highly immunosuppressive. Antibody blockade of the receptor for colony stimulating factor 1 (CSF-1) – highly expressed by TAMs – can reprogram them toward an M1 phenotype (Räihä and Puolakkainen, 2018). M1-like macrophage is desired for its enhanced antigen presentation, promotes stronger anti-tumor T cell responses and synergizes with checkpoint blockade (Räihä and Puolakkainen, 2018).

Immunogenic chemotherapy

Promotes immunogenic cell death (ICD) through presentation of "eat me signals" via the translocation of the chaperone proteins, calreticulin, from the endoplasmic reticulum to the surface of the tumor cells leading to the activation of DCs and recruitment of TILs (Wu and Waxman, 2018).

Oncolytic viruses

The use of oncolytic viruses results in the direct killing of tumor cells. These viruses can be designed to specifically target cancer cells and result in the induction of innate and adaptive immune response (Kaufman et al., 2015). Upon infection, tumor cells release ROS and Type I IFNα; upon lysis, DAMPs and PAMPs. Oncolytic viruses such as in the drug, T-VEC, have been gene edited to integrate immunomodulatory genes like cytokines, chemokines, and T-cell stimulatory molecules (Kaufman et al., 2015).

Targeting tumor vasculature

Tumor vasculature normalization allows the vessels to be more permissive to tissue perfusion and delivery of oxygen, drugs, Antibodies, and T cell infiltration following treatment (Lanitis et al., 2015). This treatment type also increases leukocyte adhesion molecules (ICAM-1 and VCAM-1), which are anti-VEGFR and anti VEGFR mAbs, and increases chemokines and cytokines like IL-10, TNF-α, and CXCL10 which leads to increased lymphocyte infiltration (Lanitis et al., 2015).

CAR T cells

Immune cells that are removed from the patient are then engineered and armed with new proteins that have the ability to recognize the cancer. Then, this newly design T cell is given back to the patient. Targets antigens found on B cells – CD19 – however, mesothelin (expressed on solid tumor cells) could be a potential target for solid tumors (Adusumilli, 2017) (Newick et al., 2016).

NK Cell activation/mobilization

The NK activating receptor is CD16 along with other cell surface receptors. Antibodies can be developed to activate this receptor thus activating NK cells and allowing them to target cancer cells without prior antigen presentation (Sharma et al., 2017).

Combined Treatment Responses

While checkpoint inhibitor treatments and cold tumor treatment have allowed for improved treatment response and survival compared to checkpoint inhibitor treatment alone, response rates are still relatively low (~30%) leaving most patients unresponsive to this combination treatment (Trillium Therapeutics, 2019). A recent combination trial run by Trillium Therapeutics, has their anti-CD47 drug – TTI-621 – exhibiting a $\geq$ 50% reduction in CAILS in 41% of patients (Table 1). The Composite Assessment of Index Lesion Severity (CAILS) ranks five aspects of lesion morphology including erythema, scaling, plaque elevation, hypo- or hyperpigmentation, and lesion size from 0 to 8. A CAILS score is a method of measure for a lesion severity and is commonly used to indicate efficacy of treatments (Olsen et al., 2011). While these results are a significant advance in cancer immunotherapy, it does not provide significant results in a majority of patients.

Table 1

Drug	**Response Rate (%)**
TTI-621(Trillium) + anti-PD-1/PD-L1	41% with a $\geq$ 50% reduction in CAILS

According to Evans *et. al.* in association with Vaccinex, a class of molecules called Semaphorins have been shown to alter cytoskeletal effects of immune cells, endothelial cells, and tumor cells influencing how they navigate around the TME. Restricted immune cell access into the TME may explain why many other cancer immunotherapy treatments fail.

Semaphorins are soluble, transmembrane proteins that are ubiquitous throughout immune cells, but have been linked to overexpression on certain cancers. Expression of a particular member of the semaphorin class – SEMA4D – has been shown to be triggered by hypoxia and other factors that are characteristic of the TME and inhibits the movement of immune cells (Evans et al., 2015). According to Evans *et. Al.,* SEMA4D acts as a physical barrier that surrounds tumors, subsequently preventing $CD8^+$ T cells from entering the tumor. If TILs are prevented from accessing the TME, then they cannot cause any damage to the tumor cells. So, while there may be an increase in TILs activated by APCs, if they cannot enter the TME then their cytotoxic effects are restricted. As evidenced by Vaccinex, SEMA4D is localized to the outer rim of the tumor bed. $CD8^+$ T cells are blocked from entering the TME as evidenced by their accumulation around the tumor margin and relatively low concentration within the tumor bed. Allowing for increased immune infiltration into the TME may result in more effective responses. Vaccinex has shown in clinical trials that patients respond to treatment with anti-SEMA4D4 (Pepinemab). Treatment with anti-SEMA4D/Mab67 (Pepinemab) effectively breaks down the barricade allowing a greater infiltration of cytotoxic immune cells.

In addition to breaking down the physical barrier SEMA4D creates, Pepinemab treatment and a checkpoint inhibitor (Avelumab) also influences the CD8+ T cell to Regulatory T cell ration (T_{eff} : T_{reg}) (Preston et al., 2013). Anti-SEMA4D treatment promotes an inflammatory response by increasing infiltration of $CD8^+$ T cells and downregulates the expression of

Regulatory T cells. These findings not only support that SEMA4D acts as a physical barrier, but also that it suppresses an inflammatory response via the regulation of T_{reg} cells.

Concluding Statement

Checkpoint therapy has provided unprecedented results for a relatively small number of patients. It works by preventing the homeostatic mechanisms that act to shut-off of the patient's immune system. In a normal and healthy patient, this regulatory mechanism works to prevent an overreaction of the immune system. Cancer cells can exploit this and overexpress either the ligands or receptors responsible for silencing immune cells. When treating cancer cells, preventing the function of immune system regulators allows for the cytotoxic immune cells to work longer on tumor cells effectively eradicating them. However, in order for checkpoint inhibitors to be effective, the tumor must be considered immunologically hot, that is have a strong interferon gamma signal, that calls various cytotoxic cells to the tumor microenvironment. A variety of methods have been employed to turn cold tumors hot and have shown to be effective working in combination with a checkpoint inhibitor. Combination treatments work in harmony to both call immune cells to the microenvironment and keep the immune system revved up to continually target and kill the tumor. Despite the strides made and the dramatic results of combination treatments, there are still patients who are unresponsive to treatment indicating that the cancer has evolved yet another way to survive and successfully propagate. Recent studies have assessed the impact of the presence of SEMA4D on the surface of cancer cells – specifically at the tumor bed boundary – effectively acting as a physical barrier preventing T cells from entering the tumor. This is an intriguing aspect to investigate and may provide an explanation as to why combination treatments do not work for a majority of patients. Patients

who do not respond to anti-CD47 and anti-PD-1 combination treatment typically fail in the non-injected lesions, while there is success in the injected lesions. This indicates that there is a sufficient immune response to eradicate the tumor. However, I postulate that non-injected tumors are not receiving this large immune response because of the SEMA4D blockade. If there is a physical barrier at the edge of the tumor bed preventing access of immune cells into the tumor microenvironment, then all other efforts to attract cytotoxic cells or keep the immune system revved up are futile. I am postulating that the blocking of SEMA4D, in combination with checkpoint inhibitors and anti-CD47, will then allow the tumor microenvironment to sustain a prolonged immune response and double the response rate of patients who otherwise were unresponsive to other combination immunotherapies. I am postulating that the augmented effect will be mediated by an increase in $CD8^+$: T_{reg} ratio in the tumor microenvironment.

Research Design and Methods

Experiment 1 – Effect of Treatment on Melanoma with Checkpoint Inhibitor, Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Anti-SEMA4D Pepinemab combination treatment

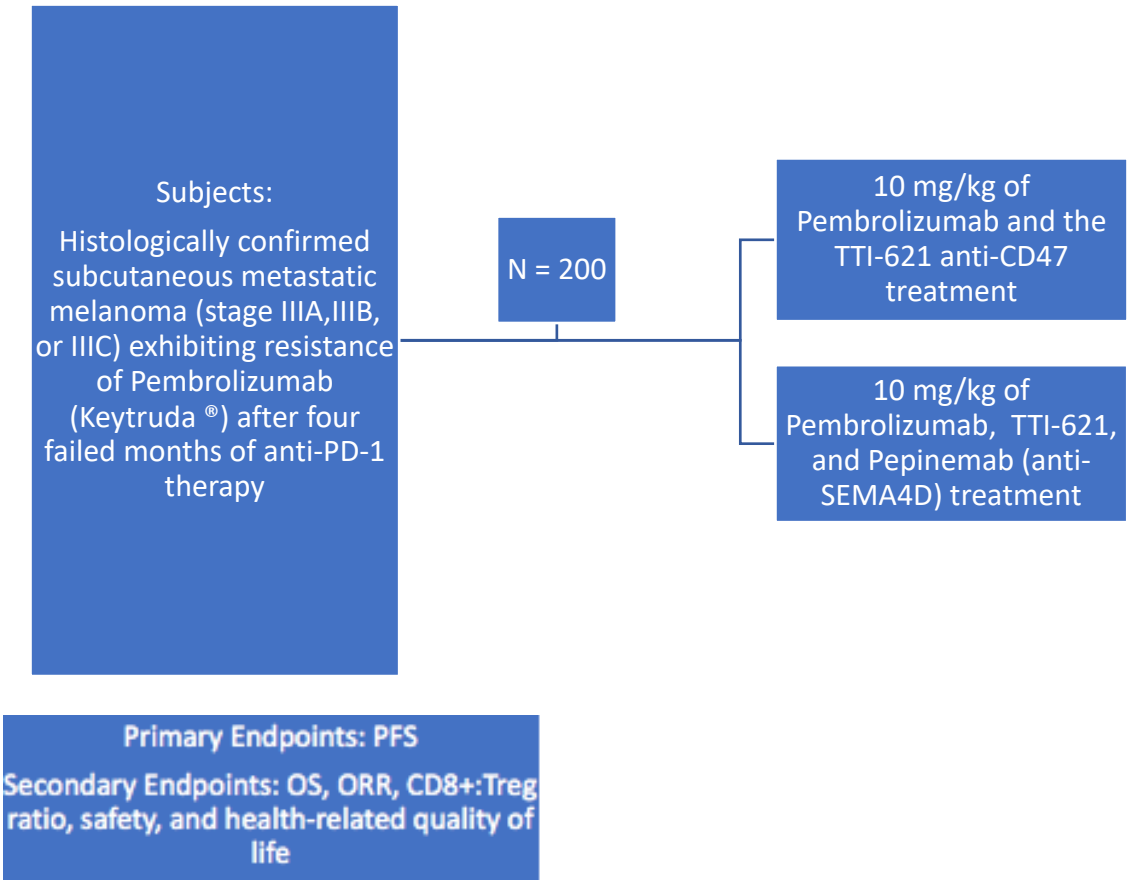


Figure 1 – Clinical Trial Design

Subjects

A sample of 200 total patients, 18 years of age or older, with histologically confirmed cutaneous melanoma with metastasis to regional lymph nodes and who also exhibit resistance to Pembrolizumab (Keytruda ®) were recruited. The patients had to have either stage IIIA melanoma, stage IIIB, or stage IIIC disease with no in-transit metastases as defined by the American Joint Committee on Cancer 2009 classification, 7th edition. Resistance to pembrolizumab indicated after four failed cycles (4 months) of previous anti-PD1 therapy. A tumor sample from melanoma-positive lymph nodes was required to be sent for central pathological evaluation of PD-L1 expression. Membranous expression of PD-L1 in tumor was assessed by means of a clinical trial immunohistochemistry assay (22C3 antibody) and was scored on a scale of 0 to 5. A score of 2 was considered to indicate PD-L1 positivity.

Trial Design

One hundred of the recruited patients were randomly placed in one of two cohorts: the control, which will receive 10 mg/kg of Pembrolizumab and the TTI-621 anti-CD47 treatment, and the experimental, which will receive 10 mg/kg of Pembrolizumab, TTI-621, and Pepinemab the anti-SEMA4D treatment. The patients received this dose every 4 weeks for a total of 12 doses (approximately 1 year) or until disease recurrence or unacceptable toxic effects occurred. The rules regarding the withholding of a dose of either the control or experimental treatment and the management of immune-related adverse events are detailed in the protocol, available at NEJM.org. The primary end point was progression-free survival. Secondary end points included overall survival, ratio of CD8+ T cells to FoxP3 T regulatory cells, safety measures, and measures of health-related quality of life (Eggermont et al., 2018) (Preston et al., 2013).

Assessments and Clinical Endpoints

Computed tomography (CT), magnetic resonance imaging (MRI) or both were performed every 12 weeks for the first 2 years. Progression or metastatic lesions had to be histologically confirmed whenever possible. The initial date of progression was also recorded.

Progression-free survival

The number of months patients survive without further progression of the disease from the time of randomization until the date of first local, regional, or distant metastasis.

Overall Survival

The number of months patients survive from the time of randomization until the date death from any cause.

Overall Response Rate

The response rate will be calculated from the number of complete responders plus the number of partial responders in both the control and experimental cohorts.

Ratio of CD8+ T cells to FoxP3 T regulatory cells

The number of CD8+ T cells and $CD4^{+}CD25^{+}FoxP3^{+}$ will be quantified and analyzed using staining and immunofluorescence analysis of tissue specimens, confocal microscopy, cell quantification, tumor-infiltrating lymphocyte isolation, and flow cytometry.

Safety

Adverse effects were assessed at every CT/MRI session with the use of the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0, and included a complete physical examination along with hematological tests. Immune-related adverse effects were determined from the *Medical Dictionary for Regulatory Activities* terms, which is periodically updated.

Consent

All methods and protocols were outlined for the patient's understanding and approval. After the patients review all necessary features of the clinical trial, written consent was obtained. Patients were told that they can withdrawal from the clinical trial at any time for any reason. The entire procedure was aligned with the guidelines outline by the Institutional Review Board (IRB) at Seton Hall University.

Demographic information

Patient demographic information was collected from each individual's personal case-report form. Details on sex, age, body mass index (BMI), disease stage, type of lymph node involvement, number of positive lymph nodes on pathological testing, ulceration, and PD-L1 expression status.

Statistical Analysis

Progression-free survival was estimated with the Kaplan-Meier method. Comparisons between the two cohorts were made using log-rank tests at two-sided alpha level. Cox proportional-hazards model was used to estimate the hazard ratio and its respective confidence interval (>95%). All analyses were made with SAS software, version 9.4. Power calculations were performed using East Software, version 6.4 (Cytel). A P-value of <0.05 is considered to be statistically significant.

Results

Combination treatment with checkpoint inhibitor, Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Anti-SEMA4D Pepinemab doubles the response rate of patients

Patients and Trial Regimen

A total of 200 patients underwent treatment in two cohorts: combination treatment with Pembrolizumab (Keytruda ®) and Anti-CD47 TTI-621 (Trillium) or Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Pepinemab. Upon initial analysis of patient demographics, looking at sex, age, body mass index, disease stage, type of lymph node involvement, ulceration, and PD-(L)1 expression (Table 2), the characteristics were found to be similar in the two groups.

Efficacy

The 24-month progression-free survival median was not reached in the experimental group with Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Pepinemab treatment (Table 3, Fig. 3). The progression-free survival median was 9 months in the control

group with Pembrolizumab (Keytruda ®) and Anti-CD47 TTI-621 (Trillium). Progression-free survival was significantly higher in the experimental group compared to the control treatment (Table 3, Fig. 3) indicated by the hazard ratio. The hazard ratio for progression-free survival was 0.4 indicating that there were significantly fewer deaths in the experimental group than there were in the control group. Same was true for overall survival where the hazard ratio was 0.25, the overall survival median was 20.4 months for the control and again, not reached for the experimental treatment. The overall response rate for the experimental treatment was 63%, more than triple that of the control, which was only 20%. (Table 3).

In addition to overall response rate, overall survival, and progression-free survival, we looked for a mechanism behind the results. Across all patients, the average ratio of $CD8^+$ T cells: $CD4^+CD25^+FoxP3^+$ regulatory cells was significantly higher in the patients receiving Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Pepinemab treatment compared to the control (0.98 compared to 0.32). The same held true when comparing the ratios of $CD8^+$:$CD4^+$ T cells (0.27 compared to 0.13 in the control) (Table 4).

Safety

Adverse effects of any grade considered to be in connection with the trial were assessed and occurred in 25% of patients in the experimental group and 37% in the control group (Table 5). The rates of fatigue or asthenia and of diarrhea were similar in the two trial groups. Adverse events of grade 3, 4, or 4 that were related to the trial regimen occurred in about the same number of patients in the triple combination group as in the control. Other immune-related adverse events of any grade occurred in roughly the same number of individuals with the most common being hypothyroidism found predominantly in the experimental group (Tables 5 and 6).

Table 2 – Demographic and Clinical Characteristics of Patients at Baseline.

Characteristic	Pembrolizumab + TTI-621+ Pepinemab (N = 100)	Pembrolizumab + TTI-621 (N = 100)
Sex		
Male	63	60
Female	37	40
Age		
Median	54	54
<50 yr	37	37
50 – <65 yr	38	38
≥65 yr	25	25
Body-mass Index		
<25	31	37
25 - <30	45	39
≥ 30	24	24
Disease Stage at Randomization		
Stage IIIA	16	16
Stage IIIB	46	46
Stage IIIC with 1 – 3 positive lymph nodes	19	18
Stage IIIC with ≥ 4 positive lymph nodes	19	20
According to AJCC 2009 Criteria		
Stage IIIA	15	15
Stage IIIB	47	46
Stage IIIC with 1 – 3 positive lymph nodes	17	19
Stage IIIC with ≥ 4 positive lymph nodes	21	20
Type of lymph node involvement		
Microscopic	36	32

Macroscopic	64	68
No of Positive lymph nodes on pathological testing		
1	44	47
2 or 3	34	33
≥ 4	22	20
Ulceration		
Yes	40	39
No	45	50
Unknown	15	11
PD-L1 Expression Status		
Positive	84	85
Negative	11	11
Indeterminate	5	4

Table 3 – Efficacy Results

Endpoint	Pembrolizumab + TTI-621+ Pepinemab 10 mg/kg every 4 weeks (N = 100)	Pembrolizumab + TTI-621 10 mg/kg every 4 weeks (N = 100)
Overall Survival (OS)		
Median	**NR**	**20.4 months**
Hazard Ratio (95% CI)	**0.25**	
p-Value (Stratified log rank)	**<0.0001**	
Progression-Free Survival (PFS)		
Median	**NR**	**9 months**
Hazard Ratio (95% CI)	**0.4**	
p-Value (Stratified log rank)	**<0.0001**	
Overall Response Rate (ORR)		
ORR (95% CI)	**63%**	**20%**
Complete Response Rate	**25**	**2**
Partial Response Rate	**38**	**18**

Table 4 – Comparisons of T Cell Ratios.

	Pembrolizumab + TTI-621+ Pepinemab 10 mg/kg every 4 weeks (N = 100)	Pembrolizumab + TTI-621 20 mg/kg every 4 weeks (N = 100)	
Ratios	**Median**	**Median**	**p-Value**
$CD8^+/CD4^+$	0.27	0.13	0.050
$CD8^+/CD4^+CD25^+FoxP3^+$	0.98	0.32	0.027

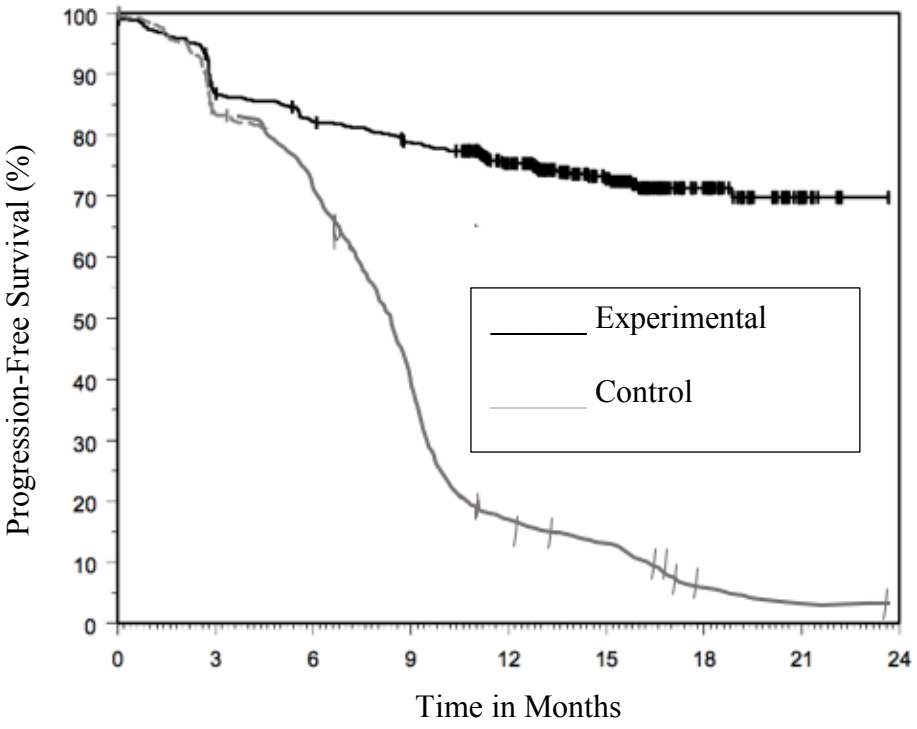
Table 5 – Adverse Reactions Occurring in ≥10% of Patients

Adverse Reaction	Pembrolizumab + TTI-621+ Pepinemab 200 mg every 4 weeks (N = 100)		Pembrolizumab + TTI-621 200 mg every 4 weeks (N = 100)	
	All Grades	Grades 3-5	All Grades	Grades 3-5
General				
Fatigue	**25**	**3**	**33**	**4**
Pyrexia	**10**	**0**	**8**	**0**
Metabolism and Nutrition				
Decreased Appetite	**17**	**2**	**21**	**2**
Respiratory, Thoracic, and Mediastinal				
Dyspnea	**17**	**2**	**11**	**1**
Cough	**16**	**0**	**11**	**0**
Skin and subcutaneous tissue				
Rash	**15**	**1**	**8**	**0**
Gastrointestinal				
Constipation	**12**	**0**	**21**	**1**
Diarrhea	**12**	**1**	**12**	**1**
Nausea	**12**	**1**	**32**	**1**
Endocrine				
Hypothyroidism	**12**	**0**	**2**	**0**
Infections				
Pneumonia	**12**	**7**	**9**	**6**
Investigations				
Weight Loss	**10**	**1**	**7**	**0**

Table 6 – Laboratory Abnormalities Worsened from Baseline in ≥ 20% of Patients.

Laboratory Test	Pembrolizumab + TTI-621+ Pepinemab 200 mg every 4 weeks (N = 100)		Pembrolizumab + TTI-621 200 mg every 4 weeks (N = 100)	
	All Grades	Grades 3-4	All Grades	Grades 3-4
Chemistry				
Hyperglycemia	**52**	**5**	**51**	**5**
Increased ALT	**33**	**5**	**32**	**3**
Hypoalbuminemia	**33**	**2**	**29**	**1**
Increased AST	**31**	**3**	**32**	**2**
Hyponatremia	**31**	**9**	**32**	**8**
Increased alkaline phosphatase	**29**	**2**	**29**	**0**
Hypocalcemia	**25**	**3**	**19**	**1**
Hyperkalemia	**23**	**3**	**20**	**2**
Increased prothrombin INR	**21**	**2**	**15**	**3**
Hematology				
Anemia	**43**	**4**	**79**	**19**
Lymphopenia	**30**	**7**	**41**	**13**

Figure 2 – Kaplan – Meier Curve for Progression-Free Survival



Discussion

Combination immunotherapies have shown to be efficacious in treating many cancers, however, there is still a relatively low response rate indicating that there must be another element that is preventing tumors from being eliminated. In order for cells from both the innate and adaptive immune branches to target and kill cancer cells, they must gain access into the tumor microenvironment. SEMA4D is a member of a class of receptors found on many immune cells, but is also exploited by many cancers, that has the ability to cause cytoskeletal changes on other immune cells effectively blocking their function and access to the tumor. So, while combination checkpoint inhibitor therapeutics like Pembrolizumab (Keytruda ®) or Ipilimumab (Yervoy ®) may be effective in bypassing homeostatic mechanisms of the immune system, if the immune cells cannot physically enter the tumor microenvironment, then these treatments will not show results since they are not targeting one of the major issues. This study postulated the effect of blocking SEMA4D in conjunction with the combination therapies in order to break down the physical barrier giving immune cells continued access to the tumor microenvironment. It was hypothesized that this triple combination approach would double response rates and be mediated by the increase in $CD8^+$ T cell to Regulatory T cell ratios.

Results indicate that the overall response rates tripled, and progression-free survival and overall survival increased significantly as well. The chances of progression-free survival in the experimental group was 60% higher than that of the control and overall survival was 75% higher compared to the control. The experimental group also had a significantly stronger overall response rate compared to that of the control indicating that these data provide evidence to support that this triple immunotherapy treatment is more effective in treating melanoma compared to other adjuvant therapies. The differences in adverse effects between the two cohorts

were statistically insignificant indicating that this new triple treatment approach is not more or less toxic than conventional adjuvant therapies. The demographic information also supports that any differences in results between the two groups is due to the different treatment methods and not attributed to any other factor.

The cytotoxic T cell to regulatory T cell ratio also increased in the experimental group indicating that more T cells are accessing the tumor bed and fewer regulatory T cells are present and acting as a negative control for the immune response. Since SEMA4D has been found to bind to T cells and influence their cytoskeletal movement, it can determine what cells are able to infiltrate the tumor bed. In order to understand the mechanism behind the improved survival rates among patients, we looked at the number of effector T cells compared to the number of regulatory T cells. The number of effector T cells increased in the cohort receiving Pembrolizumab (Keytruda ®), Anti-CD47 TTI-621 (Trillium), and Pepinemab treatment. This was expected since blocking the SEMA4D receptor prevents the altered movement of infiltrating T cells thus allowing them to move into the tumor microenvironment.

To determine how anti-SEMA4D affected T cell movement, we first calculated the ratio of CD8+/CD4+ T cells. CD4+ represent all T helper cells, which includes regulatory T cells. T helper cells are responsible for the release of cytokines that can both promote and shut off an immune response by activating or inactivating certain immune players. The ratio of CD8+/CD4+ T cells was larger in the experimental cohort compared to the control as expected. This increased ratio indicates that there are more cytotoxic CD8+ T cells compared to CD4+ T helper cells. In order to further narrow down the mechanism, we looked specifically at the CD8+/regulatory T cells ($CD4^+CD25^+FoxP3^+$) ratio where we also see a significant difference between the two groups with more cytotoxic CD8+ T cells present after therapy with anti-

SEMA4D. These data suggest that anti-SEMA4D works by allowing for an increased infiltration of cytotoxic T cells effectively suppressing the functioning of regulatory T cells that would otherwise downregulate the immune response.

The results of this clinical trial support the importance of the presence of TILs into the tumor microenvironment. Anti-SEMA4D, in combination with other immunotherapies, has shown to be an effective defense against the tumor's effort to shut off the host immune system. Based off of these results, this clinical trial can be expanded and increase sample size to 1,000 individuals for study. Further study may also be done in other cancers as well as determining the exact molecular cascade mechanism SEMA4D uses to affect T cell migration. This may be useful in the development of future therapeutics.

References

Burugu, S., Dancsok, A. R., & Nielsen, T. O. (2018). Emerging targets in cancer immunotherapy. *Seminars in Cancer Biology*, *52*, 39–52.

Castellanos, J. R., Purvis, I. J., Labak, C. M., Guda, M. R., Tsung, A. J., Velpula, K. K., & Asuthkar, S. (2017). B7-H3 role in the immune landscape of cancer. *American Journal of Clinical and Experimental Immunology*, *6*(4), 66–75.

Chae, W.-J., Ehrlich, A. K., Chan, P. Y., Teixeira, A. M., Henegariu, O., Hao, L., … Bothwell, A. L. M. (2016). The Wnt Antagonist Dickkopf-1 Promotes Pathological Type 2 Cell-Mediated Inflammation. *Immunity*, *44*(2), 246–258.

Charles A Janeway, J., Travers, P., Walport, M., & Shlomchik, M. J. (2001). Principles of innate and adaptive immunity. *Immunobiology: The Immune System in Health and Disease. 5th Edition*.

Chen, X., Lu, P.-H., Liu, L., Fang, Z.-M., Duan, W., Liu, Z.-L., … He, W.-T. (2016). TIGIT negatively regulates inflammation by altering macrophage phenotype. *Immunobiology*, *221*(1), 48–55.

D'Amico, L., Mahajan, S., Capietto, A.-H., Yang, Z., Zamani, A., Ricci, B., … Faccio, R. (2016a). Dickkopf-related protein 1 (Dkk1) regulates the accumulation and function of myeloid derived suppressor cells in cancer. *The Journal of Experimental Medicine*, *213*(5), 827–840.

Decker, W. K., da Silva, R. F., Sanabria, M. H., Angelo, L. S., Guimarães, F., Burt, B. M., … Paust, S. (2017). Cancer Immunotherapy: Historical Perspective of a Clinical Revolution and Emerging Preclinical Animal Models. *Frontiers in Immunology*, *8*.

Eggermont, A. M. M., Blank, C. U., Mandala, M., Long, G. V., Atkinson, V., Dalle, S., … Robert, C. (2018). Adjuvant Pembrolizumab versus Placebo in Resected Stage III Melanoma. *New England Journal of Medicine*, *378*(19), 1789–1801.

Evans, E. E., Jonason, A. S., Bussler, H., Torno, S., Veeraraghavan, J., Reilly, C., … Zauderer, M. (2015). Antibody Blockade of Semaphorin 4D Promotes Immune Infiltration into Tumor and Enhances Response to Other Immunomodulatory Therapies. *Cancer Immunology Research*, *3*(6), 689–701.

Han, S., Wang, Y., Shi, X., Zong, L., Liu, L., Zhang, J., … Zhang, Y. (2018). Negative roles of B7-H3 and B7-H4 in the microenvironment of cervical cancer. *Experimental Cell Research*, *371*(1), 222–230.

He, Y., Peng, H., Sun, R., Wei, H., Ljunggren, H.-G., Yokoyama, W. M., & Tian, Z. (2017). Contribution of inhibitory receptor TIGIT to NK cell education. *Journal of Autoimmunity*, *81*, 1–12.

Immuno Oncology – A 30-year Overnight Success Story. (2014, February 20). Retrieved May 5, 2019, from BIOtechNow website: https://www.biotech-now.org/health/2014/02/immuno-oncology-a-30-year-overnight-success-story

Information, N. C. for B., Pike, U. S. N. L. of M. 8600 R., MD, B., & Usa, 20894. (2016). *The innate and adaptive immune systems*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK279396/

Ishihara, J., Ishihara, A., Potin, L., Hosseinchi, P., Fukunaga, K., Damo, M., … Hubbell, J. A. (2018). Improving Efficacy and Safety of Agonistic Anti-CD40 Antibody Through Extracellular Matrix Affinity. *Molecular Cancer Therapeutics*, *17*(11), 2399–2411.

Jansen, C. S., Prokhnevska, N., & Kissick, H. T. (2018). The requirement for immune infiltration and organization in the tumor microenvironment for successful immunotherapy in prostate cancer. *Urologic Oncology: Seminars and Original Investigations*.

Joseph Gulfo. (2018, May 2). Turning “cold tumors hot” – rationale, major setback, and promise | Cancer Biology.

Kagey, M. H., & He, X. (2017a). Rationale for targeting the Wnt signalling modulator Dickkopf-1 for oncology: Targeting DKK1 for oncology. *British Journal of Pharmacology*, *174*(24), 4637–4650.

Kaufman, H. L., Kohlhapp, F. J., & Zloza, A. (2015). Oncolytic viruses: a new class of immunotherapy drugs. *Nature Reviews Drug Discovery*, *14*(9), 642–662.

keytruda_pi.pdf. (n.d.).

Knee, D. A., Hewes, B., & Brogdon, J. L. (2016). Rationale for anti-GITR cancer immunotherapy. *European Journal of Cancer*, *67*, 1–10.

Kramer, K., Kushner, B. H., Modak, S., Pandit-Taskar, N., Smith-Jones, P., Zanzonico, P., … Cheung, N.-K. V. (2010). Compartmental Intrathecal Radioimmunotherapy: Results for Treatment for Metastatic CNS Neuroblastoma. *Journal of Neuro-Oncology*, *97*(3), 409–418.

Lanitis, E., Irving, M., & Coukos, G. (2015). Targeting the tumor vasculature to enhance T cell activity. *Current Opinion in Immunology*, *33*, 55–63.

Malladi, S., Macalinao, D. G., Jin, X., He, L., Basnet, H., Zou, Y., … Massagué, J. (2016). Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. *Cell*, *165*(1), 45–60.

Mastelic-Gavillet, B., Balint, K., Boudousquie, C., Gannon, P. O., & Kandalaft, L. E. (2019). Personalized Dendritic Cell Vaccines—Recent Breakthroughs and Encouraging Clinical Results. *Frontiers in Immunology*, *10*.

Murata, Y., Saito, Y., Kotani, T., & Matozaki, T. (2018). CD47-signal regulatory protein α signaling system and its application to cancer immunotherapy. *Cancer Science*, *109*(8), 2349–2357.

Negative roles of B7-H3 and B7-H4 in the microenvironment of cervical cancer. (2018). *Experimental Cell Research*, *371*(1), 222–230.

Newick, K., Moon, E., & Albelda, S. M. (2016). Chimeric antigen receptor T-cell therapy for solid tumors. *Molecular Therapy - Oncolytics*, *3*, 16006.

Olsen, E. A., Whittaker, S., Kim, Y. H., Duvic, M., Prince, H. M., Lessin, S. R., … Vonderheid, E. C. (2011). Clinical End Points and Response Criteria in Mycosis Fungoides and Sézary Syndrome: A Consensus Statement of the International Society for Cutaneous Lymphomas, the United States Cutaneous Lymphoma Consortium, and the Cutaneous Lymphoma Task Force of the European Organisation for Research and Treatment of Cancer. *Journal of Clinical Oncology*, *29*(18), 2598–2607.

Prasad-Adusumilli.pdf. (n.d.).

Preston, C. C., Maurer, M. J., Oberg, A. L., Visscher, D. W., Kalli, K. R., Hartmann, L. C., … Knutson, K. L. (2013). The Ratios of CD8+ T Cells to CD4+CD25+ FOXP3+ and FOXP3- T Cells Correlate with Poor Clinical Outcome in Human Serous Ovarian Cancer. *PLoS ONE*, *8*(11), e80063.

Räihä, M. R., & Puolakkainen, P. A. (2018). Tumor-associated macrophages (TAMs) as biomarkers for gastric cancer: A review. *Chronic Diseases and Translational Medicine*, *4*(3), 156–163.

Roberts, N. J., Zhang, L., Janku, F., Collins, A., Bai, R.-Y., Staedtke, V., … Zhou, S. (2014). Intratumoral injection of Clostridium novyi-NT spores induces antitumor responses. *Science Translational Medicine*, *6*(249), 249ra111.

Sharma, P., Kumar, P., & Sharma, R. (2017). Natural Killer Cells - Their Role in Tumour Immunosurveillance. *Journal of Clinical and Diagnostic Research : JCDR*, *11*(8), BE01–BE05.

Simon, S., & Labarriere, N. (2017). PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *Oncoimmunology*, *7*(1).

Streby, K. A., Geller, J. I., Currier, M. A., Warren, P. S., Racadio, J. M., Towbin, A. J., … Cripe, T. P. (2017). Intratumoral Injection of HSV1716, an Oncolytic Herpes Virus, Is Safe and Shows Evidence of Immune Response and Viral Replication in Young Cancer Patients. *Clinical Cancer Research*, *23*(14), 3566–3574.

Teng, M. W. L., Ngiow, S. F., Ribas, A., & Smyth, M. J. (2015). Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Research*, *75*(11), 2139–2145.

Weinberg, R. A. (2014). *The biology of cancer* (Second edition). New York: Garland Science, Taylor & Francis Group.

Wu, J., & Waxman, D. J. (2018). Immunogenic chemotherapy: Dose and schedule dependence and combination with immunotherapy. *Cancer Letters*, *419*, 210–221.